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A novel surgical organ perfusion method for effective ex vivo and in vivo gene transfer into renal glomerular cells

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Abstract In an attempt to develop gene therapy for Alport syndrome, we have evaluated surgical methods for gene transfer into pig kidneys. For gene transfer we used an adenovirus expressing the *Escherichia coli* β-galactosidase gene as a reporter gene. The viral preparation was first infused in vivo into the porcine renal artery. Then explanted kidneys were perfused ex vivo at body temperature for 12 hours with the viral solution and, finally the kidney perfusions were carried out in vivo via laparotomy for 60 and 120 minutes. Gene transfer was determined visually on histological cryosections after 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal) and periodic acid-Schiff (PAS) staining. Perfusion of whole porcine kidneys ex vivo resulted in strong expression in about 80% of glomeruli. The in vivo kidney perfusion via laparotomy for 120 minutes resulted in reporter gene expression of about 75% of the glomeruli examined after 4 days. Expression was observed almost exclusively in glomeruli, while little if any expression was found in other renal structures. The present results suggest that operatively performed kidney perfusion may be used for gene transfer in treatment of glomerular disease. This surgical approach may also prove useful for somatic gene therapy of other organs.

Key words Kidney perfusion · Glomeruli · Gene transfer · Alport syndrome · Basement membrane

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Introduction

Advances in recombinant DNA technology have made the introduction of therapeutic genes into somatic cells possible. In recent years several clinical trials involving human gene therapy have been accepted by regulatory agencies. The first, already initiated, gene therapy trials aimed at treating both inherited diseases, such as severe combined immunodeficiency disease caused by lack of adenosine deaminase in peripheral T-lymphocytes, cystic fibrosis and familial hypercholesterolemia, as well as noninherited diseases such as cancer [2, 9, 10, 15].

The development of suitable, safe and effective gene transfer systems is a major goal of research in gene therapy. Thus far, viruses have extensively been used as vectors for that purpose [4]. Retroviruses have been widely used, but their disadvantage is that they only apply to dividing cells and, additionally, they cannot accommodate large inserts. Replication-defective adenovirus has been successfully used for transfer of a variety of genes into cells in culture and in vivo. Adenovirus can accommodate larger inserts than retrovirus, but its disadvantage is that its extrachromosomal expression only lasts for a few weeks. Herpes viruses particularly have been used in gene transfer trials to the central nervous system. They can carry foreign DNA of large sizes and stay latent for long periods of time. In spite of the availability of replication-defective viruses, concerns about their safety and efficiency have provoked interest in the development of other nonviral systems. Among those are cationic liposome–DNA complexes which have been shown to be often readily taken up by cells both in vitro and in vivo [1, 4, 8].

Another major task of gene therapy research is the development of methods for targeting the gene transfer to appropriate cells and tissues. Ex vivo gene transfer into explanted cultured cells and subsequent implantation of the treated cells has been used for treatment of hematopoietic tissue [9]. Also, direct injection into tissues, intravenous or intra-arterial administration, inha-

lation or topical application have been used. The drawbacks of these methods are that the transduction is not highly selective and that significant amounts of the therapeutic gene-containing vector may be needed. Besides, these methods may not allow for an effective enough gene transfer into the targeted tissue cells.

As part of our studies aimed at gene therapy of Alport syndrome, we have developed a surgical organ perfusion system for efficient gene transfer into kidney glomeruli [6]. Alport syndrome is an inherited kidney disease caused by mutations in the genes for the alfa3, alfa4 and alfa5 chains of type IV collagen [3, 11, 14]. These chains are essential components of the glomerular basement membrane (GBM) that constitutes the glomerular filtration barrier [7]. Alport syndrome usually progresses to end-stage renal disease due to the GBM defect. Extrarenal manifestations, such as hearing loss and eye lesions are not life threatening. Since the disease manifests itself mainly as glomerular disease, it is an excellent candidate for gene therapy targeted to the glomeruli.

In the present study we have developed and evaluated a surgical ex vivo and in vivo kidney perfusion method for highly efficient adenovirus-mediated gene transfer into glomerular cells, using pigs as experimental animals. This method may apply for gene therapy of Alport syndrome and also for somatic gene transfer into other organs for the treatment of other genetic diseases or for other purposes.

Materials and methods

Reporter gene virus

A replication defective recombinant type 5 adenovirus (Ad-CMVIacZ) containing the cytomegalovirus promoter and the *Escherichia coli* β-galactosidase gene was used as gene transfer vector. Sequences in the El A, El B and E3 regions were deleted. Expression of the reporter gene serves to identify cells where successful transduction has occurred.

Experimental animals

Experimental animals were young 22–35 kg farm pigs which were treated according to institutional guidelines. In this study operative gene transfer trials were made to 25 animals. The animals were under general anesthesia during the operation. Azaperon (Stresnil, Janssen Pharmaceutical) 4 mg/kg was first administered as intramuscular injection. For induction medetomidine (Domitor, Lääkefarmos) 80 μg/kg, ketamin (Ketalar, Parke-Davis) 4 mg/kg and atropine (Atropin, Leiras) 0.05 mg/kg were given intramuscularly. Thiopenthal (Pentothal Natrium, Abbott) 5 mg/kg was then given intravenously, the animals were intubated and the anesthesia was continued under a combination of nitrous oxide and oxygen (l:l) and 1.5% enflurane (Efrane, Abbott).

Intra-arterial adenoviral infection in vivo

In the first series of 11 animals the virus vector was injected into the renal artery during laparotomy in the presence or absence of vasodilators (Table l). In the first experiment 2.5 ml [4×10^9 plate-forming units (pfu)] of adenoviral preparation were injected through a 0.1-mm butterfly needle into the lower branch of the left renal artery. The animal was killed on the fourth postoperative day and nephrectomy was made on the left. Samples from the left kidney were taken for histologic examination and expression of the *lacZ* gene was examined by staining with 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal). Because the infusion was made to the lower pole of the kidney, the upper pole could be used as control.

Table 1 Experimental infusions of adenovirus particles intra-arterially into renal artery with vasodilative agents

Experimental animal	Vasodilative agent	Number of adenovirus particles (pfu/ml)
A1	None	2×10^9
A2	Papaverin 30 mg	4×10^9
A3	Papaverin 20 mg	4×10^9
A4	Papaverin 10 mg	4×10^9
A5	Alprostadil 5 microg	4×10^9
A6	Verapamil 2 mg	4×10^9
A7	Enalapril 0.5 mg	4×10^9
A8	Lidocaine 100 mg	4×10^9
A9	Papaverin 60 mg	4×10^9
A10	NaCl 10 ml	4×10^9
A11	Papaverin 20 mg + NaCl 10 ml	4×10^9

pfu plate-forming unit

Nine of the 11 animals were operated on in a similar fashion to the above, except that vasodilative pharmacologic agents were infused intra-arterially into the lower branch of the renal artery shortly prior to infusion of the virus preparation; this was to diminish the potential vascular resistance in the kidney. Five commonly used vasodilative agents were applied in different trials: papaverin, alprostadil, enalapril, verapamil, and lidocaine (Table l). One animal received 0.9% saline prior to viral infusion.

Kidney perfusion system

In order to extend the infection time of kidney cells with the virus, we developed a closed-circuit perfusion system for continuous circulation of the virus solution in the intact kidney ex vivo as well as in vivo. This system consisted of a reservoir for the perfusate, a pump, an artificial membrane lung, and the kidney to be perfused, all connected by silicon tubing with an inside diameter of 3 mm (Fig. l). The reservoir was a 300-ml glass bottle placed in a ther-

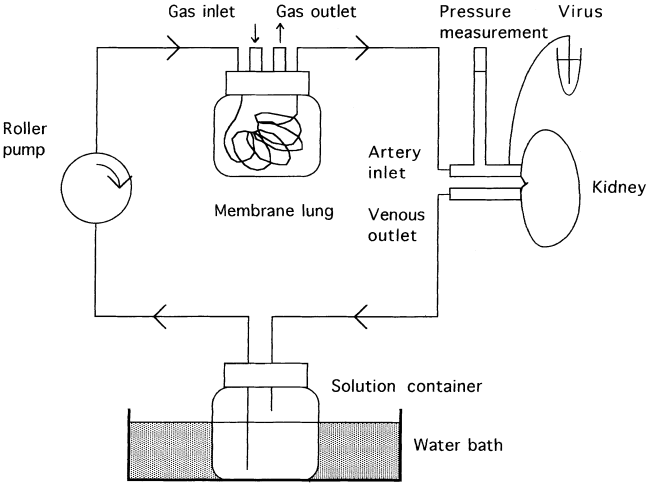

Fig. 1 Schematic presentation of the perfusion system for ex vivo and in vivo gene transfer into kidney (see text for details)

mostat-controlled 37°C water bath. The pump was derived from the portable organ perfusion machine PF-3A model peristaltic pump (Gambro) with roll per minute (rpm) control to control the flow rate. The membrane lung consisted of 8 m of 1.47-mm inside diameter silicon tubing in an 2000-ml glass container gassed with carbogen gas (95% oxygen, 5% carbon dioxide) at a pressure of 15 mmHg, according to Hamilton et al. [5]. The kidney was attached to the perfusion system by cannulating the renal artery with 14G cannula and the renal vein with 12G cannula. The venous effluent was collected directly in the reservoir. The perfusate had a total volume of 350 ml and contained previously separated red blood cells at a hematocrit value of 17% in Krebbs-Ringer solution. Additionally, 25 000 IU heparin and antibiotics were added. For ex vivo perfusion of explanted kidneys, the perfusate also contained 20 000 IU penicillin, 20 000 μg streptomycin as antibiotics and 5 ml MEM amino acid solution (Gibco BRL). For the in vivo perfusions, the perfusate contained 250 mg cefuroxim as antibiotic. The flow rate was adjusted to enable adequate diuresis. The pH and oxygen saturation in the perfusate were measured by laboratory blood gas analysis from the perfusate.

Kidney perfusion experiments

Before connecting the kidney to the perfusion system, a 10-ml lidocain-heparin solution (190 mg lidocain + 5 000 IU heparin) and 0.9% saline were infused through the renal artery until the venous effluent was clear. The adenoviral preparation (1×10^{11} pfu in 20 ml Krebbs-Ringer solution) was then infused into the arterial inlet, and the perfusion was immediately initiated the flow rate was set at 100–120 ml/minute.

At first the ex vivo perfusion experiments were made at room temperature using two explanted porcine kidneys, but when no reporter gene expression was found in those kidneys, the perfusion temperature was raised to 37°C in the subsequent experiments. A total of four kidneys were perfused ex vivo at the 37°C temperature, the average perfusion time being 12 hours. Following the experiments, tissue samples were taken for histologic analysis as described above. Kidneys of eight animals were perfused in vivo via laparotomy. One of these in vivo perfusion experiments was made without viral preparation as a control. The animals were given prophylactic antibiotic (750 mg cefuroxim) intravenously prior the operation, 250 mg cefuroxim being added to the perfusate. While the kidney was connected to the perfusion system, it was isolated from the systemic circulation by clamping the renal artery and vein proximally. At the end of the perfusion 0.9% saline was infused into the renal artery. The perfusion pressure was measured directly from the arterial inlet and the flow rate was adjusted to the level of 75/67 mmHg. Five in vivo viral perfusions and the control perfusion experiments were carried out for 60 minutes, and two viral perfusions for 120 minutes. (Table 2). Hydrocortisone 50 mg was administered intramuscularly after the operation.

Four animals were killed on the fourth postoperative day and the whole perfused kidney was removed for histologic examination. After perfusion three animals and the control animal had open biopsy of the perfused kidney on the seventh day to examine the transduction. The animals were maintained for up to 4 weeks postoperatively, after which they were killed and both the left kidney and the untreated right kidney were taken for histologic examination. Excretory urograms and serum creatinine levels were taken both before the perfusion and 4 weeks thereafter. Anti-β-galactosidase antibodies and anti-AdV-antibodies were examined by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF) respectively before the perfusion and 2 and 4 weeks thereafter.

Table 2 Viral gene transfer into porcine kidneys using an in vivo closed-circuit perfusion method. Summary of procedures and gene transfer efficacy

Number of animals	Perfusion time (min)	Following time (days)	% Glomeruli infected (mean)
2	60	4	9–58 (47)
3	60	7 (biopsy)	38–66 (48)
2	120	4	23–75 (58)
1 (control)	60	Biopsy on day 7	0
		28	0

Histochemical analysis

Efficiency of adenoviral gene transfer was monitored by visual analysis of *lacZ* gene expression on cryosections. Sections of 5 μm thickness were first fixed for 10 minutes in 4% glutaraldehyde. Following extensive washings with 1 × phosphate-buffered saline (PBS), the sections were incubated in a detergent solution containing 0.01% sodium deoxycholate, 0.02% NP40 and 2 mM magnesium chloride in PBS for 10 minutes. The sections were incubated in an X-gal solution (detergent solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal for 3 hours or overnight at 37°C and subsequently counterstained with periodic acid-Schiff (PAS). Sections from the untreated right kidney were used as a control. Possible cytotoxic and inflammatory changes were evaluated by histologic examination of formaldehyde-fixed paraffin-embedded tissue sections after PAS-hematoxylin staining.

Results

Gene transfer by arterial infusion into kidney in vivo

The first set of experiments involving injection of the virus into the renal artery in vivo did not lead to succesful gene transfer into kidney cells. *LacZ* gene expression was observed in only a few scattered cells in the kidney cortex, no signs of expression being seen in the glomeruli (data not shown). Several vasodilative agents, used in order to diminish possible intrarenal vascular resistance, immediately prior to injection of the virus had no visible effect on efficiency on gene transfer. Even up to three consecutive injections with 4-minute intervals did not seem markedly to increase the efficiency of gene transfer.

Gene transfer by kidney perfusion ex vivo

Perfusion of explanted kidneys with the adenovirus at room temperature for up to 17 hours did not result in β-galactosidase-positive kidney cells. At room temperature the cells in the explanted kidney are not able to efficiently express viral proteins such as β-galactosidase. Therefore when the perfusion was carried out at 37°C for 12 hours, marked expression of the *lacZ* gene could be observed in glomerular cells. By histologic examination, *lacZ* gene expression was observed in approximately 80% of the glomeruli, and in several glomeruli most mesangial and endothelial cells as well as epithelial podocytes appeared to be positive (Fig. 2). Only a little staining was seen in endothelial cells elsewhere in blood vessels, and epithelial cells of the tubuli did not exhibit any staining.

Gene transfer into kidney by perfusion in vivo

The first two in vivo perfusions were carried out for 60 minutes. Four days postoperatively *lacZ* gene expression was seen almost exclusively in glomerular cells. Expression was found in 9%–58% of the glomeruli, the mean value being 47% as determined from 32 sections taken randomly from various sites (upper, middle, and lower pole) of the kidney (Table 2). Within the glomeruli themselves between 2% and 50% of the cells were estimated to exhibit expression. Expression was not observed in cells of other segments of the kidney, except for some vascular endothelial cells.

Two other in vivo perfusions were performed for 120 minutes. Four days later *lacZ* gene expression was observed in 23%–75% of the glomeruli, the mean being 58% determined from 10 sections (Table 2). Individual glomeruli expression was seen in most mesangial and endothelial cells as well as in the epithelial podocytes. In certain segments all glomeruli appeared to be positive with seemingly all cells exhibiting expression (Fig. 3). In this experiment expression was only seen in scattered endothelial cells elsewhere in the vascular system and epithelial cells of proximal and distal tubuli were negative.

Four animals perfused for 60 minutes were maintained postoperatively for 4 weeks. One kidney perfused with the virus solution showed some shrinkage after 4 weeks, while the other three were macroscopically normal. The reason for the shrinkage of one kidney is not clear, but it might have been caused by the adenovirus perfusion procedure, or possibly by thrombosis. The *lacZ* gene expression was seen in biopsies taken on day 7, but after 4 weeks the expression had diminished markedly. After 4 weeks no remarkable titer could be measured for anti-β-galactosidase antibodies, (data not shown), only one animal showing slight elevation of IgG -type anti-AdV-antibodies. Serum creatinine levels were within normal reference value. Normal kidney function was detected on excretory urogram weeks after perfusion. By histologic analysis the control perfusion had no effect on cell morphology, but following viral perfusion there were some inflammatory changes, such as mononuclear cell clusters mainly around blood vessels (Fig. 4).

Discussion

In the present study we have evaluated the efficiency of adenovirus-mediated gene transfer into kidney cells following intra-arterial injection or continuous closed-circuit perfusion. Gene transfer into kidney cells after intra-arterial injections was insignificant. Even the use of high pharmacologic amounts of a number of vasodilative agents did not notably improve the uptake of virus into the kidney cells. In contrast, the organ perfusion system developed in this study resulted in efficient gene transfer into glomerular cells both using ex vivo and in vivo perfusion. The kidneys could be maintained viable ex vivo for up to 12 hours when the perfusion being carried out 37°C with a Krebbs-Ringer solution containing oxygenated red blood cells at 17% hematocrit. Under those conditions gene transfer was achieved in up to 80% of the glomeruli. Perfusions were carried out in vivo for up to 2 hours with gene transfer being observed in up to 75% of glomeruli. Gene transfer was highly specific for glomerular cells. Significant expression was not seen in endothelial cells of arteries and veins nor in epithelial cells of the proximal or distal tubuli.

The results obtained with the kidney perfusion technique can be considered significant advances compared with other methods previously used for gene transfer into kidney cells. Moullier et al. [12] showed some adenovirus-mediated transfer of the *lacZ* gene into rat tubular, but not glomerular cells following a combination of infusion of the virus into renal artery and retrograde infusion into the ureter. Those results agree with the present results showing insignificant transfer after intra-arterial infusion. It appears that simple infusion of soluble virus solution does not provide a long enough time for the virus to bind to and be taken up by cells. Somewhat better results were obtained by Tomita et al. [13] who infused a complex of Sendai virus and liposomes into the rat renal artery in vivo. This resulted in expression of the marker gene in about 15% of the glomerular cells which is, however, considerably less than that achieved with the perfusion method developed here.

The organ perfusion method developed here for gene transfer seems to work well for the kidney. However, our results suggest that despite its benefits the adenovirus vector may cause harmful reactive changes in the organ. A further disadvantage is that the expression is transient. Zsengeller et al. [17] found that an E1a-E3-deleted recombinant Ad5 adenoviral gene transfer was associated with inflammation and cytotoxic cell-mediated immune clearance of respiratory epithelial cells in the lungs of mice, which may partly explain the diminishing gene expression. In our experiments no remarkable humoral immunity was elicited, but mononuclear cell clusters were seen in the renal interstitium. To circumvent these problems other nonimmunogenic vectors should be tested.

The present results are particularly promising when considering potential gene therapy for Alport syndrome, since the genes (i.e. C0L4A3, C0L4A4) to correct the kidney vascular system with a viral vector may not allow for treatment of diseases affecting tubular epithelial cells.

The perfusion system may prove applicable for transfer of genes into other organs using virus vectors or synthetic systems such as liposome–DNA complexes. The prerequisite is that the organ has a suitable blood circulation system. The perfusion of gene transfer solutions is more efficient if the organ has one end artery, and it may be possible to use this technique even though there are two or more end arteries. Organs that might

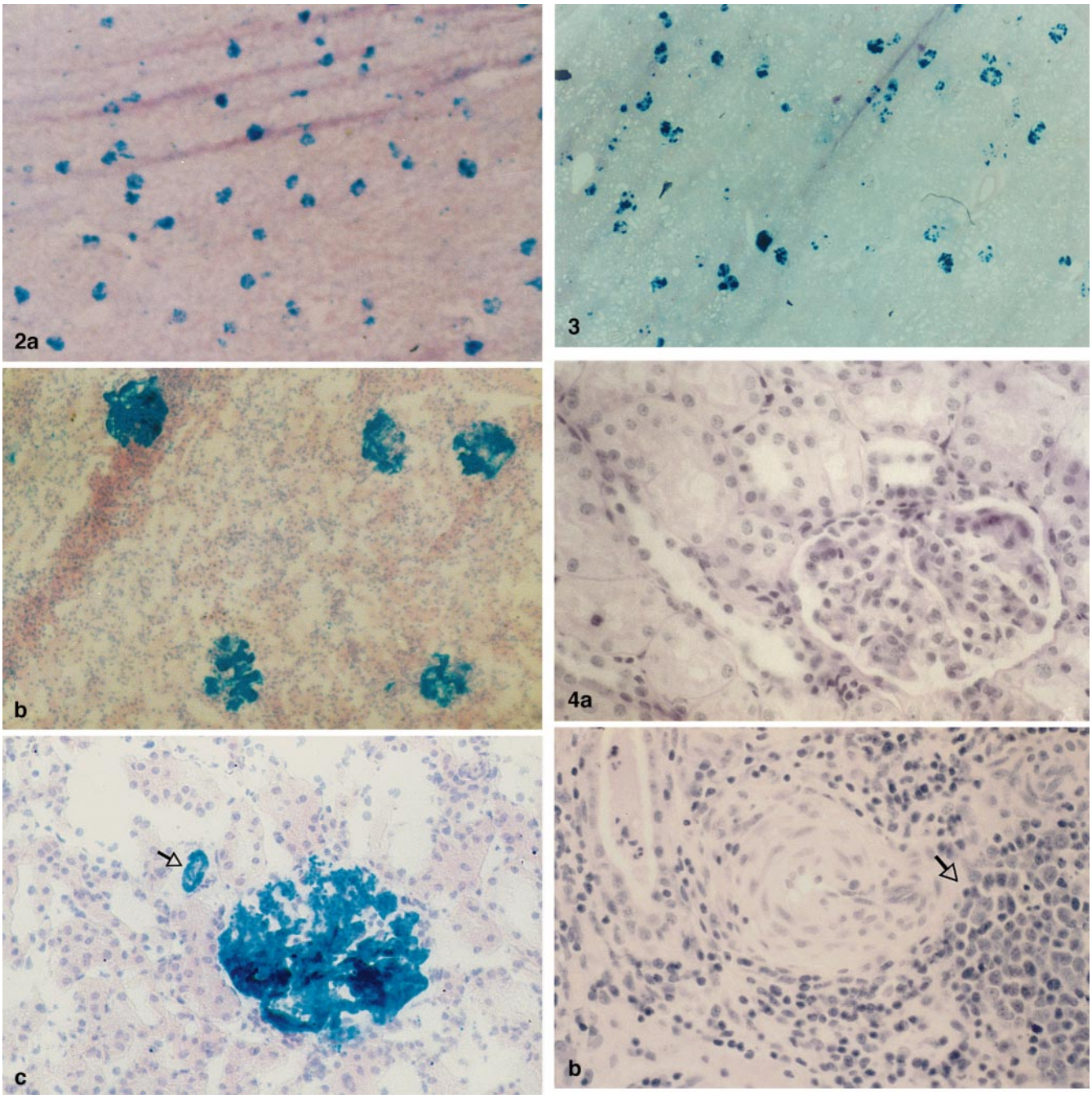

Fig. 2(a–c) Expression of the *lacZ* gene in explanted porcine kidney following ex vivo perfusion. (**a**) Almost all glomeruli are positive for expression as determined by 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal) and periodic acid-Schiff (PAS) staining of a cryosection. Magnification ×21 (**b**) Magnification ×86. (**c**) Expression in all cell types of a glomerulus, and also in endothelial cells of an adjacent capillary (*arrow*). Magnification ×214

Fig. 3 Expression of the *lacZ* gene following porcine kidney perfusion in vivo. Almost all glomeruli are positive for expression of the reporter gene. (X-gal and PAS staining, ×21)

Fig. 4(a, b) Paraffin-embedded kidney section 4 weeks after viral perfusion (PAS hematoxylin staining, ×351) (**a**) A normal appearing glomerulus. (**b**) Mononuclear cell cluster in the interstitium (*arrow*)

potentially be treated with the perfusion system include liver, spleen, lung, and mammary gland. However, organs such as intestine, pancreas with several venous outlets may be difficult to perfuse because collection of the viral preparation for recirculation may be impossible.

The most obvious benefits of the in vivo perfusion method, as opposed to intra-arterial injections, are highly organ-specific gene transfer and the possibility of using only small amounts of potentially expensive vector materials. Furthermore, the extracorporeal perfusion system diminishes the risk of administering large amounts of foreign genetic and other material to an

immunocompetent human, since after the organ perfusion the excess transgene material can be washed from the organ. We believe that the use of a surgical method such as organ perfusion can become a useful addition to the repertoire of methods that can be used for gene transfer in the future. As the technique is better optimized, and especially as different vectors and conditions are tested, it can become a realistic alternative for delivery of genes in somatic gene therapy and other cases where organ gene transfer or administration of other therapeutics are needed.

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