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Molecules and Cells

Gene Delivery into Rat Glomerulus Using a Mesangial Cell Vector

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To develop an effective protocol of gene transfer into glomeruli, an *ex vivo* gene delivery system using rat mesangial cells (RMC) as a vector was examined. RMC genetically engineered with a retrovirus harboring the *Escherichia coli* β-galactosidase gene was used to estimate the efficacy of gene delivery and the location of the cells within the kidney. The RMC expressing β-galactosidase, RMCLZ1, was cultured *in vitro* and the cells were injected into the left kidney through the renal artery of a normal Sprague Dawley rat. At least 1×10^6 RMCLZ1 was required for effective gene delivery into glomeruli. One hour and 1, 4, and 14 d after injection, glomeruli were isolated from the left kidneys injected with the cells and the expression of β-galactosidase in each glomeruli was evaluated. One hour and 1 d after injection, more than 90 and 80%, respectively, of glomeruli from the left kidney showed strong β-galactosidase activity, while no activity of β-galactosidase was found in the glomeruli from the right kidneys. The number of glomeruli stained by X-gal and the intensity decreased with time. Fourteen days after injection, about 35% of the glomeruli retained the RMCLZ1. X-gal and periodic acid–Schiff staining of frozen sections obtained 14 d after injection allowed the estimation of the site where the mesangial cells injected were located. The mesangial cells were found mainly in two different locations, the glomerular capillary and the mesangium. The majority (about 90%) of the mesangial cells were located in the glomerular capillary and about 9% of the cells were in the mesangial area. Occasionally, the positive staining was found in proximal tubules and the interlobular artery. Although additional methods are required for the site-specific targeting of the mesangial area, the *ex vivo* gene transfer to glomeruli is feasible and may be a useful tool for future investigations in the pathological mechanisms of glomerular injury.

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Introduction

Glomerulosclerosis characterized by excessive accumulation of extracellular matrix expansion in the mesangium is considered a final common pathway of progressive renal diseases. The progressive mesangial expansion results in the encroachment on neighboring glomerular capillaries with the decrease in filtration surface area and is the main renal structural lesion responsible for the loss of renal function.

During the last decade, various bioactive molecules, including transforming growth factor beta, have been found to mediate glomerulosclerosis mainly from *in vitro* cell culture studies, and most of the studies focused on the alteration of expression of various genes under pathological conditions. To understand the pathophysiology of glomerulosclerosis induced by diabetes mellitus and other causes, however, the evaluation of a specific gene expression and the function of the gene product in glomerulus is required. In this context, gene transfer technology would be a promising method to allow evaluation of the *in vivo* function of a specific gene as well as the value of intervention in diseases.

The first trial of gene transfer into kidney was reported in 1991 (Koseki *et al.,* 1991). Thereafter, various somatic gene delivery systems were examined for the transfer of a target gene into kidney at the experimental level. The main targeting area in kidney for the gene transfer was the glomerulus (Arai *et al.*, 1995; Heikkila *et al.*, 1996), the proximal tubule (Bosch *et al.*, 1993; Lien and Lai, 1997), and the tubular interstitium (Zhu *et al.*, 1996). Both *in vivo* (Bosch *et al.*, 1993; Heikkila *et al.*, 1996; Moullier *et al.*, 1994; Tomita *et al.,* 1992; Zhu *et al.*, 1996) and *ex vivo* gene delivery systems (Kitamura and Sütö 1997; Kitamura *et al.*, 1994; Naito *et al.*, 1996) have been tried to transfer the

reporter or specific genes into kidney. The results from these studies suggested that the *in vivo* gene delivery system may be less effective for specific targeting of glomeruli because glomeruli are small structures and are scattered throughout the renal cortex. Therefore, a gene delivery system more suitable for glomerular targeting is required. To this end, *ex vivo* gene delivery to the mesangial area in the glomerulus using the mesangial cell vector was successful when mesangial proliferation was induced (Kitamura *et al.*, 1994).

We evaluate an *ex vivo* gene transfer system targeting the mesangial area in normal kidney using a renal mesangial cell vector engineered with a retrovirus harboring β-galactosidase.

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), calf serum, fetal bovine serum (FBS), calf serupenicillin G, and streptomycin were purchased from GIBCO Life Technologies (Grand Island, NY), and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal), puromycin, and G418 were obtained from Sigma (Sigma Chemicals, St. Louis, MO). Male Sprague Dawley (SD) rats weighing 250 g were used for the receiver of the RMC expressing β-galactosidase.

Isolation and culture of rat mesangial cells Mesangial cells were obtained from a culture of glomeruli isolated from male SD rats weighing 150–200 g by a sieving method as described previously (Kim *et al.*, 2000). Briefly, isolated glomeruli were incubated in DMEM containing 20% of FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Cells from outgrowth of glomeruli showing a stellate shape and positive immunofluoresence staining for desmin and vimentin were selected as mesangial cells.

Construction of retrovirus LZβPG Retroviral vector LZRS-LacZ(A) (Kinsella and Nolan, 1996) harboring *lac Z* of *E. coli* was kindly provided by Dr. Nolan. The region of pLNCX2 (Clonetec Laboratory Inc., CA) containing the G418 resistant gene and the CMV promoter was amplified by PCR and *Bam* HI and *Hin*d III sites were introduced at the 5′ and 3′ end of the PCR product, respectively. The *Bam* HI and *Hin*d III treated PCR product was introduced at the same restriction sites of LZRS-LacZ(A) and then LZβPG was obtained. In this retroviral vector, the G418 resistant gene and *lacZ* are transcribed by LTR and CMV promoter, respectively.

Production of retrovirus LZβPG Transfection of amphotropic packaging cell PT67 (Clonetec Laboratory, CA) with LZβPG was carried out with FuGene 6 (Boehringer Mannheim) in accordance with the manufacturer's description. After transfection, the cells were incubated at 37°C for 24 h in DMEM containing 10% FBS, L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) without selectable antibiotics. The cells were divided and transferred into five 100-mm culture dishes and then cultured in the same media containing antibiotics, puromycin (3 μg/ml) and G418 (0.5 mg/ml). The media were changed every 3 days. After colony formation, each colony was cloned using a cloning cylinder and amplified in a 60-mm culture dish with the same media until the cells reached 80% confluence. The respective cells were transferred to two 60-mm dishes and cultured under the same condition. When the cells in one dish reached 80%, the medium was removed and X-gal staining was carried out. The cells presenting stronger staining with X-gal were selected and amplified. From the culture, the supernatant was obtained as a viral stock and the viral titer was estimated using NIH3T3 (Kim *et al.*, 1998).

Transduction of rat mesangial cells with LZβPG After the sixth passage, mesangial cells (5×10^5) were plated and cultured for 16 h in a 100-mm culture dish. The cells were infected with retrovirus LZβPG in the presence of 8 μg/ml of polybrene. Selection of transductants was started after 2 d of infection in the same media containing 0.5 mg/ml of G418. After colonies had formed, the respective colony was amplified in a 60-mm culture dish until the cells reached confluence. The cells were divided in two 60-mm culture dishes and then cultured again to confirm whether the cells were expressing β-galactosidase. Cells from one colony presenting the highest β-galactosidase activity were isolated and named RMCLZ1.

Introduction of RMCLZ1 into the kidney The RMCLZ1 cells cultured were trypsinized, washed twice with phosphate buffered saline (pH 7.3), and suspended in 1 ml of serum-free DMEM. The cells were injected into a normal left kidney through the renal artery using a 29-gague needle at a rate of 50 μl/s. To prevent bleeding after injection of the RMCLZ1, the renal artery was constricted with a thread for 5–10 min and then the blood was allowed to re-perfuse.

Estimation of glomerular targeting and localization of RMCLZ1 in the kidney To estimate the glomerular targeting of the RMCLZ1 injected, the glomeruli were isolated separately from both kidneys of the rats 1 h, 1 d, 4 d, and 14 d after injection, and the glomeruli were stained with X-gal to confirm the existence of the cells injected. Fourteen days after injection, frozen sections were prepared from a central area of the kidneys to estimate the location of the RMCLZ1 cell injected. The staining with X-gal of the glomeruli isolated and frozen sections was performed by the methods described by Kitamura *et al.* (1994).

Results

Isolation of rat glomerular mesangial cells Rat mesangial cells were characterized by stellate shape, an ability to grow in medium lacking D-valine, and positive immunofluoresence staining of desmin and vimentin (data not shown). Figure 1A shows the isolated rat mesangial cells after staining with Diff-Quik (International Reagents, Japan).

Production of retrovirus LZβPG Fifty different colonies were cloned and each clone was transferred to a separate 60-mm culture dish. As the cells reached 70% confluence, the respective clone was divided into two

60-mm dishes and cultured again. The cells in one of the two dishes were stained with X-gal and eight different clones were selected by the intensity of β-galactosidase activity. The cells cultured in the counter dish of the selected clones were amplified. The viral titer in the supernatant was estimated using NIH3T3 (Kim *et al.*, 1998). The viral titer was from 1×10^5 to 2×10^6.

Selection of rat mesangial cells expressing β-galactosidase by infection with retrovirus LZβPG Rat mesangial cells were infected with 1×10^5 c.f.u./ml of LZβPG. Two days after infection, the mesangial cells were divided into four 60-mm culture plates and the colonies resistant to G418 were screened. Ten colonies were isolated and the transductant showing the highest β-galactosidase activity was amplified and was named RMCLZ1 (Fig. 1B). The RMCLZ1 was cultured to 80% confluence and was used for injection into the left kidney.

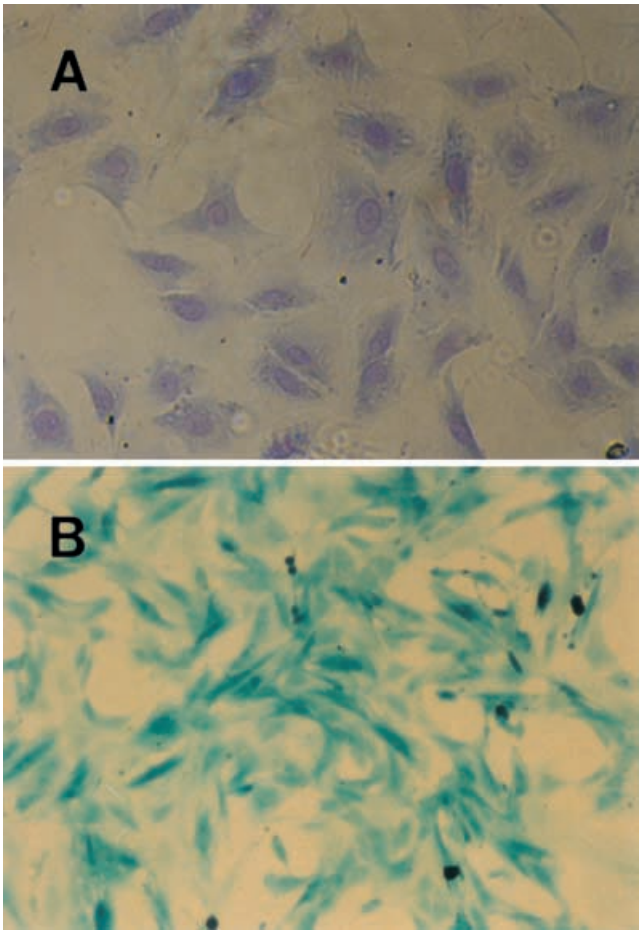

Fig. 1. Rat renal mesangial cells before and after infection with retrovirus LZβPG. Rat renal mesangial cells were isolated as described in **Materials and Methods**, and stained with Diff-Quik (International Reagent, Japan). The cells show stellate shape (**A**). The cells isolated were infected with retrovirus LZβPG and G418 resistant clones were selected. The cells from one colony showing the highest activity of β-galactosidase were obtained and were named RMCLZ1 (**B**).

Introduction of RMCLZ1 into the kidney RMCLZ1 cells were introduced into a normal left kidney by injection through the renal artery. To evaluate the appropriate cell numbers for sufficient gene delivery, 1×10^4, 1×10^5, or 1×10^6 cells respectively, were injected, into three different rats of each group. One day after injection, the rats were sacrificed and glomeruli were isolated from both kidneys and stained with X-gal. When 1×10^4 transductants were injected, no glomeruli presenting β-galactosidase activity were found in 2000 glomeruli selected randomly from three rats. Less than 10% of 2000 glomeruli showed β-galactosidase activity when 1×10^5 cells were injected. On the other hand, 80 ± 8% of 1000 glomeruli from three different rats stained positively with X-gal when 1×10^6 cells were injected (Table 1 and Fig. 2C). These data suggest that at least 1×10^6 cells are required for the efficient delivery of mesangial cells into the kidney.

Estimation of glomerular targeting of the mesangial cell vector To estimate the survival and localization of the RMCLZ1 injected into the kidney, glomeruli isolated from both kidneys were stained with X-gal. Glomeruli isolated from the right kidney served as a control (Fig. 2A). One hour after injection of RMCLZ1 into the left kidney, 91 ± 6% of the glomeruli expressed β-galactosidase activity (Fig. 2B). Four days after injection, about 53 ± 4% of the glomeruli retained β-galactosidase activity (Fig. 2D), and 38 ± 2% of glomeruli showed β-galactosidase activity 14 d after injection (Fig. 2E). The intensity of the color staining became weaker with time after 4 d (Table 2). Fourteen days after injection, frozen sections were stained with X-gal and Periodic acid–Schiff to determine the location of

Table 1. Efficacy of gene delivery to glomerulus according to the number of cells injected.

Number of RMCLZ1 cells injected into the left kidney	Percentage of glomeruli expressing β-galactosidase (N, n)
1×10^4	ND (3, 2×10^3)
1×10^5	6 ± 2% (3, 2×10^3)
1×10^6	80 ± 8% (3, 1×10^3)

ND: no detection.
RMCLZ1 cells were cultured, harvested, and then injected into the left kidneys of normal rats. One day after injection, glomeruli were isolated from the kidneys and the numbers of glomeruli presenting positive staining with X-gal were counted. N represents the number of kidneys injected with RMCLZ1 and n shows the number of total glomeruli isolated from the left kidneys and examined to confirm the β-galactosidase activity.

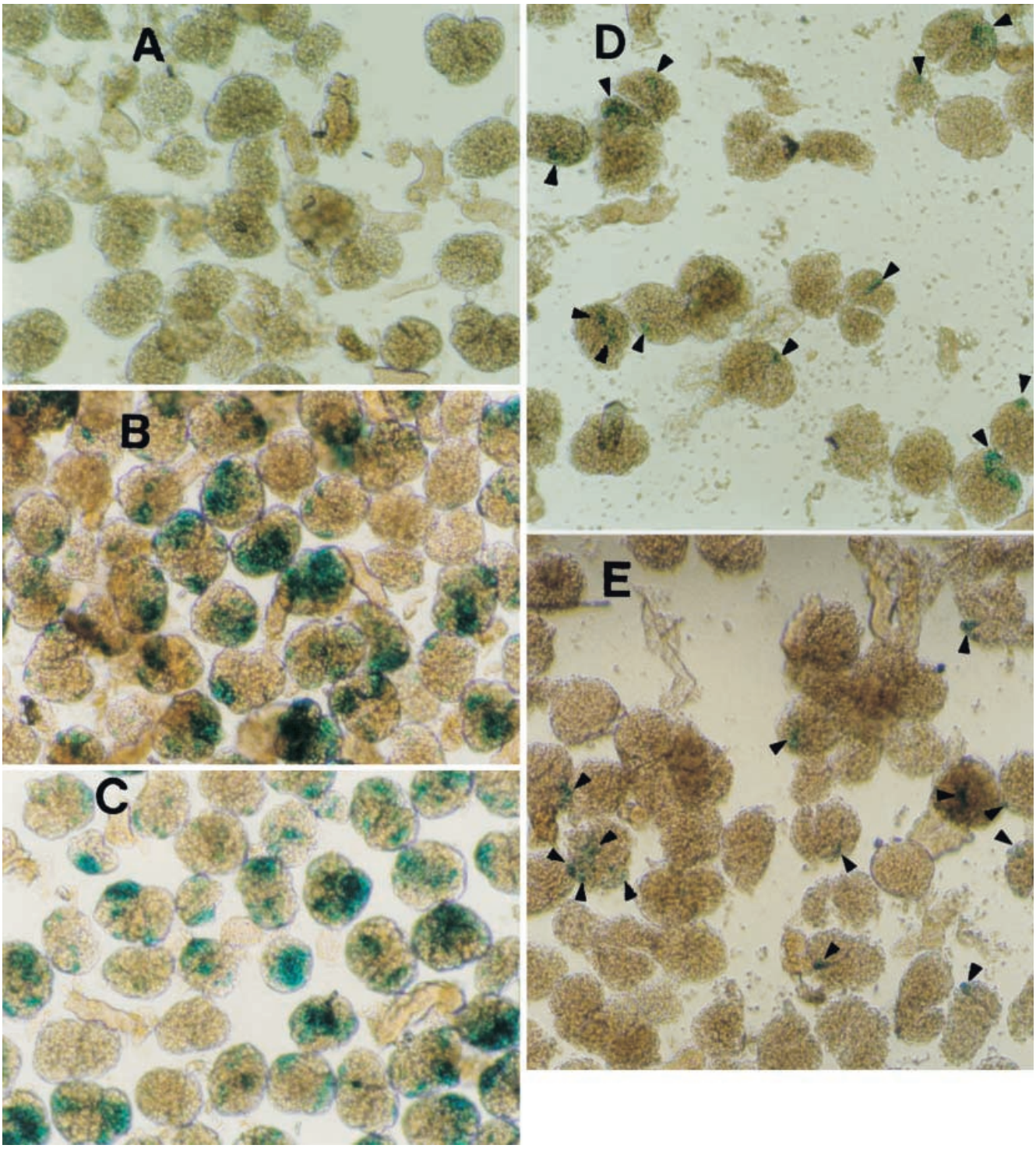

Fig. 2. X-gal staining of glomeruli isolated from kidneys injected with RMCLZ1.The glomeruli were isolated from the right kidney (**A**) as a control and from the left kidney 1 h (**B**), 1 d (**C**), 4 d (**D**) and 14 d (**E**) after RMCLZ1 injection. The glomeruli isolated were stained with X-gal to confirm the existence of RMCLZ1 injected.

the mesangial cells injected. The location of the cells are summarized in Table 3. The RMCLZ1 cells were located in different sites, the mesangium (Fig. 3A), the glomerular capillary (Fig. 3B), proximal tubules (Fig. 3C), and the interlobular artery (Fig. 3D). In almost all findings, the mesangial cells transferred into the kidney were located in the glomerular capillary (89 ± 5%) and in the mesangium (8 ± 2%). Occasionally the X-gal staining was found in proximal tubules.

Table 2. Ratio of glomeruli presenting β-galactosidase activity and intensity of blue color staining by X-gal at the experimental period indicated.

Experimental period (time after injection of RMCLZ1)	Percentage of glomeruli expressing β-galactosidase (N, n)	Intensity of blue color by X-gal staining
1 h	91 ± 6 (3, 5×10^2)	++++
1 d	80 ± 8 (3, 1×10^3)	++++
4 d	53 ± 4 (3, 1×10^3)	+++
14 d	38 ± 2 (3, 1×10^3)	+(+)

At different times after injection, glomeruli were isolated from the left kidneys and the numbers of glomeruli presenting positive staining with X-gal were counted. N represents the number of kidneys injected with RMCLZ1 and n shows the number of total glomeruli isolated from the left kidneys and examined to confirm the β-galactosidase activity. The intensity of the blue color of the positive staining was scored arbitrarily.

Table 3. Location of RMCLZ1 injected in the kidney.

Location	Percentage of cell found (N)
Mesangial area	8 ± 2 %[a] (50)
Glomerular capillary	89 ± 5 %[a] (50)
Proximal tubules	< 0.2%[b] (50)
Interobular artery	< 0.05%[b] (50)

Frozen sections were prepared from left kidneys 14 d after injection of RMCLZ1 and X-gal and periodic acid-Schiff staining were undertaken. The percentage ratio of the cells in the mesangial area and the glomerular capillary was obtained from 2000 glomeruli. N represents the numbers of frozen sections of the left kidneys from three experimental animals.
[a] The percentage means the number of positively stained cells at the indicated location per total positive X-gal staining in the glomeruli.
[b] The percentage means the number of positively stained cells per total number of the cells stained in 50 frozen sections. Four and one positive X-gal stainings in proximal tubules and the renal interobular artery were found, respectively.

Discussion

The evaluation of a specific gene expression and the function of the gene product in the glomerulus is required to understand the precise role of a gene or gene product in the pathophysiology of glomerulosclerosis induced by diabetes mellitus and other causes; therefore, a specific gene transfer to the mesangial area in the glomerulus may be a promising technique to understand the *in vivo* function of the specific gene.

Several methods targeting glomeruli, including *in vivo* or *ex vivo* gene transfer, have been developed during the past years (reviewed by Kitamura, 1997). Since the glomerulus is the end of the renal artery, it was suggested that the renal circulation might be the most reasonable and accessible route of the exogenous gene delivery to the glomerulus. Although *in vivo* gene delivery using a conventional liposome or viral vector was less effective by this access, hemagglutinating virus of Japan (HVJ)-liposome was more efficient in the transfer of the target gene to the glomerulus (Tomita *et al.*, 1992); however, less than 35% of glomeruli were targeted and the duration of the transgene expression was not extended over 1 week. On the other hand, an *ex vivo* trial using a mesangial cell vector was more successful (Kitamura *et al.*, 1994), with the kidneys pretreated with anti-Thy 1 antibody to induce proliferation of mesangial cells. We will repeat our experiment with *ex vivo* gene transfer into normal kidney without any pretreatment to evaluate the efficacy of gene delivery and the potential problems to be solved in a future clinical trial. The kinetics of RMCLZ1 in the glomeruli was very similar to that reported previously (Kitamura *et al.*, 1994); however, almost all cells injected were found in the glomerular capillary (about 90%) rather than in the mesangial area (less than 10%) in our experiment. Even though the duration of our experiment was 2 weeks, Kitamura *et al.* (1994) observed the transgene expression for 4 weeks after injection, suggesting that the efficacy and duration of transgene expression by the *ex vivo* system may be better than that by the *in vivo* system with HVJ-liposome. Pretreatment of anti-Thy 1 antibody leading to masangiolysis followed by transient and specific proliferation of mesangial cell increased not only the number of the mesangial cells expressing a reporter gene but also the duration of transgene expression up to 8 weeks after injection (Kitamura *et al.*, 1994). This suggests that this gene transfer system may be more efficient for mesangial proliferative disorder.

We need to address at least three problems in future studies. The first one is the low efficiency of targeting of the cells to the mesangial area in the normal kidney. Our data showed less than 10% of the cells were located in the mesangial area and others were mainly in the glomerular capillary, suggesting that the endothelial lining of the glomerular capillary functions as a barrier. The application of an infiltration system of immune cells and/or treatment with vascular endothelial growth factor to enhance the permeability of capillary may improve the efficiency. The next problem is the duration of transgene expression. Although transgene expression was longer than that of the HVJ-liposome trial, it was reduced with time. The use of a promoter functioning at the pathological condition rather than a strong viral promoter may be an alternative; however, no promoter has been found for this purpose. The last problem is the removal of the cells injected by the immune system of the recipient. This may not be the most important problem if autologous cells are used for gene transfer because mesangial cells can be isolated and cultured from the biopsy sample of the patient.

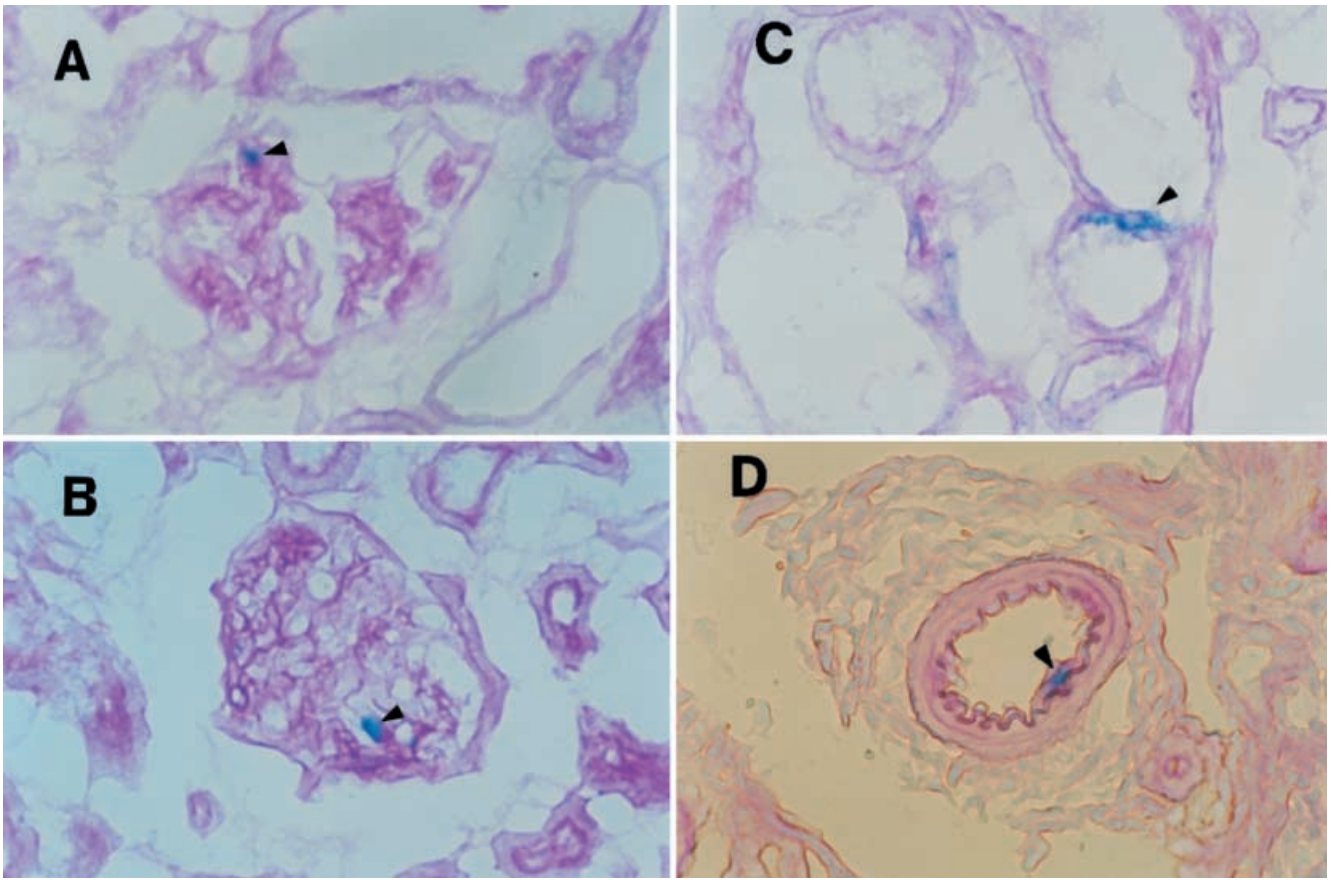

Fig. 3. Location of RMCLZ1 injected kidneys**.** From the rats at 14 d after injection of RMCLZ1, frozen sections of the left kidney were prepared and X-gal and PAS staining were undertaken. The cells were mainly located in the mesangial area (**A**) and on the glomerular capillary (**B**). Occasionally, the cells were found in the tubular interstitial area (**C**) and a few cells were located on the interobular artery (**D**). Arrowheads indicate the cell expressing β-galactosidase.

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