TABLE 2. Clinical Data at the Time of Diagnosis and During Observation: Comparisons According to Each Combined Genotype

	Patients With *ACE II* Genotype			Patients With *ACE ID* or *DD* Genotype		
	GW or *GG* (n=79)	*WW* (n=27)	*P*	*GW* or *GG* (n=127)	*WW* (n=43)	*P*
At time of diagnosis						
Gender, male, %	48.1	59.3	0.3167	44.9	41.9	0.7302
Age, y	37.2±12.8	40.1±15.8	0.3512	36.2±13.6	36.6±12.3	0.8947
SBP, mm Hg	128.3±18.7	133.8±17.4	0.2188	126.6±18.6	127.3±17.8	0.5821
DBP, mm Hg	78.3±13.9	79±12.3	0.6233	76.3±13.2	77.2±14.3	0.4704
UP, g/d	1.5±1.6	1.6±1.3	0.2353	1.3±1.2	1.1±1.2	0.3732
sCr, mg/dL	1.0±0.5	1.2±1.0	0.2474	1.0±0.7	0.9±0.3	0.7276
CCr, mL/min per 1.73 m^2	87.8±38.6	75.1±27.7	0.2288	92.0±31.4	91.0±29.0	0.6924
Hypertension, %	32.9	55.6	0.0370	33.9	34.9	0.9024
During observation						
Observation, mo	99.0±62.1	69.1±45.2	0.0412	107.6±67.6	89.3±73.1	0.0612
Reached end point, %	36.7	48.1	0.2941	27.6	20.9	0.3910
Mean SBP, mm Hg	130.1±17.0	133.7±14.9	0.3433	128.1±17.4	124.5±16.2	0.3327
Mean DBP, mm Hg	79.6±12.1	80.3±10.3	0.6934	76.9±11.6	73.9±12.4	0.2100
Corticosteroids, %	22.9	26.9	0.6783	23.1	32.5	0.2702
ACEI/ARB, %	34.2	48.1	0.1960	45.7	34.9	0.2168

UP indicates urinary protein excretion. All other abbreviations are the same as in text.

carrying the *II* genotype of *ACE*. In patients with other *ACE* genotypes, the *ADD1* polymorphism had no influence on renal survival. There is a possibility that the association between the *ADD1* polymorphism and renal disease progression observed in this study was secondary to its effect on blood pressure regulation, because for patients with the *ACE II* genotype, the frequency of hypertension in the *ADD1 460WW* genotype was higher than that in other genotypes. However, the prognostic effect of *ADD1 460WW* was significant even after adjusting for other clinical risk factors, including urinary protein excretion, hypertension, and no administration of ACEI/ARB. In this study *ACE II* carriers presented with numerically but not significantly higher blood pressures at the time of diagnosis and the lowest frequency of ACEI/ARB administration. Although this study is based on a retrospective observation and we cannot clearly define the reason for these indistinct deviations, the results of the Cox regression model, which takes these variables into account, show an independent prognostic effect of the genetic polymorphism *ADD1 WW* only in patients with the *ACE II* genotype.

To investigate the risk factors for progression of renal dysfunction, this study used a time-to-event approach rather than an analysis of mean slope of renal function, because a substantial proportion of our patients had stable or slowly declining renal function. In fact, only one third of the patients progressed to the end point during the follow-up duration in this study. It has been suggested that an analysis of mean slope of renal function is favored when a negligible proportion of patients have stable or slowly declining renal function.[29]

The present study could not detect any independent effect of the *ACE I/D* polymorphism solely in terms of clinical manifestations and renal prognosis in patients with IgAN. This negative result for the role of the genetic polymorphism of *ACE I/D* alone on renal prognosis is compatible with a previous report in a large-cohort, multicenter trial in Japanese patients with IgAN.[12]

Interestingly and unexpectedly, our data suggest that the *ACE* genotype has an influence on sensitivity to the *ADD1* polymorphism and that the effect of the *ADD1* polymorphism might be enhanced in cases with lower activity of the RAS, because there is general agreement that the *D* allele of *ACE* is associated with increased plasma and tissue levels of ACE. We do not have data to explain the precise molecular mechanism of the observed interaction between the *ADD1* and *ACE* polymorphisms. However, it is not surprising that patients with low RAS activity are more susceptible to the effect of the *ADD1* polymorphism than are those with high RAS activity, because it is well known that RAS has a direct influence on salt sensitivity.[30] Patients with the *ACE D* allele might be resistant to the effect of the *ADD1* polymorphism because of their high salt sensitivity owing to high RAS activity.

Adversely, there is a possibility that patients with low α-adducin activity, which is known to be associated with the *ADD1 460G* allele, would be more susceptible to the influence of the *ACE I/D* polymorphism. Although our study could not confirm this notion, Nicod et al[19] have recently reported, in an unselected renal population, a more rapid progression with the *ACE DD* genotype compared with *ACE ID* and *II* genotypes in patients homozygous for the *ADD1 460G* allele. In their study, although the *ADD1 460WW* genotype was observed in only 10 of 260 patients in their population, they also found that the average time from diagnosis to the onset of ESRD tended to be shorter in the

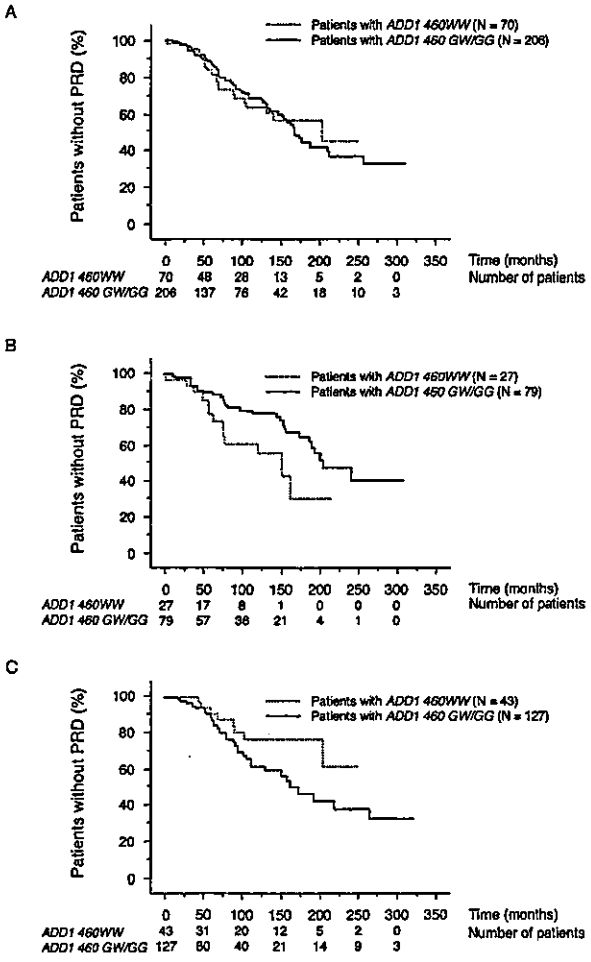

Figure 1. Renal survival rate in IgAN patients with *460WW* and other genotypes of *ADD1* polymorphism. In all patients studied (A), there was no difference between patients with *460WW* (dotted line; n=70) and those with *460GW* or *GG* genotypes (solid line; n=206; Kaplan-Meier, log rank test, χ^2=0.655; *P*=0.9958). Within patients carrying the *ACE II* genotype (B, n=106), however, survival of patients with the *WW* genotype (dotted line; n=27) was significantly worse than in those with other *ADD1* genotypes (solid line; n=79; Kaplan-Meier, log rank test, χ^2=6.062, *P*=0.0138). By contrast, in patients with *ACE ID* or *DD* (C, n=170), patients with *460 WW* (dotted line; n=43) tended to have a better but not significantly better survival curve than those with other *ADD1* genotypes (solid line; n=127; χ^2=2.238, *P*=0.1386). Numbers of available observations for each genotype are indicated. The primary endpoint (PRD; progressive renal disease) was defined as the date when the sCr level was double that at the time of diagnosis or when patients underwent their first renal replacement therapy.

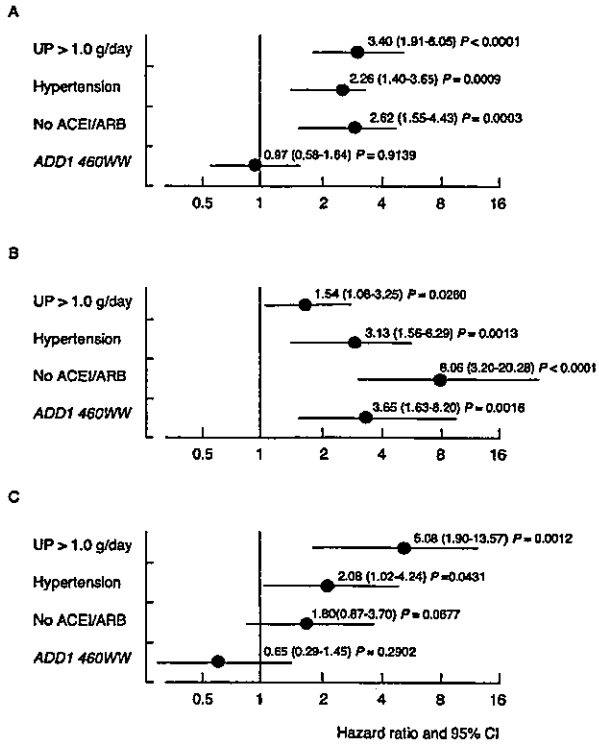

Figure 2. Cox proportional-hazards regression model to test significance of clinical covariates and genotypes of the *ADD1 G460W* polymorphism as predictors of renal survival. The end point was defined as the date when the sCr level was double that at the time of diagnosis or when patients underwent their first renal replacement therapy. Clinical covariates were selected by a stepwise backward method, and analyses were performed in all patients (A, n=276), in patients with the *ACE II* genotype (B, n=106), and in patients with the *ACE ID* or *DD* genotypes (C, n=170). HRs and 95% CIs are indicated. UP indicates urinary protein excretion.

presence of the *ADD1 460WW* than with the *GW* and *GG* genotypes. These partly differing results between our study and those of Nicod et al are likely to be a consequence of the differences in genotype distribution of *ACE I/D* and *ADD1 G460W* polymorphisms between Japanese and whites. It is well known that a polymorphism with a higher allele frequency has more statistical power in an association study.[31] In fact, the frequencies of *ACE I* and *ADD1 460W* alleles in our population were 0.629 and 0.525, respectively, and both were much higher than in Nicod's study (0.525 and 0.185, respectively). Thus, the population in this study might be more suitable to investigate a possible interaction between these 2 loci. Conversely, there is a possibility that our negative data in patients with the *ACE D* allele might be a consequence of its low frequency in the population studied. In addition, the numbers of patients with the combined genotypes shown in Table 2 might be insufficient to draw a conclusion. Therefore, further investigation with a large-scale population of other ethnic origins is needed. This study could not provide data on plasma renin and angiotensin II levels in patients with each genotype. However, RAS is mostly a paracrine and autocrine system, and the plasma level of its constituents poorly reflects its activity, particularly in local kidney tissues.

A limitation of the present study is that we could not make any adjustment for other possible factors that might have had an influence on the prognosis of renal function, such as smoking, dietary factors, insulin sensitivity, and other medications, including 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, fish oil administration, antiplatelet agents, and diuretics. However, the major clinical risk factors established by previous studies were investigated as confounding factors for disease progression. In addition, the reduced sample size over time shown in Figure 1 might

indicate that an even longer observation period and a larger sample size are necessary for confirming the present results. However, overall renal survival was significantly associated with the *ADD1* polymorphism in patients with the *ACE II* genotype, and it was confirmed by the multivariate Cox proportional-hazards regression model.

Perspectives

Although a prospective, randomized, control study with a fixed medication protocol and sufficiently long-term observation is necessary to confirm the present results, this study suggests an interaction between genetic polymorphisms of *ACE I/D* and *ADD1*, not only on blood pressure regulation but also on the progression of renal dysfunction in Japanese patients with IgAN. Despite the fact that the effect of each single polymorphism might not be sufficiently significant, this study supports the notion that interindividual variation in susceptibility to the effect of a single-gene polymorphism of *ADD1 G460W* can be modified by the *ACE I/D* polymorphism. Genotyping both polymorphisms might have provisional importance in Japanese patients with IgAN.

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ORIGINAL ARTICLE

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Association of single-nucleotide polymorphisms in the polymeric immunoglobulin receptor gene with immunoglobulin A nephropathy (IgAN) in Japanese patients

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Abstract Immunoglobulin A nephropathy (IgAN) is a primary glomerulonephritis of common incidence world-wide whose etiology and pathogenesis remain unresolved, although genetic factors are assumed to be involved in the development and progression of this disease. To identify genetic variations that might confer susceptibility to IgAN, we performed a case-control association study involving 389 Japanese IgAN patients and 465 controls. Genome-wide analysis of approximately 80,000 single-nucleotide polymorphisms (SNPs) identified a significant association between IgAN and six SNPs located in the *PIGR* (polymeric immuoglobulin receptor) gene at chromosome 1q31-q41. One of them, PIGR-17, caused an amino-acid substitution from alanine to valine at codon 580 ($\chi^2 = 13.05$, $P = 0.0003$, odds ratio [OR] $= 1.59$, 95% confidence interval [95% CI] $= 1.24$–2.05); the OR of minor homozygotes to others was 2.71 (95% CI = 1.31–5.61). Another SNP, PIGR-2, could affect promoter activity ($\chi^2 = 11.95$, $P = 0.00055$, OR = 1.60, 95% CI = 1.22–

Electronic database information: URLs for the data in this article are as follows: Online Mendelian Inheritance in Man (for IgAN, MIM161950): http://www.ncbi.nlm.nih.gov/Omim/JSNP (for SNPs and primers): http://snp.ims.u-tokyo.ac.jp/

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2.08); the OR of minor homozygotes to others was 2.08 (95% CI = 0.94–4.60). Pairwise analyses demonstrated that all six SNPs were in almost complete linkage disequilibrium. Biopsy specimens from IgAN patients were positively stained by antibody against the secretory component of PIGR, but corresponding tissues from non-IgAN patients were not. Our results suggest that a gene associated with susceptibility to IgAN lies within or close to the *PIGR* gene locus on chromosome 1q in the Japanese population.

Keywords Single-nucleotide polymorphism · IgA nephropathy · Polymeric immunoglobulin receptor

Introduction

Immunoglobulin A nephropathy (IgAN; MIM161950), characterized by the deposition of immunoglobulin A at the mesangium of glomeruli (Berger and Hinglasis 1968) is the most common form of glomerulonephritis worldwide and is a major cause of end-stage renal disease. Surveys of patients with primary glomerulonephritis conducted in 1985 and 1993 by the Research Group on Progressive Renal Diseases in Japan revealed a high prevalence of IgAN and relatively poor prognosis for patients (Koyama et al. 1997). The rate of survival for the 502 cases of IgAN followed in that study, for which renal-related death was the end-point, was 61% at 20 years from the time when renal dysfunction was first detected. Strong evidence of a role for genetic factors in the development and progression of IgAN has come from descriptive reports of familial aggregation and from analyses of affected sib-pairs and parent-child pairs from multiple ethnic groups (Hsu et al. 2000). Sporadic IgAN is difficult to evaluate, however, because it probably involves a combination of various genes and diverse environmental factors (Schena 1998). We chose therefore to look for potential IgAN-susceptibility genes that might correlate with the development and/or progression of this disease.

Polymeric immunoglobulin receptor (PIGR) is an integral membrane protein on the basolateral surface of secretory epithelial cells. It mediates the transport of polymeric immunoglobulins (pIg) across epithelia, particularly dimeric IgA or polymeric IgM (Brandtzaeg and Prydz 1984). Expression of *PIGR* mRNA can be detected in most human secretory epithelia, e.g., in the intestine, bronchus, salivary glands, renal tubule, and uterus (Krajci et al. 1989). PIGR neutralizes extracellular and intracellular pathogens in mucous membranes by transporting dimeric IgA-pathogen complexes across epithelia and then excreting them via epithelial transcytosis by means of a domain known as the secretory component (SC), although the restriction site of PIGR that binds to the pIg-pathogen is not clarified completely (Mostov et al. 1984). *PIGR*-knockout mice develop increased levels of serum IgA because of the interruption of the trans-epithelial transport of dimeric IgA (Shimada et al. 1999). On the basis of these findings, one may ascribe the primary abnormalities of IgAN to the impairment of PIGR-mediated transport of IgA; therefore, PIGR represents a potential candidate for the mediation of IgA binding to mesangial cells.

Recent approaches to the discovery of disease-susceptibility genes have focused on the identification of single-nucleotide polymorphisms (SNPs) in the human genome. SNPs are likely to be useful for the identification of genes involved in complex diseases through association studies and, as such, serve as markers for various genetic analyses (Kruglyak 1999). Because SNPs are the most frequent type of genetic variation in human DNA, we have screened gene-based SNPs on a genome-wide scale to detect possible associations with susceptibility to IgAN. Earlier, we reported a case-control association study that identified SNPs in the L- and E-selectin genes on chromosome 1q24–25 as potential susceptibility factors for IgAN in the Japanese population (Takei et al. 2002). Furthermore, we reported that the HLA-DRA locus on chromosome 6p was a susceptibility gene for IgAN in the Japanese population (Akiyama et al. 2002). For the present work, we designed a case-control association study with approximately 80,000 SNPs found on genome-wide scans and estimated haplotypes that might serve to identify SNPs on chromosome 1q31-q41 that might be responsible for IgAN phenotypes in the Japanese population.

Materials and methods

Materials

IgAN in our study population was diagnosed by renal biopsy as a mesangial proliferative glomerulonephritis with predominant IgA and C3 depositions in the mesangium. All biopsies were performed after 1976 at the following surgical centers in Japan: Department of Urology, Iwate Medical University; Department of Urology, Sanai Hospital; Department of Urology, Iwate Prefectural Ofunato Hospital; Department of Medicine, Kidney Center, Tokyo Women's Medical University; Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences. Patients with Henoch-Schonlein purpura or secondary IgAN as liver cirrhosis were excluded from analysis. Peripheral blood samples were obtained from 389 IgAN patients (222 females and 167 males, mean age: 38.5 ± 14.4 years) for extraction of DNA. The mean value of serum creatinine at the time of renal biopsy was 0.98 ± 0.33 mg/dl; the mean value of 24-h proteinuria at the time of renal biopsy was 0.89 ± 1.01 g/day. As controls, we analyzed DNA from 465 healthy Japanese volunteers (278 females and 187 males, mean age: 54.4 ± 14.5 years). These controls were randomly selected; none showed hematuria, proteinuria, or renal dysfunction. Written informed consent was obtained from all participants, and DNA was prepared from each blood sample according to standard protocols.

Screening of SNPs

SNPs in *PIGR* gene were screened according to methods described previously (Iida et al. 2001). Information about each SNP that we

have discovered can be obtained from the Japanese SNP Web site (JSNP) at http://snp.ims.u-tokyo.ac.jp/.

Multiplexed polymerase chain reaction

We amplified multiple genomic fragments by using 20 ng genomic DNA for each polymerase chain reaction (PCR), as described previously (Ohnishi et al. 2001). Sequences of the primers used in this study are available at JSNP (http://snp.ims.u-tokyo.ac.jp/). Each PCR was performed in a 20-μl solution containing 50 pmol each primer, 10 U Ex-*Taq* DNA polymerase (TaKaRa Shuzo, Tokyo, Japan), and 0.55 μg *Taq*Start (Clontech Laboratories, Palo Alto, Calif., USA) in a GeneAmp PCR system 9700 (PE Applied Biosystems). Initial denaturation was at 94°C for 2 min, followed by 37 cycles of amplification at 94°C for 15 s and annealing at 60°C for 45 s, with final extension for 2 min at 72°C.

Genotyping

We genotyped all participants for a total of 21 SNPs present in the *PIGR* gene (PIGR 1–21; Fig. 1) by means of the Invader assay, which combines a structure-specific cleavage enzyme with a universal fluorescent resonance energy transfer (FRET) system (Mein et al. 2000). FRET probes were labeled with either FAM or VIC corresponding to each allele. Signal intensity was indicated as the ratio of FAM or VIC to ROX, an internal reference. Each total reaction volume of 5 μl contained 0.25 μl signal buffer, 0.25 μl each FRET probe, 0.25 μl structure-specific cleavage enzyme, 0.5 μl allele-specific probe mix, and 2 μl PCR product diluted 1:10. Samples were incubated in a GeneAmp PCR system 9700 (95°C for 5 min and then at 63°C for 15 min) and were analyzed on an ABI Prism 7700 sequence detector.

Immunohistochemistry for SC and IgA

Tissues from renal biopsies were fixed in 10% formalin, routinely processed, and embedded in paraffin. Paraffin sections (3 μm thick) were immunostained for SC and IgA. The avidin-biotinylated-peroxidase complex method was performed by using a Histofine SAB-PO KIT (Nichirei, Tokyo, Japan). Briefly, the sections were de-paraffinized in xylene and soaked in absolute methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. After being washed in phosphate-buffered saline for 20 min, the sections were incubated with antibodies overnight. The primary antibodies consisted of rabbit anti-human secretory component (1:200 dilution, DAKO, Glostrup, Denmark) and rabbit anti-IgA (1:50 dilution, Lip Sandon-Lipshow, Detroit, Mich.). The sections were treated with biotinylated anti-rabbit immunoglobulin for 30 min at room temperature and then allowed to react with peroxidase-labeled avidin for 30 min. All sections were rinsed with TRIS-buffered saline after each step. After incubation of the sections in 0.05 mol/l TRIS-HCl buffer (pH 7.6) containing 0.005% 3–3-diaminobenzidine, nuclei were stained with hematoxylin.

Statistical analysis

Genotype distributions and allele frequencies of each selected SNP were compared between cases and controls by means of the χ^2 test. Significance was judged according to the guidelines of Lander and Kruglyak (1995). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Woolf's method. Hardy-Weinberg equilibrium of alleles at individual loci was assessed by χ^2 statistics (Nielsen et al. 1998). Linkage disequilibrium (LD) coefficients were calculated and expressed as $D' = D/D\max$ (Devlin and Risch 1995).

Results

We performed a case-control association study by genotyping approximately 80,000 SNPs scattered throughout the genome. When the *P*-value in the statistical analysis was less than 0.01, we increased the number of IgA cases. The distributions of genotypes that we observed in IgAN patients and controls did not differ from the expected frequencies under the assumption of Hardy-Weinberg equilibrium (data not shown). However, comparison of allelic frequencies in IgAN patients versus controls disclosed a significant association of the disease with one SNP present in the *PIGR* gene (PIGR-17 in exon 7). In view of the strong association found at this locus, we screened for additional SNPs within the *PIGR* gene.

Among the 20 new SNPs that we discovered within the *PIGR* gene, we found significant association of five more of them with the IgAN phenotype: PIGR-2 in the 5′ flanking (promoter) region, PIGR-5 in intron 1, PIGR-9 in exon 4, PIGR-13 in intron 4, and PIGR-19 in intron 10 (Table 1). The most significant associations with IgAN at single SNP loci were observed for PIGR-9, PIGR-13, and PIGR-17; these three SNPs were in complete LD. The C-to-T polymorphism of PIGR-17 substitutes valine for alanine at codon 580 of PIGR, and the frequency of the minor allele (T) of PIGR-17 was significantly higher in patients with IgAN than in con-

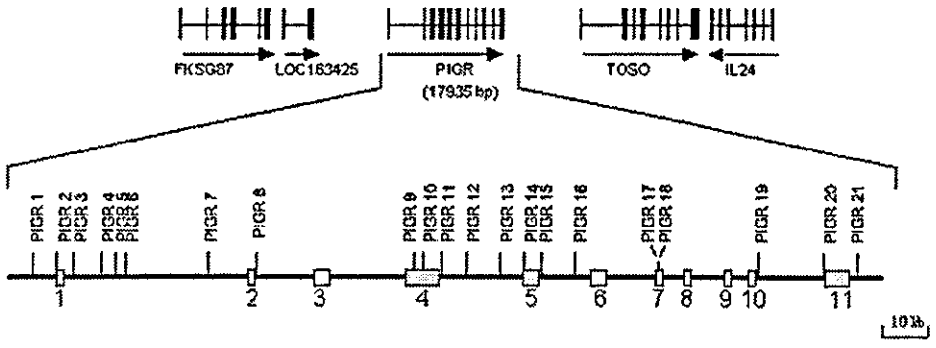

Fig. 1 Genomic organization of the human polymeric immunoglobulin receptor (*PIGR*) gene at chromosome 1q31–41 (*rectangles* exons, *horizontal lines* introns). Locations of the 21 SNPs identified in this study are indicated *above* the gene

Table 1 Genotype data and association tests of SNPs on the *PIGR* gene

	PIGR-2	PIGR-5	PIGR-9	PIGR-13	PIGR 17	PIGR-19
SNP information						
Location	5' flanking	Intron 1	Exon 4	Intron 4	Exon 7	Intron 10
Position	−46	1259	549	1015	1740	29
Genetic variation	G → T	C → T	A → G	T → C	C → T	A → G
Substitution			Val 183 Val		Ala 580 Val	
IgAN						
Major allele [%]	635 [81.62]	635 [81.62]	617 [79.31]	617 [79.31]	617 [79.31]	623 [80.08]
Minor allele [%]	143 [18.38]	143 [18.38]	161 [20.69]	161 [20.69]	161 [20.69]	155 [19.92]
Total	778 [100.00]	778 [100.00]	778 [100.00]	778 [100.00]	778 [100.00]	778 [100.00]
Major homozygous [%]	263 [67.61]	263 [67.61]	252 [64.78]	252 [64.78]	252 [64.78]	256 [65.81]
Heterozygous [%]	109 [28.02]	109 [28.02]	113 [29.05]	113 [29.05]	113 [29.05]	111 [28.53]
Minor Homozygous [%]	17 [4.37]	17 [4.37]	24 [6.17]	24 [6.17]	24 [6.17]	22 [5.66]
Total	389 [100.00]	389 [100.00]	389 [100.00]	389 [100.00]	389 [100.00]	389 [100.00]
Controls						
Major allele [%]	815 [87.63]	815 [87.63]	799 [85.91]	799 [85.91]	799 [85.91]	802 [86.24]
Minor allele [%]	115 [12.37]	115 [12.37]	131 [14.09]	131 [14.09]	131 [14.09]	128 [13.76]
Total	930 [100.00]	930 [100.00]	930 [100.00]	930 [100.00]	930 [100.00]	930 [100.00]
Major homozygous [%]	360 [77.42]	360 [77.42]	345 [74.19]	345 [74.19]	345 [74.19]	347 [74.62]
Heterozygous [%]	95 [20.43]	95 [20.43]	109 [23.44]	109 [23.44]	109 [23.44]	108 [23.23]
Minor homozygous [%]	10 [2.15]	10 [2.15]	11 [2.37]	11 [2.37]	11 [2.37]	10 [2.15]
Total	465 [100.00]	465 [100.00]	465 [100.00]	465 [100.00]	465 [100.00]	465 [100.00]
χ^2 [*P*]						
Genotype frequency (2×3 table)	11.20 [0.00369]	11.20 [0.00369]	12.73 [0.00172]	12.73 [0.00172]	12.73 [0.00172]	11.60 [0.00302]
Allele frequency (major vs minor)	11.95 [0.00055]	11.95 [0.00055]	13.95 [0.00030]	13.95 [0.00030]	13.95 [0.00030]	11.63 [0.00065]
Major homozygous vs others	10.33 [0.00131]	10.33 [0.00131]	8.92 [0.00282]	8.92 [0.00282]	8.92 [0.00282]	7.93 [0.00487]
Minor Homozygous vs. Others	3.41 [0.06486]	3.41 [0.06486]	7.80 [0.00523]	7.80 [0.00523]	7.80 [0.00523]	7.21 [0.00723]
Odds ratio [95% CI]:						
Major homozygous vs others	0.61 [0.45–0.82]	0.61 [0.45–0.82]	0.64 [0.48–0.86]	0.64 [0.48–0.86]	0.64 [0.48–0.86]	0.65 [0.49–0.88]
Minor homozygous vs others	2.08 [0.94–4.60]	2.08 [0.94–4.60]	2.71 [1.31–5.61]	2.71 [1.31–5.61]	2.71 [1.31–5.61]	2.73 [1.28–5.83]
Minor allele vs minor allele	1.60 [1.22–2.08]	1.60 [1.22–2.08]	1.59 [1.24–2.05]	1.59 [1.24–2.05]	1.59 [1.24–2.05]	1.56 [1.21–2.01]

Table 2 Linkage disequilibrium (LD) coefficients between 6 SNPs in *PIGR* gene. LD coefficients were calculated and expressed as D' = D/Dmax (Devlin and Risch 1995)

	PIGR-5	PIGR-9	PIGR-13	PIGR 17	PIGR-19
PIGR-2	1.000	0.901	0.901	0.901	0.947
PIGR-5		0.901	0.901	0.901	0.947
PIGR-9			1.000	1.000	0.949
PIGR-13				1.000	0.949
PIGR-17					0.949
PIGR-19					

trols (case: 20.7% vs controls: 14.1%, $\chi^2 = 13.05$, $P = 0.00030$). The OR for patients with IgAN versus controls was estimated to be 1.59 (95% CI = 1.24–2.05) for the minor allele versus major allele.

The PIGR-2 SNP (G → T) in the 5' flanking region could affect promoter activity. The frequency of the minor T allele at the PIGR-2 locus was significantly higher in patients with IgAN than in controls (case: 18.4% vs controls: 12.4%, $\chi^2 = 11.95$, $P = 0.00055$). The OR for patients with IgAN versus controls was 1.60 (95% CI = 1.22–2.08) for the minor allele versus the major allele. Pair-wise LD between each pair of all six SNPs was calculated on the basis of case and control subjects. LD coefficients among the six SNPs indicated quasi-complete LD (D' > 0.9; Table 2).

Further analysis indicated that individuals having the TT genotype (homozygotes for the minor allele) of PIGR-17 were likely to have a higher risk of IgAN because the OR of the genotype (TT vs CC or CT) was as high as 2.71, with a 95% CI of 1.31–5.61. We compared clinical data between patients with the TT genotype and those with the CC or CT genotypes but found no significant differences with respect to age, sex, levels of serum IgA or serum creatinine, or 24-h urinary excretion of protein at the time of renal biopsy (Table 3).

Finally, we performed immuohistological staining of polymeric immunoglobulin receptor in biopsy specimens in order to investigate a potential role of this molecule in the pathogenesis of IgAN. When sections of normal colonic mucosa were used as a positive control, strong positive staining for SC was observed in the mucosa of the colon. Although no staining was observed in any of the glomeruli in the normal kidney, positive staining was seen for SC and IgA at the mesangial area of glomeruli in patients with IgAN (Fig. 2). When we examined immunostaining for SC on a total of nine speciemens (about three of each genotype), SC deposition were detected in all nine speciemens (100%). However, no significant correlation for staining of SC was determined with genotyping.

Discussion

We have demonstrated here a significant association of six SNPs in the *PIGR* gene with IgA nephropathy. IgAN is a complex disorder whose etiology involves immuno-

Table 3 Clinical and biological variables at the time of renal biopsy. Results are given as mean ± SD (*NS* not significant)

	All patients	PIGR-17 (CC+CT)	PIGR-17 (TT)	*P*-value
No. of patients	389	365	24	
Age in years	38.5 ± 14.4	37.9 ± 14.7	42.3 ± 6.7	NS
Gender (M/F)	167/222	158/207	9/15	NS
Serum IgA (mg/dl)	338.6 ± 261.9	342.8 ± 273.0	346.5 ± 156.1	NS
Serum creatinine (mg/dl)	0.98 ± 0.33	0.98 ± 0.33	1.07 ± 0.41	NS
24-h Proteinuria (g/dl)	0.89 ± 1.01	0.88 ± 1.01	1.14 ± 1.30	NS

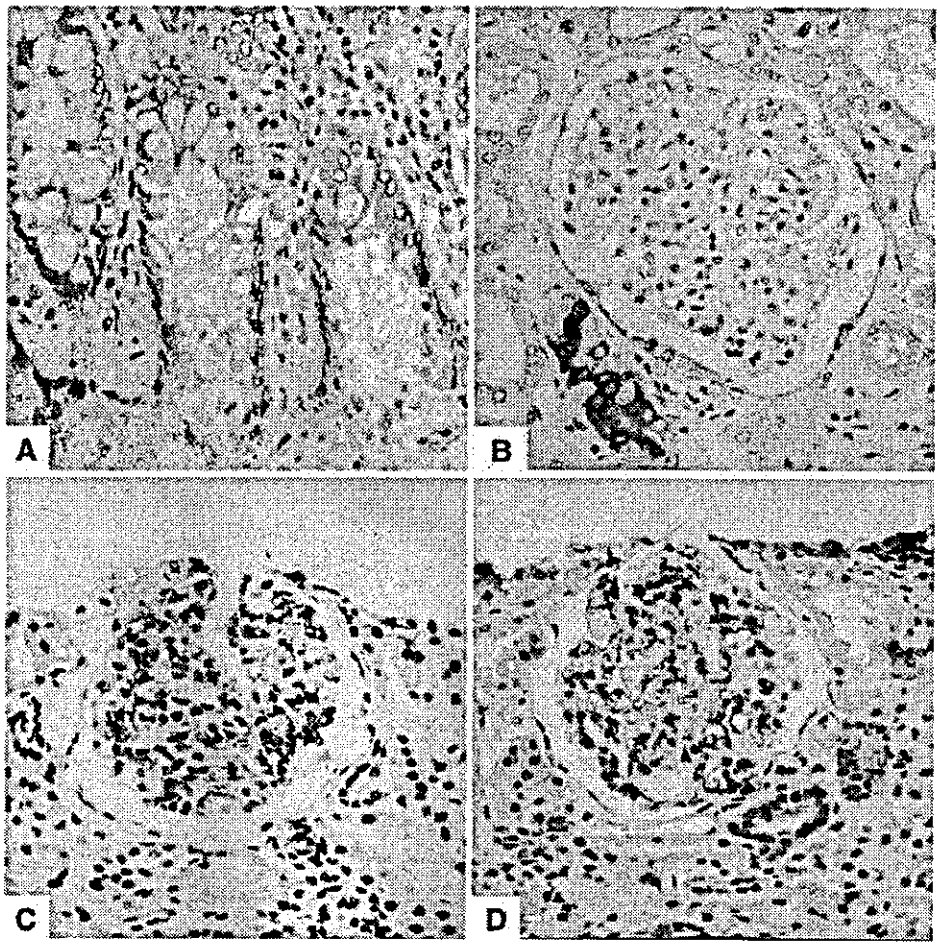

Fig. 2A–D Immunohistochemical localization of IgA and secretory component in biopsy specimens of patients with IgAN and control tissues obtained from patients who had undergone colectomy because of colon cancer or nephrectomy because of renal cell carcinoma. Staining for secretory component (**D**) was carried out on the section next to that stained for IgA (**C**). **A** Positve control: colonic mucosa. **B** Negative control: normal kidney. **C** Anti-IgA antibody. **D** Anti-secretory component. ×250

logical, environmental, and genetic factors (Hsu et al. 2000). Gharavi et al. (2000) have reported a genome-wide analysis of familial cases, but the great majority of patients with IgAN have no apparent familial history. Hence, most of the genetic investigations reported to date on IgAN have been association studies that have searched for susceptibility genes in a given population. Our study is the first to report a whole-genome association study; this appears to have been an efficient approach for identifying major susceptibility genes for this disease in the Japanese population.

We found significant association between IgA nephropathy and homozygotes for minor alleles at six SNP loci in the *PIGR* gene: PIGR-2, PIGR-5, PIGR-9, PIGR-13, PIGR-17, and PIGR-19. These showed strong LD with each other. Among them, PIGR-17, which substitutes valine for alanine at codon 580, and PIGR-2, which is located in the promoter region, are likely to influence the quality or the quantity of the gene product. Haplotype analyses for SNPs that may affect gene products often increase the significance of associations; however, since the two SNP loci in question showed only quasi-complete LD (data not shown), analyses of this particular two-locus haplotype (PIGR-2 and PIGR-17) yielded no increase of significance in comparison with our single-locus tests.

Increases in serum IgA levels have been observed in 50%–70% of IgAN patients (Lopez-Trascasa et al. 1980). Elevated serum IgA, particularly when associated with mucosal infections, such as respiratory pathogens and dietary components, suggest that mucosal immunity might play a critical role in the pathogenesis of this disease (Suzuki et al. 1990). Activation of the mucosal immune system presumably increases the production of polymeric IgA (pIgA) in the bone marrow (De Fijter et al. 1996). Indeed, up-regulation of pIgA synthesis in the bone marrow and an increase of pIgA-producing plasma cells have been described in patients with IgAN (Harper et al. 1996; Van den Wall Bake et al. 1988).

PIGR encodes a type I transmembrane protein with five immunoglobulin superfamily-homology domains constituting its N-terminal extracellular region, a single membrane-spanning region, and a short cytoplasmic C-terminal tail. The extracellular ligand-binding portion (from amino acid 550 to amino acid 764 in human PIGR), known as the secretory component (SC), is proteolytically cleaved from the apical surface of epithelial cells and released together with polymeric immunoglobulins such as dimeric IgA or tetrameric IgM. Association with SC is believed to protect IgA from degradation by bacterial proteases (Kilian et al. 1983). In the present study, we found a significant increase in the frequency of valine alleles at codon 580 in IgAN patients and assume that 580V-PIGR affects the binding of pIgA-PIGR, decreases the cleavage and production of SC, and then elevates the serum IgA level. However, since we discerned no relationship between this polymorphism and serum IgA levels, we speculate that the mechanism of serum IgA elevation in IgAN patients is likely to be complex and cannot be explained by the alteration of a single factor.

Although functional studies with biochemical and cellular-biological techniques will be required to clarify the relationship between the genetic variations reported here and IgAN, the results of our genetic investigation suggest that an allele conferring susceptibility to this disease lies within or close to the *PIGR* locus on chromosome 1q31–41.

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RESEARCH ARTICLE

Gene transfer into the liver by plasmid injection into the portal vein combined with electroporation

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Abstract

Background We investigated the optimum conditions for gene transfer into the liver by injection of plasmid via the portal vein combined with electroporation.

Methods In anesthetized 7-week-old male Shionogi Wistar rats, a tapered 3Fr. catheter was inserted through a branch of the colonic vein and its tip was secured into the portal vein. After clamping the vessels around the right and caudal lobes of the liver, FITC-oligodeoxynucleotide (ODN), green fluorescence protein (GFP) plasmid, or pCAGGS-luciferase was injected into the right and caudal lobes during electroporation using electric pulses applied to the caudal lobe only.

Results Transfected cells were identified by FITC-ODN and GFP fluorescence, and transfection efficacy was estimated by measurement of luciferase activity. FITC-ODN and GFP were detected in the caudal lobes of the liver. FITC-ODN was particularly present in hepatocytes surrounding the Glisson capsule and central veins, while GFP was detected in only hepatocytes surrounding the Glisson capsule. Luciferase activity in the liver was dependent on the plasmid concentration and voltage of the electric pulse, but independent of the volume of plasmid solution. Luciferase activity was limited in the right lobe, in which plasmid solution was injected but no electroporation was applied.

Conclusions Our results showed local expression of the induced gene at the electroporation site, suggesting it is potentially useful for liver function support during recovery after extensive liver surgery and for the treatment of chronic and/or diffuse liver diseases. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords electroporation; *in vivo*; local gene therapy; rat liver

Introduction

Gene transfer can be efficiently induced by techniques using various viruses [1,2] but such methods carry various risks, such as insertional mutagenesis, chemokine induction, and immune response [3]. On the other hand, nonviral methods of gene transfer carry less risk and are more advantageous at present for clinical usage. Several groups have already reported the use of nonviral methods for gene transfer [4–8]. Especially in electroporation-mediated gene transfer, the introduced gene is locally expressed in the target organ, such as skin [4], muscle [8], and liver [5]. We have previously examined the efficacy of gene transfer into the muscle by

electroporation *in vivo* [8] in several injury models, with respect to organ preservation, stimulation of regeneration, and prolongation of survival rate [9,10]. Furthermore, we have also established a new procedure for direct gene transfer into the kidney by electroporation *in vivo* [11], which showed a higher efficacy than the hemagglutinating virus of Japan (HVJ) liposome (nonviral) method.

In the liver, several groups have applied direct gene transfer using *in vivo* electroporation combined with injection of plasmid by liver puncture [5,12,13]. This combination of local DNA injection and *in vivo* electroporation resulted in highly efficient gene expression; however, the gene expression was observed in a limited area of the liver around the site of injected DNA. To apply this simple gene transfer technique to support liver function after major tissue loss such as following extensive liver surgery, the genes should be expressed diffusely throughout the liver. Zhang *et al.* [6] produced diffuse gene expression in the liver by injecting plasmid into the liver through afferent and efferent blood vessels. However, for such effect, large amounts of plasmid solution need to be injected under high perfusion pressure. Since liver injury could easily occur as a result of high perfusion pressure, the above technique should be avoided [14]. Electroporation might be more advantageous with regard to the transfection efficacy in cases with low perfusion pressure, and results in diffuse expression of the gene at the site of electroporation in the target organ.

In the present study, we describe a modification of the method of gene transfer into the liver by injecting plasmid via the portal vein combined with electroporation.

Materials and methods

Animals

Male Shionogi-Wister rats (WS; Shionogi Aburabi Laboratory, Shiga, Japan, 6 weeks old) weighing 150–170 g were used in the following experiments. The rats were kept in a temperature-controlled room with a 12-12 hour light-dark cycle and acclimated for at least 7 days before experiments. All animals had free access to water and standard laboratory diet (Oriental Yeast Co., Tokyo, Japan). All procedures were performed under pentobarbital anesthesia, and all experiments were carried out in compliance with the guidelines on the care and use of laboratory animals of Osaka University.

Operative procedure and electroporation *in vivo*

After mobilization of the liver, we inserted a catheter (tapered 3Fr., ATOM intravenous catheter; ATOM Medical Co., Tokyo, Japan) through a branch of the colonic vein, and its tip was secured in the portal vein. After clamping the portal vein, saline was injected through the catheter to wash out blood cells. Next, we clamped the inferior and superior vena cavae, and the Glisson capsule between the branches of the middle and left lobes and right and caudal lobes (Figure 1). Immediately after vascular occlusion, the plasmid solution was injected very slowly through the catheter while electrodes (a pair of stainless steel with round pincers 10 mm in diameter) were applied to the caudal lobe at a distance of 4–5 mm. Electric pulses were delivered to the liver by a pulse generator (square electroporator CUY 21; Tokiwascience, Japan). We used a square pulse of constant voltage (see below) throughout electroporation. Three pulses of a preselected voltage followed by three more pulses of the same voltage but opposite polarity were applied at each injection site at 1 pulse/s and a pulse duration of 100 ms.

FITC-labeled oligodeoxynucleotides

The 20-base-long phosphorothionate oligodeoxynucleotides (ODNs) labeled with FITC (FITC-ODN) at the 5′ end (5′-FITC-CCGCGTGATGATCTCCTCCG-3′) were purchased from Gliner (Tokyo, Japan). The ODNs were deprotected on the column, dried, resuspended in TE buffer, and quantified by spectrophotometry.

Confocal laser scanning microscopy

To identify the cells into which FITC-ODN had been incorporated, we used a confocal laser scanning microscope (LSM410; Carl Zeiss, Germany) to examine the lobe in which the plasmid had been injected. We used the left lateral lobe as control. The transfected caudal

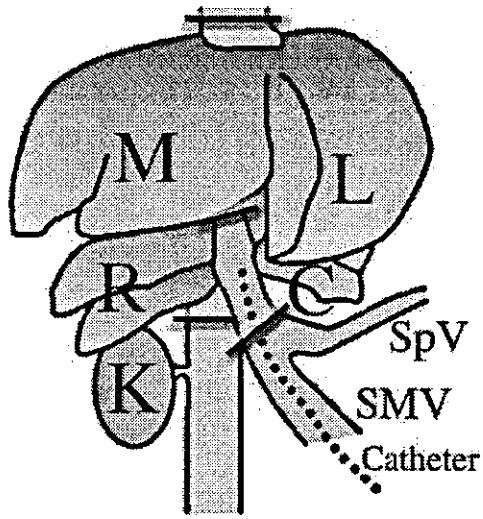

Figure 1. Sites of vascular occlusion during plasmid injection and electroporation. The locations of vascular clamps are shown as bold solid lines. Inserted catheter is shown as a dotted line. The tip of the catheter was positioned in the portal vein, and plasmid solution was injected into the right and caudal lobes. R, right lobe; M, middle lobe; L, left lateral lobe; C, caudal lobe; K, right kidney; SMV, superior mesenteric vein; SpV, splenic vein

lobes of the livers of five rats were dissected out, 10 min after *in vivo* electroporation using 50 V applied during injection of 100 μg/ml of 1.0 ml FITC-ODN. The resected lobes were embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IL, USA) and frozen in liquid nitrogen. Serial sections (6 μm) were cut with a cryostat and placed on slide glasses. A confocal laser scanning microscopy system (CLSM) equipped with an argon-ion laser (LSM410; Zeiss, Germany) was employed in this study. The images acquired by the photomultiplier tube detector were averaged eight times in real time to reduce noise, and the average was digitized to 256 gray levels using an analog-to-digital converter. Images of 512 × 512 pixels were employed. All images were prepared at the same contrast and brightness under the same magnification.

Plasmid DNA

Green fluorescent protein (GFP) plasmid was obtained commercially (Wako Pure Chemical Industries, Osaka, Japan) [10]. *pCAGGS-luc* plasmid, which was constructed by cloning luciferase into *pCAGGS* [8,11], which contains a CMV enhancer and beta-actin promoter, was kindly provided by Dr. Isaka (Osaka University Graduate School of Medicine, Osaka, Japan). Plasmids were grown in *Escherichia coli* DH5alfa strain and extracted by a Concert™ high-purity plasmid Maxiprep system (GIBCO™, Invitrogen Corporation). DNA was dissolved in TE buffer, and its quantity and quality were assessed by measuring the optical density at 260 and 280 nm.

Fluorescence microscopy of GFP

To identify the cells expressing the transferred gene, we examined the resected specimen and frozen section of the liver lobe injected with GFP by fluorescence stereoscopic microscopy and fluorescence microscopy. We examined the transfected caudal lobes of livers obtained from five rats in each group, 4 days after *in vivo* 50-V electroporation combined with injection of 100 μg/ml of 1.0 ml GFP plasmid. *PCAGGS*-transfected liver was used as control. The resected lobes were examined by fluorescence stereoscopic microscopy after embedding in OCT compound and frozen in liquid nitrogen. Serial sections (8 μm) were cut with a cryostat and placed on slide glasses. We also examined the sections by fluorescence microscopy. Under the same magnification, all images were exposed using the same exposure time. For estimation of the transfection efficacy, GFP-positive areas, including vascular areas, were analyzed using MacSCOPE software version 2.5.6 (Mitani Corporation). For each slide, we randomly selected five sections, each 1,278 × 996 pixels in area, using a fluorescence microscope at a magnification of ×100. The GFP-positive area was expressed as a percentage of the total area [(GFP positive area/total area) × 100].

Luciferase assay

Five rats in each group were examined for liver luciferase activity, several days after transfection. Firstly, rats were sacrificed on day 4 after gene transfer using different conditions for gene transfer. Secondly, rats were sacrificed on day 1, 2, 4, 6, 10, or 14 after gene transfer following 75-V electroporation combined with injection of 1.0 ml of 100 μg/ml *pCAGGS-luc*, to examine the time course of luciferase activity. Finally, we obtained the right liver lobe, middle liver lobe, small intestine, pre-tibial muscle, kidney, heart, lung, brain, and bone marrow, to determine the gene expression at other organs apart from the electroporated caudal lobes of the liver, on day 1 or 4 after injection of 1.0 ml of 200 μg/ml *pCAGGS-luc* into the right and caudal lobe via the portal vein under electric pulses of 75 V. The protocol used for the processing of tissue homogenates for luciferase assay system was similar to that described previously [15]. Briefly, the transfected liver lobe was dissected out under anesthesia, cut by forceps into small pieces, and stored at −80 °C until luciferase assay. The liver tissue were treated with 0.7 ml of 1× luciferase cell culture lysis buffer (Promega, Tokyo, Japan), re-stored at −80 °C, and centrifuged at 12 000 rpm for 2 min at 4 °C after dissolution. The supernatant of the lysate was used for luciferase assay and determination of protein concentration. Luciferase activity was measured using a luciferase assay system (Promega) in a luminometer (Lumat LB9501; Berthold, Wildbad, Germany). The enzyme activity was expressed relative to liver protein concentration, which was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) [16]. The data were expressed as the corrected luciferase activity (luciferase activity/protein concentration) for each individual transfected liver. Experiments were repeated at least three times.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Statview J software (version 5.0). Student's t-test was used for comparisons of luciferase activity, and Spearman's correlation coefficient by rank was used to examine the correlation between various gene transfer conditions and luciferase activities. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Oligonucleotides transfer in the liver using FITC-oligo

To determine the possible transfer of a foreign oligonucleotide into the liver, we selectively infused FITC-ODN into the caudal and right lobes of the normal rat livers

via portal vein after clamping other vessels before infusion (Figure 1), combined with 50-V electroporation of the caudal lobes. Although the electrodes used for electroporation did not cover the entire surface of the caudal lobe, FITC-ODN was distributed diffusely in almost all hepatocytes and fibroblasts around the Glisson capsule including the edge of the liver (Figure 2B). On the other hand, no fluorescence was detected in the left lateral lobe (Figure 2A). FITC-ODN accumulated in the nuclei of cells around the Glisson capsule (Figure 2C) as well as those around the central veins (Figure 2D).

GFP expression in the liver by direct electroporation

To confirm the expression after gene transfer, we used GFP plasmid as positive control, and pCAGGS plasmid as negative control (Figure 3). A weak green fluorescence was detected in the background of the whole liver specimen when examined under a fluorescent microscope in negative control. In positive control, GFP expression was noted especially in the Glisson capsule cells and the surrounding hepatocytes (P), but a lower expression was noted in cells around the central veins (V). The GFP-positive area was $12.6 \pm 4.9\%$ of the total area.

Effects of electroporation conditions on gene expression

To determine the optimal parameters for gene transfer, including the electric field intensity, we perfused the right and caudal lobes of the liver with various volumes of various concentrations of luciferase reporter gene, *pCAGGS-luc*, and then only the caudal lobe was electroporated using various voltages. First, we determined the optimal plasmid concentration using the same volume of plasmid and electroporation voltage (1.0 ml and 75 V). On day 4, luciferase activity increased with increased plasmid concentration and reached a plateau at 200 μg/ml ($1.73 \pm 0.87 \times 10^3$, $7.85 \pm 2.69 \times 10^3$, $10.5 \pm 0.12 \times 10^3$, and $31.0 \pm 0.84 \times 10^3$ RLU/mg protein at 25, 50, 100, and 200 μg/ml, respectively). No further increase was noted in luciferase activity when plasmid concentration was increased from 200 to 400 μg/ml ($25.0 \pm 1.10 \times 10^3$ at 400 μg/ml, $P = 0.36$; Figure 4A). We also analyzed

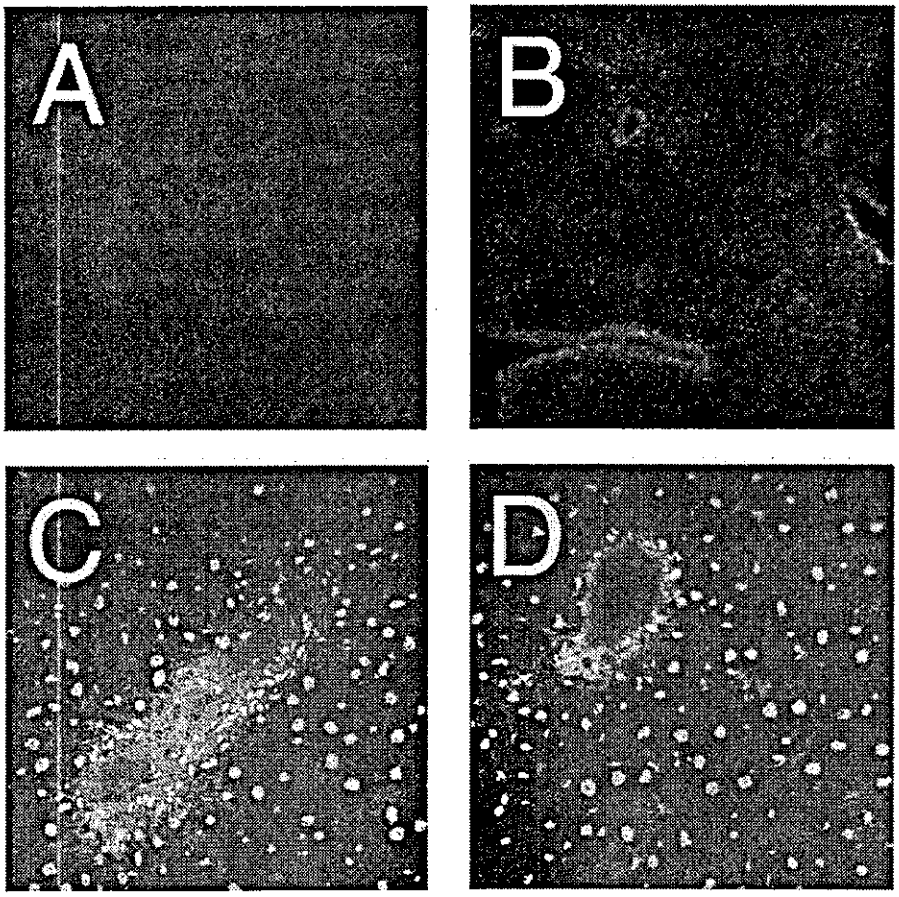

Figure 2. Representative confocal laser scanning micrographs of FITC-labeled oligodeoxynucleotide (FITC-ODN)-transfected liver lobes. Note the accumulation of FITC-ODN mainly in the nuclei of fibroblasts and hepatocytes around the vessels (B, C, D). No fluorescence was observed in the left lateral liver lobe (A). Magnification: ×100 for A and B; ×400 for C and D

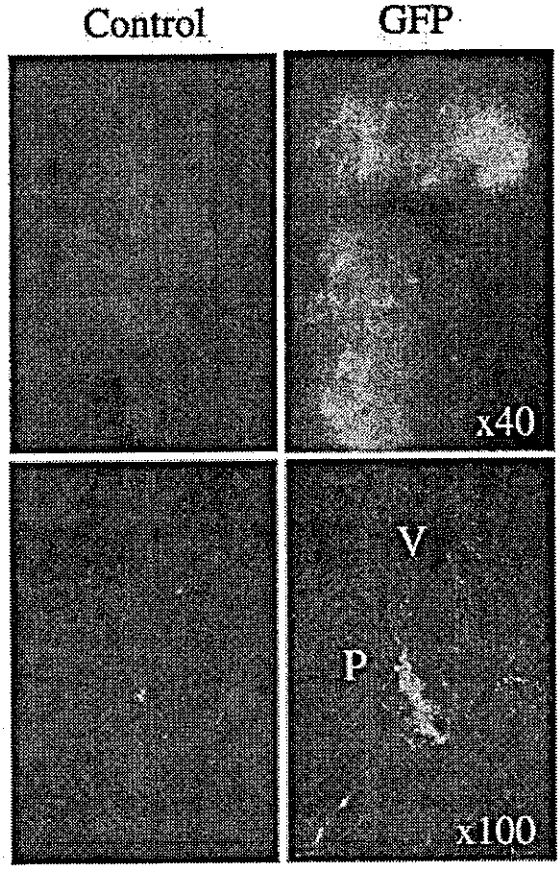

Figure 3. Microscopic findings after gene transfer of *GFP* gene with electroporation. Top: liver specimen examined by fluorescence stereoscopic microscopy (×40). Bottom: 8-μm thick frozen section examined by fluorescence microscopy (×100). The liver specimens were resected 4 days after GFP transfection and *pCAGGS* gene transfection as control. P, portal triad; V, central vein

the relationship between luciferase activity and plasmid concentration. Luciferase activities correlated significantly with plasmid concentration between 25 and 200 μg/ml (luciferase activity vs. plasmid concentration $r = 0.60$, $P < 0.01$).

We then determined the optimal volume of plasmid using the same plasmid concentration and electroporation voltage (100 μg/ml and 75 V). On day 4, no difference was noted in luciferase activity when plasmid was injected at 0.5, 1.0, or 1.5 ml (Figure 4B). However, luciferase activity was significantly lower when 2.0 ml of plasmid were injected, compared with other volumes ($P < 0.01$; Figure 4B). Finally, we determined the optimal electric field intensity using constant plasmid volume and concentration (100 μg/ml and 1.0 ml). On day 4, luciferase activity increased with increased applied voltage from 50 to 100 V (Figure 4C), and decreased between 100 and 125 V (Figure 4C, $P < 0.01$). We also analyzed the relationship between luciferase activity and voltage. However, luciferase activity did not correlate with the applied voltage (luciferase activity vs. voltage, $r = 0.48$, $P = 0.67$). Burn injuries were noted on the liver surface at electrode locations, especially when 100 and 125 V were used.

Time course of luciferase activities

We also evaluated the time course of changes in luciferase activity following direct gene transfer into the liver via *in vivo* electroporation on days 1, 2, 4, 6, 10, and 14. In these experiments, 1.0 ml of 100 μg/ml *pCAGGS-luc* was injected into the right and caudal lobes, and only the caudal lobe was electroporated using 75 V. Luciferase activity reached a peak level on day 1, and decreased gradually with time (Figure 5).

Luciferase activity in other organs

These studies confirmed the lack of gene expression in other organs apart from the electroporated caudal lobe. In these experiments, 1.0 ml of 200 μg/ml *pCAGGS-luc* plasmid was injected into the right and caudal lobes via the portal vein, with 75-V electroporation applied to the caudal lobe only. Luciferase activity was estimated on days 1 and 4 in the caudal lobe, right lobe, middle lobe, small intestine, pre-tibial muscle, kidney, heart, lung, brain, and bone marrow (Figure 6). Luciferase activities in organs other than the liver were <0.1% of the electroporated caudal lobe on both days 1 and 4. In right liver lobe injected with plasmid only (no electroporation), luciferase activity was also <0.1% of that in the caudal lobe, although it was significantly higher than the control (caudal lobe in *pCAGGS*-transfected rats) on day 1 (right lobe vs. control on day 1, $3.30 \pm 2.20 \times 10^2$, $5.6 \pm 2.2 \times 10^1$, $P < 0.05$). In the middle lobe, where no *pCAGGS-luc* was injected, luciferase activity was also higher than the control on day 1 (middle lobe vs. control on day 1, $1.17 \pm 0.13 \times 10^2$, $5.6 \pm 2.2 \times 10^1$, $P < 0.05$).

Discussion

In the present study, we first identified the site of the gene transferred by electroporation *in vivo*. Our results showed diffuse distribution of FITC-ODN in the liver, especially in hepatocytes of the Glisson capsule and around the central veins. GFP was also expressed diffusely in the liver, especially in hepatocytes surrounding the Glisson capsule, but not those around the central veins. Comparison of FITC-ODN and GFP expression sites showed that cells around the central veins expressed FITC-ODN but not GFP. This could be due to the lesser effects of low perfusion pressure and/or dilution of the plasmid solution around the central veins compared to the area around the Glisson capsule.

Previous studies showed that not all hepatocytes in the liver could be transfected by electroporation and gene injection; only approximately 5–35% of hepatocytes

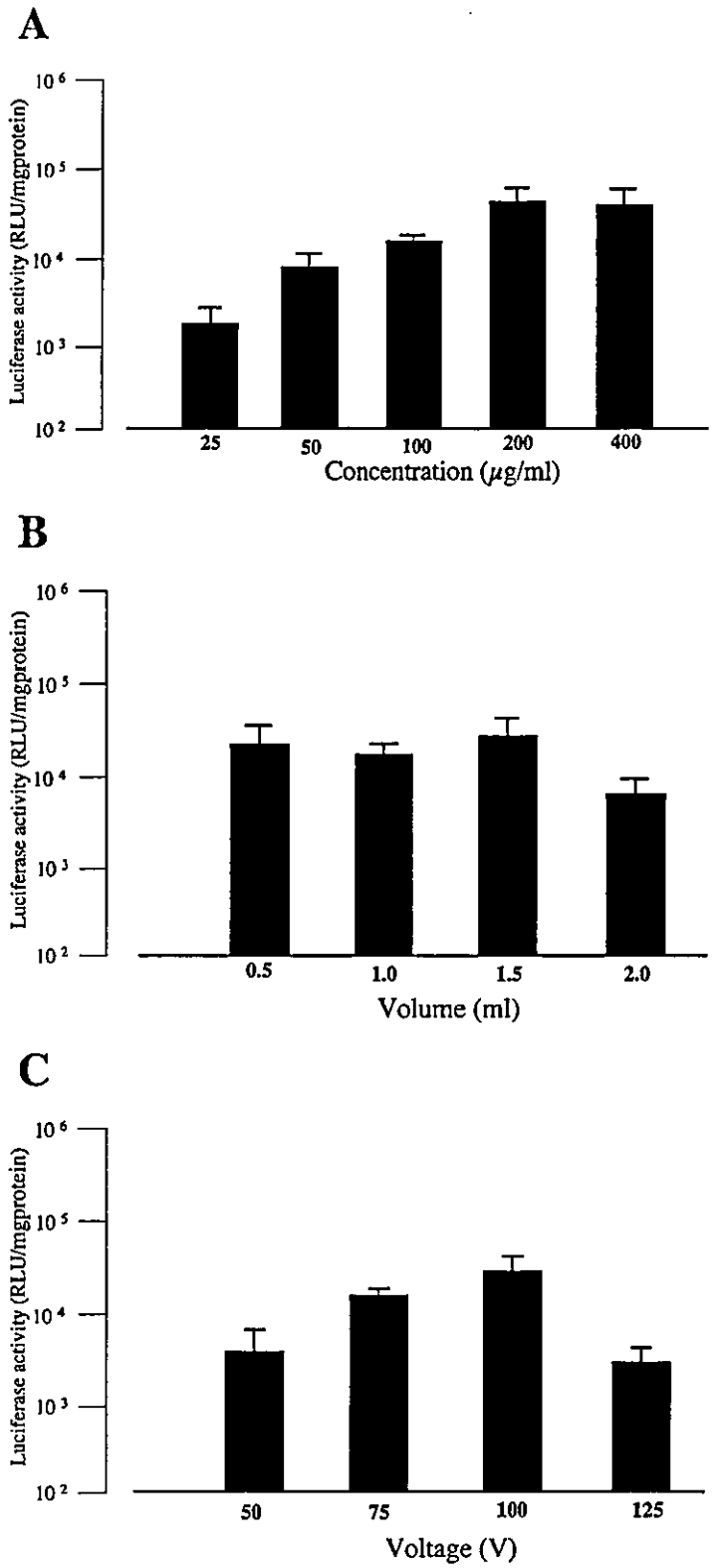

Figure 4. Effects of electroporation conditions on luciferase activity. Liver specimens were obtained on day 4 after *pCAGGS-luc* plasmid transfer combined with electroporation. (A) Luciferase activity at various concentrations of the plasmid. The voltage of the electric pulse and volume of plasmid were constant (75 V, 1.0 ml, respectively). (B) Luciferase activity at various volumes of the plasmid. The voltage of the electric pulse and concentration of plasmid were constant (75 V, 100 μg/ml, respectively). (C) Luciferase activity at various voltages. The concentration and volume of plasmid were constant (100 μg/ml, 1.0 ml, respectively). Each value represents the mean luciferase activity ± SD of five rats

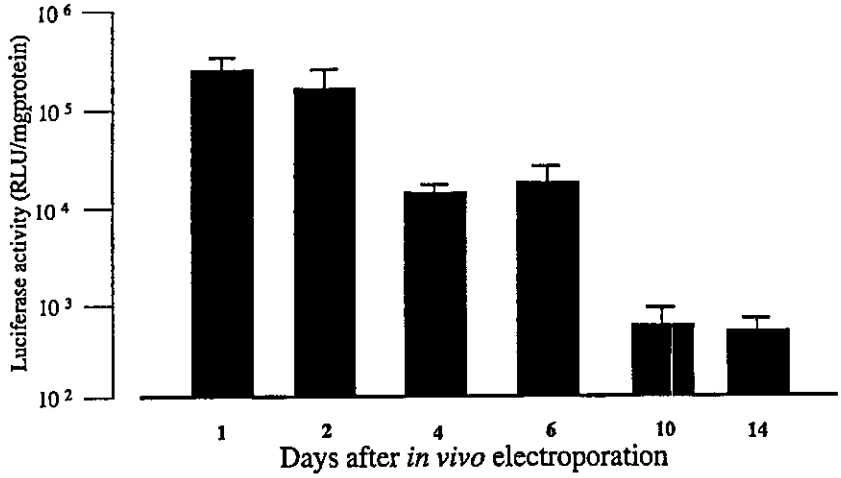

Figure 5. Time course of luciferase activity after transfer of *pCAGGS-luc* by *in vivo* electroporation. The *pCAGGS-luc* plasmid (1.0 ml of 100 μg/ml) was injected into the caudal and right lobes of the liver, and an electric pulse of 75 V was delivered to the caudal lobe. Each value represents the mean luciferase activity ± SD of five rats

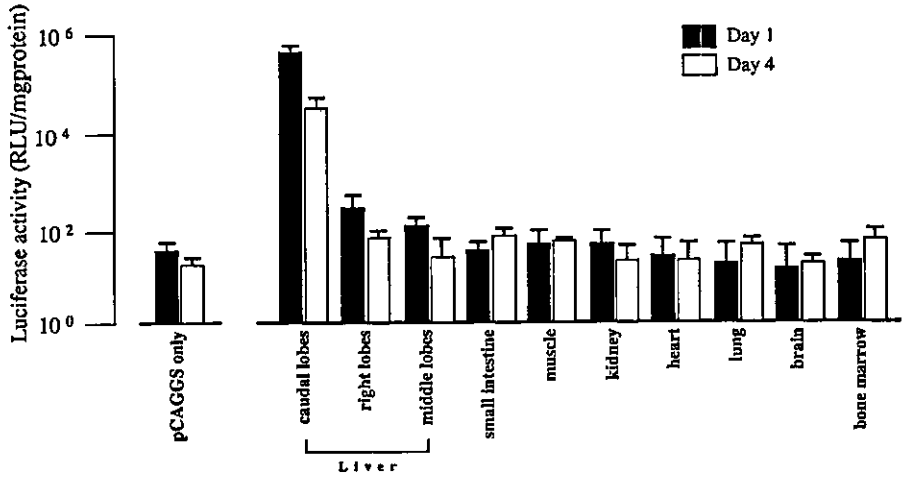

Figure 6. Luciferase activity in other organs. The *pCAGGS* or *pCAGGS-luc* plasmid was injected (1.0 ml of 200 μg/ml) into the caudal and right lobes of the liver, and an electric pulse of 75 V was delivered to the caudal lobe. Liver specimens were then obtained on days 1 and 4. Control luciferase activity was determined in the caudal lobe of *pCAGGS*-transfected rats. Each value represents the mean luciferase activity ± SD of five rats

were transfected, as demonstrated by lac Z staining [5,6]. Thus, it is difficult to achieve 100% efficacy using electroporation. When the excretory-type protein is expressed by the gene, such as hepatocyte growth factor, it is more likely to be adequate physiologically, because such proteins are secreted by the cells in the Glisson capsule, not hepatocytes [17]. Thus, these methods are likely to introduce high concentrations of the protein especially around the Glisson capsule, and such a result seems adequate compared with transfection of all hepatocytes.

In gene transfer to the liver, the use of small quantities of the plasmid, low portal pressure, and low electroporation voltage are desirable. In the present study, we investigated the efficacy of the gene transfer method by measuring luciferase activity. Our results regarding the importance of plasmid concentration and electroporation voltage in the gene transfection are in agreement with those of previous studies [5,12]. The results showed that plasmid volume, which depends on the injection pressure in a closed space, did not correlate with luciferase activity, a finding different from those of previous studies [6,18]. In our study, the liver lobes were perfectly filled with 500 μl of plasmid solution, and the transfected liver lobes expressed adequate luciferase activity under these conditions and approximately the same activity was noted when a larger amount of plasmid solution was used. Under various conditions, luciferase activity might not correlate with the injection pressure, i.e., gene transfection into the liver could be achieved using a lower injection pressure.

Although our results showed adequate transfection into the liver by *in vivo* electroporation for the secretion-type protein and that the highest luciferase activity was noted within 24 h after gene transfer, expression of the gene decreased within 2 weeks to levels less than the control,

reflected by luciferase activity. This finding indicates that while our method provides beneficial results in cases with acute injury of the liver, the efficacy of the gene is transient and limited to the period preceding recovery from injury. In chronic liver injury, there might be a need to perform the gene transfer procedure more than once.

In our experimental model, we confirmed the efficacy of expressing two different genes under the same conditions; with and without electroporation. Plasmid solution was injected through the portal vein into the right and caudal lobes, and electric pulse was only applied to the caudal lobe. Our results also showed that transfection was possible in the right lobe by gene injection and by a combination of gene injection and electroporation in the caudal lobe. Our results also demonstrated that electroporation *in vivo* resulted in 100–1000-fold increase in luciferase activity compared with gene injection alone. Our observations of lack of effects of portal pressure level and low luciferase activity associated with plasmid injection without electroporation emphasize the important effect of electroporation on gene transfection into cells and at the same time less importance on local pressure of plasmid solution [19].

Efficient gene transfer and diffuse liver expression of the transferred gene injected via the portal vein (without requiring high injection pressure) combined with electroporation is an attractive approach for treatment of chronic liver diseases, diffuse liver diseases, and especially liver function support during the recovery period after extensive liver surgery.

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RESEARCH ARTICLE

Electroporation-mediated *ex vivo* gene transfer into graft not requiring injection pressure in orthotopic liver transplantation

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Abstract

Background We investigated optimum conditions for *ex vivo* gene transfer into liver grafts by plasmid injection via the portal vein combined with electroporation in rat liver transplantation.

Methods Anesthetized 9-week-old male Shionogi-Wistar rats were used as donors and recipients. After harvest of the liver graft from the donor rat, a tapered 3Fr. catheter was inserted into the portal vein of the liver graft *ex vivo*. After clamping the afferent vessels around the right and caudal liver lobes, *pCAGGS-luciferase*, which was diluted with one of several osmotic pressure solutions, or *pCAGGS-green fluorescence protein* (GFP) plasmid was injected into these lobes to keep the efferent vessels patent. Electrical pulses were applied to the liver graft during cold preservation in lactated Ringer's solution, University of Wisconsin solution, and histidine-tryptophan-ketoglutarate solution.

Results Transfection efficacy was estimated by measurement of luciferase activity. Luciferase activity in the liver was dependent on both the voltage and electric current of the electrical pulse, and also on the type of preservation solution and plasmid osmotic pressure. Luciferase activity was noted only in plasmid-injected lobes of the liver graft. GFP-transfected cells were identified by GFP fluorescence. GFP was observed predominantly in perivascular cells, including hepatocytes.

Conclusions We have demonstrated successful *ex vivo* gene transfection into liver grafts without injection pressure by using a non-viral method.

Keywords electroporation; *ex vivo*; liver transplantation; rat liver

Introduction

Several attempts have been made to induce gene expression in liver grafts during liver transplantation using viral vectors such as adenovirus and retrovirus, with the aim of preventing rejection and enhancing liver regeneration [1,2]. However, these methods have many associated risks, such as insertional mutagenesis, chemokine induction, and induction of immune responses [3]. This is particularly a concern during transplantation, when recipients usually require immunosuppression for prevention of graft rejection, and as yet there is no irrefutable evidence that viral vectors cannot

affect transplant recipients. The risks that non-viral gene transfer methods would pose are also still unknown. However, non-viral gene transfer methods have been investigated and developed for clinical use [4,5], because they seem to have lower risks to the host compared with viral vectors.

Several groups have attempted electroporation-mediated gene transfer *in vivo* into target organs, such as skin, kidney, muscle, and liver [6–10]. This method permits control of the transfected gene expression in the target organ by change of the electroporation site. For application of electroporation-mediated gene transfer to transplantation grafts, electroporation could be performed *ex vivo* to the graft only, and thus gene expression could be limited to the transplantation graft.

In the present study, we developed a new experimental model involving *ex vivo* gene transfer into liver grafts using electroporation during rat orthotopic liver transplantation. We determined the optimal conditions for this procedure in the context of two problems that could occur during gene transfer by electroporation: firstly to reduce loss of electrical intensity in the graft, and secondly to introduce-plasmids, independent of the pressure gradient around the plasmid injection site.

Materials and methods

Animals

Male Shionogi-Wistar rats (WS; Shionogi Aburabi Laboratory, Shiga, Japan, 9-week-old, RT1k) weighing 300–350 g were used as donors and recipients in the following experiments. The rats were kept in a temperature-controlled room with a 12/12-h light/dark cycle and acclimatized for at least 7 days before the experiments. All animals had free access to water and a standard laboratory diet (Oriental Yeast Co., Tokyo, Japan). All procedures were performed under pentobarbital anesthesia, and all experiments were carried out in compliance with guidelines on the care and use of laboratory animals of Osaka University.

Syngeneic liver transplantation

Orthotopic syngeneic liver transplantation (OSLT) was performed between WS rats bred in the same environment of the same age and weight using the Kamada cuff technique [11] with some modifications [12]. The hepatic artery was not reconstructed. After death of the liver donor by exsanguination, the liver grafts were flushed with chilled normal saline solution until the venous effluent became clear.

Gene injection into the liver graft

A cuff was set on the portal vein and infra-hepatic vena cava of the liver graft in saline at 4 °C, and then the portal triad between left and middle liver lobes and caudal and right liver lobes was clamped. We inserted a catheter (tapered 3Fr., ATOM intravenous catheter; ATOM Medical Co., Tokyo, Japan) into the portal vein and clamped the end of the portal vein (Figure 1). Plasmid solutions (1 ml) were injected into the liver graft very slowly through the catheter and all clamps were removed after injection. All procedures were performed at 4 °C in saline solution.

Ex vivo electroporation

Electrical pulses were delivered to the liver by a pulse generator (Square electroporator CUY 21; Tokiwascience, Japan), which administers 0.00–4.00 A actual electric current and 1–500 V actual voltage. For safety reasons, the electric current was limited to under 4.0 A between 0–125 V and under 1.8 A between 125–500 V by a pulse generator. We used a square pulse of constant voltage (see below) throughout electroporation. Six pulses of a preselected voltage were applied at 1 pulse/s with a pulse duration of 100 ms.

We constructed an *ex vivo* electroporator with two parallel 6 × 2 cm stainless steel electrodes (Figure 2) for gene induction into liver grafts. The distance between the electrodes was 6 cm. The internal volume of the electroporator was 150 ml, and 80 ml solution perfectly covered the liver graft. We performed all experiments at 4 °C in 80 ml of electroporation solution.

After injection of plasmid solution and removal of all clamps, we placed the liver graft into the electroporator at 4 °C. After confirmation that the solution covered the liver graft and that the graft did not touch the electrodes, electric pulses were administered to the graft by the electroporator. Immediately after electroporation, the liver graft was removed from the electroporator, and stored at 4 °C in saline before transplantation. All procedures were performed on ice, and, after

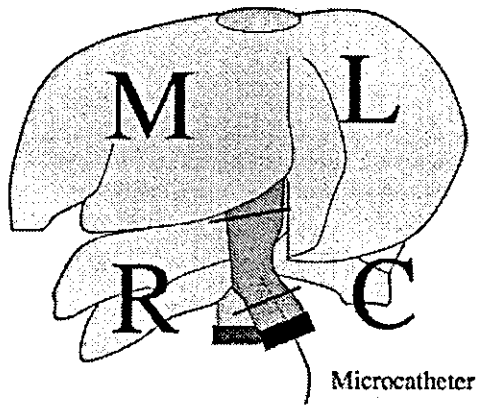

Figure 1. Sites of vascular occlusion during plasmid injection. The locations of vascular clamps are shown as bold solid lines. The inserted catheter is shown as a solid line. The tip of the catheter was positioned in the portal vein, and plasmid solution was injected into the right and caudal lobes. R, right lobe; M, middle lobe; L, left lateral lobe; C, caudal lobe