Genomic DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes with the QuickGene DNA whole blood kit (Fujifilm, Tokyo, Japan), according to the manufacturer's instructions. Primers for polymerase chain reaction (PCR) amplification of *FOXP3* gene coding, promoter, 3′-untranslated region (3′UTR) and polyadenylation region sequences were designed as previously described [4].

FOXP3 expression analysis

Mononuclear cells (MNCs) were isolated from heparinized blood by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, Inc., St. Louis, MO, USA). FOXP3 expression was analyzed by flow cytometry according to the protocol described previously [5, 6]. Briefly, fresh MNCs were washed in phosphate-buffered saline (PBS) solution, fixed in 1 ml of PBS with 1% paraformaldehyde and 0.05% Tween-20, and kept overnight at 4°C. The cells were treated twice with 0.5 ml of DNAse 100 Kunitz units/ml according to the manufacturer's instructions (Sigma-Aldrich). The cells were incubated with a murine anti-human-FOXP3 monoclonal antibody (mAb) (clone150D/E4), previously described [5], for 1 h at room temperature, washed with fluorescence activated cell sorting (FACS) buffer (PBS, 3.0% fetal calf serum, 0.5% Tween-20, and 0.05% azide). FOXP3 staining was detected with Alexa Fluor-488 goat anti-mouse-IgG antibody (Molecular Probes; Invitrogen, Eugene, OR, USA) and washed as described above. Cell surface staining was then performed with PC5-conjugated anti-human-CD4 (Immunotech, Marseilles, France) and phycoerythrin (PE)-conjugated anti-human-CD25 (Miltenyi Biotec, Bergisch Gladbach, Germany) mAbs for 20 min at room temperature. Cells were analyzed with a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

Results

The genomic DNA analysis of the *FOXP3* gene identified a 3 bp deletion (c.748–750delAAG, p.250K.del) in exon 7 of this gene (Fig. 2). This mutation was in the leucine zipper domain, which is critical for dimerization and DNA binding of the FOXP3 protein [6]. The patient's grandmother, mother, and elder sister were found to be carriers. Although Wildin et al. reported that the same mutation of the *FOXP3* gene was causative of IPEX syndrome [7], we also carried out flow cytometric analysis to examine the effect of this mutation on the FOXP3 protein level. We found that the patient's percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells (0.86%) was much lower than that of other family members and a normal

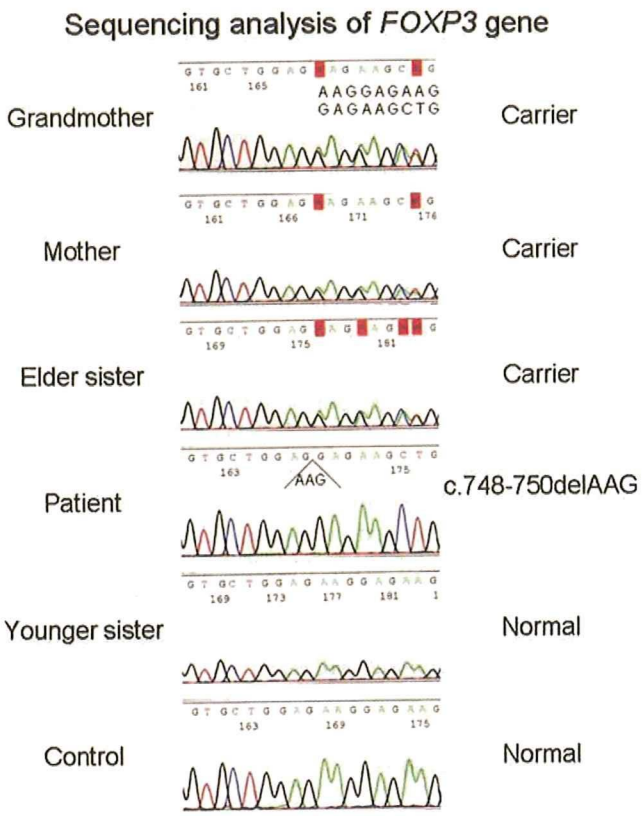

Fig. 2 *FOXP3* mutation analysis. Analysis of amplified genomic exon 7 sequence of the patient showed 3 bp deletion of nucleotide 748–750. The patient's grandmother, mother, and elder sister were found to be carriers

control [normal range of 4–6-year olds, n=12, 5.33±1.68% (3.39–8.60%)] [8] (Fig. 3), indicating that this mutation had resulted in the aberrant expression of FOXP3 protein.

Discussion

The immune system must be capable of mounting an effective response against foreign/microbial agents, but it must not be self-reactive. Mechanisms at both the central and peripheral levels exist to maintain tolerance against self-antigens. Centrally, self-reactive clones are eradicated during thymocyte differentiation, and, peripherally, various mechanisms exist to eliminate the self-reactive clones that have escaped central tolerance. Among these, $CD4^+$ $CD25^+$ (IL-2Rα) Treg cells play a critical role in limiting autoimmune processes and inflammatory responses [6].

The transcription factor FOXP3 has been identified as a molecular marker as well as a key regulatory gene for the development and function of regulatory T cells [9]. Mutations in the *FOXP3* gene cause the IPEX syndrome [10], which is characterized by deficiency of Treg cell function, due to FOXP3 malfunction, highlighting the importance of FOXP3 in Treg cell development [6].

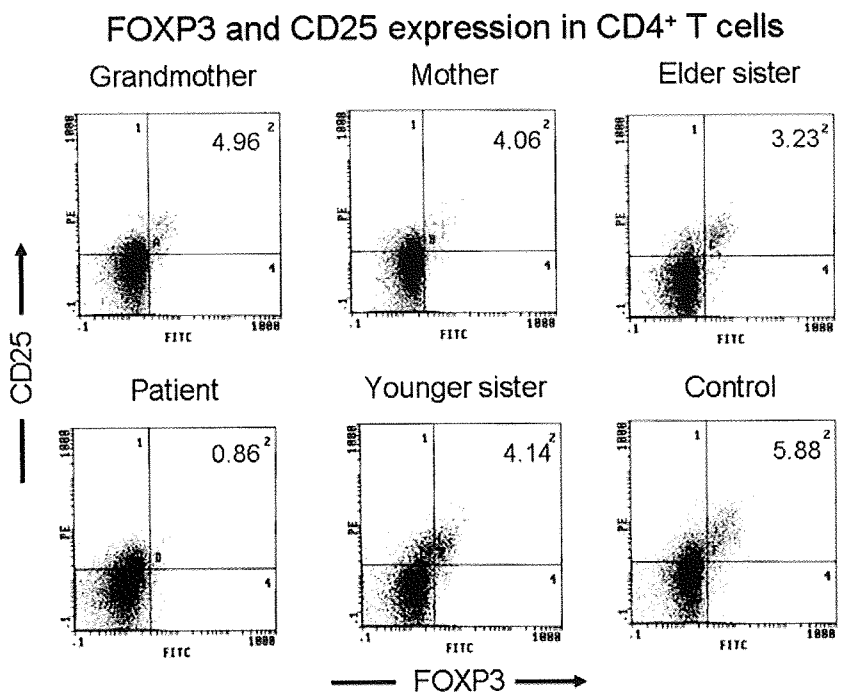

Fig. 3 Flow cytometric analysis of FOXP3 expression in circulating $CD4^+$ T cells. Peripheral blood mononuclear cells were stained simultaneously for CD4, FOXP3, and CD25, and analyzed by flow cytometry for the expression of FOXP3 and/or CD25. *The numbers in each quadrant* indicate the percentage of the respective subpopulation. The percentage of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells in the patient was much lower than that in other family members and a normal control

More than 50 patients with IPEX syndrome or IPEX-like diseases have been reported [7]. Most of them had developed T1DM, failure to thrive and diarrhea, and several patients had also developed eczema (atopic dermatitis), thrombocytopenia, hemolytic anemia, hypothyroidism or lymphadenopathy [7]. Thus, IPEX syndrome is characterized by a wide spectrum of clinically manifested autoimmune diseases. It has been reported that membranous nephropathy and tubulointerstitial damage are also associated with IPEX [11–13]. So far as we could determine, however, there have been no reports on patients who had developed MCNS complicated by IPEX syndrome. The longer or more severe the reduction of $CD4^+$ Treg cells is, the higher the incidence of a particular autoimmune disease becomes, and the wider the spectrum of clinically manifested autoimmune diseases in a genetically determined hierarchical pattern. Many autoimmune or chronic inflammatory diseases in mice that are induced by severe Treg depletion resemble, both clinically and immunologically, human autoimmune/inflammatory diseases [14, 15]. In addition, the individual's susceptibility to, and spectrum of, particular autoimmune diseases appears to be genetically determined for every mouse host strain [14, 16]. This means that a combination of a profound Treg deficiency and a specific host genetic background is able to produce an autoimmune disease in mice that corresponds to its human equivalent. Our patient had survived a relatively long time for a victim of IPEX syndrome, probably because of successful immunosuppressive therapy with cyclosporine. The combination of our patient's comparatively longer clinical course and a specific but unknown genetic background might have caused the rare clinical manifestation of MCNS in association with IPEX syndrome.

It is well known that children with MCNS show a higher incidence of allergic disorders such as asthma, allergic rhinitis and atopic dermatitis [17–20]. Also, several studies have reported higher mean serum IgE levels and B cell counts in MCNS [20, 21]. In addition, Goldman et al. [22] reported on four children with steroid-sensitive nephrotic syndrome and T1DM. These findings suggest that particular immunological conditions inducing allergic disorders, high serum IgE levels and T1DM, which are seen in IPEX syndrome, might also lead to the development of MCNS. Furthermore, our patient's condition suggests that MCNS with allergic disorders, high serum IgE levels or T1DM might be, at least in part, due to FOXP3 malfunction caused by polymorphisms or mutations of the *FOXP3* gene, although further studies are needed. Also, the above findings, such as increased IgE levels in MCNS, might indicate that B cell activation, as well as T cell activation, was induced in MCNS as previously described [23].

Although there have been no patients with *FOXP3* mutations in autoimmune diseases except IPEX syndrome so far, there have been several reports on the association

between *FOXP3* polymorphism and disease susceptibility in several autoimmune diseases such as psoriasis, primary biliary cirrhosis and type 1 diabetes [24–26]. It is, therefore, possible that *FOXP3* polymorphism may be associated with disease susceptibility of childhood MCNS. Further studies are needed to examine the above possibility.

In the previous report [7], there was a patient with IPEX syndrome who had a *FOXP3* mutation identical to that of our patient. He had developed enteritis, T1DM, polyarticular arthritis and idiopathic thrombocytopenic purpura but not MCNS. He had been treated with several immunosuppressive agents such as cyclosporine, tacrolimus and rituximab. It is therefore possible that the development of MCNS had been prevented by the strong immunosuppression. Moreover, it is well known that patients with identical mutations do not necessarily show the same symptoms in many hereditary diseases. Therefore, the fact that the previously reported patient with the identical mutation of *FOXP3* had not developed MCNS does not rule out the possibility that *FOXP3* mutations cause MCNS.

Recently, Rubio-Cabezas et al. reported that a boy with IPEX syndrome having a *FOXP3* mutation (V408M) developed steroid-sensitive nephrotic syndrome at 6 years old [27]. Strictly speaking, it is unclear whether their patient suffered from MCNS, because he did not undergo renal biopsy. Nevertheless, this finding strongly supports our conclusion that MCNS can be complicated by IPEX syndrome.

It is well known that cyclosporine is effective for the treatment of children with MCNS. Indeed, our patient remained in remission from nephrotic syndrome as a result of cyclosporine treatment. It was believed until recently that the beneficial effect of cyclosporine depended on the inhibition of the nuclear factor of activated T cells (NFAT). However, Faul et al. [28] reported that the antiproteinuric effect of cyclosporine was due to the direct inhibition of synaptopodin, a novel calcineurin substrate of podocytes, not to NFAT inhibition. It is therefore possible that the *FOXP3* mutation may directly affect the actin cytoskeleton and the function of podocytes. Therefore, we evaluated the expression of the *Foxp3* gene in mouse glomerulus, both by blast search against mouse glomerular expressed sequence tag libraries [29] and by signal intensities on Affymetrix gene arrays [30, 31]. The *Foxp3* gene was not detected by either technique. In addition, UniGene Electronic Northern showed that only spleen and thymus in normal human tissues express *FOXP3*. Collectively, it is likely that podocytes do not express *FOXP3*, although further studies on FOXP3 expression of human podocytes, both in normal and disease states, should be carried out.

Taken together, these findings strongly suggest that aberrant development of Treg may be crucial for the development of MCNS, although the final target of effector cells or cytokines in MCNS may be kidney podocytes.

Acknowledgments This study was supported by a Grant in Aid for Scientific Research (C-18590920) (to K.I.) from the Japan Society for the Promotion of Science. The authors thank Ms. Yoshimi Nozu for her help with the genetic analysis.

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Pediatr Nephrol (2009) 24:1773–1774
DOI 10.1007/s00467-009-1122-0

LETTER TO THE EDITORS

Detection by multiplex ligation-dependent probe amplification of large deletion mutations in the *COL4A5* gene in female patients with Alport syndrome

Kandai Nozu · Rafal Przybyslaw Krol · Koichi Nakanishi · Norishige Yoshikawa · Yoshimi Nozu · Yasufumi Ohtsuka · Kazumoto Iijima · Masafumi Matsuo

Received: 28 November 2008 / Revised: 12 December 2008 / Accepted: 15 December 2008 / Published online: 24 January 2009

Sirs,

Recently, we reported on two female subjects with X-linked Alport syndrome (XLAS) whose genetic test findings were negative by the direct sequencing method [1]. However, we could detect their large heterozygous deletion mutations with the semi-quantitative polymerase chain reaction (PCR) method, which we frequently use these days because the technique is very simple, consisting of PCR and capillary electrophoresis [1–3]. On the other hand, this method needs sophisticated equipment for capillary electrophoresis, and every exon analysis must be conducted separately.

In connection with the first submission of our last article, one of the reviewers suggested we should validate the results with the multiplex ligation-dependent probe amplification (MLPA) method, because the MLPA kit for XLAS has become commercially available. Although, at that time, there were no reports on the use of MLPA for XLAS, Hertz et al. have recently reported on the usefulness of this method [4]. This prompted us to conduct the MLPA analysis according to Hertz's recommendation, using the SALSA P191/192 Alport MLPA assay (MRC-Holland, Amsterdam, The Netherlands) for the patients we reported on last time, and this confirmed our results.

Peaks for each PCR product were obtained after capillary sequences, and the peak of every sample was determined from the relative peak area (PRA). The individual peak areas in relation to the total of all peak areas of several other parts on the X-chromosome, except for the *COL4A5* and *COL4A6* genes, were calculated, and the resultant values were compared to those of normal controls. We obtained values for the normal controls by calculating separately the average values for three healthy male and female subjects. A reduction in the ratio to 0 in male subjects and 0.5 in female subjects indicates a deletion in hemizygous and heterozygous form, respectively. A normal MLPA ratio is >0.7 and <1.3 [4].

We have previously detected with semi-quantitative PCR that patient 1 had an exon1-51 entire heterozygous deletion of the *COL4A5* gene, patient 2 an exon 37 to 51 hemizygous deletion, and the mother of patient 2 a heterozygous deletion [1]. The MLPA results were exactly the same as those for semi-quantitative PCR (The schema is shown in Fig. 1). Moreover, the deletion in patient 1 extended to *COL4A6* exons 1 and 2.

XLAS associated with diffuse leiomyomas [DL-AS; online Mendelian inheritance in man (OMIM) catalogue no. 308940] has been reported previously in more than 30 cases. This phenotype is caused by deletion of the *COL4A5* and extends into the second intron of the *COL4A6* gene, and it should be noted that defects in the *COL4A6* gene alone do not appear to cause AS or leiomyomas [5, 6]. *COL4A5* and *COL4A6* are arranged head-to-head at Xq22.3

K. Nozu (✉) · R. P. Krol · Y. Nozu · K. Iijima · M. Matsuo
Department of Pediatrics,
Kobe University Graduate School of Medicine,
Kusunokicho 7-5-1, Chuo,
Kobe 650-0017 Hyogo, Japan
e-mail: nozu@med.kobe-u.ac.jp

K. Nakanishi · N. Yoshikawa
Department of Pediatrics, Wakayama Medical University,
Wakayama, Japan

Y. Ohtsuka
Department of Pediatrics, Saga Medical School,
Saga, Japan

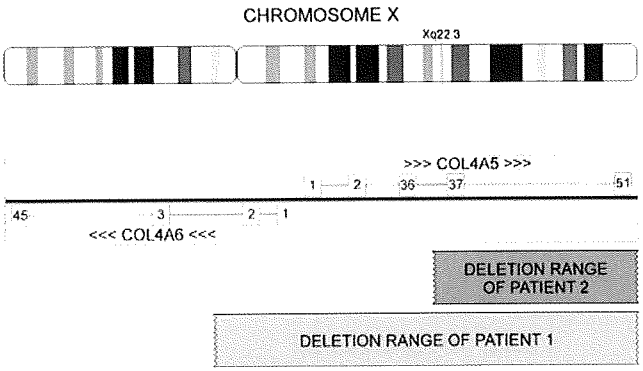

Fig. 1 Schema of the results of the multiplex ligation-dependent probe amplification (MLPA) analysis. Patient 1 has a deletion at least from exon 2 of *COL4A6* to exon 51 of *COL4A5*. Patient 2 has a deletion from exon 37 to exon 51 of *COL4A5*

and share the same promoter region (Fig. 1). The large deletion extending from *COL4A5* to *COL4A6* exons 1 and 2 usually leads to DL-AS. It is, therefore, very important for genetic counseling of female sufferers with deletions including *COL4A5* exon 1 for one to clarify whether the deletion extends to *COL4A6*. However, if the deletion extends into exon 3, no leiomyomas are observed [7]. The current commercially available kit for *COL4A5* MLPA analysis includes only the probe for *COL4A6* exons 1 and 2. Patient 1 had a heterozygous deletion into *COL4A6* exons 1 and 2, but exon 3 could not be examined. We hope this kit will be improved to include a probe for the analysis of *COL4A6* exon 3.

Our study made it clear that both semi-quantitative PCR and MLPA are quite useful for detecting large heterozygous deletions in cases that show negative results when analyzed with the standard analytical methods of PCR and direct sequencing.

The advantages of the semi-quantitative PCR method are: (1) the same primer pairs can be used for PCR and direct sequencing analysis; (2) the technique very simple; (3) the cost is very low. The disadvantages are: (1) every exon must be analyzed separately; (2) capillary electrophoresis equipment is needed. On the other hand, the advantages of MLPA analysis are: (1) almost all exons and some neighboring gene exons (for example, *COL4A6* exon 1 and 2 analysis can be conducted with the SALSA P191/192 Alport kit) can be analyzed at the same time; (2) for the screening of patients over several decades, the cost would be cheaper because 50 patients can be analyzed with one kit. The disadvantage of MLPA analysis is that a commercial kit is needed because it is not easy for the probes or universal primer pairs to be personally designed in this method. However, several MLPA kits are currently available at the MRC-Holland web site (http://www.mrc-holland.com/pages/indexpag.html).

Our results indicate that MLPA analysis and semi-quantitative PCR are two useful methods for detecting large heterozygous deletions, not only in female subjects with X-linked diseases but also in people with autosomal dominant or recessive diseases with no mutations detected by standard genetic analytical methods.

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Pediatr Nephrol (2009) 24:2177–2185
DOI 10.1007/s00467-009-1264-0

ORIGINAL ARTICLE

Cyclosporine and steroid therapy in children with steroid-resistant nephrotic syndrome

Yuko Hamasaki · Norishige Yoshikawa · Shinzaburo Hattori · Satoshi Sasaki · Kazumoto Iijima · Koichi Nakanishi · Takeshi Matsuyama · Kenji Ishikura · Nahoko Yata · Tetsuji Kaneko · Masataka Honda · for Japanese Study Group of Renal Disease in Children

Received: 31 March 2009 / Revised: 10 June 2009 / Accepted: 26 June 2009 / Published online: 28 August 2009

Abstract We conducted a prospective, multicenter trial to evaluate the efficacy and safety of a 12-month course of cyclosporine in children with steroid-resistant nephrotic syndrome (SRNS). Thirty-five patients were enrolled, of whom 28 had minimal change or diffuse mesangial proliferation (MC/DMP), and seven had focal segmental glomerulosclerosis (FSGS). All patients received cyclosporine and prednisolone; patients with FSGS additionally received methylprednisolone pulse therapy (MPT). The dose of cyclosporine was adjusted to maintain a trough level of 120–150 ng/ml during the initial 3 months of treatment, followed by 80–100 ng/ml during months 4–12. The primary end point was the remission rate at month 12. Remission was achieved in 23 of 28 (82.1%) patients in the MC/DMP group and in six of the seven (85.7%) patients in the FSGS group. Follow-up renal biopsies were performed in 26 patients (nine at month 12, 17 at month 24), and cyclosporine-related nephrotoxicity was detected in one (3.8%). Major adverse events comprised severe bacterial infections (two patients) and posterior reversible encephalopathy syndrome (one patient). In conclusion, a high remission rate was achieved in our patient cohort using a combined cyclosporine/prednisolone treatment regimen in children with SRNS who had MC/DMP and a combined cyclosporine/prednisolone plus MPT regimen in children who had FSGS.

Keywords Children · Clinical trial · Cyclosporine · Methylprednisolone · Nephrotic syndrome · Steroid-resistant

Y. Hamasaki (✉) · K. Ishikura · N. Yata · M. Honda
Department of Pediatric Nephrology,
Tokyo Metropolitan Kiyose Children's Hospital,
1-3-1 Umezono,
Kiyose-city, Tokyo 204-8567, Japan
e-mail: yu.hama@chp-kiyose-tokyo.jp

N. Yoshikawa · K. Nakanishi
Department of Pediatrics, Wakayama Medical University,
Wakayama, Japan

S. Hattori
Department of Fundamental Medicine,
Kumamoto Health Science University,
Kumamoto, Japan

S. Sasaki
Department of Pediatrics,
Hokkaido University Graduate School of Medicine,
Sapporo, Japan

K. Iijima
Department of Pediatrics,
Kobe University Graduate School of Medicine,
Kobe, Japan

T. Matsuyama
Department of Pediatrics, Fussa Hospital,
Tokyo, Japan

K. Ishikura · N. Yata
Department of Clinical Research,
Tokyo Metropolitan Kiyose Children's Hospital,
Tokyo, Japan

T. Kaneko
Department of Biostatistics/Epidemiology
and Preventive Health Sciences, School of Health
Sciences and Nursing, Graduate School of Medicine,
the University of Tokyo,
Tokyo, Japan

Abbreviations

DMP diffuse mesangial proliferation
FSGS focal segmental glomerulosclerosis
MC minimal change
MPT methylprednisolone pulse therapy
PRES posterior reversible encephalopathy syndrome
SRNS steroid-resistant nephrotic syndrome

Introduction

Steroid-resistant nephrotic syndrome (SRNS) is the most common glomerular disease in children that progresses to end-stage renal failure [1]. After a follow-up of 10 years, end-stage renal failure develops in 30–40% of children with SRNS [2, 3].

Despite many clinical trials, the optimal treatment regimen for children with SRNS remains unclear. The Cochrane analysis of randomized and controlled prospective trials of SRNS revealed that remission rates after cyclosporine pharmacotherapy were significantly higher than those after other immunosuppressive agents [4]. Cyclosporine has recently been designated as a first-line treatment for SRNS in children [4–6]. Randomized controlled trials have shown that children with SRNS who have completed a 6-month course of cyclosporine have a significantly increased rate of complete remission compared to those treated with placebo [7] or supportive therapy [8]. The efficacy of cyclosporine for SRNS has also been evaluated in many other single-center trials [1, 5, 9–13]. Niaudet et al. and others have reported that cyclosporine combined with alternate-day or low-dose steroids produces a higher response rate than cyclosporine monotherapy [5, 10, 14, 15]. Several studies have reported a favorable response to methylprednisolone pulse therapy (MPT) in patients with focal segmental glomerulosclerosis (FSGS) [16–19].

We have conducted a prospective, multicenter trial to evaluate the efficacy and safety of a 12-month course of cyclosporine-based treatment in children with SRNS. All patients received cyclosporine and prednisolone. Patients with FSGS additionally received MPT because steroid-resistant FSGS is associated with poor outcomes [2, 3, 20].

Methods

Patients

To be eligible for enrollment, patients had to have SRNS (see Definitions). The pathological diagnosis was established by renal biopsy and was classified into three groups: minimal change (MC), diffuse mesangial proliferation (DMP), and FSGS. At study entry, the pathological findings were evaluated by a pathologist at each hospital. Patients aged 1–18 years were eligible for enrollment.

Patients were excluded if they had: (1) previously received MPT or immunosuppressive agents, (2) clinically significant hematuria, (3) uncontrolled hypertension, (4) severe liver dysfunction, (5) serious infection, or (6) impaired renal function (creatinine clearance <60 ml/min per 1.73 m^2). Patients who had FSGS with more than 20% global sclerosis were also excluded.

The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki Principles, and informed consent was obtained from all patients or their parents.

Definitions

Nephrotic syndrome was diagnosed if the urinary protein/creatinine ratio was ≥1.8 and the serum albumin level was ≤2.5 g/dl. SRNS was diagnosed if no remission (serum albumin level ≤2.5 g/dl) was achieved after treatment with 2 mg/kg prednisolone daily for 4 weeks.

The response to treatment was classified as complete remission, partial remission, or non-remission. Complete remission was defined as negative or trace proteinuria (on the dipstick method or a urinary protein/creatinine ratio ≤0.20) on urinalysis and a serum albumin level of >2.5 g/dl. Partial remission was defined as a serum albumin level of >2.5 g/dl, but persistent proteinuria on urinalysis. In the present trial, remission was defined as complete remission and partial remission. Non-remission was defined as persistent nephrotic syndrome.

The relapse of nephrotic syndrome in patients who once achieved complete remission was defined as the reappearance of proteinuria (2+ on the dipstick method) for 3 consecutive days. The relapse of nephrotic syndrome in patients who once achieved partial remission was defined as increased proteinuria and a serum albumin level of ≤2.5 g/dl. Frequently relapsing nephrotic syndrome was defined as three relapses within any 6-month period or four or more relapses within any 12-month period.

Late non-response to steroids was defined as an initial response to steroid therapy but not during a subsequent relapse.

Protocol treatment

All patients received oral cyclosporine (Neoral; Novartis, Basel, Switzerland) divided into two equal daily doses plus prednisolone. The total duration of treatment was 12 months, and the follow-up period was 12 months. The dose of cyclosporine was adjusted to maintain a whole-

blood trough level of 120–150 ng/ml for the initial 3 months, followed by 80–100 ng/ml for months 4–12. Prednisolone was given in a dose of 1 mg/kg per day, divided into three equal daily doses for the first 4 weeks, followed by a reduced dose of 1 mg/kg every other day in a single dose for months 2–12. Patients with FSGS additionally received methylprednisolone by intravenous infusion in a dose of 30 mg/kg per day (maximum prescribed amount 1 g) for 3 consecutive days at weeks 1, 2, 5, 9, and 13 (Fig. 1).

The response to treatment was evaluated at the end of month 4 of the protocol treatment. In patients who achieved complete remission or partial remission, treatment was continued until month 12. For patients with MC or DMP (MC/DMP) who did not achieve remission within 4 months, treatment was restarted with the regimen of the FSGS group (cyclosporine, prednisolone, and MPT). Patients with FSGS who did not achieve remission within 4 months were given off-protocol treatment chosen at the discretion of the physician. Patients who progressed to SRNS or frequently relapsing nephrotic syndrome after once achieving remission also received off-protocol treatment.

At the completion of the protocol treatment, the dose of cyclosporine was tapered by 0.5–1.0 mg/kg per day every week, and all patients were scheduled to undergo renal biopsy. An independent investigator at the coordinating center who was blinded to patients' information also reviewed the histological sections. Arteriolar changes, tubular atrophy, and interstitial fibrosis were graded semiquantitatively according to a scale of 0 to 3+ as follows: 0, none; 1+, mild; 2+, moderate; 3+, intense. A diagnosis of cyclosporine-related nephrotoxicity required arteriolopathy (arteriolar hyalinosis, hyperplasia) with or without striped interstitial fibrosis and tubular atrophy.

Concomitant treatment with drugs known to affect the pharmacokinetics of cyclosporine was avoided. Antihypertensive drugs (calcium-channel blockers, angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers), diuretics (furosemide), anticoagulants, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) were permitted.

Treatment for relapses of nephrotic syndrome

Patients who had relapses of nephrotic syndrome after achieving complete remission or partial remission received 2 mg/kg per day of prednisolone in three divided doses (maximum dose 80 mg/day). For patients who responded to prednisolone, the dose was reduced to 2 mg/kg in a single dose every other day for 2 weeks after 3 consecutive days of negative proteinuria on the dipstick method, followed by 1 mg/kg in a single dose every other day until the end of month 12.

Pretreatment evaluation and follow-up

The following measurements and tests were performed at baseline, after 2 weeks, after 1 month, and every month thereafter: body weight and height; blood pressure; blood analysis (complete blood cell count, blood chemistry); urine tests (urinalysis, quantitative proteinuria); estimated creatinine clearance as determined by the Schwartz equation. The trough level of cyclosporine was measured by monoclonal radioimmunoassay.

The primary end point was the remission rate at month 12. The secondary end point was the remission rate at month 4, change in renal function, and adverse events.

Recommended treatment

Since May 2003, the Safety Committee has recommended that for patients who completed the 12-month protocol

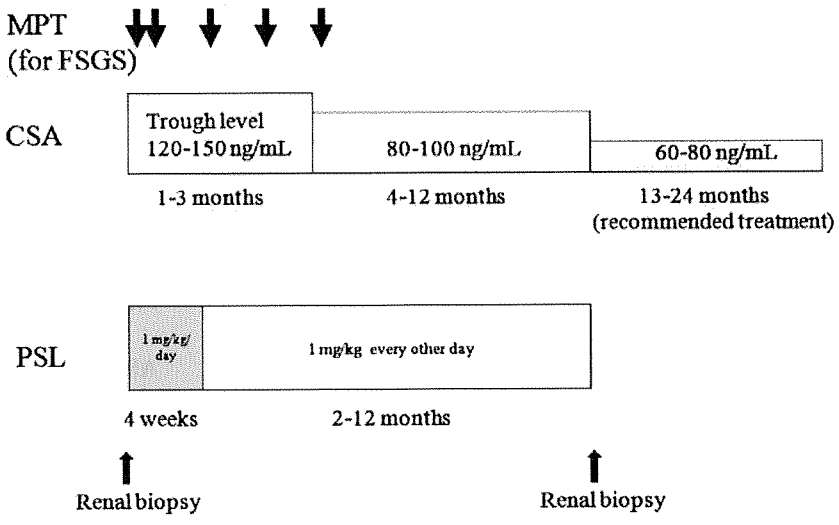

Fig. 1 Protocol for patients with steroid-resistant nephrotic syndrome. *FSGS* Focal segmental glomerulosclerosis, *MPT* methylprednisolone pulse therapy, *CSA* cyclosporine, *PSL* prednisolone

treatment, the follow-up treatment with cyclosporine should be a dose producing a whole-blood trough level between 60 and 80 ng/ml. This treatment was given from month 13 to month 24.

Registration

This study has been registered in a public trials registry, UMIN (the University Hospital Medical Information Network, ID C000000009 http://www.umin.ac.jp/ctr/index.htm.)

Results

Patient characteristics

This prospective study was conducted by Japanese Study Group of Renal Disease in Children. Between January 2001 and December 2007, a total of 35 children (21 male and 14 female) were enrolled at 14 centers. The patients' characteristics are shown in Table 1. At the start of the protocol treatment, the median age of the patients was 2.7 years (range 1.4–15.0 years); 20 of the 35 patients (57.1%) were <3 years. The mean duration from the onset of nephrotic syndrome to the start of the protocol treatment was 3.4 months (range 1.0–25.0 months). Twenty-six patients had an initial non-response to steroids, while nine had a late non-response to steroids. The mean serum total protein level and albumin level at the start of treatment were 4.1 and 1.7 g/dl, respectively.

Pathological examination (initial biopsy for all patients) at study entry revealed that 23 patients had MC, five had DMP, and seven had FSGS.

Cyclosporine dosage and trough level

The mean trough level and mean dose of cyclosporine during months 1–3 of treatment were 110.2 ng/ml (range 71.0–159.7 ng/ml) and 6.0 mg/kg per day (range 3.1–10.4 mg/dl), respectively. During months 4–12, the mean trough level and the mean dose of cyclosporine were 88.6 ng/ml (range 61.0–136.5 ng/ml) and 5.1 mg/kg per day (range 3.0–8.1 mg/dl), respectively.

Response to treatment

A flow diagram of the patients, summarizing the numbers of patients enrolled, followed up, and included in the analyses, is shown in Fig. 2. The response to treatment was analyzed separately in patients with MC/DMP and in those with FSGS on renal biopsy. Thirty-five patients were enrolled: 28 patients in the MC/DMP group and seven in the FSGS group.

The response to treatment within 4 months after initiation of the protocol is shown in Table 2. Remission (including complete remission and partial remission) was achieved during the first month of treatment in 60.7% (17/28) of the patients in the MC/DMP group and 71.4% (5/7) of those in the FSGS group. The remission rate at the end of month 4 was 92.9% (26/28) in the patients with MC/DMP, with 23 patients having complete remission and three having partial remission; two had non-remission. In contrast, the remission rate was 85.7% (6/7) in the patients with FSGS, with five patients having complete remission and one having partial remission; one patient had non-remission. Two patients with MC/DMP who had non-remission received the regimen for the FSGS group. One patient with FSGS who had non-remission received off-protocol treatment.

The results of the evaluation at month 12 are shown in Table 3. Twenty-three patients completed the 12-month course of protocol treatment in the MC/DMP group, with 22 of these achieving complete remission and one achieving partial remission. Five patients received off-protocol treatment during months 5–12. The remission rate at month 12 was thus 82.1% [23/28; 95% confidence interval (CI) 0.63–0.93]. Of the 22 patients who had complete remission, two received treatment that violated the protocol: one patient received mizoribine and the other received MPT at the first relapse after achieving remission. If these patients are excluded from analysis, the remission rate was 80.8% (21/

Table 1 Characteristics of the patients

Characteristic	Level	MC/DMP (n=28) n (%)	FSGS (n=7) n (%)
Age at entry (years)	0 to <3	15 (53.5)	5 (71.4)
	≥3 to <7	5 (17.9)	1 (14.3)
	≥7 to <11	4 (14.3)	1 (14.3)
	≥11 to <15	3 (10.7)	0 (0.0)
	≥15	1 (3.6)	0 (0.0)
Sex	Male	17 (60.7)	4 (57.1)
	Female	11 (39.3)	3 (42.9)

MC, Minimal change; DMP, diffuse mesangial proliferation; FSGS, focal segmental glomerulosclerosis

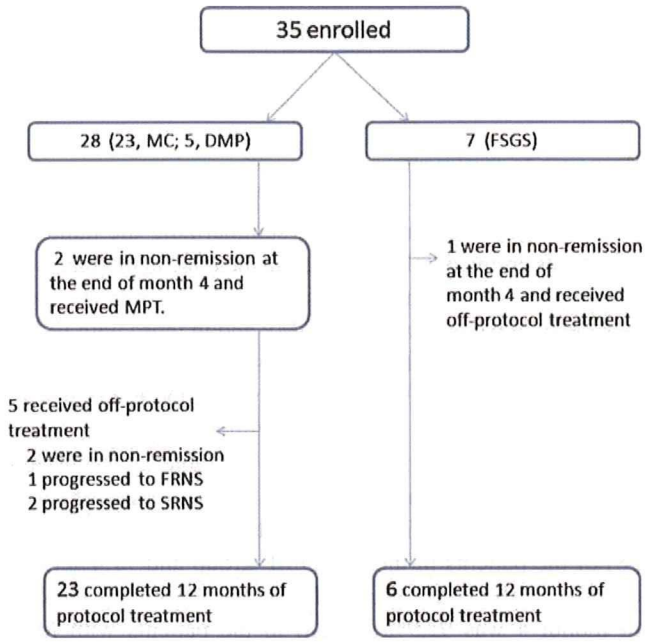

Fig. 2 Flow diagram of patients. *MC* Minimal change, *DMP* diffuse mesangial proliferation, *FRNS* frequently relapsing nephrotic syndrome, *SRNS* steroid-resistant nephrotic syndrome, *FSGS* focal segmental glumerulosclerosis

26; 95% CI 0.60–0.93). Six patients completed the 12-month protocol treatment in the FSGS group, with five achieving complete remission and one achieving partial remission. The remission rate was 85.7% (6/7; 95% CI 0.42–0.99).

Among the 22 patients with complete remission in the MC/DMP group at month 12, 15 patients continued to show complete remission during months 5–12, and relapse occurred during this period in seven patients, but was steroid-sensitive. Among the five patients with complete remission in the FSGS group at month 12, three patients continued to show complete remission during months 5–12 and relapse involving steroid-sensitive nephrotic syndrome occurred in two patients during the same period. The status at month 12 of the patients who received off-protocol treatment was as follows: two patients had complete remission, three patients in the MC/DMP group had nephrotic syndrome, and one patient in the FSGS group had partial remission.

Adverse events

Follow-up renal biopsies were performed in 26 patients (nine at month 12 and 17 at month 24). Mild nephrotoxicity attributable to cyclosporine occurred in one patient (3.8%) who had mild arteriolar hyalinosis and mild interstitial fibrosis with mild tubular atrophy. No patient in this series had striped interstitial fibrosis. Four patients had mild tubular atrophy without interstitial fibrosis, and four had mild tubular atrophy with mild interstitial fibrosis. These findings were considered to reflect the natural course of SRNS rather than an adverse effect of cyclosporine.

Other adverse events are summarized in Table 4. Severe adverse events occurred in three patients with MC/DMP: one patient had bacterial peritonitis, one had sepsis with disseminated intravascular coagulation and multiorgan failure, and one patient had posterior reversible encephalopathy syndrome (PRES). The two patients with infections received antibiotics and supportive therapy. The infections resolved immediately and uneventfully, and treatment according to the protocol was resumed. The patient with PRES was in complete remission when the event occurred at the end of the protocol treatment. Cyclosporine was discontinued after the development of PRES, and the patient completely recovered after receiving antihypertensive and supportive therapy. This patient did not receive cyclosporine again. Hypertension was present in ten of 35 patients (28.6%), but blood pressure was easily controlled with antihypertensive therapy. Transient arrhythmias (sinus bradycardia) associated with MPT were observed in two patients. No patient had elevated serum creatinine levels during cyclosporine treatment. The mean creatinine clearance was similar at the start of cyclosporine treatment (119.9 ml/min per 1.73 m^2) and at month 12 (117.7 ml/min per 1.73 m^2). The mean standard deviation (SD) scores for height at the start of the protocol treatment and at month 12 were –0.38 and –0.08, respectively. The mean difference in the SD scores for height was 0.30 (95% CI 0.10–0.50). Other complications, such as aseptic necrosis of the femoral head, diabetes, and pancreatitis, were not observed.

Table 2 Response to treatment within 4 months after the initiation of protocol treatment

Pathology	Patients (*n*) who went into remission at:					Patients with non-remission[a] (*n*)	Total (*n*)
	0–1 months	1–2 months	2–3 months	3–4 months	Subtotal		
MC/DMP	17	4	3	2	26	2	28
FSGS	5	1	0	0	6	1	7

Values are given as the number of patients

[a] Patients with persistent nephrosis at the end of month 4

Table 3 Evaluation of patients with steroid-resistant nephrotic syndrome at month 12

Pathology	Patients who completed 12-month protocol treatment (*n*)		Patients who received off-protocol treatment[a] (*n*)	Total (*n*)
	Complete remission	Partial remission		
MC/DMP	22[b]	1	5	28
FSGS	5	1	1	7

NS, Nephrotic syndrome; FRNS, frequently relapsing nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; MPT, methylprednisolone pulse therapy

[a] Includes patients with persistent nephrosis at month 4 and those who progressed to FRNS or SRNS during months 5–12

[b] Two patients achieved complete remission with additive immunosuppressive agents (MPT or mizoribine) at the first relapse

Clinical course during months 13–24

After 12 months of treatment, cyclosporine was discontinued in four patients with FSGS in 2003. SRNS recurred immediately in all patients. There was no relapse of SRNS in the MC/DMP group; however, progression to frequently relapsing nephrotic syndrome occurred in four (80%) of the five patients. In May 2003, the Safety Committee therefore recommended that patients who completed the 12 months of protocol treatment should receive an additional 12 months of treatment with cyclosporine.

The status of the patients at month 24 is shown in Table 5. Seventeen patients with MC/DMP received the recommended treatment; of these, 11 patients continued to have complete remission, and six had relapses with steroid-sensitive nephrotic syndrome between months 13 and 24. Two patients with FSGS received the recommended treatment; remission (complete remission or partial remission) continued in both patients from months 13 to 24.

One patient with MC/DMP who received off-protocol treatment had progression to end-stage renal failure at month 24. The patient had persistent nephrotic syndrome, with no response to treatment, including to cyclosporine and prednisolone plus MPT.

Discussion

This prospective, multicenter trial was conducted in children with SRNS with the aim of evaluating the safety and effectiveness of a 12-month course of cyclosporine and prednisolone in children with MC/DMP and a 12-month course of cyclosporine and prednisolone plus MPT in children with FSGS. Both groups of patients had high remission rates, and end-stage renal failure developed in only one of 35 patients during the 2 years of follow-up. Renal biopsy at the end of treatment showed that the protocol treatment was safe in terms of nephrotoxicity.

In our trial, all patients received prednisolone combined with cyclosporine based on the hypothesis that prednisolone improves the effectiveness of cyclosporine in patients with SRNS. The 12-month protocol treatment was com-

Table 4 Adverse events

Adverse events	Total (*n*=35)	MC/DMP (*n*=28)	FSGS (*n*=7)
Peritonitis	1 (2.9)	1 (3.6)	0 (0.0)
Sepsis	1 (2.9)	1 (3.6)	0 (0.0)
PRES	1 (2.9)	1 (3.6)	0 (0.0)
Hypertrichosis	18 (51.4)	13 (46.4)	5 (71.4)
Hypertension	10 (28.6)	9 (32.1)	1 (14.3)
Hyperlipidemia	5 (14.3)	3 (10.7)	2 (28.6)
Obesity	3 (8.6)	2 (7.1)	1 (14.3)
Gastric pain	3 (8.6)	3 (10.7)	0 (0.0)
Gingival hypertrophy	2 (5.7)	1 (3.6)	1 (14.3)
Alopecia	2 (5.7)	2 (7.1)	0 (0.0)
Sinus bradycardia	2 (5.7)	0 (0.0)	2 (28.6)
Glaucoma	1 (2.9)	1 (3.6)	0 (0.0)
Acne	1 (2.9)	1 (3.6)	0 (0.0)
Hyperuricemia (>8 mg/dl)	1 (2.9)	1 (3.6)	0 (0.0)

Values are given as the number of patients, with the percentage in parenthesis

PRES, Posterior reversible encephalopathy syndrome

Table 5 Status of patients with steroid-resistant nephrotic syndrome at month 24

Status of patients	Patients who completed 12-month protocol treatment (*n*)				Patients who received off-protocol treatment[b]		Total
	Recommended treatment (+)		Recommended treatment (-)[a]				
	MC/DMP	FSGS	MC/DMP	FSGS	MC/DMP	FSGS	
CR	16[c]	1	6	4[c]	2[c]	1	30
PR	0	1	0	0	0	0	1
NS	0	0	0	0	2	0	2
ESRF	0	0	0	0	1	0	1
Unknown	1[d]	0	0	0	0	0	1
Total	17	2	6	4	5	1	35

CR, Complete remission; PR, partial remission; ESRF, end-stage renal failure

[a] Patients in whom treatment was left to the discretion of their physicians after 12 months

[b] Includes patients with persistent nephrosis at month 4 and those who had FRNS or SRNS during months 5–12

[c] Includes one patient each with FRNS at month 24

[d] Patients who were lost to follow-up; data were unavailable

pleted in 23 of 28 patients in the MC/DMP group and six of seven patients in the FSGS group. High remission rates were obtained at month 12: 82.1% (80.8% after excluding the two patients with protocol violations) in the MC/DMP group and 85.7% in the FSGS group. Previous studies have shown that remission is generally achieved in 20–50% of patients with SRNS who receive cyclosporine monotherapy [7, 8, 13]. A multicenter trial of cyclosporine with low-dose prednisolone in children with SRNS, conducted by the French Society of Pediatric Nephrology, reported that remission was achieved in 23 (51.1%) of 45 patients with MC and eight (40.0%) of 20 with FSGS, suggesting that prednisolone enhanced the remission rate [5]. Several other studies also have provided evidence that combining prednisolone with cyclosporine considerably improves outcomes in comparison to cyclosporine monotherapy [10, 14, 15, 21]. These results are supported by our findings.

The patients with FSGS received MPT in addition to cyclosporine and prednisolone; to the best of our knowledge, this is the first prospective, multicenter trial to evaluate combined therapy with MPT and cyclosporine in children with FSGS. An excellent remission rate (85.7% at month 12) was obtained. Several retrospective studies have reported that a combination of MPT with cyclosporine and prednisolone produces better outcomes than those previously obtained in patients with FSGS, with remission rates ranging from 84 to 90% [20–22]. Other studies have shown that MPT alone is an effective treatment in children with SRNS [17, 18]. These results suggest that MPT combined with cyclosporine and prednisolone may improve the remission rate in children with FSGS. However, randomized controlled trials are needed to confirm these findings.

Cyclosporine-related nephrotoxicity, which is characterized by arteriolopathy (arteriolar hyalinosis and hyperplasia), with or without striped interstitial fibrosis and tubular atrophy [23, 24], is the most important factor limiting the long-term or high-dose use of cyclosporine [25–29]. One study reported that 2 years of treatment with cyclosporine in a dose that maintained the trough level at 100 ng/ml caused tubulointerstitial changes in seven of 13 patients with steroid-dependent nephrotic syndrome [29]. Only a few studies have evaluated nephrotoxicity in children with SRNS. We therefore performed renal biopsy at month 12 or 24 to evaluate the safety of treatment with cyclosporine in 26 patients and found that only one patient showed mild signs of cyclosporine-related nephrotoxicity. This low frequency of nephrotoxicity may be attributable to the management and adjustment of the cyclosporine dosage by trough-level monitoring. As mentioned above, we believe that our protocol treatment did not affect long-term cyclosporine-related nephrotoxicity in most of the patients.

Other serious adverse events were infections and PRES. There were two episodes of severe bacterial infections (sepsis and peritonitis) in the MC/DMP group, both of which responded to treatment with antibiotics. Nephrosis predisposes patients to bacterial infections [30], and cyclosporine treatment may increase the risk. Therefore, patients who receive cyclosporine should be closely monitored for bacterial infections. PRES is a distinct and potentially serious complication of cyclosporine treatment or hypertension [31] that may develop in children, including those with nephrotic syndrome [32, 33]. In our trial, PRES developed in one patient at the end of 12 months of the protocol treatment; this patient fully recovered in response to antihypertensive treatment and the withdrawal

of cyclosporine. Meticulous control of blood pressure is required to prevent PRES, particularly in patients given cyclosporine.

The optimal duration of cyclosporine treatment in children with SRNS remains unclear. In our trial, an excellent remission rate was achieved with 12 months of treatment; however, after the withdrawal of cyclosporine, most children had severe relapses, with SRNS developing in the FSGS group and frequently relapsing nephrotic syndrome developing in the MC/DMP group. In May 2003, the Safety Committee therefore proposed that patients should receive an additional 12 months of treatment with cyclosporine in a dose producing a whole-blood trough level between 60 and 80 ng/ml after the initial 12 months of protocol treatment. The trough level was based on the results of a previous study showing that this level was effective and relatively safe in children with frequently relapsing nephrotic syndrome [34]. Although even 2 years of treatment with cyclosporine may not be long enough to prevent relapses after the discontinuation of this agent, more than 2 years of treatment has been shown to be an independent risk factor for cyclosporine-related nephrotoxicity [35]. A total cyclosporine treatment period of 2 years may therefore be reasonable.

During the 12 months of treatment, five of 28 patients in the MC/DMP group and one of seven patients in the FSGS group received off-protocol treatment. Most of these patients were resistant to the protocol treatment, and end-stage renal failure finally developed in one patient at 2 years following treatment initiation. Renal function in the other patients may have been maintained by concurrent treatments, such as cyclophosphamide, plasma exchange, frequent MPT, and mizoribine combined with cyclosporine (data not shown). The establishment of new strategies for patients who do not respond to treatment is awaited.

In addition to the remission rate after 12 months of the protocol treatment, outcomes were also evaluated 24 months after the start of treatment. Similar to the results at 12 months, remission rates remained high in both groups, and most relapses of nephrosis were steroid-sensitive. Although 24 months of follow-up may not be adequate, our treatment protocol may result in long-term remission and prevent the development of end-stage renal failure in children with SRNS. To evaluate long-term outcomes of treatment, a 5-year follow-up study is currently being conducted.

An important limitation of our trial was the lack of a control group. The need for MPT in children with SRNS and the associations between pathological diagnosis (i.e. MC/DMP and FSGS) and outcomes have to be confirmed in future studies. Another limitation is that renal biopsy was not performed after treatment in nine patients. In six patients, the attending physicians decided not to perform follow-up renal biopsies because of associated risks; two patients received short-term cyclosporine before discontinuing the protocol treatment, and one patient was lost to follow-up. Because these reasons did not indicate a general trend, we believe that the incidence of cyclosporine-related nephrotoxicity was not affected by the lack of follow-up biopsy data in some patients.

In conclusion, this prospective, multicenter trial in children with SRNS showed that high remission rates were obtained after 12 months of treatment with cyclosporine and prednisolone in patients with MC/DMP and after 12 months of treatment with cyclosporine and prednisolone plus MPT in patients with FSGS. Maintenance cyclosporine treatment may be required to prevent relapses after the initial 12 months of treatment. Renal biopsy performed after treatment according to the protocol showed that cyclosporine was safe in terms of nephrotoxicity. Results from randomized controlled trials are necessary to confirm the need for MPT.

Acknowledgments The authors would like to thank the patients and physicians who participated in this trial:

M. Goto (Tokyo), R. Hamada (Tokyo), K. Hasegawa (Tokyo), H. Hataya (Tokyo), H. Hayakawa (Niigata), T. Igarashi (Tokyo), M. Ikeda (Tokyo), S. Ito (Tokyo), K. Kamei (Tokyo), A. Kobayashi (Hyogo), S. Kodama (Fukuoka), T. Nakamura (Kanagawa), K. Nozu (Hyogo), R. Ochiai (Shizuoka), T. Sakai (Tokyo), M. Sako (Tokyo), T. Sekine (Tokyo), R. Suzuki (Tokyo), R. Tanaka (Hyogo), N. Wada (Shizuoka), H. Wakaki (Kanagawa), K. Yoshidome (Kagoshima).

Disclosure This study was supported in part by the Kidney Foundation, Japan.

Norishige Yoshikawa has received research grants from Novartis, Japan.

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Am J Physiol Renal Physiol 296: F598–F604, 2009.
First published December 23, 2008; doi:10.1152/ajprenal.00074.2008.

Abnormal autoregulation and tubuloglomerular feedback in prediabetic and diabetic OLETF rats

Seiji Hashimoto,[1] Kanji Yamada,[1] Tetsuya Kawata,[1] Toshio Mochizuki,[1] Jurgen Schnermann,[2] and Takao Koike[1]

[1]*Hokkaido University Graduate School of Medicine, Internal Medicine II, Sapporo, Japan; and* [2]*National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland*

Submitted 24 August 2008; accepted in final form 16 December 2008

Hashimoto S, Yamada K, Kawata T, Mochizuki T, Schnermann J, Koike T. Abnormal autoregulation and tubuloglomerular feedback in prediabetic and diabetic OLETF rats. *Am J Physiol Renal Physiol* 296: F598–F604, 2009. First published December 23, 2008; doi:10.1152/ajprenal.00074.2008.—The mechanisms underlying the development and prevention of diabetic nephropathy are still not fully understood. In the present study in the Otsuka Long-Evans Tokushima Fatty (OLETF) model of type 2 diabetic rats, we investigated whether renal hemodynamic abnormalities exist and whether they precede the onset of diabetes. Using OLETF rats in both prediabetic and diabetic stages, we assessed autoregulatory responses of total renal blood flow (RBF) and of superficial (SBF) and deep renal cortical (DBF) blood flow to stepwise reductions of renal perfusion pressure (RPP) induced by a manual clamp on the abdominal aorta. During clamp-induced reductions of RPP by 10 or 20 mmHg, RBF fell significantly more in OLETF rats than in lean control [Long-Evans Tokushima Otsuka (LETO)] rats. Whereas SBF showed no significant changes in either OLETF rats or LETO rats during mild clamping, DBF decreased significantly more in OLETF rats than LETO rats. Reduced autoregulatory efficiency in OLETF rats was observed in both prediabetic and diabetic stages. Micropuncture studies showed that tubuloglomerular feedback (TGF) responses of stop flow pressure are reduced in prediabetic (−7.3 vs. −25.7%) as well as in diabetic OLETF rats compared with LETO control rats (−4.4 vs. −18.8%). Renal corticotomy was performed to measure glomerular capillary pressure (P_{gc}) directly. P_{gc} of deep cortical glomeruli was higher than superficial glomerular P_{gc} in both strains of rats, but the difference was especially pronounced in OLETF rats (deep 78 ± 2 vs. superficial 57 ± 4 mmHg). This study demonstrates reduced autoregulatory adjustments and impaired TGF efficiency in prediabetic OLETF rats. Thus abnormal RBF regulation precedes the onset of diabetes and is especially pronounced in the deep cortical region.

type 2 diabetes; hemodynamics; deep nephrons; corticotomy; micropuncture; Otsuka Long-Evans Tokushima Fatty

ALTHOUGH DIABETIC NEPHROPATHY is a leading cause of end-stage renal disease in many countries, the complex mechanisms underlying the development of diabetic nephropathy are still controversial. Dysregulation of renal hemodynamics is well supported as a factor in the etiology of diabetic glomerular sclerosis, and it may also act as one of the progression factors. Earlier studies have shown that intraglomerular pressure elevation and glomerular hyperfiltration precede glomerular disease in diabetes and high blood pressure states (6). Another open question is whether the mechanisms responsible for the glomerular disorder differ between superficial and deep nephrons. This is a distinct possibility since there are considerable differences between superficial and deep cortical glomeruli in both structure and function (11, 17, 22). In fact, there is limited evidence to show that the glomerulosclerosis observed in spontaneously hypertensive rats is enhanced in the juxtamedullary cortex (10) and that this may be related to hemodynamic dysregulation in this region of the kidney (21).

Streptozotocin (STZ)-induced diabetes has been widely used in rats as an experimental model of type 1 diabetes mellitus, whereas some strains such as Goto-Kakizaki (GK) rats (13) and Zucker diabetic fatty rats (28) are known as models of type 2 diabetes. However, the disease form in most diabetic models is different from human diabetes and diabetic nephropathy, so that the conclusions drawn are not easily transferable. Recently, a new rat strain with type 2 diabetes [Otsuka Long-Evans Tokushima Fatty (OLETF)] has been described that had a similar mature onset of type 2 diabetes mellitus than observed in humans. Because of the closer resemblance to human diabetic nephropathy, we used these rats to assess whether autoregulatory and tubuloglomerular feedback (TGF) responses as suspected underlying mechanisms of diabetic nephropathy are abnormal, and whether such abnormalities precede the onset of type 2 diabetes mellitus.

OLETF rats show insulin resistance at an early stage, late-onset hyperglycemia, and relatively mild obesity and hyperlipidemia, thus resembling the development of human type 2 diabetes (26). Comparable to the human disease, insulin resistance and overweight become apparent at 10–12 wk of age preceding the appearance of frank hyperglycemia at ~20 wk of age that reaches blood glucose levels of >250 mg/dl at 30 wk. Diabetic nephropathy and renal insufficiency are late symptoms. Like in humans, control of dietary food intake can delay the onset of diabetes. It has been reported that systolic blood pressure and heart rate of OLETF rats begin to rise at ~20 wk (12). The excess of insulin in type 2 diabetes mellitus resulting from insulin resistance or insulin-like growth factor-I may upregulate the Na^+/H^+ transporter of the proximal tubule, thereby increasing Na^+ reabsorption and causing a volume-dependent increase of blood pressure (9). Furthermore, there may be a stimulation of the sympathetic nervous system, but detailed examination in the OLETF rat has not been reported. A previous report by Uriu et al. (26) indicates that hyperfiltration in OLETF rats precedes the development of diabetes and persists until about the 40-wk age range. Urine volume tends to decrease with aging in nondiabetic control [Long-Evans To-

Address for reprint requests and other correspondence: S. Hashimoto, Hokkaido Univ. Graduate School of Medicine, Internal Medicine II, North 15, West 7, Kita-ku, Sapporo, 060-8638, Japan (e-mail: seijinih@med.hokudai.ac.jp).

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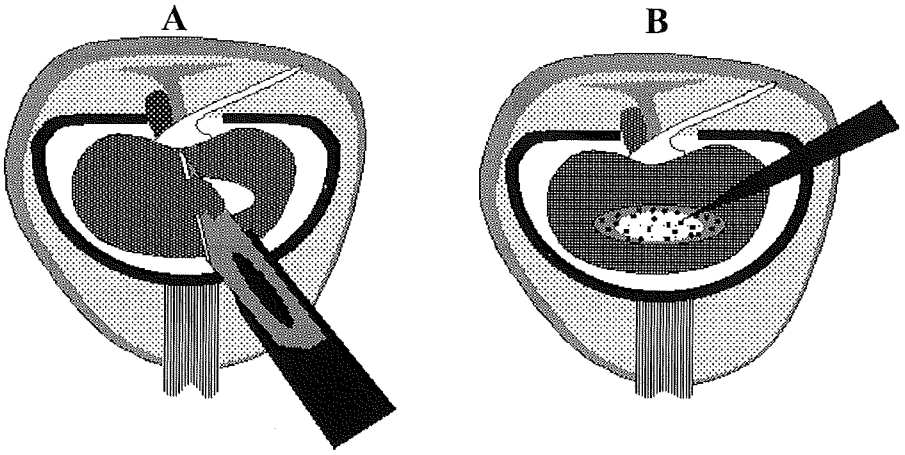

Fig. 1. Procedure of corticotomy and direct puncture of glomerulus. Schema depicts an overview of the left kidney placed in a plastic cup through a flank incision. *A*: center part of the kidney surface was gently sliced away in ca. 2-mm thickness using a surgical blade. *B*: blood clots were cleaned, and the exposed viable glomeruli were punctured under microscope to measure glomerular capillary pressure.

kushima Otsuka (LETO)] rats, whereas a marked diuresis and reduced osmotic urine concentration prevail in OLETF rats. Although some studies have attempted to clarify the underlying mechanisms, a number of questions have remained. For example, it is unclear whether physiological or pathological abnormalities exist already in the prediabetic stage.

The present study demonstrates the presence of hemodynamic abnormalities in OLETF rats at both prediabetic (~11 wk of age) and diabetic stages (~40 wk of age) stages. In addition, OLETF rats exhibit pathological features of renal injury similar to those of human type 2 diabetes (18, 25). These data suggest that renal vascular dysregulation precedes and accompanies diabetic nephropathy.

METHODS

All studies were approved by and performed in compliance with the guidelines and practices of Hokkaido University Graduate School of Medicine.

Animals and preparation. Male 4-wk-old OLETF rats and LETO rats (genetic control of OLETF rats) were supplied by Otsuka Pharmaceutical (Tokushima, Japan). Rats were kept on standard rodent chow and tap water. Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). Blood glucose was measured by Medisafe mini GR 102 (TERUMO, Tokyo, Japan). Rats were anesthetized with 100 mg/kg thiobutabarbital (Inactin) intraperitoneally. Body temperature was maintained at 38.0°C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated, and a stream of 100% oxygen was blown toward the tracheal tube throughout the experiment. Cannulas were placed in the trachea, in the femoral artery for measurement of arterial blood pressure, and in the femoral vein for an intravenous saline infusion at a rate of 1.0 $ml \cdot h^{-1} \cdot 100$ g body wt^{-1}.

Measurements of total renal blood flow and superficial and deep cortical renal blood flow. Experiments were performed in male OLETF and LETO rats both in the prediabetic (10–12 wk) and diabetic (30–40 wk) age range. The femoral artery was catheterized for measurement of arterial blood pressure and blood withdrawal. Mean arterial pressure monitored in the lower abdominal aorta was regarded as renal perfusion pressure (RPP). RPP was set to the desired level by a manual clamp placed above the branching sites of both renal arteries. RPP was reduced in two stages by tightening the clamp mildly or more severely. The left renal artery was approached from a flank incision and carefully dissected free to permit placement of a Doppler blood flow transducer (internal diameter, 1.0 mm; HDP-10, Crystal Biotech, Northborough, MA) connected to a 20-MHz module (PD-20; Crystal Biotech) and dedicated amplifier (VF-1; Crystal Biotech). Because the preparation of the renal artery may interfere with renal nerve integrity, we complemented determinations of total renal blood flow (RBF) with measurements of superficial blood flow (SBF) and deep cortical blood flow (DBF), often in the same rats, but also in independent groups of animals. Regional blood flow of the left kidney was monitored with two glass fiber probes connected to a real-time dual laser Doppler flowmeter (PeriFlux System 5000; Perimed, Stockholm, Sweden). For recordings of superficial and deep cortical flow signals, the probes were held in place at the surface and at a depth of ~3 mm, respectively, and regarded to register SBF and DBF. RBF, SBF, and DBF signals were digitized and analyzed using MacLab software (AD Instruments, Colorado Springs, CO).

Glomerular capillary pressure. Glomerular capillary pressure (P_{gc}) was measured by direct puncture of glomeruli exposed by partial corticotomy using the method of Aukland et al. (1). Although partial kidney cortex removal was obviously traumatic, compression of the cut surface usually caused the bleeding to stop within 15 min, and it was not excessive as judged from unaltered arterial blood pressure. An intravenous injection of FD&C no. 3 green dye was given to identify individual glomeruli as rapidly clearing green spheres. Glomeruli slightly below the surface were chosen for micropuncture to reduce

Table 1. *Profile of OLETF and LETO rats*

	Body Wt, g	Left Kidney Wt, g	Systolic Blood Pressure, mmHg	Blood Glucose, mg/dl
LETO (G1)	331±13	1.1±0.07	110±3	106±6
OLETO (G1)	400±29*	1.5±0.13*	111±2	113±8
LETO (G2)	532±15	1.21±0.03	117±2	118±11
OLETO (G2)	678±11*	2.34±0.15*	139±3*	426±28*

Values are means ± SE. LETO, Long-Evans Tokushima Otsuka; OLETO, Otsuka Long-Evans Tokushima Fatty; G1, 11–13 wk or age; G2, 30–40 wk of age. Body wt, kidney wt, systolic blood pressure, and blood glucose at G1 and G2 are shown. *$P < 0.05$, OLETF vs. LETO rats.

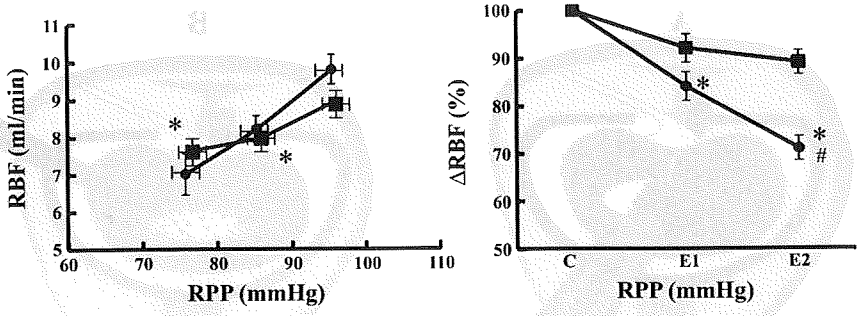

Fig. 2. Renal blood flow (RBF) and percent change from baseline (ΔRBF) during steady state and following a change in renal perfusion pressure (RPP) in 30- to 40-wk Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *Left*: effect of stepwise reductions in RPP on RBF. *Right*: RBF and ΔRBF during experimental periods [baseline (B), time control (C), mild clamp (E1), and severe clamp (E2)] were significantly altered by the change in RPP in OLETF rats (●: $n = 7$) compared with LETO rats (■: $n = 7$). $P <$ 0.05 vs. control (*) and vs. LETO (#).

the risk of selecting nephrons with interrupted proximal tubules. Because of the kidney curvature, glomeruli in the center of the exposed area are at a greater depth, and they were considered deep glomeruli, whereas glomeruli at the edges of the exposed area were taken as superficial glomeruli. The preparation is shown schematically in Fig. 1. Experiments were performed in male OLETF and LETO rats in the 30- to 40-wk age range.

TGF. Experiments were performed in rats of two age groups, 10–12 and 30–40 wk of age. The left kidney was fixed in a Lucite cup with 2% agar, and the kidney surface was immersed in warm saline (37°C). Tubules for study were identified by injecting a small amount of saline colored with lissamine green in a randomly chosen proximal tubular segment with a micropipette. At the identification site, the proximal tubule was then completely blocked by solid wax (Merck, Darmstadt, Germany) injected by a hydraulic pressure system (Effenberger, Attel, Germany). A widened proximal segment upstream from the wax block was gently punctured with a micropipette (OD, 2 μm) filled with 2 M NaCl solution stained with lissamine green and mounted in a servo-null micropressure system (900A; WPI, Sarasota, FL). Measurements of stop flow pressure (P_{sf}) were performed during loop of Henle perfusion to assess TGF function. When P_{sf} had stabilized, loop of Henle perfusion rate was altered between 40 and 0 nl/min in a random fashion. The tubular perfusion solution was an artificial tubular fluid containing 140 mM Na^+, 140 mM Cl^-, 4 mM K^+, 4 mM Ca^{2+}, 8 mM HCO_3, 7.5 mM urea, and 100 mg/100 ml FD&C green.

Glomerular pathology. All rats were killed at 30–40 wk of age. The kidneys of OLETF rats ($n = 5$) and LETO rats ($n = 5$) were removed and fixed in 10% formalin solution, embedded in paraffin, and sliced into 1-mm sections in the transverse plane. Two sections from each rat were stained with periodic acid-Schiff. The renal cortex was equally divided into three zones, and the glomeruli in the outer or the inner zone were regarded as superficial or deep glomeruli, respectively. Fifty glomeruli per rat (25 in superficial cortex and 25 in juxtamedullary cortex) were evaluated with a computer-assisted quantification method using NIH-image software, and average values were obtained for each rat. Total 250 glomeruli of each strain of rats were assessed. The mean glomerular area was measured with a digital imaging analysis that used the Image Analyser LAS-4000 (Fujifilm, Tokyo, Japan). Glomeruli judged to be cut at or near the equatorial plane were selected for morphometric analysis.

Pathological changes were quantified by determining glomerular volume and sclerosis index. Glomerular volume was assessed in hematoxylin and eosin satin tissue sections using the standard stereological technique of Weibel (29) where glomerular volume = $\text{area}^{1.5} \times 1.38/1.01$ (10^6 μm^3) with area = mean glomerular profile area.

Glomerular sclerosis index was quantified as described by Raij et al. (19). Sclerosis area occupied in each glomerular area was scored as 0 (no sclerosis), 1 (0–25% sclerosis), 2 (25–49% sclerosis), 3 (50–74% sclerosis), or 5 (>75% sclerosis).

Statistical analysis. Unpaired *t*-test was used to compare two values between different groups. Multiple groups were analyzed with ANOVA. A P value <0.05 was considered significant.

RESULTS

Blood pressure, body weight, kidney weight, and blood glucose. At 10–12 wk of age, body weight of OLETF rats was greater compared with LETO rats (Table 1), whereas there was no significant difference in blood glucose between both strains of rats. In contrast, at 30–40 wk of age, OLETF rats showed higher blood glucose levels than LETO rats (Table 1). Systolic blood pressure remained unaltered in LETO rats, whereas the hyperglycemia in 30- to 40-wk-old OLETF rats was accompanied by an elevated blood pressure.

Total blood flow, SBF, and DBF at the diabetic stage. RPP at baseline was not significantly different between OLETF rats and LETO rats (OLETF rats: 95.4 ± 2.0 mmHg, LETO rats: 96.0 ± 1.2 mmHg). RPP fell by ~10 mmHg (OLETF rats: 10.2 ± 0.7 mmHg, LETO rats: 9.8 ± 0.8 mmHg) with mild clamping of the abdominal aorta (E1) and by ~20 mmHg (OLETF rats: 19.7 ± 0.8 mmHg, LETO rats: 19.5 ± 0.8 mmHg) with moderate clamping (E2) in both groups of rats. There were no significant differences between either group at the E1 and E2 period. Continuous recordings of RBF, SBF, and DBF showed that there were no significant differences between

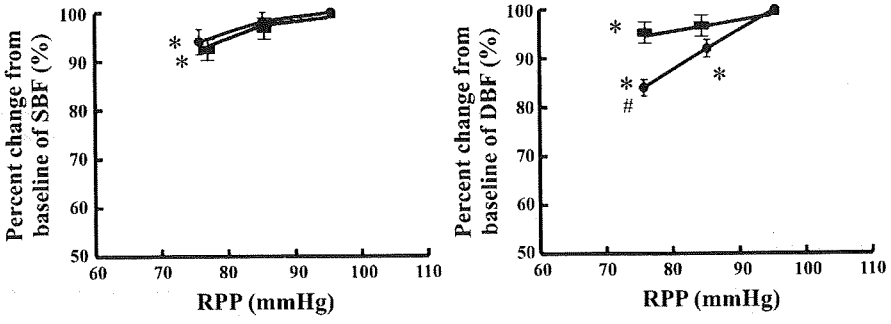

Fig. 3. Percent change from baseline of superficial cortical blood flow (ΔSBF) and deep cortical blood flow (ΔDBF) during steady state and change in RPP in 30- to 40-wk LETO rats and OLETF rats. During experimental periods, the regional blood flow was significantly affected by the change in RPP in OLETF rats (●: $n = 7$) compared with those in LETO rats (■: $n = 7$). ΔDBF was more vulnerable than ΔSBF in OLETF rats. $P < 0.05$ vs. control (*) and vs. LETO (#).

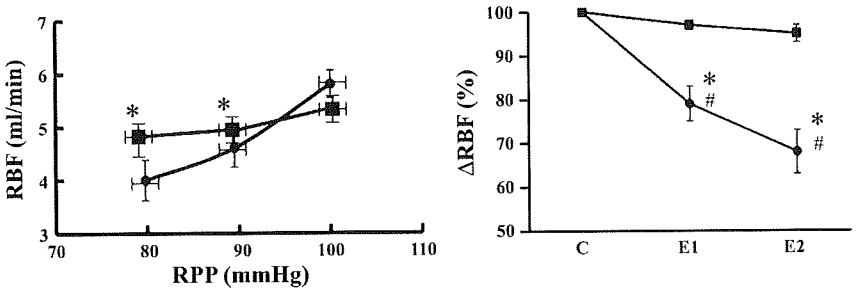

Fig. 4. RBF and ΔRBF during steady state and following a change in RPP in 10- to 12-wk LETO rats and OLETF rats. *Left*: effect of stepwise reductions in RPP on RBF. *Right*: RBF and ΔRBF during experimental periods were significantly altered by the change in RP in OLETF rats (●: $n = 6$) compared with LETO rats (■: $n = 7$). $P < 0.05$ vs. control (*) and vs. LETO (#).

RBF of OLETF rats (9.8 ± 0.43 ml/min) and LETO rats (8.6 ± 0.44 ml/min) under basal conditions, although there was a tendency for a higher RBF in the OLETF group. RBF of OLETF rats fell significantly at the E1 (to 8.2 ± 0.35 ml/min) and E2 (to 7.0 ± 0.55 ml/min) stage. In contrast, RBF of LETO rats did not change significantly at the E1 stage (7.9 ± 0.4 ml/min) but fell significantly at the E2 stage (to 7.6 ± 0.4 ml/min). Expressed as percent change from baseline, RBF of OLETF rats fell to 84.0 ± 4.3% at E1 and to 71.3 ± 4.8% at E2. RBF in LETO rats at E2 fell to 88.5 ± 2.5% of baseline. Autoregulation curves are shown in Fig. 2.

Measurements of regional renal hemodynamics are summarized in Fig. 3. With baseline values set at 100%, there were no significant changes of SBF at the E1 level of clamping. At the E2 level, SBF fell in both OLETF (to 94 ± 2.6%) and LETO (to 91 ± 3%) rats, with differences between both rat strains not being significant. In contrast, DBF of OLETF fell significantly to 92.2 ± 1.8% at the E1 stage and to 84 ± 1.7% at the E2 stage, whereas DBF of LETO rats only fell modestly at the E2 level (to 95.0 ± 3.7%). The fall of DBF at the E2 level was significantly greater in OLETF rats than LETO rats.

Total, SBF, and DBF at the prediabetic stage. RPP at baseline was not significantly different between OLETF rats and LETO rats (OLETF rats: 100.5 ± 0.6 mmHg, LETO rats: 99.5 ± 2.6 mmHg). RPP fell by ~10 mmHg (OLETF rats: 11.0 ± 0.7 mmHg, LETO rats: 11.7 ± 2.0 mmHg) with mild clamping of the abdominal aorta (E1) and by ~20 mmHg (OLETF rats: 20.7 ± 0.9 mmHg, LETO rats: 21.7 ± 1.3 mmHg) with moderate clamping (E2) in both groups of rats, changes of RPP similar to those induced in the older animals. There were no significant differences between RBF of OLETF rats (5.8 ± 0.26 ml/min) and LETO rats (5.1 ± 0.29 ml/min) under basal conditions (Fig. 4). RBF of OLETF rats fell significantly at the E1 (to 4.6 ± 0.36 ml/min) and E2 (to 4.0 ± 0.38 ml/min) stage. In contrast, RBF of LETO rats did not change significantly at either the E1 stage (5.0 ± 0.31 mmHg) and E2 stage (to 4.9 ± 0.29 ml/min). Expressed as percent change from baseline, RBF of OLETF rats fell to 80.0 ± 3.9% at E1 and to 69.0 ± 5.2% at E2. RBF in LETO rats at E2 fell only to 95.3 ± 2.0% of baseline ($P > 0.05$).

Measurements of regional renal hemodynamics are summarized in Fig. 5. With baseline values set at 100%, there were no significant changes of SBF at the E1 level of clamping. At the E2 level, SBF fell significantly in OLETF (to 92 ± 4.5%), whereas there was no significant reduction of SBF in LETO rats (to 97 ± 3.0%). DBF of OLETF fell significantly to 89 ± 3.7% at the E1 stage and to 80 ± 4.8% at the E2 stage, whereas DBF of LETO rats only fell modestly at the E2 level (to 95 ± 5.4%). The fall of DBF at the E2 level was significantly greater in OLETF rats than LETO rats.

P_{gc}. With the use of the method of partial corticotomy, measurements of P_{gc} were performed in 9 superficial and 12 deep cortical glomeruli in 4 OLETF and 4 LETO rats (Fig. 6). Blood pressure did not show a significant change before and after partial corticotomy. P_{gc} in OLETF rats was significantly higher than in LETO rats in both superficial and deep renal cortex (deep cortex: 78.5 ± 2.7 vs. 58.5 ± 1.6 mmHg; superficial cortex: 57.5 ± 4 vs. 48.2 ± 2 mmHg). In addition, deep cortical P_{gc} was higher than superficial P_{gc} in both strains of rats.

TGF. Micropuncture evaluation of TGF was conducted in four OLETF and four LETO rats at 10–12 wk of age and in four OLETF and four LETO rats at 30–40 wk of age. Thirteen nephrons were studied in each of the four groups. At 10–12 wk, arterial blood pressure averaged 106 ± 1.0 mmHg in OLETF and 107 ± 1.3 mmHg in LETO rats. P_{sf} of OLETF rats was 41.0 ± 2.5 mmHg, not significantly different from the average of 41.6 ± 1.9 mmHg in LETO rats. During perfusion of the loop of Henle with artificial tubular fluid at 40 nl/min, P_{sf} fell to 37.7 ± 2.1 mmHg in OLETF rats (a reduction of 7.2 ± 1.8% compared with zero perfusion) and to 30.7 ± 1.3 mmHg in LETO rats (a reduction of 25.0 ± 2.3%). Thus TGF responses were significantly attenuated in prediabetic rats (Fig. 7). Identical experiments were performed in OLETF and LETO

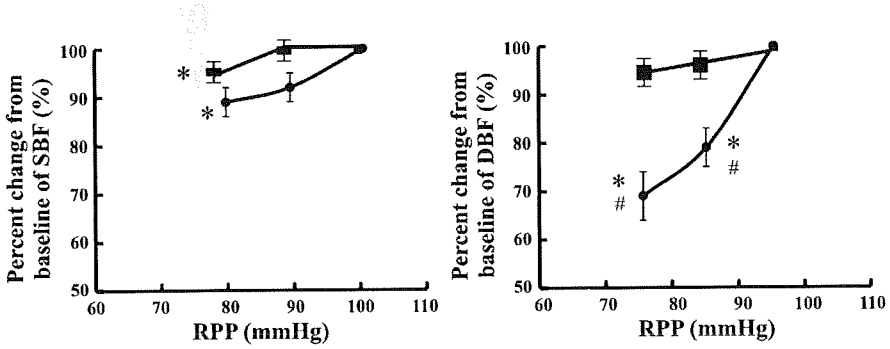

Fig. 5. Percent change from baseline of ΔSBF and ΔDBF during steady state and change in RPP in 10- to 12-wk LETO rats and OLETF rats. During experimental periods, the regional blood flow was significantly affected by the change in RPP in OLETF rats (●: $n = 6$) compared with those in LETO rats (■: $n = 7$). ΔDBF was more vulnerable than ΔSBF in OLETF rats. $P < 0.05$ vs. control (*) and vs. LETO (#).