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IV. 研究成果の刊行物・別刷

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Clinical Importance of *Stenotrophomonas maltophilia* Nosocomial Pneumonia Due to its High Mortality in Hemodialysis Patients

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Abstract: Patients undergoing hemodialysis are immunocompromized and can suffer from pneumonia with various pathogens in nosocomial conditions. We investigated the fundamental information on the characteristics of hemodialysis inpatients and nosocomial pneumonia. We surveyed 1803 hemodialysis patients admitted to our university hospital between 2001 and 2007. The mean patient age was 64.8 years and the average period of hospitalization was 28.1 days, which was considerably longer than the average stay in our hospital (14.2 days). Patients were admitted to many different departments and for various reasons. We isolated 391 microorganisms from the sputum of 120 pneumonia patients undergoing hemodialysis, including *Candida albicans*, methicillin-resistant *Staphylococcus aureus*, and *Staphylococcus epidermidis*, which were the leading three isolates. From these 120 patients, a total of 199 pathogens were identified as being responsible for the pneumonia. Multi-drug resistant *Stenotrophomonas maltophilia* was found to be susceptible to a new fluoroquinolone, but is resistant to older generation quinolones. Out of the 120 patients with pneumonia, 12 out of 18 patients infected with *S. maltophilia* died, indicating the highest fatality rate for this pathogen. In this survey, we found that hemodialysis patients were hospitalized for long periods, and for various reasons in many departments. They suffered from nosocomial pneumonia caused by multi-drug resistant pathogens, including *S. maltophilia*. For pneumonia due to *S. maltophilia*, new generation fluoroquinolones can be the treatment of choice, although *S. maltophilia*-related pneumonia should be treated very carefully because of its high fatality rate. **Key Words:** Fatality, Hemodialysis, Multi-drug resistance, Nosocomial pneumonia, Quinolone, *Stenotrophomonas maltophilia*.

Infectious diseases are important causes of morbidity and mortality among patients with end-stage renal disease (ESRD), which is ranked as the second leading cause of death in Japanese hemodialysis (HD) patients, following cardiovascular diseases. It has been reported that among the various infectious diseases, approximately 20% is attributed to pneumonia (1). In addition, the mortality rate of pulmonary infection in hemodialysis patients has been reported to be 14- to 16-fold higher than in the general population (1). An increased susceptibility to infection has been ascribed partly to old age, a high prevalence of diabetes mellitus, defective phagocytic function of granulocytes, and frequent exposure to potential infectious risk factors during the normal course of dialysis therapy (2). Anemia and malnutrition also contributed to the immunocompromized status of HD patients. In addition, HD patients usually have problems other than infection, such as cardiovascular or musculoskeletal disorders and are frequently hospitalized because of operations and for reasons other than infection, where they are treated with various antibiotics over a long period of time. These typical clinical courses of HD patients have some influence on the clinical characteristics and microbiological features of pneumonia in HD patients. In this article, we describe the prevalence and microbiological characteristics of nosocomial pneumonia in HD patients admitted to Keio University Hospital. Our surveillance revealed that patients

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undergoing HD were susceptible to nosocomial pneumonia by multi-drug resistant microorganisms, which included methicillin-resistant *Staphylococcus aureus* (MRSA) and *Stenotrophomonas maltophilia*. The fatality rate of patients infected with *S. maltophilia* was 66.7%, which indicates that *S. maltophilia*-related respiratory tract infection calls for careful attention because of its high fatality rate.

PATIENTS AND METHODS

Patients

Between April 2001 and March 2007, 1803 patients underwent hemodialysis in the hemodialysis center of Keio University Hospital. These patients were admitted to our hospital for HD from outpatient clinics or HD was initiated in our hospital because of acute renal failure for various reasons, including surgical operations and chemotherapy cancer treatment.

Diagnosis of nosocomial pneumonia

We checked the past medical records and surveyed the results of sputum culture examination obtained from all patients suspected to suffer from an upper respiratory infection. This investigation aimed to elucidate the clinical pictures of the patients undergoing chronic dialysis therapy. Patients with acute renal failure were immunocompromized, not because of renal insufficiency itself, but because of cancer chemotherapy or malnutrition for surgical procedures; therefore, patients with acute renal failure who were had begun HD were excluded from further analysis.

Specimens were obtained twice from each patient to confirm the diagnosis. The diagnosis of infectious pneumonia combines clinical findings, laboratory microbiological data, and confirmatory chest radiographic findings. A compatible clinical picture included fever, cough, and/or auscultatory findings such as rales, and/or evidence of pulmonary consolidation. Sputum specimens were washed, Gram stained and cultured quantitatively (3). Since it is difficult to identify the etiologic agent for pneumonia and the purpose of this study is to survey the related organisms for pneumonia in HD patients, all the organisms present in the specimen when the patients were clinically diagnosed with pneumonia were regarded as potential pathogens; therefore, in some cases several organisms were related to pneumonia in the same patient. These surveillance protocols were approved of by the ethical committee of the School of Medicine of Keio University.

Microbiological data

All antimicrobial agents were obtained from their manufacturers in the form of standard laboratory powder. In the disk diffusion sensitivity test, Sensidisks (Nippon Becton Dickinson, Tokyo, Japan) were used according to the Kirby–Bauer method (4). The minimal inhibitory concentration (MIC) was determined by a standard agar-dilution method. The antibiotics examined included the following: ceftazidime (CAZ), cefpirome (CPR), cefepime (CFPM), cefotaxime (CTX), amikacin (AMK), gentamicin (GM), aztreonam (AZT), imipenem/cilastatin (IPM/C), meropenem (MEPM), ciprofloxacin (CPFX), and levofloxacin (LVFX). The MIC was taken as the lowest concentration showing no visible growth (5).

RESULTS

Patients characteristics

Of the 1803 patients examined, 67.6% of patients were male and the mean patient age was 64.8 years. Figure 1A shows the distribution of patient ages, which indicates that approximately 70% of the patients were over 60 years of age. Diabetic patients made up 28.8% of all patients. The average period of hospitalization was 28.1 days, ranging from one day to 478 days, which was longer than the average inpatient stay of 14.2 days for our hospital. As shown in Figure 1B, approximately half of the patients were hospitalized for more than 15 days. Since our hospital is a general hospital, patients were admitted to

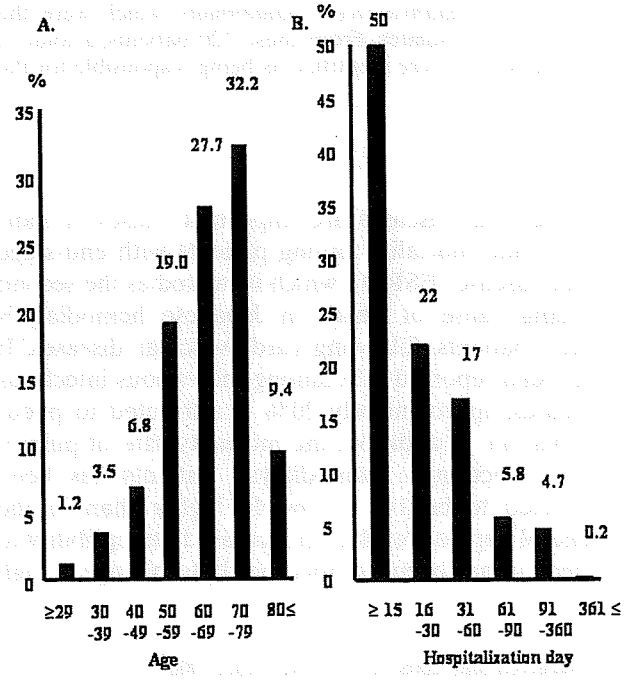

FIG. 1. Distributions of age and hospitalization periods. Distributions of (A) age and (B) hospitalization periods were investigated in 1803 patients who underwent hemodialysis in the Keio University Hospital between April 2001 and March 2007.

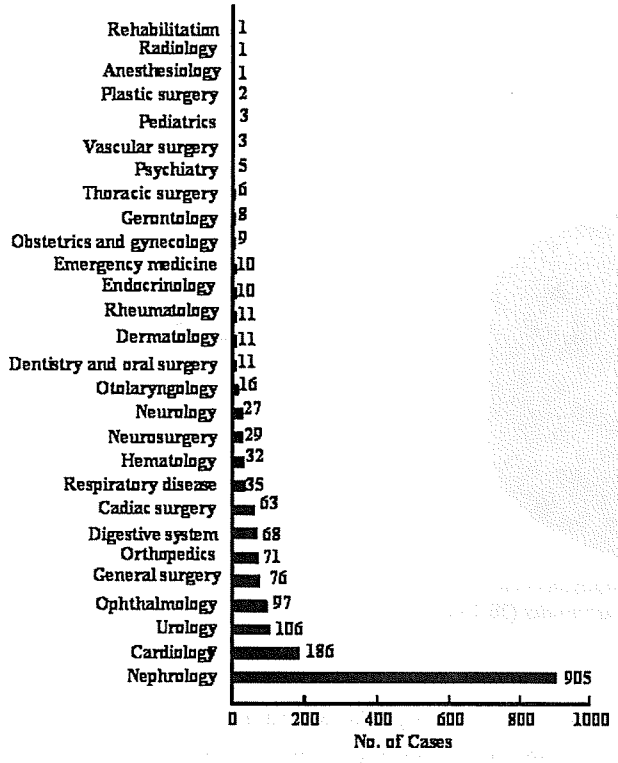

FIG. 2. The different medical departments where the 1803 patients who underwent hemodialysis at the Keio University Hospital were admitted between April 2001 and March 2007.

various departments and for various reasons, including the departments of internal medicine (70.7%), surgery (6.4%), orthopedics (3.9%), ophthalmology (5.4%), and so forth, as shown in Figure 2.

Sputum culture

Of the 1803 patients, sputum specimens were collected from 120 patients (6.7%) who were diagnosed with pneumonia. Figure 3 shows the microbiological spectrum of the specimens from these 120 patients (330 microorganisms were isolated in total). Of all the isolates documented, *Candida albicans* was most frequently detected. As with gram-positive bacteria, MRSA and *Staphylococcus epidermidis* were frequently isolated; and in terms of gram-negative bacteria, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *S. maltophilia* were detected frequently. Figure 4 illustrates the percentages of each organism associated with clinically diagnosed pneumonia. A total of 199 from 330 organisms were identified as pathogens from the sputum specimens of the 120 pneumonia patients. Of these organisms 21.6% were *C. albicans* and 18.1% were MRSA. Gram-positive bacteria (MRSA and *S. epidermidis*) accounted for 38.2% of all the detected organisms. Gram-negative bacteria (*Enterococcus faecalis*, *P. aeruginosa*, *S. maltophilia*, *Serratia marcescens*) accounted for 36.6%. Of all the organisms detected, the multi-drug resistant bacteria were MRSA and *S. maltophilia*, which accounted for 27.1%.

S. maltophilia-related pneumonia in HD patients

We examined the relationship between the number of deaths of HD patients and these pathogens. As shown in Table 1, the fatality for MRSA-infected HD patients was 41.9%, and that for *C. albicans* was 41.7%. Of note, 12 out of 18 patients infected with *S. maltophilia* died, which indicates the highest fatality (66.7%). The average HD duration of patients with *S. maltophilia* infection was 5.6 ± 2.3 years. Therefore, it can be said that *S. maltophilia*-related pneumonia occurred after long-term maintenance HD.

Antibiotic susceptibility of *S. maltophilia*

The antibiotic susceptibility of *S. maltophilia* in our hospital was examined. Most of the cephalosporins and carbapenems showed poor activity, although ceftazidime exerted some in vitro activity (Table 2). Aminoglycosides show some activity, which was a unique characteristic of *S. maltophilia* in this study. Of

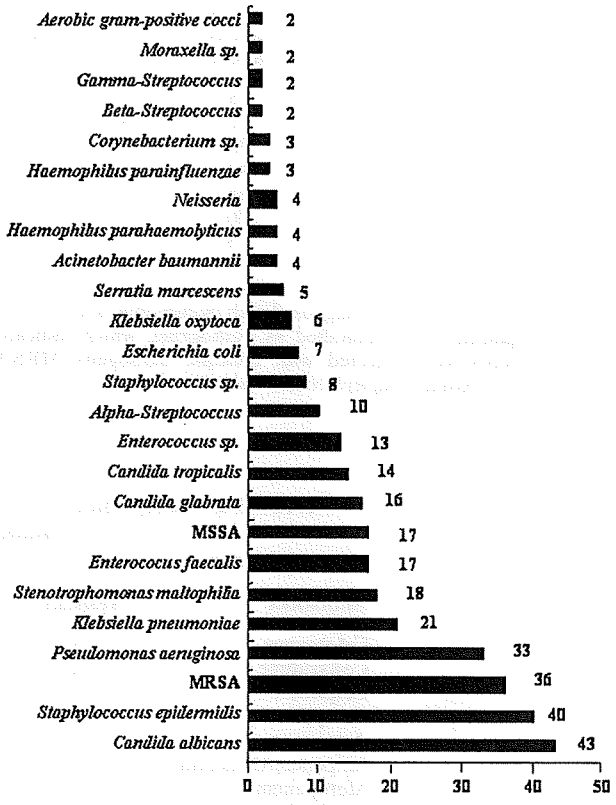

FIG. 3. The microbiological spectrum of the sputum specimens for the 120 patients who were suspected of and diagnosed with pneumonia. MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*.

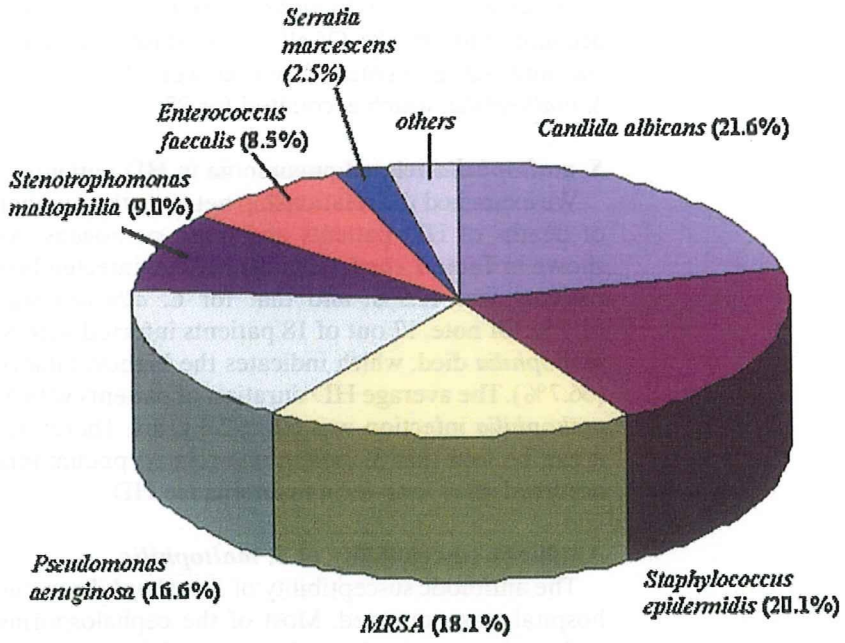

FIG. 4. The percentage of organisms associated with clinically diagnosed pneumonia. MRSA, methicillin-resistant *Staphylococcus aureus*.

TABLE 1. *Fatality of nosocomial pneumonia by various microorganisms*

Organism†	No. of infected patients	No. of deaths	Fatality (%)
Stenotrophomonas maltophilia	18	12	66.7
Enterococcus faecalis	17	9	52.9
Pseudomonas aeruginosa	33	14	42.4
Candida albicans	43	18	41.9
MRSA	36	15	41.7
Staphylococcus epidermidis	40	10	25.0
Serratia marcescens	5	1	20.0
Others	7	0	0

†From the 120 patients diagnosed with pneumonia, a total of 199 microorganisms were identified as pathogenic, which indicates that patients were infected with multiple pathogens. MRSA, methicillin-resistant *Staphylococcus aureus*.

note, the *S. maltophilia* found in this study is resistant to older generation quinolones, such as ciprofloxacin, but is susceptible to new generation fluoroquinolones, such as levofloxacin, which is similar to a previous report (6,7).

DISCUSSION

In this study, we examined the characteristics of nosocomial pneumonia in patients undergoing HD. One of the characteristic clinical courses of HD patients is the long hospitalization periods, a factor that would contribute to the high probability of nosocomial infection and the wide variety of the organisms in the sputum cultures in this study. Regarding pneumonia, the typical community-acquired pathogens such as *Streptococcus pneumonia* and *Haemophilus*

TABLE 2. *Susceptibility of* Stenotrophomonas maltophilia *to selected antimicrobial agents*

Antibiotics	Resistant	Intermediate	Susceptible	% Isolates susceptible
Cefotaxime	19	4	4	15
Ceftazidime	11	5	10	38
Cefpirome	20	2	1	4
Cefepime	15	7	2	8
Aztreonam	20	1	1	5
Imipenem/Cilastatin	22	0	0	0
Meropenem	24	0	0	0
Amikacin	6	1	6	46
Gentamicin	5	0	6	55
Ciprofloxacin	8	2	1	9
Levofloxacin	2	2	6	60

influenzae were not documented in our list of microorganisms, while MRSA and *C. albicans* were most frequently detected. In addition, HD patients were admitted to various sections of the hospital, which indicated that the reasons for the hospitalization included many disorders. Hemodialysis patients suffer from various complications, including cardiovascular, infectious, and metabolic bone diseases. This clinical feature of multiple complications was attributed to the high frequency of hospitalization and the frequent use of antibiotics, which also affected the flora of the existing microorganisms and their susceptibility to antibiotics. We described all the microorganisms found in the sputum culture, which revealed that multi-drug resistant organisms such as MRSA and *S. maltophilia* were frequently detected. These results were probably because of the prior frequent use of broad-spectrum antibiotics in previous hospitalizations.

S. maltophilia is one of the increasingly important organisms complicated in numerous nosocomial infections. The clinical manifestations include bacteremia, respiratory infections, endocarditis, urinary tract infections and gastrointestinal infections. This microorganism has the propensity to cause serious infections in immunocompromized hosts, such as patients undergoing a transplantation or patients with cancer (8). Our survey revealed the high mortality of *S. maltophilia*-infected HD patients. Patients undergoing hemodialysis are susceptible to infectious diseases, which may lead to life-threatening complications. The immune defect in uremia is multifactorial, mainly related to uremic toxicity, anemia, iron overload, dialyzer membrane biocompatibility, and malnutrition. Moreover, *S. maltophilia* easily becomes multi-drug resistant. In the previous paper, a number of risk factors were reported to be associated with the acquisition of *S. maltophilia* infections, including the prior use of carbapenems or quinolones and admission to an intensive care unit within 30 days of isolation of the pathogens (9). Other risk factors for infection by *S. maltophilia* include mechanical ventilation and severe mucositis (10). Although 17% (3 patients out of 18) of patients in our study were treated in an intensive care unit, approximately 40% (7 patients) of the patients underwent treatment with a mechanical ventilator. These backgrounds contributed to the high morbidity of *S. maltophilia*-related pneumonia in this study. The high fatality of *S. maltophilia* infection has been also reported in another study where 50% of immunocompromized patients with pneumonia due to *S. maltophilia* patients died (11). This might be due to the lack of effective antibiotics or because this organism was dominant when patients were immunocompromized and severely ill. In our survey, HD patients had various complications other than pneumonia, were hospitalized for long periods, and were frequently diabetic. These factors altogether attributed to the high prevalence and mortality of *S. maltophilia* infection.

Susceptibility of *S. maltophilia* to early examples of the quinolone class of antimicrobial agents, including ciprofloxacin, varies in different clinical settings. However, both superinfection by *S. maltophilia* in patients receiving quinolones and therapeutic failures of *S. maltophilia* infection by these quinolones have been reported (12,13). On the other hand, newer quinolones such as clinafloxacin and sparfloxacin appear to be more active against the bacterium than the older agents in this class (14). It has been reported that 93% of 25 ciprofloxacin-resistant strains were susceptible to clinafloxacin (15). The present data have also shown that only 5% of *S. maltophilia* isolated from HD pneumonia patients were susceptible to ciprofloxacin, while 57% were susceptible to levofloxacin. There are scant reports on the effective use of the newer quinolones in clinical settings towards *S. maltophilia*-related infection and prospective studies are awaited in the future. Several mechanisms were demonstrated for the acquisition of quinolone resistance for *S. maltophilia*. For instance, the inhibition of DNA gyrase II or DNA topoisomerase by bacterial production was responsible for the resistance to earlier generation quinolones. Gene mutation of these enzymes is also another mechanism for the resistance to quinolones. The reason why patients with HD acquire resistant strains is also considered to be attributable to the characteristics of HD patients; that is, the frequent use of antibiotics, frequent hospitalizations, immunocompromized state, and so on. These data warranted us to use antibiotics carefully for patients undergoing maintenance HD.

CONCLUSION

Our survey of 1803 HD patients revealed that these patients frequently suffer from nosocomial pneumonia with multi-drug resistant pathogens. Among the 199 pneumonia patients undergoing maintenance hemodialysis, 66.7% patients who were infected with *S. maltophilia* died, which is the highest fatality rate for all the pathogens identified. *S. maltophilia*-related infection should be treated with the utmost care as *S. maltophilia* is multi-drug resistant and its fatality rate is high.

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Original Article

Brachial-Ankle Pulse Wave Velocity Predicts Silent Cerebrovascular Diseases in Patients with End-Stage Renal Diseases

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Aim: Cerebrovascular disease (CVD) is a major determinant of the prognosis in end-stage renal diseases (ESRD). The purpose of this study was to examine whether factors associated with arterial stiffness contributed to the development of CVD in patients with ESRD.
Methods: CVD (lacunes and carotid/intracranial artery stenosis) was evaluated with brain magnetic resonance imaging (MRI), and carotid/intracranial artery magnetic resonance angiography (MRA) in 44 pre-dialytic patients. The severity of CVD was evaluated by the number of lacunes and the degree of stenosis, respectively. The association between CVD and atherosclerotic parameters was evaluated.
Results: Patients with severe lacunes ($n=18$) manifested older age, lower diastolic blood pressure, serum creatinine and albumin, and higher CRP and serum calcium than those with absent-moderate lacunes ($n=26$). When assessed by multivariate analysis, only baPWV was adopted as an independent risk factor for severe lacunes. Furthermore, baPWV and i-PTH were associated with the severity of carotid/intracranial artery stenosis, both of which were independent of other risk factors, including age and diabetes.
Conclusions: Arterial stiffness may constitute a novel determinant predicting the severity of CVD in pre-dialytic patients besides classical risk factors.

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Key words; End-stage renal disease, Lacunar infarction, Carotid/intracranial artery stenosis, Malnutrition-inflammation-atherosclerosis syndrome

Introduction

Cerebrovascular disease has been established as one of the major causes of death among patients with chronic kidney disease[1, 2]. Several lines of studies have revealed a high incidence of cerebrovascular accidents in chronic dialysis patients[3-5] and kidney transplant recipients[6-8]. Furthermore, silent cerebral infarction, including lacunes, is frequently observed in patients on hemodialysis, which is recognized as a predictive marker for major stroke and vascular events[9]. Nevertheless, factors responsible for the progression of atherosclerosis and their associated markers for the detection of cerebrovascular disease in pre-dialytic and pre-transplant chronic kidney disease (CKD) patients remain undetermined.

Although atherosclerosis represents the pathological processes of the vascular wall, several lines of investigation have delineated functional vascular stiffness using a more clinically available procedure, brachial-ankle pulse wave velocity (baPWV). With this method, previous studies showed that the level of PWV paralleled the incidence of coronary heart disease in hypertensive patients, and suggested PWV as a useful independent predictor of cardiovascular disease[10, 11]. The role of baPWV or other atherogenic factors in the development of cerebrovascular disease, however, has not been examined fully in patients with end-stage renal disease (ESRD).

In the present study, we attempted to characterize cerebrovascular damage in pre-dialytic and pre-transplant patients. We hypothesized that baPWV

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predicted silent cerebrovascular damage as well as coronary heart disease[10, 11], and therefore examined the relationship between cerebrovascular damage and baPWV in patients with ESRD.

Subjects and Methods

Study Protocol

This study was a cross-sectional study performed in Saiseikai Nakatsu General Hospital (Osaka, Japan) and Keio University Hospital (Tokyo, Japan). Fifty-three subjects with ESRD (CKD stage 5) but not receiving dialysis therapy gave written informed consent between September 2002 and September 2008. Among these patients, nine candidates were excluded from the study due to episodes of major stroke in their medical history, clinically apparent symptoms related to cerebrovascular disorder (i.e. transient ischemic attacks; TIA) or peripheral artery disease (defined as an ankle brachial pressure index <0.9) and sustained atrial fibrillation. In the remaining 44 patients, the effects of various factors, including baPWV, on cerebrovascular damage were evaluated. The medical history was obtained for all patients; ischemic heart disease was defined as having angina pectoris and old myocardial infarction was confirmed with ECG/coronary angiography and medical history.

Brain MRI and carotid/intracranial MR angiography (MRA) were examined to assess cerebrovascular damage, including lacunes and carotid/intracranial artery stenosis. Although the potential occurrence of nephrogenic systemic fibrosis prevented the use of gadolinium-containing contrast medium[12], clinically reliable high resolution images were still obtainable[13-15]. The prevalence of lacunes and carotid/intracranial artery stenosis was further compared between the diabetes (DM) group and the non-DM group. Lacunes and stenosis were evaluaed by experienced stroke neurologists and confirmed by radiologists, both performed in a blind fashion without the patients' clinical information. The institutional ethics committee approved the protocol.

Evaluation of baPWV and Cerebrovascular Damages

The baPWV was evaluated using a pulse pressure analyzer (model BP-203RPE; Nihon Colin, Komaki, Japan). Prior to measurement of baPWV, patients were advised to have a 15-minute rest, during which their heart rate settled to 60 to 80 beats/min.

Silent cerebral infarction and lacunar infarction were evaluated with MRI whereby brain images were obtained in 10 mm thick slices. Lacunes were defined as small (diameter <15mm) high-intensity lesions on T2-weighted images, with a corresponding distinctive low-intensity area on T1-weighted images[16]. The degree of lacunes has been reported to be classified into 3 grades; normal-mild (total number of lacunes: 1-3), moderate (4-10) and severe (>10)[17]. In this study, however, normal-mild and moderate groups were combined because the patients with normal-mild lacunes were very few in our ESRD patient population.

MRA was obtained using the 3-dimensional time-of-flight gradient echo technique for intracranial and carotid arteries[18]. Evaluation of arterial stenosis was conducted in five arteries, i.e., bilateral middle cerebral arteries, bilateral internal carotid arteries and basilar artery. The levels of stenotic changes were initially classified into the following grades; normal, mild (signal reduction $<50\%$), moderate (signal reduction $\geqq 50\%$), severe (focal signal loss with the presence of distal artery signal) and occlusion[13, 18]. Because very few subjects fell into the normal, severe and occlusion groups, the patients were divided into two groups (normal-mild group and moderate-severe stenosis and occlusion group).

Data Analysis

Statistical analysis was performed using the SPSS 12 software package (SPSS, Chicago, USA). All variables were tested for distribution normality using the Shapiro-Wilk test. Normally and non-normally distributed variables were expressed as the mean ± SD and the medians (25 to 75 percentiles), respectively. The clinical features were analyzed by unpaired t-test for normally distributed continuous variables, by the Mann-Whitney U test for non-normally distributed variables and by χ^2 test for categorical variables. Variables showing significant differences between the normal to moderate group and severe group by univariable analysis were entered into multilinear regression analysis to elucidate the influence of each variable on the development of lacunes. Similarly, multivariate logistic regression analysis was performed to determine whether each variable contributed to the progression of moderate to severe stenosis and occlusion without the influence of other variables. Differences with $p<0.05$ were considered significant.

Results

Cerebral Lacunes

Table 1 shows the profiles of the patients enrolled in this study. Among 44 patients enrolled, 18 patients (i.e., 40.9%) had severe lacunes, and were older than those with absent to moderate lacunes ($p=0.006$).

Table 1. Profiles of patients with various degrees of lacunes

Parameters	Patients with absent to moderate lacunes ($n=26$)	Patients with severe lacunes ($n=18$)	p
Age (y/o)	60.8±10.7	69.9±9.1	0.006
Gender (male/female)	22/4	13/5	NS
BMI (kg/m^2)	21.4±3.6	21.3±3.5	NS
Smoking (%)	50.0	52.9	NS
Diabetes (%)	46.2	61.1	NS
Ischemic heart disease (%)	23.1	44.4	NS
SBP (mm/Hg)	145 (140-155)	140 (114-158)	NS
DBP (mm/Hg)	87±10	75±16	0.009
Pulse pressure (mm/Hg)	59.5 (55-66)	66 (53-76)	NS
CTR (%)	51.1±7.5	54.2±4.8	NS
baPWV (cm/s)	1759.0±238.9	2068.1±463.9	0.019
BUN (mg/dL)	63.1 (81.8-91.4)	46.8 (56.0-82.5)	NS
Serum creatinine (mg/dL)	8.2±2.5	6.7±1.8	0.035
eGFR	5.9(4.6-7.4)	6.9 (5.9-8.2)	NS
Hct (%)	27.5±4.1	27.5±4.7	NS
Serum Albumin (g/dL)	3.8±0.4	3.5±0.4	0.018
CRP (mg/dL)	0.09 (0.04-0.29)	0.30 (0.11-0.58)	0.042
Serum cCa (mg/dL)	8.4±0.9	9.1±0.9	0.038
Serum phosphorus (mg/dL)	5.6±1.7	5.0±1.1	NS
cCa×P (mg^2/dL^2)	46.6±13.8	46.2±13.5	NS
i-PTH (pg/mL)	220.0 (158.0-299.0)	173.0 (94.0-279.5)	NS
HbA1c (%)	5.3±0.5	5.1±0.9	NS
Total cholesterol (mg/dL)	165.5 (134.0-197.3)	169.0 (133.0-187.5)	NS
LDL-cholesterol (mg/dL)	97.2±29.5	90.2±24.8	NS
Triglyceride (mg/dL)	120.5 (100.8-151.5)	172.0 (91.0-284.8)	NS

BMI, CTR, eGFR and Hct indicate body mass index, cardiothoracic ratio, estimated glomerular filtration rate and hematocrit, respectively. Serum cCa and cCa×P mean albumin-corrected calcium and calcium-phosphorus product, respectively. Values are the mean±SD for normality and median (25-75 percentiles) for non-normality.

There was no predilection for DM or ischemic heart disease in patients with severe lacunes ($p=0.329$ and $p=0.135$, respectively). The rate of the use of antihypertensive agents, including ACE inhibitors, angiotensin receptor blockers (ARB) and Ca antagonists, as well as other drugs (statins and calcium-based phosphate binders) did not differ between these two groups (data not shown).

The patients with severe lacunar infarction had greater levels of baPWV ($p=0.019$), serum albumin-corrected calcium (cCa, $p=0.038$) and CRP ($p=0.042$), and manifested lower DBP ($p=0.009$), serum creatinine ($p=0.035$) and serum albumin ($p=0.018$, **Table 1**). SBP or other factors, such as total cholesterol, LDL-cholesterol and triglyceride, did not affect the level of lacunes significantly. The number of lacunes was correlated with baPWV ($r=0.349$, $p=0.022$, **Fig. 1A**) as well as age ($r=0.375$, $p=0.012$, **Fig. 1B**). Similarly, the number of lacunar infarctions was inversely associated with DBP ($r=-0.352$, $p=0.019$, **Fig. 1C**). Serum albumin or CRP levels, however, did not correlate with the number of lacunar infarctions ($p=0.117$ and 0.791, respectively; **Fig. 1D, 1E**).

To elucidate the specific contribution of each parameter to lacunar infarction, multilinear regression analysis was conducted. Thus, the number of lacunes was significantly associated with baPWV (coefficient=0.926, $p=0.039$; **Table 2**). In contrast, the number of lacunar spots was not directly correlated with other parameters, including age and DBP.

Carotid/Intracranial Artery Stenosis

Among forty-four patients enrolled, eleven patients (25.0%) had moderate to severe stenosis and occlusion of the carotid/intracranial artery. There was no significant difference in the use of anti-hypertensive drugs between the normal to mild stenosis group and moderate to severe and occlusion group (data not

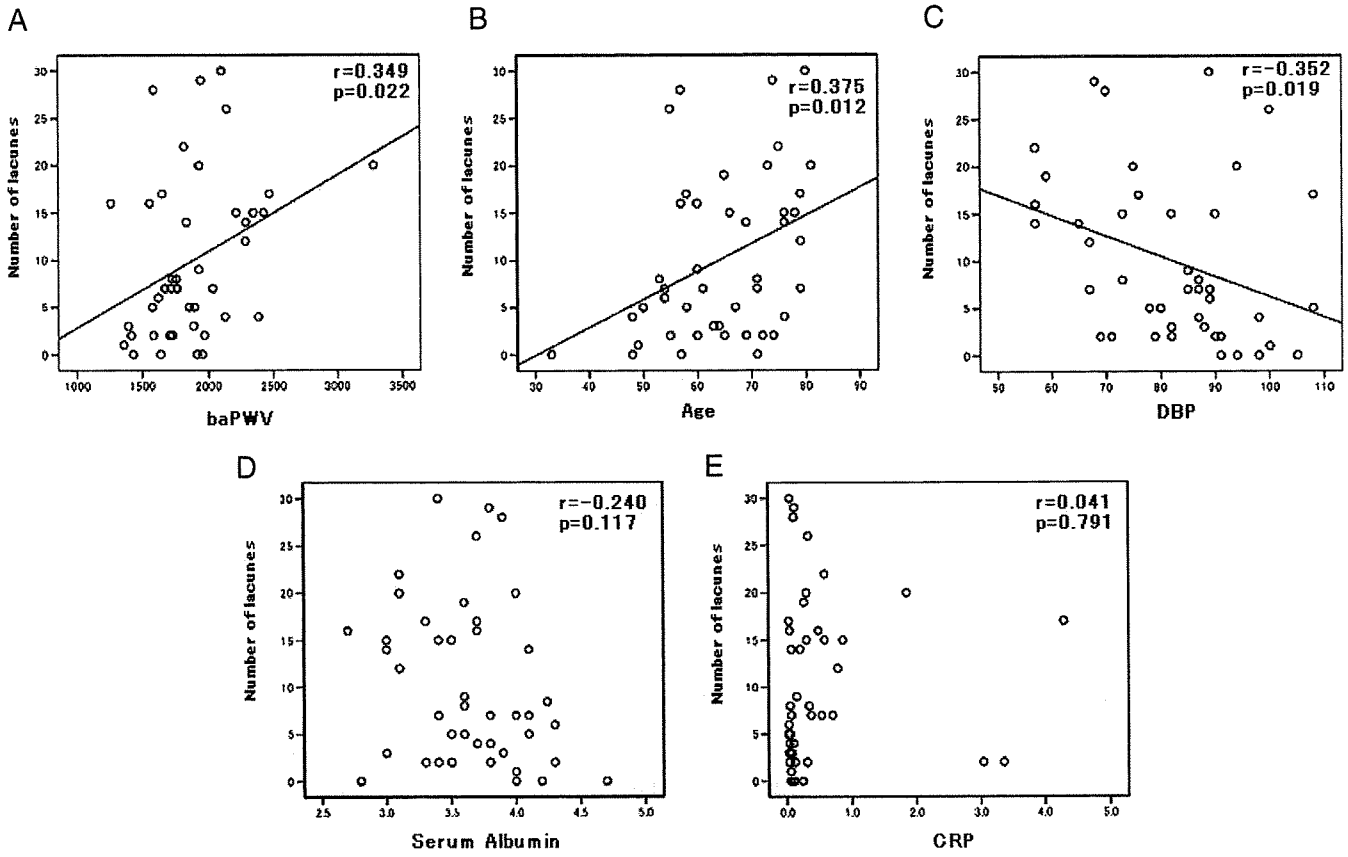

Fig. 1. Correlation between lacunar infarction and other parameters.

The number of cerebral lacunes was correlated positively with baPWV (A) and age (B) and negatively with diastolic blood pressure (DBP; C). The number of lacunes tended to be associated with serum albumin levels (D) and CRP (E).

Table 2. Multilinear regression analysis of predictors of the number of lucunes

Variable	coefficient	Standardized coefficient	*t*-value	*p*-value
Age (y/o)	0.047	0.062	0.251	0.804
Gender	−4.338	−0.212	−1.400	0.171
PWV ($\times 10^2$ cm/s)	0.926	0.410	2.149	0.039
SBP ($\times 10$ mm/Hg)	−0.554	−0.156	−0.458	0.650
DBP ($\times 10$ mm/Hg)	−1.420	−0.233	−0.633	0.532
Serum creatinine (mg/dL)	−0.268	−0.071	−0.469	0.642
ALB ($\times 10^{-1}$ mg/dL)	−0.307	−0.162	−0.982	0.334
Serum cCa (mg/dL)	0.590	0.068	0.450	0.655
CRP ($\times 10^{-1}$ mg/dL)	−0.099	−0.108	−0.676	0.504
Carotid/intracranial artery stenosis ≧50%	−0.911	−0.048	−0.295	0.770

shown). As shown in **Table 3**, a higher prevalence of DM was noted in the moderate to severe stenosis and occlusion group ($p=0.004$). BaPWV was markedly greater and serum intact-parathyroid hormone (i-PTH) levels were lower in the moderate to severe stenosis and occlusion group than the normal to mild stenosis group ($p=0.016$ and $p=0.036$, respectively). Other parameters, however, did not differ between these two groups.

The results of multivariate logistic regression analysis are shown in **Table 4**. The level of PWV was significantly associated with the development of severe

Table 3. Clinical features of patients with various degrees of carotid/intracranial artery stenosis

Parameters	Patients with Normal to Mild Stenosis (n=33)	Patients with Moderate to Severe Stenosis and Occlusion (n=11)	p
Age (y/o)	63.5±11.3	67.8±9.8	NS
Gender (male/female)	27/6	8/3	NS
BMI (kg/m^2)	21.5±3.4	20.9±4.0	NS
Smoking (%)	43.6	45.5	NS
Diabetes (%)	39.4	90.9	0.004
Ischemic heart disease (%)	30.3	36.4	NS
SBP (mm/Hg)	144±21.0	151±31.0	NS
DBP (mm/Hg)	82.0±14.0	80.0±16.0	NS
Pulse pressure (mm/Hg)	62.0±14.0	71.0±18.0	NS
CTR (%)	52.9 (47.1-55.9)	54.2 (52.6-60.6)	NS
baPWV (cm/sec)	1,727.0 (1,612.0-1,948.5)	2,128.0 (1,856.0-2,317.5)	0.016
Serum creatinine (mg/dL)	7.5 (6.7-8.7)	6.0 (5.8-7.9)	NS
BUN (mg/dL)	79.8 (57.6-91.5)	62.1 (46.0-78.2)	NS
eGFR (ml/min)	6.2 (4.7-7.1)	6.9 (5.7-8.6)	NS
Hct (%)	27.3±44.2	28.0±4.8	NS
Albumin (g/dL)	3.7±0.4	3.4±0.4	NS
CRP (mg/dL)	0.12 (0.05-0.34)	0.30 (0.06-0.53)	NS
Serum cCa (mg/dL)	8.7±0.9	8.9±1.0	NS
Serum phosphorus (mg/dL)	5.5±1.5	4.8±1.3	NS
cCa×P (mg^2/dL^2)	47.5±12.7	43.1±16.1	NS
i-PTH (pg/mL)	229.0 (159.0-324.0)	157.0 (90.5-219.0)	0.036
HbA1c (%)	5.1±0.6	5.3±0.8	NS
Total cholesterol (mg/dL)	161.3±38.2	170.7±31.7	NS
LDL-cholesterol (mg/dL)	91.4±26.0	103.2±31.7	NS
Triglyceride (mg/dL)	105.0 (90.5-153.5)	104.0 (81.5-113.0)	NS
Number of lacunes	7 (2-15)	15 (6-16.5)	NS

Table 4. Multivariate logistic regression analysis of predictors of moderate to severe stenosis and occlusion

Variable	Standardized coefficient	Adjusted odds ratio	95% confidence interval	p-value
Age (y/o)	−1.047	0.910	0.759-1.091	0.307
Gender	−0.748	0.162	0.006-4.254	0.275
PWV(×10^2 cm/s)	1.621	1.544	1.015-2.348	0.042
SBP (×10 mm/Hg)	1.289	1.721	0.595-4.977	0.316
DBP (×10 mm/Hg)	−1.388	0.367	0.055-2.456	0.301
i-PTH (×10 pg/mL)	−1.748	0.875	0.773-0.989	0.033
Diabetes	0.998	7.219	0.487-107.040	0.151
ALB(×10^{-1} mg/dL)	−1.261	0.753	0.538-1.053	0.097
CRP (×10^{-1} mg/dL)	−0.830	0.914	0.714-1.171	0.477

stenosis and occlusion, with an adjusted odds ratio of 1.544 (1.015-2.348, p=0.042). Similarly, i-PTH levels contributed negatively to severe stenosis and occlusion (odds ratio; 0.875, p=0.033). The adjusted odds ratio for DM was 7.219 (0.487-107.040), but it did not achieve statistical significance (p=0.151). Serum albumin levels tended to be negatively associated with severe stenosis and occlusion (odds ratio; 0.753, p=0.097).

Discussion

A growing body of evidence has been accumulated that CKD constitutes a critical determinant of

the progression of arterial stiffness and cardiovascular disease[19]. This clinically important issue has been focused on intensively because of the recently well-recognized topic, i.e., cardio-renal continuum[20]. Numerous factors are supposed to contribute to the development of cerebrovascular disease and atherosclerosis, including glucose/lipid metabolism, calcium-phosphorus handling and blood pressure in ESRD. Nevertheless, the contribution of these factors to the development of cerebrovascular events has not been fully determined. Furthermore, it remains unclarified whether other diagnostic parameters reflect the severity of cerebrovascular disease.

The present study has demonstrated that patients with ESRD exhibit various degrees of lacunar infarction. Furthermore, univariate analysis shows that the number of lacunes is correlated positively with baPWV ($r=0.349$) and age ($r=0.375$), and negatively with DBP ($r=-0.352$, **Fig. 1**). When confounding factors such as age and DM are eliminated by multilinear regression analysis, only PWV is adopted as a significant risk factor for the development of lacunar infarction ($p=0.039$, **Table 2**). These findings lend support to the premise that arterial stiffness and the subsequent insults are linked to lacunar infarction. In this regard, Van-Popele *et al.*[21] reported in a population-based study targeting elderly subjects that the carotid-femoral PWV was strongly associated with atherosclerosis at various vascular sites, and suggested that aortic stiffness could be used as an indicator of generalized atherosclerosis. Although lacunar infarction results from the occlusion or severe stenosis of small penetrating cerebral arteries, our finding could indicate that baPWV is an appropriate marker for damage to cerebral arteries and consequently lacunar infarction in patients with ESRD. Further studies are required, however, to confirm this hypothesis.

Of note, reductions in DBP are associated with the number of lacunes (**Fig. 1C**). It is well established that atherosclerotic changes in the aortic wall reduce DBP, which is deemed an ominous sign of developing cardiovascular accidents[22, 23]. To the extent that low DBP jeopardizes cerebral perfusion[24, 25], silent lacunar infarction is a predictor of cardiovascular events[9]. Similarly, patients with severe lacunes manifest greater CRP levels than those with absent to moderate lacunes (**Table 1**), thus suggesting vascular inflammation and loss of integrity of vascular function[26]. Nevertheless, multivariate analysis shows no correlation between DBP and the number of lacunes or between CRP and the number of lacunes (**Table 2**). In this regard, the number of lacunes is well correlated with age (**Fig. 1B**). Furthermore, in our preliminary analysis, we found that both DBP and CRP are significantly associated with age (DBP; $r=-0.452$, $p=0.002$, CRP; $r=0.343$, $p=0.023$). Collectively, although our current study fails to demonstrate gender, DBP and CRP per se as a strong independent risk factors for the development of lacunes (**Table 2**), these factors could contribute to lacunar infarction through multifaceted mechanisms that are modified by age.

Several lines of studies have disclosed that nutritional status not only constitutes a critical determinant of the development of cardiovascular events but also contributes to the prognosis of hemodialysis patients[27]. Furthermore, the idea has emerged that malnutrition, inflammation and atherosclerosis serve in concert to promote the development of cardiovascular disease (MIA syndrome)[28]. In the present study, we have shown that serum albumin levels are lower and CRP is higher in ESRD patients with severe lacunes (**Table 1**). Indeed, serum albumin levels per se are inversely correlated with an increased risk for cardiovascular death in ESRD[29], and a strong association has been reported in peritoneal dialysis patients between arterial stiffness and nutritional markers, of which serum albumin is representative[30]. Furthermore, evidence has accrued that atherosclerosis reflects the vascular inflammatory process[31] and CRP is recognized as a surrogate marker of atherosclerosis[32] and a predictor of ischemic stroke[33]. Together, the nutritional status presumably contributes to the development of lacunes, and serum albumin levels may be regarded as a novel marker of cerebrovascular disease in ESRD patients. To the extent that cerebral infarction is a consequence of atherosclerosis, the association between CRP and lacunar infarction is reasonably anticipated in patients with ESRD as well as patients on hemodialysis[34].

In the present study, we have found that baPWV is higher in patients with moderate to severe carotid/intracranial artery stenosis or occlusion than in those with normal to mild stenosis ($p=0.016$, **Table 3**). Multivariate regression analysis also reveals a greater odds ratio for PWV in moderate to severe stenosis or occlusion (i.e., 1.544, $p=0.042$; **Table 4**). Moreover, DM is more frequently seen in patients with moderate to severe stenosis and occlusion ($p=0.004$, **Table 3**) though the odds ratio for DM does not attain statistical significance (7.219, $p=0.151$; **Table 4**). Although multiple factors mediate the structural changes in carotid/intracranial artery stenosis, the underlying mechanism is supposed mainly to be atherosclerosis, resulting in plaque formation and intima-media wall thickening (IMT). Indeed, PWV is reported to be positively correlated with carotid artery IMT and negatively with luminal diameter[35]. Our present findings

therefore suggest that atherosclerotic changes contribute to carotid/intracranial artery stenosis and occlusion; baPWV serves substantially to detect silent cerebral damage that may be a prodrome of major stroke in patients with ESRD. Furthermore, the presence of DM apparently favors the carotid/intracranial artery stenosis and occlusion.

The role of PTH in cerebrovascular injury merits comment. Although the consensus on Ca metabolism in ESRD suggests optimal ranges of Ca/phosphate/PTH[36], this issue has not been endorsed by compelling evidence. Previous studies showed the deleterious effects of excess PTH on cardiovascular events in ESRD[37]. In the present study, serum i-PTH levels are lower in moderate to severe stenosis and occlusion than in normal to mild stenosis (**Table 3**). Moreover, low i-PTH levels are a risk factor for severity of stenosis independent of DM and age (**Table 4**). It has been shown that low i-PTH is linked to coronary calcification in dialysis patients[37], which is based on the notion that low bone turnover due to low PTH reflects reduced bone Ca-phosphate buffering capacity[38]. Indeed, serum Ca was higher in patients with i-PTH <150 pg/mL than in those with i-PTH ≥ 150 pg/mL (data not shown). Our findings are therefore compatible with this opinion and would provide crucial information on the status of carotid/ intracranial artery.

In conclusion, the present study has revealed the close association between cerebrovascular damage and arterial stiffness, as expressed by the elevation in PWV, in patients with ESRD. Furthermore, novel parameters (DBP, serum albumin, CRP and i-PTH) in addition to classical risk factors (age and DM) serve to promote the progression of cerebrovascular disorder. Our observations therefore suggest that these parameters constitute predictive markers for the development of cerebrovascular accidents, and would further provide a reliable guide for the establishment of a therapeutic strategy for patients with ESRD.

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Kidney-specific Overexpression of Sirt1 Protects against Acute Kidney Injury by Retaining Peroxisome Function[S]

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Sirt1, a NAD-dependent protein deacetylase, is reported to regulate intracellular metabolism and attenuate reactive oxidative species (ROS)-induced apoptosis leading to longevity and acute stress resistance. We created transgenic (TG) mice with kidney-specific overexpression of Sirt1 using the promoter sodium-phosphate cotransporter IIa (Npt2) driven specifically in proximal tubules and investigated the kidney-specific role of Sirt1 in the protection against acute kidney injury (AKI). We also elucidated the role of number or function of peroxisome and mitochondria in mediating the mechanisms for renal protective effects of Sirt1 in AKI. Cisplatin-induced AKI decreased the number and function of peroxisomes as well as mitochondria and led to increased local levels of ROS production and renal tubular apoptotic cells. TG mice treated with cisplatin mitigated AKI, local ROS, and renal tubular apoptotic tubular cells. Consistent with these results, TG mice treated with cisplatin also exhibited recovery of peroxisome number and function, as well as rescued mitochondrial function; however, mitochondrial number was not recovered. Immunoelectron microscopic findings consistently demonstrated that the decrease in peroxisome number by cisplatin in wild type mice was restored in transgenic mice. In HK-2 cells, a cultured proximal tubule cell line, overexpression of Sirt1 rescued the cisplatin-induced cell apoptosis through the restoration of peroxisome number, although the mitochondria number was not restored. These results indicate that Sirt1 overexpression in proximal tubules rescues cisplatin-induced AKI by maintaining peroxisomes number and function, concomitant up-regulation of catalase, and elimination of renal ROS levels. Renal Sirt1 can be a potential therapeutic target for the treatment of AKI.

Sir2 (silent information regulator 2), a family of NAD-dependent protein deacetylases, can deacetylate various substrates involved in metabolism, acute stress resistance, and longevity (1). Sirt1, a mammalian homologue of Sir2, acts on several nuclear proteins, including p53 (2) and forkhead family proteins (FoxOs) (3), and prevents apoptosis. Calorie restriction or resveratrol, which activates Sirt1, is reported to protect from reactive oxygen species (ROS)-induced[2] organ insult (4, 5). Additionally, the overexpression of Sirt1 itself also strengthens the stress resistance. For example, in mice with heart-specific Sirt1 overexpression, ROS-induced cardiac apoptosis was attenuated (6). Furthermore, we have recently demonstrated that Sirt1 in cultured proximal tubular cells alleviates H_2O_2-induced apoptosis (7). However, the direct relationship between kidney-specific Sirt1 and renal tissue survival *in vivo* has not been elucidated.

Several mechanisms have been suggested for the role of Sirt1 in apoptosis and/or oxidative stress. We have elucidated that Sirt1 activates catalase, which helps protect against ROS-induced injury (7). Transgenic (TG) mice overexpressing catalase in proximal renal tubular cells manifest attenuated renal tubular apoptosis (8). Liver-specific Sirt1 knock-out mice showed that Sirt1 activates fatty acid oxidation (FAO) (9). Because both peroxisomes and mitochondria play a significant role in FAO, and catalase is mainly localized in peroxisomes, it is surmised that Sirt1 also influences not only mitochondria but also peroxisomes. However, the direct relationship between Sirt1 and peroxisomes has not been examined thus far. Furthermore, Sirt1 has been reported to preserve mitochondria function by inducing PGC-1α, which increases mitochondrial number (10). Because both peroxisome and mitochondrial functions are compromised by pathophysiological conditions, including cisplatin-induced (11, 12) and ischemic/reperfusion (I/R)-induced acute kidney injury (AKI) (13, 14), it is anticipated that Sirt1 is capable of alleviating renal injury by modulating peroxisome and mitochondrial function.

To examine the role of Sirt1 in renal tubules, we produced TG mice overexpressing Sirt1 specifically in proximal tubules. These mice were subjected to cisplatin- or I/R-induced AKI to investigate whether Sirt1 rescued renal insults. We also attempted to elucidate the role of peroxisome as well as mitochondria in mediating the mechanisms for the renal protective effects of Sirt1 in AKI.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables SI–SIII and Figs. SI–SV.

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[2] The abbreviations used are: ROS, reactive oxidative species; TG, transgenic; AKI, acute kidney injury; WT, wild type; FAO, fatty acid oxidation; I/R, ischemic/reperfusion; BUN, blood urea nitrogen; mtDNA, mitochondrial DNA; PMP70, 70-kDa peroxisomal membrane protein; ACOX1, peroxisome acyl-CoA oxidase; MCAD, medium chain acyl-CoA dehydrogenase; PPAR, peroxisome proliferator-activated receptor; PGC-1α, PPARγ cofactor-1α; 4-HNE, 4-hydroxy-2(E)-nonenal; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; immuno-EM, immunoelectron microscopy; CR, calorie restriction; NC, normal chow; FACS, fluorescence-activated cell sorter; AQP4, aquaporin 4.

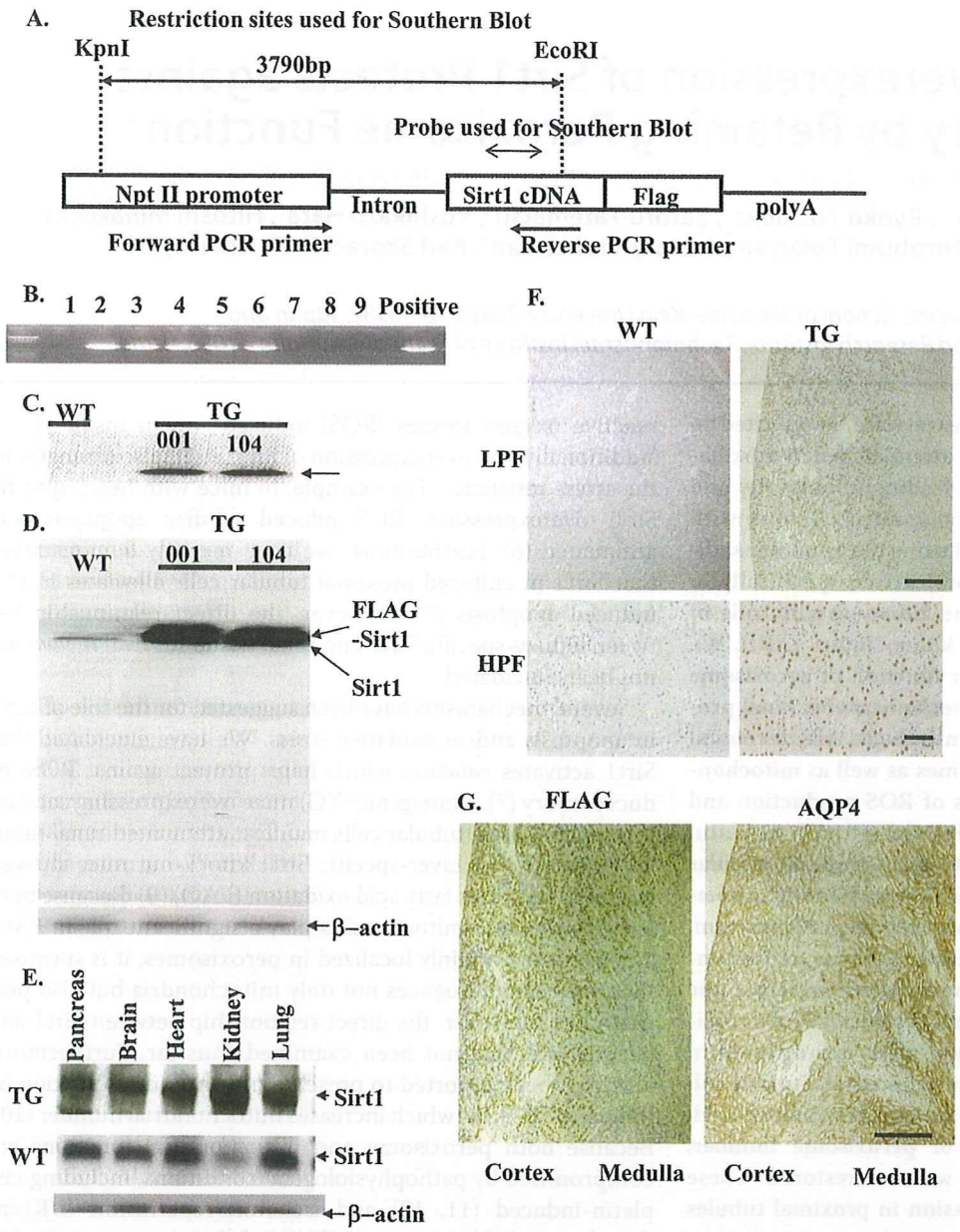

FIGURE 1. **Foundation of renal tubule-specific Sirt1 TG mice.** *A*, the construct used for the foundation of kidney-specific Sirt1 TG mice contains a fragment from the Npt II promoter, a human Sirt1 cDNA fragment, a FLAG tag sequence, an intron, and β-globin polyadenylation sequences. The restriction sites or probes used in the Southern blot and PCR primers are indicated. Sequences of primers designed for the of Southern blot probe are listed in supplemental Table SI. *B*, a PCR analysis for genotyping of TG mice. We used the transgenic vector including human kidney cDNA as a positive control. *C*, Southern blotting shows the introduction of Sirt1 transgene in mice of line 001 and 104. An *arrow* indicates a band corresponding to transgene-derived Sirt1. *D*, immunoblotting for Sirt1 indicates that TG mice express approximately five times the level of Sirt1 as compared with WT mice. *E*, Sirt1 expressions in various tissues from TG mice. *F*, immunohistochemical localization of Sirt1 shows predominant expression in proximal tubules in kidneys of TG mice. Kidneys from TG and WT mice were immunostained for FLAG epitope as described under "Experimental Procedures." Original magnification was ×100 (*LPF*) or ×400 (*HPF*). *Scale bars* indicate 500 μm (low power field; *LPF*) and 100 μm (high power field; *HPF*). *G*, kidneys from mice overexpressing Sirt1 demonstrate that Sirt1 is overexpressed preferentially in the cortical area (proximal S1 + S2 segments). The immunohistological examination reveals no overlap between Sirt1 and AQP-4 staining areas (proximal S3 segments). They are from sequential sections labeled with anti-Sirt1 and anti-AQP4 antibody. *Scale bar*, 500 μm.

EXPERIMENTAL PROCEDURES

Experimental Protocol for AKI—Eight-week-old male mice were used in these experiments. For the induction of cisplatin-induced AKI, the mice were given intraperitoneal saline or cisplatin infusion (20 mg/kg/day; Sigma) for 3 days. On the third day, the kidney was harvested for histological analysis, and blood was taken for laboratory assay.

For the induction of I/R renal injury, the kidney was subjected to ischemia by clamping both renal pedicles for 60 min with vascular clips (Roboz, Gaithersburg, MD), during which the mice were kept at constant temperature (37 °C) with a warm blanket and well hydrated. Then the clip was removed, which allowed for reperfusion. After 24 h of renal ischemia, the kidney tissues were removed, and serum samples were obtained. A sham operation was performed by manipulation of the renal pedicles without clamping. Four animals from each respective genotype were used for each experiment. Serum levels of blood urea nitrogen (BUN) and creatinine were measured with Fujifilm DRI-CHEM3500V autoanalyzer (Fuji Photo Film). These studies as well as the following animal studies were performed in accordance with the animal experimentation guidelines of Keio University School of Medicine.

Experimental Protocol for Calorie Restriction—Calorie restriction was performed as described previously (15). Ten male C57BL/6 mice were randomly assigned to two groups of five. From two months of age, one group was fed normal chow, and the other group was put on a caloric restriction diet (70% of calorie intake adjusted to body weight) for three months. At the end of five months, the effects of cisplatin on the kidneys from calorie-restricted mice were evaluated. The mice were sacrificed, and the kidneys were removed for use in immunoblotting and histological experiments as described below.

Plasmids and Constructs—Human Sirt1 cDNA containing its full-length open reading frame was cloned as described previously (16). In constructing the transgenic expression vector, the cDNA for Sirt1 was followed by an eight-amino acid FLAG epitope (Invitrogen). To generate renal tubular epithelium-specific TG mouse, the sequence for human Sirt1 cDNA with FLAG epitope was ligated with mouse sodium phosphate cotransporter IIa (Npt2) promoter (17), and this clone was designated as Npt2-Sirt1-FLAG (see Fig. 1*A*).

Generation of Renal Tubular-specific Sirt1 TG Mice—To generate TG mice, the SalI-NotI fragment of Npt2-Sirt1-FLAG was microinjected into one-cell fertilized mouse embryos

obtained from superovulated C57BL/6 × C3H mice (see Fig. 1*A*) (18). Founder mice were identified by Southern blot analysis of KpnI/EcoRI-digested tail genomic DNA with the human Sirt1 cDNA as a probe. The PCR primers for making Southern blot probes are listed in supplemental Table SI. The positive TG founders were then crossed with wild type C57BL/6 mice (Charles River Japan Inc.) to obtain the F1 generation. Genomic DNA was isolated from tail biopsies at 3 weeks of age using a DNeasy kit (Qiagen) and subjected to Southern blot analysis to identify the transgene. Southern blots were performed using a ^{32}P-labeled probe of human Sirt1 cDNA. Genomic DNA digested by KpnI and EcoRI yielded a single band of the expected size in TG mice only (see Fig. 1*C*). Additional screening of genomic DNA samples was performed by polymerase chain reaction using the transgene-specific oligonucleotide primers CTATGCTGAGGCCCTAGGTTC TAT (Npt2 promoter side) and GTCGTCGTCTTCGTCGTACAAGTTG (Sirt1 gene side) (see Fig. 1*A*), which amplify a 1310-bp region spanning the junction between the Npt2 promoter and the Sirt1 gene (see Fig. 1*B*). We used the transgenic vector as a positive control.

Quantification of Mitochondrial DNA (mtDNA)—Total RNA from mouse kidneys were prepared as described previously (19). The LightCycler Instrument (Roche Applied Science) was used for the quantification of mtDNA with hybridization probes in a total volume of 20 μl, containing 0.5 μM of each primer, 0.2 μM of each probe, 5 mM $MgCl_2$, 2 μl of Light Cycler-DNA Master hybridization probes (Roche Applied Science), and 20 ng of DNA. The cycling conditions were set as follows: initial denaturation 95 °C for 30 s (temperature transition rate, 20 °C/s) followed by 45 cycles of 95 °C for 0 s (20 °C/s), 53 °C for 5 s (20 °C/s), and 72 °C for 11 s (2 °C/s). The standard curve was calculated by analyzing serial dilutions (0.64–5.0 × 10^4 pg/μl) of plasmid DNA inserted with the target PCR product. The mtDNA contents of kidney tissues were normalized to the total amount of DNA in each sample. The primers and probes were as follows: sense primer, 5′-ACCATTTGCAGACGCCATAA-3′; antisense primer, 5′-TGAAATTGTTTGGGCTACGG-3′; 3′-fluorescein-labeled anchor probe, 5′-CATCTAGCCTATCAGTTTACTCCATTCT-3′; and 5′-LC Red 640-labeled sensor probe, 5′-TGATCAGGATGAGCCTCAAACTCCAAAT-3′. These primers and probes were designed to amplify the mouse mtDNA designed in reference to the *Mus musculus* mitochondrion genome and not to amplify the mtDNA-like sequence in the nuclear genome.

Immunoblotting—Immunoblotting was performed as described previously (19) using specific antibodies against Sirt1 (Millipore, Bedford, MA), catalase (Calbiochem, La Jolla, CA), 70-kDa peroxisomal membrane protein (PMP70; Affinity Bioreagents, Golden, CO), peroxisome acyl-CoA oxidase (ACOX1; Abcam Inc., Cambridge, MA), medium chain acyl-CoA dehydrogenase (MCAD; Cayman Chemicals, Ann Arbor, MI), and PPARγ cofactor-1α (PGC-1α; Santa Cruz Biotechnology, Santa Cruz, CA). The anti-Sirt1 antibody was generated from rabbit and recognizes both human and mouse Sirt1. The β-actin band recognized by specific antibody (Sigma) was used as a loading control. For immunoblotting, 30 μg of protein from each sample was loaded into the individual lanes of the gels.

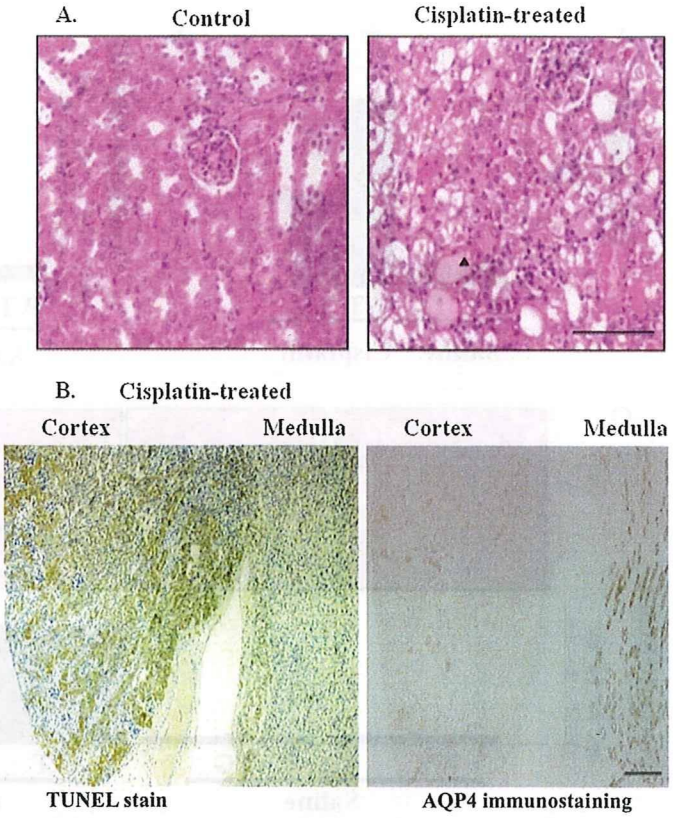

FIGURE 2. **Effects of cisplatin on renal histology and apoptotic process.** *A*, renal histological changes induced by cisplatin-induced acute kidney injury. AN *arrowhead* indicates the areas of cisplatin-induced damages. *Scale bar*, 100 μm. *B*, TUNEL-positive cells are detected in cortical areas, which barely overlap with areas containing cells expressing AQP4 (a marker for proximal S3 segments) in mice treated with cisplatin. They are from sequential sections labeled with anti-AQP4 antibody and stained with TUNEL. *Scale bar*, 200 μm.

Immunohistochemistry—Four-μm paraffin sections of kidneys were used to stain for aquaporin 4 (AQP4), 4-hydroxy-2(E)-nonenal (4-HNE), Sirt1, and FLAG, using primary antibodies including anti-AQP4 antibody (Santa Cruz Biotechnology), 4-HNE (Japan Institute for the Control of Aging), Sirt1 (Millipore, Bedford, MA), and FLAG (Sigma). Tissue sections of 4-μm thickness were obtained and fixed in 3% formaldehyde in phosphate-buffered saline for 15 min at room temperature and treated as described previously (20). Briefly, the sections were sequentially blocked for endogenous biotin binding using a Vector blocking kit (Vector Laboratories, Burlingame, CA) and for endogenous peroxidase activity with 1.5% H_2O_2, 0.2 mol/liter Na_3N in Hanks' balanced salt solution with 0.01 mol/liter HEPES (HBSS-HEPES) with 0.1% saponin. Nonspecific binding was blocked with 10% normal goat serum. After incubation with preimmune or immune sera overnight, the sections were stained with biotin-labeled goat anti-rabbit IgG (Vector) or biotin-labeled anti-mouse IgG (Vector) and then treated with the Vecstatin Elite ABC Kit (Vector). The slides were washed with HBSS-HEPES and developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (0.5 mg/ml) in 0.05 mol/l Tris, pH 7.4, 0.0075% H_2O_2 (Sigma).

RNA Isolation, Reverse Transcription, and Quantitative PCR—Total RNA was isolated using TRIzol reagent (Invitrogen). RNA (400 ng) was reverse transcribed to cDNA using the SuperScript II

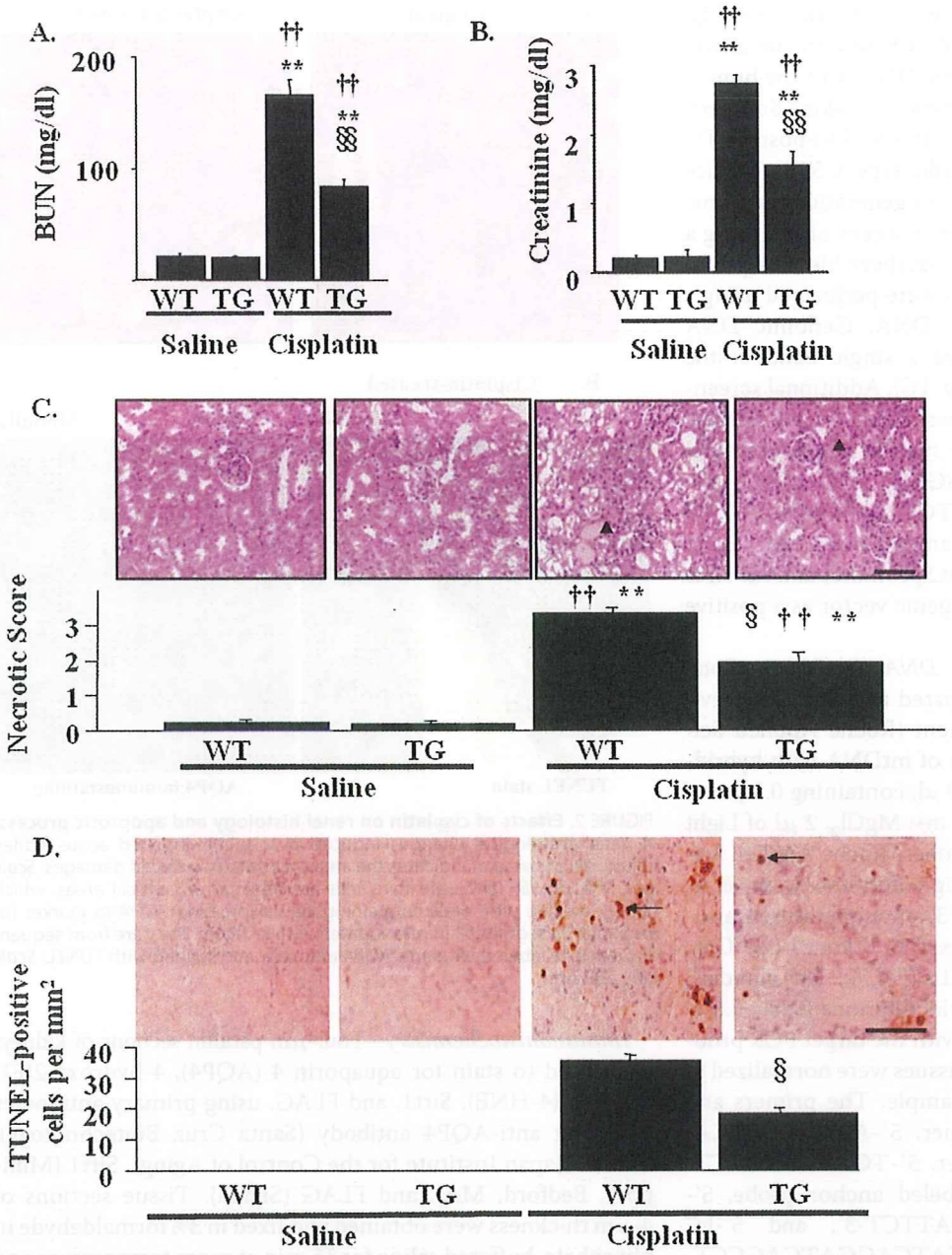

FIGURE 3. **Effects of kidney-specific Sirt1 overexpression on renal function and histology in cisplatin-induced acute kidney injury.** *A* and *B*, WT or TG mice was treated with cisplatin as described under "Experimental Procedures." Serum levels of BUN (*A*) and creatinine (*B*) were measured 3 days after cisplatin injection. *C*, representative hematoxylin-eosin staining of kidney sections from WT and TG mice in cisplatin-induced AKI. An *arrowhead* represents lysed tubules. Pathological scores of tubular damages as described under "Experimental Procedures" are shown in the *lower panel*. *Scale bar*, 100 μm. *D*, representative images of the TUNEL assay show cisplatin-induced renal tubular cell apoptosis in WT mice, which is attenuated in TG mice. An *arrow* indicates TUNEL-positive cells with condensed or fragmented nuclei. The numbers of TUNEL-positive cells are shown in the *lower panel*. *Scale bar*, 100 μm. *, $p < 0.05$ *versus* saline-infused WT mice; **, $p < 0.01$ *versus* saline-infused WT mice; †, $p < 0.05$ *versus* saline-infused TG mice; ††, $p < 0.01$ *versus* saline-infused TG mice; §, $p < 0.05$ *versus* cisplatin-infused WT mice; §§, $p < 0.01$ *versus* cisplatin-infused WT mice ($n = 4$).

RNase H-Reverse Transcriptase system (Invitrogen), and specific transcripts were quantitated by real time PCR using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) and the SYBR GREEN system (Applied Biosystems). Sequences of the primers are listed in supplemental Tables SII and SIII. The relative mRNA levels for the specific genes were normalized to 28 S rRNA levels. The reference gene (28 S rRNA) was not affected by cisplatin treatment.

Renal Histology—After the kidneys were removed, they were fixed in 4% parformaldehyde for paraffin embedding. Paraffin-embedded tissues were sectioned at 4 μm for hematoxylin and eosin staining. Renal histology was examined in a blinded fashion. The following histological changes were evaluated: the percentage of renal tubules that displayed cell lysis, the loss of brush border, and cast formation. The development of tissue damage was scored as follows; 0, no damage; 1, <25% damage; 2, 25–50% damage; 3, 50–75% damage; 4, >75% damage. Pictures of representative fields were also recorded.

TUNEL Assay—Apoptosis of renal tubular cells was identified by TUNEL assay as described previously (18) with an *in situ* cell death detection kit (Roche Applied Science).

Quantification of Peroxisomes and Mitochondria by Immunoelectron Microscopy—For postembedding immunoelectron microscopy (immuno-EM), sliced kidney sections were fixed for 1 h at room temperature with 4% formaldehyde and 0.25% glutaraldehyde in 0.1 M HEPES/NaOH, pH 7.4. After rapid dehydration in ethanol, the sections were embedded in LR White embedding resin. Thin sections on nickel grids were preincubated on a drop of 0.5% bovine serum albumin in phosphate-buffered saline. The sections were incubated overnight in the 1/1000 diluted primary antibody, an anti-PMP70 antibody. After being rinsed with phosphate-buffered saline, the sections were incubated for 30 min on a drop of protein A-gold (15 nm) prepared as described previously (21). The sections were briefly contrasted with uranyl acetate and lead citrate before examination. Eight random ultrastructural photomicrographs taken on the microscopic fields corresponding to the kidney cortex area from each mice at a magnification of ×12,800 were chosen for observation. Then peroxisomes and mitochondria were numbered. By the above postembedding protein A gold labeling, anti-PMP70 antibody gave specific signals on the membranes of peroxisomes, whereas mitochondria were negative on staining with the anti-PMP 70 antibody.