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Suppressed Expression of Superoxide Dismutase in Dialyzed Kidneys

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Accumulation of oxidative DNA damage in the dialyzed kidney, particularly in acquired cystic disease of the kidney (ACDK), was previously demonstrated. Here, we assessed expression in dialyzed and normally functioning kidneys of superoxide dismutases (SODs), which catalyze the dismutation of O_2^- to start the reactive oxygen species chain reaction. We evaluated expression of SODs in renal tissue at the protein and mRNA levels using enzyme-linked immunosorbent assay and RT-PCR, respectively, as well as its localization using immunohistochemistry for 45 patients (maintenance hemodialysis, 29 and normal kidney function, 16). Levels of Cu- and Zn-SOD, but not Mn-SOD mRNA expression, were significantly lower in dialyzed kidneys than in normally functioning kidneys. At the protein level, however, Mn-SOD expression was lower in dialyzed kidneys than in normally functioning kidneys. Immunohistochemically, the majority of tubules in dialyzed kidneys were shown to have discontinuous or patchy staining patterns for both SODs, and the density of SOD-positive cells was lower compared to normally functioning kidneys. Decreased expression of SODs in kidneys of dialysis patients suggests impairment of O_2^- scavenger reactions, which may induce oxidative DNA damage and subsequently increase the risk of neoplastic lesions in these patients.

Key words: superoxide dismutase, acquired cystic disease of the kidney, autosomal dominant polycystic kidney disease, dialyzed kidney, semiquantitative RT-PCR

Introduction

Patients with endstage renal disease (ESRD), especially those on maintenance dialysis therapy, often develop multiple cysts in their remnant kidneys. The condition is known as acquired cystic disease of the kidney (ACDK). The most critical problem in ACDK is a high incidence of renal tumors including adenocarcinoma[1]. Additionally, Gregoire's detailed histopathological study revealed a high incidence of proliferative lesions including hyperplasia and adenoma in renal tissues of dialysis patients with autosomal dominant polycystic disease (ADPKD) as well as in patients with ACDK[2]. The frequency of renal malignant neoplasm in dialysis patients has been estimated to be 57 to 134 times greater than in the general population[3]. The mechanism underlying the development of neoplastic lesions in kidneys of dialysis patients remains unsolved. Our previous studies, however, showed that an accumulation of oxidative DNA damage may contribute to kidney carcinogenesis in dialysis patients, especially in association with ACDK[4].

In general, oxidative damages including DNA modifications are thought to develop when the generation of reactive oxygen species (ROS) overwhelms the defense system against ROS. Patients with ESRD are constantly exposed to an environment that favors ROS generation from uremic toxins and extracorporeal circulation[5], although whether ROS generation is actually enhanced in these patients remains controversial[6][7]. Even though this point is not clarified, most physiological functions, including defense mechanisms against ROS, are supposed to be deteriorated in kidneys of

dialysis patients, who also show disorganized morphological features in the kidney.

ROS production is normally regulated by antioxidation enzymes including superoxide dismutases (SODs) and endogenous antioxidants. SODs are metalloenzymes that catalyze the dismutation of O_2^- to dioxygen and H_2O_2. In mammals, they consist of three forms: copper, zinc-SOD (Cu, Zn-SOD), manganese-SOD (Mn-SOD), and extracellular SOD[8]. Some investigators showed that in glomerulonephritis, the activity and expression of Cu, Zn-SOD are declined in proportion to disease severity while Mn-SOD activity remains normal[9]. Depressed SOD activity may result in accumulation of toxic O_2^-. Additionally, O_2^- accumulation may lead to excess formation of H_2O_2 through disproportional O_2^- reaction independent of SOD. On the other hand, increased SOD activity may also directly generate excessive H_2O_2[10]. Therefore, either a deficiency or an excess of SOD in dialyzed kidneys may induce imbalance in the regulation of ROS generation and contribute to oxidative DNA injury.

In this study, we sampled renal tissues of patients on dialysis and evaluated the expression of Cu, Zn-SOD and Mn-SOD at protein level using enzyme-linked immunosorbent assay (ELISA) and at mRNA level using semiquantitative RT-PCR, and also investigated their localization by immunohistochemistry.

Subjects and Methods

1. Patient

Forty-five patients who underwent nephrectomy were enrolled in this study. They were divided into three groups according to the underlying renal diseases. Group I was composed of 15 dialysis patients with renal cell carcinoma (RCC) associated with ACDK, and their underlying renal diseases were chronic glomerulonephritis in 7, nephrotic syndrome in 2 and unknown causes in 6. Group II was composed of 14 dialysis patients, including 11 with ADPKD and 3 with renal diseases unrelated to ACDK. Group III was composed of 15 patients with RCC in normally functioning kidneys.

The mean ages of the subjects were 51.7 ± 8.5, 49.6 ± 11.9 and 61.2 ± 10.4 years in groups I, II and III, respectively (Kruskal-Wallis' test, $p = 0.017$). All the patients in groups I and II started maintenance hemodialysis before surgery. The duration of hemodialysis therapy until surgery was 199.9 ± 81.8 and 63.9 ± 95.7 months in groups I and II, respectively (Mann-Whitney's U-test, $p = 0.002$).

This study was carried out according to the regulation of Tokyo Women's Medical University institutional review board and of institutional review board regarding gene analysis study.

2. Preparation of specimen

Immediately after nephrectomy, blocks were cut out of the extracted kidneys to distinguish between cancerous from non-cancerous lesions. Each block was taken from the part close to the renal capsule, which was expected to be compatible to one part of renal cortex. Some of the blocks were frozen in liquid N_2 until use and some were fixed in 10% phosphate-buffered formalin solution.

3. Semiquantitative RT-PCR

Total RNA was extracted from frozen non-cancerous tissues by the standard AGPC method[11]. Complementary DNA was synthesized from 1 μg of total RNA using a RNA PCR kitTM (TaKaRa, Shiga, Japan) with 0.125 μM oligo dT-adaptor primer, according to the manufacturer's protocol. The cDNA targets were semiquantitated with Quantum RNATM (Ambion Inc, Austin, USA) according to the manufacturer's instructions[12].

First we determined the standard PCR protocol as follows. The 20-μl PCR mixture was composed of 1× PCR buffer [50 mm Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin], 0.2 mM dNTPs, 0.5 unit of Ampli Taq polymerase TM (Roche, Foster City, USA), 0.25 μM primers for Cu, Zn-SOD or Mn-SOD gene with and without primers TM and competimer TM for 18S rRNA gene. The mixture was overlaid with 20 μl of mineral oil. The primer set specific to Cu, Zn-SOD gene was 5′-GCCTTCTG GACAATCTTTCC-3′ (sense) and 5′-GGTAGGTG ACATCATCAAGC-3′ (antisense), and that specific to Mn-SOD gene was 5′-GCCTTCTGGACAATCT TTCC-3′ (sense) and 5′-GGTAGGTGACATCATC AAGC-3′ (antisense), which were expected to yield fragments of 393 and 365 bp in length, respectively.

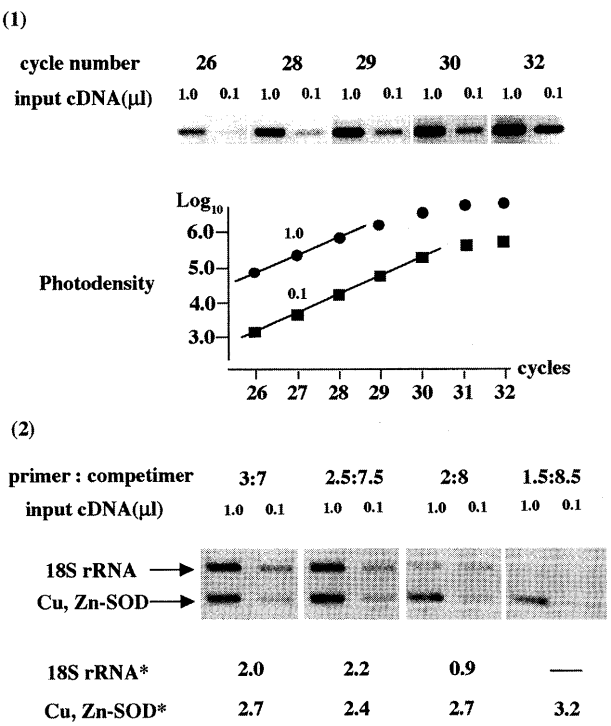

Fig. 1 Optimal number of PCR cycles for SOD and optimal ratio of primer to competimer in RT-PCR analysis of SOD mRNA expression

(1) The optimal number of PCR cycles was 27 ± 1 for Cu, Zn-SOD, which allowed gene amplification to be terminated during the exponential phase.

(2) The optimal ratio of primer to competimer was 2.5 : 7.5 for both Cu, Zn-SOD and 18S rRNA, at which the amount of PCR product was proportional to the quantity of input cDNA under standard conditions.

* : Ratio = Amount of PCR product obtained from 1.0 μl of input cDNA/Amount of PCR product obtained from 0.1 μl of input cDNA.

The 18S rRNA gene primer set was expected to amplify a 488 bp fragment. The standard cycling condition was 94 ℃ for 1 min, 60 ℃ for 2 min, and 72 ℃ for 2 min, followed by a 5-min extension at 72 ℃ after the last cycle using a Zymoreactor IITM thermal cycler (Atto, Tokyo, Japan).

We first determined the optimal PCR conditions (Fig. 1). Using our standard protocol, we found that 27 and 30 cycles were optimal for Cu, Zn- and Mn-SOD gene amplification, respectively, for various amounts of input cDNA. These conditions allowed termination of PCR reaction during the exponential phase of amplification. Subsequently, we determined that the optimal ratios of 18S rRNA gene primers to competimers were 2.5 : 7.5 and 1.5 : 8.5 for Cu, Zn- and Mn-SOD gene, respectively, using the standard protocol with 27 and 30 cycles. Under these conditions, the amounts of PCR product were proportional to the quantities of input cDNA for 18S rRNA, Cu, Zn-SOD and Mn-SOD genes (Fig. 1).

In the main step, 18S rRNA gene and each SOD gene were amplified together in a single tube in a standard reaction mixture containing 1.0 or 0.1 μl of cDNA, which corresponded to 10 or 1 ng of total RNA, respectively.

PCR products were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, visualized under ultraviolet light, and recorded on instant B/W film FP-3000BTM (Fuji Film, Tokyo, Japan). The photographs were converted to digital images by a flat bed scanner GT6000TM (Epson, Tokyo, Japan). The optical density of each band was semiquantitated by densitometric method using the NIH Image 1.54 program. Since rRNA occupies the majority of the RNA and its expression remains essentially constant independent of tissue and cell type, 18S rRNA expression adjusted by competimersTM can be regarded as an internal control for comparing the degree of SOD gene expression among renal tissues. Thus, we calculated the ratio of the photodensity of SOD gene to that of modulated 18S rRNA gene, which was expected to represent the relative amount of SOD gene expression in each patient.

In the preliminary study, PCR products were extracted from the agarose gel and subsequently cloned into pGEM-T vectorTM (Promega, Madison, USA). Single strands were sequenced employing the Taq Cycle Sequencing kitTM and automated DNA sequencer 373TM (both from Applied Biosystems, Foster City, USA). The resulting sequences corresponded to published data for the gene products of Cu, Zn-SOD, Mn-SOD and 18S rRNA (data not shown).

4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out using the Mn-SOD ELISA System (Amersham Life Science, Buckinghamshire, England) according to the manufacturer's manual. In brief, whole tissue protein was extracted from frozen non-cancerous tissues by standard methods

as described previously[13].

For the assay, 100 μl of serially diluted standard Mn-SOD or sample was added to each well of the microtiter plates precoated with anti-Mn-SOD polyclonal antibodies, and incubated for 1 hr at room temperature. After washing three times with a washing buffer, 100 μl of peroxidase-labeled anti-Mn-SOD monoclonal antibody was added to each well and incubated for 1 hr at room temperature. After rinsing three times, enzyme reaction was carried out at room temperature for 10 min with 100 μl of a substrate solution containing 0.4 mg/ml of *o*-phenylenediamine and 0.03% H_2O_2. The reaction was stopped by adding 50 μl of 2N H_2SO_4. Finally, absorbance was measured at 492 nm using a Plato 1300 (Aloka, Tokyo, Japan), and the Mn-SOD content of each sample was estimated from the standard curves prepared using serially diluted Mn-SOD standard.

5. Immunohistochemical analysis

The avidin-biotin-peroxidase complex (ABC) technique was used for immunohistochemical staining. Briefly, 4-μm paraffin sections on glass slides were deparaffinized with xylene and rehydrated in graded ethanol. After treating with 1% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity, the sections were incubated in a 1 : 200 solution of sheep polyclonal antibodies against human Mn-SOD or Cu, Zn-SOD (Calbiochem-Novabiochem Corporation, San Diego, USA) for 120 min at room temperature. After washing twice with 0.01 M pH 7.2 Tris-buffer (TBS) for 10 min, the sections were treated with biotinylated anti-sheep antibody diluted 1 : 2,000 (Chemicon, Temecula, USA) and ABC reagent (Vector Laboratories, Burlingame, USA). Color was developed with diaminobendizine containing 0.02% hydrogen peroxide in 0.05 M Tris-HCl buffer at pH 7.2 for 2 to 5 min. The sections were finally counterstained with hematoxylin. As control, non-immunized sheep serum was used instead of the primary antibody.

6. Statistical analysis

Statistical analysis was done with Statview 4.0 (Abacus Concepts, Inc, Berkeley, USA). The results are expressed as mean ± SD. Since the data were not normally distributed, non-parametric statistics; Kruskal-Wallis' test and Dunn's procedure were used to compare the means of arbitrary ratios of PCR products in the three groups. Results were considered to be statistically significant at $p < 0.05$.

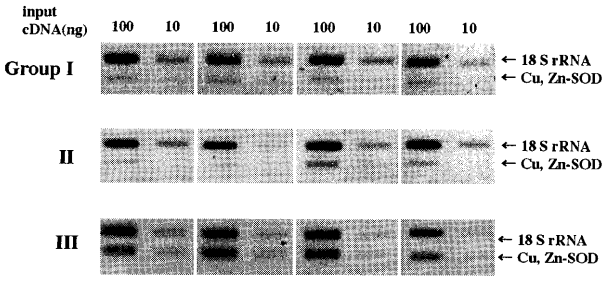

Fig. 2 Representative data of PCR products for Cu, Zn-SOD and 18S rRNA in four patients in normally functioning renal tissue (group III) and dialyzed kidney with and without ACDK (group I and group II, respectively)
The upper line represents the volume of input cDNA in 20-μl PCR reaction. The photodensities of PCR products for Cu, Zn-SOD and 18S rRNA in each patient were proportional to the amounts of input cDNA.

Results

1. SOD expression determined by semiquantitative RT-PCR

On each electrophoretic gel, 2 PCR products of different sizes were visible in all patient (Fig. 2). The larger product corresponded to the fragment of 18S rRNA which is 488 bp in length, and the other product to that of Mn-SOD which is 365 bp or Cu, Zn-SOD which is 393 bp. In addition, the photodensities were proportional to the amounts of input cDNA, that is, 1.0 or 0.1 μl of cDNA.

We compared the ratios of the amounts of PCR product of SODs to those of 18S rRNA among groups (Fig. 3). The ratios for Cu, Zn-SOD were 0.35 ± 0.11, 0.25 ± 0.09, 0.79 ± 0.24 in groups I, II and III, respectively. A significant difference was found between groups I and III, and between groups II and III, but not between groups I and II, which indicated that the level of Cu, Zn-SOD expression was significantly lower in dialyzed kidneys than in normally functioning kidneys.

On the other hand, the ratios for Mn-SOD were 4.14 ± 2.53, 4.03 ± 1.84, 4.03 ± 2.93 in groups I, II and III, respectively, with no significant differences

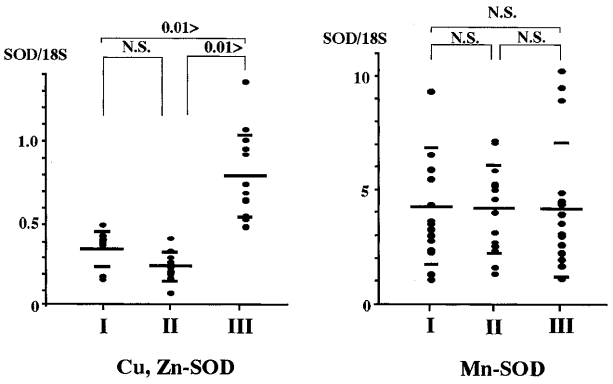

Fig. 3 Comparison of arbitrary ratios of Cu, Zn- and Mn-SOD mRNA expression among normally functioning kidney (group III) and dialyzed kidney with and without ACDK(group I and group II, respectively)
The arbitrary ratios(photodensity of SOD/photodensity of 18S rRNA) for Cu, Zn-SOD mRNA expression were significantly higher in group III than in other groups. On the contrary, those of Mn-SOD showed no significant differences among the groups. The horizontal bars represent the mean ± SD.

among the three groups (Fig. 3). This meant that Mn-SOD expression in dialyzed kidneys was similar to that in normally functioning kidneys, unlike the results for Cu, Zn-SOD expression.

2. Mn-SOD content determined by ELISA

We evaluated Mn-SOD expression at protein level in renal tissues by ELISA. The mean Mn-SOD content in group I was comparable to that in group II (0.36 ± 0.09 vs 0.31 ± 0.16, $p = 0.197$). On the contrary, the mean level of Mn-SOD in group III was approximately 3-fold and significantly higher than that in dialyzed kidneys including groups I and II (1.09 ± 0.32 vs 0.34 ± 0.12, $p < 0.01$).

3. Immunohistochemical study

Immunohistochemistry was used to examine the localization of SOD proteins. In subjects with normally functioning kidneys (Group III), the cytoplasm of the epithelial cells lining the entire length of the tubules is stained for both Cu, Zn- and Mn-SOD without exception. Additionally, Mn-SOD is also localized in epithelial cells of the glomerulus and Bowmann's capsule. The majority of tubules in dialyzed kidneys, including dilated tubules, were stained for both Cu, Zn- and Mn-SOD, which indicated that localization of SOD proteins in dialyzed kidneys was comparable to that in normal tissues. However, a discontinuous or patchy staining pattern was observed in the dialyzed kidneys (Fig. 4). In addition, most glomeruli in dialyzed kidneys were distorted and lost SOD staining.

In the present study, we could not compare the intensity of staining for SOD among groups because there were no components in the renal tissues which could be used as an appropriate internal control for comparison.

Discussion

Kashem et al reported that in human glomerulonephritis which did not result in hemodialysis, Cu, Zn-SOD activity decreased depending on the degree of tissue damage at both protein and mRNA levels whereas both protein and mRNA levels of Mn-SOD did not differ in intact and damaged tissues[9]. In the present study, Mn-SOD mRNA expression in dialyzed kidneys (groups I and II), which included the terminal stage of glomerulonephritis, was at the same level as in normally functioning kidneys (Group III). However, Mn-SOD expression at protein level was lower in dialyzed kidneys. In addition, immunohistochemical study also demonstrated that tubular epithelial cells had a discontinuous or patchy staining pattern for Mn-SOD in dialyzed kidneys whereas uniform staining was observed in normally functioning renal tissues. The plausible reasons for this discrepancy between protein and mRNA expression of Mn-SOD are as follows: ① there were no significant differences in intensity of gene expression of Mn-SOD because there is wide variation in each group, ② even though the mean mRNA expression of SOD per cell is not changed, protein expression per unit tissue may be decreased due to a decrease in density of SOD-positive cells per unit tissue as a result of tissue distortion, and ③ protein expression may be impaired at the post-transcriptional level even if mRNA was synthesized without any hindrance. Berkovich et al reported that the efficiency of Mn-SOD translation is decreased by certain substance such as pertussis toxin[14]. This supports the latter possibility although it is still unknown whether inhibitors of Mn-SOD expression exist in the uremic

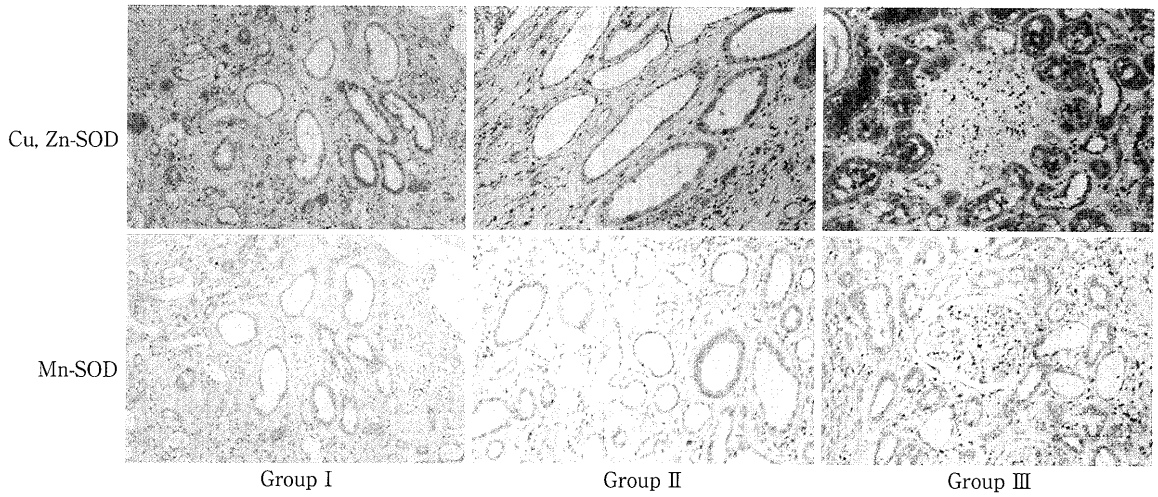

Fig. 4 Immunohistochemical staining for Cu, Zn-SOD and Mn-SOD in normally functioning renal tissue (group III) and dialyzed kidney with ACDK (group I) and ADPKD (group II) (×100)
In normally functioning kidney, proximal and distal tubules were positive for both Cu, Zn- and Mn-SODs. In dialyzed kidney, most tubules including dilated ones were also positive for both SODs, but they showed a discontinuous or patchy staining pattern. Additionally, in dialyzed kidneys, SOD-positive cells apparently decreased in number with distortion of renal tissues.

state.

In the present study, we did not perform ELISA for Cu, Zn-SOD or measure the total SOD activity because we did not remove erythrocytes, which contain a lot of Cu, Zn-SOD protein, from the specimens. However, our results of RT-PCR and immunohistochemical study for Cu, Zn-SOD, and those of ELISA and immunohistochemistry for Mn-SOD demonstrated that their expression at protein level was suppressed in dialyzed kidneys, which suggested that the total activity of SODs was lowered in dialyzed kidneys compared with intact tissues.

Decreased SOD activities may induce impairment in the scavenger reaction of toxic O_2^-, which subsequently may result in excessive O_2^- accumulation. In general, excess O_2^- reacts with H_2O_2, which is also formed through disproportional reaction of O_2^-, and subsequently gives rise to hydroxyl radical (·OH), a potent toxic radical, in the presence of transition metal ions such as ferric and copper ion[15]. This highly reactive radical may promote peroxidative damage in cellular components including DNA, as represented by the generation of 8-hydroxydeoxyguanosine (8-OHdG)[16] that is generally observed to accumulate in the renal tissues of patients under maintenance hemodialysis, especially in ACDK tissues[4]. Additionally, O_2^- itself has been reported to directly induce DNA strand scission and DNA nicking[17][18].

Moreover, O_2^- reacts with nitric oxide (NO) to form a strong oxidant, perioxynitrite ($ONOO^-$), which also causes cellular injury including DNA damages, generating 8-OHdG, nitrotyrosine and other products. In a previous study, we demonstrated using immunohistochemistry that expression of inducible nitric oxide was enhanced in cystic epithelial cells as well as in macrophages and other stromal cells more frequently in ACDK than in other renal cystic diseases. These findings imply that nitric oxide (NO) is generated ectopically in the locality of dialyzed kidney, especially in ACDK, and reacts with O_2^-.

Excess O_2^- accumulation, therefore, may give rise to various oxidative DNA damages directly and indirectly, and cause mutations and subsequently a high incidence of neoplastic lesions in ACDK and ADPKD patients under dialysis[1][2].

Conclusion

In conclusion, our results showed decreased expression of SODs in kidneys of dialysis patients in-

cluding those with ACDK and ADPKD, and suggest that this may result in impairment of the O_2^- scavenger reaction. It is possible that subsequent O_2^- excess may induce oxidative DNA damage and mutation, which is a possible cause of the high incidence of neoplastic lesions in dialyzed kidneys.

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透析腎における Superoxide Dismutase 発現の抑制

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透析腎における酸化的 DNA 損傷の蓄積，特に後天性嚢胞性腎疾患（ACDK）患者に伴う酸化的 DNA 損傷の蓄積については既に報告されている．今回我々は，透析腎および正常機能の腎において，活性酸素の最上流にあるスーパーオキシド（O_2^-）を消去する反応を触媒するスーパーオキシド・ジスムターゼ（SOD）の発現について検討した．免疫組織化学法を用いて SOD の局在性を評価すると同時に，酵素免疫測定法と RT-PCR 法を個々に使用し，腎組織中における SOD の発現について蛋白質レベルと mRNA レベルで検討した．本研究の対象は維持血液透析患者 29 名と正常腎臓機能を有していた患者 16 名の合計 45 名であった．mRNA レベルでの Cu および Zn-SOD の発現は，正常機能腎よりも透析腎において有意に低かったが，Mn-SOD の発現は正常機能腎と同等であった．しかしながら，蛋白質レベルでの Mn-SOD の発現は正常機能腎よりも透析腎において有意に低下していた．免疫組織化学的には，透析腎の大多数の尿細管において，Cu および Zn-SOD と Mn-SOD がともに不連続性または斑点状の染色パターンを示し，SOD 陽性細胞密度は，正常機能腎よりも透析腎において低かった．透析患者の腎臓における SOD 発現の低下は，O_2^-スカベンジャー反応の障害を示唆し，同障害によって酸化的 DNA 損傷が誘導され，その結果としてこれらの患者において腫瘍の発生リスクが増大するものと考えられる．