Free Radical Research, 2003 Vol. 37 (4), pp. 373–379

Taylor & Francis
healthsciences

Targeting Superoxide Dismutase to Renal Proximal Tubule Cells Attenuates Vancomycin-induced Nephrotoxicity in Rats

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Accepted by Professor E. Niki

(Received 10 September 2002; In Revised form 21 October 2002)

Vancomycin hydrochloride (VCM), a glycopeptide antibiotic, has a broad spectrum against methicillin-resistant *Staphylococcus aureus* (MRSA). As it is known to induce renal dysfunction, the dose and the duration of its administration are limited. Moreover, the mechanism of VCM-induced renal dysfunction remains to be unclear.

To evaluate the involvement of free radical on VCM-induced renal dysfunction, we carried out analysis with a hexamethylenediamine-conjugated superoxide dismutase (AH-SOD) which rapidly accumulates in renal proximal tubule cells and inhibits oxidative injury of the kidney. Male Wistar rats (weighing 200–210 g) were intraperitonealy administered with 200 mg/kg of VCM twice a day for 7 days. AH-SOD 5 mg/kg/day was subcutaneously injected 5 min before every VCM injection. VCM induced renal injury dose-dependently. Biochemical analyses revealed that plasma levels of blood urea nitrogen and creatinine significantly increased in the VCM-treated group by an AH-SOD-inhibitable mechanism. VCM simultaneously elicited an increase of 8-OHdG levels and chemiluminescence intensity of free radical generation in the kidney. Histological examination revealed that VCM also elicited a marked destruction of glomeruli and necrosis of proximal tubules. AH-SOD inhibited these phenomena in the kidney. These results suggested that oxidative stress might underlie the pathogenesis of VCM-induced nephrotoxicity and targeting SOD and/or related antioxidants to renal proximal tubules might permit the administration of higher doses of VCM sufficient for eradication of MRSA without causing renal injury.

Keywords: Renal dysfunction; Hexamethylenediamine-conjugated superoxide dismutase; Oxidative stress; Vancomycin

INTRODUCTION

Vancomycin hydrochloride (VCM), a glycopeptide antibiotic, has been effective against methicillin-resistant *Staphylococcus aureus* (MRSA).[1] Unfortunately it is also known that VCM has the potential to cause nephrotoxicity.[2–5] As VCM may cause nephrotoxicity in the patient population as an adverse reaction, administration in large doses and usage duration are limited. Thus, prevention of the side effects of VCM is an important clinical problem in treating patients with MRSA infection. The molecular mechanism of VCM-induced nephrotoxicity and the methods for preventing the side effect of this antibiotic remain unclear.

Reactive oxygen species (ROS) and related metabolites have been postulated to underlie the pathogenesis of renal dysfunction.[6–8] ROS easily react with various molecules including proteins, lipids and DNA. For example, ROS have been found to mediate cell injury after ischemia in the kidney,[7] and are considered to be important mediators of gentamicin-mediated nephrotoxicity.[8]

We therefore investigated whether VCM-induced renal dysfunction is based on free radical injury. If VCM directly or indirectly generates ROS, it is not surprising that it exhibits cytotoxicity to renal cells and tissues. Superoxide dismutase (SOD) and other

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000061002

scavengers often inhibited cell injury by superoxide and related metabolites *in vitro*.[9] However, intravenously injected SOD often failed to inhibit oxidative tissue injury *in vivo* presumably because of its rapid excretion in urine. Intravenously injected Cu/Zn-SOD disappears from the circulation with a half-life of 5 min predominantly via the renal filtration mechanism and rapidly appears in the urine in its intact form. Therefore, the effective dismutation of superoxide radicals cannot be achieved *in vivo* by simply administering the enzyme. To scavenge superoxide radicals effectively in and around renal tubule cells, we synthesized hexamethylenediamine-conjugated SOD (AH-SOD),[10] which could inhibit the renal dysfunction caused by ROS.[10–12] AH-SOD rapidly undergoes renal glomerular filtration, electrostatically binds to the brush border membranes of renal proximal tubule cells and localizes in kidney for a fairly long time without being excreted in the urine. AH-SOD disappears from the circulation with a half-life of 3 min.[10] On the other hand, more than 80% of AH-SOD accumulates in the kidney and the half-life of AH-SOD in the kidney is 3.5 h.[10]

To determine whether superoxide radicals are involved in the pathogenesis of the VCM-induced nephrotoxicity, the effects of AH-SOD and SOD were tested in rats treated with VCM.

MATERIALS AND METHODS

Chemicals

VCM, hexamethylenediamine and L-012 were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). VCM was dissolved in distilled water and administered to the dosage of 20–400 mg/kg. Cu/Zn-type recombinant human SOD (3000 units/mg protein) was kindly received as a gift from Nippon Kayaku Co. (Tokyo, Japan). AH-SOD (2700 units/mg protein) was synthesized by conjugating the enzyme with hexamethylenediamine as described previously.[10] All other chemicals used were of analytical grade.

Animals and Treatments

Male Wistar rats (Japan SLC Inc., Shizuoka, Japan) weighing 200–210 g, were housed on a 12 h light/dark cycle and were provided rat chow and water *ad libitum*. All animals used were cared for according to the guidelines outlined in the Guide for Care and Use of Laboratory Animals approved by the authorities of the Osaka City University Medical School on experimental animal research. Under light ether anesthesia, these rats were intraperitonealy injected with VCM or saline at the corresponding dose twice a day for 7 days. Five minutes before each 200 mg/kg of VCM injection, 5 mg/kg of AH-SOD or SOD or saline (control group) was administered subcutaneously. Sham rats were treated with saline alone. To examine the effect of single administration of VCM, the same animals were injected with 200 mg/kg of VCM and 24 h later they were sacrificed.

Measurement of Plasma Levels of Blood Urea Nitrogen (BUN) and Creatinine

On day 8, animals were anesthetized by the intraperitoneal administration of ethyl carbamate (1 g/kg). Blood samples were obtained from the abdominal aorta and centrifuged at 11,000*g* for 5 min. Plasma levels of BUN and creatinine were measured by diacetylmonoxime and Jaffe's methods, respectively.

Chemiluminescence Intensity of Free Radical Generation

Free radical generation from renal tissues 24 h after 200 mg/kg of VCM injection was analyzed using a chemiluminescence technique. The kidney obtained from these rats was cut into eight pieces and incubated at 37°C in 0.5 ml of 0.9% NaCl solution containing 10 mM phosphate buffer (pH 7.4), 6 mM KCl and 6 mM $MgCl_2$ in the 400 μM L-012 as chemiluminescence probe.[13] During incubation, chemiluminescence intensity was continuously recorded for 20–40 min using Luminescence Reader (BLR-201, Aloka Co., Ltd, Tokyo, Japan).[13,14]

Analysis of DNA Oxidation

The DNA base-modified product 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the most commonly used markers for the evaluation of oxidative DNA damage in various tissues.[15] The obtained kidney was homogenized in 10 volumes of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100) at 4°C. After incubation for 20 min, 100 μl of the mixture was centrifuged at 10,000*g* for 20 min. The supernatant fraction was incubated with RNase at 37°C for 1 h and subsequently with 40 μg proteinase K for 1 h. DNA was precipitated with isopropanol and extracted from the nuclear fraction. 8-OHdG and deoxyguanosine (dG) were determined by means of a high performance liquid chromatography–electrochemical detector (HPLC–ECD, Coulochem II, ESA Inc., MA, USA) and UV detector system, SPD-6D (Shimadzu Co., Kyoto, Japan).[16] The levels of 8-OHdG in each DNA sample were determined as the molar ratio of 8-OHdG to 10^6 dG.

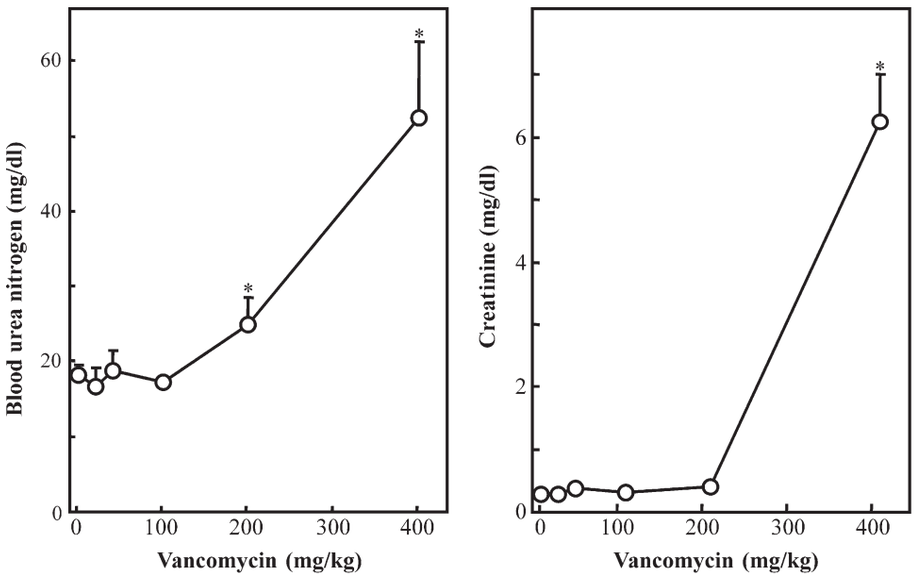

FIGURE 1 Effect of vancomycin on serum BUN and creatinine levels in rats. After intraperitoneal administration of 20–400 mg/kg VCM for 7 days, blood sample were obtained from aorta. BUN and creatinine levels in serum samples increased dose-dependently. Values are means ± S.E. ($n = 3$–4). *$P < 0.05$ vs. VCM-nontreated rats.

Histological Examination

Tissue specimens were fixed in 10% formaldehyde and then embedded in paraffin. The sections (4 μm) were stained with hematoxylin–eosin.

Data Analysis

All data are expressed as means ± S.E. Statistical analyses were performed using the analysis of variance (ANOVA) P value less than 0.05 was considered statistically significant.

RESULTS

Effect of SOD and AH-SOD on Vancomycin-induced Renal Dysfunction

The plasma levels of BUN increased dose-dependently in VCM-treated rats (Fig. 1). The plasma levels of creatinine increased in correlation with the changes in BUN. In 400 mg/kg of VCM-treated rats, the plasma levels of creatinine significantly increased up to 6.25 ± 0.76 mg/dl.

AH-SOD or SOD treatment did not affect kidney weight (Fig. 2). In 400 mg/kg, but not 200 mg/kg, of VCM-treated rats, kidney weights significantly increased two-folds. AH-SOD significantly inhibited the increase in kidney weight.

On day 8, the plasma levels of BUN and creatinine were significantly increased in VCM-treated rats ($P < 0.05$) (Fig. 3). Administration of AH-SOD significantly decreased the plasma levels of BUN and creatinine in VCM-treated rats. However, SOD failed to inhibit the VCM-induced increase of these levels. Administration of AH-SOD or SOD alone had no appreciable effect on plasma levels of BUN and creatinine (data not shown).

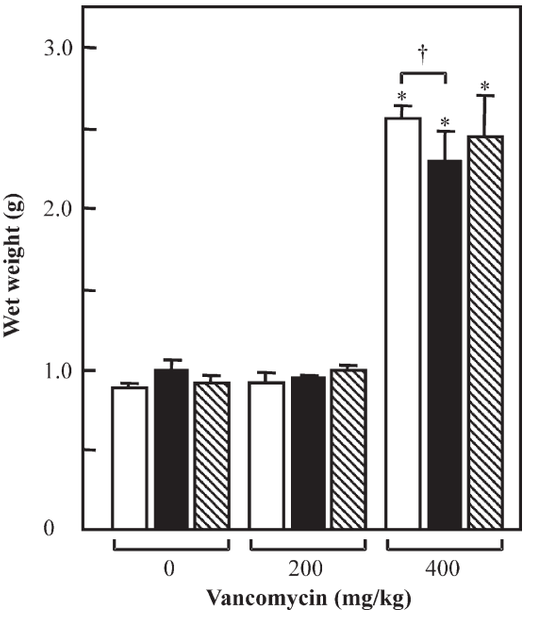

FIGURE 2 Changes in kidney weights after vancomycin injection. Animals were treated as described in Fig. 1. Each SOD/AH-SOD sample (5 mg/kg) was subcutaneously administered to rats twice a day for 7 days. Open column, control rats; closed column, AH-SOD-treated rats; oblique column, SOD-treated rats. Values are means ± S.E. ($n = 5$–6). *$P < 0.05$ vs. VCM-nontreated rats, respectively. †$P < 0.05$.

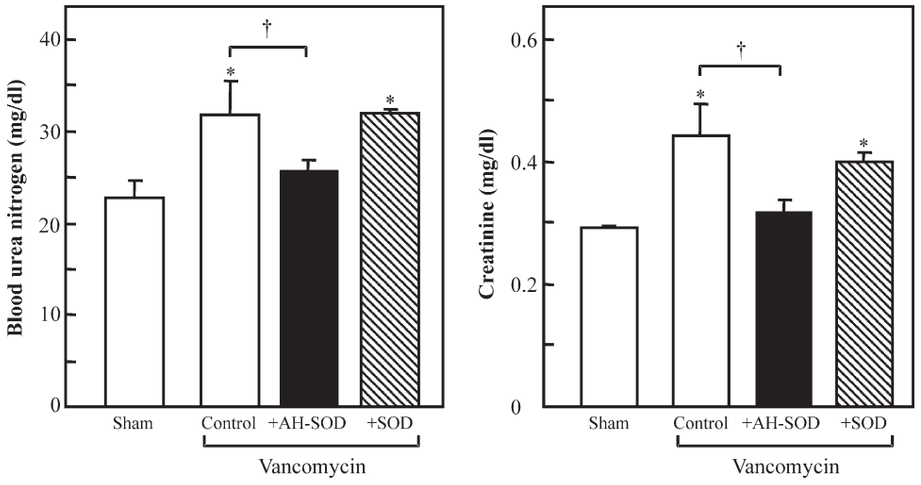

FIGURE 3 Effect of AH-SOD and SOD on the plasma levels of blood urea nitrogen and creatinine in vancomycin-treated rats. VCM (200 mg/kg) was intraperitonealy administered to rats twice a day for 7 days. Other experiments were performed as described in Fig. 2. The plasma levels of BUN and creatinine were measured. Open column, control rats; closed column, AH-SOD-treated rats; oblique column, SOD-treated rats. Values are means ± S.E. ($n = 5–6$). $^{*}P < 0.05$ vs. sham rats. $^{\dagger}P < 0.05$.

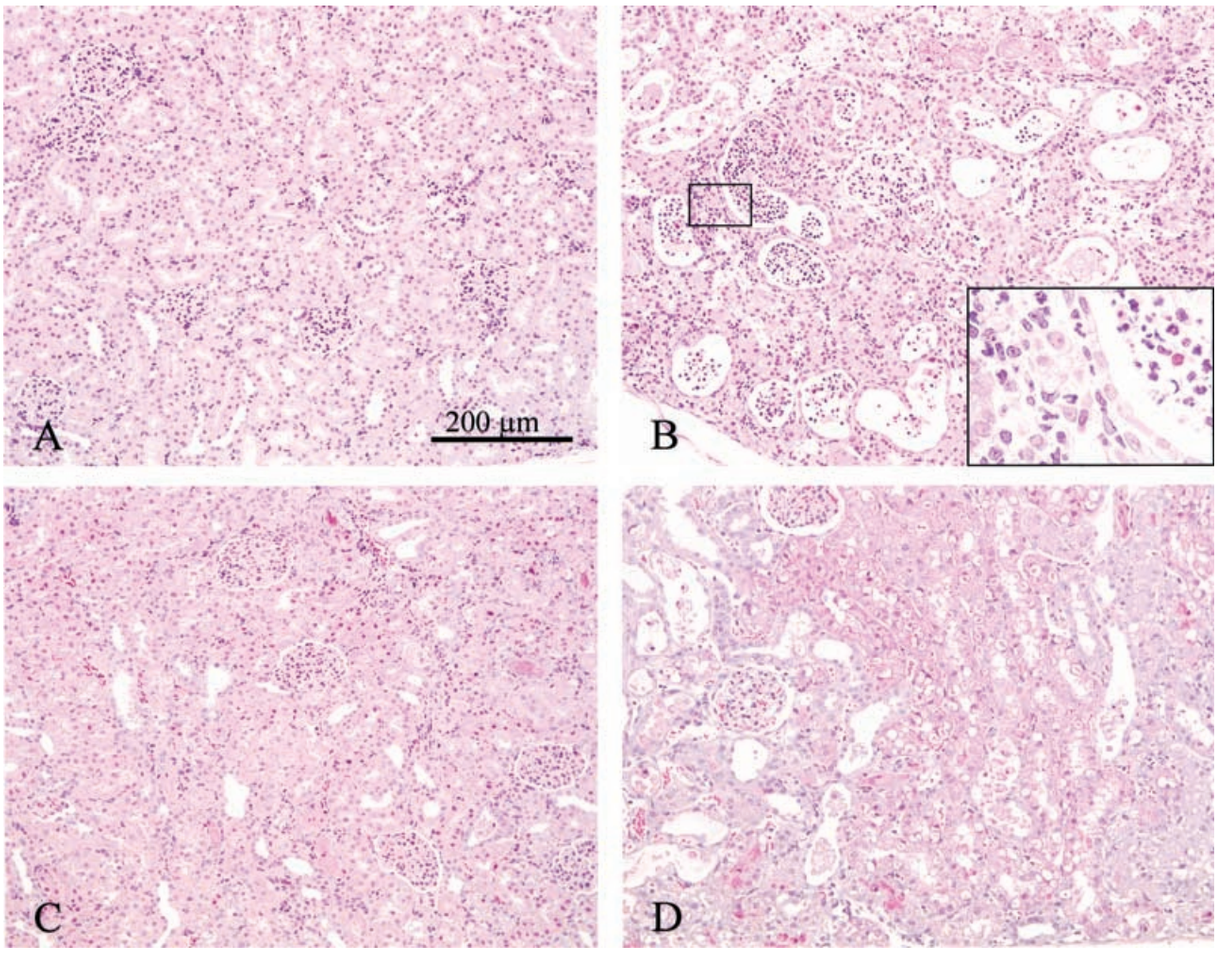

FIGURE 4 Effect of AH-SOD and SOD on vancomycin-induced renal injury. No damage was found in sham-treated rats (A). In VCM-treated rats, most of the proximal tubular cells became swollen and focal epithelial cells turned necrotic (B). Inserted figure is a same section at higher magnification × 1000. In AH-SOD-treated rats these renal tissue damages were inhibited (C). SOD could not reverse these damages (D). Sections were observed at original magnification × 200.

Effect of AH-SOD and SOD on Vancomycin-induced Renal Injury

Histological findings revealed that most of the proximal tubular cells in VCM-treated kidney became swollen and focal epithelial cells turned necrotic (Fig. 4). VCM elicited a marked destruction of glomeruli, the infiltration of polymorphonuclear leukocytes (PMN) and necrosis of proximal tubule cells (Inserted figure). AH-SOD inhibited these renal tissue damages. However, SOD did not change these damages.

Effect of AH-SOD and SOD on Vancomycin-induced Free Radical Generation

Chemiluminescence intensity of renal tissue in VCM-treated group was markedly higher than that in sham operated rats (Fig. 5). AH-SOD, but not SOD, significantly inhibited the intensity of chemiluminescence.

Effect of AH-SOD and SOD on Renal Levels of 8-OHdG

VCM significantly increased the 8-OHdG levels of renal tissues (Fig. 6). AH-SOD inhibited the increase of 8-OHdG levels.

Effect of Single Administration of VCM

Twenty four hours after a single administration of VCM did not affect plasma levels of BUN, creatinine, formation of renal 8-OHdG levels and histological findings (data not shown).

DISCUSSION

The present work demonstrates that AH-SOD, targeting SOD to renal proximal tubule cells, effectively inhibited VCM-induced renal injury. Most free radicals rapidly impair biological functions of cellular constituents at or near the sites of their generation; hence antioxidants and related enzymes should be targeted to subcellular sites of their generation for preventing free radical injury.

As VCM did not directly generate free radicals when renal tissue homogenate was incubated with VCM (data not shown), VCM-induced nephrotoxicity might be caused by indirectly generating reactive oxygen species associated with inflammatory events *in vivo*. In fact, VCM increased the infiltration of PMN in the histological study. Furthermore, VCM increased 8-OHdG levels as well as chemiluminescence intensity in the kidney. These results might indicate that the reactive oxygen species were produced in and around the kidney of VCM-treated rats.

SOD did not appreciably inhibit the renal dysfunction induced by VCM. SOD derivative by conjugating with polystyrene(co-maleic acid)butyl ester (SM-SOD), which binds to albumin in the circulation and remains circulating for a long time,[17–19] failed to inhibit VCM-induced nephrotoxicity (data not shown). These observations suggest that it is of critical importance to target SOD at the site of superoxide generation before achieving inhibition of oxygen toxicity induced by VCM.

Our work clearly demonstrates that AH-SOD inhibited the VCM-induced renal nephrotoxicity and supports the hypothesis that superoxide radicals are involved in its pathogenesis. It has been reported that because AH-SOD rapidly accumulated in and around proximal tubule cells,[10] it effectively inhibited other tubular injuries induced by agents such as cisplatin.[11,12] These results indicate that the major site of VCM-induced nephrotoxicity may be in and around the proximal tubule cells rather than in the glomeruli or other sites. Previous articles have shown that VCM accumulates in proximal tubular cells.[20,21]

Therefore, VCM might at the beginning cause inflammation in and around proximal tubule cells and cause the infiltration of PMN and the generation

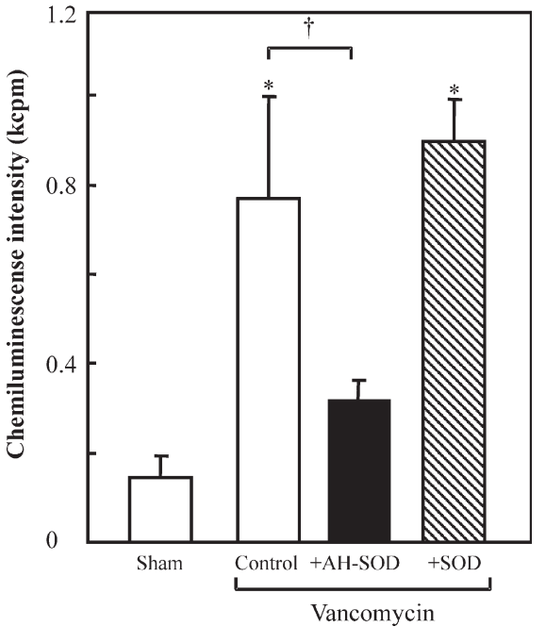

FIGURE 5 Effect of AH-SOD and SOD on vancomycin-induced free radical generation in the kidney. VCM (200 mg/kg) was intraperitonealy administered to rats. AH-SOD or SOD was subcutaneously injected before VCM injection. Twenty four hours after VCM injection renal tissues were incubated in reaction mixtures as described in the "Methods" section. The indicated values are the peak of chemiluminescence intensity. Open column, control rats; closed column, AH-SOD-treated rats; oblique column, SOD-treated rats. Values are means ± S.E. ($n = 4–9$). *$P < 0.05$ vs. sham rats. †$P < 0.05$.

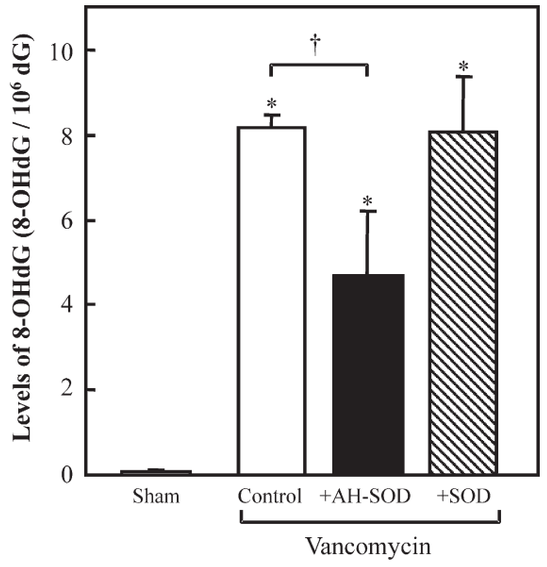

FIGURE 6 Effect of AH-SOD and SOD on renal levels of 8-OHdG. Animals were treated as described in Fig. 3. DNA was extracted from kidney samples. The levels of 8-OHdG/10^6 dG were determined by HPLC-ECD method. Values are means ± S.E. ($n = 4–6$). $^*P < 0.05$ vs. sham rats. $^\dagger P < 0.05$.

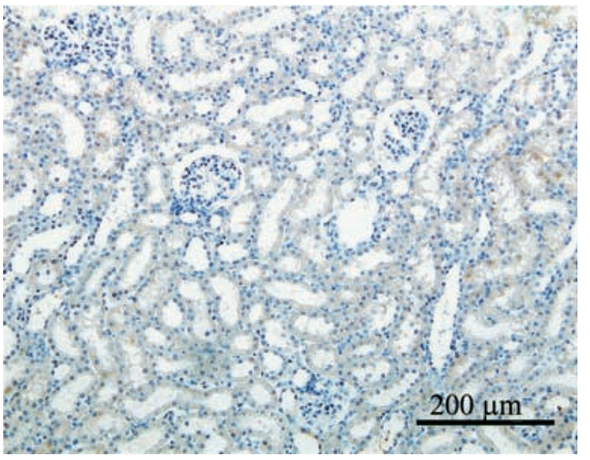

FIGURE 7 Immunohistochemistry of the effect of catalase on the kidney. Samples fixed with formalin were embedded in paraffin. The 4 μm thin sections were deparaffinized with xylol and ethanol and were treated with 0.3% H_2O_2 in methanol, and normal rabbit serum. The sections were treated overnight at 4°C with polyclonal antibody for catalase (× 200). The specimens were stained by the bridged immunoperoxidase method.

of ROS, and consequently result in glomerulus injury. Consistent with this notion is the fact that AH-SOD rapidly accumulated and was maintained within the endosomes of renal proximal tubule cells for a fairly long time,[10] and dismutated the superoxide generated by PMN. Moreover, proximal and distal tubular cells showed significant staining for catalase (Fig. 7). Therefore, AH-SOD may be concerted with the co-localized peroxidases (catalase and others)[22] and then totally inhibit the VCM-induced renal dysfunction.

Although the morphological changes in rats administered 200 mg/kg of VCM was apparently less severe, this dose of VCM markedly induced oxidative damage in the kidney. These results suggest that because VCM-induced oxidative damage starts prior to morphological damage, various indices related to free radical generation may be effective in the renal handling of VCM-treated patients. Furthermore, VCM is used for MRSA infection and MRSA-infected patients under severe inflammatory status such as endotoxemia, surgical stress, and shock, and the incidence of renal complications is high in these patients. Therefore, the magnitude of VCM-induced renal impairment in pathological status might be greater than that noted for the dose of VCM in normal condition. The effects of VCM treatment in pathological conditions therefore demands further study as well as possible involvement of superoxide radicals in the pathogenesis of VCM-induced renal injury in human subjects.

In conclusion, we have demonstrated that oxidative stress underlies the pathogenesis of VCM-induced nephrotoxicity and this involves AH-SOD-inhibitable mechanisms. Therefore, related antioxidants assembled to renal proximal tubule cells could enable the administration of higher doses and longer duration of VCM sufficient for eradication of MRSA without causing severe renal injury.

Acknowledgements

The authors greatly thank Ms Kazuyo Yoshida of Osaka City University for excellent technical assistance and Dr David O. Kennedy for the preparation of this manuscript.

References

[1] Scorrell, T.C., Packham, D.R., Shanker, S., Foldes, M. and Munro, R. (1982) "Vancomycin therapy for methicillin-resistant *Staphylococcus aureus*", *Ann. Intern. Med.* **97**, 344–350.
[2] Appel, G.B., Given, D.B., Levine, L.R. and Cooper, G.L. (1986) "Vancomycin and the kidney", *Am. J. Kidney Dis.* **8**, 75–80.
[3] Aronoff, G.R., Sloan, R.S., Dinwiddie, C.B., Glant, M.D., Fineberg, N.S. and Luft, F.C. (1981) "Effects of vancomycin on renal function in rats", *Antimicrob. Agents Chemother.* **19**, 306–308.
[4] Wood, C.A., Kohlhepp, S.J., Kohnen, P.W., Houghton, D.C. and Gilbert, D.N. (1983) "Toxicology of vancomycin in laboratory animals", *Rev. Infect. Dis.* **30**, 20–24.
[5] Marre, R., Schulz, E., Anders, T. and Sack, K. (1984) "Renal tolerance and pharmacokinetics of vancomycin in rats", *J. Antimicrob. Chemother.* **14**, 253–260.
[6] Edelstein, C.L., Ling, H., Wangsiripaisan, A. and Schrier, R.W. (1997) "Emerging therapies for acute renal failure", *Am. J. Kidney Dis.* **30**, S89–S95.
[7] Sener, G., Sehirli, A.O., Keyer-Uysal, M., Arbak, S., Ersoy, Y. and Yegen, B.C. (2002) "The protective effect of melatonin on renal ischemia–reperfusion injury in the rat", *J. Pineal Res.* **32**, 120–126.

[8] Walker, P.D. and Shah, S.V. (1988) "Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats", *J. Clin. Investig.* **81**, 334–341.
[9] Münzel, T., Hink, U., Yigit, H., Macharzina, R., Harrison, D.G. and Musch, A. (1999) "Role of superoxide dismutase in *in vivo* and *in vitro* nitrate tolerance", *Br. J. Pharmacol.* **127**, 1224–1230.
[10] Inoue, M., Nishikawa, M., Sato, E., Matsuno, K. and Sasaki, J. (1999) "Synthesis of superoxide dismutase derivative that specifically accumulates in renal proximal tubule cells", *Arch. Biochem. Biophys.* **368**, 354–360.
[11] Nishikawa, M., Nagatomi, B., Chang, B.J., Sato, E. and Inoue, M. (2001) "Targeting superoxide dismutase to renal proximal tubule cells inhibits mitochondrial injury and renal dysfunction induced by cisplatin", *Arch. Biochem. Biophys.* **387**, 78–86.
[12] Nishikawa, M., Nagatomi, H., Nishijima, M., Ohira, G., Chang, B.J., Sato, E. and Inoue, M. (2001) "Targeting superoxide dismutase to renal proximal tubule cells inhibits nephrotoxicity of cisplatin and increases the survival of cancer-bearing mice", *Cancer Lett.* **171**, 133–138.
[13] Imada, I., Sato, E.F., Miyamoto, M., Ichimori, Y., Minamiyama, Y., Konaka, R. and Inoue, M. (1999) "Analysis of reactive oxygen species generated by neutrophils using a Chemiluminescence Probe L-012", *Anal. Biochem.* **271**, 53–58.
[14] Scheuer, H., Gwinner, W., Hohbach, J., Gröne, E.F., Brandes, R.P., Malle, E., Olbricht, C.J., Walli, A.K. and Gröne, H.J. (2000) "Oxidant stress in hyperlipidemia-induced renal damage", *Am. J. Physiol. Renal Physiol.* **278**, F63–F74.
[15] Laws, G.M. and Adams, S.P. (1996) "Measurement of 8-OHdG in DNA by HPLC/ECD: the importance of DNA purity", *Biotechniques* **20**, 36–38.
[16] Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S. and Loeb, L.A. (1992) "8-hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions", *J. Biol. Chem.* **267**, 166–172.
[17] Ogino, T., Inoue, M., Ando, Y., Awai, M., Maeda, H. and Morino, Y. (1988) "Chemical modification of superoxide dismutase. Extension of plasma half life of the enzyme through its reversible binding to the circulating albumin", *Int. J. Peptide Protein Res.* **32**, 153–159.
[18] Inoue, M., Ebashi, I., Watanabe, N. and Morino, Y. (1989) "Synthesis of a superoxide dismutase derivative that circulates bound to albumin and accumulates in tissue whose pH is decreased", *Biochemistry* **28**, 6619–6624.
[19] Nishimura, Y., Nakayama, M., Sato, T., Tomita, T. and Inoue, M. (1995) "Inhibition of puromycin-induced renal injury by a superoxide dismutase derivative with prolonged *in vivo* half-life", *Nephron* **70**, 460–465.
[20] Sokol, P.P. (1991) "Mechanism of vancomycin transport in the kidney: studies in rabbit renal brush border and basolateral membrane vesicles", *J. Pharmacol. Exp. Ther.* **259**, 1283–1287.
[21] Beauchamp, D., Gourde, P., Simard, M. and Bergeron, M.G. (1992) "Subcellular localization of tobramycin and vancomycin given alone and in combination in proximal tubular cells, determined by immunogold labeling", *Antimicrob. Agents Chemother.* **10**, 2204–2210.
[22] Muse, K.E., Oberley, T.D., Sempf, J.M. and Oberley, L.W. (1994) "Immunolocalization of antioxidant enzymes in adult hamster kidney", *Histochem. J.* **26**, 734–753.