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Edaravone Inhibits Acute Renal Injury and Cyst Formation in Cisplatin-Treated Rat Kidney

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Background: Although *cis*-diamminedichloroplatinum (II) (cisplatin) is an effective anticancer agent, its clinical use is highly limited predominantly due to its adverse effects on renal functions. The present work examined the therapeutic potential of edaravone, a free radical scavenger, for inhibiting cisplatin-induced renal injury.

Methods: Edaravone, 3-methyl-1-phenyl-pyrazolin-5-one, was administrated intravenously at a dose of 30 mg/kg of body weight to male Wistar rats (200–220 g). After 30 min, cisplatin was injected intraperitoneally at a dose of 5 mg/kg of body weight. At the indicated times after the treatment, functions and histological changes of the kidney were analyzed. To test the therapeutic potential of edaravone in chemotherapy, its effect on the anticancer action of cisplatin was examined in ascites cancer-bearing rats.

Results: We found that cisplatin rapidly impaired the respiratory function and DNA of mitochondria in renal proximal tubules, thereby inducing apoptosis of tubular epithelial cells within a few days and chronic renal dysfunction associated with multiple cysts one-year after the administration. Administration of edaravone inhibited the cisplatin-induced acute injury of mitochondria and their DNA and renal epithelial cell apoptosis as well as the occurrence of chronic renal dysfunction and multiple cyst formation. The anticancer effect of cisplatin remained unaffected by intravenous administrating of edaravone.

Conclusions: These results indicate that edaravone may have therapeutic potential for inhibiting the acute and chronic injury of the kidney induced by cisplatin.

Keywords: Cisplatin; Cysts; Mitochondria; Nephrotoxicity; Free radical

Abbreviations: BUN, blood urea nitrogen; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; 4-HNE, 4-hydroxy-2-nonenal; ABC, avidin-biotin-peroxidase complex; mtDNA, mitochondrial DNA; RCI, respiratory control index

INTRODUCTION

Although *cis*-diamminedichloroplatinum (II) (cisplatin) is one of the most effective anticancer agents for the treatment of various types of tumors,[1–3] it often exhibits early and late adverse effects, such as acute renal injury,[4] chronic tubulointerstitial nephropathy and cyst formation.[5,6] Thus, prevention of the early and late adverse effects of cisplatin is one of the major issues in treating patients with cancer. Although various methods to prevent the occurrence of the adverse effects of cisplatin have been tested,[7–9] effective methods available for the clinical use remain to be established.

The cytotoxity of cisplatin has been postulated to occur via mitochondrial dysfunction and/or direct injury of nuclear DNA by generating reactive oxygen species such as superoxide and hydroxyl radicals.[10–13] Over production of reactive oxygen species in and around mitochondria oxidizes the critical dithiols in adenine nucleotide translocase and causes the release of cytochrome *c* into cytosol, thereby triggering the sequence of events leading to cell death.[14–17] Thus, selective protection of mitochondria against reactive oxygen species generated by cisplatin in intact tissues, such as the kidney, is of critical importance in the chemotherapy of patients with cancer.

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In fact, we previously demonstrated that hexamethylenediamine-conjugated superoxide dismutase (AH-SOD) preferentially accumulated in renal proximal tubule cells and inhibited cisplatin-induced renal dysfunction.[7] Furthermore, L-carnitine rapidly accumulated in the kidney also inhibited lipid peroxidation and mitochondrial injury induced by cisplatin.[18]

Edaravone is a potent scavenger for hydroxyl radical and peroxyl radical.[19] This compound has been shown to inhibit the oxidative damage of cultured endotherial cells[20] and to protect the brain from postischemic reperfusion injury.[21,22] Recent reports also showed that edaravone inhibited the acute renal dysfunction induced by cisplatin.[23,24] However, the mechanism by which edaravone inhibited the acute renal injury caused by cisplatin and the role of mitochondria in its pathogenesis remain to be elucidated. Furthermore, effects of edaravone on the chronic dysfunction and cyst formation of the kidney induced by cisplatin are not known.

The present work describes the mechanism-based protection of the kidney by edaravone against early and late adverse effects of cisplatin. The results indicate that edaravone substantially inhibits the cisplatin-induced mitochondrial injury, thereby suppressing the occurrence of acute and chronic renal injury. Thus, edaravone may have therapeutic potential to minimize the adverse effects of cisplatin in the kidney of patients during chemotherapy.

MATERIALS AND METHODS

Materials

Cisplatin was obtained from Nippon Kayaku (Tokyo, Japan). Edaravone was provided by Mitsubishi Pharma Corporation (Tokyo, Japan). Blood urea nitrogen (BUN) and creatinine test kits were purchased from Wako Pure Chemical (Osaka, Japan). Apoptosis detection kit was purchased from Takara Shuzo (Kusatsu, Japan). Other reagents used were of the highest grade commercially available.

Animal Experiments

Male Wistar rats (200–220 g) and Donryu rats (150–160 g) were obtained from Japan SLC Inc. (Shizuoka, Japan) and Oriental Yeast, Co., Ltd. Tokyo, Japan respectively. They were fed laboratory chow and water *ad libitum* and used for experiments without prior fasting. Under light ether anesthesia, either edaravone (30 mg/kg of body weight) or saline was intravenously administrated to rats. After 30 min, cisplatin was injected intraperitoneally at a dose of 5 mg/kg of body weight. At the indicated times after the treatment, body weight was measured and blood samples and kidneys were obtained from intact and cisplatin-treated animals. BUN and plasma creatinine were measured for monitoring renal function.

Ascites hepatoma (AH-130) cells were inoculated intraperitoneally to Donryu rats (1×10^7 cells/animal). On day 5 after AH-130 cell inoculation, animals were administered with cisplatin (5 mg/kg of body weight) intraperitoneally. Edaravone (30 mg/kg of body weight) was intravenously administrated 30 min before cisplatin-administration. At the indicated times after the inoculation, ascites were obtained from the peritoneal cavity and centrifuged at 500*g* for 5 min. The precipitated cells were suspended in phosphate-buffered saline and used freshly for experiments.

Histological Analysis

Five days after cisplatin administration, the kidney was perfused with 5 ml of ice-cold 0.9% NaCl solution, fixed in 5% formalin, and embedded in paraffin. Sections of tissue specimens, 4-μm thick, were stained with hematoxylin and eosin. Pathological examination was carried out under a light microscope. The degree of apoptosis was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) by using an *in situ* Apoptosis Detection Kit. To analyze the possible occurrence of oxidative stress, 4-hydroxy-2-nonenal (4-HNE) adducts were immunohistochemically detected by using the avidin-biotin-peroxidase complex (ABC) method with a vestastain elite ABC kit (Vector Laboratories Inc., Burlingame, CA). Briefly, after inactivation of endogenous peroxidase with a methanol solution containing 0.3% H_2O_2 and blocking with normal blocking serum, the sections were incubated with monoclonal mouse anti-rat-4-HNE antibody (100 × dilution; Japan Institute for the Control of Aging, Shizuoka, Japan) at 4°C for 1 h. After three times washing with PBS solution (pH 7.4), the sections were incubated with the biotinylated second antibody at room temperature for 30 min and subsequently treated with ABC reagent for 30 min. Peroxidase activity was developed with 0.025% 3, 3′-diaminobenzidine tetrahydrochloride in PBS solution (pH 7.4) containing 0.015% H_2O_2.

Analysis of Mitochondrial Respiration and DNA

Renal cortical mitochondria were isolated according to the method of Jung and Pergande.[25] Briefly, kidneys were perfused with ice-cold 0.9% NaCl solution and rapidly excised and the medullae were removed. The remaining cortex was minced and homogenized in 210 mM mannitol containing 70 mM sucrose, 0.5 mM EDTA, and 4 mM Tris–HCl (pH 7.4)

in a Teflon homogenizer. The homogenate was centrifuged at 800*g* and 4°C for 10 min. The supernatant was centrifuged at 12,000*g* for 5 min. The pellet (mitochondrial fraction) was then resuspended in the medium at 10–20 mg protein/ml and stored on ice until use. Protein concentration was determined by the method of Bradford[26] using bovine serum albumin as a standard.

Respiration of renal mitochondria was determined polarographically using a Clark-type oxygen electrode at 25°C.[27] Isolated mitochondria were suspended in a reaction medium consisting of 210 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4 and 60 mM Tris–HCl (pH 7.4) at a concentration of 0.25 mg protein/ml. Oxygen consumption was monitored in the presence of 5 mM succinate and 300 μM ADP. Mitochondrial function was examined by measuring state III respiration, ADP/O ratio and respiratory control index (RCI).

Mitochondrial DNA (mtDNA) was isolated from 50 mg of renal cortical mitochondria by using a mtDNA extraction kit purchased from Wako Pure Chemical (Osaka, Japan). The amount of mtDNA obtained from 50 mg of renal cortical mitochondria was about 2 μg. The mtDNA samples were subjected to 1% agarose gel electrophoresis at 100 V using TAE solution (40 mM Tris–acetate, pH 8.0, 1 mM EDTA) as a running buffer. The gel was stained with 0.1 μg/ml of ethidium bromide and visualized under ultraviolet light.

Analysis of Long-term Effects of Cisplatin on the Kidney

One year after the treatment, rats were housed in metabolism cages for 24 h and urine samples were obtained and creatinine clearance was determined. Then, plasma and kidneys were obtained. The number of renal cysts larger than 1 mm in diameter was counted and the area of renal cysts was measured using an NIH image. The respiration of renal mitochondria and the base sequence of mtDNA were also analyzed.

Statistical Analysis

Values are expressed as mean ± SD. Statistical analysis was performed by unpaired Student's *t*-test and significance was put at $P < 0.05$.

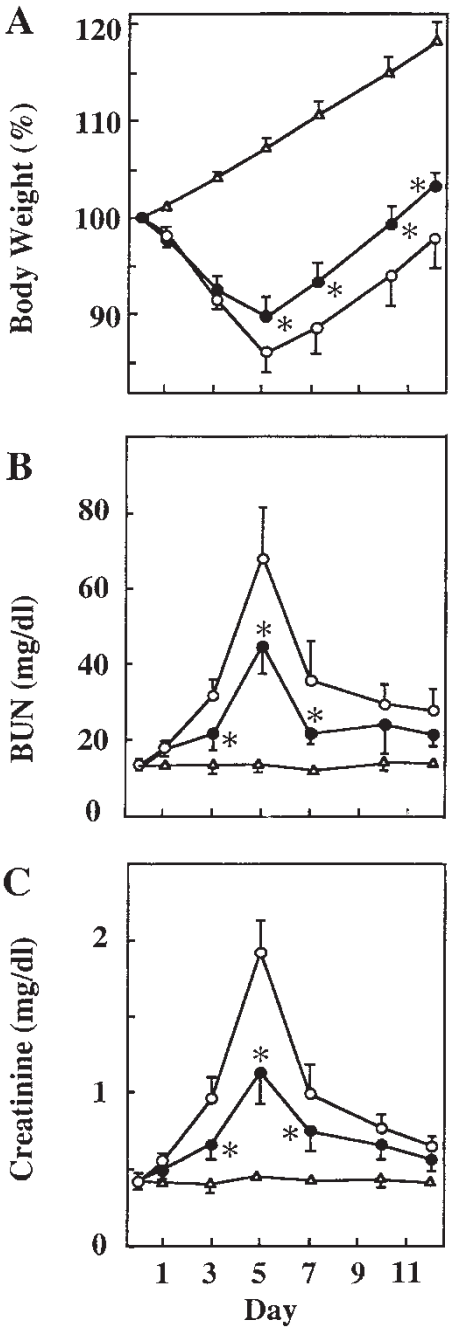

FIGURE 1 Effect of edaravone and cisplatin on the body weight and renal functions. Thirty minutes after intravenous injection of edaravone (30 mg/kg body weight), cisplatin was administered intraperitoneally (5 mg/kg body weight). At the indicated times, body weight (A) and plasma levels of BUN (B) and creatinine (C) were measured. Open circles, cisplatin-treated group; closed circles, cisplatin and edaravone-treated group; open triangles, control group. Data are expressed as means ± SD ($n = 8$). *$P < 0.05$ compared with cisplatin-treated group.

RESULTS

Effect of Cisplatin and Edaravone on the Body Weight and Renal Functions

The effect of edaravone on cisplatin-induced loss of body weight and renal injury was analyzed (Fig. 1). Body weight of control rats continuously increased, while that of cisplatin-treated animals decreased by 14 ± 3% within 5 days and then recovered thereafter. The body weight of animals treated with both cisplatin and edaravone also decreased during the first 5 days and recovered thereafter. The recovery of the body weight was faster with edaravone-treated group than with untreated

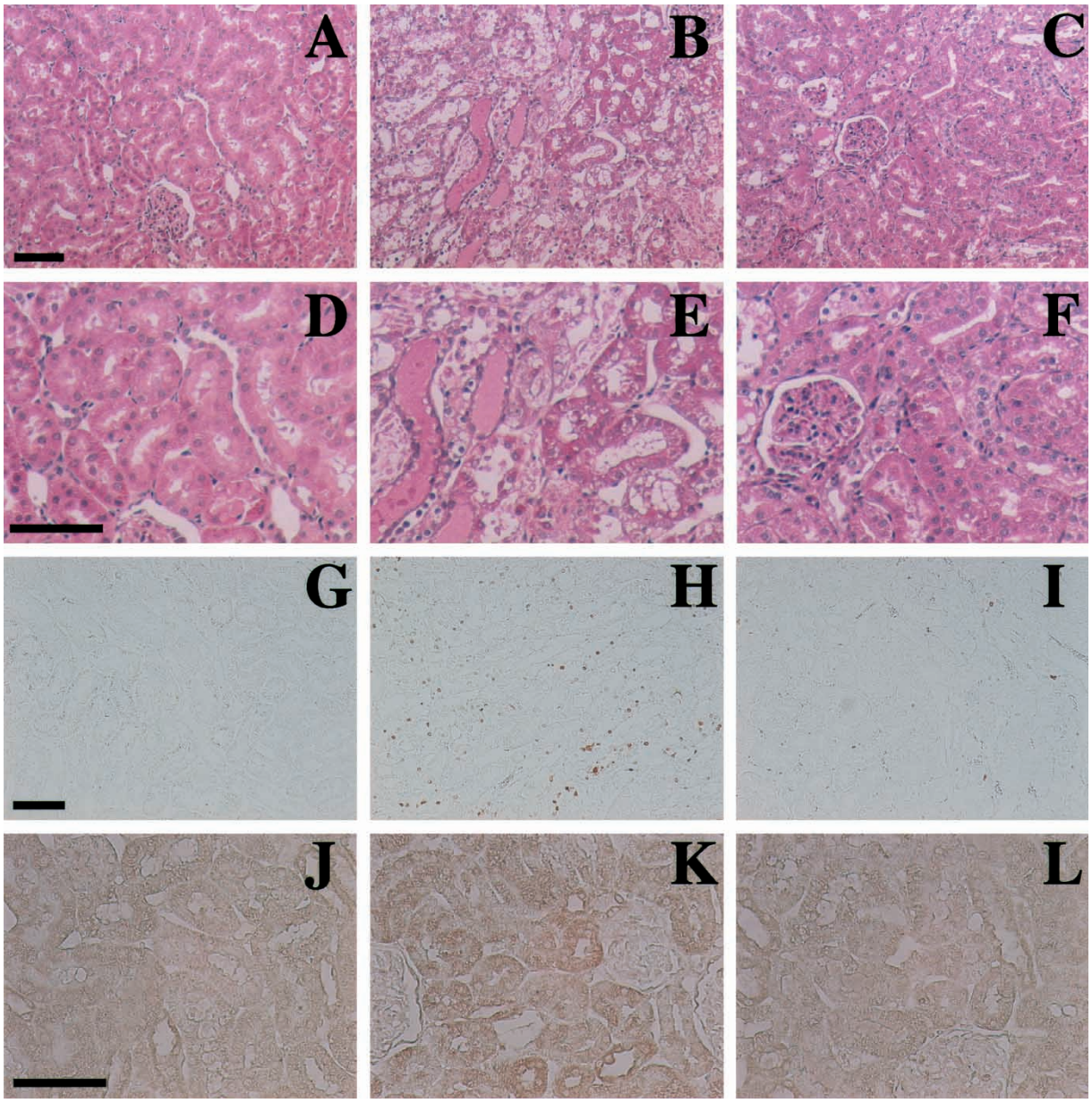

FIGURE 2 Effects of edaravone on cisplatin-induced renal injury and TUNEL-positive cells. Effects of cisplatin and edaravone on renal structure on day 5 (A–F) and the occurrence of apoptosis (G–I) and lipid peroxidation (J–L) on day 3 were examined. A, D, G and J, control group; B, E, H and K, cisplatin-treated group; C, F, I and L, cisplatin and edaravone-treated group. Magnification, A–C, G–I, ×100; D–F, J–L, ×200 (Bar, 100 μm).

group. After administration of cisplatin, plasma levels of both BUN and creatinine increased transiently, peaked on day 5, and recovered thereafter. The cisplatin-induced increase of plasma levels of BUN and creatinine was suppressed by edaravone. Administration of edaravone alone had no appreciable effects on the body weight and plasma levels of BUN and creatinine (data not shown).

Effect of Edaravone on Cisplatin-induced Changes in Renal Histology

Administration of cisplatin induced structural injury of the kidney (Fig. 2). On day 5, cytoplasmic vacuolization, swelling of tubular epithelial cells, dilation of tubular lumens, focal tubular necrosis, cast formation in the lumen, and some cells with pyknotic nuclei in the proximal tubules was apparent. Administration of edaravone markedly inhibited the cisplatin-induced renal injury; luminal cast formation and cells with pyknotic nuclei were observed minimally.

We also analyzed the effect of edaravone on day 3 when cisplatin-induced structural injury was not apparent. Although TUNEL-positive cells were not apparent in the kidney of control rats, their number increased significantly in the proximal tubules of the cisplatin-treated group by some

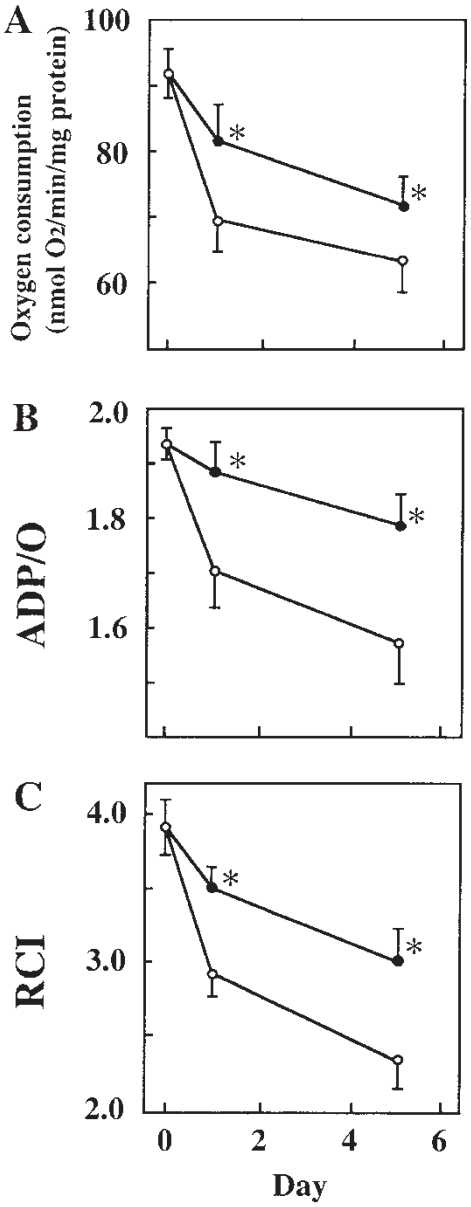

FIGURE 3 Effect of edaravone on cisplatin-induced mitochondrial dysfunction. Animals were intravenously injected either 30 mg/kg of edaravone (closed circles) or 1 ml of saline (open circles). After 30 min, 5 mg/kg of cisplatin was injected intraperitoneally. On days 1 and 5, renal mitochondria were isolated and their respiration was monitored at 25°C in 10 mM Tris–HCl buffer (pH 7.4) containing 5 mM succinate and 300 μM ADP. Mitochondrial function was examined by measuring oxygen consumption (A), ADP/O ratio (B) and respiratory control index (RCI) (C). Data are expressed as means ± SD ($n = 5$). *$P < 0.05$ compared with cisplatin-treated groups.

edaravone-inhibitable mechanism. Thus, edaravone inhibited the occurrence of cisplatin-induced apoptosis of renal proximal tubule cells. Although 4-HNE-positive cells were apparent in renal proximal tubules of cisplatin-treated animals, they decreased significantly in edaravone-treated group. Thus, edaravone inhibited cisplatin-induced lipid peroxidation in renal proximal tubules.

Effect of Edaravone on Cisplatin-induced Renal Mitochondrial Dysfunction

Mitochondrial dysfunction is an initial event in cisplatin-induced renal injury.[7,18] Thus, we analyzed the effect of edaravone on mitochondrial respiration of cisplatin-treated rats (Fig. 3). Kinetic analysis revealed that state III respiration, ADP/O ratio and RCI were markedly decreased with renal mitochondria obtains on day 1 after the cisplatin-treatment. On day 5, mitochondrial functions decreased further.

Effect of Edaravone on Cisplatin-induced mtDNA Injury in the Kidney

To test the possible involvement of mtDNA in the pathogenesis of cisplatin-induced renal dysfunction, we analyzed the effect of edaravone on renal mtDNA isolated from renal cortex of cisplatin-administrated rats (Fig. 4). The amount of intact mtDNA electrophoresed as a major band (16.5 kb) markedly decreased one day after cisplatin-treatment, while it recovered slightly on day 5. Significant fractions of mtDNA remained intact in edaravone-treated group. Thus, edaravone inhibited the cisplatin-induced fragmentation of mtDNA and/or enhanced its recovery in the kidney.

Effect of Edaravone on the Anticancer Action of Cisplatin

Prevention of adverse effects of anticancer agents without decreasing their anticancer activity is one of

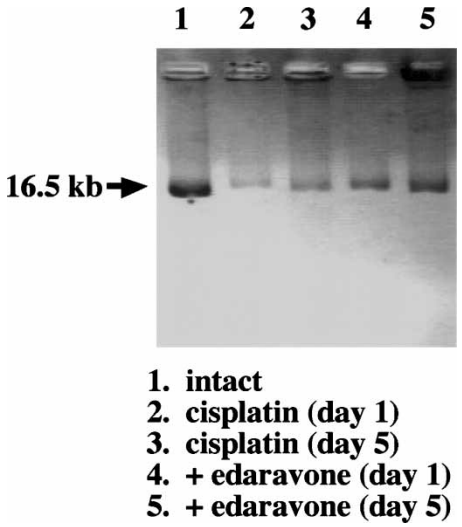

FIGURE 4 Effect of edaravone on cisplatin-induced renal mtDNA. Animals were intravenously injected 30 mg/kg of edaravone (lane 4 and 5) or 1 ml of saline (lane 2 and 3). After 30 min, 5 mg/kg of cisplatin was injected intraperitoneally. On days 1 (lane 2 and 4) and 5 (lane 3 and 5), mtDNA extracted and subjected to agarose gel electrophoresis as described in the text. Lane 1 shows the intact renal mitochondrial DNA.

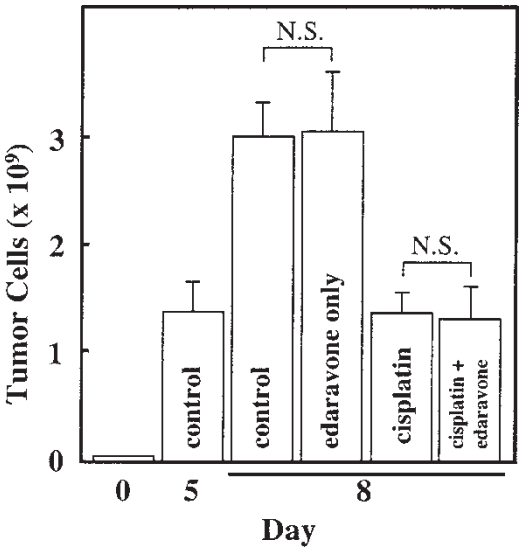

FIGURE 5 Effect of edaravone on the anticancer action of cisplatin. Animals were intraperitoneally inoculated with AH-130 cells (1×10^7/rat). On day 5, edaravone was administered intravenously (30 mg/kg body weight) 30 min before intraperitoneal administration of cisplatin (5 mg/kg body weight). On day 5 and 8 after cell inoculation, the number of AH-130 cells in the peritoneal cavity was counted. Data are expressed as means ± SD ($n = 5$).

the major subjects in the chemotherapy of patients with cancer. To test the therapeutic potential of edaravone in cancer chemotherapy, its effect on the anticancer action of cisplatin was examined in AH-130 cell-bearing rats.

On day 8 after the inoculation, the number of AH-130 cells in the peritoneal cavity increased markedly (Fig. 5). The number of peritoneal cancer cells strongly decreased in cisplatin-treated animals. Administration of edaravone alone has no effect on AH-130 cell growth. The anticancer action of cisplatin remained unaffected by edaravone.

Effect of Edaravone on the Cisplatin-induced Renal Cyst

No significant difference in plasma creatinine levels was found among control, cisplatin-treated and cisplatin plus edaravone-treated rats one year after the administration. However, the value of creatinine clearance was significantly decreased in cisplatin-treated animals with concomitant elevation of plasma BUN (Fig. 6). The cisplatin-decreased creatinine clearance and increased BUN were within normal ranges in edaravone-treated group. To get further insight into the long-term effect of edaravone, pathological examination of the kidney was performed one year after the administration (Fig. 7). Macroscopic observation of the kidney revealed the presence of a large number of cysts in cisplatin-treated group (21.5 ± 8.0 cysts/kidney) but not in edaravone-treated group (2.0 ± 1.7 cysts/kidney);

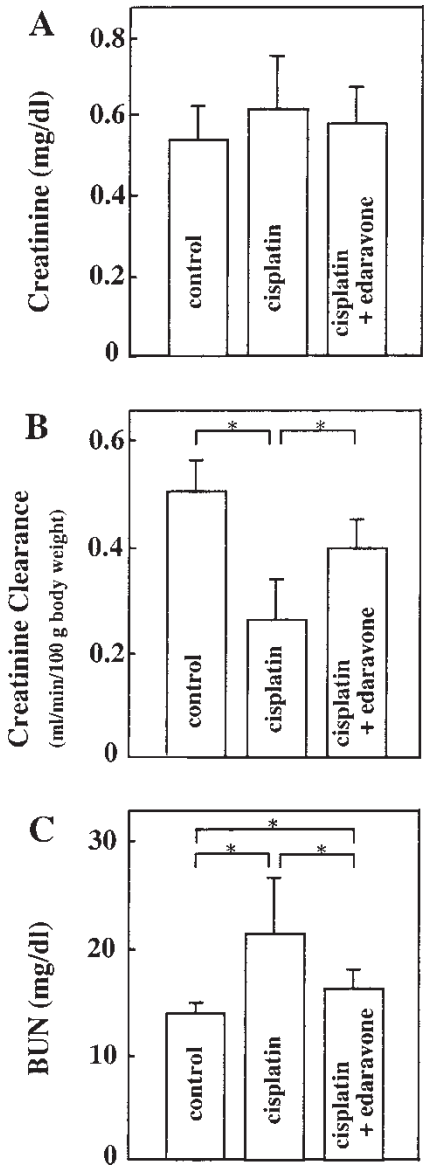

FIGURE 6 Effects of edaravone on cisplatin-induced chronic renal dysfunction. One year after the treatment, plasma levels of BUN (A) and creatinine (B) and creatinine clearance (C) were measured as described in the text. Data are expressed as means ± SD ($n = 5$). *$P < 0.05$.

percent of renal area occupied by cysts in cisplatin-treated and cisplatin and edaravone-treated group was 21.1 ± 7.1 and 0.7 ± 0.8, respectively. Histological examination revealed that, in addition to multiple cyst formation, infiltration of inflammatory cells, particularly lymphocytes, and interstitial fibrosis were apparent in the kidneys of cisplatin-treated rats. Furthermore, atrophic tubules with thick basement membranes, cystically dilated tubules and hyaline casts were also observed in the cortex of cisplatin-treated rat kidney. Administration of edaravone strongly inhibited the occurrence of pathological changes induced by cisplatin. Thus, edaravone was also effective in inhibiting the long-lasting effects of cisplatin.

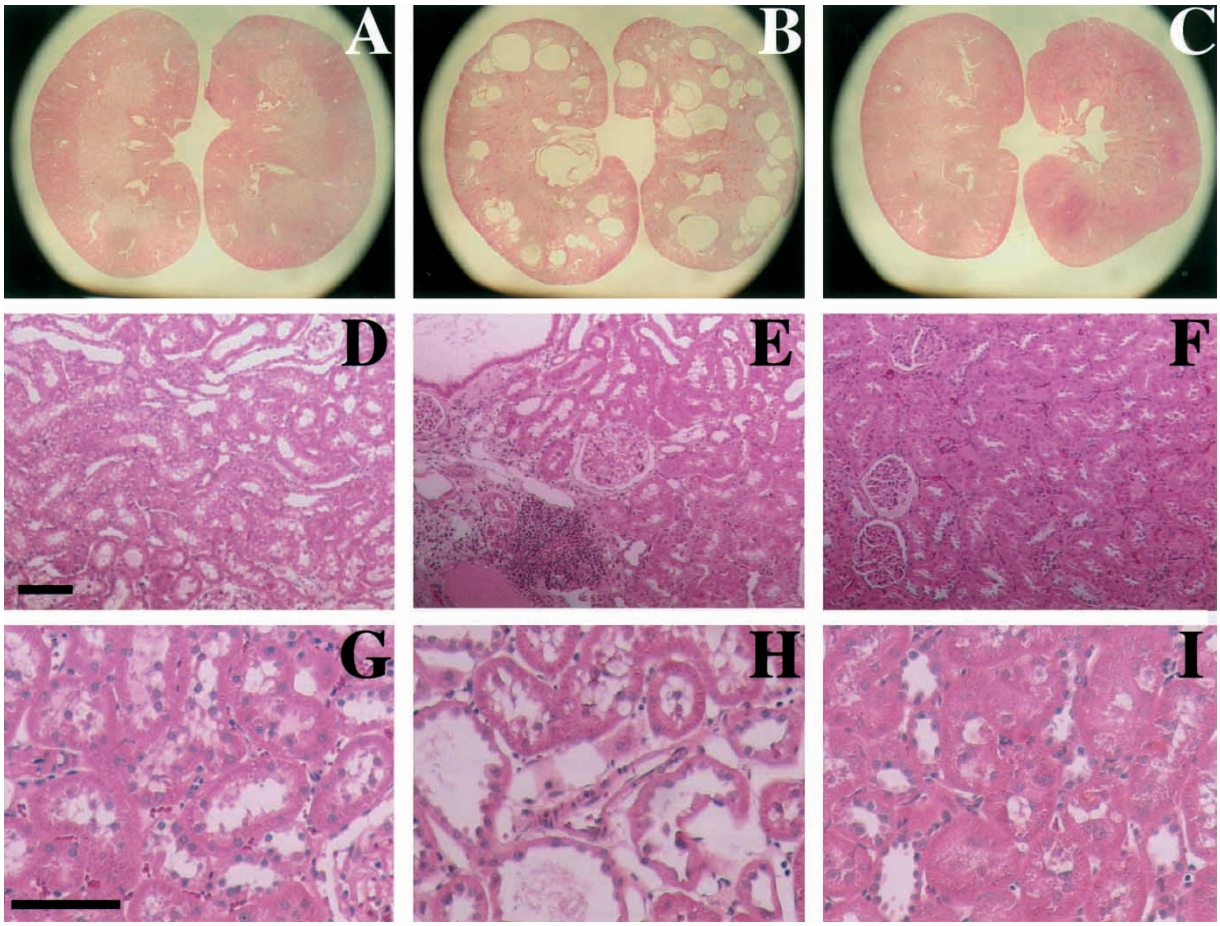

FIGURE 7 Effects of edaravone on cisplatin-induced renal injury and cyst formation. One year after the treatment, kidneys were obtained from control (A, D, G), cisplatin-treated (B, E, H) and cisplatin and edaravone-treated rats (C, F, I). Magnification, A–C, × 1; D–F, × 100; G–I, × 200 (Bar, 100 μm).

DISCUSSION

The present work demonstrates that cisplatin rapidly impaired the renal tubular functions and their mtDNA within 24 h and induced the occurrence of TUNEL-positive cells and histological injury that caused the long-lasting events leading to the formation of multiple cysts associated with renal dysfunction by some edaravone-inhibitable mechanism.

The reports about the protective effects of edaravone on the cisplatin-induced renal injury are conflicting. Sueishi *et al.*[23] reported that simultaneous administration of edaravone with cisplatin failed to protect renal functions, while it showed therapeutic effect when administered 24 and 36 h after the treatment with cisplatin. In contrast, Satoh *et al.*[24] reported that edaravone showed protective effect on renal injury even if the agent was administered simultaneously with cisplatin. The present work clearly demonstrates that cisplatin impaired renal mitochondria and mtDNA within 24 h by a mechanism that was inhibited by pretreating animals with a single dose of edaravone. We previously showed that targeting SOD to renal proximal tubule cells strongly inhibited the renal injury induced by cisplatin and suggested the importance of the superoxide radical and/or its reactive metabolites for the pathogenesis of cisplatin nephropathy.[7] Furthermore, simultaneous administration of L-carnitine also inhibited the cisplatin-induced injury of the kidney and small intestine.[18] Because renal accumulation of edaravone and L-carnitine access transiently after their administration,[28] the early phase of oxidative injury of renal mitochondria induced by cisplatin might seem to be the critical event leading to apoptosis of proximal tubule cells followed by the long-lasting nephropathy.

It has been well documented that antioxidants, such as GSH, vitamin E and ascorbic acid, play critical roles in inhibiting oxidative tissue injury by potentiating their activity through interaction with each other to form redox-cycles that regenerate the active (reduced) form of each compound.[29] Antioxidants lacking such property to regenerate themselves by some redox-cycling mechanism have low activity to inhibit oxidative tissue injury because

such suicide scavengers can detoxify limited (stoichiometric) amounts of reactive oxygen species.

Cisplatin has been shown to generate reactive oxygen by interaction with DNA.[13] It has been well documented that the hydroxyl radical is generated from hydrogen peroxide by the metal-catalyzed Fenton reaction and that this reaction can be maintained by a redox cycling of transition metals.[30,31] Because the superoxide radical catalyzes the reduction of oxidized transition metals,[31,32] cisplatin might undergo redox-cycling to regenerate its active form. Furthermore, renal proximal tubules are highly enriched with γ-glutamyltransferase that degrades GSH to form cysteine, a potent reducer.[33,34] Thus, it is not surprising that nephrophilic xenobiotics having redox-cycling activity preferentially exhibit their toxicity in the kidney.[35] Under physiological conditions, edaravone behaves as an organic cation and, hence, preferentially accumulates in the kidney presumably via organic cation transport system in renal proximal tubule cells.[28,36] Thus, pharmacological actions of both cisplatin and edaravone are expected to appear preferentially in the kidney that transiently accumulates these agents to secrete into urine. In fact, the present work clearly shows that the therapeutic effect of edaravone was apparent within 24 h after cisplatin treatment. Although edaravone has been known to show strong activity to scavenge free radicals *in vitro*,[20] it is not clear at present whether the agent inhibited the cisplatin toxicity through its putative antioxidant activity or not. In this context, ascorbic acid and edaravone have been shown to inhibit the peroxidation of phosphatidylcholine *in vitro*.[37] Although the presence of both edaravone and ascorbic acid strongly inhibited the peroxidation reaction, it is not clear whether they exhibited the inhibitory effect additively or synergistically. The possibility that ascorbic acid can regenerate the active form of edaravone through its redox-cycling activity should be studied further. Analogously, possible involvement of the redox cycling by endogenous antioxidants, such as cysteine and GSH in the protective mechanism of edaravone against nephrotoxicity of cisplatin should be studied further.

The present work also shows that administration of edaravone to AH-130 cell-bearing rats did not suppress the anticancer action of cisplatin. It should be noted that tissues distribution of edaravone and cisplatin changes depending on the time after and routes of their administration, and the presence of their transport systems. Because the intravenously administered edaravone preferentially accumulated in the kidney,[28,36] anticancer effects of intraperitoneally administrated cisplatin was affected minimally by edaravone.

Cisplatin induces not only acute but also chronic types of renal injury including multiple cyst formation.[5,6,38] In fact, the present work also shows the occurrence of multiple cysts in the kidney one year after the administration of cisplatin. To our surprise, a single dose of edaravone just before the administration of cisplatin was sufficient for the inhibition of multiple cyst formation in the kidney. Because edaravone is excreted in urine fairly rapidly and its half-life in the circulation is shorter than 30 min,[39] inhibition of the tubular injury at an early period after cisplatin administration seems to be responsible for its long-lasting effect to inhibit the occurrence of multiple cysts in cisplatin-treated rat kidney. This notion is consistent with the hypothesis that edaravone should be accumulated in renal tubules cells with pharmacologically high concentrations when the toxic action of cisplatin operates. Preliminary experiments in this laboratory revealed that the respiratory functions of renal mitochondria and their mtDNA sequence obtained from cisplatin-treated animals with multiple cysts were not impaired. Histochemical examinations also showed no significant sign of oxidative stress. This observation is consistent with the notion that edaravone inhibited acute renal injury induced by cisplatin, thereby suppressing the long-lasting pathologic processes leading to the formation of multiple cysts in the kidney. To elucidate the mechanism of edaravone action, effects of antioxidants including ascorbic acid, vitamin E and reduced glutathione should be studied on the effect of cisplatin to generate renal multiple cysts should be studied further. Apart from the mechanism of edaravone action, the combined administration of this agent with nephrotoxic drugs used clinically, such as anticancer agents, may have therapeutic potential in human subjects.

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