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ORIGINAL PAPER

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Cisplatin-induced apoptosis in human proximal tubular epithelial cells is associated with the activation of the Fas/Fas ligand system

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Abstract The role of Fas and Fas ligand (Fas-L) in the apoptotic cell death process in cisplatin (CP)-treated human proximal tubular epithelial cells (PTECs) was examined. The human PTECs were treated with various concentrations (20–80 μM) of CP for 24 h, and the incidence of apoptosis in CP-treated cells was assessed by trypan blue staining, propidium iodide staining, in situ end labeling, and electron microscopy. The expression of Fas and Fas-L was detected by immunofluorescence microscopy. The results showed that: (1) CP-treatment resulted in a decreased number of live human PTECs and an increased number of dead cells, (2) CP-treated human PTECs showed an increased rate of apoptosis with its typical morphological features, and (3) expression of both Fas and Fas-L was upregulated in CP-treated human PTECs. These results indicate that CP treatment induces apoptosis in human PTECs and the activation of the Fas/Fas-L system may play an active role in the induction of the apoptotic cell death process.

Introduction

Cisplatin (CP)-induced tubulointerstitial nephritis is one of the major chronic problems encountered in clinical practice. CP-induced renal injury is associated with damage to tubular epithelial cells (mainly proximal), interstitial inflammatory cell infiltration, and interstitial fibrosis (Choie et al. 1980; Goldstein and Mayor 1983). The CP-induced structural changes of the kidneys subsequently lead to impaired renal function. The exact mechanism by which CP induces tubulointerstitial damage in the kidney remains mostly unknown. Although several reports have documented the increased rate of apoptosis during tubulointerstitial damage in various renal diseases (Gobe and Axelsen 1987; Truong et al. 1996), very little is known about the involvement of apoptosis in CP-induced tubulointerstitial nephritis, and even less information is available about the association between apoptosis and the Fas/Fas ligand (Fas-L) system.

Apoptosis occurs in a wide variety of different biological systems in various physiological and pathological conditions, including the repair process of ischemic tubular necrosis (Shimizu and Yamanaka 1993). Cell deletion by apoptosis was also noted in tubular atrophy in experimental hydronephrosis (Gobe and Axelsen 1987) and experimental chronic obstructive uropathy (Truong et al. 1996). Despite a large volume of literature describing the ability of CP to induce apoptosis in various tumor cells (Dixit et al. 1997; Evans and Dive1993; Henkels and Turchi 1997), no report concerning the ability of CP to induce apoptosis in human tubular epithelial cells has been found. Clearly, there is potential importance to investigate the involvement of apoptosis in CP-induced tubular damage, since apoptosis implies the possibility to be manipulated (Gobe and Axelsen 1987; Shimizu and Yamanaka 1993; Truong et al. 1996). This study was undertaken, therefore, to evaluate the ability of CP to induce apoptosis in human PTECs, and, if so, to explore the possible involvement of the Fas/Fas-L system in the apoptotic cell death process.

Materials and methods

Cell cultures

Normal human proximal tubular epithelial cells (PTECs) were obtained from Clonetis, USA (CC 2553) and cultured in renal epithelial growth medium supplied by Clonetis (CC 3190).

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Experimental design

Human PTECs were seeded in T-flasks, multiwell plastic plates, and on coverslips at a density of 2500 cells/cm^2 and incubated at 37°C in a humidified atmosphere of 5% CO_2. When the cells became approximately 80% confluent, they were treated with CP at a concentrations of 20, 40, and 80 µM for 24 h and then the cells on the coverslips were fixed with 10% formalin for 10 min for morphological studies. The cells in the T-flasks and multiwell plastic plates were treated with trypsin/EDTA and pelleted. The cell viability was assessed by the trypan blue (TB) dye exclusion test. A part of the cell pellet was immersed in 2.5% glutaraldehyde for 48 h at 4°C for electron microscopic examination.

Morphological studies

Both control and CP-treated human PTECs grown on the coverslips were stained by H&E for routine morphological studies.

Cell viability

Cell viability was assessed by the TB exclusion test. Briefly, cell counts were performed in a hemocytometer using equal volumes of cell suspension and TB (0.3% in PBS). Cells with membrane permeability allowing uptake of TB were interpreted as non-viable.

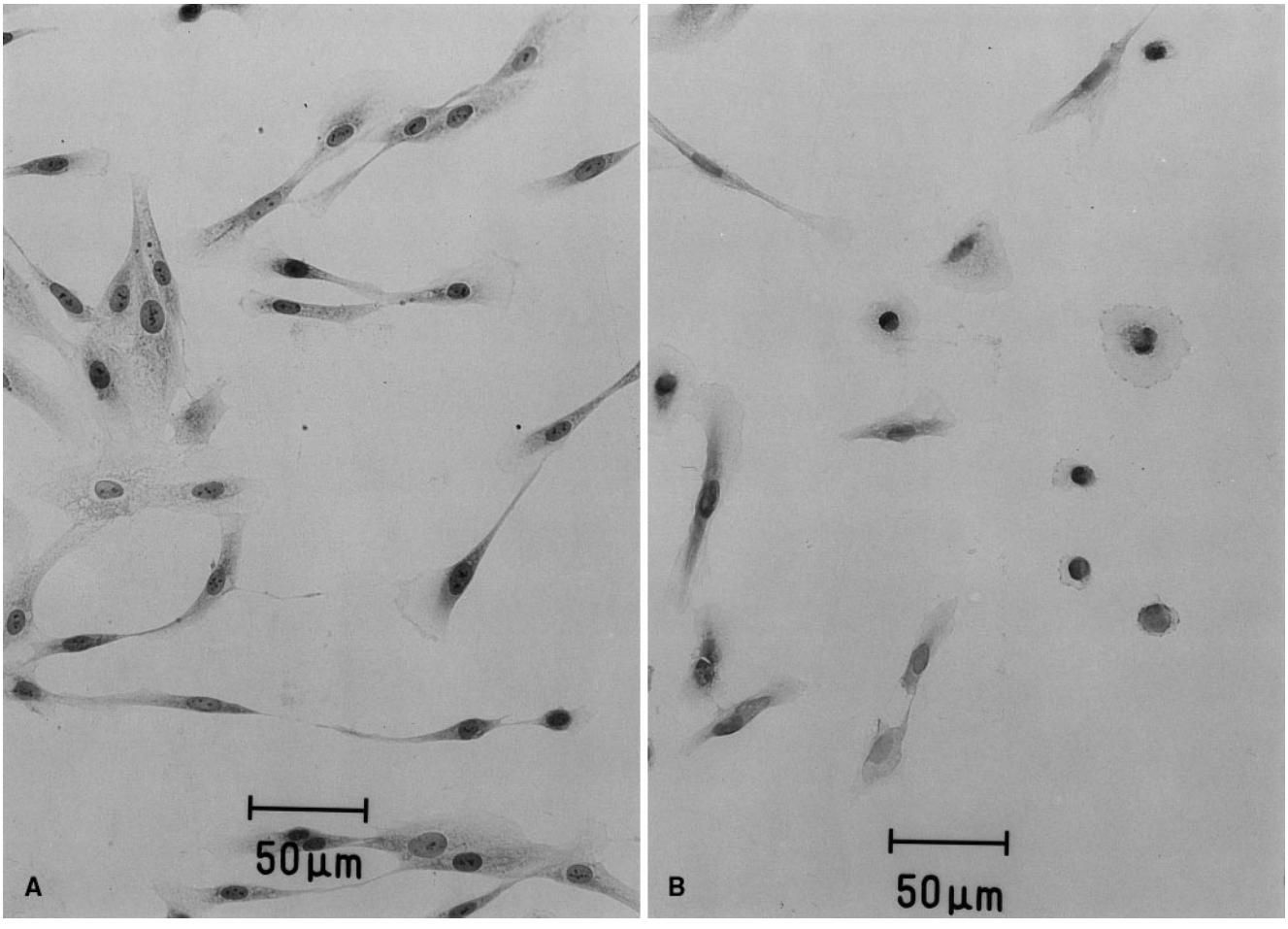
In situ end labeling

In situ end labeling was performed using an Apoptag kit (Oncor, USA). Briefly, formalin-fixed cells grown on glass coverslips were rinsed in 0.01 M PBS (pH 7.2) twice for 5 min and then soaked in 0.3% hydrogen peroxide in methanol for 15 min at room temperature. The coverslips were then covered with equilibration buffer for 30 s, followed by incubation with terminal deoxynucleotidyltransferase and digoxigenin-11-dUTP from the Apoptag kit, as recommended by the manufacturer, at 37°C for 1 h. The reaction was terminated using the Apoptag stop buffer. The labeled nuclei were detected with a peroxidase conjugated anti-digoxigenin antibody and the coverslips were developed with 3,3'-diaminobenzidine and H_2O_2. As a control, cells grown on glass coverslips were reacted with TdT without digoxigenin-11-dUTP. The TdT-positive nuclei were counted in at least 15 randomly selected fields from the stained coverslips under the microscope at a magnification of ×200.

Propidium iodide(PI) staining

PI staining was performed as described by Coles et al. (1993) with a slight modification. Briefly, formalin-fixed cells grown on glass coverslips were rehydrated through 0.01 M PBS (pH 7.2) twice for 5 min and were incubated in PI (4 mg/l) with RNase A (100 mg/l) in PBS at 37°C for 1 h. Then, the coverslips were washed with PBS, mounted, and examined with a fluorescence microscope (wavelength 568 nm). The cells with condensed and/or fragmented nuclei were interpreted as apoptotic.

Fig. 1 **A** Human proximal tubular epithelial cells (PTECs) without cisplatin (CP) treatment. **B** Morphological features of human PTECs 24 h after treatment with 20 µM of CP. Note that in contrast to the control cells (**A**), CP-treated cells are mostly rounded with condensed hyperchromatic nuclei (**B**). H&E staining. *Bars*=50 µm



Electron microscopy

For electron microscopy, glutaraldehyde-fixed cells were processed using the conventional staining method. Briefly, cell pellets were immersed in 2.5% glutaraldehyde for 48 h at 4°C, postfixed with 1% osmium tetroxide, dehydrated, and embedded in Epon. The ultrathin sections contrasted with uranyl acetate and lead citrate were examined with a Jeol 1200 transmission electron microscope.

Immunofluorescence microscopy

Immunofluorescence staining was performed on formalin-fixed cells grown on glass coverslips. Briefly, the cells on coverslips were rehydrated thoroughly with PBS. The sections were then incubated with either 10% goat serum (30 min), followed by incubation with either anti-Fas antibody (×400; Koji et al. 1994) or anti-Fas-L antibody (×200; Hakuno et al. 1996) for 1 h. As immunohistochemical controls, the antibodies were replaced with 0.01% PBS or with rabbit serum. After washing with PBS, the sections on coverslips were further incubated with FITC-conjugated secondary antibody. They were washed with PBS, mounted, and examined with a fluorescence microscope. The specificity of the Fas and Fas-L antibodies were detailed in earlier reports (Hakuno et al. 1996; Iwamoto et al. 1996; Koji et al. 1994). The Fas- and Fas-L-immunostained cells were counted in at least ten randomly selected fields under the microscope at a magnification of ×200.

Statistical analysis

When appropriate, the statistical analysis was assessed by the use of parametric ANOVA and calculated with StatView software (for Apple Macintosh).

Results

Cell morphology

CP-treatment resulted in structural changes of the human PTECs at the light microscopic level. Compared to the control cells (Fig. 1A), an increased number of rounded cells having condensed hyperchromatic nuclei were noted in CP-treated cells (Fig. 1B).

Cell viability

The TB staining revealed that the numbers of living human PTECs were decreased, in contrast to the increased number of dead cells in the CP-treated cells (Fig. 2, 3).

Identification of apoptosis

By PI staining, compared to the control cells (Fig. 4A), an increased number of cells with fragmented nuclei and/or condensed nuclei, resembling apoptotic bodies, was noted in CP-treated human PTECs (Fig. 4B). Also, the number of cells with positive nuclear staining by in situ end labeling was increased in CP-treated human PTECs. The average numbers of TdT-positive nuclei in control and CP-treated cells are shown in Table 1. Compared to the untreated cells, a significantly increased number of TdT-positive apoptotic cells ($P<0.0001$) was noted in CP-treated cells. By electron microscopy, in comparison to the control cells (Fig. 5A), typical ultrastructural features of apoptotic nuclei with condensation

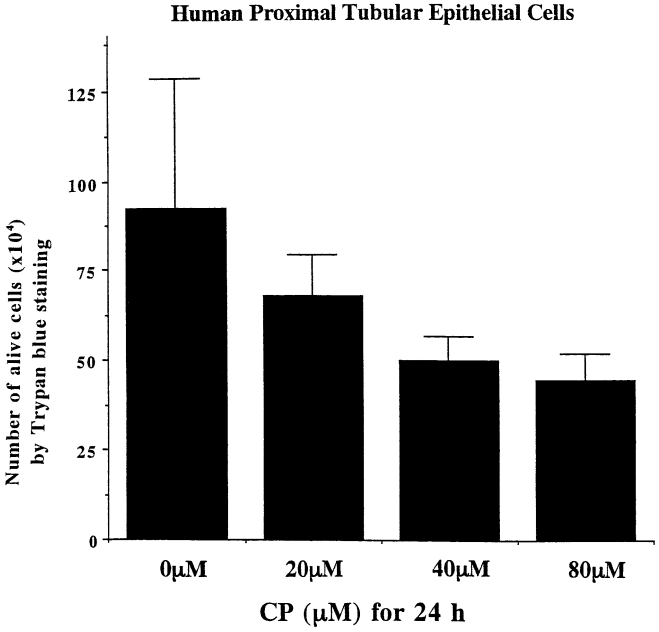


Fig. 2 Number of live cells determined from trypan blue staining after various concentrations of cisplatin (CP) treatment for 24 h. Note that the number of live cells decreased in a dose-dependent manner in CP-treated cells

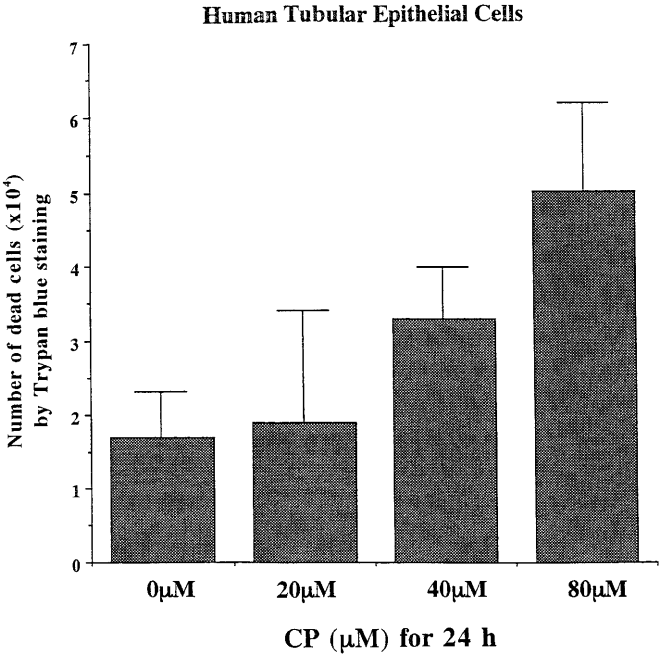


Fig. 3 Number of dead cells determined from trypan blue staining after various concentrations of CP treatment for 24 h. Note that the number of dead cells increased in a dose-dependent manner in CP-treated cells

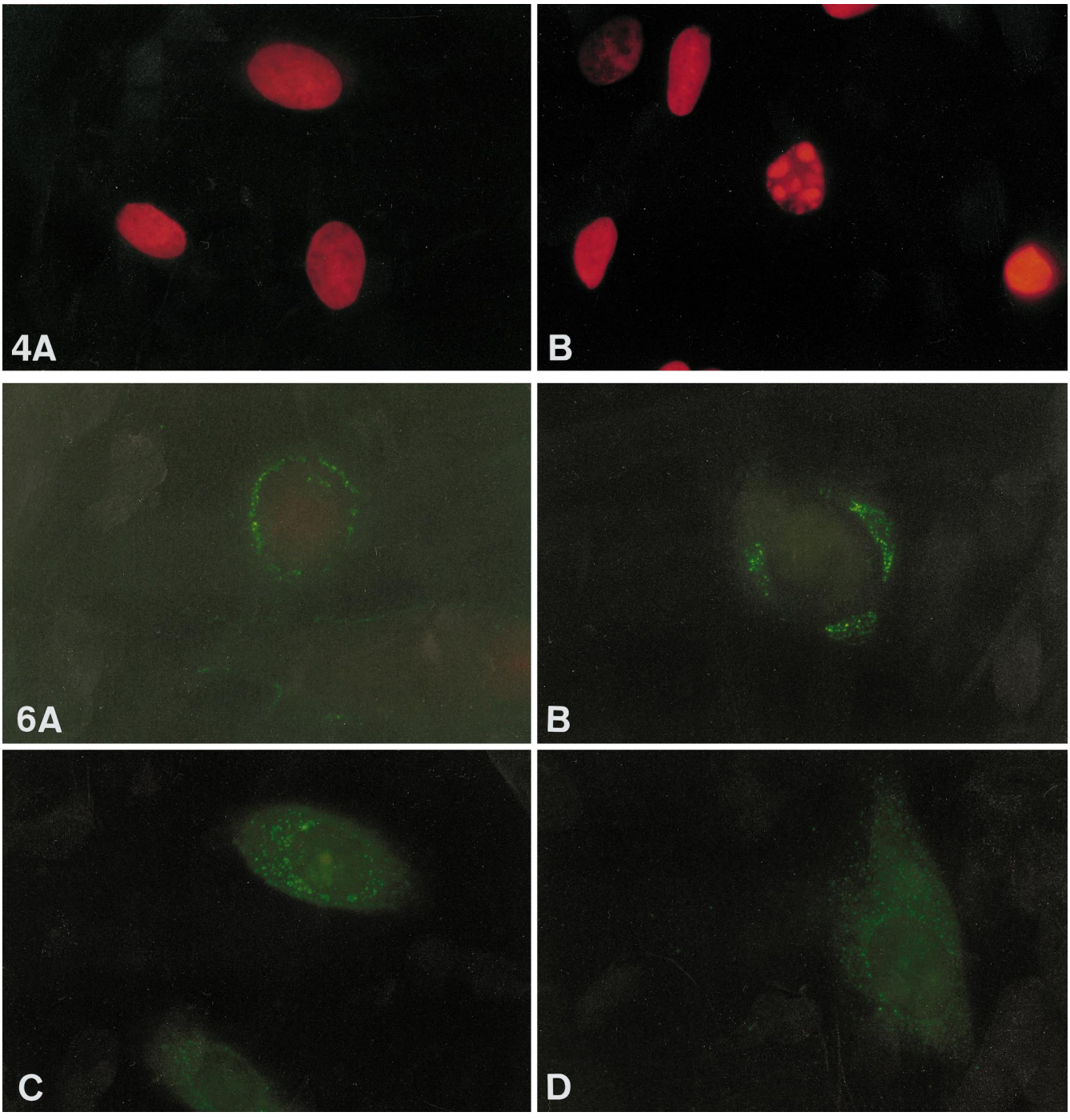


Table 1 Average numbers of TdT-, Fas-, and Fas ligand (Fas-L)-immunopositive human proximal tubular epithelial cells (PTECs) in control and after various concentrations of CP treatment for 24 h. Note that compared to the non-treated control cells, the numbers of TdT-, Fas-, and Fas-L-immunopositive cells are significantly (P<0.0001) increased in CP-treated cells

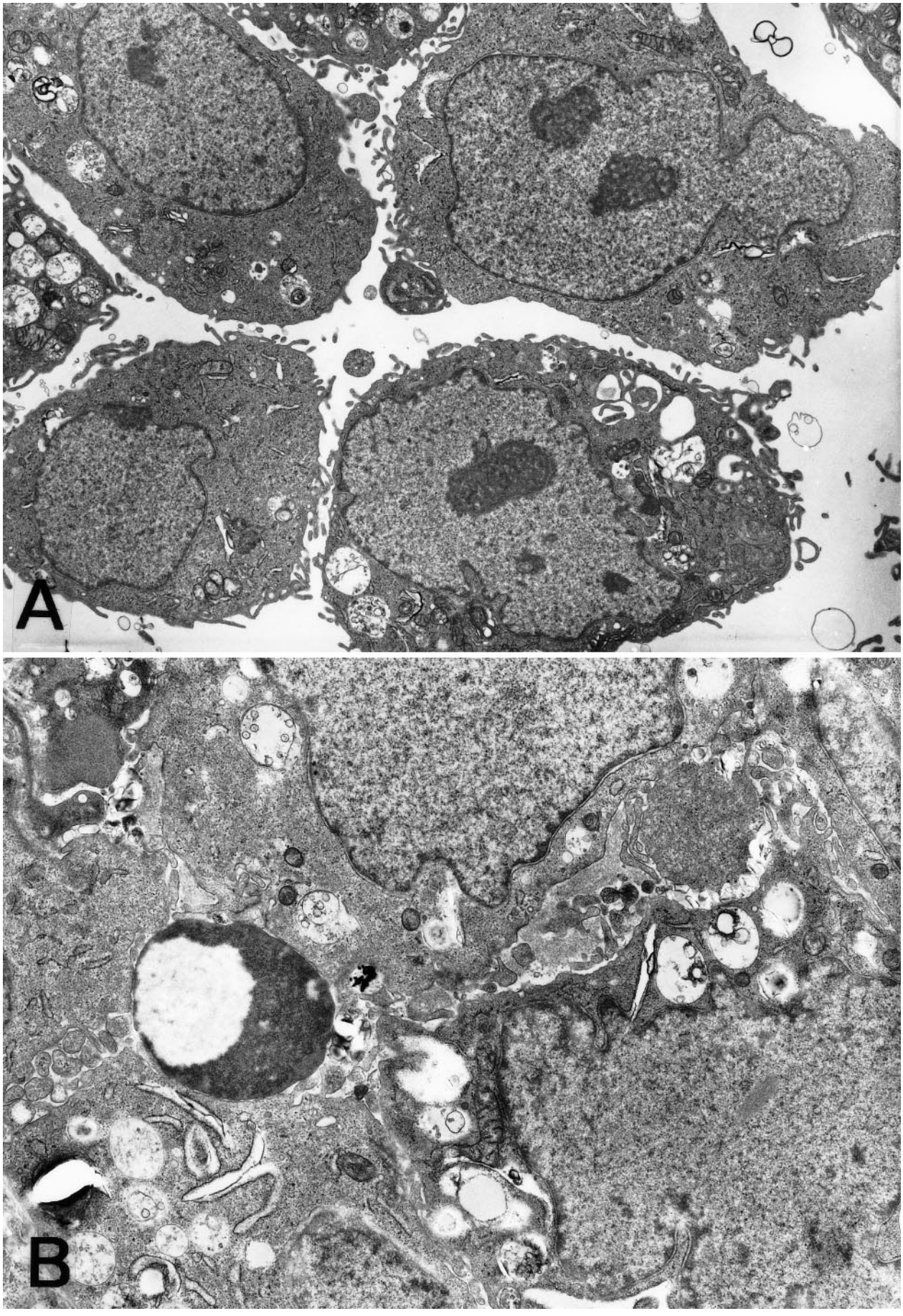
	TdT	Fas	Fas-L
Control	1.8±1.3	0.7±0.6	1.8±0.6
20 μM	3.5±1.3**	4.8±1.5**	3.5±2.1**
40 μM	6.2±2.1**	5.6±1.4**	5±1.5**
80 μM	12.5±3.2**	5.9±1.7**	5.3±1.7**

**Statistically significant (P<0.0001) vs control

Fig. 4 Propidium iodide staining in control (**A**) and CP -treated (80 μM for 24 h) cells (**B**). Fragmentation of nuclei with apoptotic body, typical feature of apoptosis, is seen in the CP-treated cells (**B**)

Fig. 6 Immunostaining of Fas (**A**, **B**) and Fas ligand (Fas-L; **C**, **D**) in CP-treated cells. Note the perimembranous immunostaining for Fas in the CP-treated cells (**A, B**). In contrast to Fas, Fas-L is showing cytoplasmic staining in CP-treated cells (**C, D**)

Fig. 5 **A** Electron microscopy of control human PTECs, showing characteristic features of microvilli. **B** Typical apoptotic nuclei with peripheral margination of chromatin are seen in cells treated with CP (80 μM for 24 h). Original magnification **A** ×5000, **B** ×3500 ▶


A
B

of chromatin and peripheral margination were noted in CP-treated cells (Fig. 5B).

Immunofluorescence staining for Fas and Fas-L

Although control human PTECs were mostly negative for both Fas and Fas-L (data not shown) immunostaining, CP-treated cells showed positive staining for both Fas (Fig. 6A,B) and Fas-L (Fig. 6C,D). Fas antigen was detected mainly in the perimembranous regions (Fig. 6A,B), while its ligand gave mainly cytoplasmic staining (Fig. 6C,D). The numbers of Fas and Fas-L-positive cells in control and CP-treated cells are shown in Table 1. Compared to the untreated cells, significantly increased numbers of Fas and Fas-L-immunopositive cells ($P<0.0001$) were noted in CP-treated cells.

Discussion

CP (*cis*-diamminedichloroplatinum) is used in the treatment of various tumors including testicular tumors (Loehrer et al. 1997). A major complication of the use of this drug is nephrotoxicity. Despite much literature describing the pathological changes in CP-treated kidney, the exact mechanisms of nephrotoxicity are still poorly understood. Apoptosis plays an important role in the pathogenesis of various human and experimental renal diseases (Gobe and Axelsen 1987; Harrison 1988; Shimizu and Yamanaka 1993; Truong et al. 1996;). However, its role(s) in CP-induced renal damage has not yet been well understood. The aim of the present study was to assess whether CP treatment can induce apoptosis in human PTECs and, if so, to explore the possible involvement of the Fas/Fas-L system in the apoptotic cell death process.

In this study, by TB staining, we have shown that CP treatment alters the cell viability (less numbers of viable cells were accompanied by increased numbers of dead cells). By PI staining, in situ end labeling, and electron microscopy, we have confirmed the presence of apoptosis in CP-treated human PTECs. An increased number of apoptotic cells with a distinct nuclear staining pattern was evident by PI staining and in situ end labeling in CP-treated cells. Morphologically, by routine electron microscopic examination, cells undergoing apoptosis were easily recognizable (the margination of chromatin with condensation and the formation of apoptotic bodies) and were found only in CP-treated cell populations. The ultrastructural changes were consistent with the typical morphological changes of apoptosis, as described elsewhere. Our results are in good agreement with the earlier reports, where CP has been shown to induce apoptosis in various tumor cells (Dixit et al. 1997; Evans and Dive 1993; Henkels and Turchi 1997).

The major finding in the present study was that CP-treatment induces the Fas/Fas-L system which was accompanied by apoptosis of the human PTECs. It should be noted that while our results showed a temporal and spatial correlation between the expression of Fas/Fas-L and the appearance of apoptotic cells in the CP-treated cells, no evidence of the association has yet been defined. However, the ability of Fas/Fas-L to induce apoptosis (Nagata 1997; Nagata and Suda 1995; Suda et al. 1993) is tempting us to speculate that the upregulation of Fas and Fas-L expression in CP-treated cells may play a significant role in the subsequent apoptotic cell death induction. Our results are similar to an earlier report where the therapeutic concentration of CP, as well as other chemotheraputic agents, was found to induce Fas/Fas-L to mediate apoptosis in chemosensitive neuroblastoma cells in vitro, and anti-Fas antibody treatment markedly reduced apoptosis induced by those drugs (Fulda et al. 1997). It appears to be, however, that not all CP-induced apoptosis is mediated by the Fas/Fas-L system. Fas/Fas-L-independent apoptosis induced by antitumor drugs including CP was found in T-acute lymphatic leukemia cells (Villunger et al. 1997). Similarly in the Jurkat human T-cell leukemia line, the antitumor drugs, including CP, induced apoptosis that was also not through the Fas-dependent pathway (Eischen et al. 1997). Thus it seems likely that, although CP can induce apoptosis in a wide variety of cells, involvement of the Fas/Fas-L system may be cell specific. Our present data provide the evidence of CP-induced apoptosis in human tubular epithelial cells with possible involvement of the Fas/Fas-L system. Our results are partly in accord with a recent study, where Fas/Fas-L-mediated apoptosis is shown in tubular epithelial cells in the ischemia/reperfusion model (Nogae et al. 1998).

To the best of our knowledge, this is the first report demonstrating that CP treatment could activate the Fas/Fas-L system, leading to apoptosis subsequently in human PTECs. Although acute CP-induced renal damage may be partly due to necrotic changes (Aggarwal 1993), recent in vivo studies have clearly shown that even a very high dose of CP (20 mg/kg) can induce apoptosis in mouse kidney (Megyesi et al. 1998). In addition, in our preliminary study, we found a single dose of CP (6 mg/kg) can induce apoptosis in the rat kidney in association with the upregulation of Fas and Fas-L expression, which is consistent with the present in vitro results. Considering all these findings and taking account of our present in vitro study, we think CP-induced renal damage may not only be related to necrosis but also to apoptosis (at least partly).

Cell deletion by apoptosis contributes significantly to the development of tubulointerstitial damage in various renal diseases, including hydronephrosis, obstructive uropathy, and in the repair process of ischemic tubular necrosis (Gobe and Axelsen 1987; Harrison 1988, Nogae et al. 1998; Shimizu and Yamanaka 1993; Truong et al. 1996). Apoptosis induced by CP may similarly play an important role in the subsequent tubulointerstitial damage found in CP nephrotoxicity in both human and experimental animals. Finally, these results strongly indicate that the interaction of Fas and Fas-L might play an active role in inducing apoptosis in human PTECs.

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