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Role of Apoptosis in Cisplatin-Induced Toxicity in the Renal Epithelial Cell Line LLC-PK$_1$

IMPLICATION OF THE FUNCTIONS OF APICAL MEMBRANES

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ABSTRACT. The role of apoptosis and the implications of the functions of apical membranes in cisplatin-induced nephrotoxicity were investigated using the kidney epithelial cell line LLC-PK$_1$. When LLC-PK$_1$ cells were treated with 30 μM cisplatin, the number of floating cells was increased markedly. However, the number was not increased by treatment with 1 mM cisplatin, suggesting that different mechanisms were involved in the toxicities of these two treatments. DNA fragmentation, condensation of nuclear chromatin, and the absence of trypan blue staining suggested that cellular toxicity following treatment with 30 μM cisplatin for 24 hr was mediated predominantly by apoptosis. Specific activities of apical enzymes (γ-glutamyltransferase, EC 2.3.2.2; and alkaline phosphatase, EC 3.1.3.1) in LLC-PK$_1$ cells were decreased markedly by treatment with 30 μM cisplatin for 24 hr, whereas neither lactate dehydrogenase (LDH; EC 1.1.1.27) release nor a decrease in cellular protein content was observed following the same treatment. In addition, concomitant treatment with reduced glutathione completely attenuated both the apoptosis and the decrease of apical enzyme activities induced by 30 μM cisplatin. Neither DNA fragmentation nor condensation of chromatin was induced by treatment with 1 mM cisplatin for 12 hr. However, LDH release and a decrease in cellular protein level were induced by 1 mM cisplatin, suggesting that the toxic effect was due to necrosis. Under these conditions, specific activities of apical enzymes were not decreased. These results suggested that apoptosis was more responsible than necrosis for the loss of apical functions in cisplatin-induced toxicity in LLC-PK$_1$ cells. BIOCHEM PHARMACOL **59**;2:195–201, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. cisplatin; nephrotoxicity; apoptosis; necrosis; LLC-PK$_1$; apical membranes

Nephrotoxicity is a major complication in drug therapy with various anticancer agents, antimicrobial drugs, and immunosuppressants. These drugs accumulate in the tubular epithelial cells through tubular secretion and/or reabsorption processes, resulting in severe nephrotoxicity. Drug-induced nephrotoxicity is accompanied frequently by morphological changes such as vacuolization of tubular epithelial cells and/or loss of brush-borders, and by decreases of tubular epithelial functions such as urine-concentrating activity and/or reabsorption of organic solutes, ions, and nutrients. In fact, thinning or focal loss of brush-borders was observed by electron microscopy in rats injected with cisplatin [1]. In patients with cisplatin nephrotoxicity, activities of brush-border enzymes, originating from brush-border membranes of tubular epithelial cells, were detected in urine [2]. In general, these morphological and functional changes in renal epithelial cells induced by nephrotoxic drugs are considered to be mediated by passive destruction of the cells, i.e. necrosis.

Recently, a distinct pathway of cell death, apoptosis, was shown to be involved in the cellular toxicity of various stimuli. It has been reported that a wide variety of potentially necrotic cell insults induce apoptosis, including cytotoxic agents, ischemia, heat shock, viral infection, oxidants, bacterial toxins, and ethanol [3, 4]. In addition, Lieberthal *et al.* [5] and Takeda *et al.* [6] reported that apoptosis participates in cisplatin-induced toxicity in epithelial cells derived from the mouse kidney in culture. However, there have been few previous studies of the involvement of renal epithelial functions in the apoptosis induced by cisplatin.

LLC-PK$_1$ is an established cell line that retains characteristics of proximal tubular epithelial cells [7]. LLC-PK$_1$ cells are useful for analyzing the functions of proximal tubular cells [8] and drug-induced nephrotoxicity [9]. We have used LLC-PK$_1$ cells for analyses of transport characteristics of organic cations [10, 11], aminoglycoside antibiotics [12, 13], and the immunosuppressant cyclosporin A [14]. In addition, we have demonstrated that various functions of apical membranes, including enzyme activities

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and Na^+-dependent hexose transport, are decreased in aminoglycoside-treated LLC-PK_1 cells [15–18]. LLC-PK_1 cells are also useful for analyzing the toxic effects of cisplatin [19–21] and cyclosporin A [22, 23]. In the present study, the role of apoptosis in the toxic effects of cisplatin and the implication of apical functions in the pathways of cisplatin toxicity were investigated, using LLC-PK_1 cells as the model system.

MATERIALS AND METHODS

Materials

cis-Platinum(II)diammine dichloride (cisplatin) was obtained from the Sigma Chemical Co. Hoechst 33258 (bisbenzimide H 33258 fluorochrome trihydrochloride), and paraformaldehyde were from Nacalai Tesque, Inc. All other chemicals were of the highest purity available.

Cell Culture

LLC-PK_1 cells (ATCC CRL-1392) obtained from the American Type Culture Collection were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum (Whittaker Bioproducts Inc.), without antibiotics, in an atmosphere of 5% CO_2–95% air at 37° [10]. The cells were subcultured every 4–7 days, using 0.02% EDTA and 0.05% trypsin. In most experiments, 35-, 60-, and 100-mm dishes, as well as 12-well plates, were seeded with 16×10^4 cells in 2 mL, 4×10^5 cells in 5 mL, 1×10^6 cells in 10 mL, or 7×10^4 cells in 0.9 mL of complete culture medium, respectively. In the present study, the LLC-PK_1 cells were used between passages 213 and 219.

Detection of DNA Fragmentation

Fragmented DNA in the floating and attached LLC-PK_1 cells was detected by the method of Lieberthal *et al.* [5] with some modifications. Confluent monolayers of LLC-PK_1 cells cultured on plastic dishes for 4–6 days were exposed to drugs for the specified periods. Culture medium was centrifuged at 870 *g* for 20 min (CF15D2, Hitachi), and then precipitates were assayed for DNA fragmentation. Attached cell monolayers were collected using a rubber policeman and then centrifuged at 870 *g* for 20 min (CF15D2, Hitachi). Floating or attached cells were lysed in 0.5% Triton X-100, 10 mM Tris–HCl (pH 7.5), and 25 mM EDTA for 1 hr at 4°. After centrifugation at 16,000 *g* for 30 min (CF15D2, Hitachi), the supernatants were treated with 100 μg/mL of RNase A and 100 μg/mL of proteinase K for 60 min at 50°, and then with phenol/chloroform. Fragmented DNAs were precipitated at −80° in the presence of sodium acetate, $MgCl_2$, and ethanol. Samples were separated by electrophoresis on 2% agarose gels in the presence of ethidium bromide.

Morphological Observation

LLC-PK_1 cells cultured on glass slides were treated with drug solution for the specified periods. Floating cells in the culture medium were collected by centrifugation at 870 *g* for 20 min (CF15D2, Hitachi). Attached and floating cells were treated with 1 μg/mL of Hoechst 33258 for 10 min at 37°. The cells were washed with isotonic phosphate-buffered saline, fixed with 4% paraformaldehyde, and then observed by fluorescence microscopy (BX-50, Olympus Optical Co.).

Enzyme Assays

After exposure of LLC-PK_1 cells to the test drugs for the specified periods, the medium was centrifuged at 870 *g* for 10 min (CF7D, Hitachi), and then the supernatant was used for LDH* (EC 1.1.1.27) assay. Attached cells were washed twice with ice-cold saline and then transferred into 1 mL of ice-cold saline. The cells were homogenized by sonication (VP-5S, Taitec) three times for 5 sec each time. LDH activities in the culture medium and cell homogenates were determined as described previously [16]. γ-Glutamyltransferase (EC 2.3.2.2) and alkaline phosphatase (EC 3.1.3.1) in the cell homogenates were measured as described previously [17, 18]. Protein was measured by the method of Bradford [24], using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with bovine γ-globulin as the standard.

Statistical Analyses

Statistical analyses were performed by one-way ANOVA followed by Dunnett's test or Fisher's *t*-test. *P* values of less than 0.05 were considered significant.

RESULTS

The effects of 30 μM and 1 mM cisplatin on the formation of floating cells in LLC-PK_1 cell cultures were examined. As shown in Fig. 1, treatment with 30 μM cisplatin for between 24 and 72 hr markedly increased the number of floating cells. However, the number of floating cells was not increased by 1 mM cisplatin during the period examined, suggesting that the toxic effects of these two concentrations of cisplatin were mediated by distinct pathways.

Next, we examined fragmentation of genomic DNA, one of the hallmarks of apoptosis, in LLC-PK_1 cells treated with or without 30 μM cisplatin. As shown in Fig. 2A, fragmented DNA was observed in floating and attached LLC-PK_1 cells at 24–120 hr after treatment. DNA fragmentation was maximal at 48 hr after cisplatin treatment and then decreased gradually thereafter. In contrast, fragmented DNA was not observed in the control LLC-PK_1 cells without cisplatin treatment during the periods examined

* *Abbreviation*: LDH, lactate dehydrogenase.

(Fig. 2B). In preliminary experiments, 3–10 μM and 1 mM cisplatin did not induce DNA fragmentation. However, 100 and 300 μM cisplatin induced DNA fragmentation at 24 and 12 hr after treatment, respectively (data not shown). These observations suggested that maximal DNA fragmentation induced by cisplatin treatment occurred between 30 and 300 μM.

Critical features of morphological changes in apoptosis are the condensation of chromatin and the fragmentation of nuclei. Therefore, we observed morphological changes in LLC-PK_1 cells induced by 30 μM cisplatin, using the fluorescent dye Hoechst 33258, which specifically binds to DNA. The nuclei of untreated control cells showed normal morphology (Fig. 3B). However, condensation of nuclear chromatin was observed in some of the attached LLC-PK_1 cells after treatment with 30 μM cisplatin for 24 hr (Fig. 3A). On the other hand, condensation of nuclear chromatin was observed in most of the floating cells from cisplatin-treated monolayers (Fig. 3C). These results suggested that apoptosis was induced in 30 μM cisplatin-treated LLC-PK_1 cells. In addition, almost none of the attached cells treated with 30 μM cisplatin for 24–72 hr were stained with trypan blue (data not shown), suggesting that apoptosis was the dominant pathway in 30 μM cisplatin-induced cellular toxicity. Condensation of nuclear chromatin in the floating cells from untreated control monolayers was not detected, because the numbers of floating cells obtained from the monolayers were insufficient for analysis.

To examine the correlation between cisplatin-induced apoptosis and alterations in apical functions, we compared the time courses of LDH release into culture medium and the decreases in cellular protein level and apical enzyme activities induced in LLC-PK_1 cell homogenates by 30 μM cisplatin. The release of LDH (Fig. 4A) and the decrease in cellular protein level (Fig. 4B) were stimulated by incubation with 30 μM cisplatin for more than 48 hr. On the other hand, specific activities (activity per cellular protein) of γ-glutamyltransferase (Fig. 4C) and alkaline phosphatase (Fig. 4D) were decreased markedly by treatment with 30 μM cisplatin for 24–72 hr.

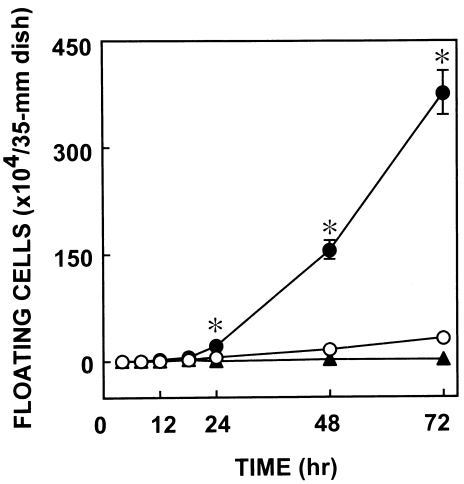

FIG. 1. Effects of 30 μM and 1 mM cisplatin on the formation of floating cells in LLC-PK_1 cell cultures. LLC-PK_1 cells were seeded on 35-mm dishes. Four days after seeding, culture medium was replaced with fresh medium (○) or medium containing 30 μM (●) or 1 mM (▲) cisplatin. At the indicated time points after treatment, the number of floating cells in the culture medium was counted according to the method described previously [15]. Each point represents the mean ± SEM of three dishes. Key: (*) indicates values significantly different ($P < 0.05$) from the respective controls by Fisher's *t*-test.

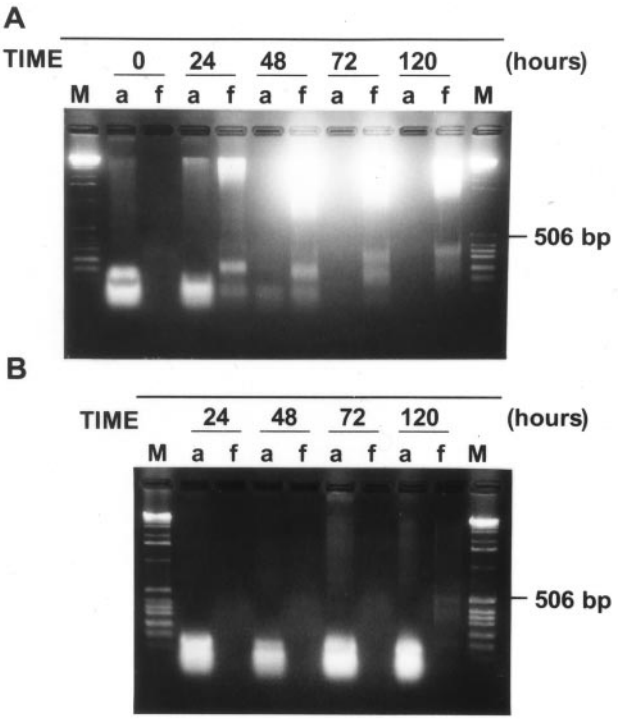

FIG. 2. Time course of DNA fragmentation by 30 μM cisplatin in LLC-PK_1 cells. LLC-PK_1 cells were seeded on 60-mm dishes. Four days after seeding, culture medium was replaced with fresh medium (B) or with medium containing 30 μM cisplatin (A). At the indicated time points after treatment, DNA extracted from attached (denoted as "a") or floating (denoted as "f") cells was mixed with ethidium bromide and then separated by 2% agarose gel electrophoresis. "M" indicates DNA size markers.

Zunino *et al.* [25] reported that concomitant administration of reduced glutathione has a protective effect against cisplatin-induced nephrotoxicity in rats. To examine the correlation between apoptosis induced by cisplatin and various markers of cellular toxicity, we tested the protective effects of reduced glutathione on DNA fragmentation, LDH release, and decreases in cellular protein and apical enzymes induced by cisplatin in LLC-PK_1 cells. As shown in Fig. 5, simultaneous treatment of LLC-PK_1 cells with reduced glutathione (0.5 to 2 mM) markedly attenuated cisplatin-induced DNA fragmentation in both attached and floating cells. On the other hand, reduced glutathione did not affect DNA fragmentation in the absence of cisplatin (data not shown). Simultaneously, reduced glutathione markedly attenuated cisplatin-induced decreases in apical enzyme activities (Fig. 6, C and D) and alterations in LDH release (Fig. 6A) and cellular protein levels (Fig. 6B).

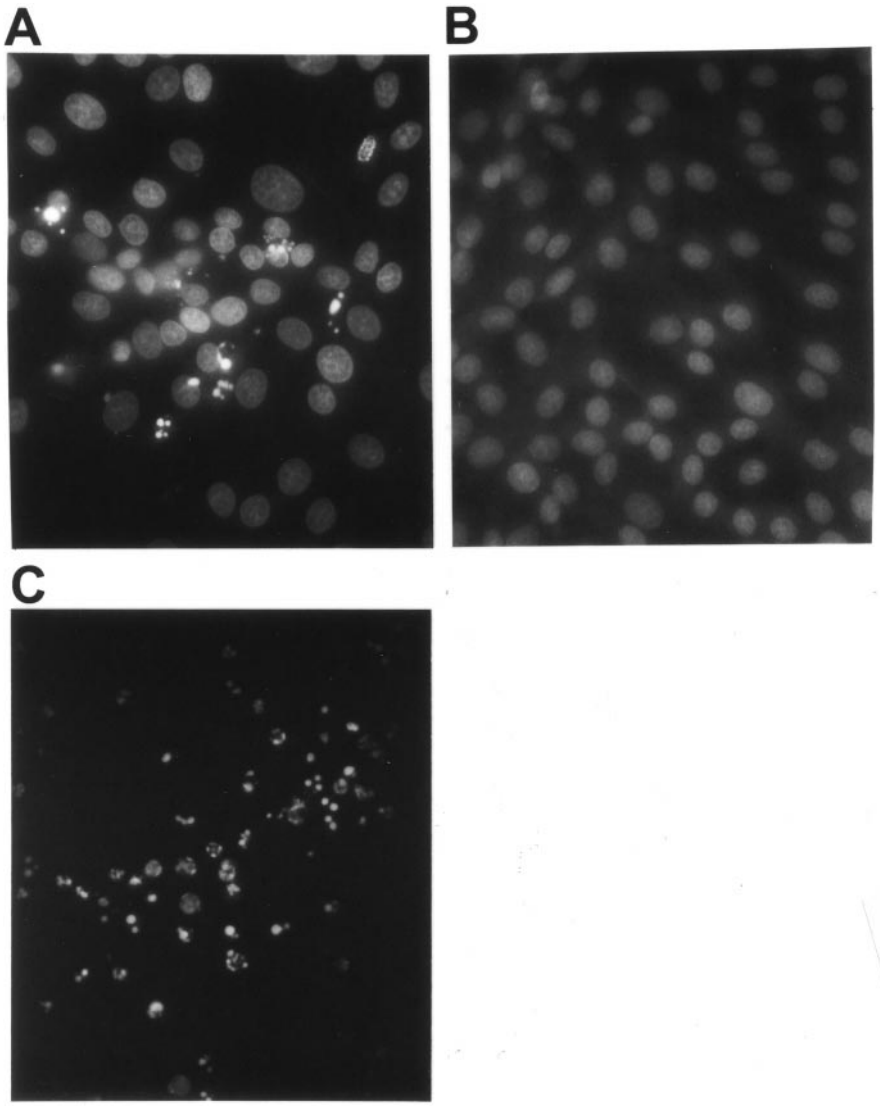

FIG. 3. Effects of 30 μM cisplatin on chromatin condensation of nuclei in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on glass slides. Six days after seeding, the culture medium was replaced with fresh medium (B) or with medium containing 30 μM cisplatin (A, C). Twenty-four hours after treatment, attached (A, B) and floating (C) cells stained with Hoechst 33258 were photographed under fluorescence microscopy (350x).

These results suggested that decreases in the functions of apical membranes were correlated with the apoptosis induced by cisplatin in LLC-PK$_1$ cells.

To examine the correlation between toxic pathways of cisplatin and decreases in apical enzyme activities induced by cisplatin, we tested the effects of 1 mM cisplatin on markers of apoptosis and apical enzyme activities in LLC-PK$_1$ cells. As shown in panels A and B of Fig. 7, DNA fragmentation and the condensation of nuclear chromatin were not induced by 1 mM cisplatin. However, the release of LDH into culture medium (Fig. 8A) and the decrease in cellular protein level (Fig. 8B) were stimulated markedly, suggesting that the cellular toxicity of 1 mM cisplatin was not mediated by apoptosis, but rather by necrosis. In addition, almost all attached cells treated with 1 mM cisplatin for 8–12 hr were stained with trypan blue, suggesting that necrosis was the dominant pathway in 1 mM cisplatin-induced cellular toxicity (data not shown). In contrast to the results observed with 30 μM cisplatin, specific activities of γ-glutamyltransferase (Fig. 8C) and alkaline phosphatase (Fig. 8D) were not decreased by 1 mM cisplatin during the time points examined.

DISCUSSION

We studied the role of apoptosis in cisplatin-induced nephrotoxicity using the kidney epithelial cell line LLC-PK$_1$ as a model system. Our results suggested that both apoptosis and necrosis were responsible for the loss of apical functions in the toxicity induced by cisplatin. However, apoptosis was more effective than necrosis for inducing the loss of apical functions in cisplatin-induced toxicity in LLC-PK$_1$ cells.

Although decreases in the activities of apical enzymes

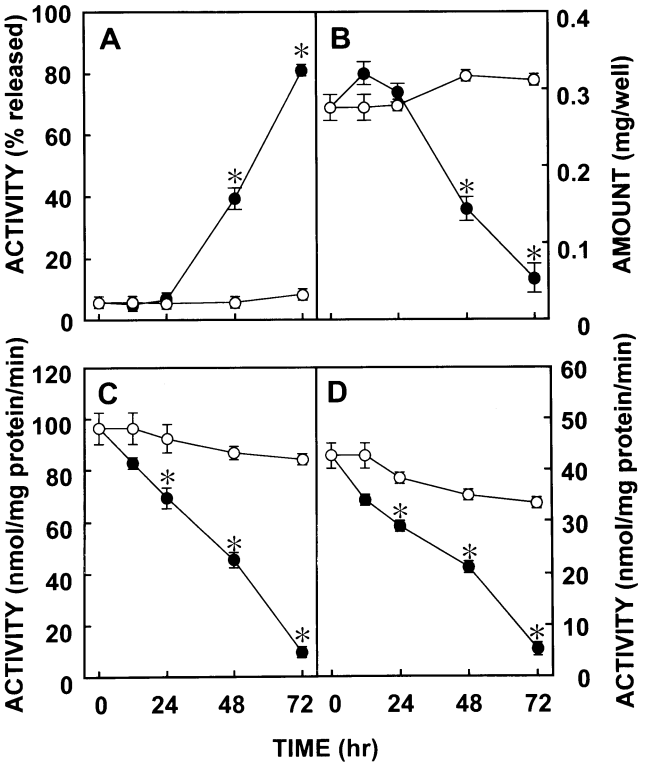

FIG. 4. Effects of 30 μM cisplatin on LDH release (A), cellular protein (B), and apical enzymes (C, D) in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on 12-well cluster plates. Four days after seeding, the culture medium was replaced with fresh medium (○) or with medium containing 30 μM cisplatin (●). At the indicated time points after treatment, LDH activity in the culture medium (A), the amount of cellular protein (B), and the γ-glutamyltransferase (C) and alkaline phosphatase (D) activities in the cell homogenates were determined. Each point represents the mean ± SEM of three dishes. Key: (*) indicates values significantly different ($P < 0.05$) from the respective control by Fisher's *t*-test.

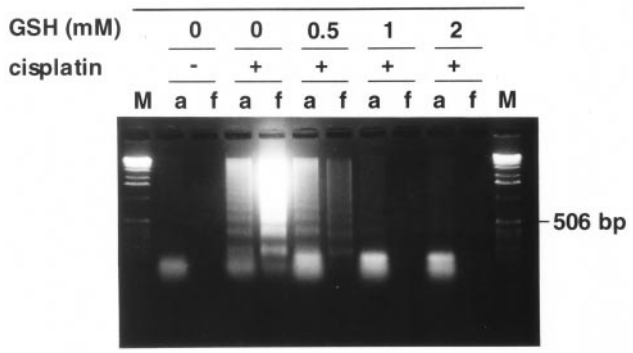

FIG. 5. Effects of reduced glutathione on DNA fragmentation induced by 30 μM cisplatin in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on 60-mm dishes. Four days after seeding, the culture medium was replaced with medium containing 30 μM cisplatin and various concentrations (0–2 mM) of reduced glutathione. Twenty-four hours after treatment, DNA extracted from attached (denoted as "a") or floating (denoted as "f") cells was mixed with ethidium bromide and then separated by 2% agarose gel electrophoresis. "M" indicates DNA size markers.

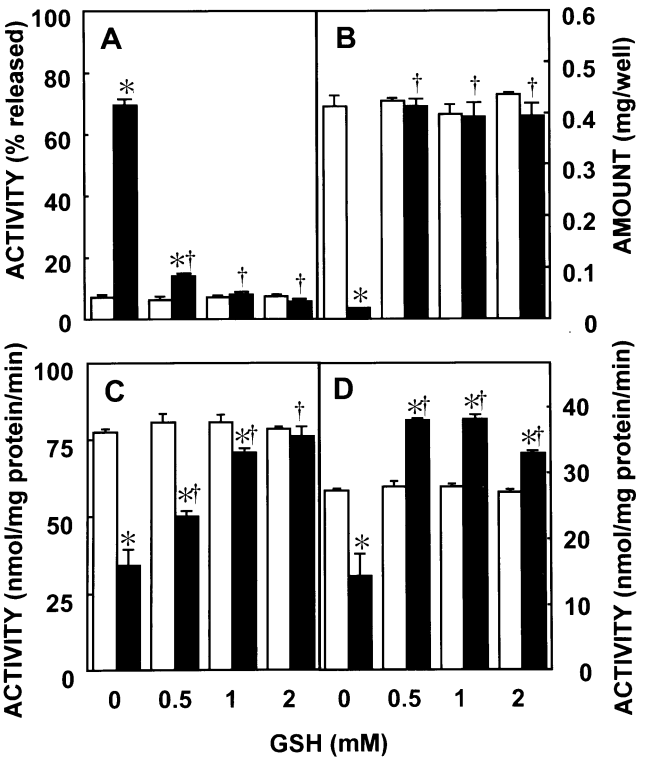

FIG. 6. Effects of reduced glutathione on alterations in LDH release (A), cellular protein (B), and apical enzymes (C, D) induced by 30 μM cisplatin in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on 12-well cluster plates. Four days after seeding, the culture medium was replaced with medium containing various concentrations of reduced glutathione with (closed columns) or without (open columns) 30 μM cisplatin. Three days after treatment, LDH activity in the culture medium (A), the amount of cellular protein (B), and γ-glutamyltransferase (C) and alkaline phosphatase (D) activities in the cell homogenates were determined. Each column represents the mean ± SEM of three dishes. Key: (*) indicates values significantly different ($P < 0.05$) from the cisplatin-untreated control, by Fisher's *t*-test; and (†) indicates values significantly different ($P < 0.05$) from the reduced glutathione-untreated control, by Dunnett's test.

induced by cisplatin treatment were observed, the correlation between these enzyme activities and toxic pathways, i.e. apoptosis and necrosis, was unclear. In the present study, specific activities of apical enzymes (γ-glutamyltransferase and alkaline phosphatase) in the cell homogenates were decreased markedly by 30 μM cisplatin. By comparing the time courses of alterations in apical enzyme activities, cellular protein level, and LDH release, it became clear that decreases in apical enzyme activities occurred earlier than the decrease in cellular protein level and the LDH release induced by 30 μM cisplatin. In contrast, 1 mM cisplatin did not reduce the specific activities of apical enzymes, although these activities per well of cells were decreased with a time course similar to those of the decreases in cellular protein level and LDH release (data not shown). These results suggested that apoptosis is more effective than necrosis in causing loss of apical functions in response to cisplatin in LLC-PK$_1$ cells. The mechanism responsible for

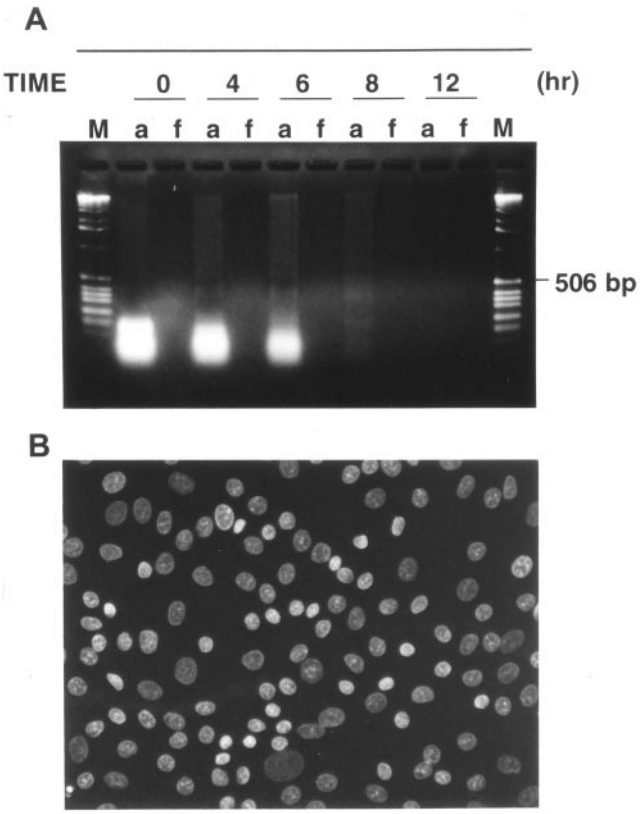

FIG. 7. Effects of 1 mM cisplatin on DNA fragmentation (A) and chromatin condensation of nuclei (B) in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on 60-mm dishes. (A) Five days after seeding, the culture medium was replaced with medium containing 1 mM cisplatin. At the indicated time points after treatment, DNA extracted from attached (denoted as "a") or floating (denoted as "f") cells was mixed with ethidium bromide and then separated by electrophoresis on 2% agarose gels. "M" indicates DNA size markers. (B) LLC-PK$_1$ cells were seeded on glass slides. Four days after seeding, the culture medium was replaced with fresh medium containing 1 mM cisplatin. Twelve hours after treatment, cells stained with Hoechst 33258 were photographed under fluorescence microscopy. (350x).

the decreased specific activity of apical enzymes is not clear, although decreased apical enzymes may be correlated with decrease of epithelial functions caused by apoptosis.

Fragmentation of DNA (Fig. 2) and condensation of nuclear chromatin (Fig. 3) were observed in the floating cells released from LLC-PK$_1$ cell monolayers treated with 30 μM cisplatin. This raised the question of whether apoptosis was induced primarily by cisplatin in attached cells or was induced secondarily by detachment of the cells from the monolayer. As shown in Fig. 2, apoptosis was observed in attached as well as floating cells treated with 30 μM cisplatin. As floating cells were exposed continuously to 30 μM cisplatin in the culture medium, we speculated that at first apoptosis occurred in the attached LLC-PK$_1$ cells and apoptotic cells were released into the culture medium, then apoptosis continued in floating cells. Lieberthal *et al.* [5] have reported that floating cells in the culture medium finally undergo necrosis (secondary necrosis). Therefore, fragmented DNA in the floating cells may also contain fragmented DNA responsible for the secondary necrosis.

Morphological changes such as condensation of nuclear chromatin and formation of apoptotic bodies are considered to be the most critical features of apoptosis. Fragmentation of genomic DNA into nucleosome-sized units is considered to be the most significant biochemical change accompanying the occurrence of apoptosis. In the present study, both chromatin condensation (Fig. 3) and DNA fragmentation (Fig. 2) in LLC-PK$_1$ cells were observed following treatment with 30 μM cisplatin for 24 hr, suggesting that apoptosis was induced by cisplatin at this concentration. On the other hand, the number of floating cells was elevated markedly by 30 μM cisplatin, but not by 1 mM cisplatin (Fig. 1). These results suggested that formation of floating cells as well as chromatin condensation and DNA fragmentation could be useful markers of apoptosis induced by 30 μM cisplatin.

After treatment with 30 μM cisplatin for 24 hr, attached cells were scarcely stained, and approximately 25% of

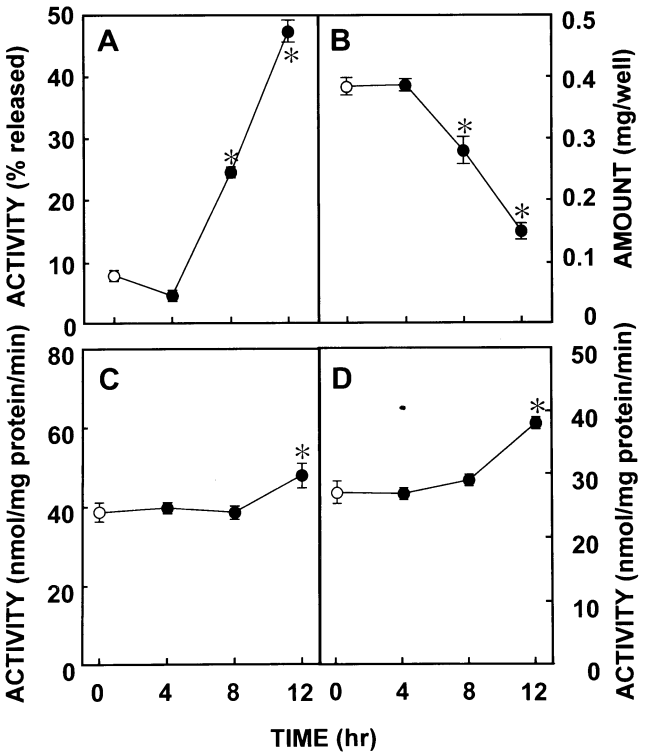

FIG. 8. Effects of 1 mM cisplatin on LDH release (A), cellular protein (B), and apical enzymes (C, D) in LLC-PK$_1$ cells. LLC-PK$_1$ cells were seeded on 12-well cluster plates. Four days after seeding, the culture medium was replaced with fresh medium or medium containing 1 mM cisplatin (●). At the indicated time points after treatment, LDH activity in the culture medium (A), the amount of cellular protein (B), and γ-glutamyltransferase (C) and alkaline phosphatase (D) activities in the cell homogenates were determined. Cell culture without replacement of medium was used as time-zero control (○). Each point represents the mean ± SEM of three dishes. Key: (*) indicates values significantly different ($P < 0.05$) from cisplatin-untreated control (time 0), by Dunnett's test.

floating cells were stained with trypan blue (data not shown), suggesting that apoptosis was the dominant pathway of cell death induced by 30 μM cisplatin. Three days after treatment with 30 μM cisplatin, residual cellular protein levels (Fig. 4B) and apical enzyme activities (Fig. 4, C and D) in the cell homogenate were very low (17, 2, and 2% of each control for cellular protein, γ-glutamyltransferase, and alkaline phosphatase, respectively). In contrast, the nuclei of almost all LLC-PK_1 cells treated with 1 mM cisplatin were stained with trypan blue and tended to be lifted off as monolayers from the culture dishes (data not shown), consistent with the observations of Lieberthal *et al*. [5]. These results suggested that excess progression of apoptosis alone induced by 30 μM cisplatin may cause loss of epithelia, which in turn may cause malfunction of renal tubules, resulting in, for example, a decrease in urine-concentrating activity.

In conclusion, both apoptosis and necrosis were responsible for the toxic effects of cisplatin in LLC-PK_1 cells. However, decreases in the activities of apical enzymes induced by cisplatin were correlated more closely with apoptosis than necrosis. These results provide useful information for the understanding of cisplatin-induced nephrotoxicity.

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