Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death

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Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death. Am J Physiol Cell Physiol 289: C1466–C1475, 2005. First published August 17, 2005; doi:10.1152/ajpcell.00265.2005.—We studied the relationship between the mitochondrial density in the cells and the cellular sensitivity to the toxicity of cis-diaminedichloroplatinum (II) (cisplatin), a potent anticancer agent. Biochemical analyses revealed that the density of mitochondria in the intestinal epithelium changed markedly along its entire length. The density was the highest at the duodenum, medium at the jejunum, and the lowest at the ileum. The sensitivity of epithelial cells to cisplatin toxicity was the highest at the duodenum, medium at the jejunum, and the lowest at the ileum as judged from the occurrence of apoptosis. Similar correlation between the cisplatin sensitivity and mitochondrial density was also observed with in vitro experiments, in which intestinal epithelial cells (IEC-6) and their \( \rho^0 \) cells with reduced number of mitochondria were used. The \( \rho^0 \) cells had a strong resistance to cisplatin compared with the control cells. Cisplatin markedly increased mitochondrial generation of reactive oxygen species in IEC-6 but not in \( \rho^0 \) cells. We analyzed the sensitivity of eight cell lines with different density of mitochondria to cisplatin and found the same positive correlation. These observations clearly show that cellular density of mitochondria is the key factor for the determination of the anticancer activity and side effects of cisplatin.

Chemotherapy; oxidative damage; apoptosis

CHEMOTHERAPY is one of the most effective treatments for patients with cancer. However, the cytotoxicity of chemotherapeutic agents to normal tissues is a critical factor that undermines the curative potential of chemotherapy. To achieve the method for selective killing of cancer cells without causing side effects of chemotherapeutic agents, it is important to elucidate the mechanism of cell death induced by the agents. Recent studies (6, 42, 47) revealed that a variety of anticancer agents induces cell death by modifying the signaling pathways involving reactive oxygen species (ROS). Mitochondrial electron transport chain is one of the major sites for ROS generation (31). When cells were exposed to chemotherapeutic agents, the rate of ROS generation increased in and around mitochondria (1, 47). In addition to synthesizing ATP required for the maintenance of aerobic life, mitochondria also play important roles in the regulation of cell death (19, 48). Mitochondrial DNA (mtDNA) is responsible for the synthesis of 13 polypeptides that constitute electron transport chains (2). Because mitochondria do not contain histone-like proteins and because they have a less efficient repair mechanism for injured DNA than the nucleus does, mtDNA is more susceptible to oxidative stress than nuclear DNA (nDNA) (56). Mitochondrial dysfunction and/or mtDNA mutations are often observed with various diseases involving mitochondria-enriched tissues, such as the brain, heart, and skeletal muscles (11, 37, 50). The density of mitochondria generally depends on cellular requirement of ATP and differs significantly from one cell type to another (52). These facts indicate that cellular density of mitochondria is one of the risk factors for oxidative cell injury and, hence, plays a role in the pathogenesis of ROS-induced cell death.

Cis-diaminedichloroplatinum (II) (cisplatin) is an effective chemotherapeutic agent widely used for the treatment of cancer patients (44, 53). Recent studies reported that, in addition to nDNA damage (43), extranuclear events involving mitochondria (14), endoplasmic reticulum (33), and lysosome (8) were also important for the induction of cancer cell apoptosis by cisplatin (33). Because an upregulation of Bel-2 was found to inhibit cisplatin-induced apoptosis of renal proximal tubular cells in the rat, the mitochondria-dependent pathway has been postulated to play critical roles in the cytotoxic action of the agent (54). We also reported that oxidative injury of mitochondrial function and mtDNA in the kidney were the early events elicited by cisplatin (38). Hence, protections of mitochondrial functions and mtDNA by nephrophilic antioxidant and/or superoxide dismutase (SOD) prevented the cisplatin-induced renal injury (7, 22). On the basis of these previous observations, we undertook this study to investigate whether there are correlations between the mitochondrial density and the cellular sensitivity to cisplatin.

MATERIALS AND METHODS

Materials. Cis-diaminedichloroplatinum (II) (cisplatin) was obtained from Nippon Kayaku (Tokyo, Japan). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine-iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). The Vectastain Elite ABC kit was purchased from Vector Laboratories (Burlingame, CA). A lactate dehydrogenase (LDH) assay kit and an in situ apoptosis detection kit were purchased from Wako Pure Chemical (Osaka, Japan) and Takara Shuzo (Kusatsu, Japan), respectively. Other reagents used were of the highest grade commercially available.

Analysis of mitochondrial enzymes in the small intestine. All experiments were approved by the Animal Care and Use Committee of Osaka City University Medical School. Male Wistar rats (200–220 g) were fed laboratory chow and water ad libitum and used for experiments. The whole length of the small intestine was perfused in situ with 10 ml of ice-cold saline, cut into nine sections, and speci-
ments of the duodenum (section 1), jejenum (sections 2–5), and the ileum (sections 6–9) were obtained. The intestinal epithelium was gently scraped from each section, and 10 µg of protein samples were subjected to 15% SDS-PAGE. The electrophoresed proteins were transferred onto nitrocellulose sheets with the use of a semidry blot system (2 mA/cm² for 1 h in 0.2 M Tris–glycine buffer) and the amounts of cytochrome c oxidase-I (COX-I), cytochrome c, MnSOD, cyclin-dependent kinase-2 (cdk2), and actin were analyzed using specific antibodies. After the sheets were incubated in TBS solution (140 mM NaCl, 50 mM Tris·HCl, pH 7.2) containing 0.1% Tween 20 and 5% lowfat milk powder at 4°C for 12 h, they were treated with anti-COX-I, anti-cytochrome c, and anti-MnSOD antibodies (1:1,000 in TBS solution containing 5% lowfat milk powder) at 4°C for 12 h. The incubated sheets were washed five times with the same buffer to eliminate nonspecific antibody binding. After incubation with horseradish peroxidase-conjugated anti-IgG antibody (1:1,000 in TBS with 5% lowfat milk powder) at 25°C for 1 h, immunoreactive spots were detected by ECL (Amersham, Chalfont-St. Giles, UK).

In situ detection of apoptosis in the small intestine. Intestinal specimens were analyzed with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) using an in situ apoptosis detection kit. Briefly, 24 h after treatment with or without intravenous administration of cisplatin (5 mg/kg body wt), nine sections cut out of the small intestine were fixed with 10% formalin and embedded in paraffin, and thin sections of the specimen were then mounted on glass slides. After deparaffinization, the specimens were incubated with 20 µg/ml proteinase K at 25°C for 15 min and subsequently with TdT enzyme and the labeling safe buffer in a humidified chamber at 37°C for 60 min. The specimens were then analyzed under a fluorescence microscope (Olympus, Tokyo, Japan). The image was processed to analyze the TUNEL-positive areas, and the number of apoptotic cells was counted using the Scion Image Beta 4.0.2 software.

Cell preparation. Epithelial crypt cells (IEC-6) from rat intestine were obtained from Riken Cell Bank (Tsukuba, Japan) and cultured at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin G, and 50 µg/ml streptomycin. MtdNA-depleted IEC-6 cells (p⁰ cells) were established as described previously through a long-term treatment of IEC-6 cells with ethidium bromide (50 ng/ml) (27). The depletion of mtDNA was confirmed by PCR amplification using several sets of mtDNA specific primers. The reduction of mitochondrial density was confirmed by staining the cells with MitoTracker red, an indicator of mitochondria.

Analysis of cell viability. IEC-6 and their p⁰ cells were incubated with various concentrations of cisplatin for 24 and 48 h. The viability of cells was evaluated under a light microscope using the Trypan blue exclusion test.

Analysis of ROS production. To detect the generation of ROS, cells were loaded with membrane-permeable 2,7-dichlorofluorescein (DCF) diacetate (DCFHDA). DCFHDA is hydrolyzed to nonfluorescent DCFH that reacts with ROS to form highly fluorescent DCF. Cultured cells were incubated with or without 10 µM cisplatin for 1, 1.5, and 3 h, and then with phenol red-free medium containing 20 µM DCFHDA at 37°C for 30 min. After being washed three times with PBS solution (pH 7.4), the cells were analyzed with a fluorescence microscope.

Analysis of mitochondrial membrane potential. Mitochondrial membrane potential (ΔΨm) was measured using a cationic fluorescent dye JC-1, which accumulates in mitochondria and changes its fluorescence spectrum from orange to green, depending on the decrease in membrane potential. Cells were incubated with 5 µM JC-1 at 37°C for 30 min and washed twice in PBS solution (pH 7.4) and then with phenol red-free medium containing 10 µM cisplatin. After 1.5 and 3 h, fluorescence spectra were analyzed under a fluorescence microscope.

Immunocytochemical analysis. To assess the presence of oxidative stress, two immunostaining methods were employed. Namely, the amounts of both 8-hydroxydeoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (HNE) in cultured cells were assayed using the avidin-biotin-peroxidase complex (ABC) method with the Vectastain Elite ABC Kit. Briefly, the cells were cultured with or without 10 µM cisplatin for 6 h and then fixed with 4% paraformaldehyde. After being treated with 0.2% Triton X-100 in PBS and subsequently with 0.3% H2O2 in methanol and then with normal blocking serum, the cells were incubated with anti-8-OHdG and anti-4-HNE monoclonal antibodies (Japanese Aging Control Institute, Shizuoka, Japan). Cytochrome c released from mitochondria to cytosol was immunocytochemically stained using the specific antibody (anti-cytochrome c monoclonal antibody provided by BD Pharmingen, San Diego, CA) at 25°C for 1 h. After being washed three times with a PBS solution (pH 7.4), the cells were incubated with the biotinylated second antibody for 1 h and then with the ABC reagent for 30 min. Subsequently, the peroxidase activity was developed by 0.025% 3,3′-diaminobenzidine tetrahydrochloride solution in the presence of 0.015% H2O2 (pH 7.4). Cell-associated immunocomplexes were then detected under a light microscope (Olympus).

Analysis of cytochrome c localization. To estimate the release of cytochrome c from mitochondria to the cytosol, the harvested cells were incubated in an ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, and 200 µg/ml digitonin in PBS) for 5 min without affecting mitochondrial integrity, as previously described (55). Next, the supernatant fraction containing cytosolic proteins and the pellet with mitochondria were obtained by centrifugation (1,000 g at 4°C for 5 min) and used for Western blot analysis with the use of anti-cytochrome c, MnSOD, and anti-actin antibody.

Analysis of DNA fragmentation. IEC-6 cells and p⁰ cells were incubated with 10 µM cisplatin for 0, 12, and 24 h. The cells were then collected and homogenized in an 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 at 4°C for 20 min, the mixture was centrifuged at 10,000 g for 20 min. The supernatant fraction was incubated with 40 µg of RNase A at 37°C for 1 h and subsequently with 40 µg/ml proteinase K at 1 h. DNA was then precipitated by incubating with a mixture of 1 volume of isopropanol and 0.2 volume of 5 M NaCl at −20°C for 12 h, followed by centrifugation at 10,000 g for 20 min. The pellets were air dried and dissolved in 10 µl of a buffer containing 10 mM Tris·HCl, pH 8.0, and 1 mM EDTA. The DNA samples thus obtained were subjected to 1.6% agarose gel electrophoresis at 100 V using a solution of 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, as the running buffer. The electrophoresed gel was stained with 0.1 µg/ml ethidium bromide and visualized under ultraviolet light.

Analysis of apoptotic cell death in cultured cells. Apoptosis of IEC-6 and p⁰ cells was assessed by TUNEL staining with the use of an in situ apoptosis detection kit. Briefly, cells were cultured with or without 10 µM cisplatin for 12 h. After fixation with 4% paraformaldehyde solution at room temperature, the cells were treated with the permeabilization buffer on ice and then with TdT enzyme and the labeling safe buffer in a humidified chamber at 37°C for 60 min. The specimens were analyzed under a fluorescence microscope.

Analysis of LDH release. The LDH activity in the culture medium was measured spectrophotometrically using a LDH assay kit. Briefly, after treatment with or without cisplatin for 24 h, the culture medium was collected and centrifuged at 1,000 g for 5 min. The supernatants were used for the LDH assay.

Analysis of cisplatin sensitivity and succinate dehydrogenase activity of cells. Six types of tumor cell lines (HeLa, HL-60, HepG2, AH-130, Ehrlich ascites tumor cells, and Lewis lung carcinoma cells) and two types of cell lines derived from normal tissues (human embryonic kidney (HEK)-293 and J774) were cultured with various concentrations of cisplatin for 24 h. The concentrations of cisplatin required for 50% cell death (LD₅₀) values for cisplatin were calcu-
lated based on their survival curve analyzed with the Trypan blue dye exclusion test.

The cultured cells of all cell lines were sonicated in an ice-cold 50 mM potassium phosphate buffer (pH 7.4) for 5 s. The cell sonicates were diluted to 2 mg protein/ml in the same buffer. The sonicates (each 50 μl) were added to 0.95 ml of an assay mixture containing 2 mM sodium succinate, 9.6 mM 2,6-dichloroindophenol, 65 mM phenazine methosulfate, and 10 mM sodium cyanide in a 1-ml cuvette. A decrease in the absorbance at 600 nm was measured at 25°C for 5 min. The enzyme activity was expressed as micromoles of 2,6-dichloroindophenol reduced per minute per milligram of protein.

**Statistics.** Values are expressed as means ± SD. Statistical differences were determined using the Student’s t-test with the significance set at \( P < 0.05 \). The correlation was estimated by the Pearson’s correlation coefficient analysis using a simple linear regression.

**RESULTS**

**Zonation of mitochondrial density along the small intestine.** To evaluate the cellular density of mitochondria, we analyzed tissue levels of mitochondria-specific proteins, such as mtDNA-encoded COX-I, nDNA-encoded MnSOD, and cytochrome c, in the mucosa of the nine segments of the small intestine. The tissue levels of these proteins and enzymes decreased monotonously from the entrance toward the exit of the small intestine (Fig. 1). The levels were highest at the duodenum, decreased gradually along the intestine, and the lowest at the distal part of the ileum. Thus the density of mitochondria shows a marked zonation along the small intestine.

**Correlation between cisplatin sensitivity and mitochondrial density in the small intestine.** We evaluated the cisplatin sensitivity of mucosal cells in the nine sections of the small intestine. Histochemical analysis revealed that the occurrence of apoptosis of epithelial cells was not apparent along the small intestine in control rats. However, the cisplatin-treated rats showed a substantial degree of apoptosis in the duodenum and the proximal part of the jejunum but a lesser degree at the distal part of the ileum (Fig. 2).

As shown in Fig. 3, a clear, positive correlation was found between the occurrence of cisplatin-induced apoptosis and epithelial level of mitochondria-specific proteins in the small intestine. The cells of the intestinal epithelium continuously regenerate from a stem cell population in the crypts. Thus...
differences of the sensitivity to cisplatin were possibly due to differentiation or proliferation of the intestinal epithelium. In this context, we analyzed the correlation between the occurrence of cisplatin-induced apoptosis and epithelial level of cell cycle protein cdk2 in the small intestine. However, no correlation was found.

**Effect of mtDNA depletion on cisplatin-induced cell death.** To elucidate the mechanism of mitochondrial density-dependent apoptosis that was found in the small intestinal epithelial cells from cisplatin-treated rats, we compared the viability of IEC-6 cells and their \( \rho^0 \) cells during the incubation with cisplatin. Cisplatin killed IEC-6 cells in dose- and time-dependent manner; the cisplatin sensitivity was higher in IEC-6 cells than in \( \rho^0 \) cells (Fig. 4).

**Effect of mtDNA depletion on cellular production of ROS.** The oxidative stress has been postulated to play important roles in the mechanism of cisplatin-induced cell death (32, 36). We therefore analyzed the effect of cisplatin on the generation of ROS in control and \( \rho^0 \) cells with the use of DCFHDA. After treatment with cisplatin for 1 h, the fluorescence intensity of DCF increased significantly with IEC-6 cells but not with \( \rho^0 \) cells (Fig. 5), suggesting that cisplatin enhanced the production of ROS in control cells but not in \( \rho^0 \) cells.

**Effect of mtDNA-depletion on \( \Delta \Psi_m \).** ROS have been reported (9, 10) to promote mitochondrial membrane permeability transition (MPT), an early event leading to apoptosis. MPT can be assessed by monitoring the change in \( \Delta \Psi_m \) of JC-1-treated cells. Thus the change in \( \Delta \Psi_m \) was monitored with IEC-6 cells during the treatment with cisplatin. As shown in Fig. 6, the fluorescence of JC-1-treated IEC-6 cells shifted from orange to green during the incubation with cisplatin, suggesting a decrease in \( \Delta \Psi_m \). The density of polarized mitochondria with orange color in JC-1-treated \( \rho^0 \) cells was significantly lower than that of IEC-6 cells. \( \Delta \Psi_m \) in \( \rho^0 \) cells seems to be partly restored at 3 h after depolarization at 1.5 h, because JC-1 staining showed the locally accumulated orange-red color at 3 h. It is likely that cisplatin stimulated the mtDNA-lacking mitochondria and fused them in \( \rho^0 \) cells.

**Effect of mtDNA-depletion on cisplatin-induced oxidative injury.** We evaluated the extent of oxidative damage of DNA and lipid peroxidation using immunocytochemical methods of staining 8-OHdG and 4-HNE, respectively, after cisplatin treatment for 6 h. The nuclear localization of 8-OHdG became apparent in control cells but not in \( \rho^0 \) cells (Fig. 7A). The generation of 4-HNE also became apparent in the perinuclear region of control cells but not in \( \rho^0 \) cells (Fig. 7B). These results suggested that the oxidative stress increased markedly in cisplatin-treated control cells but not in \( \rho^0 \) cells.

**Effect of mtDNA-depletion on cisplatin-induced cytochrome c release.** Cytochrome c is released from mitochondria into cytosol through the MPT pore during an early period of apoptosis. To evaluate the degree of cytochrome c release in cisplatin-treated cells, we used anti-cytochrome c antibody. As shown in Fig. 8A, mitochondria-enriched perinuclear regions of untreated control cells were stained clearly with the antibody. However, perinuclear localization of cytochrome c decreased markedly after cisplatin treatment. The perinuclear localization of cytochrome c was more apparent with control cells than with \( \rho^0 \) cells. It remained unchanged with \( \rho^0 \) cells even after treatment with cisplatin. Western blot analysis revealed that a significant amount of cytochrome c was de-
detected in the cytosolic fraction of the control cells but not in \( \rho^0 \) cells (Fig. 8B).

**Effect of mtDNA depletion on cisplatin-induced DNA fragmentation.** To evaluate the occurrence of apoptosis, TUNEL staining, and analysis of DNA fragmentation were conducted with cisplatin-treated IEC-6 cells and \( \rho^0 \) cells (Fig. 9). The DNA fragmentation became apparent with control cells during the incubation with cisplatin, but not with \( \rho^0 \) cells. Cisplatin significantly increased the number of TUNEL-positive IEC-6 cells, but not of \( \rho^0 \) cells.

**Effect of mtDNA depletion on cisplatin-induced LDH release.** The release of LDH into the culture medium has been used as a marker for cell necrosis. As shown in Fig. 10, the LDH activity in the culture medium of control cells increased significantly 24 h after the treatment with cisplatin. However, the release of the enzyme was low in cisplatin-treated \( \rho^0 \) cells. Thus necrosis also participates in the mechanism of cisplatin-induced cell death more markedly with IEC-6 cells than with \( \rho^0 \) cells.

**Correlation between mitochondrial density and sensitivity to cisplatin.** To evaluate the relationship between the sensitivity of cells to cisplatin and cellular activity of succinate dehydrogenase (SDH), LD50 for cisplatin was determined with six types of tumor cell lines and two types of nontumor cell lines with different density of mitochondria (Fig. 11). In all of the eight cell lines tested, the SDH activities were strongly correlated with the LD50 values for cisplatin, namely, the higher the density, the higher the sensitivity. Thus the cellular density of mitochondria seems to determine the sensitivity of cells to cisplatin.

**DISCUSSION**

The present work demonstrates that cisplatin exhibits its cytotoxic effect depending on the cellular density of mitochondria in both in vivo and in vitro experiments. In essence, the higher is the mitochondrial density, the stronger is the cytotoxic effect of cisplatin.
We found the presence of a marked zonation of mitochondrial density along the small intestinal epithelial cells; the density was the highest at the duodenum and decreased gradually along the intestine and was the lowest at the distal portion of the ileum. The small intestine is the principal site for the digestion and absorption of nutrients. It should be noted that the hydrolysis of macromolecular nutrients, such as proteins and polysaccharides, occurs via ATP-independent mechanisms, whereas the absorption of low molecular weight nutrients across IECs requires a large amount of energy. The presence of the marked zonation of the mitochondrial density in mucosal epithelial cells along the intestine suggests a difference among the activities of duodenum, jejunum, and ileum in transporting nutrients. From the duodenum to the distal part of the ileum, the length of epithelial villi gradually decreases along the intestine. Because the jejunum is the potential site for the absorption of nutrients, a significant fraction of low molecular weight nutrients is taken up at the proximal portion of the jejunum. On the other hand, the absorption of water and electrolytes is the major function of the ileum and the large intestine. Recent studies (49) also revealed that UDP-glucuronosyltransferases and related phase II enzymes had the highest activities at the duodenum, but the activities decreased along the jejunum and ileum and were the lowest at the large intestine. The mucosal levels of ghrelin also shows a similar zonation in the order of the duodenum > jejunum > ileum (21). Thus the physiological significance of the marked zonation of mitochondrial density, enzymes, and other proteins in epithelial cells along the small intestine should be elucidated further.

Injury of the gastrointestinal tract is one of the major side effects of chemotherapy (3).Mitochondria have been demonstrated to play important roles in the induction of cell death (15). The present work clearly shows that the density of mitochondria and the cellular sensitivity to cisplatin toxicity are closely correlated. Mitochondria-enriched mucosal cells in the duodenum and jejunum undergo a greater degree of apoptosis after exposure to cisplatin than do cells in the distal portion of the ileum. Furthermore, a similar correlation be-

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**Fig. 6.** Effect of cisplatin on mitochondrial membrane potential (ΔΨm) in IEC-6 and ρ0 cells. IEC-6 (top) and ρ0 cells (bottom) were treated with 10 μM cisplatin for indicated times (0, 1.5, and 3 h) in the presence of 5 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine-iodide (JC-1), and changes in ΔΨm were observed by fluorescence microscopy. The experiments were repeated 3 times with similar results.

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**Fig. 7.** Effect of cisplatin on oxidative damage of IEC-6 and ρ0 cells. IEC-6 and ρ0 cells were treated with 10 μM cisplatin for 6 h. Oxidative injury of cells was evaluated by immunocytochemical stainings of 8-hydroxydeoxyguanosine (8-OHdG) (A) and 4-hydroxy-2-nonenal (4-HNE) (B). These typical fluorescence images were observed in 3 separate experiments.
tween the cisplatin sensitivity and mitochondrial density was observed with in vitro experiments in which intestinal epithelial IEC-6 cells and their \( \rho^0 \) cells with reduced number of mitochondria were used. The \( \rho^0 \) cells had a strong resistance to cisplatin compared with the control cells. Cisplatin-related chemotherapy has been reported to induce gastroduodenal mucosal injury (45), although it is unclear whether upper gastrointestinal tract epithelial cells are more susceptible. To elucidate the correlation of the present data with clinical data pertaining to the side effects of cisplatin in inducing intestinal epithelial cell death is extremely important.

Cisplatin is hydrolyzed to generate a positively charged metabolite (25). Because the membrane potential of mitochondria is inside negative (\( \sim 180 \text{ mV} \)), they readily accumulate membrane-permeable cationic compounds, including the cationic metabolite of cisplatin. Thus the amount of cisplatin uptake seems to increase proportionally to both the mitochondrial density and the membrane potential. Relatively more cisplatin is accumulated in the cells, which are enriched with mitochondria, and especially with those having higher membrane potential. Consistent with this hypothesis, cisplatin-resistant cancer cells have been shown to have mitochondria with low membrane potential (18).

Cisplatin exerts its cytotoxicity through the formations of ROS and cross-links in DNA (43). MtDNA appears to serve as one of the primary targets for cisplatin because of 1) preferential formation of adducts, 2) a low activity of decomposing the cisplatin-mtDNA adducts (39), and 3) a higher rate of mutation in mtDNA than in nDNA (46). In this context, we and others previously reported that mitochondrial dysfunction and related apoptosis play critical roles in the mechanism of cisplatin-induced cell death (7, 38, 41). Thus cells with higher density of mitochondria seem to accumulate a large amount of cisplatin and exhibit enhanced mtDNA injury and the subsequent dysfunction of mitochondria. In fact, cisplatin-induced cell death occurred more strongly in mitochondria-enriched duodenal epithelial cells than in ileal mucosal cells.

The present findings also suggest that the decrease in the mitochondrial density might increase the resistance of tumor cells to cisplatin. Consistent with our hypothesis that the mitochondrial density determines the sensitivity of cells to cisplatin, the decrease in the mitochondrial density lowered the sensitivity both in cultured cells and in small intestinal epithelial cells. Although \( \rho^0 \) cells are characterized by a low density of mitochondria with defective respiration, the mitochondrial potential is maintained in \( \rho^0 \) cells (5, 13, 28). Hence, \( \rho^0 \) cells also undergo apoptosis through the pathway that releases cytochrome \( c \) (24). Experiments using \( \rho^0 \) cells revealed that mitochondria also play important roles in the induction of cell death by anticancer agents (17, 20, 51).

ROS generation has been shown to underline the mechanism of cisplatin-induced cell injury (4, 36). For energy production, cells in metabolically active tissues depend mainly on their mitochondria. Mitochondria-rich cells seem to have a higher tendency to produce ROS under pathological conditions. Cisplatin generates a significant amount of ROS and impairs mitochondrial respiratory chain (30, 34). Uncontrollable production of ROS triggers the opening of the MPT pore and induces apoptosis and/or necrosis (16, 26, 29). Because the release of cytochrome \( c \) from mitochondria to cytosol activates the mitochondria-dependent pathway leading to apoptosis, lower densities of both mitochondria and cytochrome \( c \) in \( \rho^0 \) cells seem to decrease the incidence of cell death.

Fig. 8. Effect of cisplatin on cytochrome \( c \) release in IEC-6 and \( \rho^0 \) cells. Cytochrome \( c \) release after cisplatin-treatment was evaluated through immunocytochemical staining (A) and Western blot analysis (B). Immunocytochemical staining was performed before and after IEC-6, and \( \rho^0 \) cells were treated with 10 \( \mu \text{M} \) cisplatin for 6 h. Western blot analysis was carried out at 0, 3, and 6 h after treatment with 10 \( \mu \text{M} \) cisplatin. Cytochrome \( c \) release from mitochondria to cytosol was detected by using anti-cytochrome \( c \) antibody as described in the text. The experiments were performed 5 times with similar results.
Recurrent tumor cells after chemotherapy generally acquire a strong resistance to a wide variety of anticancer agents (MDR). It has been postulated that high expression of MDR genes is responsible for this multidrug resistance. Because mitochondria and mtDNA are highly sensitive to anticancer agents (7, 38), the recurrent cancer cells may have defective genes, including those in mtDNA. In fact, it has been well documented that the activity of glycolysis in malignant cancer cells is generally high, and, hence, they grow rapidly independently of energy supply from mitochondria. Such a property of cancer cells might also participate in the mechanism of MDR.

The present work also shows that the sensitivity of eight types of cells to cisplatin was dependent on the cellular activity of SDH. SDH is expressed exclusively in mitochondria, and, hence, it is used as a marker for the presence of mitochondria and for the potential for oxidative phosphorylation in cells and tissues (12, 40). SDH has been postulated to participate in the generation of ROS in both complexes I and III (23). Therefore, a higher density of mitochondria would result in the enhanced generation of ROS, particularly when the electron transport system is perturbed. The reduction of mitochondrial density decreased the sensitivity of cells to cisplatin and lowered the incidence of cell death. Whereas the analytical method for measurement of mitochondrial density was different from our present study, it was reported (35) that a cisplatin-resistant lung cancer cell line showed an increased number of mitochondria compared with the parental cell line by using electron microscopy. Thus, in addition to the biochemical parameters shown in the present study, differences in ultrastructural morphology should be studied further.

Fig. 9. Effect of cisplatin on the apoptosis of IEC-6 and $\rho^0$ cells. Before and 6 h after treatment with 10 $\mu$M cisplatin, apoptosis of control and $\rho^0$ cells was evaluated by electrophoresis of DNA (A) and TUNEL staining (B). The experiments were performed 5 times with similar results.

Fig. 10. Effect of cisplatin treatment on lactate dehydrogenase (LDH) release. After treatment of cells with 10 $\mu$M cisplatin for 24 h, the supernatants of culture medium were collected and analyzed for LDH activity. Data show means ± SD (n = 7) and are expressed as percentages of spontaneous release of the enzyme. The experiments were performed four times with similar results. Open bars, control cells; solid bars, $\rho^0$ cells. *P < 0.05 vs. control cells.

Fig. 11. Correlation between succinate dehydrogenase (SDH) activity and sensitivity to cisplatin. Various tumor cell lines and two types of cell lines derived from normal tissues were cultured with various concentrations of cisplatin for 24 h. The concentrations of cisplatin required for 50% cell death (LD50) were calculated from the cell survival curves analyzed with the Trypan blue exclusion test. SDH activities of these cells were determined as described in the text. Correlation coefficient ($r = 0.8637$) was determined by using simple linear regression and Pearson’s correlation coefficient analysis. HEK293, human embryonic kidney-293; EATC, Ehrlich ascites tumor cells.
In conclusion, we established a positive correlation between mitochondrial density and cellular sensitivity to cisplatin, not only in normal tissue but also in tumors. Reduction in mitochondrial density increases the cellular apoptotic and necrotic thresholds, and hence confers a selective advantage for cells to survive in the presence of chemotherapeutic drugs. ROS generation and ROS-mediated metabolic pathways are considered to be important in mitochondrial density-dependent cell death induced by cisplatin, due to different ROS-generating potential among cell types with different mitochondrial densities. With regard to chemotherapeutic intervention, this implies that design of more efficacious therapeutic clinical regimes, in which cisplatin chemotherapy is used only for patients with mitochondria-rich tumors. Design of novel therapeutic strategies may also allow alleviation of the side effects of chemotherapy by taking advantage of the tissue distribution patterns of mitochondrial density.

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