Mitochondria Determine the Efficacy of Anticancer Agents that Interact with DNA but Not the Cytoskeleton

Kenjirou Hara, Emiko Kasahara, Nozomi Takahashi, Masami Konishi, June Inoue, Mika Jikumaru, Shuji Kubo, Haruki Okamura, Eisuke Sato, and Masayasu Inoue

Department of Biochemistry and Molecular Pathology, Osaka City University Medical School, Osaka, Japan (K.H., E.K., N.T., M.K., J.I., M.J., E.S., M.I.); Iwata Chemical Co., Ltd, Iwata, Shizuoka, Japan (K.H.); and Laboratory of Host Defenses, Institute for Advanced Medical Sciences, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan (E.K., S.K., H.O.)

Received January 19, 2011; accepted March 14, 2011

ABSTRACT

Although chemotherapy is an important method for the treatment of patients with cancer, its efficacy is limited because of different sensitivities of tumor cells to anticancer agents and/or side effects on normal tissues. The present work demonstrates that mitochondria play a crucial role in the apoptosis of cancer cells induced by anticancer agents that interact with DNA but not with the cytoskeleton. Agents that interact with DNA selectively enhanced generation of reactive oxygen species (ROS) in mitochondria, released cytochrome c, and activated caspase-9 and caspase-3 to induce apoptosis of mesothelioma H2052 cells but not their \( \rho^0 \) cells, which lack mitochondrial DNA (mtDNA). The sensitivity of a variety of cells to the agents showed positive correlation with the amounts of their mitochondria. In contrast, agents that selectively affect the cytoskeleton activated caspase-8 and caspase-3 and equally induced apoptosis of both H2052 and their \( \rho^0 \) cells by a mitochondria-independent mechanism. The results suggest that mtDNA is a potential target for the anticancer agents that interact with DNA to induce ROS-dependent apoptosis of cancer cells, whereas agents that affect the cytoskeleton induce cell death by a mitochondria- and ROS-independent mechanism. The present observation is important for the selection of medicine for chemotherapy of patients with cancer.

Introduction

Although chemotherapy is an important method for treating patients with cancer, its efficacy is limited partly because of the different sensitivities of tumor cells to anticancer agents and side effects of the agents on normal tissues and cells. The mechanism of action of anticancer agents can be grouped depending on their affinity to cellular constituents, such as nuclear DNA (nDNA) (Momparler et al., 1976; Pacheco et al., 1989; Peters et al., 2002; Fuertes et al., 2003; Akiyama et al., 2008), the cytoskeleton (Chen and Horwitz, 2002), hormonal receptors, and specific epitopes on the surface of cancer cells (Mendelsohn and Baselga, 2000; Hudis, 2007).

Some anticancer agents that impair structure and function of DNA have been postulated to generate reactive oxygen species (ROS) and induce apoptosis of tumor cells (Gewirtz, 1999; Ravid et al., 1999; Caporossi et al., 2003; Wang et al., 2005). Because mitochondria are potential sites of ROS generation (Lenaz, 1998) and are highly enriched in mtDNA, oxidative injury occurs more rapidly and significantly with mtDNA than with nDNA (Saitou, 1991; Yakes and Van Houten, 1997). Therefore, agents that interact with DNA oxidatively impair mtDNA and/or the electron transport system, thereby increasing ROS generation in mitochondria to form a vicious cycle leading to apoptosis. Based on such a concept, we tested the effect of cisplatin on a variety of cancer cells and found that the amount of mitochondria in IEC-6 cells positively correlated with cellular sensitivity to the agent (Qian et al., 2005). Thus, we hypothesized that the amount of mitochondria may be a key factor determining the efficacy of anticancer agents that interact with DNA. To test this hypothesis, we analyzed the effects of several anti-
cancer agents that impair either structure and function of DNA or the cytoskeleton on mesothelioma H2052 cells and their \( \rho^0 \) cells lacking mtDNA. We also compared the toxicity of the two types of anticancer agents in a variety of cancer cells with different amounts of mitochondria. The present work demonstrates that the amounts of mitochondria and mtDNA in cancer cells are the important factors determining cell sensitivity to agents that preferentially impair structure and function of DNA but not the cytoskeleton.

**Materials and Methods**

**Anticancer Agents.** Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO). Bleomycin, doxorubicin, methotrexate, busulfan, fluorouracil, paclitaxel, colchicine, and vinblastine were purchased from Wako Pure Chemicals (Osaka, Japan).

**Cells.** Human mesothelioma cell lines, H2052 and H2452, were obtained from the American Type Culture Collection (Manassas, VA). HeLa cells (human cervical cancer cell line), HepG2 cells (human hepatocyte cancer cell line), HL-60 cells (human leukemia cell line), LLC (murine lung cancer cell line), and HEK293 cells (human kidney embryonic cell line) were obtained from the Riken Cell Bank (Tsukuba, Japan). B16 cells (murine melanoma cell line) were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured at 37°C under 5% CO₂ in Dulbecco’s modified Eagle’s medium or RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (Nacalai Tesque, Kyoto, Japan).

mtDNA-depleted \( \rho^0 \) cells were established through long-term treatment of H2052 cells with ethidium bromide (25 ng/ml) as described previously (King and Attardi, 1989). Depletion of mtDNA in the \( \rho^0 \) cells was confirmed by the polymerase chain reaction amplification method using a set of mtDNA-specific primers. Amounts of mitochondria in cells were analyzed by staining with MitoTracker Green FM (Invitrogen, Carlsbad, CA).

**Evaluation of Cell Death.** Viability of cells was evaluated by the trypan blue exclusion test. After treatment of cells with various anticancer agents for 24 h in the presence or absence of 100 \( \mu \)M manganese(III) tetrakis(4-benzoic acid)porphyrin (Mn-TBAP; Enzo Life Sciences Inc., Farmingdale, NY), both floating and adherent cells were counted by trypan blue exclusion as described previously (Waterhouse et al., 2001). The incubated membranes were washed five times with Tris-buffered saline solution containing 0.1% Tween 20 and 5% low-fat milk.

**Analysis of Released Cytochrome c.** To estimate the amount of cytochrome c released from mitochondria to cytosol, the harvested cells were incubated in an ice-cold cell lysis buffer (250 mM sucrose, 70 mM KCl, and 200 \( \mu \)g/ml digitonin in phosphate-buffered saline) for 5 min as described previously (Waterhouse et al., 2001). The supernatant and pellet fractions containing cytosolic and mitochondrial proteins, respectively, were subjected to SDS-PAGE and analyzed by using anticytochrome c antibody (BD Pharmingen, San Diego, CA).

**Isolation of Mitochondria.** Mitochondria were isolated from male C57BL/6J mice liver as described previously (Halestrap et al., 1997). In brief, the liver was homogenized in an ice-cold 5 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose and 0.1 mM EDTA. Subcellular fractions were separated using sucrose density gradient centrifugation. The mitochondrial fraction was suspended in a 5 mM Tris-HCl buffer, pH 7.5, containing 250 mM

**Role of Mitochondria in Cancer Chemotherapy**

**Anticancer agents induced ROS generation and cell death.** A, H2052 cells were incubated with increasing concentrations of cisplatin and paclitaxel for 24 h and analyzed for viability by trypan blue exclusion as described under Materials and Methods. B, after incubation of H2052 cells with 50 \( \mu \) M cisplatin or 100 nM paclitaxel, ROS generation was analyzed using DHE and flow cytometry. Data are expressed as means \( \pm \) S.D. derived from three independent experiments. \( * \), \( p < 0.05 \) versus paclitaxel.
sucrose. Mitochondria were used immediately after isolation with good conditions; respiratory control index of mitochondria was approximately 4 to 6.

**Analysis of Mitochondrial ROS Generation and ΔΨm.** Isolated mitochondria were loaded with DHE to analyze ROS generation. Mitochondria were incubated with 5 μM DHE at 37°C for 1 h in the dark in the presence or absence of 50 μM cisplatin and 100 nM paclitaxel. ROS generation was analyzed by using Spectra Max Gemini (Molecular Devices, Sunnyvale, CA). Isolated mitochondria were incubated with 50 μM cisplatin and 100 nM paclitaxel at 37°C. After incubation times, 50 nM JC-1 was added to the medium and ΔΨm was analyzed after 5 min using Spectra Max Gemini.

**Statistical Analysis.** All experiments were repeated at least three times with similar results. Data were expressed as means ± S.D. and analyzed by Student's t test (two-tailed), and P < 0.05 was considered statistically significant.

**Results**

**Effect of Anticancer Agents on Mitochondrial Generation of ROS and Cell Death.** Because ROS is important in the induction of apoptosis (Kane et al., 1993), we analyzed the effects of various anticancer agents on ROS generation and cell death using H2052 cells. A dye exclusion test revealed that incubation of cells with either cisplatin or paclitaxel for 24 h dose-dependently induced cell death (Fig. 1). However, ROS generation was enhanced time-dependently by cisplatin but not by paclitaxel. Analysis using 5 μM MitoSOX also revealed similar results (data not shown). To elucidate the relationship between ROS generation and cell death, we determined the LD50 of the two types of agents that affect structure and function of either DNA or the cytoskeleton (Table 1). Using LD50 concentrations, we determined the activity of the agents to induce ROS and cell death after incubation for 24 h (Fig. 2). Agents that interact with DNA, such as cisplatin, bleomycin, doxorubicin, methotrexate, busulfan, and fluorouracil, strongly enhanced ROS generation. In contrast, agents that affect the cytoskeleton, such as paclitaxel, colchicine, and vinblastine, failed to induce ROS.

To determine whether ROS is critical in inducing cell death, the effect of Mn-TBAP, a membrane-permeable superoxide scavenger (Faulkner et al., 1994), on cell death induced by the agents was investigated after incubation for 24 h. Mn-TBAP effectively suppressed the cell death induced by agents that interact with DNA. In contrast, Mn-TBAP failed to inhibit cell death induced by agents that affect the cytoskeleton. Thus, ROS generation seems to be responsible for the induction of cell death by agents that interact with DNA but not the cytoskeleton.

**Effect of Anticancer Agents on Mitochondrial Membrane Potential and Cytochrome c Localization.** Overproduction of ROS often induces membrane permeability transition, an early event leading to apoptosis (Green and Kroemer, 2004). Thus, we analyzed mitochondrial membrane

---

**TABLE 1**

Sensitivity of H2052 cells to anticancer agents

<table>
<thead>
<tr>
<th>Target</th>
<th>Agent</th>
<th>LD50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Cisplatin</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td>Bleomycin</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Methotrexate</td>
<td>105.1</td>
</tr>
<tr>
<td></td>
<td>Busulfan</td>
<td>1704.4</td>
</tr>
<tr>
<td></td>
<td>Fluorouracil</td>
<td>5681.4</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Paclitaxel</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Colchicine</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Vinblastine</td>
<td>0.04</td>
</tr>
</tbody>
</table>

---
permeability transition using a fluorescence probe, JC-1, during the incubation of cells with either 50 μM cisplatin or 100 nM paclitaxel for 24 h. Incubation with cisplatin but not paclitaxel increased the number of H2052 cells showing low ΔΨm (Fig. 3A). Kinetic analysis revealed that H2052 cells having low ΔΨm increased time-dependently during the incubation with cisplatin but not paclitaxel (Fig. 3B). Using LD50 concentrations of anticancer agents, we also determined their activity to increase cells having low ΔΨm. Agents interacting with DNA increased the number of cells showing low ΔΨm, whereas agents affecting the cytoskeleton failed to increase depolarized cells (Fig. 3C). Under identical conditions, cytochrome c was released from mitochondria to cytosol by agents interacting with DNA but not by agents affecting the cytoskeleton (Fig. 3D).

Effect of Anticancer Agents on H2052 Cells and Their ρ0 Cells. To elucidate the possible involvement of mitochondria in apoptosis induced by anticancer agents, we analyzed the effect of the agents on the viability of H2052 cells and their ρ0 cells using trypan blue exclusion (Fig. 4). The cytocidal activity of agents interacting with DNA was significantly higher in H2052 cells than in ρ0 cells. In contrast, agents affecting the cytoskeleton induced apoptosis equally in H2052 and ρ0 cells. Analysis using annexin V also showed similar results (data not shown).

To determine signaling pathways leading to apoptosis, we analyzed the effect of agents on the activation of caspases. Western blotting analysis revealed that the agents that interact with DNA selectively activated caspase-9 but not caspase-8 in H2052 cells. Under identical conditions, the agents failed to activate caspase-9 in ρ0 cells. Agents affecting the cytoskeleton selectively activated caspase-8 but not caspase-9 in H2052 and their ρ0 cells. Although caspase-3 was activated by agents that interact with DNA in selectively H2052 cells, the enzyme was activated equally by agents that affect the cytoskeleton in H2052 and ρ0 cells.

Effect of mtDNA Depletion on ROS Generation Induced by Anticancer Agents. We compared the effect of the two types of anticancer agents on cellular generation of ROS using H2052 and their ρ0 cells (Fig. 5). In the absence of anticancer agents, both types of cells generated small amounts of ROS, although their generation was slightly higher with H2052 cells than with ρ0 cells. Agents that interact with DNA increased ROS generation in H2052 cells but not in their ρ0 cells. In contrast, agents that affect the cytoskeleton did not increase ROS generation in both types of cells (P = 0.5389–0.9525).

Correlation between the Amounts of Cellular Mitochondria and Drug Sensitivity. To test the hypothesis that mitochondria are potential targets for agents that interact with DNA, we analyzed the relationship between the amounts of mitochondria and sensitivity of cells to the agents (Fig. 6). The result revealed that amounts of cellular mitochondria positively correlated with the sensitivity of cells to agents that interact with DNA, the higher the amount of mitochondria, the higher the sensitivity of cells to the agents. Cellular sensitivity to agents that affect the cytoskeleton did not correlate with the amounts of cellular mitochondria.

Discussion

The present work demonstrates that mitochondria play a crucial role in apoptosis induced by agents that interact with DNA but not with the cytoskeleton. The sensitivity of cells to the former type agents correlated positively with the cellular contents of mitochondria (P = ~0.0060–0.0228).

Our hypothesis that mitochondria are potential targets for the agents interacting with DNA is consistent with the present findings that the agents specifically increased ROS, released cytochrome c into cytosol, and induced apoptosis by a mechanism inhibited by Mn-TRAP. The presence of membrane-permeable N-acetylcysteine (10 mM) also inhibited apoptosis induced by agents interacting with DNA but not the cytoskeleton (data not shown). Furthermore, agents that interact with DNA failed to induce ROS generation and apoptosis of ρ0 cells. Some studies reported that superoxide generation by NADPH oxidase underlies the mechanism of apoptosis (Gilleron et al., 2009; Kim et al., 2010). However, pretreatment of H2052 cells with 10 μM apocynin, an inhibitor of the oxidase, for 2 h failed to suppress apoptosis induced by all agents tested (data not shown). Furthermore, the presence of membrane-impermeable superoxide dismutase 1 (100 unit/ml) and catalase (100 unit/ml) showed no appreciable effect on apoptosis of H2052 cells induced by the agents interacting with DNA (data not shown). Thus, intracellular ROS generated by mitochondria might be responsible for the induction of apoptosis by agents interacting with DNA.

The present work suggests that mitochondria are the primary target for the agents interacting with DNA. mtDNA is responsible for the synthesis of 13 subunits of mitochondrial electron transport chain (Anderson et al., 1981). Because turnover of mitochondrial proteins occurs independently (Rammachandran et al., 2002), imbalance and/or impairment of the electron transport system caused by mtDNA injury may facilitate leakage of electrons to generate the superoxide radical (Han et al., 2001). Although rotenone (50 μM) and antimycin (50 μM) instantaneously increase mitochondrial ROS (data not shown), its generation induced by the agents interacting with DNA increased fairly slowly (see Fig. 1B). We also tested the effects of anticancer agents on isolated mitochondria. Antimycin rapidly, but transiently, increased ROS generation by mitochondria, particularly during early periods of incubation (~3 h) (see Fig. 7). In contrast, under identical conditions, both cisplatin and paclitaxel failed to induce ROS generation. Although mitochondrial membrane potential decreased rapidly after adding carbonyl cyanide m-chlorophenyl hydrazone, it decreased slowly over 6 h irrespective of the presence of cisplatin and paclitaxel. It should be noted that cisplatin, but not paclitaxel, increased ROS generation (see Fig. 1B) and the number of depolarized cells (see Fig. 3B) after incubation for longer than 6 h. Thus, fairly long-term incubation is required for the induction of ROS and decrease in membrane potential. This observation suggests that the lag for the production of mitochondrial ROS and decrease in membrane potential reflects the time required for impairment of mitochondrial electron transport proteins supplied by both mtDNA and nDNA. This possibility requires further study.

The agents that interact with DNA selectively activated caspase-9 in H2052 cells but not in ρ0 cells, whereas those affecting the cytoskeleton preferentially activated caspase-8 in both types of cells. ROS generated in mitochondria has been shown to oxidize membranous cardiolipin to release cytochrome c (Petrosillo et al., 2003; Rostovtseva and Bezru...
Pro-caspase-9 is activated by cytochrome c-containing apoptosisome (Li et al., 1997). Thus, agents that interact with DNA seem to activate this pathway, resulting in apoptosis. Caspase-8 interacts with microtubules and induces apoptosis by a mitochondria-independent pathway (Wieder et al., 2001; Mielgo et al., 2009). These observations are consistent with our finding that the agents equally induced apoptosis of both H2052 and their \( \rho^0 \) cells by a ROS-independent mechanism. Studies have demonstrated that paclitaxel activated caspase-8 to generate a cleaved form of Bid (Bhalla, 2003; von Haefen et al., 2003) that induced Bax-dependent apoptosis of HeLa cells and breast cancer cells (Luo et al., 1998; Yin et al., 1999; Kutuk and Letai, 2008). However, paclitaxel and other agents affecting the cytoskeleton failed to induce mitochondria-dependent apoptosis of H2052 cells under the present experimental conditions. The reason for the discrepancy of the involvement of mitochondria in the action mechanism of paclitaxel remains unknown. Possible involvement of mitochondria in the action mechanism of anticancer agents interacting with the cytoskeleton should be studied further.

Multidrug resistance of cancer cells is a critical factor determining efficacy of chemotherapy of patients with cancers (Tomek et al., 2003). In this context, ATP-binding cassette transporters, including multidrug resistance-associated proteins, participate in the mechanism for resistance to a wide variety of anticancer agents (Baldini, 1997). However, the expression of ATP-binding cassette transporters is not

Fig. 4. Effect of agents on apoptosis and caspase activation. A, H2052 and \( \rho^0 \) cells were incubated with anticancer agents for 24 h, and viability was analyzed by dye exclusion. B, under identical conditions, caspase activation was analyzed by SDS-PAGE and Western blotting using antibodies against caspase-3, caspase-8, and caspase-9. Other conditions were as described under Materials and Methods. Other conditions were as in Fig. 2. *, \( p < 0.05 \) versus control.

Fig. 5. Effect of agents on cellular generation of ROS in H2052 and \( \rho^0 \) cells. After incubation of H2052 and \( \rho^0 \) cells with the agents for 24 h, ROS generation was analyzed using DHE and flow cytometry. The percentage of DHE-positive cells (high staining group) are expressed as means \( \pm \) S.D. derived from at least three independent experiments. Other conditions were as in Fig. 2. *, \( p < 0.05 \) versus control.

Fig. 3. Effect of the agents on membrane potential and cytochrome c localization. A, H2052 cells were incubated with 50 \( \mu \)M cisplatin or 100 nM paclitaxel for 24 h, and \( \Delta \Psi_m \) was determined using JC-1 and flow cytometry. B, during the incubation with the agents \( \Delta \Psi_m \) was determined. C, cells were incubated with various agents for 24 h and \( \Delta \Psi_m \) was determined. *, \( p < 0.05 \) versus control. D, the released cytochrome c into cytosol was analyzed by Western blotting. Data shows mean \( \pm \) S.D. derived from at least three independent experiments. Other conditions were as in Fig. 2.
sufficient to explain all mechanisms responsible for multidrug resistance (Ogretmen et al., 1998; Soini et al., 2001). Preliminary experiments using specific antibody to multidrug resistance protein 1 revealed that \( \text{H9267} \) cells used in the present experiments did not express this protein (data not shown). We reported previously that cancer cells enriched in mitochondria showed higher sensitivity to cisplatin than did other cells having smaller amounts of mitochondria (Qian et al., 2005). The present work also demonstrates that malignant mesothelioma \( \text{H2052} \) cells exhibited higher sensitivity to all agents interacting with DNA than did their \( \rho^0 \) cells. This observation indicates that reduction in the amount of mitochondria in cancer cells underlies, at least in part, the mechanism for multidrug resistance.

The present work demonstrates that anticancer agents used in the experiments can be classified into those affecting mtDNA that induce caspase-9 activation and mitochondria-dependent apoptosis and those affecting the cytoskeleton that induce caspase-8 activation and mitochondria-independent cell death. Studies suggest that mitochondrial factors,
including Bel-2, superoxide dismutase, and other antioxidant enzymes, may also be potential targets for anticancer agents (Fulda et al., 2010). Thus, analysis of mitochondrial status in cells is important for the selection of drugs for chemotherapy of patients with cancer. The present work also suggests that anticancer agents interacting with mtDNA may produce side effects in normal tissues that are enriched with mitochondria. This hypothesis is consistent with the fact that mitochondria-enriched renal proximal tubules and intestinal mucosal cells are critical sites for the occurrence of side effects of cisplatin (Chang et al., 2002). Thus, such a possibility should also be taken into account for the chemotherapy of patients with cancer.

Acknowledgments

We thank Prof. Irwin M. Arias for valuable suggestions during the preparation of this article.

Authorship Contributions

Participated in research design: Hara, Kasahara, Takahashi, Konishi, J. Inoue, Jikumaru, Okamura, Sato, and M. Inoue.

Conducted experiments: Hara, Kasahara, Takahashi, Konishi, and J. Inoue.

Performed data analysis: Hara, Kasahara, and M. Inoue.

Wrote or contributed to the writing of the manuscript: Hara, Kasahara, Takahashi, Kudo, Sato, and M. Inoue.

References


Hara, Kasahara, and M. Inoue.


Hara, Kasahara, Takahashi, Kudo, Sato, and M. Inoue.

Wrote or contributed to the writing of the manuscript: Hara, Kasahara, Takahashi, Kudo, Sato, and M. Inoue.

References


