

Modulation of Anti-apoptosis by Endogenous IAP Expression in MKN45 Human Gastric Cancer Cells

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Abstract. *Background:* This study was designed to clarify differences in apoptotic signal transduction between gastric cancer cells and leukemia cells. *Materials and Methods:* In order to study apoptotic signal transduction of gastric cancer cells, MKN45 gastric cancer cells expressing the wild-type p53 gene and U937 myeloid leukemia cells expressing a mutated p53 gene were prepared. Cisplatin (CDDP) was used to induce apoptosis. We compared apoptotic signal transduction downstream to mitochondria between those two lines. *Results:* In contrast to U937 cells, MKN45 gastric cancer cells revealed delayed response in release of mitochondrial cytochrome c into the cytosol following caspase 3 activation. In signal pathways downstream of caspase 3 cleavage, of the three substrates detected, poly (ADP-ribose) polymerase (PARP) and PKC (protein kinase c) δ were not activated in MKN45 cells compared with U937 cells, resulting in delayed appearance of DNA ladder formation during CDDP-induced apoptosis. MKN45 constitutively expressed cIAP1, regardless of CDDP treatment, compared with no expression in U937. Drug sensitivity testing showed that MKN45 was more resistant to CDDP than U937 cells. *Conclusion:* We demonstrated that there is a delayed mitochondrial response and incomplete activation of caspase 3 in MKN45 gastric cancer cells compared with U937 leukemia cells. In addition, there was endogenous cIAP1 expression in MKN45 cells, which may be a factor in the presumed anti-apoptotic system in these human gastric cancer cells.

Gastric cancer is one of the most common cancers in Japan and a number of other countries. Survival rates, especially in advanced cases, are poor because only surgical curative resection has been effective against this malignancy. Gastric

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cancer is strongly refractory to various chemotherapeutic agents, a phenomenon known as intrinsic multidrug resistance (MDR), and many studies have been performed to elucidate the underlying mechanisms (1-4). One explanation for MDR lies in the activity of drug efflux pumps like P-glycoprotein and MRP (multidrug resistance related protein) (1, 2). These proteins function as ATP-driven drug efflux pumps and directly pump various drugs from the cytosol. Another possible explanation is that anti-apoptosis occurs as a cellular response to diverse anticancer agents. Although p53 status is closely associated with cellular apoptosis, most chemotherapeutic agents induce mitochondria-mediated apoptotic signal transduction, as described in previous studies (5, 6).

According to specific stimuli associated with anti-cancer agents, cytochrome c release from mitochondria into the cytosol is associated with an Apaf-1 caspase-9 complex, which activates the caspase cascade downstream, triggering cellular apoptosis (5-7). Recent studies have shown that several anti-apoptotic proteins play a role in drug-induced, mitochondria-mediated apoptosis (8-16). Overexpression of the anti-apoptotic Bcl-xL proteins (members of the Bcl-2 family) block cytochrome c release and activation of caspase-3 (8-10), and overexpression has also been reported in gastric cancer (11) as a main factor in the anti-apoptotic system, although details are vague. In addition, researchers have found that baculoviral p35 IAP (inhibitor of apoptosis) protein blocks the apoptotic response of mammalian cells to genotoxic stress. Eight human IAP-like proteins have been identified and designated as XIAP, cIAP1, cIAP2, NIAP, survivin, apollon, ILP2 and livin (12-16). These proteins are characterized by the presence of a caspase recruitment domain and an NH₂-terminal baculovirus inhibitor of the apoptosis repeat motif. Of the eight IAP-like proteins, XIAP, cIAP1, cIAP2 and survivin have been shown to prevent apoptosis by binding to caspases, thereby inhibiting the direct effector of apoptosis (13, 14, 16). Furthermore, cIAP1 appears to inhibit apoptosis through noncaspase mechanisms by activation of nuclear factor κ B (NF κ B) (15) and c-Jun-NH₂-terminal kinase (SAPK/JNK) (17).

Although many forms of leukemia are sensitive to various chemotherapy agents, and there is the possibility of cure with chemotherapy (often combined with other treatment), chemoresistance has been the main barrier to effective treatment of advanced or inoperable cases in gastric cancer. Research has not advanced beyond progression and improvement of anti-cancer agents (18,19). If chemotherapy were as effective in gastric cancer as in leukemia, prognosis of the former disease would be remarkably improved. In the present study, apoptotic signal transduction in MKN45 human gastric cancer cells was investigated and findings were compared with those for U937 leukemia cells; differences between cell lines were examined to elucidate the anti-apoptotic system in gastric cancer cells.

Materials and Methods

Cell culture. The human gastric cancer cell line MKN45 (RIKEN Cell Bank, Tsukuba, Japan) and human U937 myeloid leukemia cells (American Type Culture Collection, Manassas, VA, USA) were maintained and grown in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, NY, USA), penicillin (100 units/mL), streptomycin (100 µg/mL) and gentamycin (40 µg/mL).

The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere and treated with 100 µM cisplatin (CDDP).

Immunoblot analysis. Preparation of cell lysates and immunoblot analysis were performed, as described previously, using C-2-10 anti-poly(ADP-ribose) polymerase (PARP) (20), anti-PKC (protein kinase c) δ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-caspase-3 (Pharmingen, San Diego, CA, USA) (21, 22). Briefly, lysates for anti-PARP and anti-PKCδ were prepared by suspending cells in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% TritonX-100, 0.2mM phenylmethylsulfonyl fluoride, 10 µg/mL each of leupeptin and pepstatin and 1 µg/mL aprotinin), and those for anti-PARP were prepared as described (20). The lysates were cleared by centrifugation and subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. Proteins were then transferred to nitrocellulose, blocked with 5% dry milk in phosphate-buffered saline containing 0.05% Tween 20, and probed with anti-heparanase monoclonal antibody. Antigen-antibody complexes were visualized by chemiluminescence (ECL detection system; Amersham Pharmacia Biotech, Tokyo, Japan).

Cytochrome c release. Cells were washed twice with phosphate-buffered saline and pellets were suspended in 5 mL ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL each leupeptin, aprotinin and pepstatin containing 250 mM sucrose). Cells were homogenized by being run through a Dounce homogenizer 14 times for U937 cells and 20 times for MKN45 cells with a sandpaper-polished pestle. After centrifugation for 5 min at 4°C, supernatants were centrifuged at 105,000 xg for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with

anti-cytochrome c (Pharmingen). The same proteins were used for anti-DFF/ICAD (DNA Fragmentation Factor/ Inhibitor of Caspase Activated DNase) (Medical & Biological Lab. Co., Ltd., Nagoya, Japan) (23, 24).

Analysis of DNA fragmentation. Cells (5 x 10⁶) were harvested, washed, and suspended in 50 µL 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS and 0.5 mg/mL proteinase K (Sigma). After incubation at 50°C for 6 h, the samples were mixed with 50 µL 10 mM EDTA (pH 8.0) containing 1% (w/v) low melting point agarose and 40% sucrose for 10 min at 70°C. DNA was separated in 2% agarose gels. After treatment with RNase, the gels were visualized by ultraviolet illumination.

Drug sensitivity test. The sensitivity of MKN45 cells and U937 cells to CDDP was determined using MTT assays, as described previously (24). Briefly, CDDP was used at concentrations of 0.625, 1.25, 2.5, 5.0 and 10.0 µM. In each cell line, 100 µL of the 2x10⁵ cell suspension were dispensed into 96-well microtiter plates and incubated at 37°C in a humidified 5% CO₂ atmosphere for 3 days in the presence of CDDP. The colorimetric reaction was initiated by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl [2H]-tetrazolium bromide (MTT) at a concentration of 0.4 mg/mL and sodium succinate in the range of 0-30 mM. The formazan crystals were dissolved in 150 µL dimethylsulfoxide, and absorbance at 540 nm was quantitated using a microtiter-plate spectrophotometer. Succinate dehydrogenase activity (SD activity) is presented as the optical density per milligram protein. The chemosensitivity of the test samples to the respective drug concentrations is given as a percentage of the SD activity in drug-treated cells to that of the control (non-treated) cells. A lower SD activity value infers a higher chemosensitivity to the drug in question.

Results

cIAP1 expression. MKN45 gastric cancer cells constitutively expressed cIAP1 and continued to express it regardless of CDDP treatment; U937 leukemia cells expressed low to undetectable levels of cIAP1 (Figure 1). Both lines expressed low to undetectable levels of cIAP2, a major member of the IAP family (data not shown).

Cytochrome c release and caspase-3 activation. To assess whether similar mechanisms for response to CDDP are involved in MKN45 and U937 cells, the release of mitochondrial cytochrome c was examined following CDDP treatment. Cells were treated with CDDP (100 µM), and cytoplasmic lysates were subjected to immuno-blotting with anti-cytochrome c antibody and whole cell lysate was analyzed with anti-caspase-3 antibody. Cytochrome c release into the cytosol was detected with a 4-h exposure to CDDP for U937 cells. However, release was detected only after 24 h for MKN45 cells. Following cytochrome c release, caspase-3 activation was recognized in both lines (Figure 2).

Substrate cleavage by active caspase-3. Cleavage of two substrates, PKCδ and PARP, by active caspase-3 was examined to study signal pathways downstream of caspase-3 for response to CDDP (21,22).

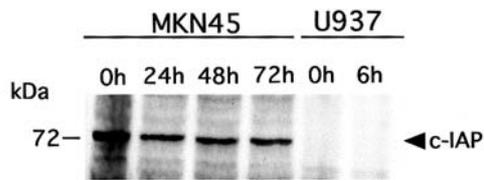


Figure 1. *cIAP1* expression in MKN45 human gastric cancer cells and U937 leukemia cells. Lysates from both lines treated with 100 μ M CDDP for the indicated durations were immunoblotted with anti-*cIAP1*.

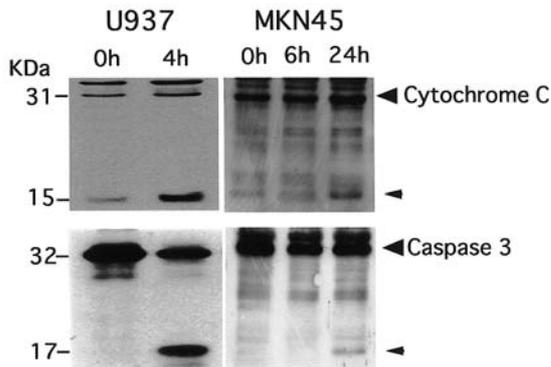


Figure 2. Cytochrome *c* release from mitochondria and caspase-3 activation in response to CDDP. Both cell lines were treated with CDDP and harvested at the indicated times. Cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-cytochrome *c* and anti-caspase-3. Cleavage products are indicated by small arrows.

Immunoblot analysis of lysates from both lines with anti-PKC δ antibody and PARP revealed that caspase-3-mediated cleavage of PARP and PKC δ were observed in MKN45 cells only at a low level despite treatment with CDDP for up to 72 h. With U937 cells, complete cleavage of both substrates was observed following caspase-3 activation (Figure 3).

DFF/ICAD cleavage by active caspase-3. DFF/ICAD is a potent substrate of active caspase-3. CAD, a product of DFF cleavage, directly binds to nuclear DNA to cause strand breakage (23, 24), which is quite different from the results of PKC δ and PARP activity. Cleavage with CDDP treatment was also examined. Immunoblot analysis of cytoplasmic lysate from MKN45 cells with anti-DFF/ICAD antibody revealed that obvious cleavage was recognized in both lines following caspase-3 activation, in contrast to the response from those of PKC δ and PARP (Figure 4).

DNA fragmentation in both cell lines. U937 cells treated with CDDP and harvested at 4 h exhibited a pattern of DNA fragmentation characteristic of apoptosis. In contrast, MKN45 cells exhibited the apoptosis pattern only after much longer treatment (more than 24 h), which was parallel to the time

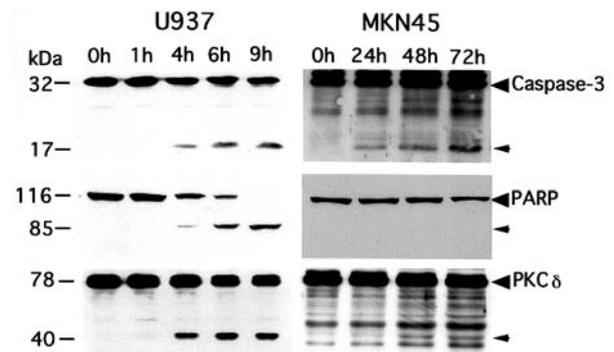


Figure 3. Substrate cleavage by active caspase-3. Lysates from both cell lines treated with CDDP for the indicated times were immunoblotted with anti-caspase-3, anti-PARP and anti-PKC δ antibody. Cleavage products are indicated by small arrow.

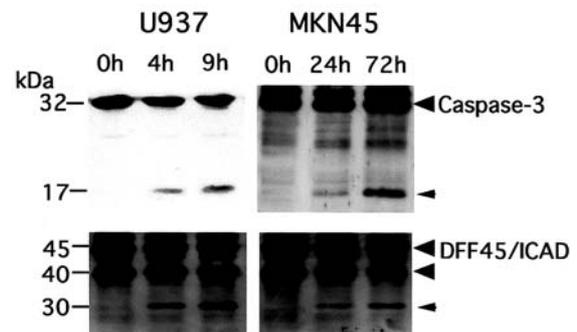


Figure 4. DFF45/ICAD cleavage by active caspase-3. Both cell lines were treated with CDDP and harvested at the indicated times. Cytosolic proteins were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-DFF45/ICAD and anti-caspase-3. Cleavage products are indicated by small arrows.

course of DFF/ICAD cleavage following caspase-3 activation and cytochrome *c* release (Figure 5).

Drug sensitivity test. To study the drug sensitivity of both cell lines, the MTT assay was performed using CDDP-induced apoptosis. The dose-dependent surviving fraction in the two lines is provided in Figure 6. MKN45 cells were clearly resistant to CDDP compared with U937 cells at all dose points.

Discussion

In the present study, differences in cisplatin (CDDP)-induced apoptosis signal transduction between MKN45 gastric cancer cells and U937 leukemia cells were demonstrated. CDDP has significant activity against a wide variety of human malignancies, especially gastric cancer (25, 26). The antitumor activity of cisplatin is thought to be mediated by its binding to DNA, resulting in formation of adducts and cross-links that lead to inhibition of DNA synthesis (27). Although CDDP binds to

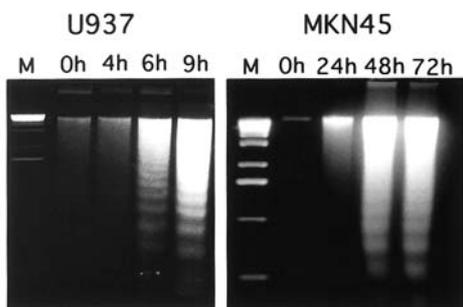


Figure 5. CDDP-induced DNA fragmentation. DNA was assayed for fragmentation by electrophoresis in 2% agarose gels. Both cell lines were treated with CDDP and harvested at indicated times; the size marker 1kb DNA ladder is shown in Lane M.

DNA directly, it also induces mitochondria-mediated apoptosis signal transduction (6, 28). Many studies have demonstrated that cytochrome c release from mitochondria contributes to this response (5-10, 28). In the cytosol, cytochrome c associates with a complex of Apaf-1 and caspase-9, thereby inducing activation of caspase-3 (5, 7). The induction of apoptosis is associated with caspase-3-mediated cleavage of poly(ADP-ribose) polymerase (PARP), protein kinase C δ (PKC δ), DFF45/ICAD, and other proteins (23, 24, 29, 30). Although leukemia is generally sensitive to various chemotherapeutic agents and gastric cancer is refractory to them, the inability to understand or manipulate chemoresistance in gastric cancers has been a severe barrier to the treatment of advanced or inoperable cases. In this study, anti-apoptotic factors were detected in CDDP-induced apoptotic signal transduction in MKN45 gastric cancer cells carrying a wild-type p53 gene in comparison with U937 leukemia cells carrying a mutated p53 gene.

Our data demonstrate that in MKN45 cells apoptosis induction was delayed, and these cells were resistant to CDDP compared with the U937 cells. Cytochrome c release was delayed and the subsequent activation of caspase-3 was also delayed in MKN45 cells. Although activation of two caspase-3 substrates, PARP and PKC δ , were blocked in MKN45 cells, all three substrates were activated in U937 cells, indicating that caspase-3 activation was partly inhibited in MKN45 cells compared with no inhibition in U937 cells. These data indicate that MKN45 resistance to CDDP-induced apoptosis depends on two factors, delayed response in mitochondria and incomplete activation of caspase-3, in comparison with U937 cells.

Previous studies have shown that overexpression of the anti-apoptotic Bcl-2 and Bcl-xL proteins block cytochrome c release from mitochondria and subsequent activation of caspase-3 (8-10). Endogenous expression of both proteins has been reported for MKN45 cells (11, 31), but not for U937 cells (32). These reports may support our first hypothesis regarding delayed mitochondrial response in MKN45 cells. As to the

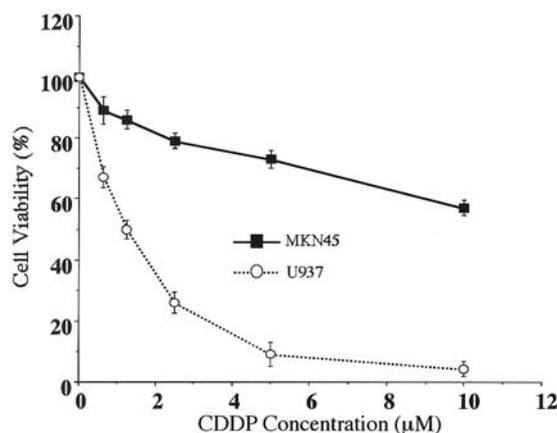


Figure 6. MTT assay were performed on both cell types: (■), MKN45; (○), U937. Points are means of triplicate determinations done in a single experiment. Standard deviation is indicated.

second hypothesis regarding incomplete activation of caspase-3, one explanation is the influence of IAPs. The IAP proteins are a new class of intrinsic cellular regulators of apoptosis, which are structurally defined by the presence of an evolutionarily conserved BIR domain (12-14). The human IAP family includes cIAP-1, cIAP-2, XIAP, NIAP, survivin, apollon, ILP2 and livin, and several members of the human IAP family including XIAP, cIAP-1, cIAP-2 and survivin have been shown to be potent inhibitors of caspase-3 and caspase-7 (13-16). It has also been reported that cIAP-1 appears to inhibit apoptosis through a non-caspase mechanism involving activation of NF κ B and c-Jun NH2 terminal kinase (15, 17). Results from previous studies have indicated that survivin is the most potent inhibitor of caspases and apoptosis in gastric cancer. Nakamura (33) reported that survivin was a predictor of CDDP sensitivity and might serve as a prognostic marker. In addition, survivin mRNA expression was significantly recognized in cancer tissue compared with adjacent normal tissue in gastric cancer (34). Endogenous expression of XIAP and survivin has been recognized in MKN45 cells (35, 36). Although our data demonstrate that cIAP-1 expression was recognized in MKN 45 cell but not in U937 cells, we confirmed there was no marked expression of XIAP and cIAP-2 in U937 cells (data not shown). Although it is unclear whether any member of the IAP family except survivin contributes to anti-apoptotic effects, endogenous IAP expression may play an important role in intrinsic chemoresistance in human gastric cancer, including the MKN45 cell line.

In conclusion, MKN45 cells revealed resistance to CDDP-induced apoptosis compared with U937 leukemia cells with regard to delayed release of cytochrome c from mitochondria and incomplete activation of caspase-3. Further research to overcome the above points may be the key to improving chemosensitivity in human gastric cancer.

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