Alleviation of the drug-resistant phenotype in idarubicin and cytosine arabinoside double-resistant acute myeloid leukemia cells by indomethacin

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Abstract. Chemoresistance to anticancer drugs is a major issue in the successful treatment of acute myeloid leukemia (AML). In this study, we developed an AML cell line (AML-2/IDAC) that is resistant to treatment with a combination of idarubicin and cytosine arabinoside (Id/AraC) by chronic exposure for more than 3 months. We then investigated the ability of indomethacin to alleviate the chemoresistance of AML-2/IDAC cells. Treatment with indomethacin alone induced growth arrest, but not the death of AML-2/IDAC cells. However, when AML-2/IDAC cells were treated with combinations of indomethacin and Id/AraC, the cell death and apoptosis rate of AML-2/IDAC cells were significantly increased in a dose- and time-dependent manner. The combined treatment with indomethacin and Id/AraC caused the collapse of the mitochondrial membrane potential and was also demonstrated to enhance the activities of caspase-3 and -8 in AML-2/IDAC cells. Furthermore, indomethacin down-regulated expression of the ABCA3 and MRP1 genes, which were over-expressed in AML-2/IDAC cells. Taken together, the results of this study suggest that indomethacin can be used to increase the therapeutic potential against drug-resistant AML when combined with anti-leukemic drugs.

Introduction

Chemotherapy has been extensively used for the treatment of patients with acute myeloid leukemia (AML), and one of the recent treatment strategies is based on a combination with cytosine arabinoside and anthracyclines (1). Despite the initial success of induction chemotherapy in AML, the remission is often maintained only for a short period of time, and most patients frequently fail to respond consistently to chemotherapy and eventually undergo a relapse (2,3). Because of this unfavorable prognosis, chemoresistance to anticancer drugs is a major issue in the successful treatment of AML.

Several mechanisms of chemoresistance have been identified in hematologic malignancies. One common mechanism is the over-expression of ATP-binding cassette (ABC) transporters (4), through which almost all anti-cancer drugs can be pumped out from cancer cells (5). Several studies have demonstrated that the drug resistance gene (MDR1/P-gp/ABCB1), multi-drug resistance-associated protein 1 (MRP1/ABCC1), breast cancer resistance protein (BCRP/ABCG2), and MRP3 (ABCC3) are associated with a poor response of AML cells to anticancer drugs (6-9). In addition, a recent study showed that ABCA3 was strongly correlated with poor prognostics of pediatric AML and that its down-regulation with siRNA sensitizes the cells to doxorubicin (10).

Another mechanism of chemoresistance is a defect of the apoptosis pathway, which is triggered by anticancer drugs (11). The activation of initiator and effector caspases by apoptotic stimuli, such as chemotherapeutic drugs, is critical in the process that leads to tumor cell death (12). However, many studies have reported that AML cells with drug-resistance can escape from the death process (13-15).

Indomethacin, a non-steroidal anti-inflammatory drug (NSAID), is a well known inhibitor of cyclooxygenases. Recently, many studies have focused on its novel anti-tumor activities (16,17). In this study, we developed an AML cell line (AML-2/IDAC) that was resistant to treatment with a combination of idarubicin and cytosine arabinoside by subjecting the cells to chronic exposure to these compounds for >3 months. We then evaluated the antitumor effect of indomethacin on alleviating the chemoresistance of AML-2/IDAC cells. The results of this study indicate that indomethacin enhances the anticancer drug-induced apoptosis of drug-resistant AML-2/IDAC cells.

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Materials and methods

Cell lines. The human leukemia AML-2/WT cell line was obtained from the Ontario Cancer Institute (Toronto, Canada) and maintained at 37˚C in a humidified 5% CO_2 atmosphere in minimum essential medium alpha (MEMα) medium (Gibco BRL, Grand island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Gibco BRL). AML-2/IDAC cells were generated from the parental drug-sensitive AML-2/WT cells by chronic co-treatment with idarubicin (Sigma, St. Louis, MO) and cytosine arabinoside (Sigma) for >3 months, which led to cross-resistance to both drugs.

Cytotoxicity and cell death assays. Exponentially growing cells were seeded into a 96-well culture plate at a density of $2x10^4$ cells/well and then cultured in the absence or presence of test drugs for various time periods at 37˚C. After treatment, the cell death induced by the drugs was accessed by a trypan blue exclusion assay. Trypan blue (Sigma) solution (0.4%) was then added to each well, and the blue-colored dead cells were counted using a hemacytometer under a light microscope.

Apoptosis detection. DNA fragmentation was used to evaluate the cell apoptosis. To accomplish this, the cells were plated on 100-mm culture dishes and then incubated in the absence or presence of test drugs, such as idarubicin, cytosine arabinoside and indomethacin (Sigma). After treatment, the cells were washed twice with PBS and then incubated for 30 min with lysis buffer (5 mM Tris-Cl, 20 mM EDTA and 0.5% Triton X-100) on ice, followed by centrifugation at 13,500 rpm for 20 min. The supernatants were then incubated for 1 h with 100 μg/ml RNase A (Sigma) at 37˚C, followed by an additional incubation with 100 μg/ml proteinase K for 1 h. The DNAs were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 0.1 volume of 3 M sodium acetate and 0.7 volume of isopropyl alcohol at -20˚C overnight. Next, the DNA pellets were resuspended in TE buffer and 5 μg of DNA from each sample was then separated on a 2.0% agarose gel and visualized by ethidium bromide staining. Apoptotic cell death was also assessed by flow cytometric analysis (FACSCalibur, BD, San Diego, CA) of propidium iodide-stained nuclei, as previously described (18). The population of cells that were in specific sub-G1 phase was analyzed by a ModiFit program and these cells were regarded as cells undergoing apoptosis.

Mitochondrial membrane potential ($\Delta\Psi m$). The collapse in $\Delta\Psi m$ was measured in cells that were stained with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$, Aldrich Chemical, Milwaukee, WI) solution, as described previously (19). Briefly, AML-2/IDAC cells were cultured in the absence or presence of indomethacin alone or in combination with antileukemic drugs for 48 h. After the treatment, the cells were washed with PBS and incubated for 15 min in 1 ml PBS containing 100 nM $DiOC_6$ at 37˚C. The cells were then resuspended in PBS, and their $\Delta\Psi m$ was determined using a flow cytometer.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from each cell culture was extracted, and cDNA was synthesized as described previously (18). PCR amplification was then conducted using the following primer sets: MRP1 (sense, 5'-ACT CAT TCA GCT CGT CTT GT-3'; antisense, 5'-GAT CCT TGG AGG AGT ACA CA-3'), ABCA3 (sense, 5'-ACC TCC TGA GTC CCG TCA AC-3'; antisense, 5'-CAT ACC CGC TGA TGT ATG CC-3'), MDR1 (sense, 5'-TTT TGG GTG TTA TTT GCT TT-3'; antisense, 5'-GCA TGC TTA AGT TCT TGC TT-3') and ß-actin (sense, 5'-AGC GGG AAA TCG TGC GTG-3'; antisense, 5'-CAG GGT ACA TGG TGG TGC C-3'). All PCR products were electrophoresed on a 1.5% agarose gel, and then visualized under a UV-transilluminator after ethidium bromide staining. The relative band densities were then determined by densitometric analysis using an ImageJ software (Wayne Rasband, NIH, USA).

Western blot analysis. The total cell lysates from each cell culture were subjected to Western blot analysis, as described previously (20), using rabbit anti-caspase 3 and anti-PARP antibodies that were obtained from Upstate (Lake Placid, NY), and goat anti-caspase-8 and anti-GAPDH antibodies that were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Colorimetric analysis of caspase activities. The activities of caspase-3 and -8 were measured by a colorimetric assay kit (Calbiochem, La Jolla, CA), according to the manufacturer's protocol. Briefly, AML-2/IDAC cells were seeded at a density of $1x10^5$ cells/ml and then cultured in the absence or presence of indomethacin alone or in combination with antileukemic drugs for 48 h. After incubation, the proteins were obtained from the cells using lysis buffer without protease inhibitors, as described above. Each protein sample (200 μg) was then mixed with 200 μM Ac-DEVD-pNA or Ac-IETD-pNA, respectively. The mixtures were then incubated for 2 h in 37˚C, and the absorbance at 400 nm was read using a microplate reader. The data are presented as a fold-change by comparing the result obtained from the treated samples with that of the untreated control sample.

Statistical analysis. The results are expressed as the mean ± SD of data obtained from three or four independent experiments performed in triplicate. Statistical significance was determined using the Student's t-test.

Results

Sensitivities of AML-2/WT and AML-2/IDAC cells to anticancer drugs. Idarubicin and cytosine arabinoside (Id/AraC) double-resistant AML-2/IDAC cells were generated from drug-sensitive AML-2/WT cells by chronic exposure to Id/AraC. To compare the susceptibility of AML-2/WT and AML-2/IDAC cells to Id/AraC, both cell lines were cultured in the absence or presence of various concentrations of Id/AraC. As shown in Fig. 1A, AML-2/IDAC cells were resistant to the cytotoxic response of Id/AraC when compared to the parental AML-2/WT cells. In addition, cellular DNA obtained from AML-2/IDAC cells exhibited no or less laddering to the

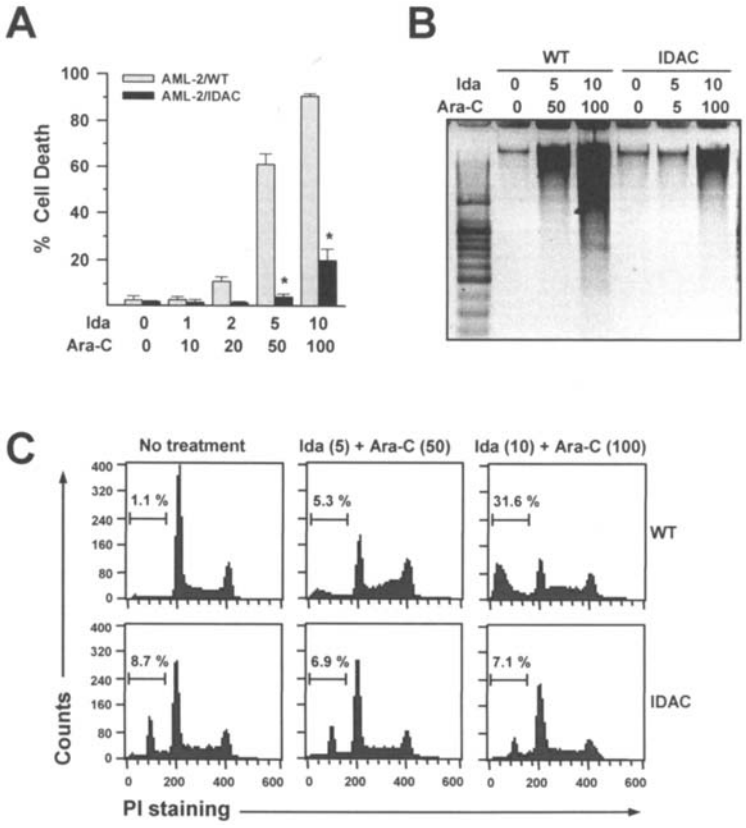

Figure 1. Susceptibility of AML-2/WT and AML-2/IDAC cells to anticancer drugs. (A) AML-2/WT and AML-2/IDAC cells were treated with both idarubicin (Ida, ng/ml) and cytosine arabinoside (Ara-C, ng/ml) for 48 h. Cell death was then evaluated by the trypan blue exclusion assay. Data are expressed as the mean ± SD of three independent experiments. *P<0.001, relative to a group of AML-2/WT cells. (B and C) At 24 h after treatment, the cells were also harvested to access the apoptotic death. Cellular DNA was extracted from the cells and separated on 2.0% agarose gel (B). Propidium iodide-stained cells were analyzed for DNA content using a flow cytometer (C). All data are representative of three independent experiments.

anticancer drugs rather than the AML-2/WT cells (Fig. 1B). Furthermore, the population of cells in the sub-G1 phase was unchanged in the drug-resistant AML-2/IDAC cells (Fig. 1C). These findings indicate that the AML-2/IDAC cells are highly resistant to the cytotoxic activities of anticancer drugs, Id/AraC.

Increase of anticancer drug-induced death of AML-2/IDAC cells by indomethacin. To alleviate the drug-resistant phenotypes of AML-2/IDAC cells, the cells were treated with a combination of Id/AraC and indomethacin, and the cytotoxic levels were determined. As shown in Fig. 2, treatment with either Id/AraC or indomethacin resulted in relatively low cytotoxic activity against AML-2/IDAC cells. Interestingly, the combined treatment of Id/AraC with indomethacin was demonstrated to effectively induce the death of AML-2/IDAC cells in a dose- and time-dependent manner (Fig. 2).

Enhancing effect of indomethacin on Id/AraC-induced apoptosis of AML-2/IDAC cells. We next examined the effect of indomethacin on anticancer drug-induced apoptosis in AML-2/IDAC cells. As shown in Fig. 3A, the cell cycle analysis revealed that treatment with a combination of Id/AraC and indomethacin strongly increased the proportion of the cell population that was in the sub-G1 phase. Additionally, co-treatment of AML-2/IDAC cells with indomethacin and Id/AraC resulted in enhanced activation of caspase-3 and -8, and also induced cleavage of PARP (Fig. 3B). Colorimetric analysis of the caspase activity also indicated that

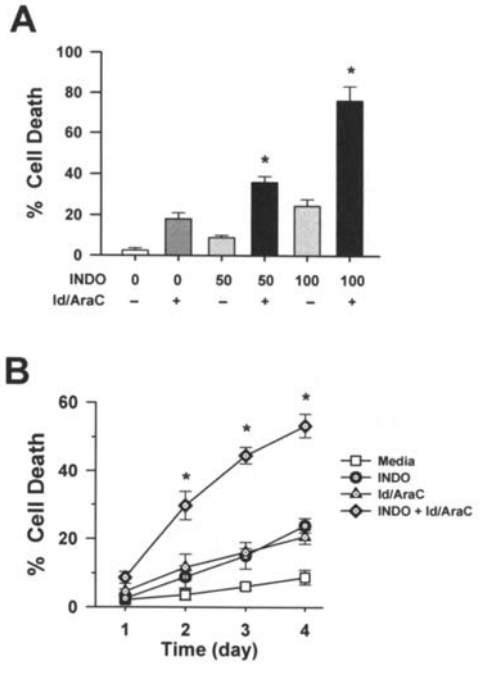

Figure 2. Effects of indomethacin on anticancer drug-induced death of drug-resistant AML-2/IDAC cells. AML-2/IDAC cells were treated with indomethacin (INDO, μg/ml) alone or in combination with 10 ng/ml idarubicin and 100 ng/ml cytosine arabinoside (Id/AraC). (A) At 48 h after treatment, cell death in each culture was evaluated by the trypan blue exclusion assay. (B) After each treatment, cell death was also accessed by the same method at various time-points. Data are expressed as the mean ± SD of four independent experiments. *P<0.001, relative to an untreated group.

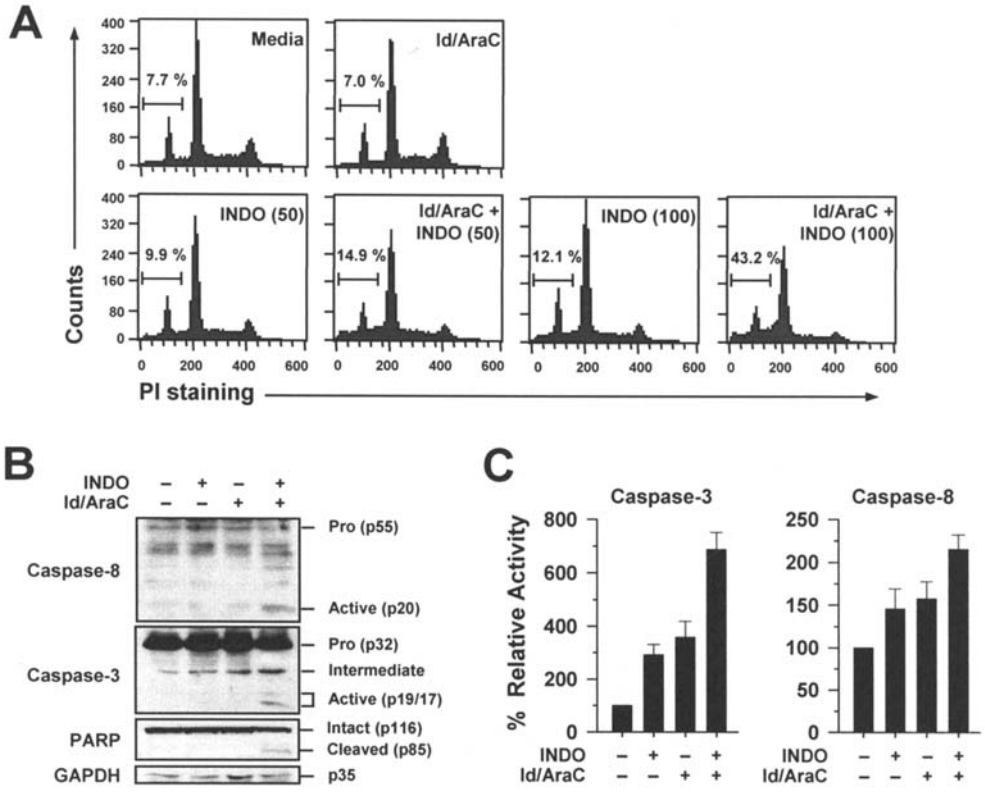

Figure 3. Effects of indomethacin in anticancer drug-induced apoptosis of AML-2/IDAC cells. AML-2/IDAC cells were treated for 48 h with indomethacin (INDO, 50 μg/ml) alone or in combination with anticancer drugs (Id/AraC), including 10 ng/ml idarubicin and 100 ng/ml cytosine arabinoside. (A) Cell death was determined by flow cytometric analysis with propidium iodide staining. The population of cells in the sub-G1 phase of cell cycle represents apoptotic death of the cells. (B) Total cell lysates were separated on SDS-PAGE gel. Western blotting was performed using specific antibodies for each protein. GAPDH was used as an internal loading control. (C) Activities of caspase-3 and -8 under each condition were accessed by colorimetric analysis using their specific substrates. Data are expressed as the mean ± SD of three independent experiments.

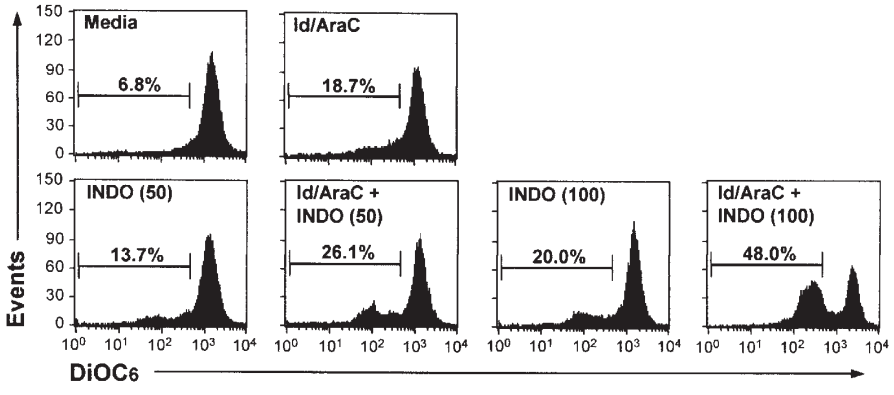

Figure 4. Changes of mitochondrial membrane potential ($\Delta\Psi$m) in AML-2/IDAC cells by the combined treatment with anticancer drugs and indomethacin. AML-2/IDAC cells were treated with indomethacin (INDO, μg/ml) alone or in combination with anticancer drugs (Id/AraC), including 10 ng/ml idarubicin and 100 ng/ml cytosine arabinoside. At 48 h after treatment, the cells were harvested and washed with PBS, followed by a staining with $DiOC_6$ solution, and the changes in their $\Delta\Psi$m were then evaluated by flow cytometric analysis.

indomethacin co-operated with Id/AraC to activate caspase-3 and -8 in AML-2/IDAC cells (Fig. 3C).

Previous studies have shown that anticancer drugs induced intrinsic apoptosis via dysfunction of the mitochondrial pathway as well as extrinsic apoptosis by caspases (21). Therefore, to determine if the apoptotic death induced by the combination of indomethacin and Id/AraC was associated with the mitochondrial pathway, the $\Delta\Psi$m changes were evaluated in AML-2/IDAC cells. As shown in Fig. 4, combination treatment with indomethacin and Id/AraC strongly promoted the collapse of $\Delta\Psi$m in AML-2/IDAC cells when compared with that of samples treated with either indomethacin or Id/AraC (Fig. 4).

Altered expressions of ABC-transporters in AML-2/IDAC cells and their modulation by indomethacin. To determine whether

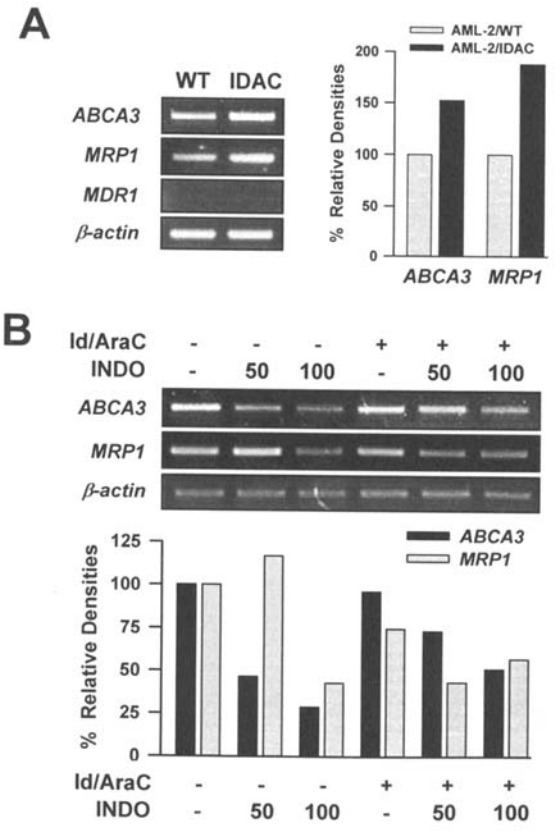

Figure 5. Altered gene expression of ABC-transporters in AML-2/IDAC cells and their modulation by indomethacin. (A) AML-2/WT (WT) and AML-2/IDAC (IDAC) cells were incubated under normal conditions for 24 h. RT-PCR analyses of the RNA samples obtained from both cells were then performed, and the products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. ß-actin was used as an internal control. The relative band densities of ABCA3 and MRP1 to ß-actin were then calculated by densitometric analysis. (B) AML-2/IDAC cells were treated for 24 h with indomethacin (INDO, μg/ml) alone or in combination with anticancer drugs (Id/AraC), including 10 ng/ml idarubicin and 100 ng/ml cytosine arabinoside. RT-PCR analyses were then conducted. ß-actin was used as an internal control. The band densities of ABCA3 and MRP1 relative to ß-actin were evaluated by densitometric analysis.

the drug-resistant ability of AML-2/IDAC cells was correlated with over-expression of ABC-transporters, the expression levels of several drug-efflux pumps were determined in both AML-2/WT and AML-2/IDAC cells. As shown in Fig. 5A, ABCA3 and MRP1 genes were over-expressed in AML-2/IDAC cells, when compared to the expression levels observed in AML-2/WT cells. However, expression of the MDR1 gene was not detected in either cell line.

We next asked if the enhanced susceptibility of AML-2/IDAC cells to Id/AraC by indomethacin was due to down-regulation of the ABCA3 and MRP1 genes, which were elevated in AML-2/IDAC cells. As shown in Fig. 5B, the expression levels of the ABCA3 and MRP1 genes were not changed by treatment with Id/AraC. Interestingly, treatment with indomethacin alone or in combination with Id/AraC inhibited expression of the ABCA3 and MRP1 genes in AML-2/IDAC cells (Fig. 5B), which indicates that indomethacin may enhance the Id/AraC-induced cell death of AML-2/IDAC cells by down-modulation of some drug-efflux pumps.

Discussion

Anticancer drug resistance is a major obstacle in the treatment of leukemia, therefore many trials evaluating leukemia treatment have been conducted (22-24). In this study, combined treatment with indomethacin was shown to potentiate a chemotherapy-induced apoptosis in drug-resistant AML-2/IDAC cells. Co-treatment with indomethacin was also found to decrease the expression levels of ABC transporter genes, such as ABCA3 and MRP1, which were over-expressed in AML-2/IDAC cells.

An increasing number of studies have indicated that NSAIDs has antitumor effects. For example, Giardiello *et al* and Sinicrope *et al* showed that sulindac can be clinically used for the treatment of colorectal malignancy (25,26). In studies of leukemia, evaluation of the anti-leukemic activity of NSAIDs has focused on attempts to overcome drug-resistance to chemotherapy (27). A major mechanism responsible for the effectiveness of NSAIDs has been shown to be the suppression of MRP1 but not MDR1 (27,28). Therefore, in the present study, we generated AML-2/IDAC cells that expressed MRP1 but not MDR1 through chronic exposure of drug-sensitive AML-2/WT cells to Id/AraC, which is extensively used in leukemia chemotherapy. Treatment with indomethacin down-regulated the MRP1 expression in AML-2/IDAC cells. Interestingly, drug-resistant AML-2/IDAC cells exhibited higher expression of ABCA3 than their parental cells. Although its involvement in drug-resistance has not been fully demonstrated, recent studies have suggested that ABCA3 is strongly correlated in the drug-response of AML cells (10,29,30). Here, we demonstrate for the first time that indomethacin can modulate expression of the ABCA3 gene, which suggests that ABCA3 is a new therapeutic target of indomethacin in cancer chemotherapy.

Another mechanism by which antileukemic activity of NSAIDs occurs is via induction of the activation of caspases, which leads to apoptosis in leukemia cells (16,31). Using our cell line models, we compared the apoptotic response to chemotherapeutic drugs in both drug-sensitive and -resistant leukemia cells. We observed a defect in drug-induced apoptosis in AML-2/IDAC cells. This phenotype was recovered by the combined treatment with indomethacin and anticancer drugs. In addition to the activation of caspase-3 and -8, the collapse of $\Delta\Psi$m suggested that the enhanced cell death induced by indomethacin was associated with an intrinsic apoptosis pathway (21).

Taken together, the results of this study indicate that indomethacin can alleviate the drug-resistant phenotype in AML cells, and that this occurs by activation of a chemotherapeutic drug-induced apoptotic pathway and suppression of some ABC transporters, such as ABCA3 and MRP1. In spite of the beneficial effect of indomethacin in the treatment of drug-resistant leukemia, further study is necessary to evaluate its clinical relevance.

Acknowledgements

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