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Resveratrol-mediated reversal of doxorubicin resistance in acute myeloid leukemia cells via downregulation of MRP1 expression

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ABSTRACT

Chemo-resistance to anti-cancer drugs is a major obstacle in efforts to develop a successful treatment of acute myeloid leukemia (AML). In this study, we investigate whether resveratrol, a common ingredient in a broad variety of fruits and vegetables, can reverse drug resistance in AML cells. Three doxorubicin-resistant AML cell lines (AML-2/DX30, AML-2/DX100, AML-2/DX300) were prepared via long-term exposure to doxorubicin for more than 3 months. DNA microarray analysis demonstrated that many genes were differentially expressed in the resistant cells, as compared with the wild type AML-2/WT cells. In particular, the expression level of the MRP1 gene was significantly increased in the AML-2/DX300 cells, as compared to that detected in AML-2 cells. Importantly, the resveratrol was shown not only to induce cell growth arrest and apoptotic death in doxorubicin-resistant AML cells, but was also shown to down-regulate the expression of an MRP1 gene. Furthermore, resveratrol treatment induced a significant increase in the uptake of 5(6)-carboxyfluorescein diacetate, a MRP1 substrate, into the doxorubicin-resistant AML-2/DX300 cells. The results of this study show that resveratrol may facilitate the cellular uptake of doxorubicin via an induced downregulation of MRP1 expression, and also suggest that it may prove useful in overcoming doxorubicin resistance, or in sensitizing doxorubicin-resistant AML cells to anti-leukemic agents.

1. Introduction

Acute myeloid leukemia (AML) is a clonal disorder occurring in a heterogeneous group of hematopoietic cells; the disorder is characterized by the uncontrolled proliferation and accumulation of clonal neoplastic cells in the bone marrow of blasts that have lost the ability to differentiate normally and have been blocked at various maturation steps, thus becoming resistant to cell death [1]. Although a number of breakthroughs have recently been made in the diagnosis of different AML subtypes and a great deal of progress has been made in therapeutic approaches, chemo-resistance and relapse remain major challenges to the successful treatment of AML [2,3]. The elucidation of the mechanisms underlying chemo-resistance in AML is an extremely important step in overcoming the obstacles to successful therapeutic intervention.

One marked impediment to chemotherapy for the treatment of AML involves the P-glycoprotein (P-gP) [4] and ATP-binding cassette (ABC) transporter, an ATP-dependent pump that effluxes cytotoxic drugs out of the cytoplasm [4–6]. Many additional cellular factors have been reported to play a role in the drug resistance of AML cells via enhanced or reduced drug metabolism [7], enhanced repair of chemotherapy-induced DNA damage, and increased cell survival [8–10]. The MRP family is comprised of a variety of ABC-related transporters that are capable of transporting structurally diverse lipophilic anions, and members of the family function as drug efflux pumps [11–13]. Previous investigations into members of this family have provided us with some profound insights into the resistance mechanisms utilized by cells against chemotherapeutic agents [14]. Multidrug resistance protein 1 (MRP1/ABCC1), a founding member of the ABC superfamily, was shown to be involved in the resistance of cancer cells against a variety of chemotherapeutic drugs including doxorubicin. Doxorubicin is one of the MRP1 substrates, and thus higher expression of MRP1 results in less accumulation of the drug in the cells [15].

Therefore, in this study we developed the doxorubicin-resistance AML cell lines by long-term (more than 3 months) co-cultures of human AML cells (AML-2/WT) with doxorubicin [16], and then assessed the mechanisms underlying their drug resistant properties. Furthermore, we attempted to determine whether resveratrol was capable of modulating the proliferation of the doxorubicin-resistance cell line (AML-2/DX300) and thus overcome its drug resistance.

Resveratrol is a phytoalexin (*trans*-3,5,4′-trihydroxystilbene), an ingredient abundantly present in grape skins, berries, and peanuts.

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Resveratrol was recognized as a potent chemopreventive agent, and blocked the initiation, promotion, and progression of tumors [17]. Furthermore, resveratrol has been previously demonstrated to inhibit the growth of a wide variety of tumor cells, including lymphoid and myeloid cancers of breast, prostate, and thyroid, melanoma, head and neck squamous cell carcinomas, and ovarian and cervical carcinomas [18,19]. The mechanisms underlying the growth inhibitory effects of resveratrol include cell-cycle arrest, apoptosis and the suppression of transcription factors such as NF-κB, and the inhibition of a variety of inflammatory gene products, such as cyclooxygenase-2 or IL-6 [20–22]. However, it remains to be determined whether or not resveratrol can reverse the resistant phenotype in drug-resistant human AML cell lines.

In this study, using doxorubicin-resistant AML cell lines selected after chronic exposure *in vitro*, we demonstrated that resveratrol can inhibit the proliferation of doxorubicin-resistant AML-2/DX300 cells and overcome the chemo-resistance of AML-2/DX300 cells, by sensitizing the cells to an anti-cancer agent via the down-regulation of MRP1 expression.

2. Materials and methods

2.1. Cell-lines and maintenance

Human acute leukemia AML-2/WT cells were obtained from the Ontario Cancer Institute (Toronto, Canada) and maintained at 37 °C in a 5% CO_2 atmosphere in minimum essential medium alpha (MEMα medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Biomeda, Foster City, CA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). AML-2/DX30, DX100 and DX300 cells, the doxorubicin-resistant sublines, were generated from parental drug-sensitive AML-2/WT cells via chronic exposure to doxorubicin (Sigma, St. Louis, MO) for more than 3 months on an intermittent dose schedule at intervals sufficient to allow for the expression of the resistance phenotypes. In order to mimic clinical situations, the cells were not exposed to any mutagens prior to selection, and were not clonally isolated.

2.2. Cell cytotoxicity, death and apoptosis

Exponentially growing cells were seeded into 96-well culture plate at a density of 4×10^4 cells/well, and then cultured in the absence or presence of the test drugs for various time periods at 37 °C. After treatment, the cell death induced by the drugs was accessed via 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 with a hemacytometer under a light microscope.

The cytotoxicity of the AML-2/WT cells and their anti-cancer drug-resistant variants was determined by MTT assay, as previously described [16].

Induction of apoptosis was determined via the detection of Annexin V (BD Pharmingen) and propidium iodide (PI, BD Pharmingen) staining using a flow cytometer (FACS Calibur, BD), in accordance with the manufacturer's introductions. In brief, 1×10^6 cells were treated for 24 h with either doxorubicin for drug sensitivity or resveratrol for cytotoxic effect at the indicated concentrations, after which the cells were washed twice with ice-cold PBS, then resuspended in 200 μl of 1× binding buffer. The cells were stained with 5 μl of Annexin V and 5 μl of PI, and then incubated for 10 min in a dark chamber at room temperature.

2.3. CFDA uptake

The CFDA uptake assay were conducted in AML-2/WT cells, drug-resistance cells, and resveratrol-treated cells, as previously described [23]. The cells were seeded on 6-well plates, then treated with 10 μM of 5(6)-carboxyfluorescein diacetate (5,6-CFDA), a substrate of MRP1, for 1 h and 30 min at 37 °C under an atmosphere of 5% CO_2. At the end of the incubation, the cells were gently washed with ice-cold PBS, after which the fluorescence was immediately assessed with a flow cytometer (FACS Calibur, BD).

2.4. RT-PCR analysis

In order to confirm the different expression levels detected on microarray analysis, the cellular RNAs were isolated from both AML-2/WT and each of the drug-resistant variants. RNA measurements were conducted with a spectrophotometer (NanoDrop Technologies, Wilmington, DE), and then the cDNAs were synthesized from 1.5 μg of total RNA using oligo-dT with a Bioneer cyclescript RT Kit (Bioneer, Daejeon Korea) for PCR. After cDNA synthesis, PCR amplification was conducted with an AccuPower PCR PreMix Kit (Bioneer). The PCR products were electrophoresed on 1.5% agarose gel, then visualized under UV-transilluminator after ethidium bromide staining.

2.5. Western blot analysis

The cells were lysed in lysis buffer and the proteins were separated on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes, as previously described [16]. The blots were then probed with mouse anti-MRP1 antibody (Chemicon, Temecula, CA), Rabbit anti-caspase-3, and anti-PARP antibodies, all of which were obtained from Upstate (Lake Placid, NY) as well as goat anti-GAPDH antibody (Santa Cruz, CA).

2.6. DNA microarray analysis

To compare the gene expression profile between AML-2/WT and AML-2/DX100 cells, we performed DNA microarray analysis using a human 10 K cDNA chip (GenoCheck, Ansan, Korea), as described previously [16]. Briefly, total RNAs from both cell lines were converted into cDNA, after which the generated cDNA samples were hybridized onto the chip. The raw data was then analyzed using GenePix Pro 4.1 (Axon, CA) and Microsoft Excel programs. Final data were expressed as a fold change in the gene expression value in AML-2/DX100 cells compared to the gene expression of AML-2/WT cells.

2.7. Confocal microscopy

Intracellular doxorubicin accumulation was evaluated with a confocal laser scanning microscope. The cells were incubated for 1 h with 50 ng/ml of doxorubicin at 37 °C in an atmosphere of 5% CO_2. At the end of the incubation, the cells were washed twice in ice-cold PBS, and then immediately observed under a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).

2.8. Statistical analysis

The results are expressed as the means ± SE of two to four independent experiments, each conducted in triplicate. The statistical significance of the data was assessed using the Student's *t*-test.

3. Results

3.1. Comparison of the sensitivity of AML-2/WT cells and their drug-resistant variants to doxorubicin

We established several AML-2/WT cell variants that acquired a drug-resistant phenotype via step-wise exposures to doxorubicin.

In order to compare the susceptibility of the parental and drug-resistant leukemia cells to doxorubicin-induced cell death, AML-2/WT cells and their drug-resistant variants were cultured in the presence of doxorubicin. As shown in Fig. 1A, doxorubicin treatment strongly induced the death of AML-2/WT cells, as determined in the trypan blue exclusion assay. However, the drug-resistant variants were significantly less sensitive to doxorubicin-induced cell death (Fig. 1A). The IC_{50} values against doxorubicin in the AML-2/WT cells and their drug-selected variants (AML-2/DX30, DX100, DX300) were 14.34, 205.5, 799.3, and 2961.0 ng/ml, respectively. As compared to the AML-2/WT cells, the drug-resistant variants exhibited resistance to the agent used for selection. Additionally, in an attempt to determine whether the difference in the susceptibility of the drug-sensitive and drug-resistant cells to cell death was attributable to apoptotic responses to doxorubicin, the cleavage of PARP and the active form of caspase-3, as an indicator of apoptosis, were assessed. Western blot analysis showed that doxorubicin induced caspase-3 activation and PARP degradation in AML-2/WT cells, but not in the AML-2/DX100 and AML-2/DX300 cells (Fig. 1B). Annexin V and PI staining analysis also demonstrated that the drug-resistant cell lines were less susceptible to doxorubicin-induced apoptotic cell death than were the AML-2/WT cells. The populations of the Annexin V-stained cells in AML-2/WT, DX30, DX100 and DX300 were 80.32%, 23.39%, 6.78% and 3.85%, respectively (Fig. 1C).

Doxorubicin has a distinctive red color in solution, and viable cells are known to intake doxorubicin. Thus, measuring red-stained cells is a way to measure drug resistance to doxorubicin in viable cells. Importantly, confocal microscopic observations revealed that the red fluorescence intensity of each stained cell was lower in the doxorubicin-resistant cells than in the AML-2/WT cells, thereby indicating that the drug-resistant cell lines may contain lower quantities of doxorubicin, owing to reduced drug intake and/or increased efflux ability (Fig. 1D). Taken together, these findings clearly show that the cell lines selected after chronic exposure to doxorubicin were significantly resistant to the cell death induced by the anti-cancer agent used for selection.

3.2. Increased activity and expression level of MRP1 in the doxorubicin-resistant variants of AML-2/WT cells

In previous studies, genome-wide approaches have fairly explained the resistant potential of tumor cells to anti-cancer drugs. Therefore, in this study, we evaluated gene expression profiles using DNA microarray analysis in order to determine whether the difference between parental AML-2/WT and doxorubicin-resistant AML-2/DX100 cells was a consequence of the altered expression of drug transport and cell apoptosis-associated genes. As shown in Fig. 2A, anti-apoptotic genes were overexpressed in AML-2/DX100 cells, whereas pro-apoptotic genes were downregulated in the doxorubicin-resistant cells. Interestingly, some of the drug efflux pump genes were markedly overexpressed in AML-2/DX100 cells. Additionally, RT-PCR and Western blot analyses showed that an increase in the gene expression of MRP1, an ATP-binding cassette (ABC) efflux pump, was observed in the doxorubicin-resistant cells, as compared to the AML-2/WT cells (Fig. 2B).

Furthermore, in order to determine whether or not changes in the expression level of MRP1 affected the ability of cells to pump

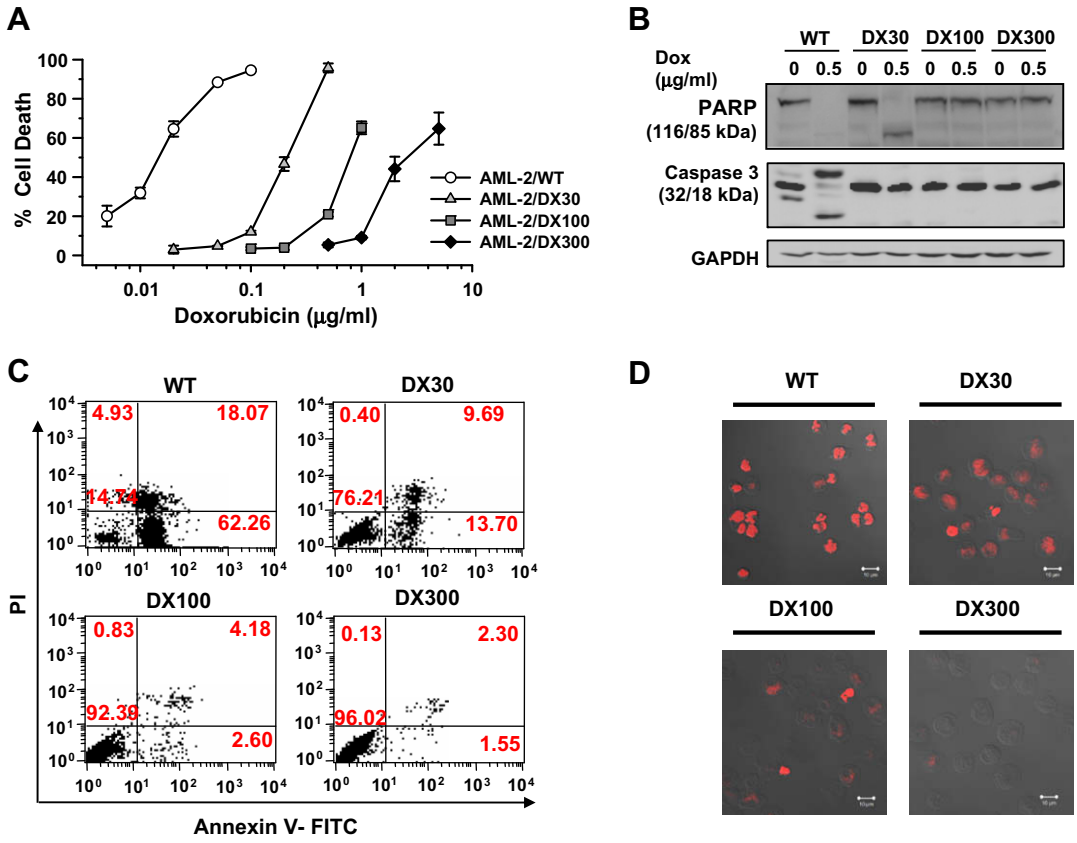

Fig. 1. Comparison of the doxorubicin sensitivity of AML-2/WT cells and their drug-resistant AML variants. (A) AML-2/WT cells and their doxorubicin (Dox)-resistant variant AML-2/DX30, DX100, DX300 cells were cultured for 48 h in the presence of the indicated concentrations of Dox, after which cell death was estimated via the trypan blue dye exclusion assay. (B) After the 24 h-incubation of the cells with 0.5 μg/ml Dox, the levels of PARP and caspase-3 were determined via Western blot analysis. GAPDH was utilized as a control. (C) After 24 h of treatment with 0.5 μg/ml of Dox, cell death was evaluated via Annexin V-PI staining. (D) Each Dox-resistant variant was treated for 12 h with 50 ng/ml of Dox, after which confocal microscopy analyses were conducted.

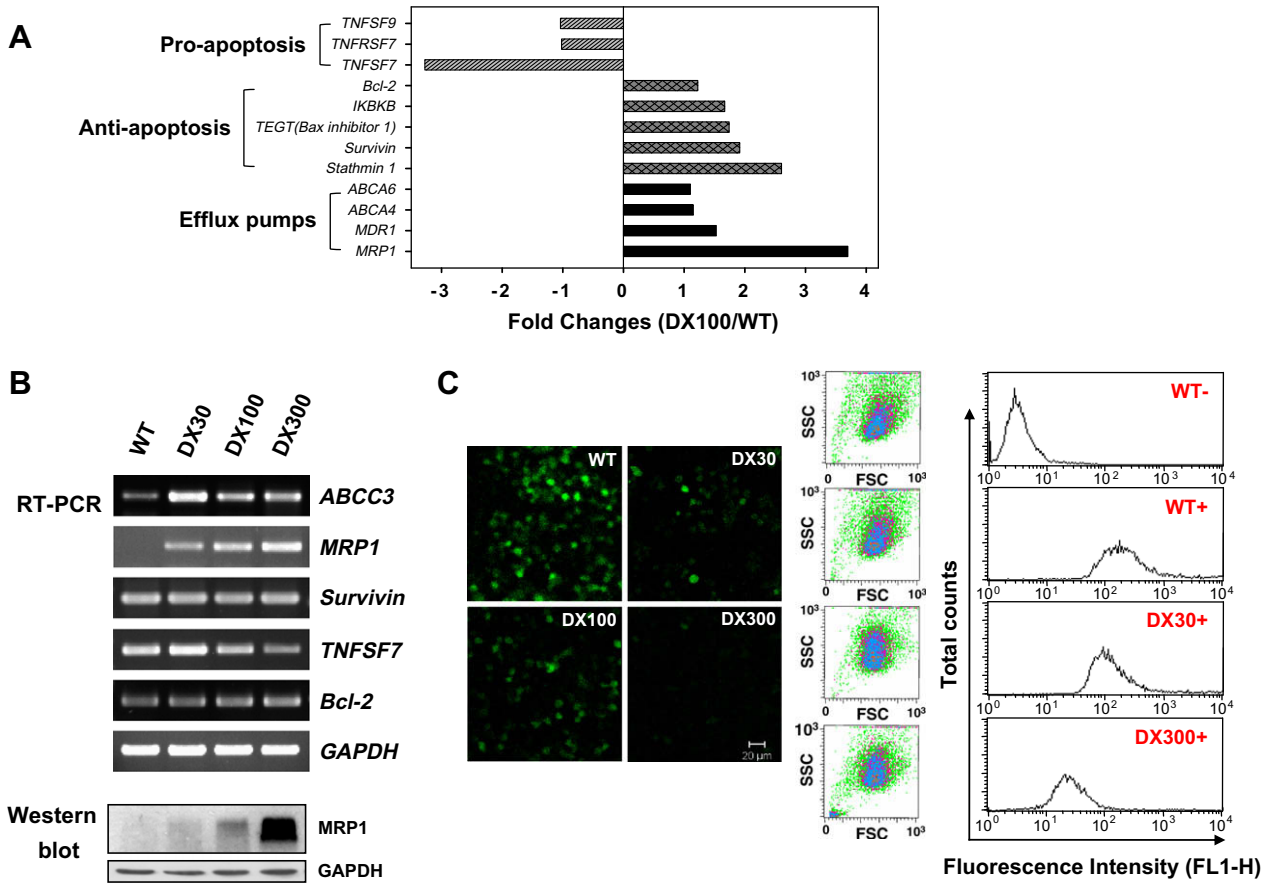

Fig. 2. Increased activity and expression level of MRP1 in drug-resistant AML-2 cell variants. (A) DNA microarray analysis of representative genes involved in the cellular activities in AML-2/DX100 cells. The data are expressed as fold-changes in the transcriptional levels of the genes in AML-2/DX100 cells (DX100) relative to those of AML-2/WT cells (WT). (B) The cellular RNAs were extracted from AML-2/WT, DX30, DX100 and DX300 cells, and the mRNA expression of each of the selected genes was analyzed by RT-PCR, and GAPDH was used as a loading control for normalization. MRP1 protein was determined by Western blot analysis. (C) Dox-resistant AML-2/DX30, 100, 300 cells were incubated for 1 h and 30 min with 10 μM 5,6-CFDA, after which the cells were cultured for 30 min in fresh media, and then washed twice in ice-cold PBS. Mean fluorescence intensity was determined using a flow cytometer.

out doxorubicin, we evaluated the activities of MRP1 in both doxorubicin-sensitive and -resistant AML-2 cell lines using 5,6-CFDA, an MRP1 substrate that emits green fluorescence. The cells were incubated with 5,6-CFDA, followed by washing in fresh media and then incubation in fresh media to allow the cells to pump out their substrate after uptake. At the end of the effluxing, the cells were washed with PBS twice and the CFDA-containing cells were evaluated via confocal microscopy and FACS analysis. As shown in Fig. 2C, the results of flow cytometric analysis revealed significantly lower numbers of the CDFA-positive cells with strong green fluorescence in the doxorubicin-resistant cell lines than in the doxorubicin-sensitive AML-2/WT cells, thereby indicating that the difference in the response to doxorubicin between the sensitive and resistant cell lines occurred as a consequence of differences in the intracellular concentration of doxorubicin. These results show that the resistance to doxorubicin noted in the AML-2/DX30, DX100 and DX300 cells is attributable to the increased activity and expression levels of MRP1, thereby resulting in enhanced doxorubicin efflux.

3.3. Reversal of drug resistance in AML-2/DX300 cell by resveratrol via the downregulation of MRP1 expression and activity

In order to determine whether or not resveratrol exerts cytotoxicity and reversal effects on a resistant phenotype of the doxorubicin-resistant cells, AML-2/DX300 cells were treated with resveratrol and cell death was measured via a MTT assay and a trypan blue exclusion assay. As shown in Fig. 3A and B, resveratrol induced significant cell death in AML-2/DX300 cells both in both a dose and time-dependent manner. Flow cytometric analysis using Annexin V and propidium iodide clearly demonstrated that resveratrol treatment induced the cell death of AML-2/DX300 cells (Fig. 3C).

Furthermore, in order to determine whether the cell death of AML-2/DX300 by resveratrol resulted from the downregulation of MRP1 expression, AML-2/DX300 cells were treated with resveratrol, and the expression levels and activities of MRP1 were evaluated. As shown in Fig. 4, the levels of MRP1 expression were higher in the doxorubicin-resistant AML-2/DX300 cells than in the AML-2/WT cells. Resveratrol treatment downregulated the expression levels of MRP1 in AML-2/DX300 cells at both mRNA and protein levels, while the expression levels of the GAPDH control were unchanged. The CFDA uptake assay using a confocal microscope demonstrated that resveratrol increased the numbers and green fluorescent intensities of the CFDA-positive cells in a dose and time-dependent manner when the cells were treated with resveratrol. These results indicate that resveratrol can overcome the drug-resistant phenotype of doxorubicin-resistant AML cells via an induced downregulation of the expression levels and activity of the MRP1 molecule.

4. Discussion

In this study we demonstrate that the expression level and activity of the MRP1 molecule were increased in AML cell lines se-

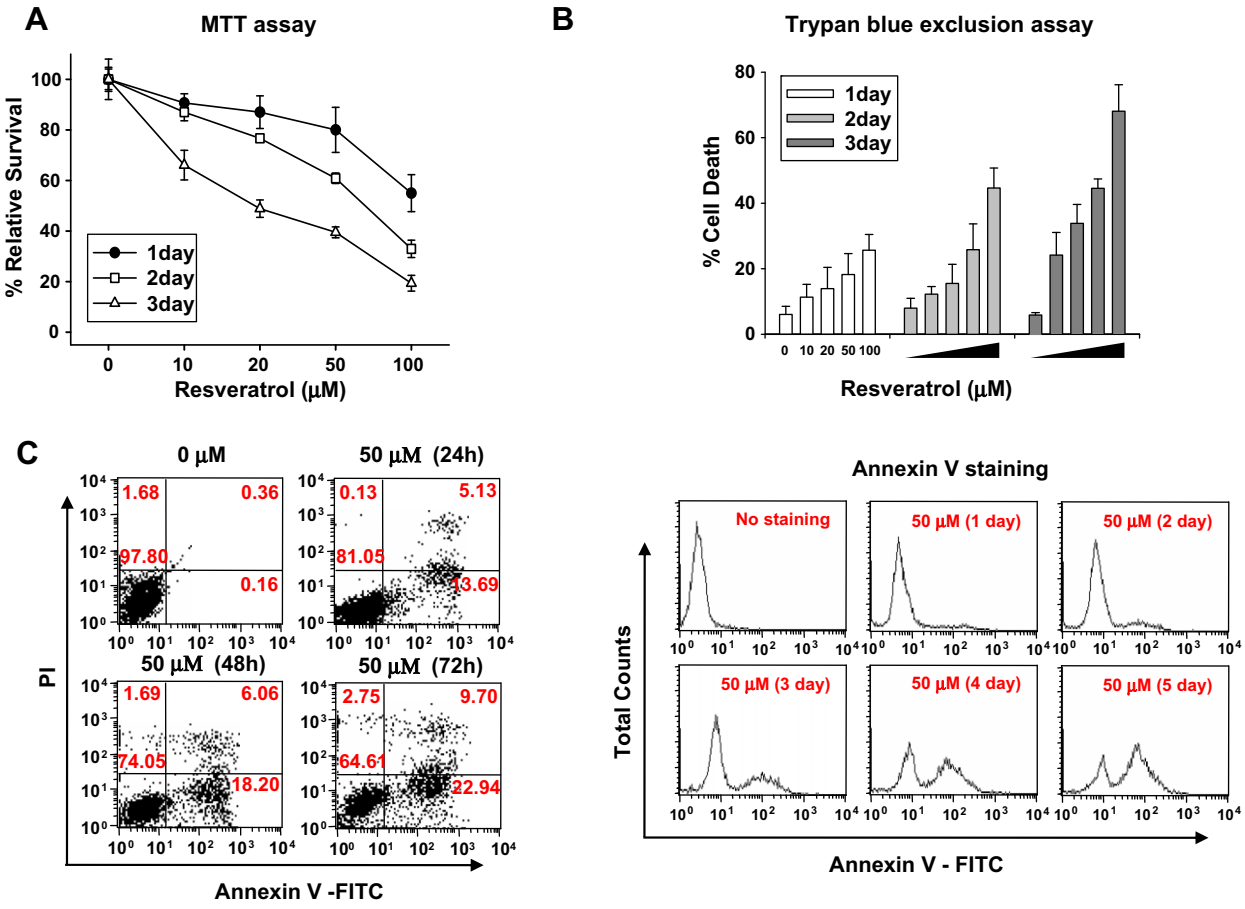

Fig. 3. Reversal of drug resistance in AML-2/DX300 cell by resveratrol. (A) AML-2/DX300 cells were cultured for 24 h with resveratrol alone at various concentrations, and the relative survival rates were determined via an MTT assay. (B) Cell death was evaluated by trypan blue exclusion assay. (C) In order to determine the effects of resveratrol on apoptosis, Annexin V and propidium iodide-stained cells were analyzed via flow cytometer after 3 days of exposure of 50 μM of resveratrol. AML-2/DX300 cells were co-cultured with resveratrol for 5 consecutive days at a concentration of 50 μM of resveratrol in a time-dependent manner, after the treated cells were stained with Annexin V-FITC antibody, and apoptosis was evaluated via flow cytometer on histogram.

lected after chronic *in vitro* exposure to doxorubicin, thereby resulting in the resistance of AML cells to the apoptotic cell death induced by the anti-leukemic drug used for selection. Importantly, resveratrol was observed to overcome the drug-resistant phenotype of doxorubicin-resistant AML cells via downregulation of MRP1 level and activity.

Doxorubicin and other anthracyclines are commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas [3,8]. Doxorubicin works by intercalating into the DNA and interfering with DNA replication, leading to cellular apoptosis and death. Their clinical use is, however, limited by a risk of cardiotoxicity and by the ability of the cancer cells to develop resistance to chemotherapeutic drugs. Therefore, assessing the drug resistant properties of cancer cells and the reversal of its resistant properties are important steps in the development of a successful treatment. The expression of the ABC transporter and P-glycoprotein has been generally correlated with AML prognosis [6]. Furthermore, although resveratrol is well-known to have chemopreventive and chemotherapeutic activities against a variety of cancer cells [24], its effects on drug-resistant AML and also on MRP1 expression have yet to be clearly elucidated. In this study, using doxorubicin-resistant AML cell lines selected by chronic *in vitro* exposure of doxorubicin, we demonstrate for the first time that resveratrol treatment reduced the expression level and activity of the MRP1 protein and reversed a resistant phenotype of doxorubicin-resistant AML cells susceptible to doxorubicin-induced apoptosis.

The mechanism whereby resveratrol can reverse a resistant phenotype of doxorubicin-resistant AML cells remains to be clearly elucidated. As resveratrol has been demonstrated to regulate the expression of genes involved in the cell cycle and apoptosis [25], its general anti-proliferative effects on cancer cells may result in the cell death of doxorubicin-resistant AML-2 cell lines used in the experiments. However, our results clearly demonstrate that the resveratrol-mediated downregulation of MRP1 is closely correlated with its reversal of a resistant phenotype in doxorubicin-resistant AML cells. First, chronic exposure of the parental AML-2 cells to doxorubicin resulted in the development of doxorubicin resistance in the AML-2 cells, and the strength of the resistance was correlated with the concentrations (50, 100 and 300 μg/ml) used for selection, as was the enhanced level and activity of the MRP1 molecule. As MRP1 functions as an efflux pump of many anti-cancer drugs, the selected AML-2 cells evidencing increased MRP1 expression pumped out doxorubicin from the cells into the extracellular medium, thereby exhibiting a resistance to doxorubicin-induced apoptotic cell death. Flow cytometric analysis demonstrated that the green fluorescent intensities of the CFDA-positive cells were lower in the doxorubicin-resistant cell lines than in the AML-2/WT cells (Fig. 2C), thereby indicating that the difference in the response to doxorubicin between the sensitive and resistant

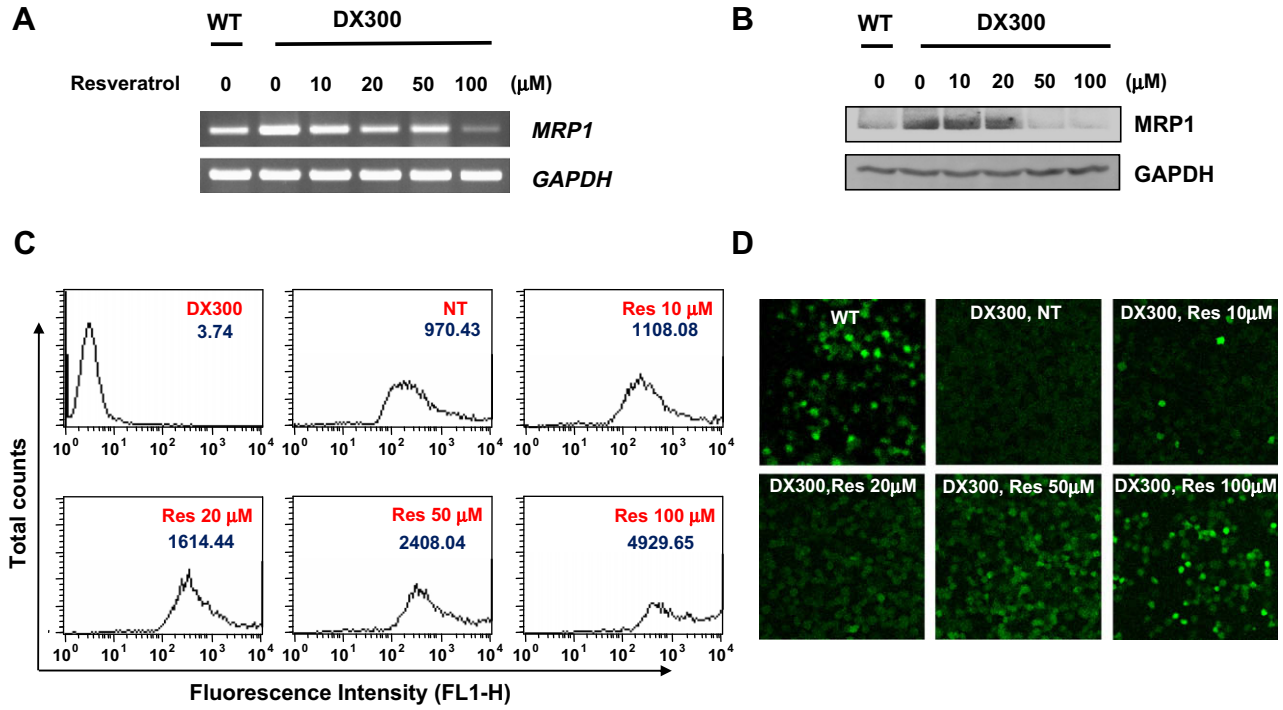

Fig. 4. Downregulation of MRP1 expression and activity in AML-2/DX300 cells by resveratrol. (A) AML-2/DX300 cells were cultured for 24 h with resveratrol. RT-PCR analyses were conducted on the RNA samples obtained from each of the cells, and the products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. (B) Western blot analysis was conducted to determine the status of MRP1. GAPDH was used as an internal control. Each of the blots shown is representative of three independent experiments. (C) After 24 h of resveratrol treatment, a CFDA uptake assay was conducted by administering 10 μM CFDA to each cell for 2 h, followed by gentle washing with ice-cold PBS.

cell lines occurred as the result of differences in the intracellular concentration of doxorubicin.

Importantly, resveratrol increased the susceptibility of the doxorubicin-resistant cells to doxorubicin-mediated apoptotic cell death and downregulated the expression levels of MRP1 in AML-2/DX300 cells at both mRNA and protein levels. Additionally, resveratrol increased the numbers and green fluorescent intensities of the CFDA-positive cells in a dose and time-dependent manner, clearly revealing that resveratrol the increased intracellular concentrations of doxorubicin, which were lowered in doxorubicin-resistant AML cells by chronic exposure to the doxorubicin used for selection. Pretreatment of AML-2/DX300 with resveratrol also enhanced susceptibility of cell death to doxorubicin at similar extents, as compared with the simultaneous treatment of AML-2/DX300 cells with resveratrol and doxorubicin (data not shown).

In conclusion, resveratrol can reverse a resistant phenotype of AML cells to anti-cancer drugs by reducing the MRP1 levels. A large number of AML patients relapse with resistant disease, even with aggressive therapy, although more than 80% of children with AML may initially achieve complete remission with current therapeutic agents [3]. As resveratrol is a naturally occurring phytochemical that is present in a wide variety of fruits and vegetables, the results suggest that resveratrol may prove useful as a subsidiary dietary supplement for patients with AML who are resistant to chemotherapeutic drugs.

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