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Monitoring the gene expression profiles of doxorubicin-resistant acute myelocytic leukemia cells by DNA microarray analysis

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Abstract

Acquired drug-resistance phenotype is a key factor in the relapse of patients suffering hematological malignancies. In order to investigate the genes involved in drug resistance, a human leukemia cell line that is resistant to doxorubicin, an anthracycline anticancer agent (AML-2/DX100), was selected and its gene expression profile was analyzed using a cDNA microarray. A number of genes were differentially expressed in the AML-2/DX100 cells, compared with the wild type (AML-2/WT). Pro-apoptotic genes such as *TNFSF7* and *p21* (*Cip1/Waf1*) were significantly down-regulated, whereas the *IKBKB*, *PCNA*, stathmin 1, *MCM5*, *MMP-2* and *MRP1* genes, which are involved in anti-apoptotic or cell cycle progression, were over-expressed. The AML-2/DX100 cells were also resistant to other anticancer drugs, including daunorubicin and camptothecin, and the expression levels of the differentially regulated genes such as *STMN1*, *MMP-2* and *CTSG*, were constantly maintained. This suggests that the deregulated genes obtained from the DNA microarray analysis in a cell line model of drug resistance might contribute to the acquired drug resistance after chronic exposure.

Keywords: Leukemia; Doxorubicin; Resistance; DNA microarray

Introduction

Despite the frequent success of chemotherapy in treating malignant hematological diseases, remission may be maintained only for a short duration and some anticancer drug resistance occurs in most patients (Chauncey, 2001; Marie, 2001). Therefore, drug resistance is a major problem and several mechanisms of drug resistance in hematological malignancies have been reported. These include the over-expression of the multi-drug resistance gene (*MDR1*) and multi-drug resistance-associated proteins (MRPs), as well as enhanced DNA repair and defects in the death systems such as the Fas–Fas ligand interaction (Chaney and Sancar, 1996; Gottesman et al., 1996; Loe et al., 1996; Mitsiades et al., 2001). The most characterized form of drug resistance is the enhanced drug export by the ATP-dependent efflux pumps including MDR1 and MRPs (Chauncey, 2001).

Although these proteins play a key role in the multi-drug resistance phenotype in cancer cells, several reports have suggested that other factors are involved (Dolci et al., 1993; Zyad et al., 1994). For example, during the treatment of a cytotoxic agent MDR1 gene-overexpressed cancer cells are more sensitive to anticancer drugs than resistant cancer cells that had been isolated by exposure to anticancer drugs. Therefore, a single or many mechanistic pathways cannot fully explain the genesis of drug resistance in cancer cells. Further studies are still needed to investigate the mechanisms for how cancer cells evade or breakdown the signals that lead to programmed cell death.

Traditional techniques for investigating drug resistance-associated gene regulation have provided new insights, but they are limited by the amount of data obtained from one

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experiment and are also time consuming (Devarajan et al., 2002; Kim et al., 2001). The DNA microarray technology is a powerful tool for examining cancer development and drug resistance by simultaneously comparing the expression levels of thousands of genes in drug-sensitive and -resistant cells (Hofmann et al., 2002). Previous studies using this method have revealed many genetic alterations including a diverse subset of gene products ranging from transcription factors to proteases and metabolites (Kudoh et al., 2000; Lamendola et al., 2003). The results from this advanced technique may provide useful information regarding drug resistant tumors in order to predict the drug responses and to design new therapeutic agents for overcoming drug resistance.

Therefore, this study monitored the differential gene expression profiles of doxorubicin-sensitive and -resistant human acute myelocytic leukemia (AML) cells using a cDNA microarray. A large number of genes related to the apoptotic and cell cycle pathways were differentially expressed in the doxorubicin-resistant cells, which might be involved in drug resistance.

Material and methods

Cell lines and maintenance

The human leukemia AML-2/WT cell line was obtained from the Ontario Cancer Institute (Toronto, Canada) and maintained at 37 °C in a 5% CO_2 using minimum essential medium (Gibco BRL, Grand Island, NY) that was supplemented with 10% heat inactivated fetal bovine serum (HyClone, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). AML-2/DX100 cells, which are a doxorubicin-resistant subline, were generated from parental drug-sensitive AML-2/WT cell via the chronic exposure to doxorubicin (Sigma, St. Louis, MO) for at least 3 months on an intermittent dose schedule at sufficient intervals to allow the expression of the resistance phenotypes. In order to mimic a clinical situation, the cells were not exposed to any mutagens prior to selection and were not clonally isolated after selection.

Determination of cell viability and morphologic studies

The level of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT, Sigma) assay. Briefly, after each treatment, 10 μl of MTT (5 mg/ml) was added to each well in 96-well plates. After 4 h incubation at 37 °C, the colored formazan product of viable cells was dissolved in 100 μl of 0.04 N HCl in isopropyl alcohol. The absorbance of each well was then read at 540 nm using a kinetic microplate reader. The morphological changes in the cells after doxorubicin treatment were examined by preparing single cell suspensions and loading 2×10^5 cells into a cyto-funnel and spun at 500 rpm in a cytospin centrifuge (Hanil, Seoul, Korea). The cells on the slides were fixed with 100% methanol and dried. The cells were then stained with a Giemsa staining solution (Sigma) for 20 min, rinsed in deionized water, and examined using an optical microscope equipped with a camera (Olympus Optical, Tokyo, Japan).

Cell cycle analysis

The effect of doxorubicin on the cell cycle phase of the AML-2/WT and AML-2/DX100 cells was determined by incubating 3×10^5 cells with various doses of doxorubicin for 3 days, fixing the cells in 100% ethanol, and treating them with 250 μg/ml RNase plus 2.5 μg/ml propidium iodide (PI) in phosphate-buffered saline (PBS) for 30 min at room temperature. The cells were then washed twice with PBS and then analyzed for their DNA content using an Epic XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

cDNA microarray analysis

The experiment was performed using a 10.0K cDNA microarrays (GenoCheck, Ansan, Korea) including a human 9217 cDNA microarray prepared, as previously described (Song et al., 2003). Briefly, a human cDNA microarray consists of 9217 cDNA spots including the Research genetics (Huntsville, AL) clones, housekeeping genes, and Arabidopsis DNA as the control. For target preparation, the total RNA was extracted from the AML-2/WT and AML-2/DX100 cells using the TRIzol reagent (Sigma) according to the manufacturer's instructions. The fluorescence-labeled cDNA probes were obtained from 30 μg of the total RNA using oligo-$(dT)_{18}$-primed polymerization with SuperScript II reverse transcriptase (Gibco BRL) in a 30 μl reaction volume. The reverse transcription mixture included 400 U Superscript RNase H-reverse transcriptase (Gibco BRL), 15 mM dATP, dTTP and dGTP, 0.6 mM dCTP and 3 mM Cy3- or Cy5-labeled dCTP (NEN Life Science Product Inc.). After the two labeled cDNAs were mixed, this mixture was then placed on a spotted slide position and hybridized for 12 h at 62 °C. The hybridized slides were washed in a series of SSC buffers at room temperature and then dried. These slides were scanned using the Axon Instruments GenePix 4000B scanner and the image was analyzed using the software program GenePix Pro 5.1 (Axon, CA) and GeneSpring 6.1 (Silicon

Table 1
Primer sequences for RT-PCR

Gene	Sense (5′–3′)	Antisense (5′–3′)	Product (bp)	Annealing condition (cycles)
MRP1	actcattcagctcgtcttgt	gatccttggaggagtacaca	299	59 °C–30 s (28)
IKBKB	agaggtggtgagcttaatga	tctgtaaccagctccagtct	341	57 °C–60 s (25)
STMN1	ttttcaatcccaattctgtc	gaaagtaacagctgacctgg	299	55 °C–60 s (25)
MMP-2	ggccctgtcactcctgagat	ggcatccaggttatcgggga	474	59 °C–30 s (28)
TNFSF7	cagctgaatcacacaggac	aaggtctcatcagtgtttcg	374	58 °C–40 s (25)
p21	ctggggatgtccgtcagaac	gagtctccaggtccacctgg	397	60 °C–60 s (30)
PDCD4	ggtggatgtgaaagatccta	ccacaaaggtcagaaagaag	279	58 °C–30 s (25)
β-actin	agcgggaaatcgtgcgtg	cagggtacatggtggtgcc	309	60 °C–30 s (20)