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Anti-oxidant adaptation in the AML cells supersensitive to hydrogen peroxide☆

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Abstract

The purpose of this study was to investigate the adaptive mechanisms of hydrogen peroxide-supersensitive AML cells against the reactive oxygen species (ROS). Their scavenging capacity against ROS was determined using a fluorometric probe in the doxorubicin-resistant AML-2/DX100 cell characterized by the down-regulation of catalase. AML-2/DX100 cells had more scavenging capacity against endogenous pro-oxidants than did the parental cells AML-2/WT, suggesting that an anti-oxidant adaptation against ROS occurred. cDNA microarrays for 8000 human genes revealed that among 21 anti-oxidant genes, each four gene was up- and down-regulated more than 1.5-fold in AML-2/DX100 compared with AML-2/WT. The mRNA expression of glutathione *S*-transferase Pi, peroxiredoxin 2, thioredoxin 2, and glutaredoxin was elevated whereas that of peroxiredoxin 3, metallothionein-1F, superoxide dismutase 2, and thioredoxin reductase 1 was depressed. The result indicates that the down-regulation of certain anti-oxidant mechanisms can be compensated for by the up- and down-regulation of the other anti-oxidant mechanisms.

Keywords: AML; Catalase; Reactive oxygen species; Anti-oxidant genes; cDNA microarray

Reactive oxygen species (ROS) are well recognized for playing a dual role both as deleterious and beneficial species. ROS are involved in a variety of cellular events, such as mutation, carcinogenesis, degenerative diseases, inflammation, apoptosis, cell cycle arrest, aging, and development [1]. The cellular events elicited by oxidative stress are regulated by reduction/oxidation (redox), and this is controlled by the ROS-defense mechanisms that include the glutathione, thioredoxin, and glutaredoxin systems [2]. The glutathione system is composed of reduced glutathione, glutathione peroxidase, and glutathione *S*-transferase [3]. The thioredoxin system is composed of NADPH, the flavoprotein thioredoxin reductase, peroxiredoxin (thioredoxin peroxidase), and thioredoxin; the glutaredoxin system is composed of NADPH, the flavoprotein glutathione reductase, glutathione, and glutaredoxin [4].

The doxorubicin-resistant AML-2 cell sublines were selected from the parental wild-type AML-2 cell line (AML-2/WT) after a chronic exposure to a 100 ng/ml concentration of doxorubicin. AML-2/DX100 was characterized by the overexpression of multidrug resistance associated protein (MRP), and this resulted from an amplification of the *MRP* gene and the down-regulation of catalase expression [5,6]. AML-2/DX100 cells were approximately 2- to 5-fold more sensitive to the pro-oxidants such as paraquat, hydrogen peroxide, and *t*-butyl hydroperoxide when compared with their parental cells [6].

The generation of ROS that was induced by hydrogen peroxide in the AML-2/DX100 cells was significantly higher than that of AML-2/WT cells when the level of

☆ *Abbreviations:* ROS, reactive oxygen species; WT, wild-type; redox, reduction/oxidation, MRP, multidrug resistance associated protein; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCFH, dichlorofluorescin; DCFH-DA, dichlorofluorescin-diacetate; SOD, superoxide dismutase.

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ROS was determined by using a fluorescent probe, dichlorofluorescin (DCFH). On the other hand, AML-2/DX100 cells had more scavenging capacity against the endogenous pro-oxidants than did the parental cells AML-2/WT, and this suggested that an anti-oxidant adaptation against ROS had occurred. In our study we report on how cells supersensitive to pro-oxidants can adapt to oxidative stress.

Materials and methods

Cell culture. The OCI-AML-2 line from the Ontario Cancer Institute (Toronto, Canada) was cultured at 37 °C in a 5% (v/v) CO_2 atmosphere using a α-MEM (Gibco-BRL, Gland Island, NY, USA) with 10% (v/v) heat inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). The cells were maintained as a suspension culture and they were then subcultured. The doxorubicin-resistant AML-2 sublines were selected from the parental cell line AML-2/WT after a chronic exposure to doxorubicin on an intermittent dosage schedule at sufficient time intervals to permit the expression of the resistance phenotypes. Doxorubicin was started from $1\times$ IC_{50} and the concentration was increased at a rate of 50%, and then finally the cells were cultured in a fixed concentration (100 ng/ml) of doxorubicin [5].

Cytotoxicity assay. The in vitro cytotoxicity of the drugs was determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO, USA] assay, as described by [7]. Ninety microliter aliquots of the cell suspensions, at 2×10^5 cells/ml in α-MEM containing 10% FBS, were seeded into a 96-well microplate which already contained 10 μl of a drug. The wells containing no drugs were used as a control of cell viability and the wells containing no cells were used for calibrating and zeroing the spectrophotometer. A stock solution of 5 mg/ml of MTT was prepared in saline and then stored at −20 °C. After the cells were incubated at 37 °C for 3 days, an aliquot of 10 μl of MTT solution was added to each well, shaken for 1 min, and the microplates were incubated for 5 h. Formazan crystals were dissolved with 100 μl of 0.04 N HCl–isopropanol alcohol. The optical density of the wells was measured with a microplate reader set at a wavelength of 540 nm. The 50% inhibitory concentration (IC_{50}) of a particular agent was defined as that drug concentration that causes a 50% reduction in the cell number versus the untreated control. The IC_{50} values were directly determined from the semilogarithmic dose–response curves. All experiments were carried out at least in triplicate.

Determination of ROS generation using a fluorometric probe. DCFH was used to measure the ROS concentration. After 2′,7′-dichlorofluorescin diacetate (DCFH-DA) crosses the cell membrane, it is de-esterified to DCFH, which is then oxidized to fluorescent DCF by the ROS [8]. Phosphate-buffered saline containing 1×10^5/ml cells was incubated with 1 μM DCFH-DA at 37 °C for 4 h. After incubation, the DCF fluorescence intensity was determined by using a fluorometer set at a wavelength of 485 nm for excitation and 530 nm for emission.

cDNA microarray. TwinChip Human-8K (Digital Genomics, Seoul, Korea) was used for the study. The total RNA was prepared from the cells by using a RNeasy midi kit (Quiagen, Hilden, Germany). The quality and integrity of the prepared total RNAs were confirmed with the use of an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA), and by spectrophotometry. Fluorescent labeled cDNA for the cDNA microarray analysis was prepared by the reverse transcription of the total RNA in the presence of aminoallyl-dUTP. This was followed by the coupling of the Cy3 dye for the controls (AML-2/WT) or Cy5 dye for the treated samples (AML-2/DX100) (Amersham–Pharmacia, Uppsala, Sweden). The TwinChip Human-8K Digital Genomics cDNA microarray was hybridized with a mixture of the fluorescent labeled cDNAs from the control cells and the treated cells at 58 °C for 16 h and then they were washed. After the washing procedure, the DNA chips were scanned using a ScanArray Lite (Perkin–Elmer Life Sciences, Billerica, MA). The scanned images were analyzed with GenePix 3.0 software (Axon Instruments, Union City, CA) to obtain the gene expression ratios (treated vs. control). Logged gene expression ratios were normalized by a LOWESS regression [9]. The genes were considered differentially expressed when the logarithmic gene expression ratios in two independent hybridizations were more than a 1.5-fold difference in the expression level.

Protein determination. Protein concentrations were determined by the method of Bradford [10].

Statistical analysis. Statistical significance of the data was determined by Student's t test. P values less than 0.05 were taken as statistically significant.

Results and discussion

Scavenging capacity of AML-2/DX100 against exogenous and endogenous pro-oxidants

The doxorubicin-resistant AML-2/DX100 cells are characterized by the down-regulation of catalase and resultantly supersensitive to pro-oxidants such as paraquat and H_2O_2 [6]. The scavenging capacity in the doxorubicin-resistant AML-2/DX100 cells against exogenous or endogenous pro-oxidants was determined using DCFH. As was expected, the generation of ROS induced by hydrogen peroxide in AML-2/DX100 cells was significantly higher than that of the AML-2/WT cells (Fig. 1). It has been demonstrated that the cells can adapt to H_2O_2 by enhancing the cell's capacity to consume H_2O_2 or with no sign of enhanced capacity to degrade H_2O_2 [11,12]. It has been shown that AML-2/DX100 cells cannot adapt to the high concentration of exogenous H_2O_2. On the other hand, AML-2/DX100 cells had more scavenging capacity against endogenous pro-oxidants than did the parental AML-2/WT cells

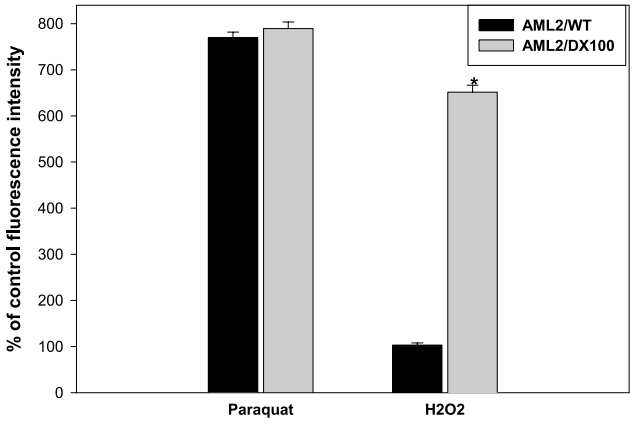

Fig. 1. Comparison of scavenging capacity against exogenous pro-oxidants between AML-2/WT and AML-2/DX100 cells. Phosphate-buffered saline containing 1×10^5/ml cells was incubated with 1 μM DCFH-DA at 37 °C for 4 h. After incubation, the DCF fluorescence intensity was determined by using a fluorometer set at a wavelength of 485 nm for excitation and 530 nm for emission. $^*P < 0.01$.

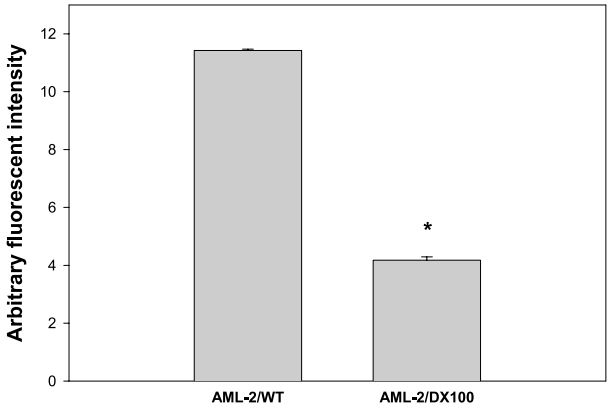

Fig. 2. Comparison of scavenging capacity against endogenous pro-oxidants between AML-2/WT and AML-2/DX100 cells. ROS was determined by the same method described in Fig. 1. $*P < 0.01$.

(Fig. 2). This result suggests that AML-2/DX100 cells were armed with adaptation to oxidative stress induced by endogenous ROS.

cDNA microarray analysis of the expression of anti-oxidant and redox genes

Advanced gene expression analysis employing cDNA microarrays has recently provided us the means to simultaneously profile the expression of thousands of genes. cDNA microarrays for 8000 human genes revealed that the expression of 100 genes was elevated but that of 279 genes was depressed in AML-2/DX100 compared to AML-2/WT. As shown in Table 1, the major cluster of genes having their mRNA expression significantly increased or decreased in AML-2/DX100 cells are the anti-oxidant and redox genes.

Among 21 anti-oxidant genes, each four gene was up- and down-regulated more than 1.5-fold in AML-2/DX100 compared with AML-2/WT. Among the significantly elevated mRNA expression of the genes reported in the literature to be associated with anti-oxidants were glutathione *S*-transferase Pi (2.11-fold), peroxiredoxin 2 (1.69-fold), thioredoxin 2 (1.59-fold), and glutaredoxin (1.55-fold). Among the significantly depressed gene expression that may play roles in the anti-oxidant mechanisms were peroxiredoxin 3 (3.07-fold), metallothionein-1F (2.06-fold), superoxide dismutase 2 (1.68-fold), and thioredoxin reductase 1 (1.60-fold). In this study, the catalase down-regulation and MRP up-regulation that were confirmed in AML-2/DX100 cells [5,6] were consistent with those results obtained by cDNA microarray analysis, a decrease of catalase mRNA (1.3-fold) and an increase of MRP mRNA (1.88-fold) (Table 1). This consistence provides the microarray expression profiles of other genes with high fidelity. In this study, the four up-regulated gene expressions in AML-2/DX100 appeared to be responsible for their increased scavenging capacity against endogenous ROS.

The depletion of GSH can reverse the chemotherapy-induced resistance and glutathione *S*-transferase can

Table 1
Expression profiles of anti-oxidant genes by cDNA microarray analysis

Gene symbol	Description of genes	Fold change vs. control	Gene function
GSTP1	Glutathione *S*-transferase Pi	+2.11	Glutathione transferase activity
PRDX2	Peroxiredoxin 2	+1.69	Response to oxidative stress
TXN2	Thioredoxin 2	+1.59	Electron transport
GLRX	Glutaredoxin (thioltransferase)	+1.55	Electron transporter activity
EPX	Eosinophil peroxidase	+1.19	Response to oxidative stress
PTGS1	Prostaglandin-endoperoxide synthase 1	+1.12	Peroxidase activity
KIAA0350	KIAA0350 protein	+1.11	Response to oxidative stress
PIP3-E	Phosphoinositide-binding protein PIP3-E	+1.08	Response to oxidative stress
GPX2	Glutathione peroxidase 2 (gastrointestinal)	+1.03	Response to oxidative stress
MPO	Myeloperoxidase	+1.03	Response to oxidative stress
PRDX3	Peroxiredoxin 3	−3.07	Unknown
MT1F	Metallothionein 1F (functional)	−2.36	Metal ion binding
SOD2	Superoxide dismutase 2, mitochondrial	−1.68	Response to oxidative stress
TXNRD1	Thioredoxin reductase 1	−1.60	Electron transport
CAT*	Catalase	−1.34	Response to oxidative stress
TXN	Thioredoxin	−1.26	Electron transporter activity
SOD1	Superoxide dismutase 1	−1.18	Response to oxidative stress
MT2A	Metallothionein 2A	−1.05	Unknown
MT1B	Metallothionein 1B (functional)	−1.04	Metal ion binding
PRDX1	Peroxiredoxin 1	−1.04	Skeletal development; cell proliferation
MT1X	Metallothionein 1X	−1.03	Metal ion binding; response to metal ion
ABCC1*	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	+1.88	Drug resistance

(+) Up-regulation and (−) down-regulation.
* Published [5,6].

play significant roles in chemoresistance [13]. Thioredoxin is a small protein having strong antioxiradical quenching capabilities and other multiple functions as well depending on the cellular redox state. There are two mammalian thioredoxins, thioredoxin 1 that is located in the cytosol and sometimes in the nucleus [14] and thioredoxin 2, which processes a mitochondrial translocation signal that is cleaved after its import into the mitochondria [15]. Thioredoxin undogoes a NADPH dependent reduction that is catalyzed by the flavoprotein thioredoxin reductase. There are three human thioredoxin reductases, the full length isoforms thioredoxin 1 in cytosol and thioredoxin 2 with a mitochondrial import sequence, and the incomplete isoform thioredoxin 3 [16,17]. AML-2/DX100 cells showed that the expression of mitochondrial thioredoxin 2 was increased, whereas that of the cytosolic thioredoxin reductase 1 decreased. It has been reported that thioredoxin protects endothelial cells from the action of H_2O_2, and it protects human leukemia U937 cells from the cytotoxic action of tumor necrosis factor that is mediated by ROS [18,19]. It is not now clear how the thioredoxin 2 and thioredoxin reductase 1 would work with respect to a defense against ROS in the AML-2/DX100 cells. On the other hand, although the thioredoxin-transfected A2780 cells showed a 1.8-fold increase in resistance to H_2O_2, resistance to adriamycin and mitomycin C known to generate ROS, was not observed in the transfectants. This suggests that thioredoxin may be necessary but insufficient to induce resistance against cisplatin, as well as other chemotherapeutic drugs [20].

Peroxiredoxins, also called thioredoxin peroxidases, are members of a newly discovered family of peroxidases, and they efficiently reduced the intracellular level of H_2O_2 produced in those cells stimulated with various cell surface ligands. Peroxiredoxins exert their protective anti-oxidant role in cells through their peroxidase activity ($ROOH + 2e^- \rightarrow ROH + H_2O$), and this is how H_2O_2, peroxynitrate, and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified [21,22]. Six isoforms of peroxiredoxin have been identified in mammalian cells [23]. Peroxiredoxins are thought to play an important role in signaling pathways in which H_2O_2 functions as an intracellular messenger because peroxiredoxins eliminate intracellular H_2O_2 produced by the growth factor thioredoxin as electron donor [21]. Although peroxiredoxin 2, located in the cytosol and nucleus, was approximately 1.7-fold more increased in AML-2/DX100 cells as compared with those of AML-2/WT cells, mitochondrial peroxiredoxin 3 was about 3.1-fold decreased, respectively, resulting in the decreased net pool of peroxiredoxins. This result suggests that the decreased expression of mitochondrial peroxiredoxin 3, as well as catalase, may be mainly responsible for the sensitivity of AML-2/DX100 cells to exogenous pro-oxidants, and especially H_2O_2. On the other hand, the decreased expression of metallothionein-1F (2.06-fold) and mitochondrial Mn-containing superoxide dismutase (SOD 2, 1.68-fold) may make AML-2/DX100 cells more sensitive to superoxide anions generated by paraquat, as compared to that of AML-2/WT cells. It could be hypothesized that AML-2/DX100 cells under oxidative stress resulting from depressed gene expression such as catalase and mitochondrial peroxiredoxin would increase their beneficial genes, such as glutathione *S*-transferase as well as decrease their detrimental genes, such as SOD 2 to generate H_2O_2. Glutaredoxin, a member of a family of thiol-disulfide oxidoreductases, is a ubiquitously expressed small cytosolic protein that acts as a cytoprotective anti-oxidant by catalyzing the reduction of protein disulfide bonds. Considering that H_2O_2 stimulates the expression of glutaredoxin in cultured human coronary vascular smooth muscle cells [24], the increased expression of glutaredoxin in AML-2/DX100 cells would be the result of H_2O_2 being less scavenged by the low level of catalase and mitochondrial peroxiredoxin.

The quinone-containing anticancer drug doxorubicin induces ROS generation following its mitochondrial reductive metabolism [25]. It is well known that the specific cardiotoxicity of doxorubicin is due to the abundance of mitochondria and the low levels of the anti-oxidant enzymes such as glutathione peroxidase and catalase in heart cells [26] and the calcium release from the sarcoplasmic reticulum [27]. Thus, AML-2/DX100 cells resemble the heart cells, as both are vulnerable to H_2O_2 because of the lower glutathione peroxidase and catalase levels of the mitochondria. Therefore, AML-2/DX100 cells can be exploited in the laboratory as an experimental model for the ROS-induced cardiotoxicity.

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