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Down-Regulation of Catalase Gene Expression in the Doxorubicin-Resistant AML Subline AML-2/DX100[1]

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A major obstacle to successful cancer chemotherapy is the development of multidrug resistance (MDR). The previous study revealed that a doxorubicin-resistant AML subline (AML-2/DX100) overexpressed an MDR-associated protein (MRP) but not P-glycoprotein. The AML-2/DX100 also showed various levels of resistance to daunorubicin and vincristine but was paradoxically sensitive to hydrogen peroxide (5-fold), t-butyl hydroperoxide (3-fold), and paraquat (2-fold) when compared to the drug-sensitive parental AML-2 cells (AML-2/WT). We compared the activities of antioxidant enzymes to detoxify reactive oxygen species (ROS), including superoxide dismutases, glutathione *S*-transferase, catalase, glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase in both AML-2/WT and AML-2/DX100. Interestingly, of these antioxidant enzymes, catalase activity of AML-2/DX100 decreased significantly to about one-third that of AML-2/WT ($P < 0.000005$). The decreased activity of catalase was due to reduced expression of the catalase gene; confirmed by Western blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses. The decreased activity of catalase was maintained even in the absence of doxorubicin for 3 months as well as by the treatment of probenecid, an MRP inhibitor. In addition, there was no difference in catalase activity between HL-60 and another MRP-overexpressing subline HL-60/Adr. Taken together, the paradoxical increase in the sensitivity of an MRP-overexpressing AML-2/DX100 in response to peroxides and paraquat is due to the down-regulation of catalase gene expression, which totally independent of overexpression of MRP. It is therefore possible that decreased catalase activity could be exploited as an Achilles' heel in resistant cells such as this. © 2001 Academic Press

A major obstacle to successful cancer chemotherapy is the acquisition of MDR by tumor cells. AML has still shown a poor prognosis due, at least in part, to the development of MDR. Anthracyline antibiotics are one of the clinically important anticancer drugs used for treating AML patients. Anthracyclines have also been known to show cytotoxicity by generating ROS, although their poisoning of DNA topoisomerase II constitutes their major antitumor action (1). It is therefore possible that resistance to an anthracycline doxorubicin could be associated with increased intracellular enzyme capacity to convert ROS into inactive metabolites. In a previous study, doxorubicin-resistant AML-2 subline (AML-2/DX100) were selected by chronic exposure to 100 ng/ml (5-fold of IC_{50} value) of doxorubicin (2). The AML-2/DX100 overexpressed MRP. This subline showed cross-resistance to doxorubicin (24-fold), daunorubicin (18-fold) and vincristine (2-fold) as compared to the AML-2/WT but not to paclitaxel. However, AML-2/DX100 sublines were paradoxically more sensitive to hydrogen peroxide, t-butyl hydroperoxide and paraquat as compared to the AML-2/WT. This study was undertaken to examine the underlying mechanisms responsible for chemosensitivity to these prooxidants. Antioxidant substances capable of detoxifying ROS, including superoxide dismutases, glutathione S-transferase, catalase, glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase were measured in both AML-2/WT and the AML-2/DX100. Interestingly, catalase activity measured in the AML-2/DX100 was about one-third that observed in AML-2/WT.

MATERIALS AND METHODS

Culture and selection for resistance to doxorubicin. The OCI-AML-2 line, obtained from the Ontario Cancer Institute (Toronto,

Abbreviations used: MDR, multidrug resistance; AML, acute myelogenous leukemia; MRP, multidrug resistance-associated protein; WT, wild type; ROS, reactive oxygen species; SOD, superoxide dismutase; RT-PCR, reverse transcription-polymerase chain reaction.

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Canada), was cultured at 37°C in a 5% CO_2 atmosphere using α-MEM medium (Gibco) with 10% heat inactivated fetal bovine serum (Sigma). The cells were maintained as a suspension culture, and subcultured. The doxorubicin-resistant AML-2 sublines were selected from the AML-2/WT by chronic exposure to doxorubicin on an intermittent dosage schedule at sufficient intervals to permit the expression of resistance phenotypes. Doxorubicin was initially administered from 1 × IC_{50}, increased at 50% increments, and then finally cultured at a fixed concentration of doxorubicin (100 ng/ml) (1). The HL-60 and its doxorubicin-resistant subline HL-60/Adr were both generously supplied by Dr. Centor (Kansas State University, U.S.A.). The latter was used as an MRP-overexpressing cell (3).

Cytotoxicity assay. The *in vitro* cytotoxicity of drugs was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay described by Pieters *et al.* (4). IC_{50} values were determined directly from semilogarithmic dose-response curves. Experiments were carried out, at least, in triplicate.

Activities of antioxidant enzymes. Cellular lysates for assays of catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and glucose-6-phosphate dehydrogenase activity, were prepared by washing with phosphate buffered saline (pH 7.4), then disrupting the cells in 50 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 0.2% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride by sonication. Unlysed cells and organelles were sedimented by centrifugation at 12,000 rpm for 15 min at 4°C using a microfuge. The activity staining for SOD was used in this study. Enzyme samples were separated on nondenaturing discontinuing polyacrylamide gels (4.3% stacking gel; 7% separating gel) with the SOD activity visualized on the gels using the *in situ* staining technique described by Beauchamp and Fridovich (5). Glutathione peroxidase was assayed spectrophotometrically at 340 nm of 1 mM reduced glutathione to oxidized glutathione at the expense of 150 μM NADPH by glutathione reductase 0.24 U/ml in 100 mM potassium phosphate buffer (pH 7.0) at 37°C (6). Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically by measuring the increase in absorbance at 340 nm as 200 μM NADP is reduced enzymatically at 25°C in 100 mM tris-HCl (pH 8.0) with the electrons derived from 0.83 mM glucose-6-phosphate (7). Glutathione reductase was assayed by recording the reduction in absorbance at 30°C at 340 nm as 100 μM NADPH is oxidized in the presence of 1 mM oxidized glutathione in 100 mM potassium phosphate buffer (pH 7.0) (8). Glutathione S-transferase was assayed spectrophotometrically at 340 nm by measuring the formation of the conjugate in the presence of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in 100 mM potassium phosphate buffer (pH 6.5) (9). Catalase was assayed spectrophotometrically by observing the catalase-induced decrease in absorbance values of 30 mM H_2O_2 at 240 nm at 25°C in 50 mM potassium phosphate buffer (pH 7.0) (10). For the activity staining of catalase, enzyme samples were separated on a nondenaturing discontinuing polyacrylamide gel (4.3% stacking gel; 7% separating gel) with catalase activity visualized on the gel using the *in situ* staining technique (11). The technique is based on local elevation of pO_2 by catalytic decomposition of H_2O_2, which in turn, inhibits the reduction of the tetrazolium by superoxide. Briefly, this method involves soaking the gels in horseradish peroxidase (50 μg/ml) in the 50 mM phosphate buffer (pH 7.0) for 45 min. H_2O_2 was then added to a concentration of 5.0 mM and gels were soaked for an additional 10 min. This was followed by rinsing twice with distilled water and subsequent soaking in 0.5 mg/ml diaminobenzidine in 50 mM phosphate buffer (pH 7.0) until staining was completed.

Protein extraction and Western blot analysis. Total cell lysates were prepared by lysing harvested cells in extraction buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) supplemented with 2 mM phenylmethylsulfonyl fluoride (Sigma) and 10 μg/ml leupeptin (Sigma). DNA was sheared by sonication and Western blotting analysis was performed using a slight modification of the method first described by Towbin *et al.* (12). Proteins were transferred onto a nitrocellulose membrane by electroblotting using a current of 60 V overnight. The membrane was incubated in blocking solution (5% skim milk) for 1 hr at room temperature, washed, and then incubated with primary mouse monoclonal antibody (1:1000, Biodesign) for catalase. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1:1000) against each IgG for hosts of primary antibodies for 1 h. The membrane was then stained using the detection reagent of the ECL detection kit (Amersham, USA).

RNA extraction and RT-PCR assay. Total RNA was extracted from the cells using the acid guanidium thiocyanate-phenol-chloroform method (13). mRNA transcripts for MRP, catalase, and *β-actin* were detected using an RT-PCR assay. MRP expression was detected with 5′ and 3′ primers corresponding to nucleotides 4180–4197 (5′-GACGGGAGCTGGGAAGTC) and 4551–4568 (3′-ACAACCTACTCCGGTGCC), respectively, of the published cDNA sequence (14), and yielded a 389-bp PCR product. Catalase expression was detected with 5′ and 3′ primers corresponding to nucleotides 314–333 (5′-TTTGGCTACTTTGAGGTCAC) and 734–753 (3′-TCCCCATTTGCATTAACCAG), respectively, of the published cDNA sequence (15), and yielded a 440-bp PCR product. *β-actin* expression, used as a control of the amount of RNA, was detected with 5′ and 3′ primers corresponding to nucleotides 1912–1932 (5′-GACTATGACTTAGTTGCGTTA) and 2392–2412 (3′-GTTGAACTCTCTACATACTTCCG), respectively, of the published cDNA sequence (16), yielding a 501-bp PCR product. RNA from each sample was reverse transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesta Research Laboratories) and oligo (dT) primer for 1 h at 37°C. The resulting cDNA was diluted in the ratio 1:5 with water and then was amplified using 2.5 units of *Taq* polymerase (Perkin-Elmer, U.S.A.) and 10 pmole of each primer in a GeneAmp PCR2400 (Perkin-Elmer-Cetus) for 21 cycles (16 cycles for *β-actin*) of sequential denaturation (at 95°C for 30 s), annealing (at 53°C for 30 s), and extension (at 72°C for 30 s). After the last cycle, all PCR products were subjected to final extension for 5 min at 72°C. PCR products were quantified by adding 5 μCi of [α-^{32}P]dCTP to each reaction mixture. The PCR products were combined and then electrophoresed on 7% nondenaturing polyacrylamide gels. The amounts of each mRNA transcript were then normalized using *β-actin* mRNA. Autoradiographic films of the RT-PCR assay were subjected to densitometric analysis using a densitometer (Pdi, U.S.A.).

Protein determination. Protein concentrations were determined using the Bradford protein assay with a Bio-Rad kit and standardized with bovine serum albumin.

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

RESULTS

Cross-Resistance of AML-2/DX100 to Various Cytotoxic Substances

The AML-2/DX100 overexpressed MRP, but not P-glycoprotein and showed a multidrug resistant phenotype (1). Paradoxically however, the AML-2/DX100 was more sensitive to hydrogen peroxide (5-fold), t-butyl hydroperoxide (3-fold) and paraquat (2-fold) when compared to the AML-2/WT (Fig. 1).

Comparison of Cellular Levels of Antioxidants between AML-2/WT and AML-2/DX100

We compared activities of antioxidant enzymes to detoxify ROS, including superoxide dismutases, gluta-

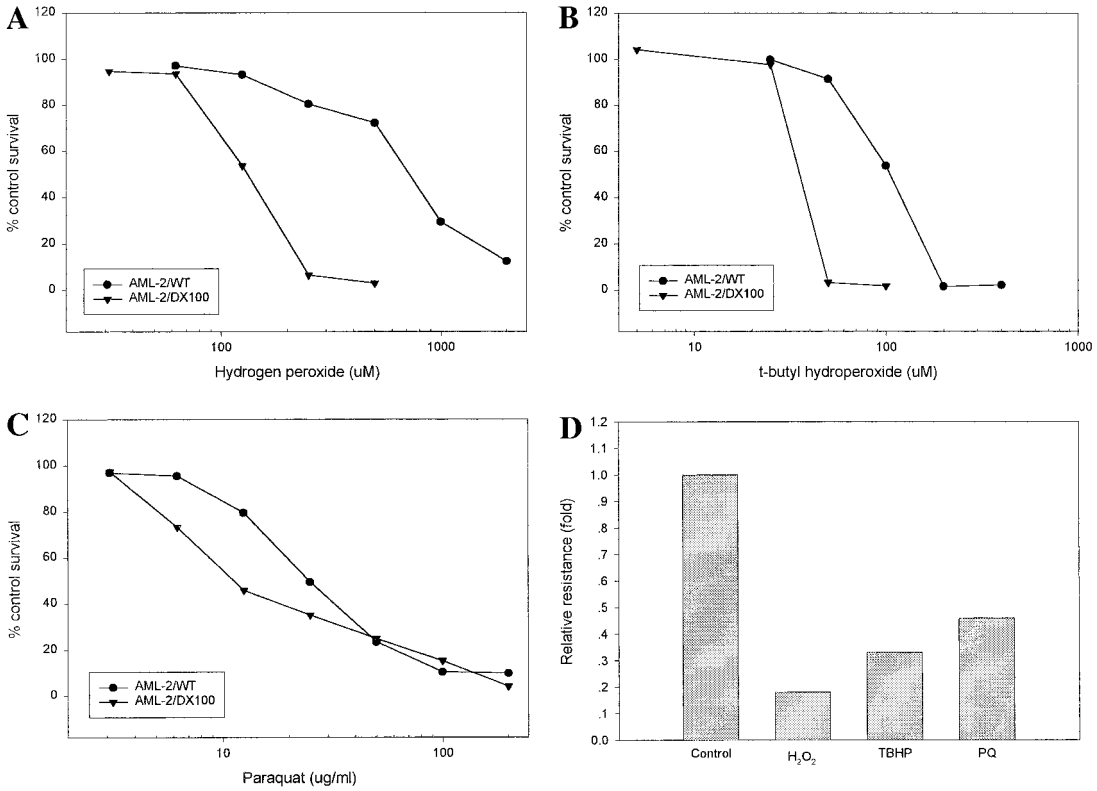

FIG. 1. Sensitivity of AML-2/WT and AML-2/DX100 to hydrogen peroxide, t-butyl hydroperoxide, and paraquat. Relative resistance was calculated as the ratio of drug concentrations (IC_{50}) which inhibit 50% growth of the cells. H_2O_2, hydrogen peroxide; TBHP, t-butyl hydroperoxide; PQ, paraquat.

thione S-transferase, catalase, glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase, in both the AML-2/WT and the AML-2/DX100. Interestingly, in the spectrophotometric assays of these antioxidants, catalase activity of the AML-2/DX100 decreased significantly to about one-third that

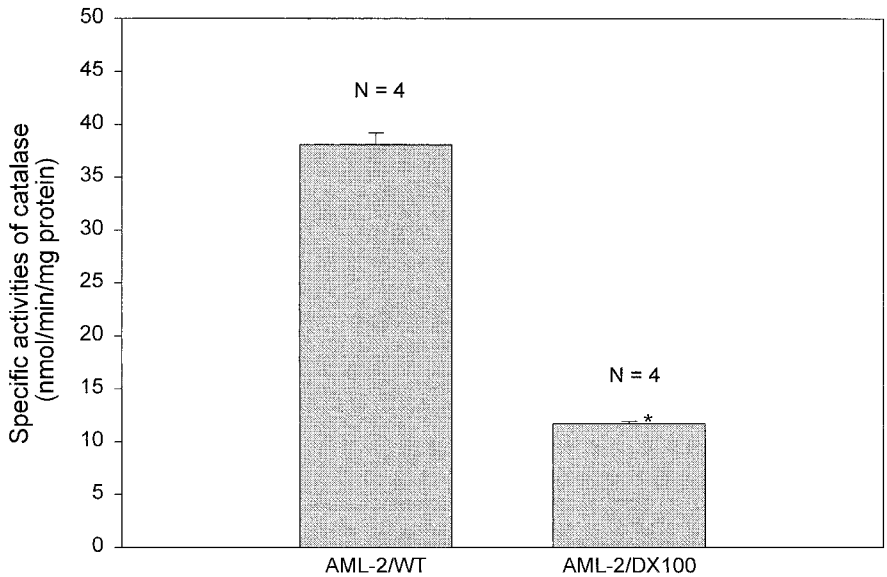

FIG. 2. Catalase activity in AML-2/WT and AML-2/DX100. Catalase activity was spectrophotometrically determined as described under Materials and Methods. N, the number of separate samples: *, statistically significant ($P < 0.000005$).

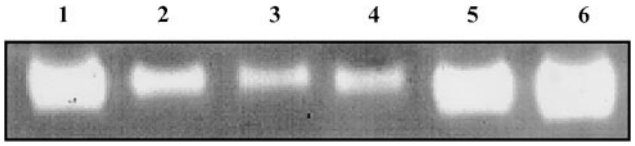

FIG. 3. Relationship of decreased catalase activity and overexpression of MRP. 10 μg of total cellular protein was separated in a 7% nondenaturing polyacrylamide gel. Activity staining for catalase was carried out as described under Materials and Methods. Lane 1, AML-2/WT; lane 2, AML-2/DX100 (an MRP-overexpressing cell); lane 3, AML-2/DX100 in the absence of doxorubicin for 3 months; lane 4, AML-2/DX100 treated with 600 μM probenecid; lane 5, HL-60/WT; lane 6, HL-60/Adr (another MRP-overexpressing cell).

of AML-2/WT ($P < 0.000005$) (Fig. 2); confirmed again by activity staining for catalase (Fig. 3). In addition, the decreased catalase activity was maintained even in the absence of doxorubicin for 3 months (lane 3 in Fig. 3). It can therefore be hypothesized that the decreased catalase activity might have something to do with the overexpression of MRP. To investigate the relationship between decreased catalase activity and the overexpression of MRP, we tested another MRP-overexpressing leukemic subline, HL-60/Adr, in terms of catalase activity as well as examining catalase activity in AML-2/DX100 selected after treatment of an MRP inhibitor in the presence of doxorubicin. No difference was observed in the catalase activity of HL-60 and its MRP-overexpressing subline HL-60/Adr (lanes 5 and 6 in Fig. 3). In addition, decreased catalase activity was maintained even in the absence of doxorubicin for 3 months as well as by the treatment of probenecid, an MRP inhibitor (lanes 3 and 4 in Fig. 3). The decreased catalase activity in AML-2/DX100 was due to the decreased amount of catalase protein (Fig. 4) as measured by Western blot analysis. Densitometric analysis showed that catalase protein levels decreased about 1.5-fold in AML-2/DX100. The steady-state level of catalase mRNA measured using the RT-PCR assay in the same cell lines also decreased in AML-2/DX100 as shown in Western data (Fig. 4).

DISCUSSION

Many anticancer drugs such as anthracycline antibiotics, mitomycin C, nitrogen mustards, ionizing radiation, and cisplatin have been suggested to cause oxidative stress, and repeated treatment with these agents is associated with the acquisition of cellular resistance to drugs as well as gene amplification (17–19). It has been proposed that the cytotoxic effects of doxorubicin is mediated through the formation of intracellular superoxide radicals. Superoxide production by doxorubicin was accompanied by the accumulation of hydrogen peroxide, and probably resulted from the transfer of electron to molecular oxygen by the doxorubicin semquinone after reduction of the drug by NADPH:cytochrome P-450 reductase (20). The gene amplification of antioxidant enzymes seems to be one mechanism whereby cells can acquire resistance to these chemotherapeutic agents (21). Chronic exposure (>200 days) of HA1 fibroblasts to increasing concentrations of H_2O_2, or O_2, results in the development of a stable oxidative stress-resistant phenotype characterized by increased cellular antioxidants including glutathione, glutathione reductase activity, glutathione peroxidase activity, superoxide dismutase activity and catalase activity (22–24). In H_2O_2- and O_2-resistant cells, catalase activity was found to be 20 ~ 30-fold higher than that observed in the parental HA1 cells and correlated with increased amounts of catalase protein and catalase mRNA levels by gene amplification (25). Compared to acute exposure, chronic exposure is particularly effective in the development of a stable and resistant phenotypes with increased catalase activity.

We found that a doxorubicin-resistant AML subline (AML-2/DX100) overexpressing the MRP (1), paradoxically exhibited increased sensitivity to prooxidants such as peroxides and paraquat. Provided that we are able to find a hidden target in the resistant cell, the potential exists for us to exploit this as an Achilles' heel in the resistant cells. Nonetheless, the increased sen-

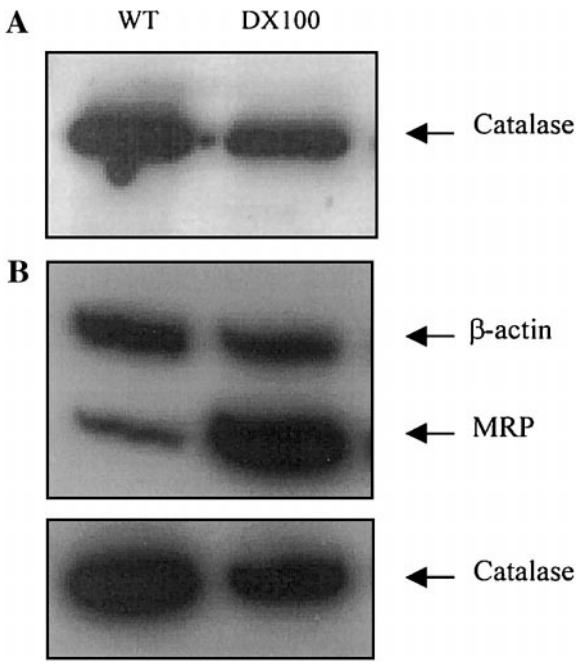

FIG. 4. Catalase gene expression in AML-2/WT and AML-2/DX100. 25 μg of total cellular protein was separated on a 12% SDS/polyacrylamide gel. Western blot analysis (A) was performed as described under Materials and Methods. Catalase mRNA levels were determined by the RT-PCR assay (B). The cDNAs reverse-transcribed from RNA were separately amplified with each primer pair for catalase and *β-actin* genes. Aliquots of each PCR reaction mixture were separated on a 7% polyacrylamide gel in Tris–acetate–EDTA. The gel was dried and exposed on X-ray film overnight.

sitivity of resistant cells to cytotoxic drugs has not been focused in the field of drug resistance. So far, there have only been two reports of decreased activity of catalase in doxorubicin-resistant leukemic sublines (26, 27). Doxorubicin-resistant P388 cells have 1.5 times the glutathion content, and 1.5 times the activity, of glutathione peroxidase measured in drug-sensitive P388 cells. No significant differences in superoxide dismutase activity between these cell lines was observed. In contrast, catalase activity measured in drug-resistant P388 cells was one-third of the activity measured in doxorubicin-sensitive P388 cells. Doxorubicin-resistant P388 cells were significantly more sensitive to X-irradiation than were drug-sensitive P388 cells (26). In doxorubicin-resistant Friend leukemic cells, the activities of the superoxide dismutase, glutathione S-transferase and glutathione reductase were higher by factors of 24, 15 and 38% respectively than when compared to their sensitive counterparts. In resistant cells however, catalase and glutathione peroxidase were reduced by 18 and 21% respectively (27). In addition to catalase, glutathione S-transferase was decreased in hemoharrintonine-selected K562 human leukemic sublines (28).

The underlying mechanism by which the AML-2/DX100 was paradoxically sensitive to prooxidants was the decreased activity of catalase resulting from the decreased gene expression. These phenomena were maintained even in the absence of doxorubicin for 3 months, suggesting that doxorubicin is not indispensable in the maintenance of decreased catalase gene expression. Thus far, the molecular mechanisms of the marked decrease in catalase activity have been investigated in some laboratories. The reduction of liver catalase activity in the livers of tumor-bearing mice has been demonstrated to be positively associated with tumor size, being restored to the normal levels after removal of the tumor. This suggests that catalase expression may be influenced by certain humoral factor(s) from the transplanted tumor (29). The decrease in catalase activity in hepatoma cells was due to negative regulation of catalase gene transcription by several cis-acting elements (30). The down regulation of catalase gene expression seen in transformed lines may occur transcriptionally rather than posttranscriptionally. It is unlikely that the striking difference in catalase gene expression observed between liver tissue and liver cells was attributed to gross structural alterations in the catalase gene, but might instead be explained by a remarkable difference in methylation status of the catalase gene (31). The decrease in catalase activity observed during SV40 transformation in human fibroblasts is related to posttranscriptional changes of regulation at early passages and to the loss of the 11p chromosome arm at later passages (32). Future work should therefore be undertaken to assess the underlying molecular mechanisms by which catalase gene expression decreased in AML-2/DX100.

Given that MRP is overexpressed in AML-2/DX100, it could be hypothesized that decreased catalase activity could be attributed to excess MRP which could pump out substances, such as inducer, from the cell. This hypothesis could be supported given that MRP has been demonstrated to be capable of transporting endogenous intracellular substances, including cystenyl leukotrience C4 and other some GSH conjugates (33, 34). However, no change in catalase activity was observed after treatment of probenecid (an MRP inhibitor). Furthermore, there was no difference in catalase activity between HL-60 and its MRP-overexpressing subline HL-60/Adr, indicating the irrelevance of MRP in decreasing catalase activity or the cell-to-cell variation.

In conclusion, the paradoxical increase in sensitivity of MRP-overexpressing AML-2/DX100 to peroxides and paraquat is due to the down-regulation of catalase gene expression, which has nothing to do with the overexpression of MRP. This could imply that the decreased activity of catalase could be exploited as an Achilles' heel of resistant cells such as this.

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