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ORIGINAL ARTICLE

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Lack of correlation of MRP and γ-glutamylcysteine synthetase overexpression with doxorubicin resistance due to increased apoptosis in SV40 large T-antigen-transformed human mesothelial cells

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Abstract *Purpose*: Evidence suggests that viral proteins such as simian virus large T-antigen (SV40 TAg) play a role in the response of cancer cells to chemotherapeutic agents. In this study, we investigated whether SV40 TAg-immortalized human mesothelial cells express drug resistance-related proteins and display resistance to chemotherapy, and whether SV40 TAg transformation affects apoptosis. *Methods*: We determined the mRNA and protein levels of the multidrug resistance-associated protein (*MRP*), γ-glutamylcysteine synthetase heavy subunit (γ-GCS_h), and P-glycoprotein (product of the *MDR1* gene) by RT-PCR and Western blotting, respectively, in normal human mesothelial (NHM) cell and SV40 TAg-transformed human mesothelial (Met-5A) cells. The effect of increasing concentrations of doxorubicin (DOX) on these cells was investigated using an MTT cytotoxicity assay, and the glutathione (GSH) content was measured spectrophotometrically. DOX accumulation in these cells was measured by treating the cells with [^{14}C]DOX followed by scintillation counting. Cytoplasmic DNA fragmentation due to apoptosis following DOX treatment of the cells was quantitated by ELISA. *Results*: We showed that the MRP and γ-GCS_h genes, but not *MDR1*, are coordinately overexpressed in Met-5A cells compared with NHM cells. Expression of MRP protein as detected by an anti-MRP antibody correlated with increased GSH levels and decreased accumulation of [^{14}C]DOX in Met-5A cells compared with NHM cells. However, Met-5A cells were twofold more sensitive to DOX than NHM cells. In addition, quantitative measurement of apoptosis when cells were treated with 0.05 and 0.5 μ*M* DOX revealed that drug-induced apoptotic cell death was increased about 1.4- and 3.0-fold, respectively, in Met-5A cells compared with NHM cells. *Conclusions*: These results suggest that increased levels of apoptosis might help overcome the DOX resistance effects of *MRP*/γ-GCS_h overexpression in SV40 TAg-transformed Met-5A cells.

Key words *MRP* · γ-*GCS* · Apoptosis · SV40 large T-Antigen · Human mesothelial cells

Introduction

The steady increase in the incidence of mesothelioma over the past 30 years has been attributed to asbestos exposure [1, 2]. It has been recently hypothesized that the monkey oncogenic virus simian virus 40 (SV40), which was introduced into a large cohort of the human population through a contaminated polio vaccine given between 1959 and 1961, also might have played a role in the increasing incidence of mesothelioma [2–4]. Support for this hypothesis has come from several recent reports [2, 5–7]. Juvenile Syrian hamsters inoculated with SV40 virus develop mesothelioma with an incidence of 100% [8]. However, SV40 is not normally infective in humans, although it can transform human cell lines in tissue culture [9]. Recent data suggest that expression of SV40 large Tantigen (TAg) in some cell lines affects their response to chemotherapeutic agents [10, 11]. Therefore, we determined whether the expression of SV40 TAg in human mesothelial cells induces chemotherapeutic drug resistance proteins, including the multidrug resistance-associated protein (MRP), P-glycoprotein (P-gp) and

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γ-glutamylcysteine synthetase (γ-GCS) heavy subunit (γ-*GCS$_h$*), and whether SV40 TAg expression affects the rate of drug-induced apoptotic cell death in these cells.

Transfection with either *MDR1* cDNA encoding P-gp or MRP cDNA has been shown to cause multidrug resistance (MDR) in sensitive cancer cells by increasing drug efflux [12, 13]. MRP-overexpressing cells display enhanced ATP-dependent glutathione (GSH) S-conjugate export (GS-X) pump activity [14-16]. This export pump is an ATP-dependent transporter for multivalent organic anions, including cysteinyl leukotrienes, GSH conjugates, GSH disulfide, and glucuronide and sulfate conjugates [14, 15]. Much evidence suggests that MRP/GS-X export pump function is linked with cellular GSH content [15, 16]. Increased cellular GSH has long been associated with tumor cell resistance to alkylating agents, cisplatin and certain anthracyclines [17]. Cellular GSH is synthesized by two enzymes, γ-GCS and GSH synthetase [17]. γ-GCS is the rate-limiting enzyme in de novo synthesis of GSH via the γ-glutamyl cycle, and cellular GSH is substantially regulated by this enzyme [17]. γ-GCSconsists of one heavy subunit (γ-*GCS$_h$*, 73 kDa) and one light subunit (γ-GCS$_l$, 28 kDa) [18-20]. γ-GCS$_h$ possesses the catalytic function, while γ-GCS$_l$ has a regulatory role in controlling the kinetic properties of γ-GCS$_h$ [18]. Transfection using expression vectors containing cDNAs encoding both the heavy and the light subunit [19] or only the heavy subunit [20] results in resistance to melphalan [19] and cisplatin [20], respectively. Overexpression of γ-GCS$_h$ following transfection increases the intracellular GSH content, which in turn leads to an increased amount of conjugated substrates for MRP, and ultimately results in lower intracellular drug concentrations.

In the study reported here, we showed that despite the overexpression of *MRP*/γ-*GCS*, increased GSH levels and lower intracellular doxorubicin (DOX) accumulation, SV40 TAg-transformed human mesothelial (Met-5A) cells were more sensitive to DOX than normal human mesothelioma (NHM) cells. Moreover, in an attempt to determine whether the increased level of apoptotic cell death is involved in DOX sensitivity, the cytoplasmic DNA-histone complexes in NHM and Met-5A cells were quantitated after treatment in the absence or presence of DOX. We believe that this study provides a useful in vitro model to evaluate the relationship between *MRP*/γ-GCS h overexpression and the levels of apoptosis in determining the drug resistance characteristics in human mesothelial cells.

Materials and methods

Cell lines and culture conditions

NHM cells were isolated from the transudative pleural effusions of three congestive heart failure patients, as described elsewhere [21]; primary cultures at passage 2–3 were used. Met-5A cells [22] were obtained from Dr. Curtis C. Harris (National Institutes of Health, Bethesda, Md.). The above cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM/F12) containing 15% fetal calf serum and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY) at 37 °C in an atmosphere containing 5% CO_2. The human breast cancer cell line MCF-7 and its MDR variant MCF-7/Adr were obtained from Dr. Kenneth Cowan (National Cancer Institute, Bethesda, Md.) and were maintained as previously described [23].

RT-PCR and PCR conditions

The mRNA levels of *MRP,* γ-*GCSand MDR1* were detected by RT-PCR as previously described [23]. In brief, total cellular RNA (1 μg) isolated by a modified SDS/phenol method was used in reverse-transcription (RT) reactions using AMV-reverse transcriptase as instructed by the manufacturer (Promega, Madison, Wis.), and then 2–3 μl of the resulting RT-cDNA sample was used in hot-start PCR [23, 24]. The DNA levels of MRP were detected by hot-start PCR using 70 ng genomic DNA samples as templates, isolated from NHM and Met-5A cells as described [23, 24]. Primers and amplification profiles were as follows: MRP (forward) 5′-TGAAGGACTTCGTGTCAGCC; MRP (reverse) 5′-CGTC CATGATGGTGTTGAGCC; γ-*GCS$_h$* (forward) 5′-GCTGCAT CTCCCTTTTACCGAG; γ-*GCS$_h$* (reverse) 5′-TGGCAACTGT-CA TTAGTTCTCCAG; *MDR1* (forward) 5′-CCCATCATTG-CAAT AGCAGG; *MDR1* (reverse) 5′-GTTCAAACTTCTGCTC CTGA.

The amplification reactions were performed at 95 °C for 45 s, 60 °C for 45 s and 72 °C for 2 min for 30 cycles. The cDNA of 28S rRNA levels were used as internal controls in RT-PCR analysis using the following primers: rRNA (forward) 5′-TTACCAAAA GTGGCCCACTA and rRNA (reverse) 5′-GAAAGATGGTGAA CTATGCC. The rRNA primers were used at a ratio of 1:6 to the other primer concentrations in PCR to achieve linear amplification conditions. The amplified fragments were then separated on 2% agarose gels and visualized by ethidium bromide (EtBr) staining. The cDNA levels corresponding to the mRNA levels of MRP and γ-GCSor rRNA on agarose gels were quantitated by densitometry using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, Md.).

Western blot analysis

Total cellular protein levels of MRP were measured by Western blotting of an Immobilon membrane containing 100 μg/lane proteins separated by 5–15% SDS-PAGE with 10 *M* urea, using the rabbit polyclonal anti-MRP antibody, 6KQ [25], at a dilution of 1:4000 (v/v) and a secondary peroxidase-conjugated antirabbit antibody at a dilution of 1:2000 (v/v) as described elsewhere [23]. Equal loading of the proteins was confirmed by Coomassie blue staining of the SDS-PAGE strips of the gels prior to blotting. The protein levels were detected using an ECL protein detection kit (Amersham, Arlington Heights, Ill.) as instructed by the manufacturer. The protein levels of MRP on Western blots were quantitated by densitometry as described above.

Cell survival (MTT) assay

The effects of increasing concentrations of DOX on the survival of NHM and Met-5A cells were detected using an MTT cell survival assay as previously described [26]. Cells (1×10^4 cells/well) were plated into 96-well plates and grown at 37 °C in an atmosphere containing 5% CO_2 for 96 h in 100 μl growth medium in the absence or presence of increasing concentrations of DOX. The cells were then incubated with 25 μl 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at 37 °C for 4 h, and then were lysed in 100 μl lysis buffer [26] at 37 °C for 4–6 h. After reading the plates in a microplate reader (Dynatech Laboratories, Chantilly, Va.) at 570 nm, the drug concentrations that inhibited cell survival by 50% (IC_{50}) compared to untreated cells were determined from the cell survival plots.

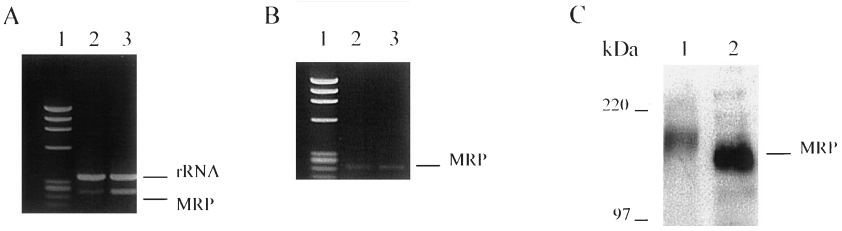

Fig. 1A–C Detection of MRP expression in NHM and Met-5A cells. **A** The mRNA levels of MRP in NHM and Met-5A cells (*lanes 2 and 3*, respectively) were measured by RT-PCR as described in Materials and methods. The 237-bp MRP cDNA and 345-bp rRNA fragments (used as internal controls) were resolved on 2% agarose gels and visualized by ethidium bromide (EtBr) staining. **B** The MRP DNA levels were measured by PCR using 80 ng genomic DNA samples from NHM and Met-5A (*lanes 2 and 3*, respectively). The 237-bp MRP DNA fragments amplified by RT-PCR were separated on 2% agarose gels and visualized by EtBr staining (*Lanes 1* in **A** and **B** contain HaeIII-cut ΦX174 DNA fragments used as molecular weight markers). **C** The protein levels of MRP in NHM and Met-5A cells were measured by Western blot analysis using the rabbit polyclonal antibody 6KQ [24] as described in the Materials and methods. The results shown are representative of at least two independent experiments

Measurement of cellular GSH levels

Cells at 70–80% confluence (about $3–5 \times 10^6$ cells) were washed with phosphate-buffered saline (PBS) and counted using a Coulter counter (Coulter Electronics, Hialeah, Fl.). After the cells were lysed with 300 µl 5% trichloroacetic acid, they were centrifuged at 10 000 rpm for 2 min. The resulting supernatant was then neutralized with 600 µl neutralization solution containing 78% trichlorotrifluoroethane and 22% tri-*n*-octylamine. GSH levels in the aqueous phase of the neutralized samples were then quantitated using the GSH-reductase method of Tietze [27] by determining the rate of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) formation spectrophotometrically at 412 nm. GSH concentrations were determined by comparison with the standard curve and expressed as nanomoles per 10^6 cells as described previously [28].

Measurement of drug accumulation

The cellular accumulation of [^{14}C]-labeled DOX in NHM and Met-5A cells was measured as described elsewhere [29]. Log-phase cultures (1×10^5 cells/well) grown in a 24-well plate were incubated with 0.5 µ*M* (60 Ci/mmol) [^{14}C]DOX (Amersham, Arlington Heights, Ill.) in 0.5 ml of the growth medium at 37 °C in an atmosphere containing 5% CO_2 for 90 min. After washing the cells three times with ice-cold Dulbecco's PBS, pH 7.1 (Life Technologies, Grand Island, N.Y.), they were trypsinized and the cellular [^{14}C]DOX content was measured by scintillation counting. For each cell line, triplicate wells per assay were used in two independent experiments. The cellular [^{14}C]DOX content was then normalized to the number of cells per well in each assay.

Apoptotic cell death

DNA fragmentation due to apoptosis was quantitated by detecting cytoplasmic DNA-histone complexes using a Cell Death ELISA kit (Boehringer Mannheim, Indianapolis, Ind.) as previously described [24]. In summary, cells (1×10^5 cells/well) were plated in 24-well plates and grown in 1 ml growth medium in the absence or presence of 0.05 or 0.5 µ*M* DOX for 96 h as previously described. Both viable and nonviable cells were then collected and counted using a Coulter counter. After lysing the cells, the supernatant containing the cytoplasmic fraction was recovered and assayed for DNA fragmentation using an ELISA as instructed by the manufacturer. Each experiment was performed in triplicate in two independent experiments. The A_{405} readings obtained from the ELISA were normalized for cell number and the results are expressed relative to untreated cells.

Results

MRP expression levels in NHM and Met-5A cells

To determine the levels of MRP mRNA, intact total RNA (intactness of RNA was confirmed by the tight bands of 28S and 18S rRNAs in each sample separated in denaturing agarose gels) was isolated from NHM and Met-5A cells using a modified SDS/phenol method [24] followed by RT-PCR as described in Materials and methods. As shown in Fig. 1A, overexpression of MRP mRNA (about three-fold) in Met-5A cells (Fig. 1A, lane 3) was detected as compared with the expression in NHM cells (Fig. 1A, lane 2) when normalized to their rRNA levels that were used as internal controls (Fig. 1A, upper band in lanes 3 and 2). In order to determine whether the overexpression of MRP mRNA in Met-5A cells was due to the amplification of the gene or was regulated at the transcriptional level, we amplified a 237-bp *MRP*-specific DNA fragment by PCR using genomic DNAs from NHM and Met-5A cells as templates. As Fig. 1B shows, the genomic MRP levels were similar in both cell lines (lanes 2 and 3), suggesting that its overexpression was mainly controlled at the transcriptional or posttranscriptional level and was not due to gene amplification in Met-5A cells. Overexpression of MRP was also detected at the protein level in Met-5A cells compared with the expression in NHM cells (Fig. 1C, lanes 2 and 1, respectively) by Western blot analysis using the rabbit polyclonal anti-MRP antibody 6KQ as described in Materials and methods. In Fig. 1C, a slight variation in the molecular weight of MRP in NHM cells and Met-5A cells is evident; the reason for this variation is unknown.

The mRNA levels of *MDR1* and γ-GCS_h and cellular GSH levels in NHM and Met-5A cells

We determined whether *MDR1* was expressed in NHM and Met-5A cells by RT-PCR as described earlier. *MDR1* mRNA expression was not detectable in either NHM cells or Met-5A cells (Fig. 2A, lanes 2 and 3), whereas the RT-PCR product, a 167-bp *MDR1*-specific

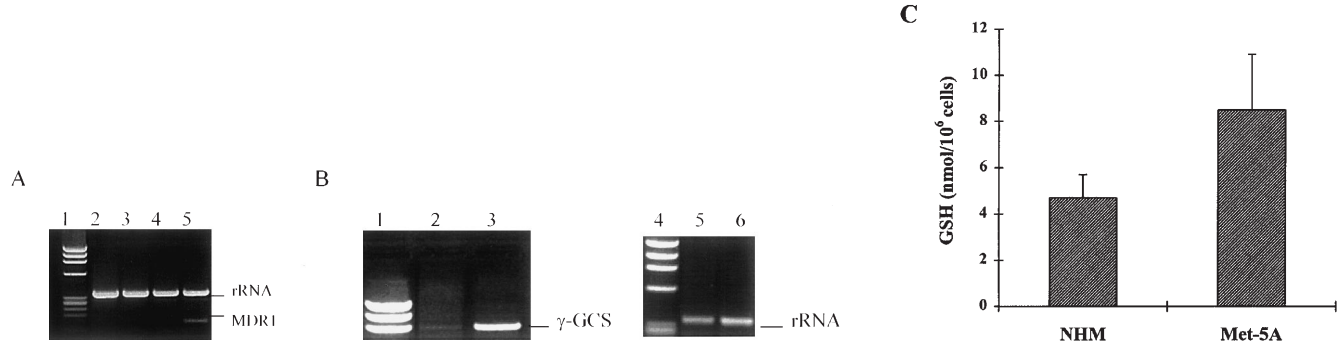

Fig. 2A–C Determination of mRNA levels of *MDR1* and γ-GCS_h and cellular GSH levels in NHM and Met-5A cells. The mRNA levels of *MDR1* (**A**) and γ-GCS_h (**B**) in NHM and Met-5A cells (*lanes 2 and 3*, respectively) were measured by RT-PCR as described in Materials and methods. The amplified 880-bp γ-GCS_h and 167-bp *MDR1* cDNA fragments were separated on 2% agarose gels and visualized by EtBr staining (*lanes 4 and 5* in **A** show the *MDR1* mRNA levels in MCF-7 and MCF-7/Adr cells which were used as negative and positive controls, respectively; *lane 1* in **A** and *lanes 1 and 4* in **B** contains HaeIII-cut ΦX174 DNA fragments used as molecular weight markers). The 345-bp rRNA-specific fragments detected by RT-PCR were used as internal controls in each amplification reaction. The results shown are representative of three independent experiments. **C** Cellular GSH levels of NHM and Met-5A cells were measured using the GSH-reductase method by determining the rate of DTNB formation spectrophotometrically at 412 nm. GSH levels in duplicate samples of three NHM cell lines, isolated independently from different individuals, and Met-5A cells were quantitated by comparison with the standard curve (*error bars* represent standard deviations)

DNA fragment, was detected in drug-resistant MCF-7/Adr human breast cancer cells that express P-gp, but not in sensitive MCF-7 cells (Fig. 2A, lanes 5 and 4, respectively).

It is known that MRP exports GSH-conjugated compounds from MDR cells. γ-GCS_h is one of the key enzymes in GSH synthesis and is involved in drug resistance by increasing cellular GSH which enhances the formation of GSH conjugates, leading to decreased intracellular drug concentrations [14–17]. Therefore, to determine whether γ-GCS_h was overexpressed in the Met-5A cells compared with the expression in NHM cells, we determined its mRNA levels in these cells by RT-PCR as described in Materials and methods. As seen in Fig. 2B, γ-GCS_h mRNA was overexpressed in Met-5A cells (lane 3) compared with the expression in NHM cells in which a very low level of γ-GCS_h mRNA was detected (lane 2). The rRNA levels of the cells were used as internal controls in the amplification of *MDR1* and γ-GCS_h (Fig. 2A, lanes 2 and 3, and Fig 2B, lanes 5 and 6, respectively) by RT-PCR as described in Materials and methods.

To investigate whether the increased levels of γ-GCS_h mRNA affect the cellular GSH level in Met-5A cells compared with NHM cells, we measured the cellular levels of GSH using the GSH reductase method as described in Materials and methods. As seen in Fig. 2C, GSH levels in NHM and Met-5A cells were 4.7 and 8.5 nmol/10^6 cells, which demonstrates that the cellular GSH level in Met-5A cells is about 1.8-fold greater than in NHM cells. The GSH content was measured in duplicate samples of three NHM cell lines isolated independently from different patients.

Cellular drug accumulation and cell survival characteristics of NHM and Met-5A cells

In order to determine whether the MRP/GS-X pump is functionally active in Met-5A cells which overexpress MRP compared with NHM cells, we measured the intracellular accumulation of [^{14}C]DOX in these cells as described in Materials and methods. As shown in Fig. 3, Met-5A cells accumulated about 73% less [^{14}C]DOX than NHM cells. The decreased drug accumulation in Met-5A cells correlated with their increased level of MRP compared with NHM cells.

To determine the impact of MRP overexpression on the survival of Met-5A cells compared with NHM cells treated with various concentrations of DOX, MTT

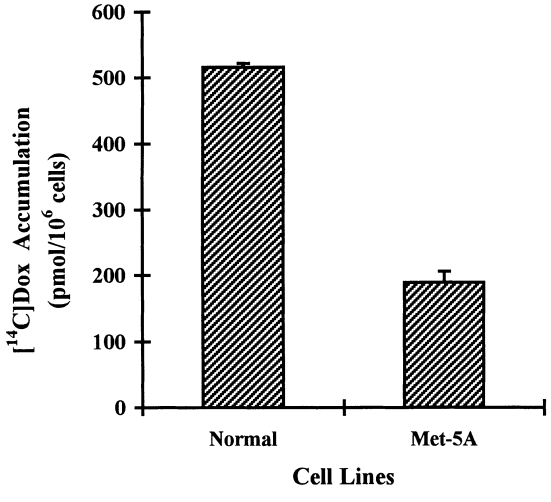

Fig. 3 The measurement of cellular accumulation of [^{14}C]DOX in NHM and Met-5A cells. The accumulation of [^{14}C]DOX in NHM and Met-5A cells was measured as described in Materials and methods. Log-phase cultures grown in 24-well plates were incubated with 0.5 μ*M* [^{14}C]DOX for 90 min at 37 °C in an atmosphere containing 5% CO_2, and the cellular accumulation of [^{14}C]DOX was measured by scintillation counting following several washes of the cells. For each cell line, triplicate samples were used in two independent experiments (*error bars* represent standard deviations)

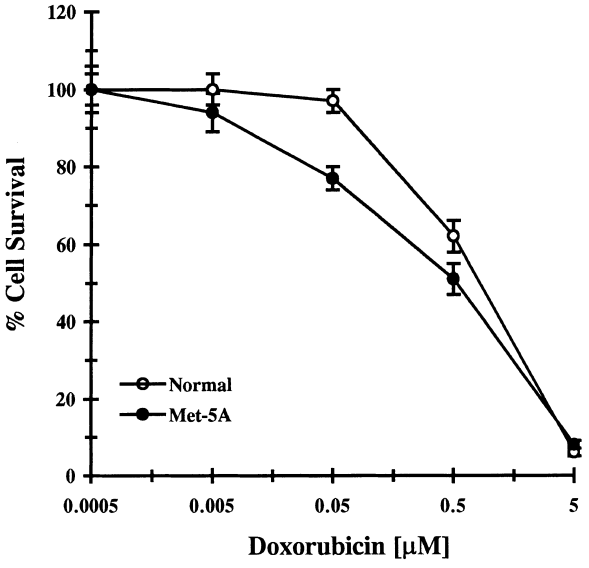

Fig. 4 Effects of increasing concentrations of DOX on cell survival in NHM and Met-5A cells. The NHM (*open circles*) and Met-5A (*closed circles*) cells were grown in the absence or presence of increasing concentrations of DOX, and the effect of DOX on cell survival was determined using an MTT assay as described in Materials and methods. Triplicate wells were used for each point in at least two independent experiments (*error bars* represent standard deviations)

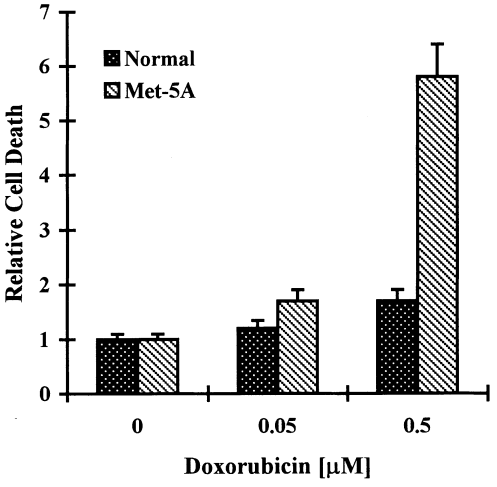

Fig. 5 Quantitative measurement of cytoplasmic DNA fragmentation due to apoptosis. Cytoplasmic DNA-histone complexes were quantitated using an ELISA in Met-5A and NHM cells grown in the absence or presence of 0.05 and 0.5 μ*M* DOX as described in Materials and methods. The A_{405} readings obtained from the ELISA were normalized for cell number and the results are expressed relative to untreated cells. Each point is the average of triplicate determinants in two independent experiments (*error bars* represent the standard deviation for each point)

cytotoxicity assays were performed as described in Materials and methods (Fig. 4). Interestingly, the results shown in Fig. 4 show that the IC_{50} values of DOX in NHM cells and Met-5A cells were 0.82 and 0.44 μ*M*, respectively, indicating that Met-5A cells were about two-fold more sensitive to DOX than NHM cells, despite our finding that Met-5A cells overexpressed MRP and accumulated less drug compared with NHM cells. These results provide evidence that there are other alterations that overcome the cellular function of MRP, leading to an overall increase in the DOX sensitivity of Met-5A cells compared with NHM cells. As controls, we used the human leukemia cell line HL-60 and its drug-resistant variant HL-60/Adr [25] which overexpresses MRP in MTT cytotoxicity assays. The IC_{50} values of DOX in HL-60 and HL-60/Adr cells were determined as 0.03 and 3.1 μ*M*, respectively, demonstrating that HL-60/Adr cells are about 100-fold more resistant to DOX than HL-60 cells (data not shown).

Apoptotic cell death in NHM and Met-5A cells

It is known that programmed cell death, or apoptosis, plays a role in determining the drug resistance characteristics of human cancer cells [24]. Therefore, we investigated whether an increased level of apoptosis is involved in the DOX sensitivity of Met-5A cells. Specifically, we measured the cytoplasmic DNA-histone complexes by ELISA in cell cultures grown in the absence or presence of 0.05 or 0.5 μ*M* DOX as described in the Materials and methods. Interestingly, as seen in Fig. 5, Met-5A cells showed about 1.4- and 3-fold increased apoptosis at 0.05 and 0.5 μ*M* DOX, respectively, compared with NHM cells. These results suggest that despite the overexpression of *MRP*/γ-GCS_h and lower intracellular drug accumulation, the high level of drug-induced apoptotic cell death is involved in the increased DOX sensitivity of these cells.

Discussion

In this study, we showed that despite the coordinated overexpression of MRP and γ-GCS_h, which correlates with higher cellular GSH levels and decreased intracellular accumulation of DOX, Met-5A cells express less DOX resistance than NHM cells. This may be due to the higher levels of apoptotic cell death induced by DOX in Met-5A cells compared with NHM cells. The finding that both MRP and γ-GCS_h are coordinately upregulated in Met-5A cells is consistent with the results of previous studies on the cisplatin-resistant derivative of HL-60 cells [16], human colorectal cancers [30] and ethacrynic acid-resistant human colon tumor cells [31]. The mechanisms of upregulation for the MRP and γ-GCS_h genes in Met-5A cells, however, remain unknown, but common factor(s) including SV40 TAg itself may be involved in the overexpression of these two genes.

Identification of the mechanisms involved in the increased level of drug-induced apoptosis in Met-5A cells due to SV40 TAg transformation remains to be determined. One possibility is that wild-type p53 tumor suppressor protein is inactivated by SV40 TAg in transformed cells, and this inactivation may contribute

to the increased DOX sensitivity of this cell line through increased apoptotic cell death. In fact, we have found inactivation of p53 with SV40 TAg in Met-5A cells in which the p53 protein and SV40 TAg were co-immunoprecipitated with an anti-SV40 TAg antibody in [^{35}S]methionine-labeled Met-5A cell extracts, followed by Western blotting using anti-p53 antibody (data not shown). Interestingly, this is also supported by a recent study by Wahl et al. [10] which demonstrated that inactivation of wild-type p53 confers increased sensitization to the anticancer agent taxol, and concurrent work by Hawkins et al. [11] showing that p53 inactivation increases sensitivity to other chemotherapeutic agents, in addition to taxol, such as carboplatin, cisplatin and melphalan. These studies used human foreskin fibroblasts and mouse embryo fibroblasts in which p53 inactivation was achieved by targeted disruption of the gene or by transformation of cells by human papilloma virus (HPV) 16E6 or SV40 TAg. Identifying the mechanisms of the increase in drug-induced apoptosis in Met-5A cells will lead to new therapeutic strategies to increase the efficacy of chemotherapeutic agents.

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