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Modulation of expression of multidrug resistance gene (mdr-1) by adriamycin

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The acquired resistance to various drugs in cancer is mediated by P-glycoprotein (P-gp) which is encoded by the mdr-1 gene. An increased level of mdr-1/P-gp was demonstrated after chemotherapy administered to treat cancer in humans. To clarify the direct effect of anticancer drugs on mdr-1/P-gp expression, we investigated the change in transport of adriamycin (ADR), and the expression of the mdr-1 gene and P-gp in an ADR-treated, multidrug-resistant leukemic cell line (K562/ADR$_{500}$). The addition of ADR induced the over-expression of mdr-1/P-gp, which led to a transient decrease in the intracellular accumulation of ADR although the difference was not statistically significant. A maximal effect was observed after 4 h incubation, returning to the baseline level after further incubation for 12–24 h. The phosphorylation of P-gp was inversely correlated with the levels of P-gp. These observations suggest that ADR itself modulates both the expression and function of P-gp. Determination of the optimal schedule for administering adriamycin is essential to achieving the optimal effect in treating cancer.

Multi-drug resistance; mdr-1; P-glycoprotein; Adriamycin

1. INTRODUCTION

Many cell lines selected for resistance in vitro to one anticancer drug also show cross-resistance to other agents which are structurally and functionally unrelated [1,2]. Most of these cell lines over-express the mdr-1 gene and its product, P-glycoprotein (P-gp) [3–6]. P-gp is thought to function as an ATP-dependent drug efflux pump [4,5]. The promoter region of the mdr-1 gene was recently isolated [7,8]. The regulatory mechanism of the expression of this gene has been studied in normal and malignant cells. Such stimuli as heat shock and differentiating agents have been shown to modify its expression of the mdr-1 gene [9–12].

The development of multidrug resistance is a major clinical problem in the chemotherapy of cancer. The increased expression of mdr-1/P-gp has been demonstrated following chemotherapy [13,14]. However, the level of mdr-1 mRNA and P-gp does not always result from gene amplification [15,16]. To clarify the mechanism involved, we investigated the direct effects of adriamycin (ADR) on the expression of mdr-1/P-gp and intracellular drug accumulation by using a multidrug resistant cell line which was treated with ADR for varying periods of time.

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2. EXPERIMENTAL

2.1. *Materials*

Adriamycin (ADR) was kindly provided by Kyowa Hakko Kogyo Co. (Tokyo, Japan). [^{125}I]Protein A (1.11 GBq/ml) and [^{32}P]orthophosphate (370 MBq/ml) were from ICN Biomedical Inc. (Costa Mesa, CA). [γ-^{32}P]ATP (370 MBq/ml) and [α-^{32}P]dCTP (370 MBq/ml) were obtained from Amersham (Buckinghamshire, UK). Monoclonal antibody against P-glycoprotein (P-gp) was from Centocore (Baltimore, MD) [17]. The oligonucleotide probe specific for mdr-1 gene, GTTCAAACTTCTGCTCCTG (residues 2734–2752, antisense strand) was prepared on an Applied Biosystem 391 automated synthesizer. The actin probe was purchased from Cosmo Bio Co. (Tokyo, Japan). Protein A-Sepharose C4-B was obtained from Pharmacia LKB (Uppsala, Sweden).

2.2. *Treatment of cells with ADR*

The multi-drug resistant subline, K562/ADR$_{500}$, was developed in vitro in our laboratory from the human myelogenous cell line, K562, and maintained with 500 nM of ADR [18]. The cells were cultured in an ADR-free medium for 3 days before these experiments. The cells were incubated in RPMI 1640 containing 10% of fetal calf serum (FCS) with 2 μM of ADR for 0, 4, 8, 12 and 24 h in 5% CO_2–95% air at 37°C. They were used in the following experiments after washing with cold phosphate-buffered saline (PBS). The viability of the cells was maintained above 90% during each treatment period.

2.3. *Intracellular transport of ADR*

The intracellular accumulation of ADR in cells incubated with this agent for specified intervals was determined by resuspending the cells (5×10^5/ml) in fresh medium with 2 μM of ADR at 37°C for 60 min. The cells were washed twice with cold PBS containing 1% bovine serum albumin (BSA), and then extracted with 0.3 N HCl–50% ethanol. The concentration of intracellular ADR was detected by spectrofluorometry (Shimadzu RF 5000) at an excitation wavelength of 470 nm and emission wavelength of 585 nm.

2.4. *Western blot analysis of P-gp*

The membrane fraction of cells treated with ADR for specified intervals was prepared by the method of Bells et al. [19]. The membrane fraction (100 μg) was separated on 8% SDS-PAGE and transferred onto a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). The filter was pre-incubated overnight at 4°C with blocking buffer (3% BSA in Tris-buffered saline) and then incubated with 1 μg/ml monoclonal antibody, C219, in blocking buffer for 3 h at room temperature. The filter was washed with 0.2% NP-40 in Tris-buffered saline, and incubated with [^{125}I]protein A at 37 KBq/ml in blocking buffer for 1 h. After extensive washing, the filter was autoradiographed with an intensifying screen at −70°C.

2.5. *Northern blot analysis of the mdr-1 gene*

Total RNA was extracted by lysis in guanidine thiocyanate and centrifugation in a cesium chloride gradient. Total RNA (10 μg) was electrophoresed on 1% agarose gels after glyoxalation, transferred onto nylon membranes (Biodyne B, Pall, East Hills, NY) and hybridized with an mdr-1 oligonucleotide probe 5' end labeled with [γ-^{32}P]ATP and polynucleotide kinase. The nylon membrane was re-hybridized with an [α-^{32}P]dCTP-labeled actin probe. Autoradiography was carried out with an intensifying screen at −70°C.

2.6. *Phosphorylation of P-glycoprotein*

After incubation with ADR for specified intervals, K562/ADR$_{500}$ cells (1×10^7) were suspended in phosphate-free Hank's balanced salt solution (HBSS) containing 10% of dialyzed FCS, and metabolically labeled with [^{32}P]orthophosphate (92.5 MBq/ml) for 4 h at 37°C. The cells were washed three times with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, and solubilized in cold lysis buffer (0.15 M NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EGTA, 2 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min. The cell lysates were clarified by centrifugation at 10,000 × *g* for 5 min and incubated with normal mouse serum for 30 min and then with protein A-Sepharose for 30 min, and the beads were removed. Immunoprecipitation was carried out with C219 antibody (1 μg/ml) for 2 h at 4°C and immune complexes were collected on protein A-Sepharose C4-B by 1 h incubation at 4°C. The beads were centrifuged and washed three times with lysis buffer, once with 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 and once with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4. Finally, the protein bound to beads was solubilized in 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 62.5 mM Tris-HCl, pH 6.8, boiled for 3 min and separated on 8% SDS-PAGE, followed by autoradiography.

3. RESULTS

Fig. 1A shows the change in the intracellular accumulation of ADR after incubation with 2 μM of ADR, a concentration which is about twice the IC_{50} for ADR in K562/ADR$_{500}$ [18], for specified intervals. The average intracellular accumulation of ADR in three experiments decreased transiently in the cells treated with ADR over 4–8 h, and recovered to the initial level in the cells treated for 24 h. A maximal inhibition of 60% of the initial level was observed at 8 h incubation. However, the difference was not significant because of the wide variation in basal values between experiments.

To clarify the mechanism for the change in ADR uptake, we studied the level of P-gp in the cells treated with ADR by Western blotting using C219 monoclonal antibody. The amount of P-gp increased by about 20% after incubation for 4 h, and reverted to the initial level after incubation for 8 h. Furthermore, it was down-regulated after incubation for 12–24 h (Fig. 2). We then examined mdr-1 gene expression by Northern blotting using the mdr-1 specific probe. The expression of the mdr-1 gene was also induced transiently in the cells treated with ADR for 4 h, and was then suppressed to the initial level (Fig. 3). These observations confirmed that the suppression of intracellular accumulation of ADR after exposure to ADR was due to the over-expression of mdr-1/P-gp.

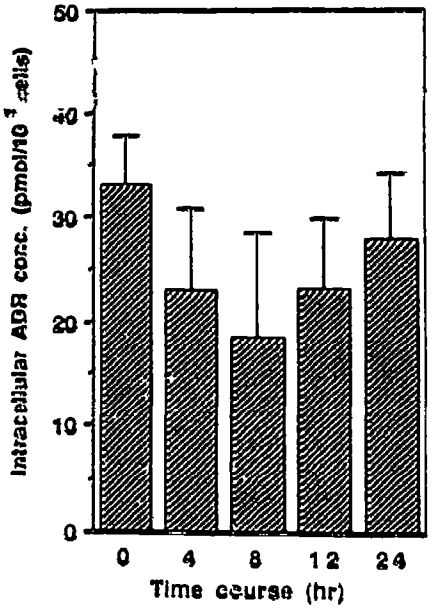

Fig. 1. Intracellular accumulation of ADR after incubation with 2 μM of ADR for specified times and resuspended with fresh medium containing 2 μM of ADR, and incubated for 60 min. The data were an average (±S.D.) of three experiments.

The phosphorylation of P-gp was enhanced after ADR treatment for 12–24 h (Fig. 4), while the amount of P-gp was decreased during these incubation periods, as shown in Fig. 2.

4. DISCUSSION

We observed that an anticancer drug, ADR, itself induced an over-expression of mdr-1/P-gp, with a decrease in the intracellular accumulation of this drug in multidrug-resistant cell lines. The expression of the mdr-1 gene appears to be correlated with the copy number of the gene in most multidrug resistant cell lines [6], but revertants of these cell lines were usually characterized by a decreased expression of this gene without a loss of amplified DNA [15]. In cancer patients, the mdr-1 gene is frequently expressed after chemotherapy and is not always amplified [20]. Our results support the previous findings that the expression of mdr-1 gene may be transcriptionally regulated by anticancer drugs [9]. This phenomenon may be one of the mechanisms for protecting the cell against an anticancer drug by pre-

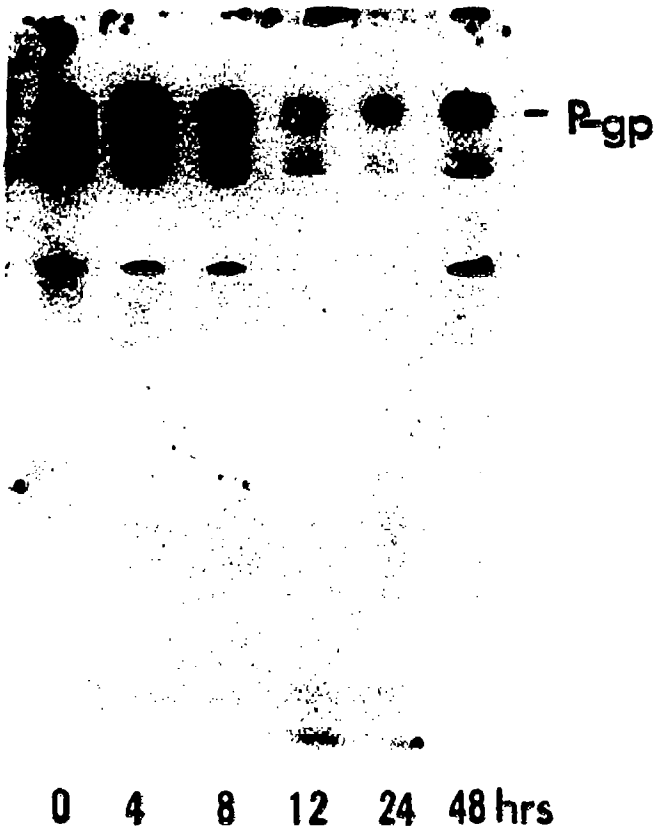

Fig. 2. Western blotting analysis of P-glycoprotein. 100 μg of membrane protein was applied to each lane, separated on 8% SDS-PAGE, and probed with monoclonal antibody C219.

serving a low level of intracellular drug concentration. However, the precise mechanism by which ADR modulates mdr-1 gene expression is unknown. Some form of stress, including differentiating agents or heat shock, are known to induce mdr-1 gene expression [10–12]. The promoter sequence of the mdr-1 gene has been identified [7]. Kohno et al. [9] reported that the mdr-1 promoter was activated by anticancer drugs, including vincristine, daunomycin, and colchicine, by using a transient CAT expression assay. Raymond and Gros [8] demonstrated the cell-specific activity of *cis*-acting regulatory elements in the promoter of the mdr-1 gene. These findings support the idea that *trans*-acting regulatory factors are constitutively or inducibly expressed in both normal cells and malignant cells.

We observed that the effect of ADR on the expression of mdr-1/P-gp appeared relatively early, within 4–8 h, and diminished after prolonged incubation with ADR. The expression of mdr-1 mRNA was also inducible by another anticancer drug, actinomycin D, in revertant cell lines after re-exposure to the drug [21]. These authors found that mdr-1 mRNA expression was enhanced following prolonged incubation (72 h) with the drug. Their use of revertant cells may explain the difference in time-course as compared with our findings. Pre-

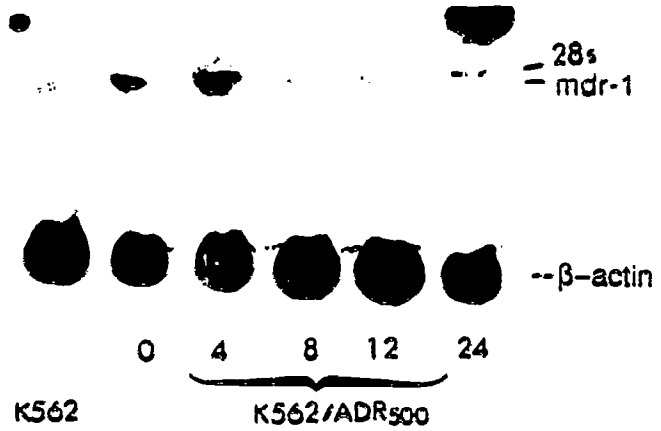

Fig. 3. Northern blot analysis of mdr-1 gene expression. Equal amounts of total RNA (10 μg) were applied to each lane and probed with the mdr-1 probe (upper) or β-actin probe (lower). The first lane shows a sample of ADR-sensitive K562 cells used as a negative control.

vious studies have shown that the level of mdr-1/P-gp expression that was induced by differentiating agents, and sustained throughout exposure, decreased rapidly with a half life of several hours after removal of the agents, returning to baseline in about 12 h [11]. In this study, the over-expression of the mdr-1 gene was transient, but continuous ADR treatment returned it to the initial level. Differentiation inducers and anticancer drugs may regulate the expression differently. It is also possible that newly expressed P-gp decreases the intracellular ADR level as a function of drug efflux pump, and thus, the effect is transient.

We showed that the level of P-gp phosphorylation

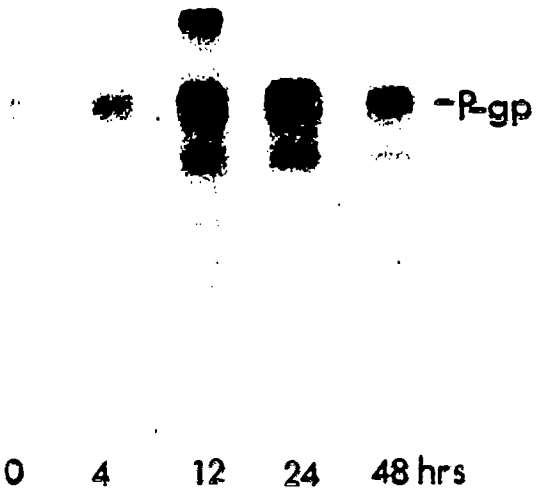

Fig. 4. Phosphorylation of P-glycoprotein after treatment with ADR. Cells were labeled with [^{32}P]orthophosphate. The phosphorylated protein was immunoprecipitated using C219 monoclonal antibody. Samples were analyzed on 8% SDS-PAGE.

induced by ADR was inversely correlated with the level of P-gp. Recent studies have demonstrated that phosphorylation plays an important role in the biological activity of P-gp [22–24]. The activation of protein kinase C by phorbol esters induces drug resistance [25], while an inhibitor of this kinase, staurosporine, inhibits P-gp phosphorylation and leads to a concomitant increase in drug accumulation as a result of the inhibition of efflux. Calcium channel blockers and calmodulin inhibitors [23,26,27], inhibitors of active drug efflux, lead to an increase in P-gp phosphorylation. These results are controversial in relation to phosphorylation and function of P-gp, although phorbol esters and calcium channel blockers are thought to induce phosphorylation at different sites of the protein [26]. Although the mechanisms by which ADR induces phosphorylation of P-gp during prolonged incubation is not known, it is likely that the phosphorylation by ADR also modifies the function of this protein.

In conclusion, exposure to ADR modified the expression of mdr-1/P-gp in a time-dependent manner. Clarification of the precise mechanism will help to avoid drug resistance and aid the efficient administration of chemotherapy to cancer patients.

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