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In Vitro Reversal by Progesterone of Multidrug Resistance in a Murine Leukemia Resistant Cell Line

TSUTOMU FUJIMURA[1], NORIKO KANEKO[2], SHINOBU FURUSAWA[2], MOTOAKI TAKAYANAGI[2], YOSHIO TAKAYANAGI[2], KEN-ICHI SASAKI[2]

[1]Division of Biochemical Analysis, Central Laboratory of Medical Sciences, Juntendo University School of Medicine*
[2]Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku College of Pharmacy**

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A major obstacle to successful cancer chemotherapy is the development of multidrug resistance (MDR) by tumor cells. Overexpression of the *mdr*1 gene product P-glycoprotein (P-170) is characteristic of such cells. In this study, *in vitro* reversion of MDR was attempted in a mouse leukemia cell line resistant to doxorubicin (DOX) using the steroid progesterone in combination wih DOX. Treatment with progesterone restored the DOX sensitivity of the P388/DOX cell line, whereas no changes in drug sensitivity were observed upon treating the parent cells with DOX. The potentiation of DOX cytotoxicity by progesterone was not due to increasing DOX accumulation and these results suggest that progesterone at noncytotoxic concentrations can enhance the cytotoxic potential of DOX without interfering with P-glycoprotein function.

Key words —— progesterone, doxorubicin, multidrug resistance, mouse leukemia cells, cytotoxicity

Introduction

The development of cellular resistance to drugs is a major impediment to successful cancer therapy. Multidrug resistance (MDR) implies that cells become simultaneously resistant to many structurally and functionally unrelated drugs, such as *Vinca* alkaloids (vinblastine and vincristine), anthracyclines (DOX), taxol, actinomycin D, and mitomycin[1-4]. Mechanisms of MDR such as overexpression or activation of P-glycoprotein[5], overexpression of glutathione S-transferase[6] or mutation of topoisomerase II[7] may play a role in MDR.

* 〒113 東京都文京区本郷 2-1-1 ; 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan
** 〒981 仙台市青葉区小松島 4-4-1 : 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan

Overexpression of P-glycoprotein, an *Mr* 170,000 plasma membrane glycoprotein encoded by the *MDR*1 gene[8-10], is a major factor implicated in the multidrug-resistant phenotype. Evidence suggests that P-glycoprotein acts as an energy-dependent drug efflux pump, resulting in reduced drug accumulation and cellular cytotoxicity. A variety of pharmacological agents, including verapamil, other calcium channel blockers, calmodulin inhibitors, and cyclosporins, have been shown to interfere with P-glycoprotein function and to successfully reverse the MDR phenotype *in vitro*[11]. However, the efficacy of these agents in animals studies and clinical trials has been disappointing due to dose-limiting toxicity and lack of specificity[12]. Progesterone-mediated modulation of MDR in cultured cell has been described by several groups[13,14]. Progesterone interacts with members of the MDR family of P-glycoproteins[15-17], although the mechanism behind this is not fully understood.

In this paper, we have investigated the interaction between progesterone and DOX associated with the MDR phenotype. Our results demonstrate that progesterone can significantly enhance DOX cytotoxicity in the MDR phenotype. Progesterone was selective for the potentiation of DOX cytotoxicity, since no modulation of DOX cytotoxicity in DOX-sensitive cells was observed. Furthermore, the modulation of DOX cytotoxicity in MDR cells was unrelated to alterations in drug accumulation.

Materials and Methods

1. Materials

Progesterone was supplied by Nacalai Tesque Co., Ltd. (Kyoto, Japan). Doxorubicin (DOX) from Kyowa Hakko Kogyo Chemical Co., Ltd. (Tokyo, Japan), Roswell Park Memorial Institute (RPMI) medium 1640 from Nipro Co., Ltd. (Osaka, Japan), fetal bovine serum (FBS) from JRH Biosciences, Inc. (Lenexa, KS, U.S.A.), penicillin G potassium and streptomycin sulfate from Meiji Seika Co., Ltd. (Tokyo, Japan). Other chemicals used in the study were of analytical grade and obtained from regular commercial sources. Progesterone dissolved in ethanol was diluted in medium to give a concentration of 10 μM. Each drug was dissolved in fresh medium and filtered through a 0.20 μm syringe filter (Iwaki Glass, Chiba, Japan).

2. Cell lines

The drug-sensitive murine leukemia cell line P388 (P388/S) used in this study was kindly supplied by the Japanese Cancer Research Resource Bank (Tokyo, Japan). Cells from this line were grown in suspension in RPMI1640 medium supplemented with 10% FBS in the presence of $5\times10^{-5}M$ 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air. The P388/DOX cells can be maintained at a high concentration of DOX in the culture medium. The cell line was maintained by passage into fresh medium three times a week.

3. Cytotoxicity assay

Cell concentrations and viability were determined by hemocytometer counts of cells excluding 0.1% trypan blue dye. Cytotoxicity was also assessed by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma)-based colorimetric assay[18]. Cells at 1×10^4/well were plated in 96-well round-

bottomed plates (Corning Co., NY, U.S.A.). To study the cyotoxic effects of progesterone alone and the combined effects of progesterone and DOX, cells were exposed to different concentrations of drugs for 48 h at 37°C in an atmosphere of 5% CO_2 in air. After completion of the drug treatment, 10 μl MTT (5 mg/ml) solution was added to each well followed by 100 μl complete medium. After incubation for 4 h at 37°C, the MTT solution was removed. The formazan crystals incorporated in viable cells were solubilized with 100 μl dimethyl sulfoxide (Nacalai Tesque, Inc., Kyoto, Japan). Cells for each experimental data point were plated into at least eight wells, and the absorbance of each well was then read at 540 nm, using an ELISA analyzer (Model ETY-96, Toyo Instruments Inc., Japan). The surviving fraction was determined by dividing the absorbance of treated wells by that of control wells.

4. DOX accumulation

Cells (1×10^6 cells/ml) were preincubated with progesterone (0.5-50 μM) for 15 min at 37°C in an atmosphere of 5% CO_2 in air. Subsequently, DOX was added to obatain a final concentration of 2 μg/ml. After a 60-min incubation period, treated cells were immediately centrifuged at $600\times g$ and washed twice with ice-cold PBS. Following this final wasing, the cell pellet was resuspended in 0.3 N HCl in 50% ethanol, sonicated and then centrifuged at $3,000\times g$. The DOX concentration in the supernatant was determined spectrofluorimetrically[19] using a Hitachi spectrofluorimeter (650-10S type, Japan). Excitation and emission wavelengths of 470 and 585 nm, respectively, were used for the fluorimetric analysis of DOX. Cellular DOX concentrations were derived from standard curves prepared using known amounts of drug. Data are expressed as the percentage of cell-associated fluorescence intensity measured in control cells after 60 min of drug accumulation.

5. Flow cytometric analysis

FACScan flow cytometric analysis was performed using a Becton Dickinson immunocytometry system (CellQuest software). Briefly, exponentially growing cells were treated with 1-10 μM DOX for 1 h. The cells were centrifuged at $600\times g$ for 10 min, and the pellet was resuspended in PBS. The cell suspension was stored at 4°C and then DOX fluorescence was measured. The experiments were repeated at least twice, and 10,000 cells were counted per sample.

6. Statistical analysis

Student's t-test was used to evaluated the statistical significance of differences between groups.

Results

The effect of progesterone on murine leukemia cell growth was examined. Progesterone was tested for its ability to inhibit proliferation of P388/S and P388/DOX cells and the results are shown in Fig. 1. The cytotoxicity of drugs after a 48-h incubation was measured by MTT assay. No significant inhibition of cell growth by progesterone was observed in P388/S and P388/DOX cells treated with a low concentration of progesterone. Progesterone slightly inhibited P388/S and P388/DOX cell growth at a concentration of 500 μM.

The ability of progesterone to modulate DOX cytotoxicity was studied. Various doses of DOX were added to the growth medium and

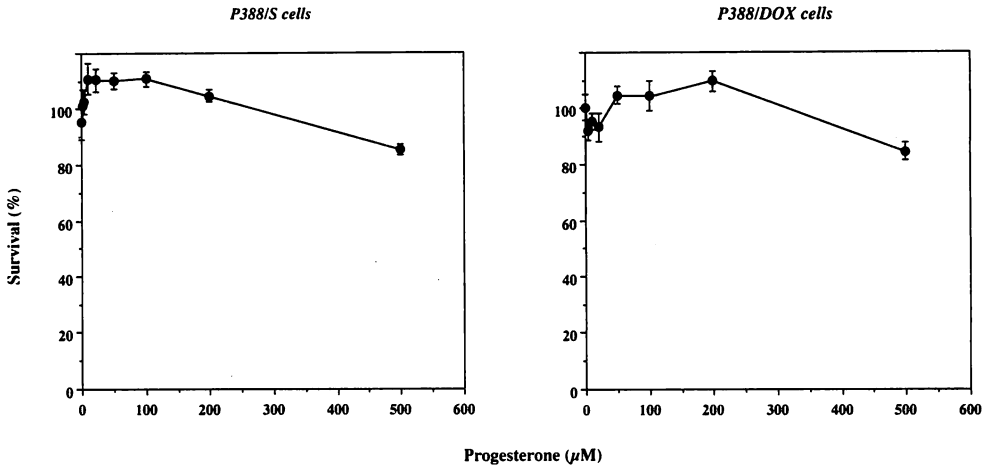

Fig. 1 Effect of progesterone on P388/S and P388/DOX cell growth. Cells were incubated with various concentrations of progesterone for 48 h. Cell survival was measured by the MTT assay in "Materials and Methods". Each point is the means±SE (n=8).

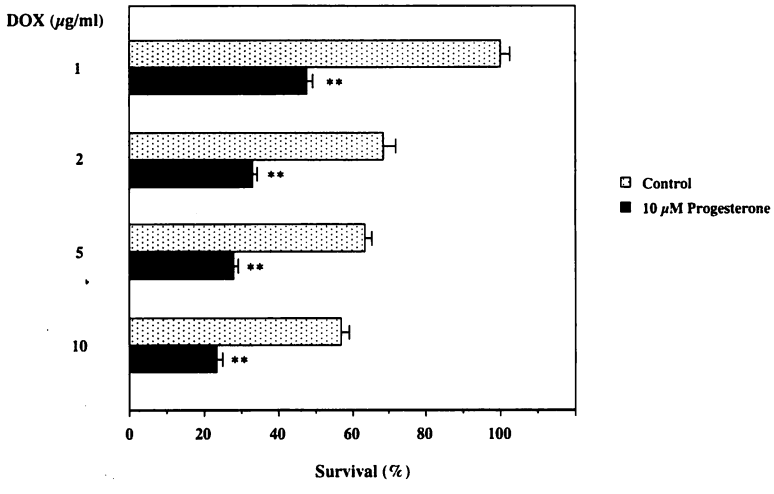

Fig. 2 Sensitizing effect of progesterone on doxorubicin (DOX) cytotoxicity in P388/DOX cells. Cells were incubated with drugs for 48h. Survival fraction of the cells exposed to different concentrations of DOX (1-10 μM) with or without 10 μM progesterone was masured by the MTT assaay. $^{**}P<0.01$, compared with DOX alone-treated group.

incubated for 48 h, with or without progesterone. The relative resistance to DOX of P388/DOX cells was 90-fold. Noncytotoxic concentrations of 10 μM progesterone increased the sensitivity to DOX by 2.1-2.4-fold in P388/DOX cells (Fig. 2) while no such increase in DOX cytotoxicity was observed in P388/S cells (data not shown). The effect of progesterone on DOX cell killing was also examined by the trypan blu dye exclusion test. The results are

shown in Fig. 3. In the case of resistant cells, the combination of DOX (5-20 μM) and 10 μM progesterone resulted in a significant ($p<0.01$) increased cell death in culture.

Flow cytometric analysis confirmed intracellular DOX fluorescence in P388/S and P388/DOX cells and a significant difference in DOX accumulation in cells was observed (Fig.

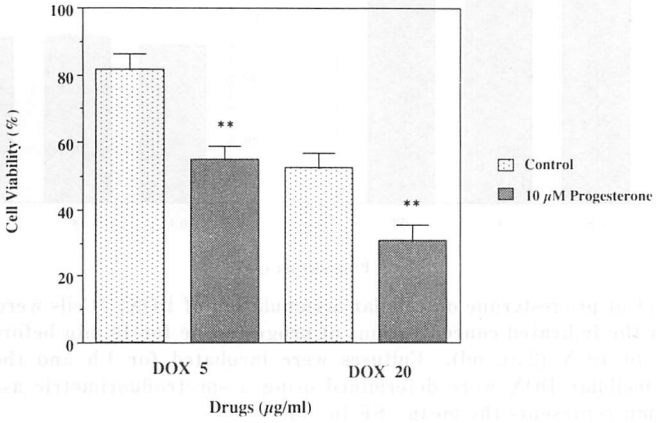

Fig. 3 Cytotoxic effect of DOX on P388/DOX cells in the presence or absence of progesterone. Cells were incubated with DOX (5-20 μM) in the presence or absense of 10 μM progesterone for 4 h. Cell viability was measured using the dye exclusion test. Each column represents the mean±SE from triplicate experiments. Each column represents the mean±SE (n=3). $^{**}P<0.01$, compared with DOX alone-treated group.

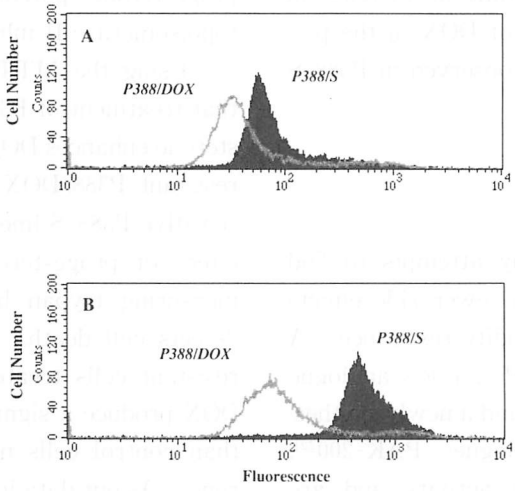

Fig. 4 DOX fluorescence remaining in P388/S and P388/DOX cells. A : Cells treated with 1 μM DOX for 1 h ; B : Cells treated with 10 μM DOX for 1 h. Cells were analyzed for intrcellular DOX fluorescence using a flow cytometer.

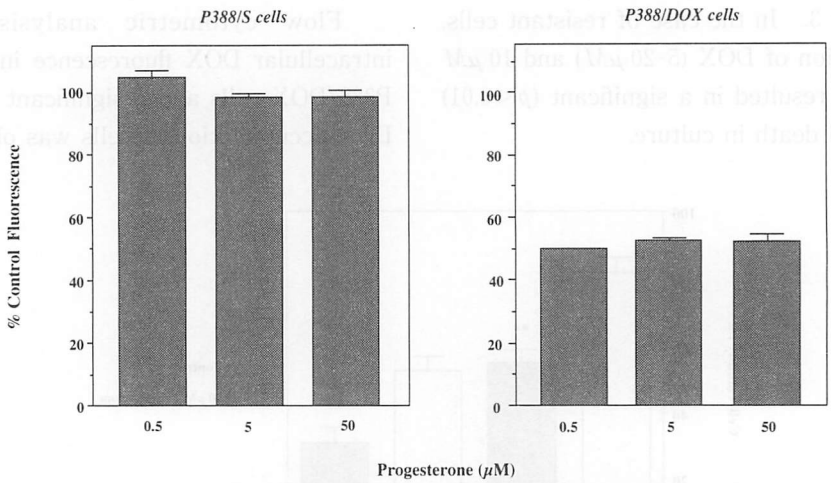

Fig. 5 Effect of progesterone on cellular accumulation of DOX. Cells were incubated with the indicated concentrations of progesterone for 10 min before the addition of DOX (2 μg/ml). Cultures were incubated for 1 h and the levels of intracellular DOX were determined using a spectrofluorimetric assay. Each column represents the mean±SE (n=3).

4). The effect of progesterone on DOX accumulation in P388/S and P388/DOX cells was also measured by analyzing of intracellular DOX fluorescence. There was a 2.5-fold reduction in the accumulation of DOX in P388/DOX cells relative to that in P388/S cells after 1 h of exposure to drug (Fig. 5) while no increase in intracellular accumulation of DOX in the presence of progesterone was observed in P388/S and P388/DOX cells.

Discussion

There have been many attempts to find new reversing agents with fewer side-effects and a greater ability to modify resistance. A new derivative of verapamil[20], a new analogue of cyclosporin, PSC-833[21], and a newly synthesized dihydropyridine analogue, PAK-200[22], have high MDR-reversing activity and are expected to have fewer side-effects than verapamil. In a previous study, we have shown that a bis-benzylisoquinoline (biscoclaurine) alkaloid (cepharanthine)[23,24], dipyridamole[25], reserpine, emetine[26,27] and tacrolimus hydrate[28] modulate anthracycline cytotoxicity and retention in MDR cells. In the present study, we demonstrated that treatment of MDR cells with progesterone potentiated the effect of the topoisomerase II inhibitor DOX.

Using the MTT assay, we demonstrated that treatment of P388 cells with 10 μM progesterone enhances DOX cytotoxicity towards the resistant P388/DOX line but not towards the sensitive P388/S line. We also determined the effect of progesterone on resistant cells by measuring trypan blue dye exclusion which detects cell death. The results indicate that resistant cells treated with progesterone and DOX produce a significantly greater cell death than control cells not treated with progesterone. As our data indicate, progesterone alone in relatively nontoxic and, even at 0.5 millimolar concentrations, it does not affect cell

viability.

Progesterone exerts many genomic actions through a nuclear receptor. Progesterone also rapidly increases intracellular Ca^{2+} concentrations and reduces cyclic AMP levels in amphibian oocytes[29] and rapidly inhibits K^+ conductance in renal epithelioid MDCK cells[30]. Recently, progesterone has been shown to bind to mouse and human P-glycoproteins and to inhibit their function[15,16]. However, according to many reports, progesterone is not transported by P-glycoprotein[31]. In our study, progesterone had no significant effect on DOX accumulation in resistant cells. The characteristics of progesterone in modulating the cytotoxic effects of DOX, in cells with the MDR phenotype, are its continued modulatory activity despite the absence of increasing DOX accumulation. On the other hand, progesterone has a biphasic effect on the pump, stimulating it at low concentrations and inhibiting it at concentrations of 10 μM or greater[32]. These facts may be important for understanding the actions of progesterone.

The results in this paper demonstrate that DOX cytotoxicity in resistant cells is enhanced by progesterone and so the interpretation of potentiation is more complex than simply a reflection of the amount of multidrug resistance pump present. In this study, we did not investigate whether the effect of progesterone in MDR cells is specific for DOX. Further studies are require to clarify the mechanism of action of progesterone in MDR cells.

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