Cyclosporin A and Verapamil Enhancement of Daunorubicin-produced Nucleolar Protein B23 Translocation in Daunorubicin-resistant and -sensitive Human and Murine Tumor Cells

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ABSTRACT

It has recently been shown that anthracycline antibiotic-resistant tumor cells are less responsive to daunorubicin-stimulated B23 nucleolar phosphoprotein translocation than drug-sensitive cells. Since cyclosporin A and verapamil reverse primary acquired and secondary cross-resistance to daunorubicin, we investigated the effect of these agents on nucleolar B23 translocation in sensitive and resistant tumors. We compared modified to baseline B23 phosphoprotein distribution between predominantly nucleolar, mixed nucleolar-nuclear, or nuclear immunofluorescence using a monoclonal anti-B23 antibody in parental drug-sensitive and multidrug-resistant acute lymphatic leukemia and in daunorubicin-sensitive and -resistant murine hepatoma. Our experiments show that cyclosporin A and verapamil enhance B23 phosphoprotein translocation, but that the addition of either agent to sensitive parental or resistant tumor sublines markedly enhances daunorubicin-stimulated translocation. This effect correlates with the correction of impaired daunorubicin inhibition of RNA synthesis by cyclosporin A and verapamil in the resistant sublines. Our observations suggest that nucleolar B23 phosphoprotein is an important site in the modulation of anthracycline antibiotic antitumor activity.

INTRODUCTION

There has been intense recent interest in the mechanism of MDR relative to cellular accumulation of natural product chemotherapeutic drugs and the control of this accumulation by M, 170,000 to 180,000 phosphorylated membrane glycoprotein, P-glycoprotein (1). Little attention, however, has been given to possible alteration of intracellular drug effects in MDR cells. In 1987 Chan et al. reported that doxorubicin and actinomycin D promoted translocation of nucleolar phosphoprotein B23, recently named nucleophosmin, from the nucleolus to the nucleus in murine tumor cells. It was noted, however, that doxorubicin and actinomycin D failed to promote nucleophosmin translocation in MDR murine P388 leukemia (2). We now report our experience with the MDR modulating agents CsA and verapamil on nucleophosmin translocation in DNR-sensitive and -resistant human acute lymphatic leukemia and murine hepatoma. Since nucleophosmin translocation correlates with pharmacological inhibition of RNA synthesis we also determined the effect of CsA and verapamil on the daunorubicin inhibition of RNA synthesis in these tumors (3).

MATERIALS AND METHODS

Tumor Lines. Human acute lymphatic leukemia, GM-3639, was obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. A MDR subline was developed by exposure of these cells to initially sublethal followed by progressively increased concentrations of vincristine as previously described (4). Parental drug-sensitive cells are designated as Lo and the resistant subline as L100. L100 cells are approximately 60-fold vincristine resistant, 5-fold daunorubicin resistant, and 10-fold VP16 resistant. Murine hepatoma 129, H-129, was obtained from the Division of Cancer Treatment tumor repository, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. A DNR-resistant subline, H-129/DNR, was developed by continuous DNR treatment of 9 sequential transfer generations of host mice as previously described (5). The P-glycoprotein expression of these resistant variants has not been defined.

Nucleophosmin Translocation Assay. Cells were washed, resuspended at a final concentration of 6 x 10^6 cells/ml in RPMI 1640 supplemented with 15% fetal calf serum, antibiotics, and 2 mM glutamine, and incubated in microtiter plate wells overnight at 37°C, 5% CO2. CsA (5.5 µM), verapamil (10 µM), and DNR (for Lo and L100, 4 µg/ml) (for H-129 and H-129/DNR, 8 µg/ml) were added 4 h prior to slide preparation. These respective DNR concentrations were chosen because they lie between the DNR IC50 value for RNA synthesis inhibition in parental sensitive and resistant sublines of each tumor. CsA and verapamil concentrations are those previously established as effective in reversal of DNR resistance (4, 6). Cell aliquots (50 µl) were cytocentrifuged onto slides, fixed using 2% p-formaldehyde, washed, and again fixed using acetone.

The first antibody (monoclonal IgG to Protein B23) was diluted 1:30 in PBS applied to slides and incubated at 4°C in a moist overnight chamber. After washing, the second antibody, fluorescein-conjugated goat anti-mouse IgG (Cappel, No. 1611-0081, 1 mg/ml) diluted 1:40 in PBS with 0.05% Evans blue dye, was applied to the slides and incubated at room temperature in a moist chamber. After washing and drying, slides were mounted in 50% glycerol in PBS (pH 9). The slides were viewed using a fluorescent microscope, and triplicate 100 cell counts were performed. Cells were graded as having predominantly Nu, mixed N/N, or Np fluorescence. Differences were analyzed by the two-tailed Student t test.

Daunorubicin Inhibition of [3H]Uridine Incorporation. Cells were counted on a hemocytometer using dye exclusion, washed, and resuspended in 1.6 x 10^6/ml. Cell aliquots of 1.6 ml were incubated with 0.2 ml of DNR (final concentration, 0 to 20 µg/ml) and 0.2 ml of CsA (5.5 µM) or verapamil (10 µM) for 1 h at 37°C, washed twice, and resuspended in 1.8 ml of RPMI. Triplicate 180-µl aliquots were placed into microtiter plates and incubated with 20 µl of [3H]uridine (specific activity 39 Ci/mmol, 1 µCi/ml) for 1 h at 37°C and 5% CO2. The wells were harvested onto glass fiber filters using a Titertek multiple sample harvester. The filters were dried and counted in 2,5-diphenyloxazole/p-bis[2-(5-phenyloxazolyl)benzene/toluene liquid scintillation system. Values are plotted as the percentage of [3H]uridine incorporation compared with control in the absence of DNR, and DNR IC50 differences are compared by the use of the two-tailed Student t test.

RESULTS

Fig. 1 presents photomicrographs, showing intense nucleophosmin immunofluorescence confined to the nucleoli of untreated control Lo (A) and H-129 (B) cells. After 4-h exposure to daunorubicin, B23 translocation occurs, and mixed N/N and predominant nuclear (Np) nucleophosmin fluorescence is noted...
in these cell lines, respectively (Fig. 1, C and D). Table 1 shows the percentage of distribution between nucleolar, mixed nucleolar-nuclear, and nucleoplasmic immunofluorescent patterns under control and experimental conditions in L0 versus L100 and H-129 versus H-129/DNR cells.

It can be seen that the addition of daunorubicin to all cells promotes nucleophosmin translocation from the nucleolus to the nucleus. This effect is greater in sensitive parental tumor cells compared to the corresponding resistant sublines. CsA and verapamil alone are essentially without effect, but the addition of each to daunorubicin markedly enhances nucleophosmin translocation in daunorubicin-resistant and -sensitive leukemia and hepatoma (P < 0.05). The data presented are confirmed by duplicate experiments which were performed with each cell line.

Table 2 presents the statistical evaluation of daunorubicin IC50 differences for inhibition of uridine incorporation into RNA compiled from triplicate experiments. It compares IC50 values between sensitive parental and resistant leukemia and hepatoma sublines and defines the modulation of daunorubicin IC50 values by CsA and verapamil. The differences between parental sensitive and resistant sublines are highly significant. The addition of CsA or of verapamil to each resistant variant reduces its daunorubicin IC50 value to that characteristic of sensitive parental tumor. In comparison, these modulators are without effect in the sensitive parental leukemia. However, CsA significantly enhances the effect of daunorubicin in sensitive parental hepatoma-129 (P < 0.05); the reduction in IC50 value produced by verapamil in this tumor approaches but does not reach statistical significance (P < 0.1, > 0.05).

Table 2 CsA and verapamil correction of DNR inhibition of RNA synthesis in resistant sublines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NaCl (control)</th>
<th>CsA (5.5 μM)</th>
<th>Verapamil (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L0</td>
<td>1.00 ± 0.0a</td>
<td>0.32 ± 0.0a</td>
<td>0.08 ± 0.0a</td>
</tr>
<tr>
<td>L100</td>
<td>3.20 ± 0.0b</td>
<td>1.20 ± 0.0b</td>
<td>0.40 ± 0.0b</td>
</tr>
<tr>
<td>H-129</td>
<td>2.00 ± 0.0c</td>
<td>1.00 ± 0.0c</td>
<td>0.60 ± 0.0c</td>
</tr>
<tr>
<td>H-129/DNR</td>
<td>1.00 ± 0.0d</td>
<td>0.50 ± 0.0d</td>
<td>0.30 ± 0.0d</td>
</tr>
</tbody>
</table>

* Mean ± SD.
** P < 0.01 versus NaCl control.
*** P < 0.05 versus corresponding parental sensitive cells.
**** P < 0.02 versus corresponding resistant sensitive cells.
***** P < 0.01 versus corresponding resistant subline control.
DISCUSSION

Early studies by Dano and by Skovsgaard noted impaired accumulation of anthracycline antibiotics and Vinca alkaloids in drug-resistant tumor cell sublines compared to corresponding drug-sensitive parental tumors. This impairment in drug accumulation was related to enhanced drug extrusion by resistant variants. It was observed that enhanced efflux is an active process, since drug accumulation normalized in the presence of metabolic inhibitors (7, 8). The modulation of MDR by a variety of compounds including verapamil and calmodulin inhibitors has been related to correction of impaired drug accumulation by inhibition of active drug extrusion (9,10). We have noted, however, that CsA corrects daunorubicin resistance in MDR human acute lymphatic leukemia without alteration of daunorubicin accumulation, and that CsA enhances the effect of daunorubicin in murine tumors in vivo with only minimal enhancement of cellular accumulation of drug (6, 11). It has also been noted that the acquisition of equimolar drug concentrations by resistant compared to sensitive cells does not restore equivalent cytotoxic drug effects (12–14). Recent studies stress the multifactorial nature of anthracycline resistance (15–17). These include, for example, the combined occurrence of earlier onset of DNA repair, increased glutathione-transferase activity, and lack of enhanced drug extrusion despite the presence of P-glycoprotein within an Adriamycin-resistant P388 leukemic subline (18).

Hindenburg et al. (19) recently noted that the cytoplasmic distribution of daunorubicin differs between sensitive and anthracycline-resistant HL-60 leukemia cells. Sensitive cells demonstrate a diffuse pattern of daunorubicin fluorescence, whereas resistant cells demonstrate punctate fluorescence corresponding to an intralysosomal distribution of drug. This pattern is corrected to that characteristic of sensitive cells by verapamil, leading the authors to suggest that heterogeneous cytoplasmic daunorubicin distribution reduces its accessibility to intracellular targets (19).

Nucleophosmin is a nucleolar phosphoprotein which is involved in the assembly of ribosomes (20–22). The protein is more abundant in tumor cells than in normal resting cells (23, 24). Electron microscopic study indicates that nucleophosmin is concentrated in the granular region of the nucleolus where ribosome assembly occurs (25). This protein forms hexamers and is associated with 60S and 80S ribonucleoproteins in the nucleolus (22, 26, 27). When cells are treated with cytotoxic agents including actinomycin D, doxorubicin, toyocamycin, luzopeptins, and mitomycin C, nucleophosmin translocates from the nucleolus to the nucleoplasm (2, 3, 22, 28, 29). It is hypothesized that these cytotoxic agents either bind to the protein or destroy its binding site in the nucleolus to cause translocation. This phenomenon has been used to detect drug-resistant cells and monitor drug efficacy (2, 3, 28, 29).

Our current study shows that CsA and verapamil enhance the translocation of nucleophosmin by daunorubicin in both daunorubicin-resistant and -sensitive tumor cells. These agents also correct impaired inhibition of RNA synthesis by daunorubicin in the resistant sublines. Their effect on daunorubicin inhibition of RNA synthesis in sensitive parental cells is less consistent. CsA significantly enhances the daunorubicin inhibition of RNA synthesis in sensitive hepatoma but not in acute leukemia. This observation is consistent with our earlier findings in which we noted CsA enhancement of daunorubicin cytotoxicity in sensitive hepatoma, and in resistant but not in sensitive acute leukemia, as measured by inhibition of DNA synthesis (6, 11). The addition of CsA to daunorubicin in vivo in the treatment of host mice bearing sensitive hepatoma significantly increased survival (11). Verapamil’s effect on RNA synthesis approaches significance in sensitive hepatoma, but it is also without effect in acute leukemia. The functional role of nucleophosmin in cell physiology is not fully understood. Although nucleophosmin translocation correlates with inhibition of RNA synthesis, recent studies (29) together with our current results indicate that additional inhibition of cellular events may occur.

Although the major focus of chemotherapy modulation has been in resistance correction, modulation can enhance the efficacy of natural product chemotherapeutic agents in sensitive tumors. Tsuruo (9) originally noted an approximately equal percentage of increase in survival of host mice bearing sensitive and resistant P388 leukemia when verapamil was added to vincristine in vivo. We noted that the addition of CsA to noneffective or to minimally effective daunorubicin regimens prolongs survival of mice bearing sensitive hepatoma-129 or Ehrlich ascites carcinoma (11).

Daunorubicin-produced nucleophosmin translocation is enhanced by CsA and by verapamil in both sensitive and resistant tumors; these agents also potentiate the efficacy of natural products in both sensitive and resistant tumors. Our observations suggest that, not only is nucleophosmin translocation an indicator of daunorubicin responsiveness, but that daunorubicin-nucleophosmin interaction may be an important target for modulator action. It is likely, however, that just as the mechanisms responsible for MDR are multifactorial, modulator action is not limited to a single mechanism. Our understanding of these mechanisms will eventually aid in their eventual exploitation for therapeutic purposes.

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