Enhancement by Cyclosporin A of Daunorubicin Efficacy in Ehrlich Ascites Carcinoma and Murine Hepatoma 129

Josephine Meador, Paula Sweet, Marie Stupecky, Martha Wetzel, Sandra Murray, Sudhir Gupta, and Lewis Slater

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ABSTRACT

Cyclosporin A abrogates pleotropic drug resistance in certain experimental tumors. Its impact on drug-sensitive tumors has not been investigated. Our studies show that in drug-sensitive Ehrlich ascites carcinoma and hepatoma 129 cyclosporin A enhances daunorubicin inhibition of DNA synthesis in vitro and prolongs survival of host mice in vivo. Of particular interest is that cyclosporin A converts ineffective daunorubicin regimens into those which result in prolongation of host mouse survival.

INTRODUCTION

A variety of agents, including calcium channel blockers, calmodulin inhibitors, tripananol analogues, and quinidine have been shown to reverse pleotropic drug resistance (1-4). The impact of these agents on chemotherapy responses in drug-sensitive tumors has not been well studied. We previously reported that CsA reverses DNR resistance in Ehrlich ascites carcinoma (5). We now report CsA enhancement of in vitro and antibiotic therapy directed against drug-sensitive as well as drug-resistant tumors.

MATERIALS AND METHODS

Tumor Lines and Treatment Regimens. EA is maintained as an ascitic tumor in BALB/c mice as previously described (6). H-129 was obtained from the DCT tumor repository, National Cancer Institute, Frederick, MD, and grown as an ascitic from the DCT tumor repository, National Cancer Institute, Frederick. Our studies show that cyclosporin A causes minimal enhancement of [3H]daunorubicin uptake without inhibition of [3H]daunorubicin efflux in both the presence and absence of interrupted active daunorubicin efflux. This suggests that the mechanism of action of daunorubicin enhancement by cyclosporin A in drug-sensitive tumors is not simply the result of increased intracellular daunorubicin accumulation.

In vivo dosages of cyclosporin A in the current study are comparable to those which can be used with reasonable safety in humans. We conclude that cyclosporin A may be useful in the potentiation of anthracycline antibiotic therapy directed against drug-sensitive as well as drug-resistant tumors.

RESULTS

Fig. 1, representative of three similar experiments, shows progressive enhancement of DNR inhibition of thymidine incorporation by 0.1-2.5 |m| CsA in both EA and H-129. From this experiment it can be seen that the enhancing effects of 2.5 and 10 |m| CsA are similar. Statistical comparison of the DNR 50% effective dose values for these three experiments in the presence of CsA versus 2.5 and 10 |m| CsA reveals P < 0.05 and < 0.02, respectively, for H-129. When the formula is used to derive a CsA index for enhancement of DNR effect in vitro, we find that 2.5 |m| CsA enhances 0.9 |m| DNR previously described (6). Cells were washed, counted on a hemacymometer using dye exclusion, and resuspended in RPMI 1640 at a concentration of 2.5 X 10^6/ml. Cell aliquots of 1.6 ml were incubated with 0.2 ml DNR HCl (final concentration, 0-18 |m|) and 0.2 ml CsA (final concentration, 0-10 |m|) for 1 h at 37°C water bath, washed twice, and resuspended in 1.8 ml RPMI. Triplicate 180-±l aliquots were plated into microtiter plates and incubated with 20 |m| [3H]thymidine (specific activity, 24 Ci/mmol; final concentration, 1 |m|Ci/ml) for 1 h at 37°C in 5% CO2. Samples were collected on glass fiber filters with a Tintertek multiple automated sample harvester unit using a deionized water wash. The filters were dried and counted in a PPO/POPPOIs liquid scintillation system. Results are expressed as a comparison of [3H]thymidine cpm in DNR containing cultures to [3H]thymidine cpm of the respective control. The daunorubicin 50% effective dose is defined as that concentration of DNR required to inhibit 50% of [3H]thymidine incorporation. In this system 2.5 and 10 |m| CsA inhibit thymidine incorporation by less than 20%.

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Other agents known to reverse pleotropic drug resistance are reported to exert their effects by increasing intracellular drug accumulation. In contrast, our studies of drug transport in drug-sensitive Ehrlich ascites carcinoma and hepatoma 129 show that cyclosporin A causes minimal enhancement of [3H]daunorubicin uptake without inhibition of [3H]daunorubicin efflux in both the presence and absence of interrupted active daunorubicin efflux. This suggests that the mechanism of action of daunorubicin enhancement by cyclosporin A in drug-sensitive tumors is not simply the result of increased intracellular daunorubicin accumulation.

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MATERIALS AND METHODS

Tumor Lines and Treatment Regimens. EA is maintained as an ascitic tumor in BALB/c mice as previously described (6). H-129 was obtained from the DCT tumor repository, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD, and grown as an ascitic tumor in C3H/HetN mice (Simonsen, Gilroy, CA). The tumor is maintained by weekly injections of 0.2 ml of undiluted ascites. Tumor cells are harvested 7-10 days after inoculation for in vitro studies. The treatment regimen consists of i.p. injections of DNR with or without CsA (10 mg/kg) beginning 24 h after the inoculation of 0.2 ml undiluted malignant ascites. The significance of difference in mean survival time is calculated by Student's t test (two-sided). The frequency of survival of tumor-bearing animals at 30 and 60 days is compared between DNR and DNR plus CsA treatment groups by x^2 analysis.

Daunorubicin Inhibition of [3H]Thymidine Incorporation. Nucleotide incorporation studies were performed by the following method as previously described (6). Cells were washed, counted on a hemacymometer using dye exclusion, and resuspended in RPMI 1640 at a concentration of 2.5 X 10^6/ml. Cell aliquots of 1.6 ml were incubated with 0.2 ml DNR HCl (final concentration, 0-18 |m|) and 0.2 ml CsA (final concentration, 0-10 |m|) for 1 h at 37°C water bath, washed twice, and resuspended in 1.8 ml RPMI. Triplicate 180-±l aliquots were plated into microtiter plates and incubated with 20 |m| [3H]thymidine (specific activity, 24 Ci/mmol; final concentration, 1 |m|Ci/ml) for 1 h at 37°C in 5% CO2. Samples were collected on glass fiber filters with a Tintertek multiple automated sample harvester unit using a deionized water wash. The filters were dried and counted in a PPO/POPPOIs liquid scintillation system. Results are expressed as a comparison of [3H]thymidine cpm in DNR containing cultures to [3H]thymidine cpm of the respective control. The daunorubicin 50% effective dose is defined as that concentration of DNR required to inhibit 50% of [3H]thymidine incorporation. In this system 2.5 and 10 |m| CsA inhibit thymidine incorporation by less than 20%.

Daunorubicin Transport Studies. [3H]Daunorubicin transport studies were performed by the following methods as previously described (7). For the determination of daunorubicin accumulation, tumor cells were resuspended at 2 X 10^6 cells/ml in RPMI 1640. Daunorubicin was added in the presence or absence of 3 |m| CsA to give a final total concentration of 0.9 |m| [3H]DNR (New England Nuclear; specific activity, 2.5 Ci/mmol) and 8.0 |m| unlabeled DNR. The cell suspensions were incubated for 120 min at 37°C. Triplicate 200-±l aliquots were plated into microtiter plates at times indicated and harvested immediately on glass fiber filters. For DNR efflux, 6-ml aliquots were removed from the RPMI 1640 cell suspensions after 35 min, centrifuged, washed, and resuspended in DNR-free RPMI 1640. Suspensions were further incubated and harvested at time points up to 90 min as described. This technique produces less than 5% nonspecific binding of [3H]DNR to the glass fiber filters.

Glucose-free buffer consisting of 57 mm NaCl, 5 mm KCl, 1.3 mm MgSO4, 9 mm NaH2PO4, and 51 mm Na2HPO4 containing 10 mm sodium azide (an oxidative phosphorylation inhibitor) was used to determine modification of DNR transport by tumor cells due to CsA without the influence of active outward transport (8). For determining the influence of active efflux on drug accumulation, cell suspensions were preincubated on ice for 10 min in glucose-free sodium azide containing buffer prior to exposure to [3H]DNR in the presence or absence of 3 |m| CsA.

RESULTS

Fig. 1, representative of three similar experiments, shows progressive enhancement of DNR inhibition of thymidine incorporation by 0.1-2.5 |m| CsA in both EA and H-129. From this experiment it can be seen that the enhancing effects of 2.5 and 10 |m| CsA are similar. Statistical comparison of the DNR 50% effective dose values for these three experiments in the presence of CsA versus 2.5 and 10 |m| CsA reveals P < 0.05 and < 0.02, respectively, for H-129. When the formula is used to derive a CsA index for enhancement of DNR effect in vitro, we find that 2.5 |m| CsA enhances 0.9 |m| DNR
ENHANCEMENT OF DAUNORUBICIN BY CYCLOSPORIN

Fig. 1. CsA effects on DNR 50% effective dose values in Ehrlich ascites carcinoma (A) and hepatoma 129 (B). Values are plotted as percentage of inhibition of 
[3H]thymidine incorporation compared with control in the absence of DNR in the absence of CsA (○) or in the presence of 0.1 μM (●), 1.0 μM (□), 2.5 μM (●), or 10 μM (♦) CsA.

Table 1: Cyclosporin enhancement of mean and long survival in host mice bearing drug-sensitive tumor

Groups of host mice bearing drug-sensitive Ehrlich ascites carcinoma or Hepatoma 129 were treated with the indicated daunorubicin regimen starting 24 h after inoculation of 0.2 ml malignant ascites.

<table>
<thead>
<tr>
<th>Daunorubicin regimen (mg/kg)</th>
<th>Mean survival time ± SD</th>
<th>Long survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CsA -</td>
<td>+</td>
</tr>
<tr>
<td>Ehrlich ascites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>18.0 ± 0.9</td>
<td>20.7 ± 6.9</td>
</tr>
<tr>
<td>0.2 for 1 day</td>
<td>23.1 ± 10.7*</td>
<td>35.9 ± 14.7</td>
</tr>
<tr>
<td>0.2 for 2 days</td>
<td>22.7 ± 3.3*</td>
<td>41.5 ± 15.7</td>
</tr>
<tr>
<td>0.3 for 2 days</td>
<td>42.3 ± 11.5*</td>
<td>54.1 ± 9.2</td>
</tr>
<tr>
<td>Hepatoma 129</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>21.9 ± 0.9</td>
<td>24.1 ± 1.5</td>
</tr>
<tr>
<td>0.2 for 2 days</td>
<td>25.2 ± 3.2*</td>
<td>35.0 ± 6.0</td>
</tr>
<tr>
<td>0.3 for 2 days</td>
<td>25.7 ± 2.7*</td>
<td>34.0 ± 8.8</td>
</tr>
<tr>
<td>0.4 for 2 days</td>
<td>27.5 ± 4.7*</td>
<td>37.0 ± 7.4</td>
</tr>
</tbody>
</table>

* P > 0.1 untreated control.

** P < 0.01 versus untreated control.

inhibition of DNA synthesis by a factor of 1.10 ± 0.15 and 9.0 μM DNR inhibition of DNA synthesis by a factor of 1.87 ± 0.37. Addition of 10 μM CsA enhances this DNA concentration by factors of 1.10 ± 0.12 and 2.05 ± 0.68, respectively. We have previously noted that this in vitro assay correlates well with in vivo survival (5, 6). Table 1 presents the statistical evaluation of mean and long-term survival of BALB/c mice bearing sensitive EA and C3H/HeN mice bearing sensitive H-129 when treated with various dose regimens of DNR with and without CsA. CsA alone has a slight effect on H-129. Significant benefit on mean survival is noted in all groups of mice treated with DNR-CsA versus DNR alone. The addition of CsA to the lower DNR regimen is effective in producing 30-day survival, whereas the addition of CsA to the 0.3 mg/kg 2-day DNR regimen enhances its ability to produce 60-day survival in EA.

Fig. 2, also representative of three similar experiments, compares [3H]DNR uptake and [3H]DNR retention by EA and H-129 tumor cells in the presence and absence of 3 μM CsA. CsA has a slight but reproducible enhancing effect on [3H]DNR uptake and fails to modify initial drug retention by either tumor. In order to further investigate any possible CsA modification of active outward [3H]DNR transport we performed similar [3H]DNR uptake studies under conditions of interrupted active transport. Fig. 3 shows that CsA enhancement of [3H]DNR uptake persists in glucose-free, sodium azide-containing buffer in both EA and H-129.

DISCUSSION

Our current studies evaluate the interaction of CsA with DNR in drug-sensitive tumors. Although Ehrlich ascites carcinoma is inherently more sensitive to DNR than H-129, we note that CsA enhances the efficacy of DNR in vitro and in vivo in both tumors. In vitro CsA produces slight but reproducible enhancement of DNR uptake in these cell lines. This effect occurs without alteration of DNR efflux in the presence of intact or impaired active membrane transport. CsA alone has minimal in vivo activity against H-129. We previously noted that a 4-fold greater CsA concentration also has minimal in vivo activity against EA (5). CsA alone fails to produce 30- or 60-day survival in either tumor but when combined with DNR produces important potentiation of DNR efficacy in vivo. The addition of CsA to DNR significantly enhances the mean and long-term survival of host mice with these tumors. Of particular interest is that the addition of CsA to DNR converts ineffective regimens of DNR against each tumor to regimens effective in

6217
ENHANCEMENT OF DAUNORUBICIN BY CYCLOSPORIN

producing 30-day survival, whereas the addition of CsA to the highest DNR regimen enhances its ability to produce 60-day survival in Ehrlich ascites carcinoma. This observation extends to human neoplasia as demonstrated by CsA enhancement of adriamycin activity against xenografted human testicular cancer recently reported by Scheulen and Osieka (9).

Cyclosporin A, a cyclic endecapeptide isolated from the fungal species Tolypocladium inflatum, has been found to possess potent immunosuppressive activity. Although its mechanism of action is incompletely understood, CsA exerts its immunosuppressive activity at least in part through its action on interleukins 1, 2, and 3 (10).

We have previously noted CsA reversal of vincristine and daunorubicin resistance in pleiotropic drug-resistant T cell acute lymphatic leukemia in vitro and daunorubicin resistance in Ehrlich ascites carcinoma in vivo (5, 7). In contrast to the reported mechanism of action of reversal of drug resistance by calcium channel blockers and calmodulin inhibitors, we have been unable to demonstrate that CsA alters initial or early drug transport in drug-resistant tumor cells in these studies (1, 2). However, Koletsky has noted marked enhancement of cellular adriamycin uptake with prolonged exposure to adriamycin by CsA drug-resistant as opposed to drug-sensitive cell lines (personal communication). Therefore additional mechanisms of action of CsA in reversal of pleiotropic drug resistance deserve investigation.

It has been shown that DNR can exert an antineoplastic effect at the level of the tumor cell membrane (11, 12). CsA, a strongly lipophilic agent, partitions into phospholipid vesicles, interferes with plasma membrane phospholipid metabolism, and depolarizes cytoplasmic membrane potentials (13–16). The results of our studies on the effect of CsA on membrane potentials in CsA reversal of anthracycline resistance relates to its action at the cell membrane. We found that DNR-resistant murine and human acute lymphatic leukemia cells have significantly lower resting membrane potentials relative to their drug-sensitive counterparts. The addition of CsA to these drug-resistant sublines results in the restoration of their membrane potentials to that characteristic of drug-sensitive parental tumors (17).

The mechanism of action of CsA enhancement of DNR efficacy in drug-sensitive experimental tumors remains to be completely elucidated. Recent studies by Saydjari et al. show that CsA alone suppresses H2T pancreatic cancer cell growth in vitro (18). This study suggests that CsA suppression may relate to inhibition of polyamine biosynthesis necessary for metabolism and function of nucleic acids. Inhibition of H2T cell growth requires prolonged in vitro exposure to CsA, suggesting a mechanism of action different from the CsA enhancement of DNR efficacy we observe in short-term exposure to CsA in vitro or the minimal prolongation of survival noted with CsA alone in host mice with hepatoma 129. Our studies demonstrating slight increases in membrane permeability to daunorubicin suggest that CsA potentiates DNR cytotoxicity in drug-sensitive as well as drug-resistant tumors by its interaction with DNR at the level of the cell membrane.
ENHANCEMENT OF DAUNORUBICIN BY CYCLOSPORIN

Since the doses of CsA utilized in our in vivo experiments are in a range which can be administered to humans with reasonable safety, we previously suggested that CsA might be successfully incorporated into treatment regimens for drug-resistant neoplasia (7). It now appears that this approach will prove beneficial in the initial therapy of patients with anthracycline antibiotic-sensitive tumors.

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We wish to thank Dr. Harry Wallerstein for his continuous encouragement.

REFERENCES

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