Modulation in vitro and in vivo of ACNU resistance in a subline of C6 glioma with reserpine

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Reserpine enhanced in vitro the cytotoxicity of 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) in both the C6 glioma and its ACNU-resistant subline, C6/ACNU. Reserpine also enhanced the chemotherapeutic effect of ACNU in C6/ACNU-bearing (C6/ACNU-meningeal gliomatosis) rats, in which ACNU resistance could be modulated by combined ACNU and reserpine therapy.

When 10 μM reserpine was added to ACNU in culture, the concentration of drug required for 50% inhibition of cell growth (IC50) of ACNU for C6/ACNU cells decreased to the level of that for C6 cells. When 20 μM reserpine was added to the culture, intracellular uptake of ACNU in C6/ACNU cells increased further and the efflux of the drug from the cells decreased. In in vivo experiments in rats, combined chemotherapy with ACNU (1 mg/kg) and reserpine (250 μg/kg) by intrathecal injection significantly increased the life span of the rats as compared to results with ACNU chemotherapy alone. The enhanced cytotoxicity of ACNU in ACNU-resistant glioma cells in vitro and in vivo may be explained by the increase of intracellular concentration of ACNU resulting from the inhibition of ACNU efflux from the resistant cells by reserpine. It was concluded that ACNU resistance could be modulated in vitro and in vivo by combined therapy with ACNU and reserpine.

KEY WORDS • ACNU • reserpine • glioma • drug resistance • rat

A NITROSOUREA derivative, 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) is widely used with other nitrosourea derivatives in treating brain tumors because it crosses the blood-brain barrier, which constitutes a serious problem in the chemotherapy of brain tumors.8 The development of this drug and other nitrosourea derivatives has advanced the chemotherapy of brain tumors. However, drug resistance, especially to ACNU, has become an issue in the treatment of brain tumors,11 and consideration of countermeasures is required. While pursuing studies of drug resistance in malignant glioma,6,7,9,11 we found enhancement of the cytotoxicity of ACNU on ACNU-resistant glioma cells with reserpine administration.6 In this communication, the enhanced cytotoxicity of ACNU combined with reserpine on ACNU-resistant glioma cells in vitro and in vivo is described.

Materials and Methods

Tumor Cultures

Male Wistar rats, each weighing 100 gm, were used for tumor production. A subline of C6 glioma resistant to ACNU (C6/ACNU) was developed by treating Wistar rats in which 1 × 107 of C6 cells were transplanted percutaneously into the cisterna magna with ACNU over successive transplant generations, as described previously.11 Both C6 and C6/ACNU cells were cultured in Falcon No. 3024 culture bottles in Eagle’s minimum essential medium (MEM), supplemented with 10% heat-inactivated fetal bovine serum, 10 μM 2-mercaptoethanol, penicillin base (50 U/ml), and streptomycin base (50 μg/ml). Stock cultures were incubated at 37°C in a humidified atmosphere supplied with 5% CO2. The cells were subcultured twice and then used for experiments. There was essentially no change in drug sensitivity or ACNU resistance during the experiments.

Cytotoxicity Assay

Culture medium (1 ml) containing 1 × 104 C6 and C6/ACNU cells/ml was transferred to Falcon No. 3047 plates. Three wells were used for each drug concentration. The cells were incubated at 37°C in a humidified atmosphere. The cultures were incubated at 37°C in a humidified atmosphere supplied with 5% CO2. The cells were subcultured twice and then used for experiments.

* Eagle’s minimum essential medium, fetal bovine serum, and penicillin and streptomycin base obtained from Grand Island Biological Co., Grand Island, New York; 2-mercaptoethanol obtained from Sigma Chemical Co., St. Louis, Missouri.
TABLE 1

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>IC50 of ACNU (μM ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>C6</td>
</tr>
<tr>
<td>control</td>
<td>26.8 ± 4.2</td>
</tr>
<tr>
<td>reserpine, 10 μM</td>
<td>13.2 ± 2.9†</td>
</tr>
<tr>
<td>reserpine, 20 μM</td>
<td>10.0 ± 2.3†</td>
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</tbody>
</table>

* The cells were treated with ACNU in the absence (control) or presence of reserpine at 10 and 20 μM. The cytotoxic activity of ACNU was measured by determining the concentration of drug required for 50% inhibition of cell growth (IC50), which was obtained by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells. SD = standard deviation. Statistical difference compared with control by Student's t-test: † p = 0.05; ‡ p = 0.001.

For the efflux experiments, the culture supernatants were aspirated off, and the cells were preloaded with 14C-ACNU (32.3 μM) for 3 hours in the absence or presence of reserpine (20 μM). The culture medium was aspirated off, the cells were washed with cold PBS, and the culture medium was replaced by 1 ml of the new medium with or without reserpine (20 μM). At various times, the culture medium was again aspirated off and the cells were washed with PBS. The adherent cells were then lysed and the radioactivity was measured in the same manner as above. Results were expressed in terms of the percentage of the radioactive drug retained; 100% values were obtained from the amounts of the retained drug at 3 hours after preincubation.

Evaluation of Antitumor Activity

One-tenth ml of diluted culture medium containing 1 × 107 C6 or C6/ACNU cells was transplanted into the cisterna magna of Wistar rats to produce brain-tumor models; these are referred to here as "meningeal gliomatosis (MG)" rats. Reserpine and ACNU were dissolved in 0.9% NaCl solution. Except as otherwise indicated, both drugs were mixed, and the mixture was administered intrathecally at a constant rate of 0.1 ml/100 gm body weight to the MG rats 1 day after tumor inoculation. Reserpine was given in a dose of 250 μg/kg and ACNU in a dose of 1 mg/kg. Antitumor activity was expressed as follows: the mean survival time of treated rats divided by the mean survival time of control rats (T/V). Ten rats were used for each experimental group.

Results

Enhanced Cytotoxicity of ACNU by Reserpine

The sensitivity of C6 and C6/ACNU cells to ACNU and the effect of reserpine on the sensitivity are illustrated in Fig. 1 and Table 1. The mean IC50 of ACNU
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for C6 and C6/ACNU cells was 26.8 and 388 μM, respectively. Reserpine at a nontoxic dose of 10 and 20 μM greatly enhanced the cytotoxicity of ACNU for C6 and especially for C6/ACNU cells. When reserpine was added at a final concentration of 10 μM to C6/ACNU cell cultures, the mean IC50 of ACNU changed from 388 to 25.9 μM. This value was almost the same as the IC50 (26.8 μM) of ACNU for C6 cells in the absence of reserpine. In the presence of 20 μM reserpine, the mean IC50 of ACNU for C6 and C6/ACNU cells was 10.0 and 8.1 μM, respectively.

Uptake and Efflux Studies

Cellular uptake of 14C-ACNU by C6 and C6/ACNU cells is presented in Fig. 2. Uptake of 14C-ACNU into cultured C6 cells increased with time of exposure to the drug. Approximately 3.2 pmol of ACNU was found at 5 hours in 10^5 C6 cells, while the amount of ACNU in C6/ACNU cells was much smaller and the level almost reached a plateau (0.8 pmol/10^5 cells) 1 hour after incubation. There was a fourfold difference in uptake between the two cell lines by 5 hours (Fig. 2).

Efflux experiments consistently showed a difference in the rates of efflux of the radiolabeled drug (Fig. 3). Retention of 14C-ACNU in C6/ACNU cells rapidly decreased with time, and about 99.5% of intracellular ACNU was lost from the cells at 5 hours, while almost 20% of intracellular ACNU was retained in C6 cells at 5 hours.

Effect of Reserpine on Transport of ACNU

Reserpine (20 μM) added to the culture greatly increased the amount of intracellular ACNU in both C6 and C6/ACNU cells (Fig. 2). Approximately fourfold accumulation of ACNU occurred in reserpine-treated C6/ACNU cells during 4 to 5 hours of incubation, while only 1.5 times the amount of ACNU was found in C6 cells treated with reserpine (Fig. 2).

The efflux of intracellular ACNU from C6/ACNU cells was significantly inhibited by reserpine, as shown in Fig. 3. At 5 hours after incubation of C6/ACNU cells with reserpine, almost 15% of the initial amount of ACNU still remained in the cells, while about 99.5% of intracellular ACNU was lost from C6/ACNU cells incubated without reserpine. The efflux of ACNU from C6 cells was also slightly inhibited by reserpine.

Combined Effect of ACNU and Reserpine In Vivo

When ACNU (1 mg/kg) was administered 1 day after the tumor inoculation, the life span (T/C value 135%) of C6 glioma-bearing (C6-MG) rats increased (Fig. 4 left and Table 2). Reserpine administered at 250 μg/kg with ACNU (1 mg/kg) further increased the life span (T/V value 112%) of C6-MG rats, although reserpine (250 μg/kg) alone showed no therapeutic effect. Neither ACNU alone nor reserpine alone given according to the schedule above had any therapeutic effect on C6/ACNU glioma-bearing (C6/ACNU-MG) rats. However, ACNU (1 mg/kg) administered with reserpine (250 μg/kg) to C6/ACNU-MG rats significantly increased the life span of the rats (T/C and T/V values
Fig. 4. Survival curves of rats with meningeal gliomatosis treated once intrathecally (it) with ACNU, reserpine, or a combination of ACNU and reserpine 1 day after intracisternal inoculation of $1 \times 10^7$ C6 (left) or C6/ACNU (right) cells. n = number of rats in each group.

142% and 143%, respectively) (Fig. 4 right and Table 2). Although this T/C value is slightly lower than that (150%) obtained in C6-MG rats treated with ACNU and reserpine, ACNU resistance was modulated in C6/ACNU-MG rats when ACNU was given with reserpine.

Discussion

In the present study, reserpine enhanced the cytotoxicity of ACNU in both C6 and C6/ACNU cells and could modulate ACNU resistance in vitro (Fig. 1).

Table 2

<table>
<thead>
<tr>
<th>Drug &amp; Dosage</th>
<th>Median Survival Time (days) ± SD (days)</th>
<th>T/C (%)</th>
<th>T/V (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6 Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>14.0</td>
<td>14.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>reserpine (250 µg/kg)</td>
<td>12.5</td>
<td>12.7 ± 2.7</td>
<td>90</td>
</tr>
<tr>
<td>ACNU (1 mg/kg)</td>
<td>19.5</td>
<td>19.0 ± 2.8</td>
<td>125†</td>
</tr>
<tr>
<td>ACNU (1 mg/kg) + reserpine (250 µg/kg)</td>
<td>22.0</td>
<td>21.2 ± 3.7</td>
<td>150‡ 112</td>
</tr>
<tr>
<td>C6/ACNU Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>14.5</td>
<td>14.2 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>reserpine (250 µg/kg)</td>
<td>13.5</td>
<td>13.8 ± 2.3</td>
<td>97</td>
</tr>
<tr>
<td>ACNU (1 mg/kg)</td>
<td>14.0</td>
<td>14.1 ± 3.0</td>
<td>99</td>
</tr>
<tr>
<td>ACNU (1 mg/kg) + reserpine (250 µg/kg)</td>
<td>19.5</td>
<td>20.2 ± 2.9</td>
<td>142‡ 143§</td>
</tr>
</tbody>
</table>

* Each group of Wistar rats was given intracisternal implants of $1 \times 10^7$ C6 or C6/ACNU cells on Day 0, and drugs were given intrathecally on Day 1. T/C = mean survival time of treated rats/mean survival time of control rats; T/V = mean survival time of treated rats/mean survival time of rats treated with ACNU alone; SD = standard deviation.

† Statistically significant (p < 0.05) by Student’s t-test as compared with that of the control group.
‡ Statistically significant (p < 0.05) by Student’s t-test as compared with that of the group treated with ACNU alone.

Reserpine also enhanced the chemotherapeutic effect of ACNU in C6/ACNU-MG rats (Fig. 4 right and Table 2). At a nontoxic dose of reserpine (20 µM), the cellular level of ACNU in C6/ACNU cells increased to almost the same extent as that in C6 cells (Fig. 2). Actually, in in vitro experiments, the sensitivities of C6 and C6/ACNU cells to ACNU were almost equal when 10 µM reserpine was added to the culture (Fig. 1 and Table 1). In vivo experiments, ACNU administered with reserpine to C6-MG rats did not significantly increase the life span of the rats compared to administration of ACNU alone, while the combined therapy significantly increased the life span of C6/ACNU-MG rats. It is interesting to speculate why this discrepancy in the effect of ACNU between in vitro and in vivo studies occurred, since reserpine enhanced the cytotoxicity of ACNU in both C6 and C6/ACNU cells in vitro. These facts suggest that the statistically significant differences observed in vitro do not correlate with results obtained in vivo because of the complicated pathophysiology in vivo and, perhaps, the existence of a blood-brain barrier in the brain tumors, as has been stressed in our previous communications.8,9

The cellular concentration of ACNU in C6/ACNU cells was approximately one-fourth that found in C6 cells, and the efflux of intracellular ACNU from C6/ACNU cells occurred more rapidly and completely than that from C6 cells. Inaba, et al.,2 reported that the mechanism of anthracycline resistance is the active efflux of the intracellular drug from the resistant sublines of P388 leukemia cells. Their findings are consistent with the results of the present experiment, as well as with the other observations previously reported by us.7,9 This led us to postulate that the decrease of intracellular uptake of ACNU in C6/ACNU cells results from an increase in efflux of the drug from the resistant cells, and that it might be possible to modulate drug resistance with drugs that inhibit the efflux from the cells. Among a number of membrane-modifying agents, calcium antagonists4 and reserpine6 were found to in-
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Hibit the efflux of the drug from the resistant cells. Although the mechanism involved in the enhancement of chemotherapeutic agents by reserpine has not been elucidated, it is well known that reserpine releases calcium from the cell membranes. Recently, Tsu"ro, et al., reported that the mechanism of drug resistance is due to both the reduced uptake of the drug and the increased efflux of the intracellular drug from the resistant cells, demonstrating the intracellular accumulation of vincristine in vincristine-resistant K562 leukemia cells by calcium antagonists. They stressed in that communication that the mechanism of drug resistance is profoundly related to the metabolism of calcium in the cell membranes. The results of the present study might be supported by their findings, if reserpine acts on the membrane as a calcium antagonist as previously reported. On the other hand, Koshiura, et al., discovered the enhanced effect of the chemotherapeutic agent, 1-(gamma-chloropropyl)-2-chloromethylpyrimidine hydrochloride (CAP-2), on AH-13 and AH-44 cells caused by reserpine, and reported that the mechanism of the enhancement of the drug is the inhibition of the damage-repairing deoxyribonucleic acid (DNA). This is in accordance with the observations made by Bodell, et al., who reported that BCNU resistance in BCNU-resistant 9L glioma cells is related to the increased damage-repairing capability of DNA in resistant cells.

There may be at least two mechanisms to explain ACNU resistance as stated above. It is suggested that the mechanism of ACNU resistance is related to both the increase of efflux and the increase in damage-repairing capability of DNA. It is difficult to explain drug resistance by a single mechanism alone, since drug resistance differs depending on the type of tumor and the drug. However, the enhanced effect of ACNU in vivo when reserpine is present, as described here, may suggest directions in the study of drug resistance, and bring about modulation of resistance to ACNU with such drugs as reserpine and calcium antagonists.

Acknowledgments

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References


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