Synergistic Effects of Hyperthermia and Intratumorous Injection of Anti-Cancer Drugs

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(Received November 22, 1993; Accepted December 10, 1993)

In an attempt to improve the combined effects of hyperthermia and anti-cancer drugs, an intratumorous (i.t.) injection of the drugs was performed and its effect compared with that obtained by intraperitoneal (i.p.) injection. Using Lewis lung carcinoma growing in the legs of BDF$_1$ mice, weakly toxic drug derivatives, Aclarubicin (ACR), a new platinum complex (DWA2114R), or Peplomycin (PEP) were injected either into the center of the tumors, or intraperitoneally, before or after usual hyperthermia in a 43.5-43.7°C water bath for 45 min. The effects on tumor growth delay and the number of lung metastases were assessed, and the enhancement ratios (ERs) due to the combination were calculated.

Tumor growth inhibition by i.t. injection was enhanced additively with ACR (ER: 1.2) and synergistically with DWA2114R (ER: 3.49) and PEP (ER: 2.4) plus hyperthermia. Hyperthermia after i.t. injections of DWA2114R (ER: 3.4) was more effective than either i.t. or i.p. injections after hyperthermia (ER: 2.4). Lung metastases were also inhibited significantly by the combination of hyperthermia and drugs, except when emulsified PEP was injected three times.

It was concluded that the i.t. injection of DWA2114R was of value when used in combination with hyperthermia.

(Key Words: Hyperthermia, Anti-cancer drugs, Tumor growth, Lung metastasis, Intratumorous injection.)

INTRODUCTION

There are some encouraging clinical reports on the combination of local hyperthermia with chemotherapy in treating cancer (4, 7, 19). However, even with this combination, it is still difficult to control advanced tumors. The combined effects are related to several physiological factors, such as pharmacokinetics, drug concentration, oxygen tension, tumor pH, etc. (10). Among these factors, the low accumulation of drugs in tumors and the time of exposure of the tumor cells are important (3, 8). Some drugs disperse rapidly from the serum and clinically intolerable doses are necessary to obtain synergism with hyperthermia (10). To overcome this problem, we have investigated less toxic drug derivatives which we administered by intratumorous injection (i.t.). It has been reported that i.t. injection of anti-cancer drugs inhibits tumor growth more effectively than intraperitoneal (i.p.) injection (2, 17).

The control of distant metastasis is also important for success. It has been reported that local hyperthermia, with or without anti-cancer drugs, inhibits lung metastases in Lewis lung carcinoma (14, 15, 16, 21). However, it is not known whether intratumoral injection of the anti-cancer drugs will act synergistically with hyperthermia to inhibit distant metastases.

In this study, we monitored the effects of hyperthermia and i.t. injection of anti-cancer drugs on tumor growth and lung metastases. The drugs used were Aclarubicin (ACR), a new platinum complex (DWA2114R), and Peplomycin (PEP), both of which have shown synergism with hyperthermia in cell survival assays (5, 6, 9, 12, 13, 16).

MATERIALS AND METHODS

Animals and tumors
 Male C57B1/6 and female BDF$_1$ mice, purchased from Charles River Japan (Atsugi, Japan), were used throughout. Groups of 8 to 10 mice were housed in plastic cages, and pro-
vided a pellet diet (CE-2; Clea Japan, Tokyo) and tap water ad libitum. Lewis lung carcinoma was maintained by subcutaneous transfer to 5 week-old C57B1/6 mice. After preparing a suspension of tumor cells as described previously (18), 2 to 3 x 10^5 cells were injected into the muscle of the right hind leg of 5 week-old BDF1 mice. After the tumors became palpable in 5 to 6 days, their diameters were measured by calipers three times/week. Tumor volumes were calculated by the following equation: (3.14/6) x tumor length x width x thickness. When the mean tumor-volume surpassed 100 mm^3, drug injections and hyperthermic procedures were performed.

Hyperthermia

Mice were put into special jigs designed for hyperthermic treatment. The tumor-bearing legs were pulled through a window in the jigs and fixed at the ankle joint by clips and tape. The legs of the mice were immersed in a 43.5-43.7°C water bath for 45 min. The temperature of the water bath was controlled to ±0.1°C with a thermoregulator (Thermo-minder; Taiyo Scientific Industrial Co., Tokyo). Temperatures were measured by an IT-18 thermometer with a copper-constantan thermocouple (Bailey Instruments Co., Inc., Saddle Brook, NJ). The tumor temperature reached a maximum 3 min after beginning hyperthermia and was kept 0.1°C lower than the temperature of the water bath. Hyperthermia at 43.7°C for 45 min elevated body temperature of mice less than 1.0°C. An electric fan was used during hyperthermia in order to blow away steam and to prevent elevation of the body temperature of the mice.

Drugs and administration

Anti-cancer drugs, 0.05 ml/mouse, were injected into the center of the tumors through a 27-gauge needle. The i.p. injection was carried out 30 min before hyperthermia, whereas the i.t. injections were either 5 or 30 min before, or 30 min after hyperthermia. The following drugs were administered at rates 10 to 30 times the clinical dose, which delayed Lewis lung carcinoma growth 1 to 2 days (Table 1).

ACR: Acalrubinic (Yamanouchi Pharmaceutical Co., Ltd., Tokyo), was given at a dose of 10 mg/kg. The 50% minimum lethal dose (LD_{50}) for mice is 30 mg/kg.

DWA2114R: (-)-(R)-2-aminomethylpyrroli- dine (1,1-cyclobutanedicarboxylato) platinum (II) monohydrate (Chugai Pharmaceutical Co., Ltd., Tokyo), was given at a dose of 60 mg/kg. The LD_{50} is 155 mg/kg.

PEP: Peplomycin (Nippon Kayaku Co., Ltd., Tokyo), was given at a dose of 10 mg/kg singly or 4 mg/kg three times. The LD_{50} is 76 mg/kg. PEP emulsion: 72% olive oil, 6.4% Sorbitan sesquioleate (Nikkol SO-15; Nikko Chemical Co., Ltd., Tokyo), 1.6% Polyxyethylene hydrogenated caster oil (Nikkol HCO-60: Nikko Chemical Co., Ltd.), and 20% PEP were mixed and given at the same dose as PEP.

Assays

Tumor growth curves were obtained from the mean tumor volumes. Tumor growth time, from the day of treatment to the day the tumor volume reached 1.5 cm^3, was compared with tumor growth and growth delay time (GD) in the control mice. The combined effect was calculated as follows: Enhancement ratio (ER)=((GD by the combined treatment)/(GD by hyperthermia alone)+GD by drug alone). An ER about 1.0 meant an additive effect, while an ER over 1.0 meant synergism. Also, the effects on tumor volume were compared between all groups using Student's t test.

Metastatic lung colonies were observed at autopsy 18 days after tumor implantation. The number of colonies was classified into 4 or 5 categories (micro; questionable small nodules, <0.5 mm, <1.0 mm, <2.0 mm, and >2.0 mm in diameter). The mean or median number of each colony was plotted according to the estimated initial development-time reported previously (18). Briefly, the time was estimated from the growth rate of the colony and the colony size at autopsy, i.e. the smaller size of colonies meant the later development of metastases, and the large ones indicated earlier development. The total number of colonies was compared between groups and statistical differences were calculated by Student's t test. The metastases inhibition ratio (MIR) for each treatment group was calculated as follows: MIR=(total number of colonies in control group)/(total number of colonies in treatment group).
RESULTS

ACR

Figure 1 shows the growth curves (upper) and metastatic curves (lower) of Lewis lung carcinoma treated with hyperthermia and/or ACR. Hyperthermia of 43.7°C for 45 min had little effect whereas i.t. injection of 10 mg/kg ACR significantly inhibited tumor growth. The combined effect was additive and the enhancement ratio was 1.2 (Table 1). There was no significant difference between the pre- and post-hyperthermia injections. The number of metastases was inhibited by both hyperthermia and by ACR (Fig 1, Table 1). The combined treatment further inhibited lung metastases (P<0.05 compared with either ACR or hyperthermia).

DWA2114R

The i.t. injection of 60 mg/kg DWA2114R inhibited tumor growth and had a greater effect than i.p. injection (p<0.05) (Fig. 2a, Table 1). When hyperthermia was combined with DWA2114R, the effect was synergistic (ER; 2.4-3.4). Injections prior to hyperthermia had a stronger effect than hyperthermia applied prior to injection (p<0.05). Both hyperthermia and DWA2114R reduced the number of lung metastases significantly (Fig. 2b, Table 1) When combined, the number of metastases was further reduced. The pre-hyperthermia injections significantly inhibited lung metas-

Table 1. Effect of drugs and hyperthermia on tumor growth delay and metastases of Lewis lung carcinoma in mice.

<table>
<thead>
<tr>
<th>Treatment Drugs (dose)</th>
<th>Hyperthermia (times)</th>
<th>GD&lt;sup&gt;a&lt;/sup&gt; (days)</th>
<th>MIR&lt;sup&gt;c&lt;/sup&gt; Hyperthermia</th>
</tr>
</thead>
<tbody>
<tr>
<td>no drug</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACR(10) i.t. → Hy</td>
<td></td>
<td>0.0</td>
<td>2.0&lt;sup&gt;+1&lt;/sup&gt;</td>
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<tr>
<td>ACR(10) i.t. → Hy</td>
<td></td>
<td>0.3</td>
<td>2.7&lt;sup&gt;+3&lt;/sup&gt;</td>
</tr>
<tr>
<td>no drug</td>
<td></td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>DWA(60) i.p. → Hy</td>
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<td>1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWA(60) i.t. → Hy</td>
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<td>1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>DWA(60) i.t. → Hy</td>
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<td>3.8&lt;sup&gt;+&lt;/sup&gt;</td>
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<td></td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>PEP(10) i.p. → Hy</td>
<td></td>
<td>1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEP(10) i.t. → Hy</td>
<td></td>
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<td>3.1&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
<td>0.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.1&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEP-E(10) i.t. → Hy</td>
<td></td>
<td>1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.3&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>no drug</td>
<td></td>
<td>0.0</td>
<td>1.3&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEP(4) i.p. → Hy</td>
<td>× 3</td>
<td>0.2</td>
<td>3.7&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEP(4) i.t. → Hy</td>
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<td>1.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
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<td>× 3</td>
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<td>4.6&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Emulsion i.t. → Hy</td>
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<td>0.0</td>
<td>1.2&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup>GD: Growth delay for treatment groups compared with control group; tumor volume 1500 mm<sup>3</sup>
<sup>b</sup>ER: Enhancement ratio by combined treatment=(GD for combined treatment)/(GD for hyperthermia alone+GD for drug alone).
<sup>c</sup>MIR: Metastasis inhibition ratio=([total number of colonies in control group]/[total number of colonies in treatment group])− or → indicates the sequence of drug injection and hyperthermia.

*<sup>1</sup>p<0.05; <sup>2</sup>p<0.01; <sup>3</sup>p<0.001 ns; not significant
tases, compared with post-hyperthermia injections (p<0.05).

PEP
The i.t. injection of 10 mg/kg PEP or PEP emulsion inhibited tumor growth as well as the i.p. injection (Table 1). PEP combined with hyperthermia synergistically inhibited tumor growth, and the PEP emulsion appeared more effective than PEP (p<0.05). The effect of the i.t. injections of PEP did not differ significantly from that of the i.p. injections. The order of administration of hyperthermia and the i.t. injections made no difference in the degree of growth inhibition. The inhibition of lung metastases by the i.t. injection of PEP was statistically significant, but that of the PEP emulsion was not. The combined treatment significantly inhibited metastasis. Three i.t. injections of 4 mg/kg of either PEP or PEP emulsion inhibited tumor growth, but the i.p. injections had no effect (Table 1). All combined therapeutic regimes inhibited tumor growth synergistically without much difference (Fig. 3). Metastases were also inhibited synergistically by the i.t. or i.p. injections of PEP. However, PEP emulsion, either alone or combined with hyperthermia, did not result in a significant reduction in the number of metastases. Pure emulsion, with or without hyperthermia, tend-
that DWA2114R, as well as cis-DDP, exhibit synergism in vitro when combined with hyperthermia (6, 9). Previously, we reported that the i.p. injection of 55.6 mg/kg DWA2114R, followed by hyperthermia in vivo, is additive (16). In the present study, we found that the i.t. injection of 60 mg/kg DWA2114R showed synergism with hyperthermia. Urano has reported the rapid disappearance of cis-DDP from plasma and the necessity of high peak concentrations to achieve enhancement (20). The reason for the superiority of the i.t. route of drug administration can be explained by the high concentration of DWA2114R within the tumor, regarded as a dose-dependent agent. Although we could not determine whether heat enhanced drug activity or the drug enhanced the effect of hyperthermia, the concomitant administration showed a greater effect than injections given after hyperthermia. This finding differed from that seen with ACR, and might be related to drug activity depending on dose (DWA2114R) or time (ACR). Further investigations are necessary to clarify the basis for our findings.

It has been reported that PEP is synergistic with hyperthermia in vitro as well as bleomycin (5, 13). However, i.t. injections did not reveal an advantage over i.p. injections. In addition, the timing of the i.t. injection was not related to its effect. One of the reasons for these findings may be that the effect of PEP depends on contact time rather than drug concentration. This would also explain the superiority of the PEP emulsion, which has a long-standing depot effect at the injection site. However, three injections did not appear superior to non-emulsified PEP. This may be due to poor diffusion of the PEP emulsion, because the tumor volume at the time of the second or third injection still had not diminished to a noticeable extent.

Lung metastases were inhibited by the i.t. injection of the anti-cancer drugs, except for PEP emulsion. Hyperthermia combined with the drugs further inhibited metastasis, especially with DWA2114R. A primary reason for the inhibition may be due to a reduction in the number of tumor cells in the blood as a result of the hyperthermia. We have shown that development of lung metastases runs parallel to the primary tumor volume in Lewis lung.

Fig. 3 Growth curves (upper) and lung metastases (lower) of Lewis lung carcinoma treated with 43.5°C of hyperthermia (Hy) for 30 min and intratumor (i.t.) injection of PEP. Saline i.t. (○), 4 mg/kg PEP intraperitoneally (●), 4 mg/kg PEP i.t. (△), or 4 mg/kg PEP-emulsion i.t. (▼) 1 injection every other day to a total of 3 injections. Mean number of metastases plotted according to 4-graded sizes (see Materials and Methods).

ed to promote lung metastases.

DISCUSSION

It has been reported that the combination of ACR and hyperthermia are synergistic in vitro, but not in vivo (12). This discrepancy has also been observed with adriamycin (10, 12). In the present study, the combined effect was additive, even though ACR was injected into the tumor. Moreover, the effect did not differ whether hyperthermia was applied before or after the injection of drug. It has been reported that the effect of ACR is time-dependent in vitro (11). The time of contact between the drug and the tumor cells might be too short to obtain an effect, because of the rapid disappearance of the i.t. administered drug.

DWA2114R, a new derivative of cis-DDP, is supposed to have the same cytotoxicity and less nephrotoxicity (1). It has been reported
carcinoma (18). The second reason may be that the i.t. injection of anti-cancer drugs kills intravascular tumor cells and metastasized cells, because most intratumorous drugs enter rapidly into the circulatory system. With the PEP emulsion, a single injection plus hyperthermia reduced the number of metastases, perhaps due to a reduction in the number of tumor cells, but three injections of PEP emulsion showed neither inhibition nor promotion of lung metastases. Mechanical pressure at the time of i.t. injection might have promoted lung metastases, because the emulsion is quite viscous. Moreover, the development of metastases can easily occur at the second or third injection, because the tumor volume is still large.

In conclusion, the i.t. injection of anti-cancer drugs, in particular DWA2114R, was shown to be of value, especially when combined with local hyperthermia.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Cancer Research, and a Grant-in-Aid for Scientific Research (C), from the Ministry of Education, Science and Culture of Japan.

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