Effects of Interleukin 2 Treatment Combined with Local Hyperthermia in Mice Inoculated with Lewis Lung Carcinoma Cells

Rong-Nian Shen, Li Lu, Bo Wu, Homayoon Shidnia, Ned B. Hornback, and Hal E. Broxmeyer

ABSTRACT

Recombinant human (rhu) interleukin 2 (IL-2) was evaluated alone and in combination with local hyperthermia (LH) in mice inoculated s.c. with 5 x 10⁶ Lewis lung carcinoma cells. Four treatment regimens were begun 6 days postinoculation at a time when the tumor had grown to approximately 8.0 mm in diameter. Treatments were: group 1, saline injected as control; group 2, LH; group 3, rhuIL-2; or group 4, LH combined with rhuIL-2. LH utilized hot water circulation by a Brann Thermomix 1420. The intratumor temperature was maintained at 43 ± 0.2°C for 30 min each on days 6 and 10 and rhuIL-2 was given s.c. at 5 x 10⁶ units twice a day for 5 days. Thirty mice in each group were sacrificed 28 days after tumor inoculation. An additional 20 mice in each group were observed for survival time. The size of primary tumor and the number of lung metastases were reduced and the survival time was prolonged in mice treated by either LH or IL-2. However, a greater antitumor effect in Lewis lung carcinoma tumor-bearing mice was observed using IL-2 therapy combined with LH. Tumor growth was associated with increased splenic granulocyte-macrophage progenitor cells and an abnormal L3T4*/Lyt-2* lymphocyte subset ratio (<1.0). Splenic granulocyte-macrophage progenitor cell numbers and the L3T4*/Lyt-2* ratio returned to normal in the group treated with combination therapy, the best responder group. The L3T4*/Lyt-2* ratio did not change in the groups treated with single therapy. These results suggest the efficacy and possible clinical relevance of combined therapy with rhuIL-2 and LH for certain metastatic tumors.

INTRODUCTION

Lymphokines are important in the development of host immunity and may be useful in modifying biological responses in cancer patients or other diseases associated with immune dysfunction (1). IL-2 is one of the earliest lymphokines considered for these types of applications (2, 3). However, a problem with IL-2 therapy is that in order to produce antitumor activity it is used in high doses, which have been associated with toxicity (4–6). It has been reported that the antitumor activity of IL-2 can be enhanced when IL-2 is used in combination with other cytokines and/or monoclonal antibodies and in combination with activated effector cytotoxic lymphocytes (7–13).

There is a biological rationale for the use of hyperthermia in the treatment of cancer (14, 15). It has been theorized that following tumor heating, absorbed necrotic cancer cell by-products provide the antigenic stimulus needed to enhance the host immune system which then leads to destruction of distant metastatic foci (16, 17). Local hyperthermia (LH) alone can induce regression of tumor (18), but better results have been obtained by combining heat with low doses of irradiation (19–22). Activities of a number of immunological cytokines can be modulated by hyperthermia (23–28).

The present study was initiated to evaluate the effects of rhuIL-2, alone and in combination with local hyperthermia, on the growth of tumors in mice inoculated with LLC cells.

MATERIALS AND METHODS

Mice. Female C57BL/6J mice, 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in plastic cages, 5/cage, and provided with water and standard laboratory diet ad libitum.

Tumor. Lewis lung carcinoma is a malignant type of epidermoid carcinoma developed spontaneously in a C57BL/6J mouse. Inoculation of tumor cells into syngeneic hosts results in the consistent appearance of metastatic foci in the lungs. Sterile tumor cell suspensions were prepared by mincing tumor fragments in RPMI 1640 and filtering the crude suspension through a fine sterile screen mesh as described previously (29). Cell viability was determined by trypan blue exclusion. Mice were inoculated s.c. with 5 x 10⁶ viable tumor cells in 0.1 ml of culture medium into the right hind limb. Six days postinoculation the tumor averaged 8.0–10.0 mm in diameter. The tumor-bearing mice were randomly divided into four groups with treatment started 6 days post inoculation: group 1, saline injected as control; group 2, LH (43 ± 0.2°C for 30 min, twice a week); group 3, rhuIL-2 (1.8 x 10⁶ IU/mg or 3 x 10⁶ Cetus units/mg; Cetus Corp., Emeryville, CA); group 4, rhuIL-2 combined with LH.

LH. Mice bearing Lewis lung carcinoma cells in their right hind limb were treated with local hyperthermia on days 6 and 10. Mice were placed on a specially designed wooden table and their tumor-bearing limbs were covered with a rubber tube and warmed with hot water circulated by a Thermomix 1420. Intratumor temperature was monitored by inserting a thermocouple temperature probe (Baily IT-1E) into the tumor. The thermocouple probe was connected to a calibrated Baily BAT-8 thermometer. Twenty-eight days after tumor inoculation, 30 mice in each group were sacrificed and an additional 20 mice in each group were kept to determine the survival time.

Measurement of Tumor Volume. The primary tumor was measured with calipers. The two perpendicular diameters and the mean diameter were used to calculate tumor volume. Volume was calculated as

\[ V = 0.4 (ab^2) \]

where \( a \) and \( b \) are two perpendicular diameters, and \( b \) is the smaller one (30).

Determination of Lung Metastases. During autopsy, the lungs were stained by infiltration with a 15% solution of India ink and fixed in Fekete's solution (31). With this technique, the metastatic nodules become prominent and are easily counted using a magnifying lens (×8).

Source of Cells. Cells obtained from femoral bone marrows and spleens of individual mice were made into single cell suspensions by filtering cells through a fine steel screen mesh. Nucleated cells were counted using a Coulter Counter (Model ZM; Coulter Electronics, Hialeah, FL).

Colony Formation by Granulocyte-Macrophage Progenitor Cells. Mouse bone marrow and spleen cells were plated, respectively, at 5 x 10⁴ cells/ml and 10⁶ cells/ml in 35-mm standard tissue culture dishes in 1 ml of 0.3% agar (Difco) culture medium containing McCoy's 5A medium supplemented with additional essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate (Gibco Lab-
IL-2-LOCAL HYPERTHERMIA IN LLC CELL-TREATED MICE

RESULTS

Primary Tumor and Lung Metastases. LLC tumor-bearing mice were routinely monitored for tumor growth twice a week and the final tumor volume was measured on day 28-post tumor cell inoculation. Table 1 shows the effects on tumor growth of LH, rhuIL-2, or LH combined with rhuIL-2. The mean primary tumor volume in mice treated with LH or rhuIL-2 is about one-third of that in the control mice. The primary volume was reduced even further in mice treated with LH combined with rhuIL-2. The number of lung metastases is significantly less in the combination therapy arm (LH- and IL-2-treated groups) compared to controls or to either therapy arm itself. The effects of either therapy alone reduced mainly the numbers of small metastases, while the numbers of both small and large metastases were reduced by combination therapy. No metastases were noted in 2 of 30 mice in the combined therapy group and no lung nodules over 4 mm were found in this group.

Survival Time. Table 2 shows the survival times for the 4 groups of mice averaging the results of 20 mice/group from a total of 2 complete experiments. Mice were examined daily. More than one-half of the saline-treated control mice had died by day 36, while at this time all mice in the treatment groups were alive. By day 45 all untreated mice had died, whereas, respectively, 80, 100, and 100% of the mice treated with LH, rhuIL-2, and LH plus rhuIL-2 survived. In all cases treatment increased survival time significantly when compared to controls.

Table 1: Influence of LH and rhuIL-2, alone and in combination, on survival of mice inoculated with LLC*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of mice</th>
<th>Survival time (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>20</td>
<td>34.5 ± 1.27</td>
</tr>
<tr>
<td>2</td>
<td>LH</td>
<td>20</td>
<td>46.9 ± 0.75</td>
</tr>
<tr>
<td>3</td>
<td>rhuIL-2</td>
<td>20</td>
<td>52.1 ± 1.16</td>
</tr>
<tr>
<td>4</td>
<td>LH + rhuIL-2</td>
<td>20</td>
<td>61.5 ± 1.35</td>
</tr>
</tbody>
</table>

Statistical analysis

* Data represent two separate experiments with the mean ± SE results for a total of 20 mice in each group. The comparison of survival of different groups were analyzed by Kaplan-Meier and log rank test.

but mice treated with combination therapy had the longest survival times.

DISCUSSION

Hyperthermia may have either a direct suppressive effect on tumor cell growth or it may act indirectly via enhancement of host immune function (34). It has been demonstrated by others that IL-2 is capable of inducing desired changes in immune response and of producing antitumor activity (4–6). Our present experiments demonstrated the most potent antitumor effect in LLC tumor-bearing mice when combination therapy with rhuIL-2 and LH was applied. The size of primary tumors and the numbers of lung metastases were reduced and the survival time was prolonged in mice treated either by rhuIL-2 or LH. However, best antitumor effects were observed using both rhuIL-2 and LH. The mechanism(s) underlying these effects are not known, but they may reflect immune associated effects as discussed below.

Mice inoculated with LLC tumor cells had increased numbers of splenic CFU-GM and a decreased proportion of spleen cells with the L3T4* phenotype with the ratio of L3T4*/Lyt-2* (<1.0) being reversed from normal. These abnormalities were corrected by combination therapy. Increased numbers of splenic CFU-GM, which may be associated with immune suppressor cell activities, may reflect a reactive process involving enhanced cytokine production by T-cells or other accessory cells, an effect counteracted by the treatment used. L3T4 is considered to the murine analogue of the human Leu-3/T4 (CD4) molecule and expression of this antigen correlates primarily with major histocompatibility complex class II antigen reactivity (35). Our
The IFN inducers tested amplified IFN production (37). A greater antitumor effect in LLC tumor-bearing mice than either polycytidylate together with local hyperthermia exerted a complement of either exogenous IFN or the IFN inducer polyinosinate-7. Others have demonstrated that combined therapy consisting of cell formation of colonies was also reduced by addition of IFN-7. Moreover, bone marrow and spleen cell formation with IFN-7. The resultant suppressor activities could be minimized by culturing the cells with IFN-7. The IL-2-LOCAL HYPERTERMIA IN LLC CELL-TREATED MICE experiments, while establishing an association between T-cells and tumor growth and metastases in animals, do not allow us to determine that there was a cause-effect relationship. However, it is intriguing to speculate that the antitumor activity of IL-2 combined with LH was at least in part mediated through L3T4+ or the ratio of L3T4+/Lyt-2+. Various cytokines, alone or in combination with other treatment modalities, have been used in clinical medicine (36). IL-2 as well as LH therapies have been associated with release of other cytokines, such as IFN-γ or the tumor necrosis factors (reviewed in Ref. 37), and these molecules may be involved in the effects noted. For example, it has been reported that LLC cells stimulate hemato poiesis in vivo and this was associated with bone marrow and splenic immune suppressor cell activities (38, 39). The resultant suppressor activities could be minimized by culturing the cells with IFN-γ. Moreover, bone marrow and spleen cell formation of colonies was also reduced by addition of IFN-γ. Others have demonstrated that combined therapy consisting of either exogenous IFN or the IFN inducer polyinosinate-polyctydylate together with local hyperthermia exerted a greater antitumor effect in LLC tumor-bearing mice than either treatment alone (40). It was pointed out that the effect of IFN was enhanced at elevated temperatures and combined LH and the IFN inducers enhanced amplified IFN production (37).

REFERENCES

Table 3 Effect of LH or rhIL-2, alone and in combination, on numbers of CFU-GM in LLC tumor-bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies/organ</th>
<th>Colonies + clusters/organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Bone marrow</td>
<td>28,435 ± 385</td>
<td>73,465 ± 5,295</td>
</tr>
<tr>
<td>Saline controls</td>
<td>36,540 ± 5,825</td>
<td>89,273 ± 17,882</td>
</tr>
<tr>
<td>LH</td>
<td>22,960 ± 280</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rhIL-2</td>
<td>20,690 ± 218</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>rhIL-2 + LH</td>
<td>20,480 ± 255</td>
<td>72,085 ± 3,235</td>
</tr>
<tr>
<td>Non-tumor-bearing controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Spleen</td>
<td>15,796 ± 5,751</td>
<td>26,098 ± 9,585</td>
</tr>
<tr>
<td>Saline</td>
<td>22,250 ± 3,745</td>
<td>35,002 ± 6,612</td>
</tr>
<tr>
<td>rhIL-2</td>
<td>3,292 ± 1,808</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>rhIL-2 + LH</td>
<td>4,895 ± 1,510</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Non-tumor-bearing controls</td>
<td>1,044 ± 185</td>
<td>2,196 ± 274</td>
</tr>
</tbody>
</table>

Table 4 Effect of LH or rhIL-2, alone and in combination, on T-lymphocyte subsets in spleens of mice bearing LLC cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of positive cells</th>
<th>Ratio L3T4*/Lyt-2*</th>
<th>Statistical Analysis of % of L3T4+-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Normal</td>
<td>19.7 ± 0.03</td>
<td>16.2 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2. LLC + saline</td>
<td>6.0 ± 0.46</td>
<td>7.0 ± 0.94</td>
<td></td>
</tr>
<tr>
<td>3. LLC + LH</td>
<td>6.9 ± 0.21</td>
<td>8.2 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>4. LLC + IL-2</td>
<td>7.5 ± 0.2</td>
<td>9.1 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>5. LLC + LH + IL-2</td>
<td>18.8 ± 2.01</td>
<td>11.6 ± 1.15</td>
<td></td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SEM from the combined average of three experiments for a total of 9 mice/group.


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