Local Adoptive Immunotherapy of Human Head and Neck Cancer Xenografts in Nude Mice with Lymphokine-activated Killer Cells and Interleukin 2

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ABSTRACT

The efficacy of local adoptive immunotherapy with human lymphokine-activated killer cells and recombinant interleukin 2 (IL-2) in growth inhibition of established squamous cell carcinoma of the head and neck (SCCHN) was evaluated in a nude mouse model. The model of xenograft SCCHN was established by s.c. injections of in vitro maintained tumor cells (2-10 x 10^6 cells/mouse) into the flank of splenectomized animals pretreated with cyclophosphamide (200 mg/kg). The SCCHN line used was tumorigenic in 95% of the appropriately conditioned nude mice. Inhibition of tumor growth by locally administered effector cells was the end point of the study, since the tumors did not metastasize within 6 weeks of tumor challenge. Either i.p. or local administration of IL-2 alone (1000 units/day) to the tumor site daily for 2 weeks resulted in a significant inhibition of tumor growth. In the absence of detectable natural killer activity in these mice, a modest dose of IL-2 had a direct antitumor effect on SCCHN cells in vivo. In addition, complete inhibition of tumor growth was achieved with 3 times weekly injections of 5-10 x 10^6 lymphokine-activated killer cells delivered to the tumor site and 1000 units of rIL-2 administered locally every day for 2 weeks. Our data indicate that local or systemic immunotherapy with IL-2 alone or local adoptive immunotherapy with an adequate dose of lymphokine-activated killer cells plus rIL-2 may be effective in preventing the growth of established SCCHN tumors in vivo.

INTRODUCTION

Tumor cell lines maintained in vitro have been extensively used in studies of the biology of human tumors (1, 2). SCCHN have been relatively difficult to establish in culture (3), and only a limited number of well characterized SCCHN lines have been available for research (4, 5). We have recently established a series of SCCHN lines from primary as well as recurrent and metastatic tumors and described their characteristics (6). These lines were tumorigenic in cyclophosphamide-pretreated athymic mice, and the histology and differentiation of the tumor xenografts were shown to be comparable to those of the original tumors used to establish the lines (6). The availability of SCCHN lines maintained in culture allowed us to develop a xenograft model of SCCHN in nude mice. The model was intended to evaluate the in vivo efficacy of AI with rIL-2 and LAK cells, injected at the site of tumor growth, in preventing or inhibiting tumor cell proliferation.

Patients with SCCHN often present with advanced unresectable disease. Recently, it has been reported that perilesional administration of natural or recombinant human IL-2 to such patients resulted in complete or partial responses in some cases (7). These results suggested that local or perilesional injections of IL-2 may be more effective and less toxic than systemic therapy, which is known to cause considerable side effects (8, 9). At this time, it is not clear whether perilesionally administered IL-2 has indirect immunostimulatory effects on the patients' antitumor effector cells, as postulated by Forni et al. (10) on the basis of in vitro experiments in a murine model, or whether it directly affects tumor growth. Furthermore, recent clinical trials in patients with solid tumors have focused on systemic adoptive immunotherapy with IL-2 in combination with LAK cells (9, 11, 12). Although results with perilesional IL-2 in patients with SCCHN have been encouraging, it seemed to us that the addition of LAK cells might result in greater antitumor effects. To explore this possibility, we established a xenograft model of SCCHN in nude mice. This report describes an initial experience with local AI of SCCHN with human LAK cells and rIL-2 in this model.

MATERIALS AND METHODS

SCCHN Lines. Over 20 SCCHN lines were established and cultured as described by us (6). All the lines tested were tumorigenic in nude mice; however, there were considerable differences in the rate of tumor growth among the different lines. The squamous cell carcinoma line (PCI-1) used in the studies described here was established from a surgical biopsy obtained from a 65-year-old male with a recurrent laryngeal carcinoma. The line was maintained in Eagle's minimal essential medium supplemented with 1% (v/v) nonessential amino acid mixture, 0.2 M glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum (all from Gibco, Grand Island, NY). The line was trypsinized and passaged at regular intervals. Its mean doubling time was 114 h, with a range of 65-185 h. The line grew as a well differentiated monolayer. In preliminary experiments, the s.c. injection of 10 x 10^6 tumor cells in suspension into nude mice pretreated with cyclophosphamide (see below) resulted in tumor growth in all the animals. The histology of the PCI-1 tumors growing in nude mice was comparable to that of the original tumor. To prepare PCI-1 cells for injections, the confluent culture was trypsinized (0.25% trypsin solution; Gibco) and the cells were washed twice, counted in trypan blue, and resuspended in 0.1 ml of HBSS to give the final concentration of 10 x 10^6 tumor cells/0.1 ml.

Nude Mice. Athymic BALB/c female 4- to 6-week-old mice were obtained from Taconic Farms (Germantown, NY) through a contract with the NIH. Mice were quarantined for 2 weeks after their arrival. They were maintained in cages placed in a laminar flow cabinet under pathogen-free conditions and were supplied with sterile water and food ad libitum. To eliminate NK activity, which is usually high in nude mice and which could contribute to resistance to tumor growth and/or to antitumor effects during therapy, it was necessary to splenectomize and immunosuppress the animals (13, 14). All mice were splenectomized through a 5-mm-long incision in the left upper flank, under systemic anesthesia, at least 2 weeks before the initiation of studies. One day prior to injection of tumor cells, the mice were pretreated with cyclophosphamide (200 mg/kg) and anti-asialo-GM1 antibody (diluted 1:20; Wako, Osaka, Japan) by i.p. injection (0.1 ml/mouse). Peripheral blood lymphocytes from splenectomized nude mice treated with cyclo-
phosphamide and anti-asialo-GM1 displayed no residual NK activity during or after systemic therapy with IL-2, as measured in in vitro cytotoxicity assays performed with cells obtained from selected animals sacrificed especially for these assays.

Adoptive Immunotherapy. SCCHN cell suspensions (10 x 10^6 cells/0.1 ml of HBSS) were injected s.c. in the right flank of nude mice conditioned as described above. Three days after the tumor challenge, mice received injections of rIL-2 (Cetus, Emeryville, CA) alone or in combination with LAK cells, the latter injected into the site of the tumor challenge.

In the first series of experiments, groups of 5–10 animals were treated daily with 1000 units of rIL-2 (0.1 ml), administered locally or i.p., for 2 weeks. Control mice received 0.1-ml injections of HBSS administered in a similar way. In subsequent experiments, groups of mice were treated with rIL-2 plus LAK cells. LAK cells (from 10^4 to 10 x 10^6) in HBSS were injected in the 0.1-ml volume 3 times weekly for 2 weeks. Also, rIL-2 (1000 units/day) was injected locally at the tumor growth site every day for 2 weeks. Mice were observed daily for tumor growth.

Measurements of Tumor Growth. Tumor xenografts growing in nude mice were measured with calipers twice weekly. The product of the two largest diameters at a 90-degree angle to each other was determined for each tumor (15). Measurements were made independently by two investigators, to ensure accuracy and minimize bias. Mice were sacrificed by cervical dislocation at various times during the course of therapy, and tumors or sites of tumor growth were harvested and fixed for histology. Tumor growth was observed for a maximum of 45 days following the start of therapy, at which time all surviving mice were sacrificed. The liver and lungs were macroscopically and histologically examined for the presence of metastases.

LAK Cells. Human peripheral blood was obtained from normal volunteers, and mononuclear cells were recovered on Ficoll/Hypaque gradients. The cells were washed, counted in trypan blue, and then cultured in the presence of 1000 units/ml rIL-2 (Cetus Corp., Emeryville, CA), in complete growth medium containing 10% (v/v) pooled human AB serum (Central Blood Bank of Pittsburgh). The initial cell concentration was 1 x 10^6/ml, and the cultures were maintained at 37°C, in an atmosphere of 5% CO_2 in air, for 5 to 10 days. Prior to harvesting of the LAK cells for therapy, their cytotoxicity and phenotype were determined as described below. Target cells were K562, Daudi, and PCI-1 lines maintained in the laboratory and tested for Mycoplasma monthly, using a GeneProbe (San Diego, CA) kit.

Cytotoxicity Assay. A 4-h ¹¹⁵Cr-release assay was used, as described earlier (16). Briefly, 5 x 10^⁶ ¹¹⁵Cr-labeled target cells (YAC-1 targets with mouse effectors and K562 targets with human effectors) were plated in wells of a microtiter plate and effector cells were added at effector:target ratios ranging from 12:1 to 1.5:1. Plates were centrifuged at 65 x g and incubated for 4 h at 37°C in 5% CO_2 in air. Supernatants were harvested using a semi-automated harvesting system (Skatron) and radioactivity was counted in a gamma counter. Spontaneous release was determined in wells containing target cells alone, and maximum release in wells containing target cells after the addition of Triton X-100. The percentage of cytotoxicity at each effector:target ratio was determined using the following equation:

\[
\text{% cytotoxicity} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100
\]

Results were expressed in LU, calculated according to the method of Pross et al. (17). One LU was defined as the number of effector lymphocytes required for 20% lysis of 5 x 10^⁶ target cells, and the number of LU present in 10⁶ effector cells was calculated.

Monoclonal Antibodies. Monoclonal antibodies for flow cytometry studies were purchased from Becton Dickinson (Mountain View, CA) and included: Leu-4 (anti-CD3), Leu-19 (anti-CD56), Leu-11a (anti-CD16), and anti-IL-2 receptor (anti-CD25). For controls, IgG1 and IgG2a isotypes were included. All antibodies were titered for optimal concentrations, prior to staining, using normal human blood mononuclear cells for flow cytometry.

Flow Cytometry. Cells were adjusted to the concentration of 2 x 10^⁶/ml and incubated with different combinations of fluorescein- and phycoerythrin-labeled monoclonal antibodies (10 μl each) for 30 min at 4 °C. Following staining, cells were washed 2 times with phosphate-buffered saline-0.1% sodium azide buffer and resuspended at the concentration of 5 x 10^⁶ cells/ml, for two-color flow cytometry on a FACStar. The gates were set for lymphocytes, and monoclonal antibody HLe-1 was used to determine the percentages of monoclonal cells in the gate. Results were expressed as the percentage of gated cells positive for each of the monoclonal antibodies used.

Statistical Analysis. Analysis of the data was performed using a one-way analysis of variance with repeated measures. Further group comparisons were tested using Fisher’s least significant difference method as a multiple comparisons procedure. The significance level was set to be P < 0.05.

RESULTS

We established an in vivo model of SCCHN in immunosuppressed nude mice in order to evaluate the effect of locally administered rIL-2 and LAK cells on the growth of a human SCCHN line. The PCI-1 SCCHN line chosen for the in vivo experiments produced tumors, within 1 to 2 weeks following s.c. injection of 1 x 10⁶ to 10 x 10⁶ tumor cells, in most (95%) splenectomized nude mice pretreated with cyclophosphamide and anti-asialo-GM1. A dose-dependent growth of the tumor was consistently observed in these animals (data not shown). Tumors achieved a considerable size by 5 weeks of growth (Fig. 1), but they did not form visible or microscopic metastases. After tumor growth for 4–6 weeks, mice bearing very large tumors tended to die, probably because of cachexia associated with tumor progression. The histology of the tumors growing in nude mice was remarkably similar to that of the original tumor (Fig. 2) from which the PCI-1 line was established.

On the basis of the preliminary experiments, we selected a
dose of $10 \times 10^6$ tumor cells for the experiments described below. A palpable tumor appeared within 1 to 2 weeks after the challenge and all mice survived for at least 5 weeks.

The administration of 1000 units of rIL-2 either locally or systemically (i.p.) resulted in significant inhibition of tumor growth (Fig. 3). There was no significant difference between the local and systemic administration routes. Therapy with rIL-2 was well tolerated by the mice, and there were no premature deaths during the observation period. Histologic examination of the tumor sites revealed only a minimal inflammatory infiltrate. None of the mice had evidence of metastases upon microscopic examination of the lungs and liver.

In preparation for local adoptive immunotherapy with human LAK cells, mononuclear cells were obtained from normal volunteers and activated in the presence of SCCHN line (PCI-1) derived from this human tumor (bottom). The line was maintained in culture and passaged 8–10 times prior to its injection into the mouse. Note histological similarity of the human and mouse tumors. × 125.

In Fig. 2, Histological sections of the original human tumor (top) and of the nude mouse tumor established by a s.c. injection of SCCHN line (PCI-1) derived from this human tumor (bottom). The line was maintained in culture and passaged 8–10 times prior to its injection into the mouse. Note histological similarity of the human and mouse tumors. × 125.

CD3 surface antigen, and only about 20% were CD56*. Although the local administration of low doses ($0.01–1 \times 10^6$) of LAK cells plus rIL-2 resulted in significant inhibition of tumor growth (Fig. 4), the effect was not significantly different from that observed after the administration of IL-2 alone (see Fig. 3 versus Fig. 4). This observation was confirmed when the local administration of low doses of LAK cells plus rIL-2 was extended to 4 weeks. In mice treated with either $5 \times 10^6$ or $10 \times 10^6$ LAK cells 3 times weekly for 2 weeks plus daily rIL-2, a nearly complete inhibition of tumor growth was

<p>| Table 1 LAK activity of human mononuclear cells cultured in the presence of rIL-2 and used for therapy of SCCHN growing in nude mice |</p>
<table>
<thead>
<tr>
<th>Days in culture</th>
<th>K562</th>
<th>Daudi</th>
<th>PCI-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>592</td>
<td>387</td>
<td>156</td>
</tr>
<tr>
<td>5</td>
<td>2846</td>
<td>2122</td>
<td>1928</td>
</tr>
<tr>
<td>6</td>
<td>3016</td>
<td>2686</td>
<td>2316</td>
</tr>
<tr>
<td>7</td>
<td>2987</td>
<td>2799</td>
<td>2448</td>
</tr>
<tr>
<td>8</td>
<td>2793</td>
<td>2493</td>
<td>2230</td>
</tr>
<tr>
<td>9</td>
<td>2875</td>
<td>2306</td>
<td>2216</td>
</tr>
<tr>
<td>10</td>
<td>2712</td>
<td>2516</td>
<td>2019</td>
</tr>
<tr>
<td>11</td>
<td>1543</td>
<td>1432</td>
<td>1461</td>
</tr>
<tr>
<td>12</td>
<td>997</td>
<td>890</td>
<td>536</td>
</tr>
</tbody>
</table>

<p>| Table 2 Phenotype of human LAK cells cultured in the presence of rIL-2 and used for therapy of SCCHN growing in nude mice |</p>
<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 8</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3*</td>
<td>81</td>
<td>33</td>
<td>57</td>
<td>77</td>
</tr>
<tr>
<td>CD56*</td>
<td>14</td>
<td>27</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>CD16*</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>CD25*</td>
<td>10</td>
<td>34</td>
<td>59</td>
<td>41</td>
</tr>
<tr>
<td>CD3<em>CD56</em></td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>4</td>
</tr>
</tbody>
</table>
achieved, and this was significantly different from effects mediated by rIL-2 alone. Control mice given injections of an equivalent dose of fresh nonactivated mononuclear cells (no rIL-2) rather than LAK cells developed tumor at the same time as the animals injected with HBSS only. Histological examination of tumor sites at days 5 and 10 and after therapy from all experimental groups showed the presence of few or no inflammatory cells (Fig. 5). There was no evidence of pulmonary or hepatic metastases by microscopic examination.

Since it was possible that the systemic therapy with rIL-2 could result in increased residual NK activity even in splencotomized and immunosuppressed animals, we sacrificed mice immediately after therapy, as well as during therapy (days 5 and 10), and measured their blood NK activity (Table 3). No NK activity was detected in the blood of animals treated with IL-2 alone or IL-2 plus LAK cells. In contrast, as expected (13), splenic lymphocytes obtained at the time of splenectomy from the nonimmunosuppressed and untreated nude mice mediated NK activity quite efficiently (Table 3). Furthermore, these immunosuppressed mice had few LAK cell precursors in the blood or bone marrow, as evidenced by minimal LAK activity generated following activation of peripheral blood lymphocytes and bone marrow cells in rIL-2 (1000 units/ml) for 4 days in culture (Table 3).

DISCUSSION

Systemic therapy of solid tumors in humans with LAK cells plus rIL-2 or rIL-2 alone has been beneficial in some cases of cancer refractory to conventional therapy (9, 11). In head and neck cancer, perilesional injections with IL-2 were recently reported to result in some complete or partial responses of considerable duration (7). The mechanisms responsible for these responses were thought to involve the in vivo generation of LAK cells and augmentation by IL-2 of a cytokine cascade in tumor-infiltrating mononuclear cells (19, 20). Alternatively, there exists a possibility that perilesional injections of IL-2 might have a direct inhibitory effect on tumor growth. To address these issues, we established an in vivo model of human SCCHN in athymic mice and used it for local adoptive immunotherapy. The availability of SCCHN lines newly established and characterized in our laboratory (6) made it possible to proceed in this direction. Furthermore, the complete suppression of NK cell function in nude mice by pretreatment with cyclophosphamide and anti-asialo-GM1 resulted in a significant and consistent improvement in successful tumor takes, as reported earlier for other tumor cell lines (13). The addition of splenectomy has further increased immunosuppression by depleting the animals of a reservoir of lymphoid effector cells (14). Indeed, in this immunosuppressed environment, SCCHN cells implanted successfully with a predictably high frequency and grew rapidly enough to satisfy the experimental design. Attempts by others to grow SCCHN lines in nude mice (21, 22), mainly to demonstrate tumorigenicity of the lines, were successful but the frequency of tumor takes was lower than that

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Immunosuppressive treatment</th>
<th>Tumor therapy</th>
<th>NK activity (LU/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen lymphocytes</td>
<td>None</td>
<td>None</td>
<td>130 ± 31</td>
</tr>
<tr>
<td>PBL</td>
<td>Splenectomy</td>
<td>None</td>
<td>8.3 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-asialo-GM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>As above</td>
<td>None</td>
<td>6.1 ± 5.8</td>
</tr>
<tr>
<td>PBL</td>
<td>As above</td>
<td>IL-2, 1000 units/ml i.p.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>As above</td>
<td>IL-2, 1000 units/day (local), LAK cells, 5 x 10⁶ (3 x weekly)</td>
<td>3.8 ± 1.0*</td>
</tr>
</tbody>
</table>

* After therapy.
* At day 5 of therapy.
* At day 10 of therapy.
in our experiments, ranging at best between 40 and 60%. The SCCHN lines we established did not metastasize in nude mice and, therefore, the end point of our model had to be the inhibition of tumor growth. Histological, immunohistochemical, and ultrastructural studies of these tumors growing in nude mice (6) confirmed the epithelial nature of the tumors and their morphological similarities to the original surgical specimen.

A xenograft model of local AI should be useful in evaluating therapeutic effects of cytokines, with or without added effector cells, on human head and neck tumors implanted into nude mice. Human SCCHN lend themselves to a local intervention and, since conventional therapy often fails to arrest tumor growth, new therapeutic modalities, including immunotherapy, need to be considered. Local immunotherapy may have an advantage of minimal side effects. In humans, systemic therapy with IL-2 and other recombinant cytokines has been associated with severe, and on occasion fatal, side effects (9, 11). The feasibility of AI and its low toxicity in patients with SCCHN has been demonstrated already in the phase I trial by Cortesina et al. (7), using low doses of natural IL-2 (7) as well as escalating doses of rIL-2. Takeda (23) evaluated the effectiveness of local AI in the treatment of a human laryngeal carcinoma established in nude mice. He showed that the local administration of IL-2 alone or of human LAK cells plus systemic IL-2 retarded tumor growth and prolonged survival of the animals (23). In this model, the tumor latency was not prolonged by locally injected LAK cells alone or by systemic IL-2 alone.

In the human clinical trial (7) and the animal studies performed by Takeda (23), it was not clear to what extent NK cells in the tumor-bearing host contributed to the observed antitumor effects (24). In our model of AI, animals were strongly immunosuppressed, so that no NK activity could be detected in the blood of animals sacrificed during or after systemic IL-2 administration. Despite the absence of detectable NK activity, locally and systemically (i.p.) administered rIL-2 significantly inhibited growth of SCCHN in vivo. This suggested two rather unexpected possibilities: either that IL-2 was acting indirectly by stimulation of host macrophages or that IL-2 had a direct antitumor effect on human SCCHN. The role for IL-2-induced host macrophages and/or adoptively transferred LAK cells in mediating antitumor effects through production of cytokines, especially tumor necrosis factor α is suggested by histological observations of some tissue necrosis at the site of tumor growth during therapy. However, the absence of a significant inflammatory infiltrate in or around the xenografts on histological examination during and immediately after therapy argues for a direct rather than effector cell-mediated effect of rIL-2 on SCCHN cells. Experiments are in progress to elucidate the mechanism of the observed sensitivity of SCCHN cells to rIL-2 in vivo and in vitro. Indeed, we have recently completed competitive binding studies with 125I-labeled IL-2 and showed that SCCHN have receptors for IL-2 and that IL-2 has a direct growth-inhibitory effect on these cells in vitro.

The combination of high doses of LAK cells (5–10 x 10⁶) and rIL-2 injected locally was significantly more effective in inhibiting tumor growth than IL-2 alone. These results suggest that the local administration of IL-2 and adequate numbers of LAK cells to patients with SCCHN might be of therapeutic benefit, particularly since IL-2 itself may both have antitumor effects and induce host immune mechanisms. In patients, little systemic toxicity is evident during its local administration (7). A potential limitation may be the relatively large number of

LAK cells required for significant tumor inhibition. In a clinical setting, however, additional LAK cells may be recruited from patients’ tumor infiltrating lymphocytes (TIL) and lymph nodes lymphocytes (LNL) by the administration of IL-2.

It is not clear at present if immunotherapy with IL-2 alone is of greater benefit than IL-2 plus LAK cells in patients with SCCHN. Our experimental system established in immunosuppressed nude mice may not be useful for addressing this issue, because it offers no possibilities for the modification of host effector function. Nevertheless, the system presents certain advantages. The availability of a xenograft model of human SCCHN for preclinical studies should allow us to determine the optimal effector cell doses and IL-2 concentrations as well as timing of the loco-regional AI in relation to tumor growth. Since the effector cells and IL-2 are delivered directly to the site of tumor growth, no problems with the localization of LAK cells to the tumor exist in our model. Further, the model lends itself to in vivo studies of purified antitumor effector cells, cytokines, and other biological and synthetic products that may directly or indirectly inhibit tumor growth.

REFERENCES


* G. Cortesina, personal communication.


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