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

Manuela Sacchi, Carl H. Snyderman, Dae Seog Heo, Jonas T. Johnson, Frank d'Amico, Ronald B. Herberman, and Theresa L. Whiteside

Departments of Otolaryngology [M. S., C. H. S., J. T. J.], Pathology [D. S. H., R. B. H., T. L. W.], and Medicine [R. B. H., T. L. W.], University of Pittsburgh School of Medicine, and Pittsburgh Cancer Institute [M. S., D. S. H., J. T. J., R. B. H.], Pittsburgh, Pennsylvania 15261, and Department of Mathematics, Duquesne University, Pittsburgh, Pennsylvania 15219 [F. D.]

ABSTRACT

The efficacy of local adoptive immunotherapy with human lymphokine-activated killer cells and recombinant interleukin 2 (rIL-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 xenografted SCCHN was established by s.c. injections of in vitro maintained (SCCHN) was evaluated in a nude mouse model. The model of xenografted 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 rIL-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 rIL-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 rIL-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 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 vivo 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 passed 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 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-
concentration was 1 x 10^6/ml. and the cultures were maintained at

ville, CA), in complete growth medium containing 10T¿ (v/v) pooled
cultured in the presence of 1000 units/ml rIL-2 (Cetus Corp., Emery

were harvested using a semi-automated harvesting system (Skatron)
harvesting of the LAK cells for therapy, their cytotoxicity and pheno-

earlier (16). Briefly, 5 x 10^6 MCr-labeled target cells (YAC-1 targets
harvested for histology. Tumor growth was observed for a maximum of 45

cotions were made independently by two
ters, to ensure accuracy and minimize bias. Mice were sacri-

largest diameters at a 90-degree angle to each other was determined for
mice were measured with calipers twice weekly. The product of the two
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
diameters was then calculated, and the tumor volume was expressed in

phosphamamide 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
ing a similar way. In subsequent experiments, groups of mice were

were treated with rIL-2 plus LAK cells. LAK cells (from 10^6 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.

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., Emery-
ville, CA), in complete growth medium containing 10% (v/v) pooled
human AB serum (Central Blood Bank of Pittsburgh). The ini-
tial cell concentration was 1 x 10^6/ml, and the cultures were maintained at
37°C, in an atmosphere of 5% CO2 in air, for 5 to 10 days. Prior to
harvesting of the LAK cells for therapy, their cytotoxicity and phen-
type were determined as described below. Target cells were K562,
Daudi, and PCI-1 lines maintained in the laboratory and tested for
Myeloplasma monthly, using a GeneProbe (San Diego, CA) kit.

Cytotoxicity Assay. A 4-h 51Cr-release assay was used, as described
earlier (16). Briefly, 5 x 10^6 51Cr-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% CO2 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 10^6 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 mononu-
clear cells for flow cytometry.

Flow Cytometry. Cells were adjusted to the concentration of 2 x 10^7/ml and incubated with different combinations of fluorescein- and phy-
coerythrin-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 con-
tcentration of 5 x 10^5 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 com-
parisons 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 immunosup-
pressed 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 10^6 to 10 x 10^6 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

Fig. 1. A SCCHN line (PCI-1) growing in a nude mouse 5 weeks after a s.c.
injection of 10 x 10^6 tumor cells.
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 rIL-2 (1000 units/ml) for 5–10 days, as described earlier (18). LAK cells harvested between days 5 and 10 of culture were tested for antitumor cytotoxicity in vitro against K562, Daudi, and PCI-1 targets. As shown in Table 1, these cells had considerable antitumor activity at the time of their administration to nude mice. The phenotypic characteristics of the LAK cells used for therapy are shown in Table 2. The majority of these cells expressed 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
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 immunosuppressed 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 deleting 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 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 splenectomized 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 immunosuppressed 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).

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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 × 10^6) 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 (LNLI) 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.

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* G. Cortesina, personal communication.


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