Antiproliferative cytokines secreted by lymphokine-activated killer cells stimulated with tumor cells

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Antiproliferative cytokine secretion by lymphokine-activated killer (LAK) cells during coculture with glioblastoma cell lines, autologous glioma cells, and nongliomatous tumor cell lines (Daudi and K562 cells) was assessed, as was the antiproliferative activity of the culture supernatants against the T98G (glioblastoma) cell line. A neutralization test using agents against interferon-γ (IFN-γ), tumor necrosis factor (TNF), and lymphotoxin (LT) showed that antiproliferative activity was due to IFN-γ, but not to TNF or LT. Nongliomatous tumor cells stimulated LAK cells to secrete cytokines, but gliomatous tumor cells did not.

It was found that there is a discrepancy between the LAK cell capability to lyse malignant glioma cells and the ability to secrete cytokines. This may be due to the factors secreted by glioblastoma cells.

KEY WORDS • lymphokine-activated killer cell • cytokine • glioma • interferon • tumor necrosis factor • lymphotoxin

LYMPHOKINE-ACTIVATED killer (LAK) cells kill a wide variety of tumor cells in vitro. Proposed immunotherapy using LAK cells for patients with various cancers, and some workers have described adoptive immunotherapy for malignant brain tumors using LAK cells. Potentially, LAK cells secrete tumor necrosis factor (TNF), lymphotoxin (LT), and interferon-γ (IFN-γ). Interferon-γ combined with TNF or LT demonstrates a considerable synergistic effect on the suppression of tumor cell proliferation.

We have shown that LAK cells secreted both TNF and LT in vitro, and that this increased when LAK cells were cultured with nongliomatous tumor cells. Glioblastoma cells, however, failed to stimulate cytokine secretion. We suggested the possibility that glioblastoma cells produce factors that suppress cytokine secretion. Therefore, malignant glioma cells may affect the function, especially the cytokine secretion, of LAK cells.

In this study, we investigated whether malignant glioma cells stimulate LAK-cell secretion of cytokines, and measured the antiproliferative activity against glioblastoma cells.

Materials and Methods

Induction of LAK Cells

Lymphokine-activated killer cells were induced as described elsewhere. Briefly, peripheral blood mononuclear cells were separated by leukaphoresis from a patient with anaplastic astrocytoma, and were further purified by density gradient centrifugation. We used a small quantity of these peripheral blood mononuclear cells for experiments and the remainder for adoptive immunotherapy. Peripheral blood mononuclear cells were cultured at 2 × 10⁶ cells/ml in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM glutamine, 5% fresh frozen human plasma (ABO blood type matched), and interleukin-2 (IL-2, 2400 U/ml). The LAK cells were collected after culture for 3 to 5 days, and demonstrated an adequate cytotoxic effect against T98G cells as determined by the standard 4-hour ⁵¹Cr release assay.

Cultures with Tumor Cells

Lymphokine-activated killer cells were displayed at 2 × 10⁶ cells/ml in complete medium containing 5% fresh frozen plasma and were cocultured with Daudi cells (a human B tumor cell line derived from a patient with Burkitt’s lymphoma) or autologous anaplastic astrocytoma cells at an LAK/tumor cell ratio of 10:1 at 37°C under 5% CO₂ for 4, 18, and 40 hours, or 4, 18, and 64 hours, respectively. After culture, the supernatants were harvested and analyzed for antiproliferative activity. The supernatants were analyzed for antiproliferative activity against glioblastoma cells, autologous glioma cells, and nongliomatous tumor cell lines (Daudi and K562 cells). The supernatants were analyzed for antiproliferative activity against glioblastoma cells.

† Ficoll-Paque density gradient centrifuge manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden.
‡ Interleukin-2 donated by Takeda Pharmaceutical Co., Ltd., Osaka, Japan.
Fig. 1. Graph showing antiproliferative activity of recombinant human interferon-γ against T98G cells. Values indicate means ± standard error of the means from four different experiments. Each experiment was done in triplicate or quadruplicate.

Fig. 2. Graphs showing antiproliferative activity of the supernatant from lymphokine-activated killer cell (LAK)/K562 cell culture (18 hours) against A172 (glioblastoma) (upper), T24 (bladder carcinoma) (center), and KAKI-I (renal cell carcinoma) (lower) cell lines. Crosses = supernatant from LAK/K562 culture, squares = RPMI 1640 medium with 5% fresh frozen plasma.

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Data were harvested and stored at −70°C until use. Some LAK cells were also cocultured with T98G (human glioblastoma cell line), Daudi, and K562 (human erythroleukemic cell line) cells§ for 18 hours, and the supernatants were harvested and stored. As LAK cells can kill tumor cells, the tumor cells (2 x 10⁵ cells/ml) were destroyed using an ultrasonic generator[ and the fluids were passed through a 0.22-μm filter and tested for antiproliferative activity against T98G cells.

Autologous anaplastic astrocytoma cells obtained at surgery were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) and were used in the experiments within seven passages. Most tumor cells tested by immunohistochemical methods were positive for glial fibrillary acidic protein.

Measurement of Antiproliferative Activity

The antiproliferative activity against T98G cells of the supernatants from the cocultures of LAK cells with T98G, Daudi, K562, or autologous anaplastic astrocytoma cells was measured by the modified procedures described elsewhere.¹⁸ Serial twofold dilutions of each supernatant were made in RPMI 1640 medium supplemented with 10% FCS in 96-well flat-bottomed microtiter plates. The T98G cells were seeded at 5000 cells/well, then incubated at 37°C under 5% CO₂. After 2 days of incubation, the cells were stained with 0.5% crystal violet in 20% methanol. The dye was eluted with 50% ethanol, and the absorbance at 570 nm was determined.* The relative percentage viability was calculated as: [optical density (supernatant-treated)/optical density (untreated)] × 100. In our preliminary study, human recombinant IFN-γ,† which has antiproliferative activity, showed maximum growth inhibition (around 50%) of T98G cells at 1000 U/ml (Fig. 1). The antiproliferative activity was therefore defined tentatively as the reciprocal of the dilution achieving 70% of the relative percentage viability of T98G. Lee, et al.,¹⁸ and our preliminary studies showed that this eluted dye intensity correlated with the cell number.

As the supernatant from the LAK/K562 cell culture showed a high antiproliferative activity against T98G cells, we also examined its activity against A172 (human glioblastoma cell line), T24 (human bladder carcinoma cell line), and KAKI-I (human renal-cell carcinoma cell line) cells using the same methods.

Antiproliferative Activity of IFN-γ Combined With TNF or LT

In our preliminary studies, human recombinant TNF and LT demonstrated a weak antiproliferative effect

§ Tumor cell lines were obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan.
† Ultrasound generator manufactured by Heat Systems Ultrasound, Inc., Farmingdale, New York.
* TiterTech Multiskan manufactured by Flow Laboratories, Inc., Helsinki, Finland.
† Human recombinant interferon-γ donated by Toray Industries, Inc., Kanagawa, Japan.
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against T98G cells. To determine whether IFN-γ combined with TNF or LT has a synergistic antiproliferative effect on T98G cells, recombinant TNF or LT was serially diluted in 96-well plates, and T98G cells were seeded with or without 100 U/ml of IFN-γ. After 2 days of incubation, cells were stained with crystal violet and eluted with ethanol, and the absorbance at 570 nm was determined.

Neutralization Test

To determine whether the antiproliferative activity was due to IFN-γ, TNF, or LT, the supernatant from culture of LAK/K562 or LAK/Daudi cells was neutralized by anti-IFN-γ, anti-TNF, and anti-LT antibodies before the proliferation assay. The supernatants from LAK/K562 or LAK/Daudi cell cultures were added to 96-well plates at 0.05 ml/well, and 0.05 ml of a monoclonal anti-IFN-γ antibody (2.56 × 10^4 neutralizing units/ml, at 1:20 and 1:200 dilutions), a monoclonal anti-TNF antibody (1.5 × 10^5 neutralizing units/ml, at a 1:100 dilution), or a polyclonal rabbit anti-LT antibody (3.2 × 10^4 neutralizing units/ml, at a 1:10 dilution) was added. Any neutralization due to the antibodies was assessed initially. After incubation of the solutions for 1 hour at 37°C, serial twofold dilutions were made in RPMI 1640 medium with 10% FCS. The T98G cells were seeded at 5000 cells/well, and incubated at 37°C under 5% CO₂. After 2 days of incubation, cells were stained with crystal violet. The dye was eluted with 50% ethanol, and the absorbance at 570 nm was determined.

The antibodies were not cross-reactive, as anti-TNF antibody did not neutralize LT and vice versa.

Results

Antiproliferative Activity

The supernatants from culture of the LAK cells and the LAK/Daudi cells showed an antiproliferative activity against T98G cells (Table 1). Supernatants from the 4-hour cultures demonstrated some activity; however, this increased markedly in the 18-hour culture of the LAK/Daudi cells. Therefore, the supernatants from 18-hour cultures of the LAK/T98G, LAK/Daudi, and LAK/K562 cells were assessed (Table 2). Supernatants from cultures of LAK/K562 and LAK/Daudi cells had high activities. The antiproliferative activity was closely correlated with the maximum percentage of growth inhibition. The activity of the supernatant from LAK/T98G cell culture was similar to that from LAK cell culture and weaker than that from LAK/K562 or LAK/Daudi cell culture.

‡ Human recombinant TNF donated by Dainippon Pharmaceutical Co., Ltd, Osaka, Japan; and human recombinant lymphotoxin donated by Kanagafuchi Chemical Industry, Hyogo, Japan.

§ Anti-IFN-γ antibody donated by Shionogi Pharmaceutical Co. Ltd, Osaka, Japan; anti-TNF antibody donated by Dr. Ohshima, Teikyo University, Tokyo, Japan; and anti-lymphotoxin antibody donated by Dr. Niki, Mitsubishi Chemical Industry, Yokohama, Japan.

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**Table 1**

Antiproliferative activity of the supernatant from LAK/Daudi cell culture

<table>
<thead>
<tr>
<th>Glial Supernatant</th>
<th>4 Hrs</th>
<th>18 Hrs</th>
<th>40 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAK/Daudi</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LAK/K562</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LAK/K562</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAK/Daudi</td>
<td></td>
<td></td>
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</tbody>
</table>

*Antiproliferative activity measured as a reciprocal of the dilution that gives 70% of the relative percentage viability of T98G cells. Values in parentheses = maximum growth inhibition (%). LAK = lymphokine-activated killer cells.

**Table 2**

Antiproliferative activity of the supernatants from LAK/tumor-cell 18-hour culture

<table>
<thead>
<tr>
<th>Culture Supernatant</th>
<th>Glial 1</th>
<th>Glial 2</th>
<th>Glial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK only</td>
<td>15 (50)</td>
<td>4 (40)</td>
<td>25 (60)</td>
</tr>
<tr>
<td>LAK/T98G</td>
<td>7 (47)</td>
<td>3 (33)</td>
<td>150 (74)</td>
</tr>
<tr>
<td>LAK/Daudi</td>
<td>28 (53)</td>
<td>37 (80)</td>
<td>383 (84)</td>
</tr>
<tr>
<td>LAK/K562</td>
<td>21 (50)</td>
<td>30 (79)</td>
<td>394 (90)</td>
</tr>
</tbody>
</table>

*Antiproliferative activity measured as a reciprocal of the dilution that gives 70% of the relative percentage viability of T98G cells. Values in parentheses = maximum growth inhibition (%). LAK = lymphokine-activated killer cells.

**Table 3**

Antiproliferative activity of the supernatant from LAK/autologous anaplastic astrocytoma cultures

<table>
<thead>
<tr>
<th>Culture Supernatant</th>
<th>4 Hrs</th>
<th>18 Hrs</th>
<th>64 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK only</td>
<td>4 (51)</td>
<td>4 (50)</td>
<td>15 (68)</td>
</tr>
<tr>
<td>LAK/autologous tumor</td>
<td>3 (33)</td>
<td>&lt; 2 (25)</td>
<td>&lt; 2 (10)</td>
</tr>
<tr>
<td>LAK/Daudi</td>
<td>5 (53)</td>
<td>24 (75)</td>
<td>305 (68)</td>
</tr>
</tbody>
</table>

*Antiproliferative activity measured as a reciprocal of the dilution that gives 70% of the relative percentage viability of T98G cells. Values in parentheses = maximum growth inhibition (%). LAK = lymphokine-activated killer cells.

† Cultured autologous anaplastic astrocytoma cells.

Lymphokine-activated killer cells were cultured with autologous anaplastic astrocytoma cells, and the supernatant was tested for antiproliferative activity (Table 3). The activity was lower than that from LAK cell culture. Supernatants from Daudi, K562, and anaplastic astrocytoma cell cultures and the fluids obtained by ultrasonic destruction of Daudi, K562, T98G, and anaplastic astrocytoma cells showed no antiproliferative activity against T98G cells. The supernatant from the LAK/K562 cell culture, which had a high antiproliferative activity against T98G cells, was also active against A172, T24, and KAKI-1 cell lines (Fig. 2).

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Interferon-γ combined with TNF or LT displayed a synergistic antiproliferative activity against T98G cells (Fig. 3). There was an additional 50% increase in antiproliferative activity when IFN-γ was added to TNF or LT.

Neutralization Tests

The antiproliferative activity of the supernatant was mostly neutralized by anti-IFN-γ antibody (Figs. 4 and 5). Anti-TNF and anti-LT antibodies, however, did not affect the activity.

Discussion

Cytokine Secretion by LAK Cells

We investigated the antiproliferative activity of the cytokines secreted by LAK cells cultured with gliomatous or nongliomatous tumor cells. Our results showed that nongliomatous tumor cells stimulate LAK cells to secrete cytokines, which inhibit the growth of tumor cells, and that the antiproliferative activity is due to IFN-γ. However, gliomatous tumor cells failed to stimulate LAK cells to secrete antiproliferative cytokines. As in natural killer cells,24 the lytic sequence of LAK cells can be divided into: 1) the binding stage; 2) the "programming for lysis" stage; 3) the secretion of lytic factors; and 4) the killer cell-independent phase of lysis. After binding to tumor cells, the cytokine secretion pathway in LAK cells may be activated. This pathway may be suppressed by factors secreted by glioma cells.

Human glioblastoma cells produce transforming growth factor-beta (TGF-β) type 2 and prostaglandin E2 (PGE2), which can suppress several immune functions.2,7,1,34 Our previous study19 showed that glioblastoma cells did not stimulate LAK cells to secrete TNF and LT. Therefore, cytokine secretion may be suppressed by factors secreted by glioblastoma cells, inhibiting the propagation of an immune reaction mediated by cytokines and allowing the tumor cells to escape from the immune surveillance mechanism.

Peripheral blood lymphocytes secrete TNF, LT, and/or IFN-γ under stimulation from IL-2, mitogen, phorbol ester, or calcium ionophore.6,21,23,28,31,32 However, cytokine secretion by LAK cells after tumor cell stimulation is not well understood. Recent reports have indicated that tumor cells stimulate LAK cells to secrete IFN-γ and TNF with synergistic cytotoxic activity against a breast carcinoma cell line,4 and that TNF, LT, and IFN-γ are present in the sera of IL-2/LAK cell-treated patients and in vitro after stimulation with tumor cell lines.12 In contrast, our study showed that glioblastoma cells failed to stimulate LAK cells to secrete cytokines, although they are sensitive to LAK cell lysis. This discrepancy may be due to the effect of TGF-β and PGE2. Transforming growth factor-β inhibits cytokine production6 but does not inhibit the cytolytic activity of LAK cells once generated.16 Prostaglandin E2 induces a considerable inhibition of T-lymphocyte activation,7 which may involve cytokine secretion by lymphocytes. Also, PGE2 inhibits the cytolytic activity of LAK cells when directly added to the cytotoxicity assay, as well as inhibiting LAK cell generation.17 Although it has been shown that significant levels of PGE2 were secreted by the tumor as early as 2 days in culture,5 it is possible that LAK cells kill glioblastoma cells efficiently in vitro before sufficient PGE2 is produced to inhibit LAK cell lysis.

Synergistic Effect of Cytokines

Previous studies have demonstrated synergistic cytostatic or cytotoxic effects among TNF, LT, and IFN-γ on tumor cells.1,10,18,30,33 Although the sensitivity of various tumor cells to these cytokines differs, some tumor cells resistant to one cytokine become sensitive to combined cytokines.18,30,33 In the present study, we assessed the antiproliferative activity of culture supernatants from LAK/tumor cells on glioblastoma cell lines. The activity was mainly due to IFN-γ, as demonstrat-
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ed by the neutralization test. The maximum percentage growth inhibition of T98G cells by some LAK/tumor cell culture supernatants exceeded that of recombinant IFN-γ. This may be due to a synergistic effect among IFN-γ, TNF, and LT.

Clinical Implications
The implications of this study have clinical interest. Local administration of LAK cells has been effective in patients with malignant glioma, however, there are still some problems. As indicated by our results, the secretion of cytotoxic or cytostatic cytokines by infused LAK cells might be suppressed by the factors produced by glioma cells. This may decrease the efficacy of LAK cell administration to patients with malignant gliomas.

Conclusions
We have demonstrated that LAK cells can secrete cytokines with antiproliferative activity against glioblastoma cells when cultured with nongliomatous tumor cells, and that this cytostatic activity is mainly due to IFN-γ. However, malignant glioma cells failed to stimulate LAK cells to secrete antiproliferative cytokines, possibly due to factors produced by the malignant glioma cells.

References


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