Administration of Recombinant Interleukin 12 Prevents Outgrowth of Tumor Cells Metastasizing Spontaneously to Lung and Lymph Nodes

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ABSTRACT

The present study investigates the ability of recombinant interleukin 12 (rIL-12) to modulate the growth of a primary tumor as well as the outgrowth of metastatic tumor cells in an ovarian carcinoma (OV-HM) model. This aggressive tumor displayed rapid growth of the primary tumor mass, high incidence of metastases to lung and lymph nodes, and invasion from the primary s.c. site to the peritoneal cavity. Starting 12 days after s.c. tumor cell implantation, several i.p. injections of rIL-12 at 2-3-day intervals resulted in regression of growing tumors. These treated mice did not show signs of metastases or tumor recurrence at the original site. One month after tumor implantation, untreated mice did not have visible lung metastasis, but some did have palpable lymph nodes. At this stage, the primary tumors of animals without palpable lymph nodes were surgically resected. When examined 2 months later, most animals had developed lymph node and lung metastases. In contrast, rIL-12 injections after tumor resection inhibited the development of metastases in both lung and lymph nodes. This contrasted with the failure of IL-2 to prevent metastases. Even for mice already showing signs of lymph node metastases or invasion of the abdominal wall, rIL-12 administration after tumor resection prevented further invasion to the peritoneal cavity and growth of metastatic tumor cells in lung. It was somewhat surprising that the IL-12 treatment of animals after 1 month of tumor growth without resection also resulted in complete tumor regression, as well as eradication of micrometastasis that would have occurred before the treatment. Moreover, they exhibited resistance to a rechallenge with the same tumor but not with a second tumor. Thus, this tumor system provides a relevant model to clinical situations in terms of treatment of advanced tumors and metastases. These results also indicate that IL-12 can induce a curative immune response, even in the face of an aggressive micrometastasizing tumor.

INTRODUCTION

IL-12 is discovered as a NK cell stimulatory factor (1, 2) and also subsequently as a cytotoxic lymphocyte maturation factor (3, 4). This cytokine exhibits a number of unique and important biological activities. These include the ability to enhance NK and CTL activity (1, 5, 6), to stimulate the proliferation of T cells and to a lesser extent, NK cells (5, 7, 8), and to induce secretion of cytokines, particularly IFN-γ, from NK and T cells (1, 9). IL-12 has also been demonstrated to influence the development of Th through facilitating a Th1-type cellular immune response and inhibiting differentiation of Th2-type lymphocytes (10, 11).

On the basis of evidence that IL-12 functions to augment the development and/or activation of Th1, CTL, and NK cells, IL-12 was examined for the capacity to induce an antitumor effect. The therapeutic activity of IL-12 has been observed in various murine tumor models (11, 12). The efficacy obtained in these models included substantial growth inhibition and prolongation of survival (12, 13). In addition, the antimetastatic effect of IL-12 was observed against experimentally induced metastases (12, 13). IL-12 treatment reduced pulmonary and hepatic metastases after i.v. injection of tumor cells (12, 13). However, therapeutic efficacy of IL-12 was not tested for the ability to inhibit spontaneous metastases. In contrast to metastasis induced experimentally in normal mice by i.v. injection of tumor cells, spontaneous metastasis is thought to occur in association with immunosuppression induced during the tumor-bearing state (14-16). Therefore, determination of the efficacy of IL-12 treatment in spontaneous metastasis models could contribute to further establishment of the antitumor therapeutic effect of this cytokine.

The present study investigated the antitumor/metastatic effect of IL-12 in a spontaneous metastasis model using an ovarian tumor cell line (OV-HM). In this model, micrometastases develop in lung and lymph nodes around 1 month after tumor implantation. Our results demonstrated that systemic administration of rIL-12 starting after tumor resection resulted in a striking inhibition of metastatic tumor growth in lung and lymph nodes. Taken together, the fact that systemic administration of rIL-12 to mice bearing a primary s.c. OV-HM tumor induced complete tumor regression and the present observations add to a growing list of evidence for the antitumor/antimetastatic effects of IL-12.

MATERIALS AND METHODS

Mice. Female (C57BL/6 × C3H/He) F1 mice were obtained from Shizuoka Experimental Animal Center (Hamamatsu, Japan) and used at 6–9 weeks of age.

Tumors. An OV-HM ovarian carcinoma clone was kindly provided by Dr. Ohtsura Niwa (Hiroshima University, Hiroshima, Japan). An ovarian tumor, OV2944, was induced in a female (C57BL/6 × C3H/He) F1 mice by giving a single whole-body neutron irradiation, and a cloned line with highly metastatic property (designated OV-HM) was isolated from the parental line (17). MCH-1-A1 was a fibrosarcoma cell clone the parental line of which was induced in a female C3H/He mouse with methylcholanthrene (18). These cloned tumor cell lines were maintained in RPMI 1640 supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO2.

Recombinant Cytokine. Murine rIL-12 was provided by Dr. B. Hubbard (Genetics Institute, Cambridge, MA) and was purified from the supernatants of Chinese hamster ovary cells transfected with the P35 and P40 cDNA plasmids. SDS-PAGE analysis indicated that the IL-12 was ≥95% pure, and endotoxin contamination was <5 enzyme units/mg IL-12, as assessed by the Limulus amebocyte assay. Murine rIL-2 was kindly provided by Shionogi Co. Ltd., (Osaka, Japan). Cytokines were administered i.p. after dilution in 0.1% mouse albumin carrier protein.

Preparation of OV-HM Tumor-bearing Mice and Surgical Resection of Tumors. OV-HM tumor-bearing mice were prepared by inoculating s.c. 5 × 106 viable OV-HM tumor cells. Tumor removal was done by surgical resection 26–36 days after tumor cell implantation. Without surgical resection of a primary tumor, all of mice died around 8 weeks after tumor cell implantation.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: IL-12, interleukin 12; NK, natural killer; Th, helper T cell; rIL-12, recombinant IL-12.

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Growth. Although tumor size slightly decreased during treatment, the systemic rIL-12 administration. We first investigated the effect of tumors. rIL-12 was i.p. administered into earlier (12-day; A) or later (32-day; B) tumor-bearing mice. Thirty-two days after tumor implantation. Even though mice treated with rIL-12 rejected a second challenge of OV-HM tumor cells (18), mice bearing similar sizes of OV-HM tumor without palpable lymph nodes (lymph node metastasis free in physical examination) were prepared, and tumor resection was performed for all mice at 4 weeks after tumor implantation. One-half of the mice were given i.p. rIL-12 12 times at 2-day intervals (Fig. 3). All mice that had not been treated with rIL-12 rejected a second challenge of OV-HM tumor cells. rIL-12-treated mice prepared according to the protocol in Fig. 1B were rechallenged with 1.5 × 10⁶ OV-HM (A) or 3 × 10⁶ MCH-1-A1 (B) tumor cells. Tumor growth was expressed as the mean ± SE of tumor diameter. O, normal mice; ●, tumor-regressed mice in Fig. 1B.

RESULTS

Complete Regression of a Primary s.c. Tumor Induced by Systemic rIL-12 Administration. We first investigated the effect of rIL-12 administration on the growth of the primary OV-HM tumor. rIL-12 was injected i.p. into mice after 12 (Fig. 1A) or 32 (Fig. 1B) days of tumor growth. The dose of rIL-12 used was 0.5 μg/mouse/time. rIL-12 was administered at 2–3-day intervals. Three or five injections were given to animals starting at 12 or 32 days, respectively, after tumor implantation. Complete tumor regression was observed for the IL-12 therapy performed at both stages of tumor growth. Although tumor size slightly decreased during treatment, the tumor was still quite large when the final rIL-12 injection was performed. However, the tumor continued to shrink in size over the next 2 weeks and every animal became tumor free 15–25 days after initiation of IL-12 treatment (Fig. 1).

Detection of Tumor-specific Immune Resistance in the IL-12-treated Mice. We confirmed that the tumor-free state continued for 1 month after complete tumor regression in mice that received the IL-12 therapy starting 32 days after tumor implantation as in Fig. 1B. These mice were rechallenged with the same OV-HM tumor cell line or with the C3H/He-derived fibrosarcoma cell line (MCH-1-A1). The results in Fig. 2 demonstrate that all mice initially bearing OV-HM tumor and treated with rIL-12 rejected a second challenge of OV-HM tumor cells. These mice did not exhibit any clinical sign of tumor metastasis or recurrence for 2 months after rejection of a primary tumor, including the period required for the second challenge experiments. However, mice challenged with the nonrelated MCH-1-A1 tumor cells did not block the growth of this tumor. We demonstrated previously the immunogenicity of the MCH-1-A1 tumor; namely, C3H/He mice that initially bore a MCH-1-A1 tumor and received surgical resection of the tumor rejected a second challenge of MCH-1-A1 tumor cells (18). We also found that the same held true when B6C3F1 mice were used as recipients (data not shown). Taken together, these results indicate that IL-12 therapy induces tumor regression and that this regression is associated with the induction of a tumor-specific immune response.

Inhibition of Development of Metastases by Postoperative Administration of rIL-12. We first confirmed the fact that OV-HM tumor-bearing mice develop a high incidence of metastasis (17). Surgical resection of a primary tumor was performed for mice at around 1 month after tumor implantation. Although no palpable lymph node was observed in these mice upon tumor resection, all of them exhibited an enlargement in lymph nodes at some or all of 4 sites examined (both sides of axillary and inguinal) within 1 month after tumor resection (data not shown). Moreover, a high incidence of nodule formation in the lung was observed at autopsy performed 2 months after tumor resection (data not shown). Histological examination revealed that the enlarged lymph nodes and lung nodules consisted of metastatic tumor cells. These results indicate that metastases to lymph nodes and lung occur at around 1 month after tumor implantation.

We asked whether systemic administration of rIL-12 after surgical resection of a primary tumor can inhibit the development of metastases. Mice bearing similar sizes of OV-HM tumor without palpable lymph nodes (lymph node metastasis free in physical examination) were prepared, and tumor resection was performed for all mice at 4 weeks after tumor implantation. One-half of the mice were given i.p. rIL-12 12 times at 2-day intervals (Fig. 3). All mice that had not been treated with rIL-12 rejected a second challenge of OV-HM tumor cells. rIL-12-treated mice prepared according to the protocol in Fig. 1B were rechallenged with 1.5 × 10⁶ OV-HM (A) or 3 × 10⁶ MCH-1-A1 (B) tumor cells. Tumor growth was expressed as the mean ± SE of tumor diameter. O, normal mice; ●, tumor-regressed mice in Fig. 1B.

Fig. 1. Systemic administration of rIL-12 induces complete regression of s.c. growing tumors. rIL-12 was i.p. administered into earlier (12-day; A) or later (32-day; B) tumor-bearing mice in a dose of 0.5 μg/mouse, at the indicated times at 2–3-day intervals. The tumor growth given as diameter (in mm) was plotted individually.

Fig. 2. Mice inducing regression of OV-HM tumor in the IL-12 therapy model reject rechallenged OV-HM cells. rIL-12-treated mice prepared according to the protocol in Fig. 1B were rechallenged with 1.5 × 10⁶ OV-HM (A) or 3 × 10⁶ MCH-1-A1 (B) tumor cells. Tumor growth was expressed as the mean ± SE of tumor diameter. O, normal mice; ●, tumor-regressed mice in Fig. 1B.

Fig. 3. Striking inhibition of lymph node metastasis development by the IL-12 treatment. Tumor resection was performed 4 weeks after tumor implantation. For one-half of mice (7 of 14), the rIL-12 treatment was started on the next day of surgical operation. Metastasis was determined by palpation of lymph nodes and diagnosed as positive by the presence of palpable lymph nodes at any of 4 sites (both sides of axillary and inguinal). O, untreated; ●, IL-12-treated.
treated with rIL-12 developed lymph node metastases within 3 weeks after tumor resection. In contrast, systemic injections of rIL-2 started on the next day after tumor resection resulted in a striking inhibition in the growth of lymph node metastasis. The ability of rIL-2 to block the development of metastasis in lung was also determined, and the results of three consecutive experiments summarized in Table 1 demonstrate that the development of metastases in lung is markedly inhibited by postoperative administration of rIL-12.

Table 2 compares the extents of metastases produced in lymph nodes and lung in a representation of three experiments for control and IL-12-treated mice. Metastases in lymph nodes at more than 2 of 4 sites (both sides of axillary and inguinal) were observed in all control mice. The lymph nodes of most control mice were quite large. Multiple visible nodules were found in the lung of most control mice as well. The results indicate that very high levels of metastases in lymph nodes and lung can be inhibited by the IL-12 treatment after tumor resection. We also confirmed that most mice treated with IL-12 after tumor resection survived for an additional 4 months without signs of tumor metastasis and/or recurrence unless sacrificed for examination.

To determine whether the potent antimetastatic effect induced in this model is specific for the IL-12 action, a comparison was made in the prevention of metastases between the treatments with the same dose of rIL-2 and rIL-12. The results obtained in one of two similar experiments are summarized in Table 3. rIL-12 again elicited potent antimetastatic effects. In contrast, rIL-2 administration failed to prevent the development of metastasis. This group of mice showed similar extents of metastases to those observed in an untreated control group. Thus, IL-12 is a specific cytokine that can induce potent inhibition of spontaneous metastases.

In the course of the present study, we found that some animals bore a large tumor mass invading abdominal wall and/or enlarged lymph nodes, probably due to metastasis at the time of surgery. We finally examined whether rIL-12 administration can protect these recipients from outgrowth of metastatic tumors in lung and/or of tumors invading abdominal wall/peritoneal cavity. For mice that bore tumors adhering to abdominal wall, as much tumor tissue as possible was resected. For mice having metastatic (enlarged) lymph nodes, only a primary tumor mass was resected without removal of enlarged lymph nodes. These treated mice were divided into two groups, one of which was treated with rIL-12. Lung metastases and i.p. invasion were examined either upon tumor death or at a later postoperative time point (Table 4). The results clearly demonstrate that rIL-12 administration is quite effective for inhibiting the development of lung metastasis as well as invasion from abdominal wall into the peritoneal cavity, even in mice not receiving radical operation of a primary tumor and/or removal of metastatic lymph nodes. Taken together, these results indicate that IL-12 treatment of animals with a large tumor burden exerts a striking inhibition of tumor growth. This includes not only cure of tumor at the primary site but also inhibition/cure of metastases already occurring at the time of tumor resection.

**DISCUSSION**

The present results demonstrate that: (a) systemic administration of rIL-12 into animals bearing a highly metastatic tumor (OV-HM) induces complete regression of a primary tumor irrespective of when the rIL-12 treatment is performed at earlier or later tumor-bearing stages; (b) whereas metastatic tumor growth was observed in lymph nodes and lung after complete resection of a primary s.c. tumor, postoperative injections of rIL-12 resulted in striking inhibition of the development of metastasis; and (c) even in mice receiving incomplete treatment, the development of metastasis was markedly inhibited by rIL-12 administration.
tumor resection due to tumor adhesion to abdominal wall or bearing enlarged (metastatic) lymph nodes, postoperative rIL-12 treatment was effective for blocking i.p. invasion of tumor and development of pulmonary metastasis. Thus, the present study establishes the therapeutic efficacy and clinical relevance of systemic rIL-12 administration using a murine spontaneous metastasis model.

Previous reports from other laboratories demonstrated the antimetastatic effect of rIL-12 in several tumor models (12, 13). However, these studies were done by i.v. injection of tumor cells to artificially induce pulmonary or hepatic metastasis in normal mice. Such an experimental system may not necessarily represent the model for metastasis spontaneously occurring in tumor-bearing hosts. The immune status of an animal bearing tumor for 1 month or more and developing spontaneous metastasis could be different from that of a naive animal receiving an i.v. tumor cell injection to artificially induce metastasis. The antitumor effect of IL-12 has been shown to involve eradication of tumor cells. These results not only support our previously obtained evidence that a massive accumulation of CD4+ and CD8+ T cells was detected around the regressing OV-HM tumor mass, and a large number of Mac-1+ cells was seen closer to tumor cells than T cells.5 In the above study, we also found that administration of anti-IFN-γ mAb before IL-12 treatment completely abrogated the antitumor effect of IL-12 as shown in a CSA1M model of our earlier study (19). Thus, it is conceivable that interactions between IFN-γ-producing T cells and Mac-1+ cells are responsible for eradicating tumor cells. These results not only support our previously obtained evidence for the efficacy of the IL-12 immunotherapy even in tumor-bearing hosts with suppressed immunity, but also provide more implications due to the property of the tumor cell type used here (see below).

One primary interest of this study was to determine whether rIL-12 administered after tumor resection would inhibit the development of spontaneous metastasis. Surgical resection of a primary tumor was performed at around 4–5 weeks after tumor implantation; then rIL-12 administration was started. At this point (4–5 weeks after tumor implantation), mice bore a large tumor mass 15–20 mm in diameter (Fig. 1B) and exhibited a decreased antitumor responsiveness.4 Spontaneous metastases to lymph nodes and lung was shown to occur at these stages of mice as tumor outgrowth in lymph nodes and lung was observed after the removal of the primary tumor. Therefore, this experimental system permitted us to ask whether the postoperative rIL-12 treatment can inhibit the development of spontaneous metastasis. The results clearly demonstrated that the rIL-12 treatment is effective for blocking the metastasis development, even when immunity of the host is already declined.

It does not appear that inhibition of metastasis development by the IL-12 immunotherapy is seen only after a primary tumor is removed. In relation to this, it should be noted that IL-12 administration to a later stage (32 day) of tumor-bearing mice induced complete regression, and that these mice exhibited no clinical sign of metastasis for 2 months after regression of the primary tumor. It is highly possible that spontaneous metastasis had already occurred in most of mice when the IL-12 treatment was started. If this is the case, the treatment could inhibit metastasis development along with regression of a primary

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**Table 4 Inhibition of lung metastasis/i.p. invasion in mice receiving nonradical operation by postoperative IL-12 administration**

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Lymph node metastasis</th>
<th>Lung metastasis</th>
<th>i.p. invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
<td>1d</td>
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<td>1.4–5.0</td>
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<tr>
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<td>4</td>
<td>1.3–4.9</td>
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<tr>
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<td>4</td>
<td>2.0–4.8</td>
<td>8</td>
</tr>
<tr>
<td>4d</td>
<td>4</td>
<td>1.4–4.4</td>
<td>10</td>
</tr>
<tr>
<td>5d</td>
<td>4</td>
<td>1.4–3.0</td>
<td>7</td>
</tr>
<tr>
<td>6d</td>
<td>4</td>
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</tr>
<tr>
<td>8d</td>
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<td>0.8–3.9</td>
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<td>0.4</td>
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<td>7d</td>
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<tr>
<td>8d</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

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* Tumor resection was performed 36 days after tumor implantation.
* All of mice were sacrificed 48 days after tumor resection. Metastasis and i.p. invasion were examined.
* Due to real tumor death, the weights of lymph nodes and number of lung nodules could not be determined.
* Mice that bore enlarged (metastatic) lymph nodes upon resection of a primary tumor.
* Mice that bore a primary tumor invading abdominal wall and therefore received incomplete tumor resection.

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4 Unpublished observations.
5 W.-G. Yu, et al. Molecular mechanisms underlying IFN-γ-mediated tumor growth inhibition induced during tumor immunotherapy with rIL-12, manuscript in preparation.
tumor (see above). This suggests that the rIL-12 administration is also effective for eradicating metastatic tumor cells in recipients whose primary tumor mass was not removed. Thus, systemic administration of rIL-12 could induce regression of a primary tumor, eradication of micrometastasis, and establishment of a tumor-specific immune response.

Our results obtained in a murine spontaneous metastasis model illustrate that IL-12 is effective in inhibiting the development of micrometastasis, which appears to have occurred before the start of treatment with this cytokine. IL-12 can induce this effect at the time when antitumor immune responses are already suppressed. Thus, the present study could provide support for clinical application of the IL-12 immunotherapy in situations where tumor metastasis has occurred.

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REFERENCES


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