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ORIGINAL ARTICLE

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Antitumor and antimetastatic effects of interleukin 12

Abstract Interleukin 12 (IL-12) has a pivotal role in controlling cell-mediated immunity through a number of important biological activities, such as secretion of interferon-γ (IFN-γ). In this review, we report our recent results regarding the antitumor and antimetastatic effects of IL-12. Five intraperitoneal injections of recombinant IL-12 (rIL-12) into mice bearing subcutaneous tumors (CSA1M fibrosarcoma) induced complete tumor regression, irrespective of whether tumors were at early or late stages of growth. Furthermore, IL-12-treated mice that had rejected the primary tumor exhibited complete resistance to rechallenge with the same tumor but did not reject a second syngeneic tumor. Immunohistochemical analyses following IL-12 treatment revealed that $CD4^+$ and $CD8^+$ T-cells had infiltrated the tumor. More importantly, IFN-γ mRNA expression was observed in fresh tumor masses from tumor-bearing mice receiving IL-12 treatment. The importance of IFN-γ was further demonstrated by the observation that systemic administration of anti-IFN-γ monoclonal antibody prior to IL-12 treatment completely abrogated the antitumor effect of IL-12. We next investigated the ability of rIL-12 to modulate the outgrowth of metastatic tumor cells in an ovarian carcinoma (OV-HM) model. This aggressive tumor showed rapid growth of the primary tumor mass, a high incidence of metastases to the lung and lymph nodes, and invasion from the primary subcutaneous site into the peritoneal cavity. At approximately 1 month after tumor implantation, primary tumors in 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 following tumor resection inhibited the development of metastases in both the lung and lymph nodes. Even in mice showing signs of lymph node metastases or invasion of the abdominal wall before primary tumor resection, rIL-12 administration following tumor resection prevented further invasion into the peritoneal cavity and metastatic tumor cell growth in the lung. Our results demonstrate that administration of rIL-12 to tumor-bearing mice results in tumor regression through mechanisms involving efficient IFN-γ production by antitumor T-cells at tumor sites in situ and the establishment of a tumor-specific protective immune response. The results also indicate that IL-12 can induce a curative immune response in the face of an aggressive micrometastasizing tumor.

Key words Interleukin 12 (IL-12) · Interferon-γ (IFN-γ) · Antitumor effect · Antimetastatic effect

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Introduction

A growing body of evidence indicates that interleukin 12 (IL-12) [8, 14] has pleiotropic effects on T-cells and natural killer (NK) cells, including stimulation of lymphokine production and promotion of cytolytic activity (reviewed in [1, 16]). Based on its biological activities, IL-12 has been examined for its capacity to induce an antitumor effect, and therapeutic activity has been observed in various murine tumor models [1, 2, 11, 12, 17]. The effects observed in these models included substantial growth inhibition and prolongation of survival [2, 11]. In addition, IL-12 was observed to have an antimetastatic effect against experimentally induced metastases; IL-12 treatment reduced the incidence of pulmonary and hepatic metastases after intravenous injection of tumor cells [2, 11]. However, IL-12 was not examined for its ability to inhibit spontaneous metastasis. In contrast to metastases induced experimentally in normal mice by intravenous injection of tumor cells, spontaneous metastasis is thought to occur in association with immunosuppression induced in the tumor-bearing state [4, 9, 15, 18].

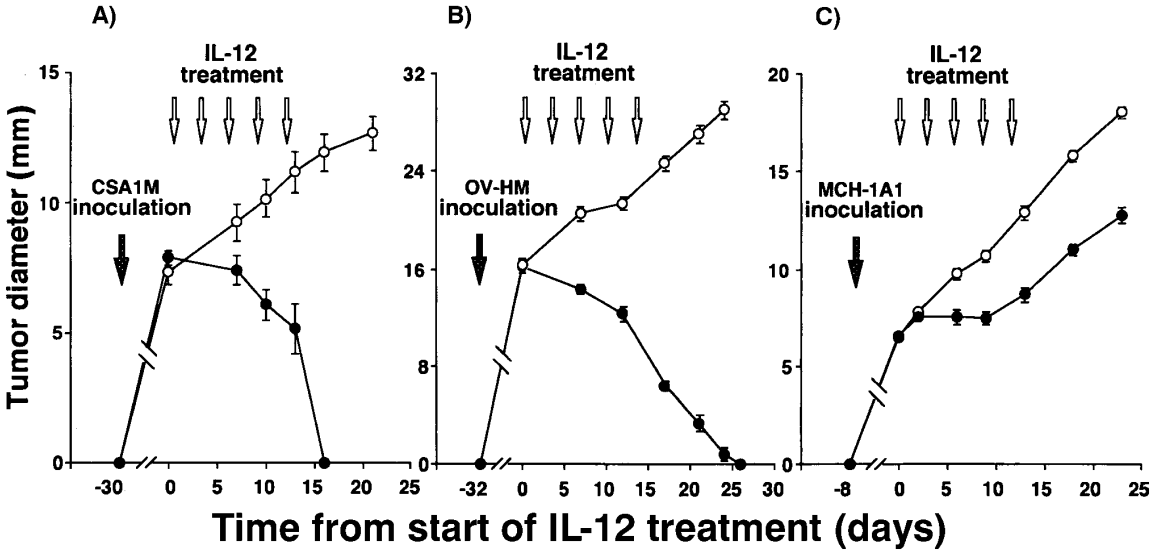


Fig. 1 Effect of in vivo IL-12 treatment on tumor growth in various tumor models. rIL-12 at 0.5 μg was given intraperitoneally to mice bearing subcutaneous syngeneic tumors at the indicated times (2- to 3-day intervals). Tumor growth was expressed as the mean diameter ± SE of 5 mice/group (*white circles* Control mice, *black circles* IL-12-treated mice)

In this review we present the results of our studies of the antitumor and antimetastatic effects of IL-12. The results show that complete tumor regression can be induced in two of three tumor models and that in a tumor model in which spontaneous metastasis occurs, administration of IL-12 following resection of a primary tumor results in marked inhibition of the outgrowth of metastasizing tumor cells. The results are discussed in the context of various factors determining the efficacy of IL-12.

Induction of complete tumor regression or tumor growth inhibition

Local or systemic delivery of IL-12 has potent antitumor efficacy in mice bearing a variety of malignancies [1, 2, 11, 12, 17]. We examined the antitumor effect of IL-12 in three tumor models: virus-induced CSA1M fibrosarcoma (BALB/c origin) [20], radiation-induced OV-HM ovarian carcinoma [(C57BL/6 × C3H/He)F1 origin] [10], and chemical carcinogen-induced MCH-1-A1 fibrosarcoma (C3H/He origin) [19]. Systemic IL-12 administration (0.5 μg on 5 occasions) induced complete regression of CSA1M and OV-HM tumors (Fig. 1A, B) [10, 20]. This regimen was effective and elicited complete regression even in CSA1M tumors growing for as long as 50 days after tumor implantation [20]. The OV-HM tumor exhibited higher growth rates, generating tumor masses as large as 16–20 mm in diameter at approximately 1 month after tumor implantation. However, these OV-HM tumors also regressed completely with IL-12 treatment.

In contrast, the same IL-12 treatment protocol failed to elicit regression of MCH-1-A1 tumors (Fig. 1C) [19]. Significant suppression of tumor growth was observed only during treatment; once treatment ended, tumor growth resumed at growth rates similar to those of control tumors. The existence of tumor rejection antigens on MCH-1-Al cells has been demonstrated in our earlier study [13]; therefore, it is unlikely that the efficacy of IL-12 treatment is due solely to the presence of tumor rejection antigens.

Tumor regression requires T-cell participation and is associated with the establishment of tumor-specific immunity

To determine the involvement of T-cells in tumor regression, we examined the effect of T-cell subset depletion on IL-12-mediated tumor regression in the CSA1M model [20]. Tumor regression was found to be inducible in either anti-CD4 or anti-CD8 monoclonal antibody (MAb)-treated mice. However, simultaneous administration of these MAbs (depletion of both CD4 and CD8 subsets) resulted in complete abrogation of the IL-12-mediated antitumor effect, indicating that this effect can be mediated by either the CD4 or CD8 T-cell population.

We next determined whether the tumor-free state continued for 1 month after complete tumor regression in mice that had received IL-12 therapy in the CSA1M and OV-HM models. CSA1M mice in which tumors had regressed were rechallenged with the same CSA1M tumor cell line or with another BALB/c syngeneic Meth A tumor cell line. All mice initially bearing CSA1M tumors that were treated with rIL-12 rejected a second challenge with CSA1M tumor cells. However, the growth of Meth A tumors was not blocked in similarly treated mice [20]. The acquisition of tumor-specific immunity was also found in mice in which the regression of OV-HM tumors had been induced [10]. These results indicate that IL-12 therapy induces tumor regression and that this regression is associated

with the acquisition of tumor-specific T-cell-mediated immunity.

Inhibition of development of metastases by postoperative administration of rIL-12

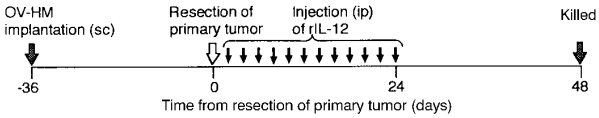
Primary OV-HM tumors were surgically resected in mice at approximately 1 month after tumor implantation. Although no palpable lymph node was observed in these mice upon tumor resection, all exhibited lymph node enlargement at some or all of the four sites examined (bilateral axillary and inguinal) within 1 month of tumor resection [7]. Moreover, a high incidence of nodule formation in the lungs was observed at autopsy at 2 months after tumor resection [7]. Thus metastasis to lymph nodes and the lungs occurs at approximately 1 month after tumor implantation in this tumor model.

We investigated whether systemic rIL-12 administration after surgical resection of a primary tumor could inhibit the development of metastases. Mice bearing OV-HM tumors of similar sizes without palpable lymph nodes (lymph node metastasis-free on physical examination) were prepared, and tumors were resected at 36 days after implantation. Half of the mice were given rIL-12 intraperitoneally 12 times every other day; the remaining mice were not treated.

Figure 2 compares the extent of metastases produced in the lymph nodes and lungs of control and IL-12-treated mice in a representative experiment. Metastases in lymph nodes at more than two of four sites (bilateral axillary and inguinal) were observed in all control mice but in only one of seven IL-12-treated mice. Lymph nodes in most control mice were relatively large. In addition, multiple visible nodules were found in the lungs of most control mice; none was found in IL-12-treated mice. These results indicate that high-level multiple metastases in the lymph nodes and lungs can be inhibited by IL-12 treatment following tumor resection. We also confirmed that most mice treated with IL-12 following tumor resection survived for an additional 4 months without showing signs of tumor metastasis and/or recurrence.

To determine whether the potent antimetastatic effect induced in this model was specific to IL-12, we compared the prevention of metastases by treatment with the same dose of rIL-2 and rIL-12. We found that whereas IL-12 elicited potent antimetastatic effects, IL-2 administration failed to prevent the development of metastases [10]. Thus, IL-12 is a specific cytokine that can potently inhibit spontaneous metastases in this tumor model.

During the course of these studies, we also found that some animals had a tumor mass invading the abdominal wall and/or enlarged lymph nodes, probably due to metastasis at the time of surgery. We examined whether rIL-12 administration could protect such mice from the outgrowth of metastatic tumors in the lungs and/or of tumors invading the abdominal wall/peritoneal cavity (Table 1). In mice that bore tumors adhering to the abdominal wall, as much tumor tissue as possible was resected. In mice with metastatic



Mouse no.		Lymph node metastasis: No. of sites	Lymph node metastasis: Weight (g)	Lung metastasis: No. of nodules
Control	1	4	1.3–5.0	36
	2	4	1.7–4.8	38
	3	4	1.0–4.9	31
	4	4	0.8–3.7	26
	5	4	0.4–3.8	13
	6	3	0.5–4.3	6
	7	2	0.9, 2.8	0
Treated	1	2	0.9, 2.1	0
	2	0	–	0
	3	0	–	0
	4	0	–	0
	5	0	–	0
	6	0	–	0
	7	0	–	0

Fig. 2 Experimental protocol and inhibition of metastasis by postoperative administration of rIL-12. Metastasis was determined by palpation of lymph nodes at 4 sites (bilateral axillary and inguinal). Palpable lymph nodes were removed and weighed individually. The metastatic nodules in both lung lobes were enumerated. Results are shown from one of three experiments conducted

(enlarged) lymph nodes, only the primary tumor mass was resected. Mice treated in these ways were divided into two groups, one of which was treated with rIL-12. Lung metastases and intraperitoneal invasion were examined either upon tumor death or at a later postoperative time point (Table 1). The results demonstrate that rIL-12 administration is effective in inhibiting the development of lung metastases and further invasion from the abdominal wall into the peritoneal cavity, even in mice not undergoing radical resection of a primary tumor and/or removal of metastatic lymph nodes. Thus, these results indicate that IL-12 treatment of animals with a large tumor burden significantly inhibits tumor growth.

Cellular and molecular mechanisms underlying the antitumor effect of IL-12

Cellular mechanisms of the antitumor effects mediated by IL-12 have been investigated in a number of tumor models [2, 11, 12, 17, 20]. The majority of studies, including ours [20], have indicated that T-cell involvement is important. One of the biological properties critical for the antitumor effects of IL-12 is the ability of this cytokine to induce interferon-γ (IFN-γ) production in T-cells and NK cells. High levels of IFN-γ have been detected in normal and tumor-bearing mice following IL-12 administration [3]. We have demonstrated that administration of IL-12 in vivo induces marked enhancement of the capacity of T-cells to produce IFN-γ [20]. This was shown by enhanced IFN-γ

Table 1 Inhibition of lung metastasis/intraperitoneal invasion by postoperative IL-12 administration in mice that had undergone non-radical primary tumor resection[a]

Expt	Mouse		Lymph node metastasis		Lung metastasis (number of nodules)	Intra-peritoneal invasion
			Number of sites	Weight (g)		
A	Control	1	4	1.4–5.0	33	–
		2	4	1.3–4.9	28	–
		3	4	2.0–4.8	8	+
		4	4	1.4–4.4	10	–
	Treated	1	4	0.1–1.2	0	–
		2	2	0.3–0.4	0	–
		3	4	0.4–1.6	0	–
		4	1	0.7	0	–
B	Control	1	4	1.4–3.0	7	+
		2	4	1.1–3.2	9	+
		3	4	0.9–4.3	4	–
		4	3	0.8–3.9	11	+
	Treated	1	2	0.4–0.5	0	–
		2	1	0.4	0	–
		3	0	0	0	+
		4	0	0	0	–

[a] Tumor resection was performed 36 days after tumor implantation. Mice in experiment (*Expt*) A had enlarged (metastatic) lymph nodes on resection of the primary tumor. Mice in Expt B had a primary tumor invading the abdominal wall and, therefore, tumor resection was incomplete. All mice were killed 48 days after tumor resection, when metastasis and tumor resection were examined

production when spleen cells from IL-12-treated mice were cultured in vitro [5, 20].

The role of IFN-γ in IL-12-induced tumor regression was examined using a neutralizing anti-IFN-γ MAb. Two injections of anti-IFN-γ MAb (1.5 mg/injection) prior to IL-12 treatment led to almost complete inhibition of the IL-12-induced antitumor effect in both the CSA1M and OV-HM models [19, 20]. These studies indicate that IFN-γ, which is produced in vivo following IL-12 administration, is required to induce tumor regression. Although the critical requirement of IFN-γ for the antitumor effect of IL-12 has been observed, the amount of IFN-γ produced in tumor-bearing animals does not correlate with the degree of IL-12 efficacy. Brunda et al. [3] have found that IL-12 induces high serum IFN-γ levels in both tumor-bearing athymic (nude) and euthymic mice. We have also observed that IL-12 induces enhanced IFN-γ production, irrespective of whether it eventually leads to tumor regression (Fujiwara and Hamaoka, unpublished observations). Consistent with these observations, treatment of tumor-bearing mice with IFN-γ alone did not cause significant tumor-inhibitory effects [3]. Thus, it appears that IFN-γ production alone, if induced only in lymphoid organs by T-cells and NK cells, is not sufficient to induce marked therapeutic effects.

In this context, our recent studies have shown that the antitumor effect of IL-12 is achieved not solely by IFN-γ production but through a series of host immune responses [5, 6, 19]. IL-12, a tumor immunity-inducing cytokine, (a) stimulates antitumor T-cells to produce high levels of IFN-γ; (b) allows these activated T-cells to migrate into tumor masses; and (c) produces an effector cytokine, IFN-γ, constitutively at tumor sites in situ. This has been demonstrated by the high levels of T-cell infiltration and constitutive IFN-γ expression within tumor masses. Furthermore, these events lead to in situ expression of several genes that are likely to inhibit tumor growth, including an inducible type of nitric oxide synthase (iNOS) [19]. That intratumoral expression of IFN-γ is important is demonstrated by the finding that anti-IFN-γ MAbs inhibit in situ expression of iNOS without affecting cellular infiltration or IFN-γ mRNA expression. Ultimately, this antibody blocks IL-12-mediated tumor regression. It is also noteworthy, again, that massive infiltration of T-cells was seen in both CSA1M and OV-HM tumor masses in IL-12-treated mice, whereas only slight cellular infiltration was observed in MCH-1-A1 tumor masses [19]. These observations indicate that tumor rejection correlates with the capacity of T-cells to migrate to tumor sites, suggesting that this process is a critical factor. The overall antitumor efficacy of IL-12 would be determined by the effective operation of each of the above-mentioned processes.

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