Cancer Chemother Pharmacol (1996) 38 (Suppl): S16–S21

REVIEW

Michael J. Brunda · Leopoldo Luistro
Leonid Rumennik · Rosemary B. Wright
Mark Dvorozniak · Anthony Aglione
Jon M. Wigginton · Robert H. Wiltrout
Jill A. Hendrzak · Alicia V. Palleroni

Antitumor activity of interleukin 12 in preclinical models

Abstract Interleukin 12 (IL-12) is a heterodimeric cytokine with a number of biological effects that are consistent with its potential role as an antitumor agent. The antimetastatic and antitumor activities of IL-12 have been demonstrated in a number of murine tumor models. Both the inhibition of established experimental pulmonary or hepatic metastases and a reduction in spontaneous metastases have been achieved by treatment with murine IL-12. Systemic treatment of mice bearing subcutaneous tumors with IL-12 results in tumor growth inhibition, prolongation of survival, and, in some models, tumor regression. The antitumor effect of IL-12 in these models is dose-dependent and can be initiated against well-established tumors. Mice cured of their tumor by IL-12 treatment are specifically immune to rechallenge with the same tumor. A series of experiments have demonstrated that both T-cells and interferon-gamma (IFN-γ) induction are necessary for the optimal antitumor effects of IL-12. However, the antitumor efficacy of IL-12 has not been observed after exogenous administration of murine IFN-γ, suggesting that additional factors may be important for the antitumor effects of IL-12. In several tumor models, IL-12 is more active or has a larger therapeutic window than either IL-2 or IFN-α, two cytokines with demonstrated antitumor activity against human malignancies. Combining IL-12 with other cytokines or chemotherapeutic drugs can improve antitumor effects.

Key words Antitumor activity · Cytokine · Interleukin 12 · Interleukin 2 · Interferon

Work presented at the 11th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium "Cytokines and New Anticancer Agents on the Horizon of Oncology", 24–25 November 1995, Nagoya, Japan

M. J. Brunda (✉) · L. Luistro · L. Rumennik · R. B. Wright · M. Dvorozniak · A. Aglione · J. A. Hendrzak · A. V. Palleroni
Department of Oncology, Hoffmann-La Roche Inc., Nutley, NJ 07110, USA

J. M. Wigginton
Biological Carcinogenesis and Development Program, SAIC Frederick, Frederick, Maryland, USA

R. H. Wiltrout
Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland, USA

Interleukin 12 (IL-12) is a heterodimeric cytokine [27, 39] originally cloned from B-lymphoblastoid cell lines [21, 47]. The major normal cell type producing IL-12 is the macrophage/monocyte [10], but other normal cells such as B-cells, granulocytes, keratinocytes, mast cells, and dendritic cells (reviewed in [22, 43]) can produce IL-12; production by T-cells has not been reported. Receptors for IL-12 are present on both natural killer (NK) and T-cells [11, 12], and are composed of two related but unique chains, designated β1 and β2 [36]. Although each chain of the IL-12 receptor can bind IL-12 with low affinity, both subunits are needed for high-affinity binding [36].

IL-12 mediates a number of biological activities (reviewed in [22, 43]), and can enhance the proliferation and cytolytic function of T-cells and NK cells [16, 17, 33]. Furthermore, IL-12 induces T-cells and NK cells to produce a number of cytokines, including interferon-gamma (IFN-γ), tumor necrosis factor (TNF), IL-2, IL-3, IL-8, IL-10, and colony-stimulating factors [19, 22, 43]. IL-12 also stimulates the generation of T-helper type 1 (Th1) effector cells during an immune response [26, 28]; some subclasses of antibodies to protein antigens are similarly enhanced by concurrent treatment with IL-12 [20]. IL-12 can also inhibit growth-factor-induced angiogenesis [45].

Based on these properties, there has been strong interest in evaluating the antimetastatic and antitumor activities of IL-12. In all of these studies, recombinant murine IL-12 has been utilized because human IL-12 is not active against murine cells [37]. IL-12 has been shown to work therapeu-

tically in a number of experimentally induced and spontaneous models of pulmonary, hepatic, and lymph node metastases [3, 18, 31, 32, 40]. Following intravenous injection of tumor cells, including B16F10 melanoma [3], M5076 reticulum-cell sarcoma [3], and MC-38 adenocarcinoma [32] cells, systemic administration of IL-12 markedly inhibited metastases and prolonged survival. IL-12 has also been demonstrated to have activity in models of spontaneous metastases. For example, following surgical resection of the primary subcutaneous OV-HM ovarian carcinoma, lymph node and lung metastases were markedly reduced in IL-12-treated mice as compared with controls [31]. Similarly, IL-12 treatment reduced spontaneous pulmonary Lewis lung carcinoma [40] and hepatic M5076 metastases [18]. Recently, in an orthotopic model of Renca renal-cell carcinoma, IL-12 improved the survival of mice that had had their primary tumor removed but died from metastases if left untreated [46].

IL-12 has been shown to be effective when used as a therapeutic agent against a large number of transplanted tumors. Included among the tumors in which the in vivo effects of IL-12 have been evaluated are B16F10 melanoma [3], Renca renal-cell carcinoma [2–5], MC-38/colon 38 adenocarcinoma [32], Lewis lung carcinoma [40, 45], colon 26 carcinoma [1], MBT-2 bladder carcinoma [30], MB49 transitional-cell carcinoma [30], OV-HM ovarian carcinoma [31], TSA breast carcinoma (G. Forni, personal communication), M5076 reticulum-cell sarcoma [3, 18], KA31 sarcoma [15], MCA-105 sarcoma [32], MCA-207 sarcoma [32], CSA1 M fibrosarcoma [44, 51], Meth A sarcoma [34], X5563 lymphoma [35], and RAW117 lymphoma [44]. In these models, IL-12 given by either local peritumoral or systemic injection induces tumor growth inhibition, prolongation of survival, and, in some models, complete tumor regression in well-established tumors. Mice cured of their tumors reject subsequent challenge with the same but not other syngeneic tumor cells [2–4, 31, 32, 51].

Fibroblasts or tumor cells transfected with IL-12 genes have also been evaluated in murine models of tumor establishment and in therapy for existing tumors. Fibroblasts genetically engineered to secrete IL-12 and injected concomitantly with tumor cells suppressed the growth of BL-6 melanoma; the efficacy was related to the amount of IL-12 expressed by the transfected fibroblasts [41]. Peritumoral injection of IL-12-transfected autologous or allogeneic fibroblasts induced regression of established MCA-207 sarcoma [50]; systemic effects against contralateral primary tumors and pulmonary metastases were also obtained by subcutaneous injection of IL-12-secreting fibroblasts [50]. Mice in which tumor regression was observed were immune to rechallenge with parental tumor cells [50]. IL-12-secreting MCA-207 sarcoma, MCA-102 sarcoma, and colon 26 carcinoma tumor cells, in contrast to non-IL-12-secreting parental cells, were rejected by immunocompetent mice [29, 42]; mice in which these tumors regressed were immune to rechallenge with parental tumor cells. A therapeutic effect has also been obtained with IL-12-transfected MCA-207 sarcoma tumor cells at up to 3 days following the injection of parental cells [42]. Greater antitumor immunity has been found with MCA-207 sarcoma or TS/A breast-carcinoma tumor cells transfected with both B7.1 and IL-12 genes [49].

Since human IL-12 is inactive against murine cells [37], work in preclinical tumor models with human IL-12 requires concurrent transfer of human effector cells. Transfer of human cytotoxic T-cells and treatment with human IL-12 significantly prolonged the survival of severe combined immunodeficiency (SCID) mice bearing human acute myelogenous leukemia as compared with treatment with either therapy alone [7]. Treatment of SCID mice bearing human melanoma tumors with human NK cells plus human IL-12 and IL-2 was more effective than treatment with NK cells and IL-2 [24]. Preincubation in vitro of a cloned cytotoxic T-cell line with human IL-12 resulted in enhanced antitumor efficacy after transfer into glioblastoma-bearing SCID mice [8]. These transfer models utilizing human effector cells and IL-12 are consistent with the effects reported for murine IL-12 in tumor-bearing mice.

Since several cytokines, in particular IFN-α and IL-2 [23], have been shown to have antitumor effects against human malignancies, the efficacy of IL-12 has been compared with that of these cytokines. In the Renca renal-cell carcinoma model, although both IL-2 and IL-12 are active, IL-2 induces tumor regression only at its maximum tolerated dose, with no long-term survivors resulting from a 2-fold lower dose; however, IL-12 is active over a 100-fold dose range [5]. In contrast to the tumor regression induced by IL-12, treatment of Renca tumor-bearing mice with IFN-α [5] or IFN-γ [4] results in inhibition of tumor growth but not in tumor regression. IL-12 has shown antitumor activity superior to that of IL-2 in mice bearing B16F10 melanoma and Lewis lung carcinomas, whereas similar effects have been found in mice bearing M5076 and colon 38 tumors [5, 40] (Brunda et al., unpublished observation). In metastasis models the efficacy of IL-12 has also been shown to be superior to that of IL-2 [31, 32]. One note of caution for these types of experiment is that it cannot be excluded that better efficacy could be achieved by some modification of IL-2 dosing that was not explored in these studies.

The mechanism(s) underlying the antitumor effects of IL-12 is just beginning to be explored. IL-12 does not have a direct antiproliferative effect on tumor cells as judged by the inability of IL-12, even at very high concentrations, to inhibit tumor cell proliferation in vitro [4, 25, 35] and by the finding that the in vivo activity of IL-12 is much lower in nude mice than in euthymic mice bearing the same tumors [3, 4]. It is nonetheless possible that IL-12 may exert direct effects on tumor cells, and it has recently been reported that IL-12 can inhibit human tumor cell attachment to matrices and growth-factor-induced invasion [25]. Although relatively few cells have been shown to have receptors for IL-12 [11, 12], other potential direct effects of IL-12 on tumor cells need to be evaluated.

Based on its biological effects, it was initially hypothesized and subsequently shown that the effects of IL-12 are mediated, at least in part, through stimulation of the immune system. Several lines of evidence have emerged

demonstrating the importance of T-cells in mediating the antitumor effects of IL-12. Large numbers of T-cells have been found within regressing tumors from IL-12-treated mice [32, 51]; the specific subpopulations vary with the tumor evaluated, with a predominance of $CD8^+$ T cells being present in MCA-207 sarcomas [32] and both $CD8^+$ and $CD4^+$ T-cells being present in CSA1 M fibrosarcomas [51].

As mentioned above, the antitumor effects of IL-12 are markedly reduced in Renca or B16F10 tumor-bearing T-cell-deficient athymic nude mice as compared with those in euthymic tumor-bearing animals [3, 4]. Depletion of T-cell subsets with monoclonal antibodies resulted in reduced efficacy of IL-12 treatment. In Renca carcinoma [3] or Meth A sarcoma [34] tumor-bearing mice, anti-CD8 antibodies markedly suppressed the efficacy of IL-12, whereas anti-CD4 antibodies had no effect. In mice bearing either MCA-207 sarcomas [32] or CSA1 M fibrosarcomas [51], depletion of either $CD4^+$ or $CD8^+$ T-cells had little effect on IL-12 efficacy; however, depletion of both subsets completely abrogated the antitumor effects. In C-26 colon carcinoma tumor-bearing mice, treatment with anti-CD4 antibodies increased the antitumor efficacy of IL-12 therapy; the increased efficacy was correlated with an increase in $CD8^+$ T-cell infiltration of tumors [29]. Overall, although specific details vary with the tumor model utilized, it is clear that T-cells are critical for optimal antitumor effects of IL-12.

In contrast to T-cells, there are few data demonstrating the involvement of other immune effector cells. Depletion of NK cells by injection of anti-asialo GM1 antibody resulted in no loss of IL-12 efficacy in Renca or MCA-207 tumor-bearing mice [3, 32]. Similarly, IL-12 treatment was just as effective in NK-cell-deficient beige mice as in normal mice bearing the B16F10 melanoma [3]. However, IL-12 activity has been demonstrated in SCID mice bearing X5563 lymphomas [35], suggesting a possible role for NK cells. Antibody depletion studies also suggest that NK cells contribute to the early phase of antitumor reactivity induced by IL-12 in gene therapy models [29, 42]. Higher numbers of macrophages infiltrate IL-12-induced regressing tumors, and there is evidence for direct or indirect activation of macrophages by IL-12 [32] (Hendrzak et al., unpublished results), but no direct role for macrophages in mediating the antitumor effects of IL-12 has been established. Evaluation of other cell types awaits further experimentation.

Since IL-12 is a potent inducer of IFN-γ [19] and IFN-γ has many biological effects [48] that could influence tumor growth, a substantial amount of work has focused on the role that IFN-γ induction by IL-12 plays in antitumor efficacy. It is clear that treatment of tumor-bearing mice with IL-12 results in enhanced serum levels of IFN-γ [4, 32] and increased levels of IFN-γ mRNA within tumors [14]. The critical role of IFN-γ has been demonstrated using neutralizing anti-IFN-γ antibodies that can inhibit the efficacy of IL-12 in several tumor models [4, 6, 32, 51] and the loss of the antitumor activity of IL-12 in IFN-γ gene knockout (gko) mice bearing B16F10 melanomas [6]. The lack of IFN-γ in gko mice results in many defects [9],

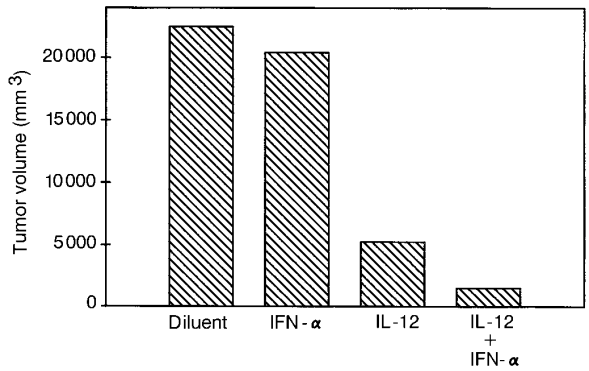

Fig. 1 Increased inhibition of M5076 tumor growth by IL-12 plus IFN-α. Mice were injected with tumor cells subcutaneously on day 0 and treatment was initiated on day 21. Intraperitoneal cytokine treatment was as follows: IL-12 at 1 μg 5 times/week, IFN-α once per week (1×10^6 U), or the cytokine combination. Tumors were measured on day 57 and their volume was calculated as described previously [3].

including a complete inhibition of IL-12-induced nitric oxide synthase and a marked reduction in the induction of lymphokine-activated killer (LAK) cell activity [6]. Although IFN-γ is necessary for the antitumor effects of IL-12, it is not clear whether induction of IFN-γ is sufficient to account for the antitumor efficacy of IL-12 or whether other factors are necessary. Several lines of evidence suggest the latter possibility. Substantially higher serum levels of IFN-γ are induced by IL-12 in tumor-bearing nude mice than in euthymic mice, but IL-12 has little antitumor activity in nude mice [4]. Treatment of Renca tumor-bearing mice with IL-12 is much more efficacious than treatment with exogenous IFN-γ [4]. However, higher levels of IFN-γ mRNA have been measured in tumors in mice treated with IL-12 [14], and this locally produced IFN-γ could mediate the antitumor effects of IL-12 through inhibition of angiogenesis [45], induction of nitric oxide [14], or other, as yet uncharacterized events. Further work is necessary to define the exact role of IFN-γ in mediating the antitumor efficacy of IL-12.

IL-12 is a potent antitumor drug when given as a single agent. However, although tumor regression is observed following IL-12 therapy in some models, in many other murine tumor models, IL-12 produces tumor inhibition rather than cure. To develop more effective therapies, we have evaluated the utility of combining IL-12 with other cytokines and chemotherapeutic drugs. In our initial combination studies, we evaluated IL-2 and IFN-α, two cytokines with demonstrated antitumor effects in animals and humans [23]. Combining IL-12 with either IL-2 or IFN-α, at optimal doses of these proteins as single agents, resulted in substantially increased toxicity and death [5]. At suboptimal doses, increased activity of the cytokine combinations as compared with the same dose/regimen of each individual cytokine was observed in some tumor models, but the antitumor effect was not superior to that achieved with the optimal dose of IL-12 alone [5]. Recently, a new regimen of IL-12 plus IL-2 administration has been developed that produces a substantial increase in antitumor

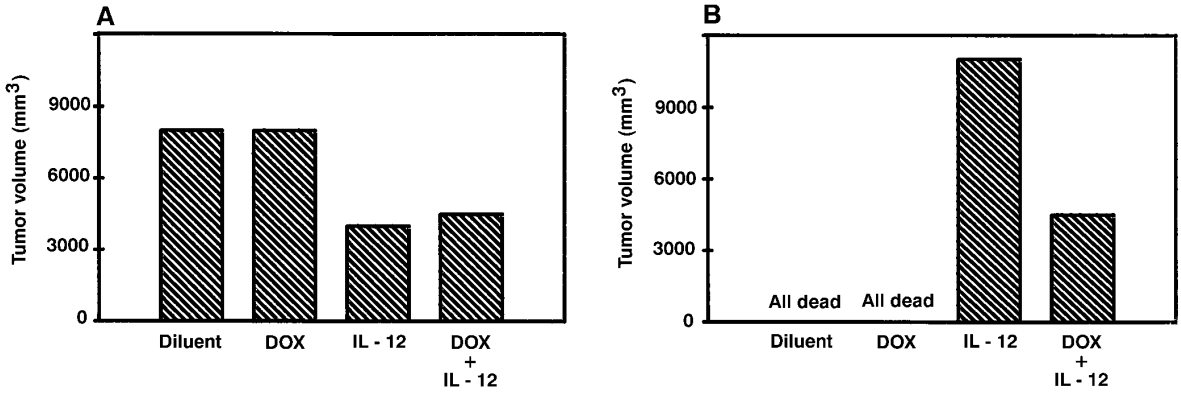

Fig. 2 A, B Increased inhibition of B16F10 tumor growth by IL-12 plus doxorubicin (DOX). Mice were injected with tumor cells subcutaneously on day 0 and treatment was initiated on day 14. Intraperitoneal treatment was as follows: IL-12 at 1 μg 5 times/week, DOX once per week (5 mg/kg), or the combination. Tumors were measured on **A** day 22 and **B** day 43, and their volume was calculated as described previously [3]. All mice in the diluent and DOX-alone groups had died by day 43

efficacy [46]. In an orthotopic model of renal-cell carcinoma, 90–100% of mice showed complete, lasting responses when treated with IL-12 given five times per week combined with IL-2 given weekly after surgical removal of the primary tumor, as compared with 10–30% of animals that underwent surgery combined with administration of one of these cytokines [46]. This combination did not produce any increase in gross toxicity. Preliminary data also show that this cytokine combination without surgical resection of the primary tumor is superior to treatment with either IL-12 or IL-2; the response observed includes the inhibition of primary tumor growth, a reduction in the number of metastases, and prolongation of survival (Dvorozniak and Aglione, unpublished observations). Similarly, in mice bearing subcutaneous M5076 tumors, the combination of daily IL-12 and weekly IL-2 resulted in a substantial improvement in survival [6]. Using a similar regimen, an improved antitumor response has been observed in M5076 tumor-bearing mice treated with IL-12 and weekly IFN-α (Fig. 1). In this model, IFN-α given weekly is not effective, but when IFN-α is given in combination with IL-12 the response is substantially improved as compared with treatment with IL-12 alone. The mechanism underlying this improved response obtained with cytokine combinations has not been characterized but is currently under investigation.

Using a similar approach, the combined therapeutic effect of IL-12 and several chemotherapeutic drugs has been evaluated in the B16F10 and M5076 models. Dosing with IL-12 five times per week combined with weekly dosing with doxorubicin resulted in greater antitumor efficacy against subcutaneous B16F10 tumors (Fig. 2). Early in the course of therapy (day 22), there was comparable inhibition by both IL-12 and IL-12 plus doxorubicin, whereas doxorubicin alone was ineffective. However, as therapy continued (day 43), tumor growth was inhibited to a greater extent in mice treated with the combination than in mice treated with IL-12 alone. In contrast to the positive results obtained with IL-12 plus doxorubicin, combining IL-12 with etoposide did not result in enhanced antitumor efficacy (Fig. 3, day 43). Combining IL-12 with several other drugs, including taxol and 5-fluorouracil, also did not

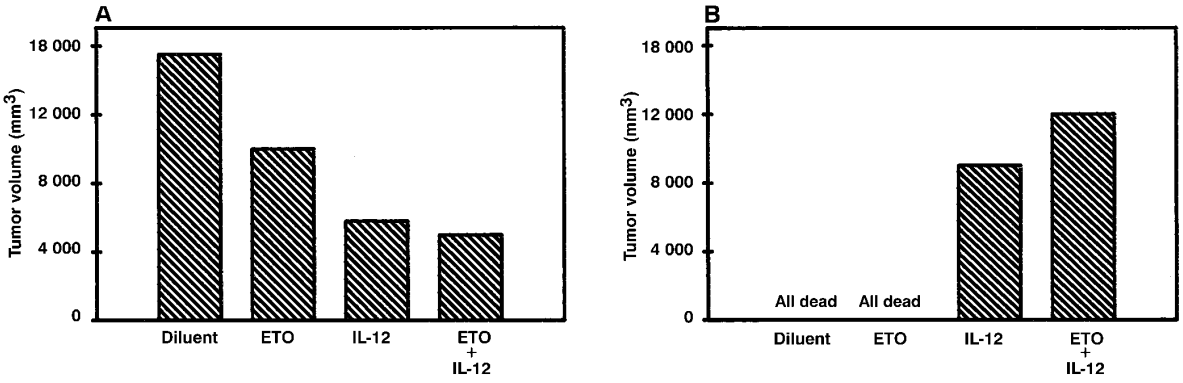

Fig. 3 A, B Treatment of B16F10 tumor-bearing mice with IL-12 plus etoposide (ETO) does not increase efficacy. Mice were injected with tumor cells subcutaneously on day 0 and treatment was initiated on day 14. Intraperitoneal treatment was as follows: IL-12 at 1 μg 5 times/week, ETO once per week (10 mg/kg), or the combination. Tumors were measured on **A** day 22 and **B** day 43, and their volume was calculated as described previously [3]. All mice in the diluent and ETO-alone groups had died by day 43

improve efficacy as compared with treatment with IL-12 alone (Luistro and Rumennik, unpublished observations). These findings contrast with the positive results obtained by combining IL-12 with radiotherapy or chemotherapy in some tumor models [13].

Doxorubicin can induce apoptosis of tumor cells [38], and the potential effect of IL-12 on this process has been evaluated in an in vitro assay. Incubation of B16F10 tumor cells with doxorubicin induced dose-dependent apoptosis; however, culture of tumor cells with doxorubicin and IL-12 did not result in increased apoptosis. Since IL-12 is a potent inducer of IFN-γ [19], B16F10 cells were cultured with the combination of doxorubicin and IFN-γ; enhanced apoptosis was observed under these conditions (R. Wright, unpublished observations). It is possible that in vivo injection of IL-12 may indirectly augment apoptosis induced by doxorubicin through induction of IFN-γ. At present it is not known whether other cytokines induced by IL-12 may induce similar effects. Additional experiments are necessary to establish whether there is a direct correlation between the in vitro and in vivo findings.

In summary, IL-12 is a cytokine that can induce multiple biological effects and is a potent therapeutic agent in murine models of tumor growth and metastasis. Both T-cells and IFN-γ are important mediators of the antitumor effects of IL-12, but the details of their involvement remain unknown. The activity of IL-12 can be enhanced by weekly pulses of other cytokines, including IL-2 and IFN-α, or doxorubicin. Future studies will determine the role of IL-12 in the treatment of human malignancies.

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