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# Antiproliferative effects of interleukin-12 treatment on human tumor colony-forming units taken directly from patients

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**Interleukin-12 (IL-12) has important immunomodulatory effects on T and natural killer (NK) cells that might be exploited in anticancer treatment. Murine IL-12 models have shown antimetastatic and antitumor effects against murine tumors *in vivo*. Data on the effects of human IL-12 on human tumors are confined to $^{51}$Cr-release assay studies showing that IL-12 increases NK activity against cancer cells. We used a human tumor cloning assay (HTCA) to investigate the effects of human IL-12 on solid tumors taken directly from patients. The HTCA is suitable to test direct, as well as immune-mediated, antitumor effects of cytokines on heterogeneous cell preparations derived from fresh tumors. Single cell suspensions prepared from 193 tumors were continuously exposed (14 days) to 10, 100 and 1000 ng/ml of human IL-12 in a capillary HTCA. Seventy-four (38%) specimens were evaluable. Inhibition of tumor growth was observed in 35 specimens (47%; concentration-related in 33 cases), including cancers of the ovary, lung, prostate, breast, colon and kidney, as well as melanoma. Antitumor effect was observed in 10 (14%), 18 (24%) and 32 (43%) tumors, at 10,100 and 1000 ng/ml of IL-12, respectively. One specimen (1%), a melanoma, showed stimulation of tumor proliferation only at 100 mg/ml of IL-12. Our results show that IL-12 has substantial *in vitro* activity against a variety of solid tumors taken directly from patients. Clinical trials of IL-12 in patients with solid tumors are warranted.**

*Key words*: Cloning assay, cytokines, interleukin-12, natural killer cell stimulatory factor.

## Introduction

Interleukin-12 (IL-12), also known as natural killer cell (NK) stimulatory factor[1] or cytotoxic lymphocyte maturation factor,[2] is a 79 kDa heterodimeric cytokine composed of p35 and p40 subunits.[1,2] The two complementary DNAs encoding p35 and p40 from both mice and humans have been cloned, and purified recombinant proteins have been produced.[3–5] Secreted by macrophages, B cells and other antigen-presenting cells,[6] IL-12 can enhance the lytic activity of NK and lymphokine-activated killer cells,[1,7–9] facilitate cytolitic T lymphocyte (CTL) responses,[9] act as a growth factor for activated T and NK cells,[7,10,11] induce the secretion of interferon (IFN)-$\gamma$ from T and NK cells,[1,12,13] and promote the induction of $T_h1$ responses.[14–17] Most of these activities have been demonstrated *in vivo* as well.[18]

The ability of IL-12 to enhance NK and CTL responses and to elicit the production of IFN-$\gamma$ provides possible mechanisms through which IL-12 might exert antitumor effects. Indeed, murine IL-12 has shown potent and reproducible antimetastatic and antitumor effects against many murine tumors.[19–22] IL-12 not only inhibits tumor growth but also, in some instances, induces regression of well-established solid malignancies.[19] These animal studies support the potential therapeutic utility of IL-12 in cancer treatment.

Since IL-12 is species specific, data on its effects on human tumors are limited to $^{51}$Cr-release assay studies showing that IL-12 increased the cytolytic activity of NK cells against established cell lines, such as K562 cells, and colon and neuroblastoma cell lines.[23–25] Although these data are encouraging, a more extensive and perhaps clinically relevant analysis of the antitumor effects of IL-12 against human malignancies would be desirable before beginning clinical trials.[26] The human tumor cloning assay (HTCA) provides a suitable model to determine the effects of lymphokines against malignant stem cells from heterogeneous cell preparations derived from fresh human tumors.[27,28] This approach might be more clinically relevant since cell lines may have different properties and growth requirements than the tumor cells from which they

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arise. In addition, single cell suspensions derived from tumors taken directly from patients contain cells other than tumor cells, including immune competent effector cells. These cells could be stimulated by IL-12 to produce a cytotoxic effect. In this study, we used an HTCA to evaluate the effects of recombinant human IL-12 treatment on single cell suspensions derived from a broad panel of solid tumors taken directly from patients.

## Materials and methods

### Recombinant human IL-12

Recombinant human IL-12 (specific activity, $6 \times 10^6$ units/mg protein) was obtained from Genetics Institute (Cambridge, MA). Stock solutions of IL-12 were prepared in Connaught Medical Research Laboratories (CMRL) 1066 medium (Irvine Scientific, Irvine, CA) and stored at −70°C until further use. On the day of an experiment the stock solution was serially diluted in enriched CMRL. IL-12 was used at final concentrations of 10, 100 and 1000 ng/ml.

### Tumor sample processing

After written informed consent was obtained according to institutional guidelines, tumor specimens were collected by sterile standard procedures as part of routine clinical measures. Solid specimens were immediately placed in McCoy's 5A medium containing 10% new born calf serum plus 1% penicillin/streptomycin (all from Gibco, Grand Island, NY). Solid specimens were minced and repeatedly passed through metal meshes with mesh widths of 0.14 mm (EC Apparatus, St Petersburg, FL) to obtain a single cell suspension. Malignant effusions were collected with preservative-free heparin (10 U/ml), centrifuged at 150 *g* for 7 min, passed through 25 gauge needles to obtain single cell suspensions and washed twice in McCoy's 5A medium with 10% fetal calf serum (FCS; Hyclone, Logan, UT). All specimens were suspended in McCoy's 5A medium containing 10% FCS, as well as 2 mM sodium pyruvate and 1% penicillin/streptomycin and 35 mg/ml L-serine (both from Gibco).

### Capillary HTCA

To evaluate the effects of IL-12 on single cell suspensions derived from human tumors we used the capillary HTCA.[29] Cells to be cloned were resuspended in 0.3% agar in enriched CMRL medium 1066 (for composition see ref. 30). Prior to plating, asparagine (100 μg/ml), glutamine (200 nM) and HEPES (10 mM; Research Organics, Cleveland, OH) were added to the cells. The concentration of IL-12 was adjusted to 10, 100 or 1000 ng/ml in the final cell suspension. Controls were incubated with equivalent volume of solvent without IL-12. One hundred microliters of the final mixture containing $5 \times 10^5$ cells/ml was placed in 100 μl glass capillary tubes (Fisher Scientific, Houston, TX) by capillary action. The ends were sealed with S/P Miniseal Clay (American Scientific, Grand Prairie, TX). A 1 mm gap was left between the clay and the agar to prevent contamination from the clay. Six capillary tubes were prepared for each data point. The capillary tubes were incubated at a 30° angle in a 7% $CO_2$ incubator at 37°C. Each experiment included a positive control with orthosodium vanadate (Sigma) at a final concentration of 200 μg/ml to assure the presence of a good single cell suspension.[31] The use of a positive control has been shown to greatly increase the reproducibility of the HTCA.[32]

### Evaluation and statistical considerations

After 14 days of incubation, the agar was extracted from the capillary tube and placed on a microscope slide and colonies were counted using an inverted microscope at ×3 magnification. An experiment was considered evaluable for analysis when the solvent control had a mean number of colonies three or above among the six control tubes and the positive vanadium control showed 30% or less colony formation compared with the solvent control. The results of the *in vitro* cloning assay were expressed as the percentage of survival to tumor colony-forming units for IL-12 relative to its control. This quantity was calculated as the ratio between the mean number of colonies surviving in the six IL-12-treated capillary tubes and the mean number of colonies growing in the six control capillary tubes. Inhibition of colony formation was defined as a reduction in growth to less than 50% of control. This cut-off point was used because it has been found to be a useful determinant of *in vitro* and *in vivo* correlative trials using the HTCA to predict patient response.[30] Growth stimulation was defined as an increase to greater than 150% of control.

**Table 1.** Effects of IL-12 on *in vitro* growth of solid tumors taken directly from patients

| Tumor Type | No. of specimens evaluable/ tested (%) | No effect[a] (%)[b] | Stimulation[a] (%)[b] | Inhibition (%)[b] | | | |
|---|---|---|---|---|---|---|---|
| | | | | 10[c] | 100[c] | 1000[c] | Total[a] |
| Ovary | 12/21 (57) | 7 (58) | 0 | 2 (17) | 2 (17) | 5 (42) | 5 (42) |
| Non-small-cell lung | 11/19 (58) | 5 (45) | 0 | 0 | 1 (9) | 6 (55) | 6 (55) |
| Colon | 11/23 (48) | 7 (64) | 0 | 1 (9) | 2 (18) | 4 (36) | 4 (36) |
| Breast | 10/50 (20 | 5 (50) | 0 | 2 (20) | 5 (50) | 5 (50) | 5 (50) |
| Melanoma | 10/20 (50) | 7 (70) | 1 (9)[d] | 1 (10) | 2 (20)[e] | 2 (20) | 3 (30) |
| Kidney | 10/24 (42) | 5 (50) | 0 | 1 (10) | 2 (20) | 5 (50) | 5 (50) |
| Prostate | 2/7 (29) | 0 | 0 | 1 (50) | 1 (50) | 2 (100) | 2 (100) |
| Others[f] | 8/29 (28) | 3 (37) | 0 | 2 (25)[e] | 3 (38)[e] | 3 (38) | 5 963) |
| Total | 74/193 (38) | 39 (53) | 1 (1) | 10 (14) | 18 (24) | 32 (43) | 35 (47) |

[a]At any concentration of IL-12.
[b]Percentage of the evaluable specimens.
[c]Concentration of IL-12 in ng/ml.
[d]This melanoma specimen showed stimulation of tumor proliferation only at 100 ng/ml.
[e]One melanoma, one carcinoma of unknown primary and one endometrial carcinoma showed inhibition only at 100, 100 and 10 ng/ml, respectively.
[f]Evaluable specimens include cancers of unknown primary, bladder, uterus (2) and gallbladder, as well as one head and neck squamous cell carcinoma, and one lymphoma.

## Results

### Growth of tumor cells in the capillary HTCA

Tumors from 193 patients were tested for the effects of IL-12. Of the 193 tumor samples tested, 74 (38%) were evaluable, as defined above. The evaluable specimens included predominantly cancers of the ovary, breast, colon, kidney and lung, as well as melanomas (Table 1).

### Response of human tumors to IL-12

Table 1 summarizes the effect of IL-12 on *in vitro* growth of the 74 evaluable human tumors. No significant effect was observed after treatment with IL-12 in 39 specimens (53%). Only one specimen (1%) showed stimulation of tumor proliferation. This single stimulated specimen was one of 10 melanomas and demonstrated enhanced growth (195% control) only at 100 ng/ml of IL-12 but not at the other concentrations (113 and 95% of control at 10 and 1000 ng/ml, respectively). Overall, inhibition of *in vitro* tumor growth with IL-12 to less than 50% control was observed in 35 of 74 evaluable specimens (47%) for at least one concentration of IL-12. According to the concentration of IL-12, antitumor activity was observed with 10 tumors (14%) at 10 ng/ml, 18 tumors (24%) at 100 ng/ml and 32 tumors (43%) at 1000 ng/ml (Table 1). In 32 of the 35 IL-12-sensitive specimens, the antitumor effect of the addition of IL-12 was concentration-related with maximum antiproliferative effects occurring at 1000 ng/ml dose (Figure 1). In contrast, one melanoma, one carcinoma of unknown primary and one endometrial carcinoma showed inhibition to less than 50% control only at 100, 100 and 10 ng/ml of IL-12, respectively, but not at the other concentrations (Figure 1D). Treatment with IL-12 showed antiproliferative activity against all tumor types tested, and in particular against cancers of the lung [non-small cell (six of 11 responses, 55%)], ovary (five of 12, 42%), kidney (five of 10, 50%), breast (five of 10, 50%) and prostate (two of two, 100%).

## Discussion

The data reported show substantial antiproliferative effects of IL-12 on single cell suspensions derived from fresh human cancers using the capillary HTCA and, thus, provide evidence that human IL-12 could be effective against a broad spectrum of solid tumors. In general, the activity of IL-12 was concentration dependent with maximum activity at the highest concentration tested. This is in agreement with the results obtained in murine tumor models.[19–21] Whether effective plasma concentrations of IL-12 are achievable in patients remains to be determined. Data obtained in animals indicate that effective doses of IL-12 can be administered *in vivo* with tolerable toxicity. Thus, after doses of IL-12 of 1

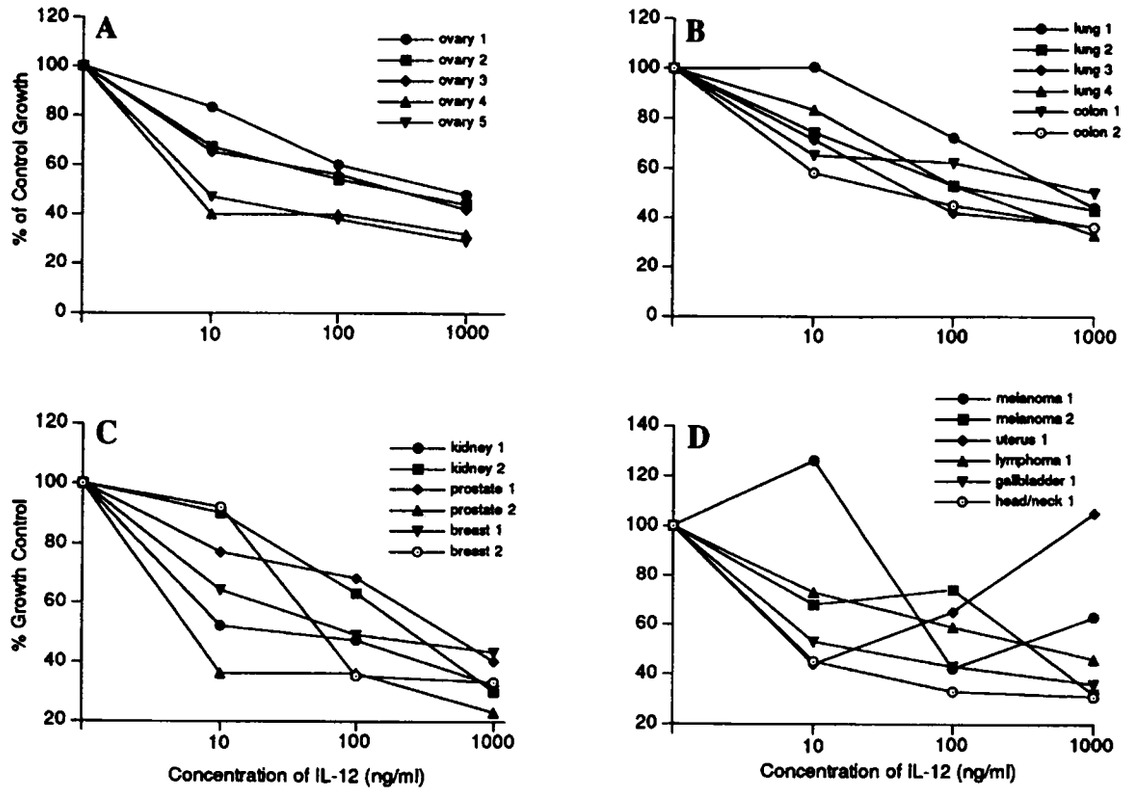


**Figure 1.** Representative dose–response curves of the effects of continuous exposure to IL-12 of human solid tumors in a human tumor cloning assay. Cancers of the ovary (A), lung and colon (B), kidney, prostate and breast (C), and melanoma and others (D). In general, the antitumor effects of IL-12 (in 32 of 35 IL-12-sensitive tumor specimens; 23 of them are represented). Only three specimens showed a non-linear dose–response curve. One melanoma, one carcinoma of unknown primary and one endometrial carcinoma showed maximum antitumor effect at 100, 100 and 10 ng/ml of IL-12, respectively [two of them are represented (melanoma, 1; uterus,1)].

μg, or even as high as 5 μg, per day in mice, no gross toxicity was evident.[19,20] In non-human primates, IL-12 was well tolerated at doses corresponding to those that have antitumor efficacy in mice.[33,34] Severe side effects, such as those resembling vascular leak syndrome, were observed only at doses 500-fold higher than doses showing positive immunomodulatory effects.[34] In the event that effective plasma concentrations of IL-12 could not be achieved in patients, the antitumor effects of IL-12 might still be clinically exploited. Low concentrations of IL-12 and IL-2 synergize in augmenting NK cell antitumor activity, CTL responses, and induction of IFN-γ, *in vitro* and *in vivo*.[2,9,12,13,22,25] Such a combination may be feasible in the clinical setting to mediate maximum antitumor effects, while circumventing the toxicity of high doses of IL-2 and, hypothetically, of IL-12.

The antitumor effects of IL-12 probably involve multiple factors, including the stimulation of immune competent effector cells. Evidence exists that T cells and production of IFN-γ by T/NK cells play a critical role in the antitumor effects of IL-12, since inhibition of either of these components substantially reduces IL-12's antitumor efficacy.[19,20] Single cell suspensions as used in the HTCA contain tumor and non-malignant cells, such as T/NK cells and macrophages, likely reflecting the tumor:effector cells (T:E) ratio present in the tumor *in vivo*. Our results suggest that the *in vivo* T:E ratio is appropriate to mediate IL-12 antitumor effects. Furthermore, since the colonies generated in the HTCA derive almost exclusively from the neoplastic population,[26] proliferation of effector cells may not be a prerequisite to mediate IL-12 antitumor effects. Similarly, proliferation of NK cells was suggested not to be necessary for their antitumor activity after IL-12 treatment.[22] IL-12 does not have direct inhibitory effects on cancer cell lines in culture or growing in nude mice.[19,21] However, cell lines display

different properties than their original tumors and we cannot exclude a direct antitumor activity of IL-12 against fresh cancer cells in the cloning assay.

The concentration-dependent antiproliferative effects of IL-12 would support this possibility. It remains to be determined whether cell types other than macrophages/B cells, and in particular fresh tumor cells from patients, express IL-12 receptors. *In vivo*, other effects of IL-12 might further contribute to its antitumor activity. Such effects include proliferation of NK/T cells,[11] chemotactic activity for NK and activated T cells,[35] and stimulation of NK interaction with endothelial cells.[35] In addition, it has been recently shown that IL-12 is a potent inhibitor of angiogenesis *in vivo*, an effect that appears to be mediated by IFN-$\gamma$.[36]

We observed stimulation of tumor proliferation, which was not concentration-related [e.g. exclusively seen at the intermediate concentration (100 ng/ml) of IL-12], in only one melanoma specimen out of 74 evaluable tumors. Since the HTCA has been useful in predicting the lack of stimulation of tumor growth by other cytokines in patients,[37] it is highly unlikely that the clinical use of IL-12 will promote the growth of solid tumors.

## Conclusion

In conclusion, our study demonstrates substantial *in vitro* activity of human IL-12 against a variety of solid tumors taken directly from patients. These results further support the information gained from animal studies supporting the use of IL-12 as a potential therapeutic agent. Phase II clinical trials of IL-12 in patients with solid tumors will be started soon. The results presented here may help in the design of these trials.

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