Microenvironment and Immunology

In vivo Antitumor Activity of a Recombinant IL-7/HGFβ Hybrid Cytokine in Mice

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Abstract

The immune cytokine interleukin (IL)-7 and the β-chain of hepatocyte growth factor (HGF) aggregate to form a naturally occurring heterodimer that stimulates the growth of common lymphoid progenitors and immature B and T lymphoid cells. We have cloned and expressed the heterodimer as a single-chain hybrid cytokine [recombinant (r) IL-7/HGFβ], which stimulates short-term hematopoietic stem cells as well as lymphoid precursors. Inasmuch as IL-7 and HGF are known to have antitumor and protumor activities, respectively, we determined here whether either of these activities is exhibited by rIL-7/HGFβ. We show that the in vivo administration of rIL-7/HGFβ markedly inhibits the growth of newly initiated and established tumors and the formation of pulmonary metastases in murine models of colon cancer and melanoma. The antitumor effect of rIL-7/HGFβ correlated with a marked increase in the number of tumor-infiltrating CD4+ and CD8+ T cells and activated dendritic cells. A major role for these immune cells in tumor suppression was indicated by the inability of rIL-7/HGFβ to inhibit the growth of tumor cells in vitro and in congenitally athymic mice. Analysis of interferon-γ–secreting T cells showed that the immune response was tumor specific. Our findings justify further evaluation of rIL-7/HGFβ as a novel experimental cancer therapy.

Introduction

We previously purified a novel hybrid cytokine consisting of interleukin (IL)-7 and HGFβ from mouse bone marrow (BM) stromal cells in a unique long-term BM culture system that selectively generated TdT– B-lineage precursors (1–3). We have cloned and constructed the IL-7/HGFβ gene, in which IL-7 and HGFβ cDNAs were connected by a flexible linker. We have expressed the gene to produce a single-chain rIL-7/HGFβ protein that stimulates the growth of day 12 spleen colony-forming units, common lymphoid progenitors, early B-lineage cells, and thymocytes in vitro (4). The rIL-7/HGFβ cross-links and induces juxtacrine interactions between the IL-7 and hepatocyte growth factor (HGF; c-Met) receptors on the cell surface. This, in turn, results in signal “cross talk,” the appearance of novel phosphorylated proteins downstream, and functional readouts that are not induced by noncomplexed rIL-7 and/or HGFβ (ref. 4; data not shown).

IL-7 is essential for the development, maintenance, and regeneration of B and T cells and is also an activation factor for monocytes, macrophages, dendritic cells (DC), and natural killer (NK) cells (5). HGF (also called scatter factor, SF) is a heterodimer consisting of a 60-kDa α-chain and a 30-kDa β-chain that regulates the growth, motility, and morphogenesis, and regeneration of many cell types (6–9). In addition, HGF plays an important role in hematopoiesis by synergizing with other cytokines such as stem cell factor, IL-3, erythropoietin, and granulocyte macrophage colony stimulating factor (6–9). Consequently, the formation of a naturally occurring rIL-7/HGFβ hybrid cytokine seems designed to regulate lymphohematopoietic stem and progenitor cell development.

Nonetheless, despite the synergistic functions of IL-7 and HGF on normal cell growth and development, IL-7 and HGF differ fundamentally with respect to their effects on cancer cells. In several animal models of neoplasia, IL-7 has been shown to have antitumor effects mediated by CD4+ and/or CD8+ T cells (10–16) whereas HGF supported the growth, invasion, and metastasis of many types of cancers (17). Given the potential therapeutic use of rIL-7/HGFβ to enhance B- and T-cell reconstitution in immunocompromised patients, especially following BM reconstitution (4), it was important to determine whether rIL-7/HGFβ positively or negatively affects tumor cell growth in vivo. Toward this end, we have tested the effect of the local and systemic administration of rIL-7/HGFβ on the growth and metastasis of murine CT-26 colon cancer and B16F10 melanoma cells. The results showed that the intratumoral injection of rIL-7/HGFβ significantly inhibited the local growth of newly initiated or established tumors, apparently by inducing the expansion and activation of tumor-infiltrating lymphocytes (TIL) and DCs. In addition, the systemic administration of rIL-7/HGFβ significantly inhibited the formation of tumor metastases in the lung.
Materials and Methods

Animals and cell lines
Murine CT-26 colon cancer and B16F10 melanoma cells were obtained from the National Cancer Institute and the American Type Culture Collection.

Murine breast cancer 66.1 cell line was kindly provided by Drs. A. M. Fulton and N. Kundu (University of Maryland). Cells were resuscitated and cultured for less than 3 months before use in the experiments. American Type Culture Collection characterized cells by using karyotyping and cytchrome c oxidase I testing, BALB/c, C57BL/6, and congenitally athymic nude mice were purchased from Harlan Sprague-Dawley and the National Cancer Institute. Mice were housed, treated, and handled in accordance with the guidelines set forth by the University of Connecticut Health Center Animal Care Committee. rIL-7 was purchased from PreproTech, eBiosciences, and R&D Systems. rHGF and rIL-7/HGFβ were cloned, expressed, and purified as we have described (3, 4).

Evaluation of local tumor growth and pulmonary metastasis
Cancer cells in the exponential growth phase were harvested and washed in PBS before in vivo injection. To induce localized tumors, 2 × 10^5 CT-26 colon cancer cells (18) or 1 × 10^5 B16F10 melanoma cells were injected subcutaneously (s.c.) into the flank of syngeneic BALB/c or C57BL/6 mice, respectively. The indicated doses of rIL-7/HGFβ, rIL-7, and/or rHGFβ (or PBS) were then injected s.c. into the tumor injection site at 2-day intervals over the indicated time period. Tumor size (volume) was determined twice weekly by caliper measurements of the shortest (A) and longest (B) diameter, using the formula V = (A × B^2)/2. To induce pulmonary metastases, 2 × 10^5 CT-26 or B16F10 cancer cells were injected into the tail vein of syngeneic mice and rIL-7/HGFβ or PBS were injected intravenously (i.v.) at 2-day intervals from days 2 to 18. The animals were euthanized at the indicated times after tumor inoculation. Metastatic tumor nodules in the subpleural regions of the lungs were counted under a dissecting microscope.

Evaluation of TILs and DCs
At the indicated time points, the s.c. tumors were excised, weighed, minced into small fragments, and digested in 1 mg/mL of collagenase IV (Sigma) and 0.1 mg/mL of DNase (Sigma) at 37°C for 1 hour. The dissociated cells were then prepared for phenotypic analysis of T cells and DCs by flow immunocytometry.

Flow immunocytometry
Single-cell suspensions from tumors and draining or non-draining lymph nodes were stained with the following fluorochrome-conjugated antibodies: CD4, CD8, CD11c, CD80, CD86, CD3, and DX5 (BioLegend or BD Biosciences). The samples were analyzed on a FACSCalibur flow cytometer (Becton and Dickinson). Data analysis was done using FlowJo software.

ELISPOT assay
Enzyme-linked immunospot (ELISPOT) assay measuring IFN-γ were used to assess the in vitro T-cell responses to stimulation with CT-26 or B16F10 cells. Splenocytes (1 × 10^5 T cells/well) and irradiated cancer cells (1 × 10^5/well) were incubated for 2 days in 96-well plates (Millipore) coated with anti-mouse IFN-γ antibody (clone R4-6A2; Biolegend) and blocked with RPMI media supplemented with 10% fetal calf serum. The wells were then washed and incubated with biotinylated anti-mouse IFN-γ antibody (clone XMIG1.2; Biolegend). Reactions were visualized and counted using the streptavidin-peroxidase system (19).

Statistical analysis
P values were based on 2-sided student’s t test. A confidence level above 95% (P < 0.05) was determined as significant.

Results
rIL-7/HGFβ treatment inhibits local tumor growth
To determine whether rIL-7/HGFβ has antitumor activity, murine CT-26 colon cancer cells were injected s.c. into syngeneic BALB/c mice and, at 2-day intervals from days 2 to 24, the mice were injected at the tumor site with different doses of rIL-7/HGFβ (2.5, 5, 15, and 30 μg/injection) or control vehicle (PBS). As shown in Figure 1A, tumor growth was inhibited in a dose-responsive manner, with no detectable effect seen by day 30 at the 2.5-μg level and greater than 80% inhibition occurring at the 30-μg level. To compare the antitumor effect of rIL-7/HGFβ with its component cytokines, BALB/c mice were injected at the tumor site with optimal and equimolar amounts of rIL-7/HGFβ (15 μg/injection), rIL-7 (5 μg/injection), and/or rHGFβ (10 μg/injection), according to the above-mentioned schedule. As shown in Figure 1B, rIL-7 alone or mixed with rHGFβ inhibited local tumor growth by ~30% by day 30 whereas rHGFβ alone had no effect. In contrast, the rIL-7/HGFβ hybrid cytokine inhibited tumor cell growth by ~76% (P < 0.01). As shown in Figure 1C, the ability of rIL-7/HGFβ to inhibit local tumor growth was not restricted to colon cancer but applied to malignant melanoma as well. Furthermore, as shown in Figure 1D, significant antitumor activity was observed in mice in which rIL-7/HGFβ treatment was initiated 10 days after melanomas had been established.

To determine whether rIL-7/HGFβ, rIL-7, and/or HGFβ directly affect the growth of tumor cells, CT-26 colon cancer cells, and B16F10 melanoma cancer cells were cultured in vitro for 2 to 7 days in the presence of 20 to 150 ng/mL of rIL-7/HGFβ (or PBS), a dose range that has been shown to stimulate the proliferation of thymocytes and early B-lineage cells (4). The rate of tumor cell growth was not significantly different at any dose level of rIL-7/HGFβ from those observed in control cultures or in cultures containing equimolar amounts of rIL-7 and/or HGFβ (data not shown). Hence, the mechanism by which rIL-7/HGFβ inhibits the growth of CT-26 and B16F10 tumors in vivo would not seem to involve direct cytotoxic or cytostatic activities. This was confirmed by the results of experiments in nude mice (see the following text).
**rIL-7/HGFβ induces significant infiltration of DCs and T cells into the tumors**

The presence of large proportions of TILs has been associated with favorable clinical outcomes in cancer patients (13, 15). We therefore determined whether rIL-7/HGFβ, rIL-7, and/or rHGFβ treatment enhanced the presence of TILs in the s.c. murine colon and melanoma tumors. To normalize for differences in tumor size at the time sacrifice, we assessed the numbers of TILs per milligram of tumor tissue. On day 30 after tumor inoculation, single-cell suspensions of tumor tissue from mice treated with equimolar amounts of rIL-7/HGFβ, rIL-7, and/or rHGFβ (see Fig. 1B) were analyzed for CD4+ and CD8+ T cells by flow cytometry. Because of the role of DCs in the activation of T cells, we also analyzed for CD11c+ tumor-infiltrating DCs. As shown in Figure 2A, rIL-7 alone or mixed with rHGFβ significantly, albeit modestly (<2-fold), increased the numbers of CD8+ and CD4+ T cells, but not DCs, in the tumors. Again, rHGFβ alone had no effect. In contrast, rIL-7/HGFβ increased the numbers of DCs as well as CD4+ and CD8+ T cells 4- to 6-fold above the levels in PBS-injected controls. Furthermore, as shown in Figure 2B, the DCs in the rIL-7/HGFβ-treated tumors expressed significantly higher levels of CD80 and CD86 than did those in rIL-7- and/or rHGFβ-treated tumors, indicating that they had undergone activation and maturation. In addition to the tumors themselves, there was a parallel increase in the numbers of CD4+ and CD8+ T cells and activated DCs in the draining lymph node (DLN) and the number of NK cells was also significantly increased (Fig. 3). Similar results were observed in rIL-7/HGFβ-treated B16F10 melanoma-bearing mice (data not shown).

**rIL-7/HGFβ treatment induces a tumor-specific immunologic response**

The above-mentioned results suggest that the mechanism by which rIL-7/HGF inhibits local tumor growth is immunologic involving TILs and activated DCs. To explore this possibility, we examined the number of IFN-γ-producing cells from the DLNs and spleen of cytokine-treated tumor-bearing mice after _in vitro_ stimulation with syngeneic homologous or heterologous tumor cells. Cultures containing single-cell suspensions of DLNs or spleen cells (normalized to 1 × 10^5 T cells/well) from day 30 CT-26 colon cancer-bearing mice that had been treated _in vivo_ with equimolar amounts of rIL-7/HGFβ, rIL-7, and/or rHGFβ (or PBS) were stimulated with irradiated CT-26 cells. Cultures stimulated with irradiated 66.1 murine breast cancer cells served as specificity controls (20). After 2 days, the numbers of IFN-γ-producing cells present were quantified by ELISPOT assays.

As shown in Figure 4A, the numbers of IFN-γ-producing cells among the cultured DLN cells from rIL-7- and/or HGFβ-treated tumors was 2- to 3-fold higher than were those from PBS-treated controls after stimulation with CT-26 cells. As expected, treatment with rHGFβ alone had no effect. In contrast, the number of IFN-γ-producing cells among the cultured DLN cells from rIL-7/HGFβ-treated tumors was elevated 6- to 7-fold (*P* < 0.01). Identical results were obtained when splenocytes from these same animals were used (Fig. 4B). However, there was no increase in IFN-γ-producing cells in any of the cultures that were stimulated with breast cancer cells. Similarly, rIL-7/HGFβ treatment significantly increased the number of tumor-specific IFN-γ-producing T cells in DLNs and spleens of B16F10 melanoma-bearing mice (data not shown). These results suggest rIL-7/HGFβ treatment...
greatly enhances both regional and systemic immunologic responses to tumor-specific antigens in vivo.

rIL-7/HGFβ inhibits the formation of pulmonary metastases in recipients of colon cancer and melanoma cells

To determine whether rIL-7/HGFβ could also inhibit metastatic disease, BALB/c mice were injected i.v. with CT-26 cancer cells to establish pulmonary metastases. The mice were then treated with 15 μg of rIL-7/HGFβ or equimolar doses of rIL-7 and/or HGFβ (or PBS). The mice were euthanized on day 21, the lungs were removed and weighed, and tumor colonies on the surface of the lung were counted. As shown in Figure 5A, rIL-7/HGFβ treatment reduced the numbers of metastatic nodules on the lungs by approximately 5.5-fold as compared with 1.3-fold after rIL-7 treatment. Proportionate decreases both in lung weights and in the sizes of the metastatic nodules that did form also were observed (data not shown). Similar antimetastatic activity was observed in the lungs of rIL-7/HGFβ–treated C57BL6 mice after i.v. injection of melanoma cells (Fig. 5B). Of interest, randomly sampled peripheral lymph nodes contained significantly elevated numbers of CD4+ and CD8+ T cells, activated DCs, and NK cells following the systemic administration of rIL-7/HGFβ similar to those observed in the DLNs after the local administration of rIL-7/HGFβ (data not shown). These results suggest that T cells, DCs, and NK cells may also be involved in the antimetastatic activity of rIL-7/HGFβ.

Inhibition of local tumor growth by rIL-7/HGFβ does not occur in nude mice

To confirm that T cells were involved in the antitumor activity of rIL-7/HGFβ, the previous experiments were repeated in congenitally athymic (nude) mice. In contrast to its effects in euthymic mice, rIL-7/HGFβ failed to inhibit local tumor growth in nude mice by both CT-26 colon cancer cells (Fig. 6A) and B16F10 melanoma cells (Fig. 6C). In contrast, the antimetastatic activity of rIL-7/HGFβ was only partly lost in nude mice (Fig. 6B and D). These results suggested that, even if the expanded T cells and activated DCs seen in rIL-7/HGFβ–treated euthymic mice inhibit the formation of metastases (Fig. 5), rIL-7/HGFβ must use a different mechanism(s) to achieve the same result in nude mice.

Figure 2. Infiltration of CD4+ and CD8+ T cells and activated CD11c+ DCs into rIL-7/HGFβ–treated tumors. BALB/c mice were injected s.c. with CT-26 colon cancer cells and treated with equimolar doses of rIL-7/HGFβ (15 μg), rIL-7 (5 μg), and/or rHGFβ (10 μg), or PBS, as in Figure 1B. Thirty days after tumor inoculation, the mice were euthanized and the tumors were removed. Single-cell suspensions from the tumors were analyzed by flow immunocytometry for (A) CD11c+ DCs and CD4+ and CD8+ T cells, and (B) the expression levels of CD80 and CD86 on the CD11c+ DCs. Data represent (A) mean numbers ± SD of positive cells per milligram of tumor tissue from 4 to 6 mice per group and (B) relative mean ± SD fluorescence intensity (MFI) of CD80 and CD86 on DCs in cytokine- or PBS-treated tumors.* P < 0.05 compared with PBS-treated group; **, P < 0.05 as compared with the rIL-7- and/or rHGFβ–treated groups.

Figure 3. Treatment of localized tumors with rIL-7/HGFβ increases the numbers of CD4+ and CD8+ T cells, CD11c+ DCs, and NK cells in the DLNs. Single-cell suspensions of DLNs from the cytokine-treated tumors (see Fig. 2) were analyzed for the numbers of CD11c+ DCs, CD4+ and CD8+ T cells, and CD3+DX5+ NK cells. Data represent mean numbers of positive cells ± SD from 4 to 6 mice per group. *, P < 0.05 as compared with the PBS-treated group; **, P < 0.05 as compared with the rIL-7- and/or rHGFβ–treated groups.
Discussion

We show here that the local injection of rIL-7/HGFβ at the site of tumor cell inoculation significantly inhibited tumor growth in murine models of colon cancer and malignant melanoma. Importantly, rIL-7/HGFβ was effective whether given at the time of tumor cell inoculation or after tumors had been established. In addition, the systemic administration of rIL-7/HGFβ inhibited the formation of pulmonary metastases in these tumor models.

The antitumor effects of rIL-7/HGFβ were associated with increased percentages and numbers of TILs and activated DCs in the tumors. In addition, rIL-7/HGFβ enhanced tumor-specific regional and systemic T-cell responses in lymph nodes and spleen cells, as shown by differential stimulation of INF-γ–secreting cells in vitro. These results suggested that the major mechanism by which rIL-7/HGFβ inhibited tumor cell growth was immunologic rather than directly cytostatic or cytotoxic. This was confirmed by the inability of rIL-7/HGFβ to prevent tumor formation in nude mice or to inhibit tumor cell growth in vitro. We presume therefore that rIL-7/HGFβ inhibits local tumor cell growth by activating (and possibly attracting) T cells and DCs, which work in concert with NK cells to inhibit tumor cell growth and survival (20–22). However, the results also suggest that an additional, T-cell–independent mechanism may also enable systemically administered rIL-7/HGFβ to inhibit the formation of metastases (as opposed to solid tumors), which continues to occur in nude mice.

Several studies have shown that IL-7 has antitumor activity (10–16). For example, tumor cell lines transfected with the IL-7 gene had reduced, T-cell–dependent tumorigenicity in vivo (10, 11). Similarly, the local or systemic administration of rIL-7 had antitumor effects (12, 13), especially when combined with cancer vaccines (15, 16). Our data also showed that rIL-7 could inhibit both tumor growth and the formation of metastases by CT-26 colon cancer cells. However, when given in the same molar amounts, our data show that rIL-7/HGFβ was significantly more effective than rIL-7 (and/or HGFβ) in inhibiting tumor formation. This difference may be qualitative as well as quantitative, as rIL-7/HGFβ could activate tumor-infiltrating DCs whereas rIL-7 did not. This is understandable, as DCs

Figure 4. Treatment of localized tumors with rIL-7/HGFβ enhances tumor-specific T-cell responses. BALB/c mice were injected s.c. with CT-26 colon cancer cells and treated with equimolar doses of rIL-7/HGFβ (15 μg), rIL-7 (5 μg), and/or rHGFβ (10 μg), or PBS as in Figure 1B. Thirty days after tumor inoculation, DLNs (A) and spleens (B) were harvested and cocultured with irradiated CT-26 colon cancer cells or 66.1 breast cancer cells. ELISPOT assays were then conducted for INF-γ+ cells. Data represent mean number of spots/1 × 10^6 T cells ± SD. *, P < 0.05 as compared with the PBS-treated group; **, P < 0.05 as compared with the rIL-7- and/or rHGFβ-treated groups.

Figure 5. rIL-7/HGFβ inhibits the formation of pulmonary metastases by colon cancer and melanoma cells. A, BALB/c mice were injected i.v. with 2 × 10^6 CT-26 colon cancer cells, followed by i.v. injections with equimolar doses of rIL-7/HGFβ (15 μg), rIL-7 (5 μg), and/or rHGFβ (10 μg) or PBS at 2-day intervals between days 2 and 18. B, C57BL/6 mice were injected i.v. with 2 × 10^6 B16F10 cells, followed by injections with rIL-7/HGFβ (15 μg) or PBS at 2-day intervals between days 2 and 18. A and B, the mice were euthanized on day 21 after tumor cell inoculation, and the total tumor nodules visible at the surface of the lungs were counted under a dissecting microscope. *, P < 0.05 as compared with PBS-treated group; **, P < 0.05 as compared with the rIL-7- and/or HGFβ-treated groups. The data are representative of 2 independent experiments, with 4 to 6 mice per group.
express the receptors for both IL-7 and HGF (5, 23–25), and, as we have previously shown in dual receptor B- and T-lineage cells (4), juxtacrine interactions secondary to receptor cross-linking by rIL-7/HGFβ may result in novel functional readouts, such as cell survival, activation, and maturation. Of course, it is also possible that rIL-7/HGFβ may indirectly affect DCs by stimulating other cell types to generate DC stimulatory factors (26). In contrast, although both rIL-7 and rHGF have been shown to individually affect DCs, they primarily influence the development of immature DCs from intrathymic precursors and peripheral blood monocytes (5, 27–30). In addition, IL-7 has been shown to downregulate the expression of MHC II on DCs and to diminish the homeostatic proliferation of CD4+ T cells in a lymphopenic setting (31). HGF can also affect DC development, although the results vary. On the one hand, HGF has been reported to induce CD34+BMMCs to generate DCs that could stimulate T-cell proliferation (23). On the other hand, HGF has been found to favor the development of tolerogenic DCs from monocytes (24) and to suppress DC functions such as antigen presentation (25).

Similarly, although many studies have shown that IL-7 can directly stimulate the survival and proliferation of T cells (5), our data showed that rIL-7/HGFβ treatment induced the appearance of significantly larger numbers of CD4+ and CD8+ T cells in the tumors and DLNs than did rIL-7 treatment (with or without rHGFβ). This may be related to the activation of DCs by rIL-7/HGFβ but not by rIL-7. Hence, rIL-7/HGFβ has distinct advantages over its component cytokines regarding not only the activation of DCs but, as a likely consequence, also the activation of antigen-specific T cells.

Importantly, many studies have shown that HGF/c-Met signaling plays a major role in stimulating the growth, invasion, and metastasis of a wide variety of cancers in vitro and that it also stimulates the proliferation of tumor cells in vivo (17). This, of course, raised concerns that HGFβ, alone or when complexed with IL-7, might do the same. However, the present study with colon cancer and melanoma show that this is not the case. Rather, our results show that rHGFβ, whether given alone or mixed with rIL-7, did not stimulate tumor cell growth in vitro, even though the cells expressed c-Met. This is consistent with reports that HGFβ binds to c-Met and is required for the mitogenic activity of HGF, but it does not by itself stimulate cell growth (32, 33). Even more reassuring, our results show that the HGFβ component of rIL-7/HGFβ seems to enhance the antitumor effects of the IL-7 component. Therefore, whether used alone or in combination with other therapeutic agents, rIL-7/HGFβ not only may prove useful in the treatment of cancer but also does not seem to pose a risk of provoking tumor growth (or recurrence) if used in the treatment of nonneoplastic lymphohematopoietic disorders.

Disclosure of Potential Conflicts of Interest

L. Lai and I. Goldschneider are named inventors on U.S. Patent Ser. No. 6,749,847 “Hybrid Cytokine of IL-7 and β-Chain of Hepatocyte Growth Factor” and U.S. Patent Ser. No. 7,679,998 “Chimeric Cytokine of IL-7 and β-Chain of HGF and Methods of Use,” which have been assigned to the University of Connecticut and are under license to Wellstat Therapeutics Corporation.

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