Interferon-γ and CXC Chemokine Induction by Interleukin 12 in Renal Cell Carcinoma

Ronald M. Bukowski, Patricia Rayman, Luis Molto, Charles S. Tannenbaum, Thomas Olencki, David Peereboom, Raymond Tubbs, Denise McLain, G. Thomas Budd, Thomas Griffin, Andrew Novick, Thomas A. Hamilton, and James Finke


ABSTRACT

Interleukin 12 (IL-12) is known to play an important role in the development of an antitumor response. Its activity has been shown to be dependent upon the intermediate production of IFN-γ and the influx into the tumor of CD8 lymphocytes. In a murine model, tumor regression induced by IL-12 treatment correlated with IFN-γ, IP-10, and Mig expression in the tumor bed and was abrogated by antibodies to both chemokines. Here we examined the effects of rHuIL-12 on IFN-γ and CXC chemokine gene expression in patients with renal cell carcinoma (RCC) in an attempt to determine whether a similar series of molecular events leading to IL-12-mediated tumor regression in mice is also detectable in humans. As in the murine RENCA model, cultured RCC cells themselves could be induced by IFN-γ to synthesize IP-10 and Mig mRNA. Explanted RCC produced IFN-γ and IP-10 mRNA in response to IL-12 treatment, which was consistent with the finding that biopsied RCC tumors from IL-12-treated patients also variably expressed augmented levels of those molecules after therapy. Although Mig mRNA was present in the majority of biopsied tumors prior to treatment, both the Mig and IP-10 chemokines as well as IFN-γ were induced in the peripheral blood mononuclear cells of IL-12-treated patients. Skin biopsies of IL-12-treated patients also all synthesized IP-10 mRNA. This study demonstrates that recombinant human IL-12 therapy of patients with RCC has the potential to induce the expression of gene products within the tumor bed that may contribute to the development of a successful antitumor response.

INTRODUCTION

IL-12 (1, 2) is a heterodimeric cytokine that exerts a variety of immunomodulatory effects, including induction of IFN-γ secretion from T and NK cells (3, 4), augmentation of cytotoxic activity of both resting NK (1, 3) and activated T cells (5), and enhancement of CD4 and CD8 lymphocyte proliferation (6, 7). In addition to synergizing with IL-2 in the generation of lymphokine-activated killer activity (2, 8), IL-12 also supports the proliferation of various lymphocyte populations including IL-2-stimulated CD56 cells (9) and suboptimally IL-2-treated resting PBLs (6). These immunostimulatory properties render IL-12 an effective therapeutic agent in the treatment of multiple parasitic (10–13) and viral (14, 15) infections and have encouraged many laboratories to assess the ability of IL-12 to promote host responses to cancer. Indeed, IL-12 is among the most potent of the numerous cytokines demonstrated to exhibit antitumor activity in rodents (16–19), mediating regression of even well-established, poorly immunogenic tumors. The ability of IL-12 to orchestrate Th1-directed responses is one mechanism by which IL-12 is thought to work (20–24), as IFN-γ is an important intermediate in IL-12-induced antitumor function (25, 26). Studies from our laboratories showed that regressing, IL-12-treated RENCA tumors synthesized augmented levels of the IFN-γ-inducible chemokines Mig (27, 28) and IP-10 (29, 30), which were demonstrated recently to elicit activated T cells expressing the CXCR3 receptor (31). Although Mig and IP-10 have additionally been characterized as molecules with antiangiogenic activity (32–36), their purported role as IFN-γ-inducible chemotactic factors in this model is consistent with the abrogation of IL-12 antitumor activity by antibodies to IFN-γ or to CD4 and/or CD8 lymphocytes (16, 25). Further supporting the involvement of Mig and IP-10 as mediators of an IL-12-initiated T-cell response to tumors are our most recent experiments in which antibodies to Mig and IP-10 inhibited both IL-12-induced T-cell infiltration into the tumor bed and antitumor activity (37). These findings tend to verify our proposed molecular model of IL-12 antitumor activity, that IL-12 induces IFN-γ, which in turn augments Mig and IP-10 expression within the tumor bed and contributes to the development of an antitumor response.
the tumor to elicit activated, effector T cells capable of mediating tumor regression.

The purpose of the studies reported here was to determine whether the same series of molecular events (19) occurring in the murine tumor model in response to IL-12 could also be detected in RCC patients undergoing similar therapy. Although IL-12 treated tumors could not always be directly assessed for modulated gene expression, data obtained from in vitro manipulated RCC cell lines and tumor explants, as well as from patient PBMCs and metastatic lesions, suggest that an IL-12-induced cytokine cascade identified in mice may also be operative in humans.

PATIENTS AND METHODS

Patient Population. The patients studied had metastatic RCC and were treated in a Phase I trial of rHuIL-12 conducted in part at the Cleveland Clinic Foundation. rHuIL-12 was supplied by Hoffman-LaRoche and had a specific activity of 5.26 × 10^8 units/mg protein. Patients eligible included those with limited prior therapy (one or fewer prior cytokine and or chemotherapeutic regimens) and adequate renal, hepatic, hematological, and cardiopulmonary function. rHuIL-12 was administered s.c. to cohorts of three to six patients at various dose levels. Two different schedules were used. The first used fixed doses of rHuIL-12 (0.1–1.0 μg/kg), and the second used escalating doses. This latter schedule involved administration of 0.1 μg/kg of rHuIL-12 initially, with escalation from 0.5 to 1.5 μg/kg per week. In patients receiving either schedule, rHuIL-12 was administered once weekly for 3 weeks, followed by a week of rest, and then for 3 additional weeks. Informed consent was obtained in accordance with institutional and Food and Drug Administration guidelines. Standard definitions of clinical response were used as described previously (38).

Isolation of PBMCs. PBMCs were isolated using Ficoll-Hypaque (Pharmacia, LKB) density gradient centrifugation. Cells were obtained at baseline and at various time points (4, 6, 8, 24, and/or 48 h) after rHuIL-12 or rIL-2 administration on day 1 of cycle one. In patients receiving escalating doses of rHuIL-12, PBMCs were also collected on days 8 and 15 of cycle one.

Skin, Tumor, and Normal Kidney Tissue Studies. Seven patients receiving rHuIL-12 underwent serial skin biopsies prior to therapy and 2–4 days after the initial treatment. A punch biopsy was used, and the tissue was maintained in gauze moistened with saline. Specimens were processed immediately, and RNA was extracted as described previously (30). Briefly, tissue or cells were hand homogenized in 0.8 ml of RNAzol B (Tel-Test, Inc., Friendswood, TX) and stored at −70°C for mRNA analysis.

Primary RCC specimens were obtained from patients undergoing nephrectomy and were used either directly for assessment of in vivo chemokine gene expression or as explants for in vitro studies of inducible gene expression. For the explant studies, 5 mm × 5 mm specimens were prepared from tumor and adjacent normal tissue using a no. 10 Bard-Parker scalpel (Becton Dickinson, San Jose, CA). The explants were then rinsed in complete RPMI 1640 with 10% FCS and transferred to 24-well plates (Falcon 3046), which contained 1.0 ml of complete RPMI. Explants were left untreated (media control) or stimulated with either rHuIL-12 (500 units/ml) or PHA (Difco Laboratories, Detroit, MI), 10 μg/ml. All experiments were set up in duplicate. After 18 h of incubation at 37°C, one sample was snap frozen in isobutanol in a dry ice/ethanol bath and stored at −70°C for immunohistological evaluation. The second was hand homogenized, and RNA extraction was performed as described previously and stored at −70°C for mRNA analysis. The presence of metabolically active cells in explants was demonstrated by measuring dehydrogenase activity (Calorimetric assay kit; Boehringer Mannheim, Indianapolis, IN).

Short-term RCC cultures were obtained from renal tumors treated with collagenase type II (3.5 mg/ml; Sigma) for 2 h at 37°C. After the digest, tumor cells were washed and plated in T75 flasks with RPMI 1640 supplemented with 10% FCS. Cells were cultured for 10–14 days before they were trypsinized (1000 units/ml; Worthington) and washed. Cells were then either plated and used directly or frozen for later use. Cells (3 × 10^6) from cultures grown to confluence 14–21 days in T75 flasks were transferred to T25 flasks containing 10.0 ml of cloning media (RPMI 1640 with 10% FCS (HyClone, Logan, UT), 200 μM L-glutamine, 10 μM MEM nonessential amino acids, 100 μM sodium pyruvate, and 50 μg/ml of gentamicin), all from Life Technologies, Inc., Grand Island, NY. Cultures were left untreated (media control) or stimulated with IFN-γ (500 units/ml) or rHuIL-12 (500 units/ml) at 37°C for 12 h.

PBMCs were obtained from a normal individual as described previously (39), and 1.0 × 10^6 cells were cultured in cloning media alone or with PHA (Difco Laboratories; 1 μg/ml) plus rIL-2 (1000 μg/ml) for 12 h to serve as positive controls. Purified T cells were prepared by depleting PBMCs of macrophages, B cells, and NK cells via negative selection using magnetic separation with microbeads coated with antibodies to CD14, CD19, and CD16/CD56, as described previously (40). The T-cell isolation procedure yielded cells that were >95% positive for CD3 as determined by three color immunocytometry. T cells were activated by culturing in the presence of PMA/ionomycin for 6 days. RNA was extracted as described above.

Cytokine Gene Expression Using RT-PCR. RNA samples stored at −70°C were thawed and processed as recommended by the RNAzol protocol. Semiquantitative RT-PCR analysis of IFN-γ, Mig, IP-10, and actin were performed as follows. Five μg of total RNA were amplified using a specific antisense primer and AMV reverse transcriptase at 42°C for 1 h. The reverse transcription reaction products were used undiluted or at a one-fifth or one-fiftieth dilution for PCR amplification using 20 μM sense and antisense primers (see below) and Taq polymerase. PCR reactions were conducted in a Perkin-Elmer/ Cetus DNA Thermal Cycler for 30–35 cycles (denaturation, 2 min, 94°C; annealing, 1 min, 60°C; amplification, 3 min, 72°C). The PCR products were separated by agarose gel electrophoresis and visualized by Southern hybridization analysis using radiolabeled oligonucleotide probes or cDNA encoding a portion of the gene of interest. The primer sequences and probes used were as follows: IFN-γ, sense (5’-TGGCTTTTCTGCTC- TGCATCGC-3’) and antisense (5’-TCGACCTTGAAACAGCA- CACGGTGTC-3’) and probe (5’-GGAGATACAGCCAAGAGAAC- CCAAAACGATGAGAAGTC-3’); IP-10, sense(5’-CCTGCAA- GCCAAATTGTCG-3’) and antisense (5’-CATTAAACCTTC-
CTACAGGAG-3') and probe (cDNA sequence from Luster et al. (32); TNF-α, sense (5'-GGAGTGACAAGCCTGTAGC-CCATGTTGTAGCA-3') and antisense (5'-GCAATGATCCAAAGTAGACCTGCCCAGACT-3'); Mig, sense (5NAGTGGTGTTCTTTTCCTCTTGGG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe [CXCR3 cDNA digested with BamHI/PstI, 750-bp fragment, courtesy of Loetscher et al. (31)]; β-actin, sense (5'-GTGGGGGCGGCCAGGCCACCA-3') and antisense (5'-GCTCTTAATGTCA-ACGCACGAGTTTTCTCTTCTCAG3N); Mig, sense (5NAGTGGTGTTCTTTTCCTCTTGGG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe [CXCR3 cDNA digested with BamHI/PstI, 750-bp fragment, courtesy of Loetscher et al. (31)]; β-actin, sense (5'-GTGGGGGCGGCCAGGCCACCA-3') and antisense (5'-GCTCTTAATGTCA-ACGCACGAGTTTTCTCTTCTCAG3N); Mig, sense (5NAGTGGTGTTCTTTTCCTCTTGGG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N); CXCR3, sense (5NCCTACTGCTATGCCCACATCCTG3N) and antisense (5NGCTTCTGGAGCCTTTTCTGGTTG3N) and probe (5NGGAAGGGCTTGGGGCAAATTGTTTAAGGTCTTTCAAG3N).}

**RESULTS**

**Inducible Cytokine and Chemokine Expression in Cultured RCC Cells and Explanted Tumors.** Previous studies from our laboratory have shown that regressing IL-12-treated murine RENCA tumors synthesize augmented levels of the IFN-γ-inducible molecules IP-10 (19, 37) and Mig (37). In vitro studies with cultured RENCA as well as in situ hybridization analysis of tumors from IL-12-treated and untreated animals indicated that the tumor cells themselves transcribed the chemokine mRNAs in response to treatment (19). To assess the ability of human RCCs to synthesize IP-10 and Mig in response to stimulation, short-term cultures of RCC (passages 1–3) were treated in vitro with or without IFN-γ or IL-12 for 12 h. As in the murine model, the chemokines were not detectable in unstimulated cultures but were induced to significant levels in seven of seven IFN-γ- but not IL-12-treated samples (Fig. 1).

RCCs are infiltrated with inflammatory cells that are not present in tumor cell cultures grown in vitro for more than several passages. To more accurately dissect the potential cellular and molecular interactions occurring within the RCC tumor bed during immunotherapy, tumor explants were stimulated or not with IL-12 in vitro immediately upon culture. Immuno-

---

**Fig. 1** Expression of Mig, IP-10, CXCR3, and GADPH in PBMCs and RCC tumor cells treated or not with IFN-γ or IL-12. PBMCs and RCC tumor cells were isolated from patients as described in “Patients and Methods” and grown in short-term cultures. Replicate plates of RCC tumor cells from seven patients were incubated in media alone or in media containing 500 units/ml IFN-γ or 500 units of rHuIL-12 for 12 h. PBMCs were cultured for 12 h in control medium or medium containing 1 µg/ml PHA + 1000 units/ml rIL-2. RNA samples from each culture were used for semiquantitative RT-PCR detection of Mig, IP-10, CXCR3, and GADPH mRNA as described in “Patients and Methods.” PCR products were separated on agarose gels, blotted onto nylon membrane, and hybridized with radiolabeled cDNAs corresponding to Mig, IP-10, CXCR3, or GADPH.
histological analysis of the explants demonstrated the presence of macrophages and CD4- and CD8-positive T cells in both stimulated and unstimulated cultures (data not shown). IFN-\(\gamma\) was rarely synthesized in tumor explants incubated in medium alone but was consistently induced after in vitro incubation with rHuIL-12 (seven of seven tumors; representative data, Fig. 2). No evidence of IFN-\(\gamma\) gene induction was detected in tissue explants from normal kidney. The expression of IFN-\(\gamma\) mRNA coincided with detectable levels of IFN-\(\gamma\) protein in two of seven tumor explants stimulated for 18 h with IL-12 (Fig. 3) and in three of four explants stimulated with PHA (data not shown).

Recent studies in the murine RENCA model demonstrated that rMuIL-12 augments the chemokine IP-10 indirectly, most probably via the intermediate induction of IFN-\(\gamma\) (19). Fig. 4 illustrates that stimulating tumor explants with rHuIL-12 induces detectable expression of IFN\(\gamma\) by 2 h and IP-10 by 4 h. Although the data depicted in Fig. 4 definitively show that IL-12 induces both IFN-\(\gamma\) and IP-10 in the RCC tumor explants, some experiments did not convincingly demonstrate a time course consistent with a secondary induction of IP-10 via the rHuIL-12-stimulated production of IFN-\(\gamma\). Additionally, when explant stimulation by IL-12 was performed in the presence of antibodies to IFN-\(\gamma\), there was no detectable abrogation of Mig or IP-10 synthesis (data not shown). These findings may reflect either the inability of the antibodies to penetrate the tissue and neutralize IFN-\(\gamma\) or another mechanism by which IL-12 mediates chemokine synthesis. Because IL-12 cannot directly induce Mig or IP-10 expression by RCC tumor cell lines (Fig. 1) and purified peripheral blood T cells were not observed to make the chemokines in response to PMA/ionomycin (Fig. 5), another IL-12-induced intermediate may have a role in stimulating chemokine expression by human RCC or tumor-infiltrating myeloid cells.

**In Vivo Expression of IFN-\(\gamma\) and the Chemokines IP-10 and Mig before and during rHuIL-12 Therapy.** Previous work has demonstrated the infrequent expression of IFN-\(\gamma\) and IL-2 in renal tumors (39). The present study extends and supports these findings for IFN-\(\gamma\), which in this trial was detected by RT-PCR in only 1 of 15 untreated renal tumors. An analysis of chemokine gene expression revealed that IP-10 was variably present in untreated RCCs (4 of 14; Fig. 6A), whereas Mig was detectable in seven of seven renal cell carcinoma tumors (Fig. 6A). Mig, however, was not detectable in adjacent normal tissue (0 of 2), although IP-10 was present in one of two control kidney specimens studied (Fig. 6A). The mRNA for CXCR3, the receptor for IP-10 and Mig, was also present in four of seven RCCs and was absent from the single normal kidney specimen examined (Fig. 6B).
Successful IL-12-mediated antitumor therapy in the murine RENCA model correlates with the induced expression of IFN-γ and the chemokines Mig and IP-10 in the treated tumors (19). Because of the difficulties encountered in performing biopsies of metastatic renal tumors, gene expression before and during rHuIL-12 therapy was monitored in the peripheral blood mononuclear cells of 15 treated patients. These individuals had an Eastern Cooperative Oncology Group performance status of 0 (n = 10) or 1 (n = 5) with 9 of 15 having received prior therapy with various cytokines. Eight received fixed doses of rHuIL-12, and seven received the escalating schedule.

Constitutive IFN-γ mRNA expression was found in the PBMCs of 6 of 15 patients. In the remaining nine patients, it was absent at baseline and induced in eight of nine during therapy (Fig. 7A). Thus, IFN-γ mRNA was present or induced in PBMCs in 14 of 15 patients, was evident as soon as 4 h after rHuIL-12 treatment, and persisted at elevated levels for at least 24 h after cytokine administration. In a patient receiving the escalating schedule of rHuIL-12, strong induction of IFN-γ was apparent during weeks 2 and 3 of treatment (Fig. 7B), although little induction was seen in this individual after the first 4 h of stimulation in week 1. It is difficult to assess from this experiment whether it was the escalating dose or the extended period of IL-12 treatment or both that resulted in the augmented levels of IFN-γ seen in PBMCs isolated after weeks 2 and 3 of therapy.

Chemokine expression by PBMCs before and after rHuIL-12 therapy was also assessed. IP-10 mRNA was absent at baseline in 12 of 15 patients but was induced in all of these individuals during treatment (Fig. 8A). The typical time required for induction was about 4 – 6 h after the first dose of rHuIL-12. Mig was present at baseline in 3 of 10 patients and was induced in the remaining seven during rHuIL-12 therapy (Fig. 8B). It is evident from the data shown in Fig. 8B that the IL-12 given during weeks 1 and 2 had a persistent effect on MIG expression by PBMCs isolated prior to the third week of therapy.

The tumors of most patients receiving IL-12 therapy were not resected subsequent to treatment; hence, tissue was not available for assessment of induced gene expression. To determine the potential ability of s.c. rHuIL-12 therapy to modulate cytokine and chemokine gene expression in other sites, serial skin samples biopsied at sites distant from the region of IL-12 administration were analyzed by RT-PCR for IFN-γ and IP-10 mRNA synthesis 2–4 days after treatment. None of the skin samples from the seven patients studied expressed IFN-γ or
IP-10 prior to IL-12 therapy. Although an IL-12 effect on IFN-γ was undetectable, skin punches of three of seven patients did synthesize IP-10 mRNA in response to treatment (data not shown). Notwithstanding the IL-12-induced chemokine expression, immunohistological analysis of skin biopsied before and during the IL-12 regimen revealed no significant alterations in inflammatory infiltrates after therapy (data not shown).

**DISCUSSION**

Numerous murine models have demonstrated the potent antitumor activity of IL-12 (16–19). Unlike some cytokines that mediate regression of only nascent experimental tumors, IL-12 has activity against even large, well-established malignancies (16, 17, 19, 41). Previous work has established the role of IFN-γ as an intermediate in IL-12 antitumor function (25, 26), and various laboratories have used cell depletion experiments and nude mice to verify the integral importance of CD4 and/or CD8 T cells as effectors of the IL-12-driven antitumor activity (16, 25). Recent studies from our laboratories showed that regressing tumors from IL-12-treated animals synthesize increased levels of the IFN-γ-inducible chemokines IP-10 (19, 37) and Mig (37), which are now known to specifically chemoattract CXCR3-bearing activated T cells (31). These results suggested a hypothetical molecular cascade from IL-12 to IFN-γ to the chemokines Mig and IP-10 to explain the observed infiltration of IL-12-treated tumors by activated, effector T cells. This scheme is supported by more recent experiments in which antibodies to Mig and IP-10, coadministered during IL-12 antitumor therapy, were found to abrogate both T-cell infiltration into the tumor bed and tumor regression (37).

The studies reported here had as their goal the determination of whether the same series of IL-12-induced molecular events leading to tumor eradication in mice also could be demonstrated in human RCC patients receiving rHuIL-12 therapy. Although experiments in the murine system have demonstrated a dose dependence for IL-12 function (25), the greater sensitivity of humans to IL-12 toxicity required that the clinical trial use significantly smaller doses per unit body weight than can be tolerated by mice. Thus, one question asked in these studies was whether the comparatively low doses of IL-12 administered s.c. throughout this trial were adequate to induce in humans the same cytokine and chemokine expression patterns observed in mice. Although IL-12 would be expected to modulate gene expression at the s.c. site of injection, synthesis of IL-12-inducible chemokines at the distant tumor itself would ostensibly be required to elicit inflammatory cells to that tissue. Although murine tumors could be directly assessed for IL-12-induced gene expression (19), most treated human RCCs were inaccessible for analysis; hence, conclusions had to be extrapolated from studies performed on PBMCs, skin biopsies, and in vitro manipulated RCC cell lines and tumor explants.

The in vitro studies with RCC cell lines demonstrated the ability of the tumor cells themselves to synthesize Mig and IP-10 mRNA in direct response to IFN-γ. These results were analogous to the findings in the murine model RENCA (19, 37) and suggested the possibility that IL-12 therapy of human patients could potentially lead to the same series of molecular events and successful immune response observed in the mouse. Thus, conclusions had to be extrapolated from studies performed on PBMCs, skin biopsies, and in vitro manipulated RCC cell lines and tumor explants.

**Fig. 7** Expression of IFN-γ mRNA in PBMCs of patients undergoing rHuIL-12 therapy. A, PBMCs were collected from patients before and 4, 6, 10, 24, and 48 h after s.c. injection of 0.1 μg/kg of rHuIL-12. RNA was isolated from the lymphocytes as described in “Patients and Methods” and subjected to RT-PCR analysis using primers to IFN-γ and actin and 35 cycles of amplification. B, PBMCs were collected from a patient receiving weekly escalating doses of rHuIL-12 (0.1 μg/kg week 1; 0.5 μg/kg week 2; and 1.25 μg/kg week 3) pretherapy and from 4 to 48 h after treatment. RNA was isolated from the PBMCs and subjected to RT-PCR analysis as described above. RT products were diluted 1:5 and 1:50 prior to amplification.
numerous inflammatory cell types (43, 44) and hence contains cells directly responsive to IL-12. Indeed, although IFN-γ mRNA was undetectable in explants incubated in medium alone, it was induced in seven of seven explant samples after treatment with IL-12. Although the cell sources of IFN-γ mRNA in these experiments were not defined, T lymphocytes infiltrating the tumor bed are likely the primary producers; a contribution from the small number of NK cells (CD56+) within the tumor, however, cannot be discounted. Previous work has shown that highly purified tumor-infiltrating T cells, when removed from the suppressive tumor environment, can produce IFN-γ after in vitro stimulation (45). The present studies indicate that lymphocytes within the tumor bed are still capable of expressing IFN-γ mRNA after stimulation, and in some cases, they can secrete detectable levels of protein. In a subset of tumor explants, however, the induction of IFN-γ mRNA did not co-incide with the secretion of detectable levels of protein. These findings are similar to those reported for tumor-infiltrating T-cells from breast cancer patients where IL-2 mRNA was expressed in the absence of detectable protein (46). It is also possible, however, that IFN-γ protein was secreted at levels undetectable by our assay but still sufficient to induce chemokine expression. The finding that IL-12-stimulated tumor explants are induced to synthesize IFN-γ and IP-10 mRNA is thus in accord with the proposed molecular cascade of IL-12-induced events established in the mouse and suggests the possibility that human RCCs respond similarly in vivo. When IL-12 was used to stimulate explants in the presence of neutralizing antibodies to IFN-γ, however, no abrogation of Mig induction was noted (data not shown). This suggests either poor penetration of explanted tissue by the antibodies or the involvement of an IFN-γ-independent mechanism of IL-12 chemokine induction. Ar-

Fig. 8 Expression of CXC chemokine mRNA in PBMCs of patients undergoing rHuIL-12 therapy. PBMCs were collected pretherapy and 2 to 48 h after treatment of one patient receiving 0.1 μg/kg IL-12 and from another patient receiving escalating weekly doses of rHuIL-12 (0.1 μg/kg week 1; 0.5 μg/kg week 2; and 0.75 μg/kg week 3). RNA was isolated from the PBMCs as described in “Patients and Methods” and subjected to RT-PCR analysis using primers to IP-10, Mig, and actin and 35 cycles of amplification. PCR products were separated on an agarose gel, blotted onto a nylon membrane, and hybridized with the appropriate cDNAs. A, IP-10. B, Mig. Data are representative of five replicate experiments.
guing against the notion that tumor-infiltrating lymphocytes might be an important direct source of IL-12-induced Mig and IP-10 was the experiment demonstrating that PMA/ionomycin-stimulated purified peripheral blood T cells synthesized abundant IFN-γ mRNA, but no or only barely detectable levels of the chemokines (Fig. 5). Although we cannot discount the possibility that under some untested stimulatory condition a T-cell population may be induced to synthesize Mig and/or IP-10, neither we nor convincing published studies have been able to demonstrate inducible Mig or IP-10 expression by purified human or murine T lymphocytes. Thus, as in the murine model, it is probable that IL-12 stimulated tumor-infiltrating lymphocytes and infiltrating NK cells are induced to synthesize IFN-γ, which in turn augments Mig and IP-10 elaboration by infiltrating myeloid cells and the tumor cells themselves.

Notwithstanding the patient to patient variability in response to IL-12 treatment, the overall trend was that IL-12 modulated gene expression in human tissues in much the same way as it did in mice. IL-12 therapy was observed to induce IFN-γ, IP-10, and Mig mRNA expression in almost all of the patient PBMCs tested. Differentiating the results obtained in this human trial from those seen in the murine IL-12 studies, however, was the extent of constitutive cytokine expression observed in some untreated patients. IFN-γ, Mig, and IP-10 mRNAs were constitutively expressed by the PBMCs of two of five, one of three, and one of five of the patients tested, respectively, and surprisingly, although not expressed by normal kidney, Mig was abundant in seven of seven untreated renal tumors. IP-10 was also present in one of two normal kidney samples and 4 of 14 untreated tumors. Inflammatory responses unrelated to cancer may explain the constitutive cytokine expression by patient PBMCs; more enigmatic, however, was the variable IP-10 and consistent Mig expression detected in RCC samples prior to IL-12 therapy, especially given the inability to detect constitutive IFN-γ mRNA in all but one of those patients. It is interesting to speculate that constitutive Mig expression by untreated RCCs has some influence on the inflammatory infiltrate characteristic of renal tumors. Measurable CXCR3 expression by patient PBMCs prior to therapy suggests the availability of cells in the periphery capable of responding to the chemotactic signal emanating from the tumor bed; the ability to actually detect CXCR3 mRNA within half of the tumors examined is consistent with the hypothesis that Mig may be involved and effective in mediating that infiltration. The inability to measure CXCR3 mRNA within all of the Mig-expressing tumors may be due to sampling, because some portions of a tumor have more or fewer viable T cells than others (47); alternatively, tumor infiltration by CXCR3-expressing T cells may be governed by additional variables. Precisely what is stimulating Mig expression in the absence of detectable IP-10 or IFN-γ within the tumor, however, remains unclear.

The data presented here indicate that rHuIL-12 therapy of RCC patients results in the initiation of a cytokine and chemokine cascade that resembles the molecular events observed in mice, where IL-12 treatment has potent antitumor effects. Given the apparent importance of Mig and IP-10 to the IL-12 antitumor response (34, 37), one can ask why RCC tumors do not respond to IL-12 therapy, notwithstanding the induction and expression of those molecules by most treated patients. Among the potential explanations are: (a) that although IFN-γ and/or IP-10 and Mig are induced in the periphery of IL-12-treated patients, such as in PBMCs, and skin, it is unclear if these cytokines are being induced at the kidney tumor itself. The toxicity of rHuIL-12 is such that on the doses and schedules administered, sufficient IL-12 may not be available at the tumor site to induce the required molecular changes; (b) it may be that IL-12-induced chemokine expression is eliciting effector T cells into the tumor bed, which are being inactivated upon arrival. Numerous mechanisms by which tumors neutralize host immunity have now been elucidated. Some tumors have reduced expression of MHC and costimulatory molecules (48), which lower the efficiency of tumor antigen presentation, and many secrete immunosuppressive molecules that inhibit T-cell function (49–52). Recent data suggest that tumors originating from diverse tissue types express Fas ligand, which upon binding to Fas receptors on activated infiltrating T cells eliminates those effectors via the induction of an apoptotic death signal (47, 53–62). Present studies are directed toward elucidating the specific immunosuppressive mechanisms of RCCs and determining whether neutralization of that activity will enhance rHuIL-12 antitumor therapy.

REFERENCES


Interferon-γ and CXC Chemokine Induction by Interleukin 12 in Renal Cell Carcinoma

Ronald M. Bukowski, Patricia Rayman, Luis Molto, et al.


**Updated version**
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/10/2780

**Cited articles**
This article cites 59 articles, 45 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/10/2780.full#ref-list-1

**Citing articles**
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/5/10/2780.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.