The CXC Chemokines IP-10 and Mig Are Necessary for IL-12-Mediated Regression of the Mouse RENCA Tumor

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The role of the non-ELR-containing CXC chemokines IP-10 and Mig in antitumor activity induced by systemic treatment with IL-12 was examined in mice bearing the murine renal adenocarcinoma RENCA. IL-12 treatment produces a potent antitumor effect that is associated with tumor infiltration by CD8+ T lymphocytes. The regression of tumor is associated with the elevated expression of the IFN-γ-inducible chemokines IP-10 and Mig within the tumor tissue. IP-10 and Mig have been shown to function as chemoattractants for activated T lymphocytes. In animals treated with rabbit polyclonal Abs specific for IP-10 and for Mig, the IL-12-induced regression of RENCA tumors was partially abrogated. This effect was associated with a dramatic inhibition of T cell infiltration. Thus, it appears that IL-12-dependent, T cell-mediated antitumor activity requires the intermediate expression of IP-10 and Mig to recruit antitumor effector T cells to the tumor site. The Journal of Immunology, 1998, 161: 927–932.

Materials and Methods

Reagents

Dulbecco’s PBS was purchased from Mediatech (Washington DC). Agarose, SDS, guandine isothiocyanate, cesium chloride, and phenol were purchased from Life Technologies (Gaithersburg, MD). Boehringer Mannheim (Indianapolis, IN) was the source of restriction endonucleases, proteinase K, nick translation kits, random primer kits, reverse transcriptase, RNase inhibitor, and Taq polymerase. [32P]dCTP was purchased from DuPont-New England Nuclear Research Products (Boston, MA). Reagents for SDS-PAGE and protein determination were obtained from Bio-Rad Laboratories (Richmond, CA). Recombinant murine IL-12 was provided by Dr. Michael Brunda (Hoffmann-La Roche, Nutley, NJ). Vector Laboratories (Burlingame, CA) was the source of biotinylated goat anti-rat IgG. Peroxidase-labeled streptavidin, biotinylated anti-rat IgG, and chromogenic substrate for immunohistology were purchased from Ventana Medical Systems (Tucson, AZ). Rat mAbs against mouse CD4 and CD8 were purchased from Becton Dickinson (Mountain View, CA), and a mAb against mouse CD31 was provided by Dr. Alberto Mantovani (Mario Negri Institute, Milan, Italy).

Animals

Male BALB/c mice, 6 to 8 wk old, were purchased from the National Institutes of Health (Bethesda, MD) and housed in a specific pathogen-free animal facility. Animals were maintained in microisolator cages with autoclaved food and bedding to minimize exposure to viral and microbial pathogens (24).

Tumors

RENSA is a spontaneously arising murine renal cell carcinoma and was isolated and maintained as described previously (25). Routinely, 4 × 103 tumor cells in 0.1 ml of PBS were inoculated s.c. Fourteen days following tumor inoculation, animals received 0.5 μg of recombinant murine IL-12 i.p. daily for the duration of the experiment, while control animals received vehicle alone. Tumor volumes were measured daily with a micrometer in two dimensions, and tumor size was estimated according to the formula: (smallest diameter)2 × (longest diameter). Tumor growth under different treatment conditions was statistically analyzed using the Wilcoxon rank sum test. The p values obtained represent the two-sided value.
Preparation of Abs

Rabbit polyclonal Abs to Mig and IP-10 were produced by Biosynthesis (Lewisville, TX) using synthetic peptides selected from the IP-10 and Mig protein sequences (CImHDGpvRMRAgK and CTSRGTtHtKsL DLkQFAPs, respectively) coupled to carrier protein KLH.

Western blot analysis

RENCA cells in 100-mm diameter petri dishes were cultured in serum- and protein-free hybridoma medium (Sigma, St. Louis, MO) with or without stimulation by IFN-γ for 24 h. Supernatant medium was dialyzed overnight against 25 mM NaPO4, pH 7.4, and then mixed with a 40-μl (bed volume) aliquot of heparin-Sepharose beads for 16 h at room temperature. The beads were washed in buffer and then boiled in the presence of 2% SDS sample buffer (26), and the eluted samples were separated by SDS-PAGE (15%). Proteins were then transferred to Nitrobind transfer membranes (Micron Separations, Westborough, MA) using a semidyed transfer cell (Bio-Rad) for 45 min at 450 mA constant current in transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol, pH 9.2). Blots were blocked with 5% nonfat milk in TBST (0.15 M NaCl, 0.1% Tween-20, and 50 mM Tris, pH 7.4) at 20°C for 2 h, then incubated overnight with rabbit polyclonal Abs against IP-10 or Mig in 5% nonfat milk TBST solution (in some reactions, peptide against which the Ab was initially raised was included as a competitor at 1 μg/ml). After washing three times in TBST, filters were incubated at room temperature for 1 h with goat anti-rabbit IgG conjugated to horseradish peroxidase and then washed again as described above. Ab binding was detected using the ECL kit from Amersham (Arlington Heights, IL).

Immunohistologic analysis

Immunohistology was performed as previously described (6, 25, 27). Tissues were snap-frozen in isopentane precooled in liquid nitrogen until sectioned. Frozen tissue sections (6 μm) were prepared, air-dried, fixed in cold reagent grade acetone for 10 min, and air dried. Rat mAbs against mouse CD8 or CD3 were applied at concentrations optimally titrated against mouse thymus or mouse lung, respectively, and linked to streptavidin-peroxidase by biotinylated rabbit anti-rat IgG using the Ventana 320 autostainer (Ventana, Tuscon, AZ). The chromogenic substrate 3,3′-diaminobenzidine/H2O2 followed by hematoxylin counterstaining was used to visualize positive reactivity. To quantify T cell infiltration into tumor tissues, the number of cells showing anti-CD8 reactivity in a series of high power fields was counted in several tumors from each experiment. Because the tumor tissue exhibited variable degrees of necrosis and variable distribution of T cell infiltrates, only fields of nonnecrotic tumor containing the highest T cell numbers for each experimental condition were included in the analysis.

Plasmids

Plasmids with inserts encoding murine IP-10 and perforin were previously described (6). A DNA fragment encoding a portion of the murine Mig mRNA sequence was obtained by RT-PCR using primers flanking the coding region and RNA derived from IFN-γ-stimulated mouse peritoneal macrophages. The PCR product was cloned into the plasmid pGEM 4Z. The methods for plasmid DNA preparation were previously described (6).

Analysis of mRNA expression in tumor tissue

Total cellular RNA was extracted from 0.3 to 0.5 g of whole tumor tissue by homogenization with a Polytron sonicator/homogenizer (Brinkmann Instruments, Westbury, NY) for 1 min in guanidine isothionate followed by ultracentrifugation through cesium chloride according to previously described methods (28, 29). Northern hybridization analysis was conducted as described previously (30, 31). Equal amounts of RNA (20 μg) were denatured, separated, and electrophoresed in an agarose-formaldehyde gel, and blotted by capillary transfer onto nylon membranes. The blots were then prehybridized 6 to 18 h at 42°C in 50% formamide, 1% SDS, 5× SSC, 1× Denhardt’s solution (0.02% Ficoll, 0.02% BSA, and 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured salmon sperm DNA, and 50 mM sodium phosphate buffer, pH 6.5. Hybridization was conducted at 42°C for 12 to 18 h with 106 rpm denatured probe. The filters were washed twice for 15 min each time at 55°C in 0.1% SDS-0.5× SSC. The blots were then exposed using XAR-5 x-ray film (Eastman Kodak, Rochester, NY) with DuPont (Wilmington, DE) Cronex Lightening Plus intensifying screens at −70°C. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control and was applied in all experiments.

Semi quantitative RT-PCR analysis of perforin mRNA was conducted as reported previously (6, 25, 32). One microgram of total RNA was amplified using an oligo(dT) antisense primer and AMV reverse transcriptase at 42°C for 1 h. The RT reaction products were used undiluted or at a 1/10 dilution for PCR amplification using 20 mM sense and antisense primers (see below) and Taq polymerase. PCR reactions were conducted in a Perkin-Elmer/Cetus DNA Thermal Cycler for 15 cycles (denaturation, 1 min, 94°C; annealing, 1 min, 60°C; amplification, 2 min, 72°C). The primer sequences used were as follows: perforin antisense primer, GGGGAGGTTTTTGTTGAC; and perforin sense primer, CAAAGTGGCAG CAAACGAGCAAG (perforin product size, 486 bp). These primers were chosen from separate exons to ensure that products derived from mRNA and contaminating genomic DNA could be distinguished. The PCR products were separated by agarose gel electrophoresis and visualized by Southern hybridization analysis using radiolabeled cDNA encoding a portion of the perforin gene sequence.

Results

BALB/c mice were injected s.c with $4 \times 10^5$ RENCA tumor cells, and IL-12 treatment (0.5 μg/mouse/day) was initiated on day 14. Tumors in animals receiving saline vehicle grew progressively, while tumors in IL-12-treated animals regressed following a modest delay, confirming previous work (6). To examine the expression of chemokines at the tumor site, total RNA was prepared from tumor tissue isolated from control or IL-12-treated animals and analyzed for the expression of the IFN-γ-inducible chemokine mRNAs by Northern hybridization (Fig. 1). IP-10 and Mig mRNA were readily detected in tumor tissue from IL-12-treated animals, but not in tumors from untreated animals.

To determine the functional importance of chemokine expression in the IL-12-mediated antitumor activity, Abs against mouse IP-10 and Mig were raised in rabbits as described in Materials and Methods. The Abs were characterized by examining reactivity in Western blots with proteins secreted by cultured RENCA cells stimulated with IFN-γ (Fig. 2). While Ab to IP-10 reacts with a single band of approximately 8 kDa present only in the medium from IFN-γ-stimulated cells, the Ab to Mig showed specific recognition of three IFN-γ-inducible peptide species of approximately 14, 10, and 8 kDa. Specificity was further demonstrated by competition with peptides used as immunogens (Fig. 2). The heterogeneity evident in the Mig protein may derive from post-translational modification and/or from degradation following secretion.

These Abs were subsequently employed to assess the roles of IP-10 and Mig in IL-12-mediated regression of RENCA tumors. BALB/c mice bearing 14-day-old RENCA tumors were treated with IL-12 (0.5 μg/animal/day) for 15 days with or without control.
or anti-chemokine Ig. Animals in groups of 12 received 1 mg of nonimmune Ig or anti-Mig and/or anti-IP-10 Ig i.p. 1 day before the initiation of IL-12 treatment and at 3-day intervals (0.5 mg each) through the completion of the experiment. Tumors grew progressively in otherwise untreated mice and in mice that were given nonimmune rabbit Ig, while growth of tumors in animals treated with IL-12 along with control or anti-chemokine Abs was analyzed for T cell infiltration by immunohistology. Tumors prepared from IL-12-treated animals were heavily infiltrated with CD8\(^+\) T cells, confirming previous findings (6) (Fig. 4A). Tumor tissue from animals receiving Abs to both IP-10 and Mig throughout the course of IL-12 treatment exhibited a dramatic reduction in CD8\(^+\) T cell infiltration (this effect was observed in all four RENCA tumors examined and in two separate experiments). Quantification of CD8\(^+\) T cell infiltration was conducted by comparing numbers of T cells in sections of tumors from different treatment conditions (Fig. 4B). In each treatment group regions of the tumor that contained the highest level of T cell infiltration were identified, and the CD8\(^+\) T cells were enumerated in a series of high power fields. Regions of the tumor that exhibited necrosis were excluded. This analysis confirms the reduction in T cell numbers within tumors from mice receiving IL-12 plus anti-IP-10 and anti-Mig compared with those in animals receiving IL-12 alone or in the presence of nongeneric Ab. Furthermore, an intermediate reduction in T cell infiltration was observed in animals receiving IL-12 and only anti-Mig Ab. The number of CD4\(^+\) T cells infiltrating the tumors in IL-12-treated animals was also reduced in animals receiving the anti-chemokine Ig (data not shown).

Previous studies from this laboratory demonstrated that perforin mRNA was strongly expressed in regressing RENCA tumors from mice treated with IL-12 (6). Perforin is a product of cytotoxic T cells and NK cells and may contribute to the antitumor activity of IL-12 (34 –37). Portions of the RENCA tumors from the experiments shown in Figure 4 were used to prepare total RNA for semi-quantitative analysis of perforin mRNA levels using RT-PCR. The results obtained provide further confirmation of the histologic data

![Analysis of IP-10 and Mig protein expression in RENCA cells in culture.](http://www.jimmunol.org/)

**FIGURE 2.** Analysis of IP-10 and Mig protein expression in RENCA cells in culture. Confluent cultures of RENCA cells in 100-mm petri dishes were not treated or were treated with 100 U/ml of murine IFN-\(\gamma\) for 18 h in serum- and protein-free culture medium. Supernatant medium from each sample was dialyzed, adsorbed to heparin-Sepharose beads, eluted, and separated by 15% SDS-PAGE. Proteins were transferred to nitrocellulose filters and analyzed separately for reactivity with Abs against IP-10 or Mig in presence or the absence of specific peptide immunogen (1 \(\mu\)g/ml) as indicated. Similar results were obtained in two separate experiments.

![Abs to IP-10 and Mig inhibit IL-12-mediated antitumor activity.](http://www.jimmunol.org/)

**FIGURE 3.** Abs to IP-10 and Mig inhibit IL-12-mediated antitumor activity. BALB/c mice were inoculated with 4 \(\times\) 10^5 RENCA cells s.c. On day 14, groups of 12 animals were treated with IL-12 alone or with control or anti-chemokine Abs as indicated. IL-12 (0.5 \(\mu\)g/animal/day) was administered daily, and Abs were given 1 day before IL-12 and at 3-day intervals thereafter. Tumor size was measured as described in Materials and Methods daily through 15 days of treatment. Results are presented as the mean \(\pm\) SEM. Similar results were obtained in two separate experiments.
Tumors from IL-12-treated mice had high levels of perforin mRNA compared with untreated tumors, in which perforin mRNA was not detectable. Tumor tissue from animals treated with IL-12 and nonimmune rabbit Ig also expressed high levels of perforin mRNA, while tumor from animals treated with IL-12 and anti-Mig/anti-IP-10 had significantly reduced perforin expression. In this experiment, the reduction in perforin mRNA from animals receiving Ab to Mig alone is also evident.

Both IP-10 and Mig have been shown to inhibit angiogenesis in vivo and in vitro (38–41). Furthermore, IL-12 has been reported to exhibit antiangiogenic activity, and this has been attributed to IL-12-dependent expression of IP-10 (42, 43). To test the possibility that our Abs were inhibiting IL-12-mediated antitumor function by blocking the inhibition of angiogenesis, we assessed the relative intratumoral density of endothelial cells based upon immunoreactivity with a mAb against CD31, a marker highly specific for vascular endothelium. The density of CD31-positive cells present in the tumor was not different between experimental groups (data not shown). Tumors from IL-12-treated mice exhibited significant necrosis, and examination of endothelial cell density was restricted to nonnecrotic areas of the tumor; the distribution and density of CD31 staining were, however, equivalent in all fields examined regardless of treatment condition. Because tumor tissue was rapidly destroyed in T cell-dependent fashion following the initiation of IL-12 treatment, this result may not reflect the angiostatic potential of IL-12 or IP-10 and Mig.

Discussion

The goal of the present study was to test the importance of the chemokines IP-10 and Mig in IL-12-mediated regression of RENCA tumor growth. Although IL-12 was originally identified...
as a stimulus of NK cells, it has since been characterized as an important cytokine in many physiologic and pathophysiologic settings (8, 14, 44–46). IL-12-stimulated antitumor function is dependent upon the induction of IFN-γ and the presence of both CD4+ and CD8+ T cells (7, 9, 10). The observation that IL-12 treatment induces high levels of IFN-γ in tumor-bearing nude mice without marked antitumor effects indicates that IFN-γ is necessary, but not sufficient, for IL-12-mediated antitumor function (7, 9, 10). We hypothesize that the remarkable antitumor efficacy of IL-12 derives from its ability both to enhance the T cell-mediated immune response to the tumor and to promote the infiltration of the tumor by activated effector T cells. Our results indicate that this latter objective is achieved via the IFN-γ-mediated production of the non-ELR-containing CXC chemokines, IP-10 and Mig, and is supported by the following observations. 1) Rabbit Abs to IP-10 and Mig, when provided in combination, reduce the antitumor activity of IL-12 against established RENCA tumors growing s.c. 2) The anti-chemokine Ab treatment results in a marked reduction in the infiltration of tumors by CD4+ and CD8+ T cells and reduced expression of mRNA encoding the cytotoxic T cell effector molecule perforin. 3) There was no detectable change in the tissue density of endothelial cells within tumors receiving any of the experimental treatments.

While the effect of Ab treatment on antitumor function is not complete, the results clearly indicate that both chemokine gene products are functionally important components of the IL-12 antitumor mechanism. Since both Mig and IP-10 bind CXCR3 and mediate T cell chemotaxis (23), it is not surprising that both Abs should be required to achieve neutralization of IL-12-mediated T cell infiltration and tumor growth inhibition. Although statistically significant effects were only observed when Abs to both Mig and IP-10 were administered, anti-Mig Ab alone appeared to produce a reduction in the action of IL-12 in several animals. In this regard Mig has also been shown to exhibit higher potency than IP-10 in T cell chemotaxis (22, 23).

The results presented here suggest that the effect of anti-chemokine Abs on IL-12-driven tumor regression is a direct consequence of their ability to block chemokine-mediated recruitment of activated T cells to the site. Such a scenario is consistent with an early report showing that IP-10 expression by tumor cells could promote a strong T cell-dependent antitumor effect (47). Both IP-10 and Mig have been shown to exhibit other nonchemotaxis-related activities that may be relevant to the antitumor effects of IL-12. Specifically, both chemokines have been reported to exhibit potent antiangiogenic activity in vitro and in vivo (38–41). Interestingly, CXC chemokines that possess an ELR amino acid motif immediately preceding the CXC motif have been demonstrated to be angiogenic agents, while those that do not have the ELR sequence are angiogenesis inhibitors (41). Indeed, the balance of expression of ELR+ and ELR− CXC chemokines within a tumor has been proposed as an important determinant of progressive tumor growth and metastasis (41, 48). This hypothesis is supported by the finding that IP-10 expression by human lung tumors in SCID mice is associated with reduced tumor growth potential, while neutralization of IP-10 enhances growth (49). In our studies, the density of endothelial cells within tumor tissue did not change in the presence of IL-12 treatment (and was not influenced by Abs to IP-10 or Mig). This result should not, however, be interpreted to mean that IL-12 did not have an angiostatic effect in RENCA tumors. Because RENCA tumors are rapidly destroyed in immunocompetent mice treated with IL-12, the angiogenesis inhibitory action of IL-12 may be masked by its potent effect on the development of antitumor T cells. Thus, these experiments do not allow a direct determination of the antiangiogenic activity of IP-10 and Mig. In addition to effects on neovascularization, both IP-10 and Mig have also been reported to cause focal tumor necrosis when injected intratumorally or when expressed by tumor cells (50, 51). As discussed above with regard to angiogenesis inhibition, we cannot determine whether IP-10 and/or Mig are responsible for enhanced tumor necrosis because of the dominant role of T cell activity in this model.

There have been numerous reports attesting to the ability of CC and CXC chemokines to promote antitumor activity when expressed as transgenes by experimental tumors (39, 52–58). While there are examples where chemokine expression alone appears to be sufficient to promote an efficacious antitumor response, chemokines may function best in cooperation with other cytokine agents. For example, tumor cells transduced to express lymphotactin, a chemokine with specificity for T cells, grew normally when injected alone, but were destroyed rapidly in mice cotreated with IL-2 (59). The results of the present study support the concept of cooperativity between chemokines and other cytokines in antitumor strategies. IL-12 is able to promote chemokine expression through the enhanced expression of IFN-γ; this may not be sufficient, however, and IL-12 also promotes expansion and activation of tumor-specific T lymphocytes. The IFN-γ-induced chemokines may cooperate by recruiting such cells to the tumor site. One consequence of increased T cell infiltration would be additional IFN-γ production and further enhancement of chemokine synthesis. It is also likely that the nonchemotactic functions of Mig and IP-10, such as inhibition of angiogenesis, can act cooperatively with the chemotactic functions to promote more efficacious antitumor function.

References
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