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Immunotherapy with Interleukin-10 Depends on the CXC Chemokines Inducible Protein-10 and Monokine Induced by IFN-γ[1]

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ABSTRACT

The cytokine interleukin (IL)-10 has potent antitumor activity in many model systems when expressed locally at very high levels from the time of tumor transplantation. We now demonstrate that systemic administration of recombinant human IL-10 to animals bearing established highly malignant mammary tumors also leads to significant growth inhibition. We had shown previously that expression of the CXC chemokines Mig (monokine induced by IFN-γ) and IP-10 (inducible protein 10) is observed in IL-10 transduced but not neo-vector control tumors. We now demonstrate that treatment of IL-10-tumor-bearing mice with antibodies to either chemokine partially reverses the therapeutic effect of IL-10. Tumor growth in animals treated with both antibodies is comparable with that of vector control tumors. Direct transduction of Mig cDNA into the parental tumor cell line before transplantation also results in smaller tumors. This tumor growth inhibition is associated with increased numbers of CD4+ cells, consistent with a T-cell chemoattractant activity for Mig. No change in vascularization, as indicated by CD31+ cells, was observed in either Mig or IL-10-transfected tumors. Thus, an antiangiogenic activity for either cytokine could not be confirmed. Mig and IP-10 are critical to the therapeutic response resulting from high levels of IL-10, and, furthermore, Mig as a single agent also has tumor-inhibitory activity in a model of breast cancer.

INTRODUCTION

Transduction of murine mammary tumor cells with murine IL-10[3] cDNA results in marked inhibition of tumor growth and metastasis (1, 2). Similar antitumor activity has been demonstrated in many model systems using histologically diverse tumors (3–6). Several immune effector cells have been implicated, including T cells, NK cells, and neutrophils (7, 8). Expression of a number of cytokines and other immune effector molecules are up-regulated in IL-10-expressing tumors that are growth inhibited. Among these, IFN-γ and nitric oxide have been demonstrated to contribute to the therapeutic effect of IL-10 overexpression (9–11). Two CXC chemokines, Mig and IP-10, are critical to the tumor-inhibitory activity of another cytokine IL-12 (12–14), and each chemokine has been shown to mediate tumor inhibition when expressed directly in tumors (15–18). Both Mig and IP-10 mRNAs are up-regulated in IL-10-expressing tumors (10, 19), and the present study examines the functional role of this increased expression. We now show that both chemokines contribute to the antitumor activity of IL-10. Furthermore, intentional overexpression of Mig cDNA in parental tumors is also therapeutic.

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[3] The abbreviations used are: IL, interleukin; NK, natural killer; IP, inducible protein; NRS, normal rabbit serum.

MATERIALS AND METHODS

Cell Lines. Tumor cell lines 410.4 and 66.1 were derived from the same spontaneously occurring mammary adenocarcinoma in a BALB/cfC3H mouse. These cell lines are highly tumorigenic and metastatic after s.c. implantation. Plasmids pBMGneo and pBMGneo.IL-10 were introduced to tumor cells, and stably transduced lines were cloned and characterized previously (1, 9).

Tumorigenicity. Tumorigenicity was evaluated by injecting IL-10-expressing or neo-vector-control cells s.c. into syngeneic BALB/cByJ female mice (Jackson Laboratory, Bar Harbor, ME). When tumors became palpable, size was determined by caliper measurement of the largest and perpendicular diameters. Tumor growth is reported as the mean of these two measurements. All animals were housed, cared for, and used strictly in accordance with USDA regulations and the NIH Health Guide for the Care and Use of Laboratory Animals.

Systemic Therapy. Recombinant Human IL-10 produced in *Escherichia coli* was a generous gift of Dr. Satwant Narula, Schering-Plough, Kenilworth, NJ. Recombinant protein was administered twice daily in two equal doses of 20 μg i.p. from day +7–13 after transplantation of 5×10^5 410.4 cells to mice.

Chemokine Antibody. Rabbit polyclonal antibodies to Mig and IP-10 were produced by Biosynthesis, Inc. (Lewisville, TX) using synthetic peptides selected from the IP-10 and Mig protein sequences (CIHIDDGPVRMRAIGK and CISTSRGTIHYKSLKDLKQFAPS, respectively) coupled to carrier protein keyhole limpet hemocyanin. Characterization of these antibodies has been reported previously (12). Antibodies or preimmune sera were administered i.p. (250 μl) to tumor-bearing mice on days +1, +4, and +7 after transplantation of 2×10^5 of 66-neo or 66-IL10c.5 cells to syngeneic mice.

Mig Transfection. Mig cDNA was amplified from IFN-γ-stimulated murine peritoneal macrophages and cloned into the EcoR1 site of the Pbabe retroviral vector (20). Briefly, primers encoding EcoR1 sites and the initial and terminal 15 bp of the Mig coding sequence were synthesized commercially (Ransom Hill Bioscience, Ramona, CA) and used in conjunction with Proof Start DNA polymerase (Qiagen, Valencia, CA) in 30 cycles of PCR amplification. The amplified product was cloned into Pbabe puro, and after verification by sequence analysis, a recombinant plasmid encoding wild-type Mig was used to transfect the ecotropic packaging cell line GP+E 86. Puromycin-resistant clones overexpressing Mig and synthesizing high titer recombinant virus were grown in bulk and used to generate infectious supernatants, which were used subsequently to transduce the parental 66.1 cell line. Tumor cells were admixed with virus in Polybrene, cultured overnight, split and cultured under puromycin selection, and cloned using standard methods. Seven clones were derived and characterized for expression of Mig mRNA by Northern hybridization. All Mig and puro-expressing clones grow *in vitro* at rates comparable with parental 66.1 cells. One million cells of 66-Mig clones 31 and 33–35, as well as three clones of 66-puromycin (clones 51–53), were transplanted to syngeneic mice, and tumor growth properties were determined.

Northern Hybridization. Total RNA was isolated from puro-vector control and Mig-expressing cell lines and hybridized with probe specific for Mig or elongation factor 1α as described previously (10).

Immunohistochemistry. Tumors derived from transplantation of various cell lines were fixed in Baker's formal calcium fixative and embedded, and 5-μm sections were prepared as described previously (19). Deparaffinized sections were reacted with trypsin and normal goat serum, followed by antibody to CD4 (L3T4; PharMingen, San Diego, CA) or CD8α (Serotec, Inc., Raleigh, NC) rat monoclonal antibody; endothelial cells were stained with antibody to CD31 (PharMingen). The appropriate isotype control antibodies were used as negative controls. Secondary biotinylated antibody (Vector Labs, Burlingame, CA) was added, slides were rinsed, and streptavidin-horseradish peroxidase (Sigma) was added. Slides were rinsed, and diaminobenzylperoxide

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was added. After rinsing, slides were counterstained in Gill's hematoxylin, coded, and randomized, and positively staining cells were quantitated. All non-necrotic areas of an entire section were analyzed, and multiple sections were examined for each tumor specimen. Five tumors of each tumor type were analyzed.

RESULTS

We have reported previously that murine mammary tumor cells transduced with murine IL-10 cDNA are growth inhibited and that the therapeutic effect is mediated immunologically (1, 9). Fig. 1*A* confirms this finding, showing that growth of the highly metastatic murine mammary tumor cell line 410.4 is virtually completely inhibited by the expression of 63 ng/2.5 $\times$ 10^5 cells/48 h murine IL-10. The same tumor line expressing only the neo-vector grows progressively, and mice succumb to pulmonary metastases. Under these conditions, IL-10 is expressed locally from the day of tumor transplantation. In contrast, under some circumstances, IL-10 is immunosuppressive, enhances tumor growth, and prevents tumor vaccination (21–23). To determine whether systemic delivery of IL-10 could mediate tumor inhibition, rather than tumor enhancement, and to test whether IL-10 can have activity against established disease, we administered recombinant human IL-10 (rHuIL-10) i.p. to mice bearing palpable (day 7) tumors. Cytokine was given at a dose of 40 μg/day and divided into two equal doses from days +7–13 post-transplantation. Fig. 1*B* shows one of two experiments in which systemic therapy with rHuIL-10 over this limited period results in significant tumor inhibition ($P < 0.02$). This latter effect is less dramatic than achieved by direct transduction of IL-10 into tumors. Nevertheless, systemic administration of IL-10 does result in less tumor growth, rather than accelerated growth, as might be predicted if IL-10 was acting solely as an immunosuppressive cytokine in this context.

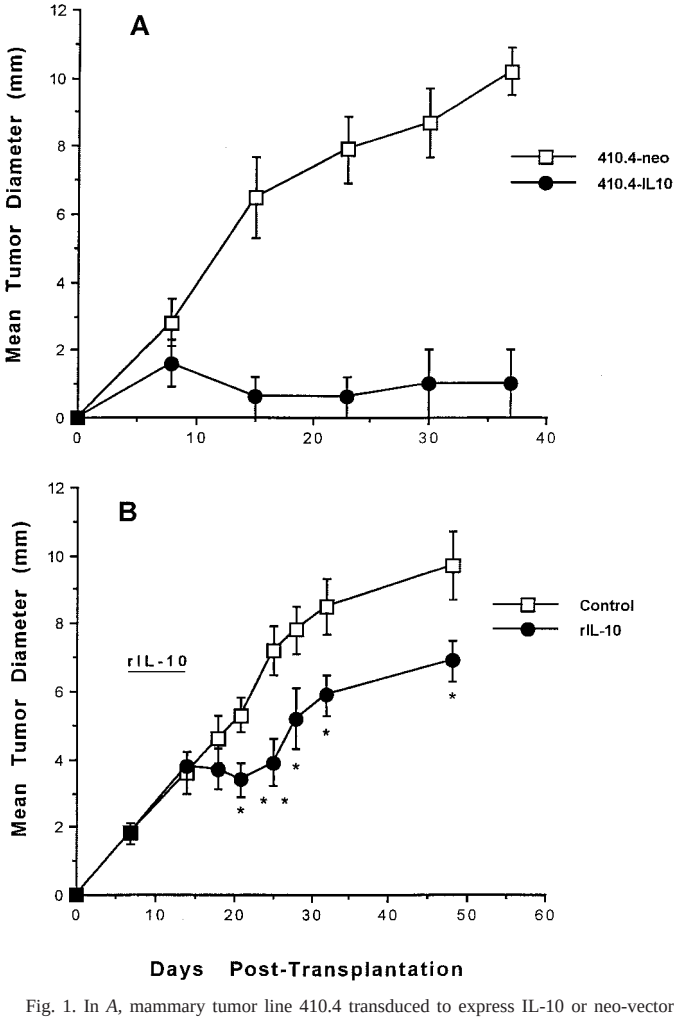

Fig. 1. In *A*, mammary tumor line 410.4 transduced to express IL-10 or neo-vector alone was transplanted to syngeneic Balb/cByJ mice. Tumor diameters were determined by caliper measurement and expressed as an average of longest diameter and perpendicular diameter ± SE of 10 mice/group. *B*, mice transplanted with parental line 410.4 and treated by daily i.p. administration of 40 μg of rHuIL-10 on days +7–13 post-transplantation. Tumor growth was determined as in *A*. Tumor size was significantly smaller ($P < 0.02$) in mice treated with rIL-10 at day +21 and all later times.

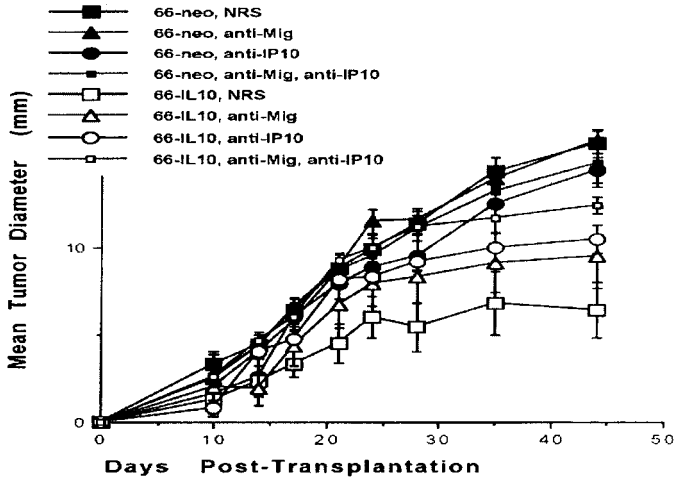

Fig. 2. Syngeneic Balb/cByJ mice transplanted with 66-neo or 66-IL10c.5 cells. On days +7, +11, and +13, mice were treated with preimmune (*NRS*) or antibody to Mig or IP-10. Mean tumor diameter ±SE of 5 mice/group. Size of 66-IL10c.5 *versus* 66-neo, NRS significantly different ($P < 0.0001$). Tumor size in mice bearing 66-IL10c.5 and treated with antibody to IP-10 or antibody to IP-10 plus Mig significantly different from mice bearing 66-IL10c.5 and treated with NRS ($P < 0.03$ and 0.003, respectively).

We have reported that the expression of the closely related chemokines Mig and IP-10 is up-regulated in IL-10-transduced tumors (10, 19). To determine whether this up-regulation is important to the mechanism by which IL-10 inhibits tumors, we examined the effect of depletion of each chemokine on the therapeutic response. For these studies, we used a tumor cell line, 66.1, which is not completely inhibited by IL-10 gene transduction. On day 0, syngeneic Balb/cByJ mice were transplanted with 2 $\times$ 10^5 of 66-neo or 66-IL10c.5 cells. On days +1, +4, and +7, mice were injected i.p. with 250 μl of polyclonal antibody to Mig, IP-10, or a combination of both antibodies. Control animals were injected with preimmune serum from the same rabbit used to prepare Mig antibody. Fig. 2 shows that, as expected, tumors derived from transplantation of 66-IL10c.5 are significantly smaller than 66-neo tumors in mice treated with NRS ($P < 0.0001$). Treatment of mice bearing 66-neo tumors with antibody to Mig, IP-10, or the combined antibodies has no significant effect on tumor size consistent with the absence of Mig or IP-10 in these tumors. In contrast, either single antibody treatment enhances growth of IL-10-expressing tumors. In the presence of the combined antibodies, 66-IL10c.5 tumors grow at a rate comparable with 66-neo tumors growing in either antibody-treated or control mice ($P < 0.08$). This latter finding suggests an additive protective effect for each chemokine, which is interesting in light of the fact that they share a common receptor, designated CXCR3 (24). In addition to the inhibition of growth of s.c. tumors, expression of IL-10 reduces the metastatic capacity of these cells. Thus, in mice bearing s.c. implants of 66-neo tumors, an average of 4.8 ± 1.3 lung tumors arises. IL-10 expression reduces this number to 1 ± 0.7 lung metastases. Treatment with chemokine antibodies does not enhance tumor metastases in mice bearing 66-neo but completely abrogates the antimetastatic

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activity of IL-10 (mean of 4.1, 6.8, and 6 metastases for anti-Mig, anti-IP-10, or combined antibody treatments, respectively). Although these latter data do not achieve statistical significance because of the small sample size, these trends are consistent with the effect of chemokine depletion on enhancement of s.c. tumor growth.

These data indicate that both Mig and IP-10 contribute to the antitumor activity of IL-10. To confirm a role for chemokines in tumor inhibition, we asked if one chemokine could mediate tumor inhibition directly, in the absence of IL-10. Using a viral vector expressing murine Mig cDNA, 66.1 cells were transfected, and stable Mig-expressing clones were derived. Fig. 3 shows a Northern hybridization of RNA extracted from these transduced cell lines. As expected, puro-vector transduced cells (66-puro.cl.51) express no detectable Mig mRNA. Four Mig-transfected clones, designated 66-Mig.cl.31 and 33–35, were selected for examination of growth properties *in vivo*. Each cell line (1×10^6) was transplanted to syngeneic Balb/cByJ female mice, and *in vivo* growth properties of Mig-expressing clones were compared with three independent puro-vector-expressing clones (66-puro.cl.51–53). Growth of each tumor expressing Mig was significantly reduced in comparison with control vector-expressing tumors (Fig. 4). RNA was extracted from these tumors and analyzed for Mig mRNA expression to confirm that differential Mig expression was maintained in transduced tumors *in vivo* (data not shown). These data indicate that Mig alone, in the absence of IL-10, can also mediate growth inhibition of a mammary tumor.

Because Mig is chemotactic for T cells, we examined the effect of Mig expression on host T-cell infiltration. Tumors were examined for CD4+ and CD8+ cells by immunohistochemistry. Consistent with the chemoattractant activity of CXC chemokines, these Mig-transduced tumors have increased numbers (3–4-fold) of CD4+ cells. Mean CD4+ cells in 66-puro.cl.51 tumors was 9.8 ± 0.2 cells/×400 field *versus* 36 ± 2, 34 ± 11, and 30 ± 7 cells in 66-Mig.c.31, 66-Mig.c.33, and 66-Mig.c.34 tumors, respectively. No effect of Mig expression on CD8+ cells was detected. Others have reported that angiostatic properties of Mig are important to tumor inhibition (16–18). To determine whether Mig transduction affected numbers of blood vessels in mammary tumors, numbers of CD31+ cells were compared in puro-vector and Mig-expressing tumors. Although a range of CD31+ cells was observed in different tumors (40–58 positive cells/field), no relationship to tumor phenotype was seen.

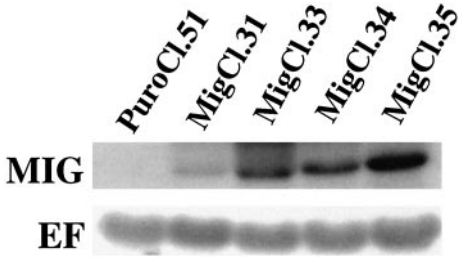

Fig. 3. Tumor cell line 66.1 transduced to express murine Mig cDNA (*Migcl.31* and *33–35*) or puro-vector alone (*Purocl.51*), and stable clones were derived. RNA was isolated from stable clones and hybridized with probes specific for murine Mig or elongation factor-1α.

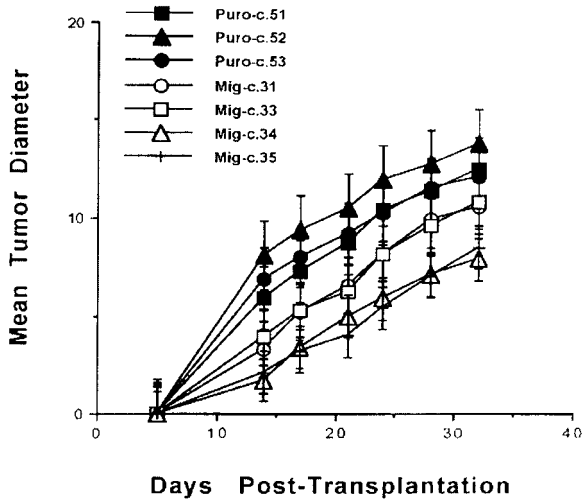

Fig. 4. Mig-transduced or puro-vector transduced clones transplanted to syngeneic Balb/cByJ mice. Mean tumor diameter ±SE of 10 mice/group. At day 21, mean tumor size of all Mig-transduced clones was significantly smaller than 66-Puro.c.51. *P*s are 66-Mig.c.31, <0.01; 66-Mig.c.33, <0.01; 66-Mig.c.34, <0.002; and 66-Mig.c.35, <0.0001.

DISCUSSION

Many laboratories (1–8) have demonstrated in a number of model systems that overexpression of IL-10 in tumor cells by gene transfer results in marked tumor inhibition that is mediated immunologically. We have confirmed these findings and have now demonstrated that recombinant HuIL-10, given to animals with established mammary tumors, also results in significant growth inhibition. These studies are consistent with a study by Berman *et al.* (4) showing therapeutic activity of recombinant protein in models of other cancers. In our hands, systemic administration was not as effective as direct transduction of IL-10 cDNA at achieving growth inhibition. There are several possible reasons for this; complete tumor ablation may require high local expression of IL-10 at the tumor site, whereas systemic administration may achieve lower local levels. Furthermore, rHuIL-10 was only administered for 6 days, and therapy was initiated when tumors were already palpable; transduced IL-10 was expressed continuously from the day of tumor transplantation.

IL-10 was first identified as a product derived from the Th2 subset of CD4+ cells that inhibits cytokine production by Th1 cells. IL-10 can inhibit alloantigen and antigen-specific T-cell proliferation and cytotoxicity (25). Many macrophage activities are also suppressed by IL-10. More recent studies have challenged the view that IL-10 acts only as an immunosuppressive cytokine, *e.g.*, IL-10 can enhance, rather than suppress, IL-2-mediated induction of cytotoxic T lymphocyte precursors (26–29). Thus, IL-10 has differential effects on CD8+ T cells depending on their activation state. IL-10 can exacerbate graft-*versus*-host disease when administered at the time of bone marrow transplantation (30), and IL-10 is also chemotactic for CD8+ cells (31).

The role of IL-10 in the behavior of malignant cells is equally complex and reflects the diverse roles of this cytokine in immune regulation. Although several laboratories have demonstrated antitumor activity of IL-10, other studies have identified an immunosuppressive role for IL-10 that contributes to the progressive growth of tumors. Many human tumor cell lines express IL-10 spontaneously (32), and this expression can, in some cases, directly support the proliferation of tumor cells (33). IL-10 production by lymphocytes can also be induced by the tumor milieu, leading to immune suppression (21, 34), *e.g.*, vaccination to either a tumor or nontumor antigen at the tumor site is prevented by tumor-induced production of IL-10 (21). Likewise, human lung carcinomas induce IL-10 production by cocultured T lymphocytes as a possible mechanism of immune suppression (34). Transgenic mice overexpressing IL-10 in the lymphocyte compartment fail to control growth of otherwise immunogenic tumors (22).

The reasons for these apparent contradictory effects of IL-10 on

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tumor behavior are not established. In most models in which therapeutic effects of IL-10 are demonstrated, tumor cells are directly transduced to express IL-10 cDNA, resulting in the expression of ng/ml quantities of IL-10. In contrast, spontaneous production of IL-10 by tumor cell lines usually results in much lower levels of IL-10 (pg/ml). The possibility that very high levels of IL-10 may actually desensitize the IL-10-receptor must be considered. Other factors that may determine the outcome of IL-10 expression on tumor behavior are the cellular compartment, in which IL-10 is expressed, *e.g.*, expression of IL-10 as a transgene in lymphocytes is clearly immunosuppressive (22). Temporal aspects of IL-10 expression are also important. Thus, IL-10 expressed as a transgene in antigen-presenting cells produced a biphasic response. Initial growth of P815 mastocytomas was enhanced in IL-10 transgenic mice, but ultimately, these tumors were rejected in transgenic, but not nontransgenic, littermates (35).

We have shown previously that IL-10-mediated tumor growth inhibition is compromised in T cell-deficient mice (1) and that regressing tumors have increased numbers of CD8+ and CD4+ cells (19). Unpublished data from our laboratory also indicate that other host cellular elements are increased in IL-10-expressing tumors as well, including B cells, dendritic cells, and polymorphonuclear cells, but that macrophage numbers were reduced.

A number of cytokines and chemokines are up-regulated in tumors undergoing regression as a result of overexpression of IL-10, including the related chemokines Mig and IP-10 (10, 19). These chemokines were identified initially in IFN-γ-activated macrophages (24, 36). Both are members of the CXC chemokine superfamily. Mig has been shown to be induced exclusively by IFN-γ, whereas IP-10 expression can be induced by IFN-α, IFN-β, or IFN-γ (24). Consistent with these properties, we have reported that induction of Mig in IL-10-expressing tumors is dependent on IFN-γ expression by the host (10) but that IP-10 is still detected in IL-10-expressing tumors transplanted to IFN-γ mutant mice.[4]

Because the antitumor activity of IL-10 depends on IFN-γ expression, and because Mig and IP-10 are induced by IFN-γ, we postulated that the up-regulation of Mig and/or IP-10 might be important to the tumor-inhibitory activity of IL-10. To answer this question, we determined the effect of neutralization of these chemokines on IL-10-mediated tumor inhibition. Using antibodies to either chemokine, we showed for the first time that both Mig and IP-10 contribute to the therapeutic activity of IL-10. Depletion of either chemokine compromised the antitumor activity of IL-10. Elimination of both chemokines resulted in tumor growth comparable with the neo-vector-transduced tumor. These data suggest that, despite the fact that these chemokines share a common receptor, they may play diverse roles in the control of tumor growth. Interestingly, immunotherapy of either RENCA or Burkitt's lymphoma with IL-12 also depends on both chemokines (12–14). Different kinetics and tissue localization for these two chemokines have also been reported during acute infections with viruses and protozoa, suggesting diverse roles during immune responses to infectious agents as well (37).

To determine whether expression of Mig alone in murine mammary tumors might also inhibit tumor growth, we transduced parental cells to express murine Mig cDNA. Although this activity has been demonstrated by other laboratories, we felt it was important to determine whether mammary tumor cells could be growth inhibited by Mig. Expression of Mig resulted in partial growth inhibition in this system. Several mechanisms have been proposed to explain the tumor-inhibitory activity of Mig (15–18). Mig and IP-10 are both chemotactic for activated T and NK cells. We have shown that the antitumor activity of IL-10 depends on both these effector cells. Furthermore, IL-10-expressing tumors have increased infiltration of both CD4+ and CD8+ cells (19). IFN-γ is up-regulated in IL-10-expressing tumors and is critical to the therapeutic activity of IL-10 (10, 11). Taken together, our data are consistent with a model in which IL-10 overexpression results in IFN-γ induction, which, in turn, activates Mig and IP-10 to attract T and NK cells to the tumor site. These effector cells may function as cytolytic cells, helper cells, and/or as producers of IFN-γ to drive the further production of these chemokines.

In addition to immune-mediated events, both Mig and IP-10 are potent inhibitors of angiogenesis. Antitumor activity of both cytokines has been demonstrated in mice lacking T-cell function (16–18). Those studies indicate that inhibition of angiogenesis and induction of tumor necrosis are important additional mechanisms. In the present model, where immune effector mechanisms are active, no effect of either IL-10 or Mig gene transduction on the number of CD31+ cells could be detected suggesting that angiogenesis was not modulated. Thus, the relative contribution of antiangiogenic and immune-targeted activities of Mig and IP-10 may be determined by the inherent immunogenicity of the tumor under study, as well as the immune capacity of the tumor-bearing host.

These studies confirm a tumor-inhibitory activity of high level IL-10, expressed either as a transgene product or administered as a recombinant molecule to hosts with established, highly malignant tumors. This therapeutic response is associated with an influx of host immune effector cells, including T (19) and B cells, dendritic cells, and polymorphonuclear cells. These studies identify two CXC chemokines as critical components in tumor inhibition and add to the literature establishing a basis for the examination of CXC chemokines for cancer therapy.

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