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Tumor Cell Responses to IFNγ Affect Tumorigenicity and Response to IL-12 Therapy and Antiangiogenesis

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Summary

Expression of a dominant negative mutant IFNγR1 in murine SCK and K1735 tumor cells rendered them relatively unresponsive to IFNγ in vitro and more tumorigenic and less responsive to IL-12 therapy in vivo. IL-12 induced histologic evidence of ischemic damage only in IFNγ-responsive tumors, and in vivo Matrigel vascularization assays revealed that while IFNγ-responsive and -unresponsive tumor cells induced angiogenesis equally well, IL-12 and its downstream mediator IFNγ only inhibited angiogenesis induced by the responsive cells. IL-12 induced angiogenesis inhibitory activity in the responsive cells, which may be attributable to production of the chemokine IP-10. Thus, IL-12 and IFNγ inhibit tumor growth by inducing tumor cells to generate antiangiogenic activity.

Introduction

Interleukin-12 (IL-12) is a cytokine with marked therapeutic effects against a wide variety of murine tumors (Brunda et al., 1993; Nastala et al., 1994). Little is known about the mechanisms of IL-12 antitumor activity other than the lymphocyte subsets required and the importance of endogenous IFNγ. Studies have shown that $CD4^+$ and/or $CD8^+$ T cells are usually required for IL-12 effectiveness (Brunda et al., 1993; Nastala et al., 1994). While the specific functions of these cells responsible for antitumor activity are unknown, IL-12 is known to induce $CD4^+$ T helper cell differentiation along the TH1 pathway (Hsieh et al., 1993) and to enhance $CD8^+$ T cell maturation and activation (Gately et al., 1992; Mehrotra et al., 1993). From this, the antitumor effect of IL-12 has been attributed to augmentation of antigen-specific antitumor immune responses. Natural killer (NK) cells are also functionally activated by IL-12 and may be important for its effectiveness (Kobayashi et al., 1989). However, the role of NK cells in IL-12 antitumor activity is unclear because NK cell ablation has variable effects on IL-12 efficacy in different tumor models (Brunda et al., 1993; Coughlin et al., 1995; Schmitt et al., 1997). In contrast, there is little question of the importance of the IFNγ induced by IL-12, because its neutralization markedly reduces or abolishes IL-12 tumor protection (Nastala et al., 1994; Coughlin et al., 1995).

IL-12 and its downstream mediator IFNγ act in various ways on both host and tumor cells that potentially favor tumor regression (reviewed in Boehm et al., 1997). Effects on the host include activation of innate and acquired immune mechanisms. IL-12 and IFNγ functionally arm cells responsible for innate immunity: IL-12 stimulates NK cell cytotoxicity and production of IFNγ, and IFNγ stimulates neutrophil and macrophage production of superoxides and nitric oxide. Acquired immune responses to antigens are directed toward cell-mediated mechanisms: IL-12 enhances the cytotoxicity of $CD8^+$ CTLs and directs TH1 differentiation of naive $CD4^+$ T cells. The IL-2 produced by TH1 cells favors CTL amplification, while the IFNγ produced induces Ig class switching by B cells toward production of opsonizing antibodies. The response of nonimmune host cells also may contribute to an antitumor effect. IL-12, through the agency of IFNγ, inhibits angiogenesis by mechanisms that are incompletely understood but involve IFNγ induction of angiogenesis inhibitory chemokines, IP-10, and Mig (Voest et al., 1995; Streiter et al., 1995; Sgadari et al., 1996, 1997). While IL-12 is known to act only on cells of the immune system, IFNγ has effects on most somatic cells, including tumor cells. Through its ability to slow cell proliferation and up-regulate expression of MHC class I and the machinery of antigen presentation, IFNγ can favor tumor regression by retarding tumor growth and enhancing immunological recognition of tumor cells and their antigens. Singly or in combination, these effects of IL-12 and IFNγ may be responsible for their activity against tumors.

We undertook the current study to examine how tumor cell responses to IFNγ affect the antitumor activity of IL-12. IFNγ stimulates cells by binding to a receptor that is widely expressed on normal and malignant cells (Boehm et al., 1997). The functional IFNγ receptor consists of two components, IFNγR1 and R2, that dimerize upon ligand binding. Receptor dimerization allows their transphosphorylation and activation of attached JAK1 and JAK2 tyrosine kinases (Kotenko et al., 1995, 1996) and phosphorylation, dimerization, and nuclear translocation of Stat1α (GAF, or gamma-interferon activated factor) (Pearse et al., 1993). Nuclear Stat1α binds gamma-

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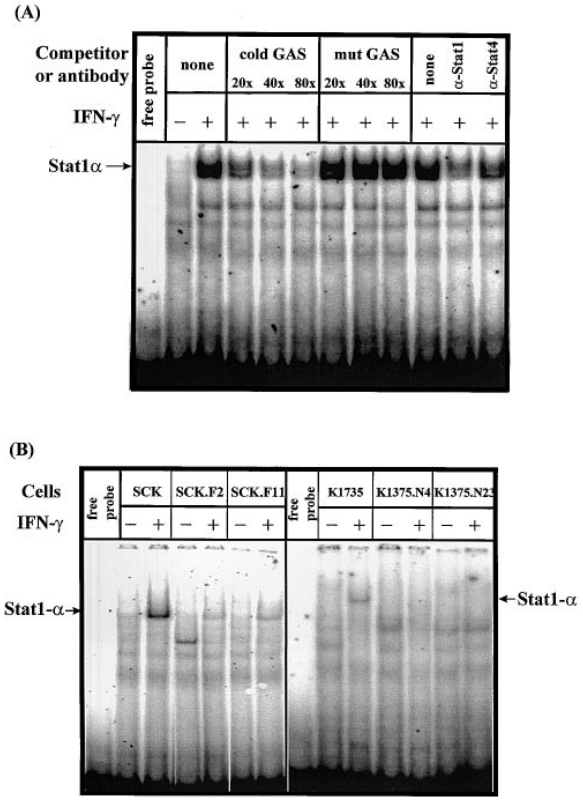

Figure 1. Stat1α Activation in SCK and K1735 Cells and Transfectants Expressing a Dominant Negative Mutant IFNγR1

EMSA was performed with end-labeled GAS oligonucleotide and nuclear extracts from SCK, SCK.F11, and SCK.F2 cells and from K1735, K1735.N23, and K1735.N4 cells. Nuclear extracts were prepared from IFNγ-stimulated (for 15 min) or -unstimulated cells as indicated. The band identified as Stat1α is indicated.

(A) EMSA with SCK extract and labeled GAS probe competed with either unlabeled GAS or mutant GAS oligonucleotides or treated with anti-Stat1 or anti-Stat4 antibodies.

(B) Stat1α activation by IFNγ was determined in SCK, SCK.F11, and SCK.F2 cells (left panel) and in K1735, K1735.N23, and K1735.N4 cells (right panel).

activated sites (GAS) in IFNγ-responsive genes to modulate transcription and bring about cellular changes associated with IFNγ (Darnell et al., 1994). Dighe et al. (1993, 1994) used a dominant negative mutant IFNγR1, truncated in its cytoplasmic domain and missing both its JAK1 and Stat1α docking sites, to create Meth A sarcoma cells that were unresponsive to IFNγ in order to study the effect of IFNγ responses on tumorigenicity. We adopted the same strategy to create IFNγ-unresponsive SCK murine mammary carcinoma and K1735 murine melanoma cells and found that the unresponsive cells formed tumors more rapidly than the parental cells and were relatively refractory to IL-12 treatment. Our studies indicated that this behavior was not due to reduced susceptibility to immunological rejection. Rather, IL-12, via IFNγ, effectively inhibited angiogenesis induced by IFNγ-responsive but not by IFNγ-unresponsive tumor cells, because only the former were induced by IFNγ to produce angiogenesis inhibitory activity that may be attributable to the chemokine IP-10 (IFNγ-inducible protein 10). These studies clearly indicate that tumor cells themselves play an important role in the inhibition of angiogenesis by IL-12.

Results

Impaired IFNγ Responses in Tumor Cells Expressing a Dominant Negative Mutant IFNγR1

Murine SCK mammary carcinoma cells and K1735 melanoma cells were transfected to express high levels of a dominant negative mutant IFNγR1. Upon staining with an antibody to murine IFNγR1 and flow cytometric analysis, transfected clones SCK.F11 and SCK.F2 expressed 12- and 8-fold higher levels, respectively, of immunoreactive IFNγR1 than SCK cells, and clones K1735.N23 and K1735.N4 expressed 25- and 21-fold higher levels, respectively, of immunoreactive IFNγR1 than K1735 cells. IFNγ signaling was inhibited in these clones when they were tested for IFNγ induction of nuclear Stat1α. Using EMSA and a radiolabeled GAS oligonucleotide (Decker et al., 1997), rmIFNγ treatment of SCK and K1735 cells resulted in the appearance of a nuclear complex that was efficiently inhibited by anti-Stat1α antibody and by cold GAS oligonucleotide but not by anti-Stat4 antibody or cold mutant GAS oligonucleotide (Figure 1A shows the results for SCK cells; not shown are similar results for K1735 cells). If rmIFNγ induction of nuclear Stat1α–GAS complex in SCK cells is 100%, complex induction in rmIFNγ-stimulated SCK.F11 and SCK.F2 cells was 8% and 17%, respectively. If rmIFNγ induction of nuclear Stat1α–GAS complex in K1735 cells is 100%, complex induction in rmIFNγ-stimulated K1735.N23 and K1725.N4 cells was 2% and 11%, respectively (Figure 1B). Downstream effects of IFNγ signaling were inhibited in SCK.F11 and F2 cells and in K1735.N23 and N4 cells. By antibody staining and flow cytometry, H-2D^d expression increased 5.2-fold with rmIFNγ treatment on SCK cells, increased 2.0-fold on treated SCK.F2 cells, and did not increase at all on treated SCK.F11 cells. H-2K^k expression increased 3.4-fold on K1735 cells exposed to rmIFNγ but did not increase on treated K1735.N23 and K1735.N4 cells (data not shown). In vitro proliferation rates in normal media of SCK, SCK.F11, and SCK.F2 cells and of K1735, K1735.N23, and K1735.N4 cells were indistinguishable. Addition of rmIFNγ inhibited the proliferation of SCK cells by 43%, determined by [^{3}H]thymidine uptake, but inhibited the proliferation of SCK.F2 and SCK.F11 cells by only 13% and 8%, respectively. A similar reduction in IFNγ-induced slowing of proliferation was seen in K1735.N4 and K1735.N23 cells compared to K1735 cells. When control transfected SCK.n and K1735.n cells expressing only the *neo*^{r} selection gene were compared to parental cells, no difference in IFNγ induction of MHC expression or slowing of cell proliferation was noted. These results show that IFNγ stimulation has little effect on SCK and K1735 cells expressing our mutant IFNγR1 and that these cells are IFNγ unresponsive to varying degrees.

IFNγ-Unresponsive Tumor Cells Are More Tumorigenic and Less Sensitive to IL-12 Therapy

To examine the tumorigenicity of IFNγ-unresponsive tumor cells, cohorts of naive A/J mice were injected at

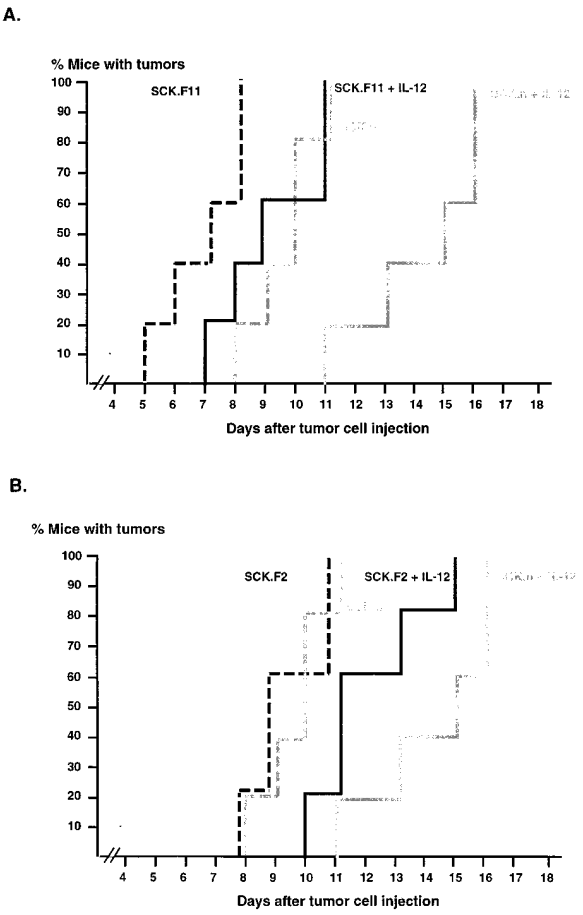

Figure 2. Tumorigenesis by IFNγ-Responsive and -Unresponsive SCK Cells and Effects of rmIL-12 Therapy

Cohorts of A/J mice were injected with 2.5×10^4 tumor cells on day 0, and rmIL-12 was administered on days 0–4 and 7–11 (0.25 μg/dose). Mice were monitored daily for tumor development. There were five mice in each group.

(A) Mice given control SCK.n or IFNγ-unresponsive SCK.F11 cells.

(B) Mice given control SCK.n or IFNγ-unresponsive SCK.F2 cells.

Black dashed lines, mice given IFNγ-unresponsive cells; black solid lines, mice given IFNγ-unresponsive cells + rmIL-12; gray dashed lines, mice given SCK.n cells; gray solid lines, mice given SCK.n cells + rmIL-12.

the same time with 2.5×10^4 viable SCK.F11, SCK.F2, or control SCK.n cells. SCK.F11 cells formed detectable tumors after a median of 7 days, while SCK.F2 and SCK.n cells formed tumors after a median of 10 days (Figure 2). Injection of 10^6 K1735.N23 (Figure 3A) and K1735.N4 (Figure 3B) cells into C3H/HeN mice produced detectable tumors after a median of 9 and 13 days, respectively, which was faster than the median of 16 days it took for K1735.n cells to form tumors. A mouse given K1735.n cells did not develop a tumor in this experiment, which is not unusual inasmuch as 10% of mice given 10^6 K1735 cells do not develop progressive tumors. These results show that IFNγ-unresponsive SCK and K1735 cells form tumors more rapidly than their IFNγ-responsive counterparts. Once they appear, however, IFNγ-responsive and unresponsive tumors enlarge at similar rates and kill the host (data not shown).

To examine the effect of tumor cell IFNγ unresponsiveness on IL-12 therapeutic efficacy, A/J mice inoculated with SCK.F11, SCK.F2, or SCK.n cells were given daily injections of rmIL-12 (0.25 μg/day × 10 over 12 days). rmIL-12 treatment delayed SCK.n tumor appearance by a median of 5 days but delayed SCK.F11 and SCK.F2 tumor appearance by a median of 2 days (Figure 2). rmIL-12 treatment of C3H/HeN mice (0.125 μg/day × 10 over 12 days) injected with K1735.n cells delayed tumorigenesis by a median of 7 days but delayed tumorigenesis by only 1 or 2 days in mice given K1735.N23 or K1735.N4 cells, respectively (Figure 3). These results indicate that IFNγ-unresponsive tumor cells are less responsive to rmIL-12 therapy. In the SCK model, cells engineered to express mIL-12 (SCK.12 cells) exert a strong local antitumor effect that inhibits tumorigenesis by colocalized SCK cells in 70% of mice (Coughlin et al., 1998). We examined whether SCK cells have to be IFNγ responsive to respond to the local antitumor effect of SCK.12 cells by injecting mice with an equal mixture of SCK.12 cells and either SCK.n or SCK.F11 cells. Whereas 3 of 10 mice given SCK.12/SCK.n cells developed tumors that appeared late, 4 of 5 mice given SCK.n/SCK.F11 cells developed tumors that were delayed by only a few days (Figure 4A). Systemically, SCK.12 cells provide poor protection against SCK tumorigenesis (only 10% of distant SCK tumors are prevented), which is substantially improved when SCK cells engineered to express mIL-18 (SCK.18 cells) are coinjected with the SCK.12 cells (70% of distant SCK tumors are prevented by SCK.12 + SCK.18 cells). This protection depends on IFNγ and is abrogated by administration of neutralizing anti-IFNγ antibody (Coughlin et al., 1998). We examined whether SCK cells have to be IFNγ-responsive to be controlled by SCK.12 + SCK.18 cells by injecting mice with this mixture of cells in one flank and with SCK.n or SCK.F11 cells in the opposite flank. Whereas 2 of 5 mice receiving SCK.n cells developed tumors that appeared very late, all 5 mice receiving SCK.F11 cells rapidly developed fatal tumors that were delayed by only 2 days (Figure 4B). These results indicate that tumor cells must respond to IFNγ to realize the benefits of secreted IL-12 or IL-12 + IL-18 therapy.

IFNγ-Unresponsive Tumor Cells Are Rejected by Immune Mice

A potential explanation for the refractoriness of IFNγ-unresponsive tumor cells to IL-12 therapy is that these cells are less susceptible to immunological attack. To explore this, we compared the ability of mice with antitumor immunity to reject IFNγ-responsive and -unresponsive tumor cells. A/J mice that had rejected a challenge of SCK.B7–1 cells and a subsequent rechallenge of SCK cells were injected a third time with either 1×10^5 SCK or SCK.F11 cells. Two of 8 mice given SCK cells developed tumors, whereas 1 of 8 mice given SCK.F11 cells developed tumors. A/J mice that had survived SCK.B7–1 cells with rmIL-12 treatment and that have a 90% probability of having protective anti-SCK immunity (Coughlin et al., 1995) were also tested. Two of 10 mice given SCK.F11 cells and 1 of 10 mice given SCK cells developed tumors. A similar experiment was performed in C3H/HeN mice

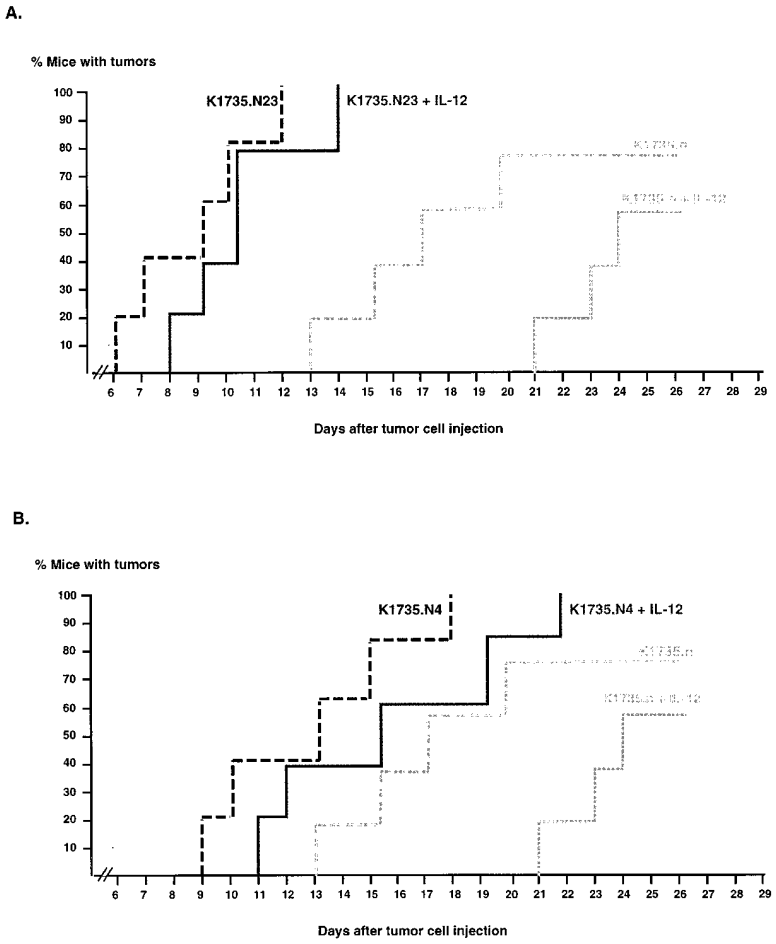

Figure 3. Tumorigenesis by IFNγ-Responsive and -Unresponsive K1735 Cells and Effects of rmIL-12 Therapy

Cohorts of C3H/HeN mice were injected with 10^6 tumor cells on day 0, and rmIL-12 was administered on days 0–4 and 7–11 (0.125 μg/dose). Mice were monitored daily for tumor development. There were five mice in each group.

(A) Mice given control K1735.n or IFNγ-unresponsive K1735.N23 cells.

(B) Mice given control K1735.n or IFNγ-unresponsive K1735.N4 cells.

Black dashed lines, mice given IFNγ-unresponsive cells; black solid lines, mice given IFNγ-unresponsive cells + rmIL-12; gray dashed lines, mice given K1735.n cells; gray solid lines, mice given K1735.n cells + rmIL-12.

previously given K1735.B7–1 cells and rmIL-12. These mice never developed tumors (Coughlin et al., 1995) and over 80% are immune to a rechallenge of K1735 cells at day 30 (Townsend et al., 1994; unpublished data). Rechallenge of these mice with either 10^6 K1735 or K1735.N23 cells resulted in 3 of 20 developing K1735 tumors and 2 of 10 developing K1735.N23 tumors. These results indicate that established antitumor immune responses do not require SCK or K1735 tumor cells to be IFNγ responsive for rejection.

Histology of IFNγ-Unresponsive Tumors

We examined the histology of SCK.n, SCK.F2, and SCK.F11 tumors to obtain clues for IFNγ actions on tumor cells that render them responsive to the antitumor effects of IL-12. Untreated SCK.n tumors had small, focal areas of coagulative necrosis 4 days after they became detectable (Figure 5A). Treatment of SCK.n tumors with rmIL-12, however, led to broad areas of coagulative necrosis (Figure 5B). In contrast, untreated SCK.F11 and SCK.F2 tumors had smaller areas of coagulative necrosis than SCK.n tumors (Figures 5C and 5E), which did not increase with rmIL-12 treatment (Figures 5D and 5F). Coagulative necrosis in rmIL-12-treated SCK.n tumors (Figure 6A; higher magnification showing hemorrhage and ghost outlines of tumor cells) resembles the pattern of necrosis seen in myocardial infarction and in other organs undergoing ischemic infarction and suggests that ischemia is the major mechanism of cell death induced by rmIL-12 treatment in SCK.n tumors. SCK.n, SCK.F11, and SCK.F2 tumors also had evidence of individual tumor cell necrosis with nuclear fragmentation consistent with apoptosis (Figure 6B), suggesting that mechanisms other than ischemia may also contribute to tumor cell death. However, the frequency of these apoptotic tumor cells did not vary signficantly among the different tumors, did not correlate with rmIL-12 treatment, and could not explain the resistance of IFNγ-unresponsive tumors to rmIL-12 treatment.

Angiogenesis Induced by IFNγ-Responsive and -Unresponsive SCK Cells

The histology of the SCK tumors led us to assess their ability to stimulate angiogenesis with in vivo Matrigel assays. Using hemoglobin content as an index of functional Matrigel vascularization (Passaniti et al., 1992), 10^5 SCK cells in the matrix induced vascularization equivalent to 10 ng recombinant basic fibroblast growth factor (rbFGF), and angiogenesis was inhibited by rmIL-12 treatment of the host. IFNγ neutralization abrogated rmIL-12 antiangiogenic activity showing that this activity was mediated by IFNγ (Figure 7A). To examine whether tumor cell responses to IFNγ are important for rmIL-12 inhibition of angiogenesis, Matrigel containing SCK,

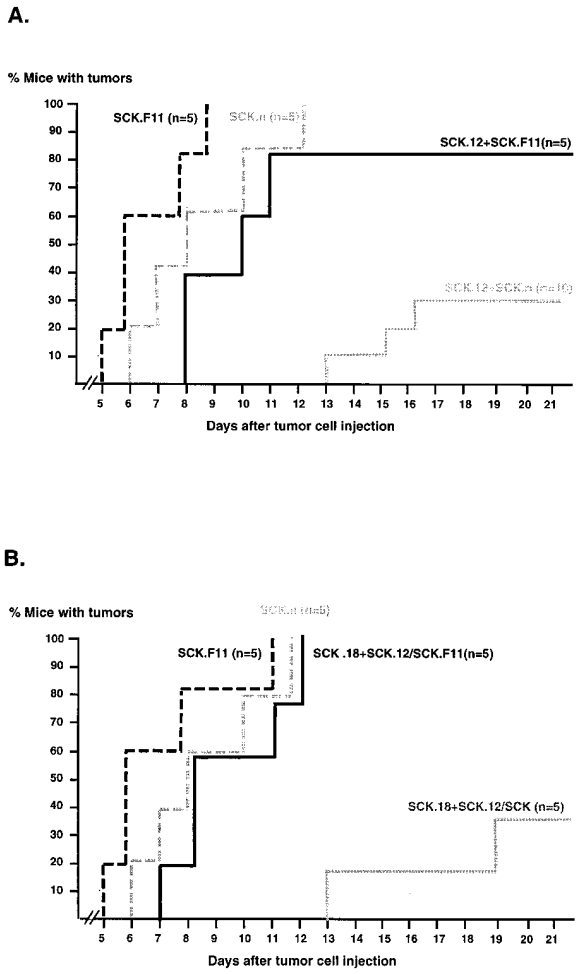

Figure 4. Effect of Secreted IL-12 and IL-18 on Tumorigenesis by IFNγ-Responsive and -Unresponsive SCK Cells

(A) Cohorts of A/J mice were injected with 2.5×10^4 SCK.n (gray dashed line) or SCK.F11 cells (black dashed line) or a mixture of SCK.n + SCK.12 (2.5×10^4 cells of each type; gray solid line) or SCK.F11 + SCK.12 cells (2.5×10^4 cells of each type; black solid line) on day 0.

(B) Cohorts of A/J mice were injected with 2.5×10^4 SCK.n (gray dashed lines) or SCK.F11 (black dashed lines) cells in the left flank. Some mice were also injected with a mixture of 2.5×10^4 SCK.12 + 2.5×10^4 SCK.18 cells in the right flank (solid lines). Mice were monitored daily for tumor development.

SCK.F11, or SCK.F2 cells was implanted. The three cell types induced equivalent vascularization (Figure 7B), but while rmIL-12 inhibited vascularization induced by SCK cells, it did not inhibit vascularization induced by SCK.F11 or SCK.F2 cells. Thus, SCK responsiveness to IFNγ does not significantly affect basal angiogenesis but is important for angiogenesis inhibition by rmIL-12. To determine whether the IFNγ-induced response in SCK cells involves reduced production of angiogenic factors or increased production of angiogenesis inhibitors, a mix of 10^5 SCK and 10^5 SCK.F11 cells was placed in Matrigel, and the recipient mice treated with rmIL-12. Angiogenesis induced by the cell mixture was effectively inhibited by rmIL-12 (Figure 7B), suggesting that SCK but not SCK.F11 cells produce angiogenesis inhibitory factors following IFNγ stimulation. IP-10, an IFNγ-inducible chemokine, has been identified as a mediator of IL-12 antiangiogenic effect (Angiolillo et al., 1995). In vitro treatment of SCK cells with rmIFNγ at doses as low as 100 U/ml induced signficant IP-10 mRNA expression, whereas treatment of SCK.F2 cells at 1000 and 10,000 U/ml induced much less IP-10 mRNA expression, and treatment of SCK.F11 cells failed to induce detectable expression (Figure 7C). Expression of another IFNγ-induced chemokine with antiangiogenic activity, Mig (monokine induced by IFNγ) (Sgadari et al., 1997), was barely detectable in mRNA from rmIFNγ-treated SCK cells (data not shown). These results suggested that IFNγ-induced IP-10 production by SCK cells contributes to rmIL-12 inhibition of SCK-induced angiogenesis, an effect that is lost in SCK.F11 cells. The importance of IP-10 as a mediator of angiogenesis inhibition was confirmed when administration of rabbit anti-mIP-10 serum, but not normal rabbit serum, abrogated rmIL-12 inhibition of SCK-induced angiogenesis (Figure 7D).

Discussion

To examine the role of tumor cell responses to IFNγ in IL-12 antitumor responses, IFNγ-unresponsive SCK and K1735 tumor cells were created by expressing high levels of a dominant negative mutant IFNγR1. These unresponsive cells formed tumors more rapidly than control cells and were relatively refractory to IL-12 therapy. To investigate the effects of IFNγ on tumor cells that contribute to IL-12 antitumor efficacy, responsive and unresponsive cells were tested in immune mice and found to be equivalently rejected, indicating that the unresponsive cells were susceptible to immunological rejection. On the other hand, histology showed that tumors formed by unresponsive SCK cells displayed little evidence of rmIL-12-induced ischemic necrosis seen in tumors formed by responsive SCK cells, and Matrigel assays indicated that rmIL-12 inhibited angiogenesis induced by the responsive but not by the unresponsive cells. Mixing experiments showed that SCK responses to IFNγ include the production of angiogenesis inhibitory activity, which may be attributable to IFNγ induction of IP-10 expression. From this we conclude that direct actions of IFNγ on tumor cells curb their tumorigenicity and that restraint may be due, at least in part, to the inhibitory effects of tumor cell IFNγ responses on angiogenesis.

An earlier study by Dighe et al. (1994) of Meth A tumor cells rendered unresponsive to IFNγ also found that these cells were more tumorigenic, but the authors came to a different conclusion about mechanism. Immunological studies indicated that these cells were less immunogenic and less susceptible to immune rejection than control cells, and thus enhanced tumorigenicity was attributed to an immunological mechanism. It is unclear why IFNγ-unresponsiveness affects immune recognition of Meth A versus SCK and K1735 cells differently, but it may be due to tumor cell or model differences, e.g., greater IFNγ stimulation of Meth A's ability to present antigen. However, whether reduced immune visibility of unresponsive Meth A cells causes accelerated tumorigenesis is a separate question. The answer depends on

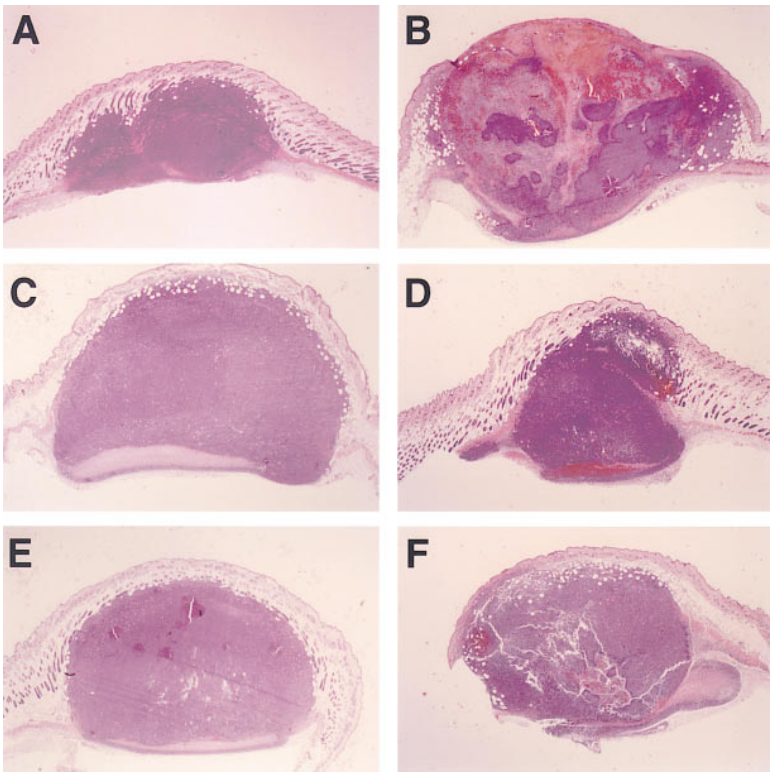

Figure 5. Histology of Tumors Produced by IFNγ-Responsive and -Unresponsive SCK Cells

SCK.n, SCK.F11, and SCK.F2 tumors were harvested from mice treated or untreated with rmIL-12 on the fourth day after the tumors appeared, fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. All sections are shown at 10× magnification. (A and B) SCK.n tumors; (C and D) SCK.F11 tumors; (E and F) SCK.F2 tumors. (A), (C), and (E) are from untreated mice, and (B), (D), and (F) are from rmIL-12-treated mice.

whether immune responses are elicited during tumorigenesis in naive mice and, if so, whether the response retards tumor growth. Our finding that IFNγ-responsive and -unresponsive SCK and K1735 cells are equally well rejected by immune mice and the poor intrinsic immunogenicity of SCK cells suggest that any immunological consequences of IFNγ unresponsiveness are unlikely to account for faster tumorigenesis by unresponsive SCK and K1735 cells.

IFNγ-unresponsive tumor cells characteristically form tumors faster than control cells in untreated mice—a slight acceleration was seen with rapidly tumorigenic SCK cells and marked acceleration was seen with more slowly tumorigenic K1735 cells. Since the behavior of IFNγ-responsive and -unresponsive cells should be indistinguishable in the absence of IFNγ, and no in vitro differences were observed without the addition of rmIFNγ, this suggests that IFNγ is present in the in vivo tumor cell environment and modifying the behavior of the responsive tumor cells. IFNγ is presumably produced by reactive host cells at low levels compared to the large amount produced in response to rmIL-12 therapy, which probably explains why therapy widens the interval between the appearance of IFNγ-responsive and -unresponsive tumors.

Mice given rmIL-12 produce high levels of IFNγ, and IFNγ effects should be more prominent. Therapy produces a marked slowing in the development of control SCK and K1735 tumors that is not seen with IFNγ-unresponsive SCK and K1735 tumors. This, together with the observation that IFNγ neutralization abrogates rmIL-12 delay of tumorigenesis (Coughlin et al., 1995), allows one to conclude that IFNγ delays tumor development and that its effects on tumor cells are at least partly responsible. Two well-known effects of IFNγ on cells, slowing of proliferation and enhancement of immunological visibility, might explain the delay. We suspect, however, that while IFNγ slowing of tumor cell proliferation may contribute to delayed tumorigenesis, it does not account for the delay. First, if cell proliferation rates

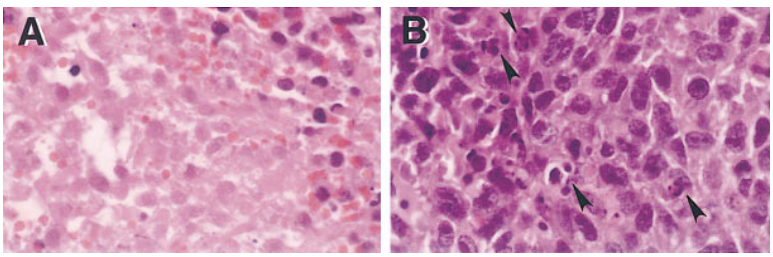

Figure 6. Effect of rmIL-12 Treatment on the Histology of SCK Tumors

Four day SCK.n tumors from mice treated with rmIL-12 were harvested, fixed in 10% buffered formalin, sectioned, and stained with hemotoxylin and eosin.

(A) A 500× magnification view showing an area of coagulative necrosis with hemorrhage and residual ghost outlines of necrotic tumor cells.

(B) A 500× view showing nuclear fragments of individual SCK tumor cells undergoing apoptotic cell death (indicated by arrowheads) scattered in areas of live tumor cells.

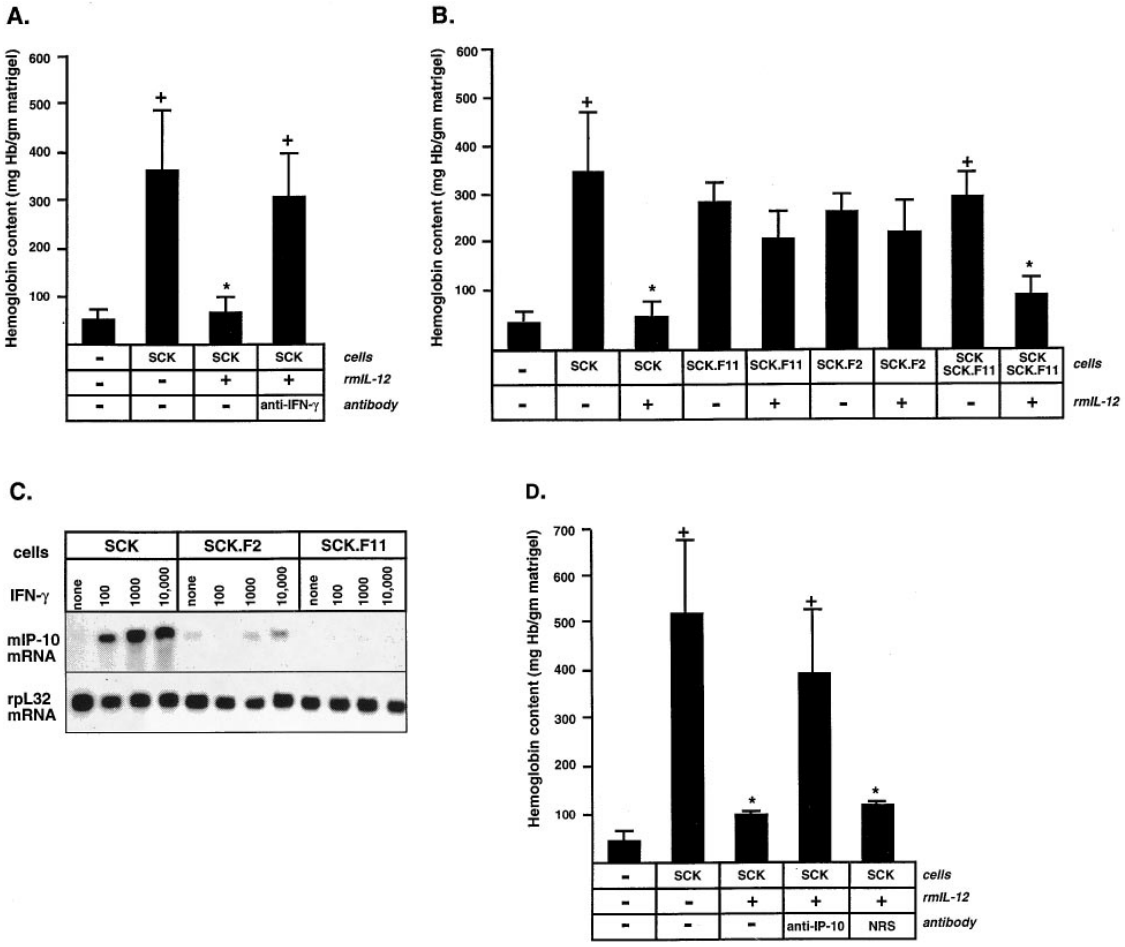

Figure 7. Angiogenesis Induced by IFNγ-Responsive and -Unresponsive SCK Cells and the Effect of rmIL-12 Therapy

(A) Mice were injected subcutaneously with Matrigel alone or Matrigel containing 10^5 SCK cells. rmIL-12 was administered i.p. where indicated (0.25 μg/dose) on days 0–4, and anti-IFNγ was administered i.p. where indicated (0.5 mg/dose) on days −1, 1, and 3. Matrigel was harvested on day 6, and angiogenesis was quantitated by determining the hemoglobin content of individual pellets.

(B) Matrigel assays were performed as described except that pellets contained 10^5 SCK, SCK.F11, or SCK.F2 cells or a mixture of 10^5 SCK.F11 + 10^5 SCK cells.

(C) Expression of mIP-10 mRNA determined by Northern analysis in SCK, SCK.F2, or SCK.F11 cells either unstimulated or stimulated with various concentrations of rmIFNγ in vitro for 48 hr. The blot was probed with either radiolabeled mIP-10 cDNA (upper panel) or ribosomal protein L32 cDNA (lower panel).

(D) Matrigel assays were performed as described. Where indicated, rabbit anti-IP-10 serum or normal rabbit serum (NRS) (0.1 ml) was mixed with 10^5 SCK cells in the Matrigel prior to implantation, and serum was given i.p. (0.3 ml/dose) where indicated on days 0, 2, and 4.

In (A), (B), and (D), each bar represents the mean hemoglobin content of three Matrigel implants with standard deviations indicated. Plus signs and asterisks indicate groups with significantly different hemoglobin content ($p < .05$) by Student t test.

determine tumor growth rates, the growth of IFNγ-unresponsive tumors during IL-12 therapy should be faster throughout their course, whereas only the early phase of tumorigenesis (tumors <1–2 mm in diameter) was obviously accelerated. Additionally, we generated a clone of IFNγ-unresponsive SCK cells that proliferates noticeably more slowly in vitro than SCK or SCK.F11 cells, yet forms detectable tumors with the rapidity of SCK.F11 cells (unpublished data), indicating that the in vitro rate of tumor cell proliferation, in or out of the presence of IFNγ, does not predict or limit the speed of tumor formation. An immunological explanation for the faster appearance of IFNγ-unresponsive Meth A tumors was proposed based on evidence of their reduced immunogenicity and enhanced ability to elude immunological attack (Dighe et al., 1994). However, no such evidence was found in the case of unresponsive SCK and K1735 tumors. Furthermore, such a mechanism would only be reasonable in our models if antigen-specific immune responses retard the appearance of the parental tumor cells. SCK cells, especially, are poorly immunogenic (Coughlin et al., 1995), which makes the induction of antigen-specific immunity improbable. Even if antigen-specific immunity were induced, it would take time to develop and be less likely to have its greatest effect very early in tumorigenesis rather than later on, which

is the opposite of what is observed. Most compelling, however, is the fact that administration of rmIL-12 at the doses and on the schedule used here induces transient but profound suppression of immunological rejection mechanisms for SCK and K1735 tumors (Kurzawa et al., 1998). For these reasons, we believe that IFNγ retards the appearance of SCK and K1735 tumors through mechanisms not involving antigen-specific immunity.

Tumor histology provided a critical clue to mechanism when it revealed notably more coagulative necrosis in IFNγ-responsive than in IFNγ-unresponsive tumors treated with rmIL-12. This type of injury often results from ischemic damage and suggests that responsive tumors may experience vascular insufficiency that the unresponsive tumors do not. Given that rmIL-12 inhibition of angiogenesis is dependent on IFNγ, these observations suggested a difference in susceptibility of IFNγ-responsive and -unresponsive tumors to IFNγ's antiangiogenic effects, which was experimentally confirmed when rmIL-12 was found to inhibit angiogenesis stimulated by SCK cells but not by SCK.F11 or SCK.F2 cells. This difference reasonably accounts for the observed difference in tumorigenic behavior between IFNγ-responsive and -unresponsive cells, because angiogenesis is needed for tumor growth beyond small nests and is often growth limiting. We hypothesize that the limited amount of IFNγ around the tumor cell inoculum in untreated mice retards angiogenesis modestly while the greater amount of IFNγ induced by rmIL-12 treatment retards angiogenesis markedly, producing significant delays in tumor development. When the cells are IFNγ unresponsive, neither spontaneously produced nor rmIL-12-stimulated IFNγ retards angiogenesis, and tumor growth in rmIL-12-treated and -untreated mice continues unabated. That rmIL-12 inhibits angiogenesis stimulated by a mix of responsive and unresponsive SCK cells but not by unresponsive cells alone suggests that refractoriness to rmIL-12 inhibition is due to failure of IFNγ-unresponsive SCK cells to produce angiogenesis inhibitors in response to IFNγ. We believe the chemokine IP-10 may be the SCK cell–produced mediator of rmIL-12/IFNγ angiogenesis inhibition. This is based on its known antiangiogenic properties (Angiolillo et al., 1995; Sgadari et al., 1996); the fact that it is induced markedly in SCK cells, weakly in SCK.F2 cells, and not at all in SCK.F11 cells; and the fact that its neutralization by antibody abrogates rmIL-12 inhibition of SCK-induced angiogenesisis. However, a role for other IFNγ effects on SCK cells during rmIL-12 angiogenesis inhibition (e.g., potential production of other angiogenesis inhibitors or reduced synthesis of angiogenesis stimulants) cannot be excluded without additional studies.

The model we are proposing implies that IL-12 is primarily effective against certain murine tumors due to inhibition of tumor angiogenesis and that tumor cell responses to treatment are important for this inhibition. It does not imply that other antitumor mechanisms are not involved. IFNγ has direct effects on tumor cells that may restrain tumorigenesis, e.g., enhanced susceptibility to killing or apoptosis and slowed proliferation, which may limit tumor growth when blood supply is abundant. IL-12 and IFNγ may effectively stimulate host innate immune mechanisms involving macrophage and NK cells and deviate and stimulate acquired immune responses (after a period of transient immune suppression by high-dose rmIL-12 [Kurzawa et al., 1998]). Additionally, while the model proposed can account for most of our observations, it does not explain all features of rmIL-12 angiogenesis inhibition. We and others (Voest et al., 1995) have shown that rmIL-12 will inhibit Matrigel vascularization induced by defined angiogenic factors such as bFGF. This inhibition is dependent on endogenously produced IFNγ and must be due to the effects of IFNγ (or its downstream mediators) on host cells. If IFNγ's effects on host cells can prevent Matrigel vascularization stimulated by bFGF, why are these effects insufficient to prevent vascularization stimulated by SCK.F2 or SCK.F11 cells? We do not have answers at this time, but we can speculate without proof that the angiogenic factors produced by SCK cells are different or more complex than bFGF and that the angiogenic mechanisms they stimulate are inadequately inhibited by the actions of IFNγ acting on host cells. Additional studies will be required to address this issue and others about the angiogenesis inhibitor(s) and inducers produced by the tumor cells.

Experimental Procedures

Mice and Cell Lines

Female A/J mice, 6–8 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME). Female C3H/HeN mice of a similar age were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were maintained in microisolator cages and handled under aseptic conditions. The SCK mammary carcinoma cell line (Song et al., 1994) is maintained in RPMI medium supplemented with 10% FCS and penicillin/streptomicin. The K1735 melanoma cell line (Kripke, 1979) is maintained in DMEM supplemented with 10% FCS and penicillin/streptomicin. In vitro culture with rmIFNγ was done in the respective media and for the times and doses indicated in the figure legends.

For MHC induction studies, SCK and K1735 cells were stimulated with 10,000 U/ml rmIFNγ for 48 hr prior to staining for flow cytometry. For proliferation studies, cells (10^4/well) in 96-well plates were treated or untreated with 10,000 U/ml rmIFNγ for 24 hr after which they were pulsed with 1 μCi [^{3}H]thymidine/well for 16 hr. Cells were harvested with a Cambridge Technology PHD Harvester (Cambridge, MA), and incorporated radioactivity was measured in triplicate cultures by scintillation counting. Percent suppression of proliferation by IFNγ $= [1 - (\text{cpm}^{+\text{IFN}\gamma}/\text{cpm}^{-\text{IFN}\gamma})] \times 100\%$.

Vectors and Cell Transfections

The plasmid pEF2.muγR is an expression plasmid that contains a truncated murine IFNγR1 cDNA under control of the eukaryotic translation elongation factor 1α promoter (Goldman et al., 1996). The truncated receptor cDNA clone was created by amplifying the mIFNγR1 cDNA from nucleotides 106 to 949 (Gray et al., 1989). This expression contruct codes for the IFNγR1 signal sequence, extracellular and transmembrane domains, and only four amino acids of the intracellular domain (Gray et al., 1989). The truncated IFNγR1 cDNA was cloned into the BamHI and EcoRI sites of the pEF2 expression vector, which contains a *neo*^{r} gene. To create SCK and K1735 cells expressing this mutant IFNγR1, parental cell lines were transfected with pEF2.muγR by the calcium phosphate method. Individual G418-resistant clones were screened by flow cytometry (described below) to identify those with the highest surface expression of the IFNγR complex. Control SCK.n cells are a pool of cells created by transfecting SCK cells with the empty pEF2 vector and selecting with G418; K1735.n cells are a pool created by transfecting K1735 cells with another *neo*^{r} empty vector, pMV6, and selecting with G418. SCK cells expressing IL-12 or IL-18 were

created previously (Coughlin et al., 1998). Briefly, SCK.12 cells were created by transfecting SCK cells with an expression plasmid, pWRG, containing both the p35 nd p40 cDNAs under control of the CMV promoter (Tan et al., 1996). Cells were plated by limiting dilution and screened by p70 radioimmunoassay. To create SCK.18 cells, the retroviral expression construct pL(IL-18)SN was transfected into ψcre cells to create L(IL-18)SN retrovirus (Miller and Rosman, 1989), which was used to infect SCK cells. Individual G418-resistant clones were screened by Northern analysis and IL-18 ELISA.

Flow Cytometry

To determine surface expression of murine IFNγR complexes, SCK or K1735 cells were stained with primary monoclonal rat anti-IFNγR1 antibody (clone GR.20, PharMingen, Inc.) or rat isotype (IgG2a) and secondary goat anti-rat IgG Fluorescein (FITC) conjugated antibody [$F(ab')_2$ fragment specific, Jackson Immunoresearch Laboratories, Inc.]. To determine surface expression of murine MHC class I molecules, SCK cells were stained with primary monoclonal mouse anti-H-$2D^d$ antibody (clone 34–5–8S, PharMingen, Inc.), and K1735 cells were stained with monoclonal mouse anti-H-$2K^k$ antibody (clone 36–7–5, PharMingen, Inc.) and secondary goat anti-rat IgG fluorescein (FITC)-conjugated antibody [$F(ab')_2$ fragment specific, Jackson Immunoresearch Laboratories, Inc.]. Fluorescence was determined on a FACScan flow cytometer using CELLQuest software (Becton Dickinson). Mean fluorescence intensity (MFI) was determined by dividing the geometric means of the specific antibody by that of the isotype antibody (MFI = geometric mean (anti-IFNγR)/geometric mean (isotype)). Induction = $MFI^{+IFN\gamma}/MFI^{-IFN\gamma}$.

Tumorigenicity Studies

Tumorigenesis studies using SCK cells were carried out by injecting 2.5×10^4 trypan blue–excluding cells (typically, 80%–90% of all cells) subcutaneously in the right flank of each A/J mouse. Tumorigenesis studies using K1735 cells were carried out similarly except that 1×10^6 trypan blue–excluding cells were injected into each C3H/HeN mouse. The cells injected were obtained from cultures established from low-passage, frozen stocks less than 1 week prior to injection. rmIL-12 was administered on a 10 dose/12 day schedule (days 0–4 and 7–11 with tumor cells injected on day 0). The dose of rmIL-12 was 0.25 μg/day for A/J mice and 0.125 μg/day for C3H/HeN mice, their respective maximum tolerated dose. Mice were monitored daily for development and growth of tumors and were euthanized according to guidelines established by the Institutional Animal Care and Use Committee.

Matrigel Assay for Angiogenesis

Angiogenesis assays were carried out by injecting A/J mice subcutaneously with 0.5 ml Matrigel (Collaborative Biomedical Products; Kleinman et al., 1982) mixed with 1×10^5 SCK, SCK.F11, or SCK.F2 cells in the abdominal midline (day 0 in all experiments). Where indicated, rmIL-12 (0.25 μg) was injected i.p. on days 0, 1, 2, 3, and 4. Where indicated, 0.5 mg anti-IFNγ monoclonal antibody (XMG.6) was injected i.p. on days −1, 1, and 3. Where indicated, rabbit anti-IP-10 serum or normal rabbit serum (0.1 ml) was mixed with the cells and Matrigel prior to implantation and (0.3 ml/dose) injected i.p. on days 0, 2, and 4. Antiserum to mIP-10 was obtained by vaccinating rabbits with purified mIP-10 produced by recombinant baculovirus bearing the Crg-2 (mIP-10) cDNA (Vanguri and Farber, 1990) in *Trichoplusia ni* cells using procedures that will be described elsewhere. The recombinant mIP-10 protein was functional and the rabbit antibody raised neutralized mIP-10 in functional assays (data not shown). To quantitate angiogenesis, Matrigel pellets were harvested on day 6, all surrounding tissue was dissected away, 0.3 ml PBS was added, and the gel liquified by incubation at 4°C overnight. Hemoglobin content of the liquefied pellets was determined by the Drabkin method (reagents from Sigma Diagnostics) as described (Passaniti et al., 1992).

Northern Blotting

Total cytoplasmic RNA was isolated as previously described (Yeilding et al., 1996). mRNA levels were determined by Northern analysis using the glyoxal method (McMaster and Carmichael, 1977). RNA was electroblotted onto Hybond N (Amersham) and UV cross-linked. Hybridizations were carried out by modifications of the methods of Church and Gilbert (1984) using probes labeled by random priming. mIP-10 was amplified from splenic RNA from a C57BL6 mouse treated with BCG and LPS using published primers (Luster and Leder, 1993).

Purification of Nuclear Extracts

Cells grown in a monolayer were treated (or untreated) with rmIFNγ for 15 min and were lysed in hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and centrifuged at 3500 rpm for 5 min to isolate nuclei. Nuclei were lysed in 0.5× volume low-salt buffer (20 mM HEPES [pH 7.9], 1.55 mM $MgCl_2$, 20 mM KCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT) and an equal volume of high-salt buffer (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 1.2 M KCl, 25% glycerol, 0.2 nM EDTA, 0.5 mM DTT). Protein from lysed nuclei was harvested by rotation at 4°C for 30 min and centrifugation at 15,000 rpm for 30 min. Protein extracts were quantitated using the MicroBCA protein assay kit (Pierce).

Gel Electrophoretic Mobility Shift Assay (EMSA)

End-labeled duplexed oligonucleotide GAS probes (Decker et al., 1997) (50,000 cpm/sample) were mixed with 12 μg crude nuclear extracts and incubated at room temperature for 20–30 min in the presence of 1 μg poly(dI-dC) in a volume of 10 μl containing 1 μl 10× binding buffer (100 mM HEPES [pH 7.9], 80 mM KCl, 50% glycerol, 0.25 dithiothreitol, 0.25 mM phenylmethylsufonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin). The mix was then fractionated through a 4% acrylamide gel in buffer containing 100 mM Tris base, (pH 7.5), 0.3 M sodium acetate, 50 mM EDTA for 1 hr at 200 V. The gel was dried and exposed to a PhosphorImager storage screen (Molecular Dynamics), scanned, and quantitated. Antibody competition experiments were carried out by preincubating the nuclear extract with monoclonal anti-Stat1 (anti-Stat91, clone C-111, Santa Cruz) or anti-Stat4 (clone L-18, Santa Cruz) antibody for 30 min at 4°C before the probe was added. Probe competition experiments were carried out using either unlabeled GAS probe (5′ AGC-TTG-TAT-TTC-CCA-GAA-AAG-GGA-TC) or a labeled probe that contains a mutated GAS-binding site (5′ AGC-TTG-TAT-GGA-TTG-TCC-AAG-GGA-TC) (Decker et al., 1997). Sp1 binding was determined by the same binding reactions using an Sp1 oligonucleotide probe. Specific Stat1α binding for a specific sample was determined by (binding activity of Stat1α)/(binding activity of Sp1). Activation of wild-type cells was normalized to 100% binding. IFNγ activation of Stat1α binding activity in wild-type and IFNγ-unresponsive clones was determined by the following equation: Stat1α activity (+ IFNγ) = [(Stat1$\alpha_{IFN\gamma}$ activity)/(Sp1$_{IFN\gamma}$ activity)]/[(Stat1$\alpha_{no\ IFN\gamma}$ activity)/(Sp1$_{no\ IFN\gamma}$ activity)].

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