Hypoxia-mediated Apoptosis from Angiogenesis Inhibition Underlies Tumor Control by Recombinant Interleukin 12


ABSTRACT

The role of angiogenesis inhibition in the antitumor activity of recombinant murine interleukin 12 (rmIL-12) was studied in K1735 murine melanomas, the growth of which is rapidly and markedly suppressed by rmIL-12 treatment. On the basis of the prediction that tumor ischemia should result from therapeutic angiogenesis inhibition, tumor cell hypoxia was evaluated as a marker of ischemia using the EFS [2-(2-nitro-1H-imidazol-1-y)-N-(2,3,3,3-pentafluoropropyl)acetamide] approach. This method measures intracellular binding of the nitroimidazole EFS, which covalently binds to cellular macromolecules selectively under hypoxic conditions. Whereas 1 week of rmIL-12 treatment effectively inhibited K1735 cell-induced angiogenesis in Matrigel neovascularization assays, 2 weeks of treatment were needed before severe tumor cell hypoxia was detected in K1735 tumors. The hypoxia that developed was regional and localized to tumor areas distant from blood vessels. The great majority of severely hypoxic tumor cells were apoptotic, and in vitro studies indicated that the degree of hypoxia present within treated tumors was sufficient to trigger K1735 apoptosis. Tumor cell apoptosis was also prevalent in the first week of rmIL-12 treatment when few cells were hypoxic. In vitro studies indicated that this non-hypoxia-related apoptosis was induced directly by IFN-γ produced in response to rmIL-12 administration. These studies reveal that rmIL-12 controls K1735 tumors initially by IFN-γ-induced apoptosis and later by hypoxia-induced apoptosis. They also establish hypoxia as an expected result of tumor angiogenesis inhibition and a mediator of its therapeutic effect.

INTRODUCTION

IL-12 has profound antitumor effects in a number of murine tumor models whether it is administered as the recombinant cytokine or secreted by engineered cells (Refs. 1–5; reviewed in Ref. 6). These effects are generally dependent upon T and NK cell production of IFN-γ in response to IL-12 stimulation. Because IL-12 and IFN-γ together induce Th1 differentiation of CD4+ T cells, enhance CD8+ T cell maturation and activation, and functionally activate NK cells (7), the antitumor activity of IL-12 has been attributed to the induction of antitumor immunity. Whereas this may be important for long-term tumor control by IL-12, its role in acute control of tumor growth is less certain. Recent studies demonstrated that therapeutically effective regimens of rmIL-12 impaired T cell-mediated immune responses during and beyond the period of cytokine administration, even as tumor growth was controlled (8, 9). Impairment was attributable to suppression of T-cell mitogenic responses by macrophage-derived nitric oxide produced in response to IFN-γ. Interestingly, the immunosuppression seen in rmIL-12-treated mice waned following cessation of therapy and was followed by enhanced antitumor immunity when this was tested at later time points.

Tumor control and regression during rmIL-12 administration may be better explained by its ability to inhibit angiogenesis. This activity has been demonstrated in several model assays of neovascularization to involve IFN-γ and IFN-γ-dependent production of antiangiogenic chemokines such as IP-10 (10–13). Production of these cytokines in turn leads to inhibition of angiogenesis by down-regulation of several proangiogenic factors, including tumor cell vascular endothelial growth factor production, matrix metalloproteinase activity (14), and expression of integrins involved in endothelial cell adhesion and survival (15). However, whereas rmIL-12 is known to inhibit angiogenesis in model assays, the issues of whether it inhibits angiogenesis within established tumors and what the consequences of tumor angiogenesis inhibition might be remain unaddressed. In this study, we assess the contribution of tumor angiogenesis inhibition to overall IL-12 antitumor efficacy.

How angiogenesis inhibition by IL-12 controls tumor growth is important to our general understanding of the effects of angiogenesis therapy because the mechanism by which angiogenesis inhibitors cause tumor regression is not currently known. The maintenance of tumor dormancy by angiogenesis inhibitor therapy has been previously shown to be associated with an increase in tumor cell apoptosis (16, 17). Whereas no formal mechanism for this increased apoptosis has been proposed, it has been speculated that angiogenesis inhibition could restrict the supply of endothelial cell-derived paracrine factors required for tumor cell survival (16). Because effective inhibition of tumor angiogenesis by definition should result in tumor ischemia, we reasoned that the signal for tumor cell apoptosis could be a downstream consequence of severe ischemia. A reasonable candidate is hypoxia, which has been shown to be a potent inducer of apoptosis by a mechanism possibly involving HIF1α-mediated stabilization of p53 (18) and inhibition of Bcl-2 (19), and hypoxia-induced tumor cell apoptosis may explain how angiogenesis inhibitors control tumor growth.

In this study, we examine K1735 murine melanomas undergoing rmIL-12 therapy to determine whether tumor hypoxia is induced by treatment and whether this plays a role in controlling tumor growth. Our results demonstrate that treatment results in severe tumor cell hypoxia, the appearance of which is delayed relative to the onset of angiogenesis inhibition. The development of hypoxia is selective, occurring only in tumor areas relatively removed from blood vessels, which reinforces its relationship with angiogenesis inhibition. The severe hypoxia that develops with therapy appears to mediate rmIL-12 antitumor activity by inducing widespread tumor cell apoptosis. Our results also reveal that, prior to the appearance of severe tumor hypoxia, rmIL-12 control of K1735 tumor growth is hypoxia inde-
pended and attributable to direct induction of tumor cell apoptosis by IFN-γ. Thus, rmIL-12 is a complex antitumor agent that uses a combination of mechanisms to suppress tumor growth, and angiogenesis inhibitor therapy, in general, may control tumor growth largely through induction of tumor cell hypoxia and hypoxic cell death.

**MATERIALS AND METHODS**

**Mice and Cell Lines.** Female C3H/HeN mice, 6–8 weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, IN). All animals were maintained in microisolator cages under sterile conditions. The K1735 murine melanoma cell line (20) was maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin. Stably transfected N23 K1735 cells containing a dominant negative IFN-γR1 were described previously (12).

**In Vivo Studies.** For tumor growth studies, 10⁴ trypan blue-excluding K1735 cells were injected s.c. into the lower left flank. Injected cells were derived from low-passage frozen stocks that had been established in culture <1 week prior to injection. rmIL-12 was administered i.p. on a five doses per week schedule (five daily injections of 125 ng, followed by 2 days of rest) for the durations indicated. Tumors were measured by calipers at regular intervals, and mice were euthanized according to guidelines established by the Institutional Animal Care and Use Committee. Serum IFN-γ levels were assayed by RIA using the polyclonal antibody AN18 as the capture antibody and the monoclonal antibody XM6G as the detection antibody. For EFS [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3-pentafluoropropyl)acetamide] studies, the mice were given an i.v. injection of 0.25 ml of 10² mM rmIL-12 in 0.9% saline 3 h prior to tumor excision. On the basis of the half-life of EFS in circulation, this assures that virtually all free EFS is cleared by the time tumors are excised and that no EFS binding is due to hypoxia resulting from interruption of circulation associated with euthanasia or tumor excision.

**Matrigel Assay for Angiogenesis.** In vivo Matrigel neovascularization assays were performed essentially as described previously (12). Briefly, C3H/HeN mice were injected s.c. along the ventral midline with 0.5 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA) mixed with 10² g of anti-IFN-γ monoclonal antibody i.p. on days –1, 1, and 3. Matrigel pellets were harvested on day 6 and reliquefied upon incubation at 4°C overnight in 300 μl of PBS. Matrigel neovascularization was quantitatively determined by measuring the hemoglobin content of the liquefied pellets (Drabkin’s method; Sigma Chemical Co., St. Louis, MO).

**Flow Cytometry.** Tumor cell suspensions were prepared and stained for EFS with the monoclonal antibody ELK 3-51 as described above (21). For single-color analysis, ELK 3-51 was conjugated to the fluorochrome Cy3, whereas for two-color analysis, it was conjugated to cyanine-5. Apoptosis was measured by TUNEL staining method according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). For dual EFS/TUNEL staining, cells were first TUNEL stained and then washed twice with PBS prior to overnight staining with ELK 3-51. Analysis of tumor cell proliferation was performed by measuring Cy3 fluorescence, whereas the FACS Calibur was equipped with blue (488 nm) and red (635 nm) lasers to excite fluorescein and cyanine-5 fluorescence, respectively. Standard collection optics were used to collect emitted fluorescence. Photomultiplier tube voltages were adjusted daily by placing standard beads in the same channel. Standardization of EFS fluorescence intensity was achieved using calibration beads (Flow Cytometry Standards Corporation, Research Triangle Park, NC). Isolation of tumor cells in cell suspensions was achieved by establishing gate parameters around in vitro cultured K1735 cells, which exhibit detectably higher forward and side scattering than erythrocytes and lymphocytes. Flow cytometric analysis of hypoxia and apoptosis was performed using CellQuest (Becton Dickinson, Mountain View, CA), whereas analysis of proliferation was performed using ModFit Version 2.0 (Verity Software, Topsham, ME).

**RESULTS**

**K1735 Tumor Growth and Tumor Cell-induced Angiogenesis Are Suppressed by IL-12 Therapy in an IFN-γ-dependent Manner.** K1735 melanoma tumors were established in syngeneic C3H/HeN mice by s.c. inoculation of 1 × 10⁶ K1735 cells. In treated mice, rmIL-12 (125 ng) was administered i.p. daily on days 0–4. Tumor cell suspensions was achieved by establishing gate parameters around a dominant negative IFN-γR1 [R&D Systems, Minneapolis, MN). IFN-γ was replaced every 48 h. Hypoxia studies were performed essentially as described previously (21). Cells (7.5 × 10⁶) were spot-plated onto 5-cm glass dishes in JRH 610 medium supplemented with penicillin/streptomycin and 10% FCS (complete JRH 610) and incubated overnight at 37°C. The following day, the dishes were placed in leak-proof aluminum chambers connected to a manifold in which the oxygen concentration was reduced to the appropriate level. For EFS hypoxia studies, EFS was added to the medium up to a final concentration of 0.10 mm immediately before induction of the appropriate level of hypoxia, and the cells were incubated for 3 h. For prolonged hypoxia studies, the medium was replaced with complete JRH610 supplemented with 20 mm HEPES, 0.1% glucose, and 0.008 N NaOH prior to hypoxic induction. pH and glucose measurements were taken at the beginning and end of the hypoxic period to ensure that adequate glucose was present in the medium and document any significant changes in acidity.

**Immunohistochemistry.** Immunohistological staining for platelet/endothelial cell adhesion molecule (PECAM) and EFS and imaging were performed essentially as described previously (22). Briefly, cryostat sections were incubated with 5 μg of rat antirabbit PECAM-1 antibody (MEC 13.3; PharMin, San Diego, CA), followed by 40 μg/ml peroxidase-conjugated mouse antirat IgG (H&L; Jackson Immunoresearch Laboratories, West Grove, PA). Following a PBS wash, sections were incubated with the chromagen 3-amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA). Sections were then stained with ELK 3-51-Cy3. Normal tissues from tumor-bearing mice were also stained for EFS to guarantee staining specificity (liver central vein hepatocytes stain positive, whereas s.c. tissues stain negative). Stained sections were imaged using an epifluorescence equipped Nikon microscope (×20 objective), digitized, and image-analyzed using Image Pro software (Version 3.0; Media Cybernetics, Silver Spring, MD). Images from adjacent microscope fields were automatically acquired and digitally combined to form a 5 × 4 montage (corresponding to 2.9 × 1.7 mm of the section). Tumors in each treatment group were size-matched for comparison.

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Hypoxic Tumor Cells Arise in Avascular Tumor Regions during rmIL-12 Therapy. Although rmIL-12 has been shown to inhibit angiogenesis in model assays, its effect on angiogenesis within tumors and the functional consequences of these effects have not been studied. The expected effect of tumor angiogenesis inhibition is vascular insufficiency that should give rise to ischemia. We used tumor cell hypoxia, measured by binding of the polyfluorinated 2-nitroimidazole, EF5 (23), as an indicator of tumor ischemia. In vitro studies measuring EF5 binding to cultured K1735 cells under various O$_2$ concentrations showed that EF5 binding is inversely correlated with O$_2$ tension, with maximal discrimination of EF5 binding occurring between 0 and 2% O$_2$ (Fig. 3). Thus, the range of O$_2$ concentrations best determined by EF5 binding to K1735 cells is physiologically...
relevant because half-maximal cellular hypoxic responses occur at an
O₂ concentration of 1.5% (24).

To assess the oxygenation of tumor cells in vivo, we injected
K1735 tumor-bearing mice treated with rmIL-12 or untreated
with EF5 i.v. 3 h prior to tumor excision. Tumor cell suspensions were
prepared, stained with a fluorescein-conjugated monoclonal antibody
directed against EF5, and analyzed by flow cytometry (Fig. 4A). Cells
from untreated K1735 tumors varying from 2- to 16-mm diameter
exhibited a uniform pattern of low EF5 staining similar to that seen in
K1735 cells cultured at 10–20% O₂; only 1–2% of cells in these
tumors showed EF5 staining at levels equivalent to that of cells
cultured at <1% O₂ (Fig. 3). Tumors treated with rmIL-12 for 1 week
also appeared to be uniformly oxygenated with minimal EF5 staining,
although there was a shift toward a lower median O₂ percentage.

Tumors receiving 2 weeks of rmIL-12 treatment, however, exhibited
a peak of EF5-staining cells (median O₂, 40% of tumor cells were apoptotic after 2 weeks of rmIL-12 therapy,
which occurred during the first week of therapy.

**Hypoxia Resulting from rmIL-12 Therapy Induces Tumor Cell
Apoptosis.** Because severe hypoxia has been shown to induce tumor
cell apoptosis (18, 19), we examined whether rmIL-12-induced tumor
cell hypoxia was associated with apoptosis by two-color flow cyto-
metric analysis using TUNEL staining to identify apoptotic cells. In
untreated tumors, only 2% of tumor cells were apoptotic. In contrast,
40% of tumor cells were apoptotic after 2 weeks of rmIL-12 therapy,
when tumors were significantly hypoxic (Fig. 6, A and B). Apoptosis
was strongly associated with hypoxia (20% of normoxic cells were
apoptotic, whereas >80% of hypoxic cells were apoptotic), so that,
although normoxic cells predominated in these tumors, the majority of
apoptotic cells were in the hypoxic population. This trend continued
in tumors treated for 3 weeks with rmIL-12, in which 67% of tumor
cells were apoptotic and, because hypoxic cells were predominant,
the vast majority of apoptotic cells were hypoxic.

The relationship between hypoxia and apoptosis during rmIL-12
treatment was examined by testing whether hypoxia could induce
apoptosis in K1735 cells in vitro. When cells were incubated under
a range of O₂ concentrations (10, 1, and 0.1% O₂) for 16 or 32 h,
marked apoptosis was seen in cells grown at 0.1% O₂ (Table I), which
was the median O₂ concentration of the hypoxic tumor cell population
in vivo after 2 weeks of rmIL-12 therapy (Fig. 4A). By 32 h, >70% of
cells in 0.1% O₂ were apoptotic, consistent with the 70–80% of
hypoxic cells observed to undergo apoptosis in vivo (Fig. 6B). In the
final 8 h of incubation in 0.1% O₂, the pH of the culture medium
decreased from pH 7.1 to 6.8. However, acidosis was not the cause of
apoptosis in these cells, because culturing K1735 cells at pH 6.8 in air
for up to 16 h did not induce significant apoptosis (data not shown).
These results strongly suggest that, in vivo, rmIL-12 treatment suppresses
K1735 tumor growth via the development of severe hypoxia,
which triggers tumor cell apoptosis.

**rmIL-12 Also Induces Early IFN-γ-dependent Tumor Cell
Apoptosis Independent of Hypoxia.** The observation that rmIL-12
suppressed tumor growth during the first week of therapy prior to the
development of severe hypoxia was still unexplained. When tumors
treated for 1 week were examined, we found that the level of apoptosis

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**Fig. 4.** rmIL-12 therapy results in delayed development of tumor hypoxia. **A,** K1735 tumors from mice receiving rmIL-12 or PBS were labeled with EF5 in vivo (i.v. injection of 0.25 ml of 10 mg EF5 3 h prior to tumor excision) and excised for flow cytometry analysis following dissociation and staining with ELK-351-Cy3 monoclonal antibody. Flow cytometry histograms of representative tumors treated with PBS or rmIL-12 for 1, 2, or 3 weeks are shown. **Top horizontal axis,** the approximate oxygen concentration based on *in vitro* correlation of EF5 fluorescence intensity versus oxygen concentration as described in Fig. 3. **Bottom horizontal axis,** absolute EF5 fluorescence intensity. **B,** histograms showing means (columns) and SD (bars) of percentage oxic (ο) and % hypoxic (<1% O₂) tumor cells from K1735 and N23 tumors treated for the indicated periods with PBS or rmIL-12 (n = 3).

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**References**

in these tumors (52%) was comparable with that present at later treatment time points (Fig. 6, A and B). However, this apoptosis occurred in the absence of hypoxia, indicating that rmIL-12 was inducing hypoxia-independent as well as hypoxia-dependent tumor cell apoptosis. Several observations led us to suspect that IFN-γ caused this hypoxia-independent apoptosis. (a) IFN-γ levels were highest in the first week of rmIL-12 treatment when this apoptosis was present. (b) This apoptosis was abrogated by antibody neutralization of IFN-γ (Fig. 6C), and non-IFN-γ-responsive N23 tumors treated with rmIL-12 for 1 week did not exhibit significant apoptosis. (c) Previous studies had shown that IFN-γ can induce apoptosis in vitro in a signal transducers and activators of transcription (STAT)-dependent manner (25). To test this hypothesis, we cultured K1735 cells with different concentrations of murine rIFN-γ for 48 or 96 h (Table 2; 96 h approximates the time that tumor cells are exposed to high IFN-γ levels in vivo during a 5-day course of rmIL-12 therapy). The percentage of apoptotic cells increased with dose and duration of rIFN-γ treatment to a high of 41% with 100 ng rIFN-γ/ml for 96 h, which approximates the percentage of apoptotic cells seen in vivo after a week of rmIL-12 treatment (Fig. 6B). That apoptosis was not induced in N23 cells under the same conditions indicates that this is likely a direct effect of IFN-γ on K1735 cells. Thus, hypoxia-independent apoptosis induced after 1 week of rmIL-12 therapy can be attributed to direct IFN-γ induction of K1735 tumor cell apoptosis.

Apoptosis involved 52, 26, and 29% of the nonhypoxic tumor cells after 1, 2, and 3 weeks of rmIL-12 treatment, respectively. The reduction in apoptotic fraction of nonhypoxic tumor cells after the first week of treatment was statistically significant (P < 0.05 for 1 versus 2 weeks and P < 0.01 for 1 versus 3 weeks by Student’s t-test) and may be explained by the significantly lower levels of IFN-γ induced by rmIL-12 after the first week (Fig. 6B). This reduction in hypoxia-independent, IFN-γ-dependent apoptosis was offset, however, by the concomitant rise in hypoxia-dependent apoptosis, so that a significant fraction of tumor cells was apoptotic throughout the 3 weeks of rmIL-12 treatment. Both apoptosis mechanisms contribute to rmIL-12 control of K1735 tumor growth, but each predominates at a different time.

To be certain that apoptosis accounted for rmIL-12 suppression of tumor growth, we also evaluated changes in tumor cell proliferation by propidium iodide staining for DNA content. These studies revealed no increase in S-phase fraction with rmIL-12 treatment to offset the increases in apoptosis (data not shown). In fact, the fraction of cells in S-phase dropped by ~35% in tumors treated for 1 or 2 weeks and by ~65% in tumors treated for 3 weeks. These decreases can be explained by the rise in the percentage of apoptotic tumor cells (which should not be cycling) observed with rmIL-12 treatment and probably do not represent therapeutic suppression of proliferation by healthy tumor cells.

**DISCUSSION**

The goal of this study was to examine the effects of rmIL-12 angiogenesis inhibition within tumors to understand how this property of rmIL-12 contributes to its overall antitumor efficacy. Hypothesizing that angiogenesis inhibition would lead to tumor cell ischemia and knowing that ischemia engenders cellular hypoxia, we examined hypoxia, measured by EF5 binding, as a possible physiological consequence of tumor angiogenesis inhibition that could be responsible for its antitumor effect. Intracellular binding of EF5, a polyfluorinated 2-nitroimidazole, is a well-established marker of cellular hypoxia (23). EF5 under hypoxic conditions is metabolically activated and forms covalent adducts with cellular macromolecules that can be detected by monoclonal antibodies. Its binding to cultured tumor cells has been shown to be a quantitative method for determining intracellular oxygen concentration (26). In addition, a recent comparison of EF5 binding with Doppler ultrasound in tumors demonstrated that
Tumor cells and the average serum IFN-γ and apoptosis cells among oxic and hypoxic cell populations. The total percentage of apoptotic from representative tumors treated with PBS or with rmIL-12 for 1, 2, or 3 weeks.

(A) Excised, and dissociated for flow cytometric analysis of hypoxia (ELK 3-51-cyanine-5) of percentage oxic and hypoxic mice bearing the K1735 tumors were also given either a normal rat antibody (NRA) or a monoclonal antibody (XMG6) against IFN-γ.

(B) RmIL-12 therapy and determined by RIA) are listed above the graph. Whereas we are confident from Matrigel neo-vascularization assays that rmIL-12 inhibits new blood vessel development, we do not know at this time whether it also has effects on existing vessels.

Our studies suggest that angiogenesis inhibition leads to tumor cell apoptosis through the agency of hypoxia. Therapeutic efficacy by antiangiogenic agents is associated with increased tumor cell apoptosis (16, 17), but the inducer of this apoptosis has yet to be identified. On the basis of the results with rmIL-12, we propose that inhibition of tumor angiogenesis produces severe tumor hypoxia (or exacerbates it if tumor hypoxia exists prior to therapy) and that hypoxia is largely responsible for the tumor cell apoptosis and tumor growth arrest seen with angiogenesis inhibitor therapy. This does not preclude a contribution from other apoptosis stimuli to tumor stasis, but hypoxia-dependent apoptosis may be an inevitable consequence of tumor ischemia from angiogenesis inhibition, provided that the tumor cells are susceptible. Of course, ischemia from angiogenesis inhibition brings about physiological changes in addition to hypoxia, such as acidosis or hypoglycemia, that may also contribute to tumor cell apoptosis. For example, acidosis has been demonstrated to induce apoptosis in a hypoxia-independent manner (28).

Table 1 Induction of K1735 apoptosis in vitro by hypoxia

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<th>Tumor cell type</th>
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<th>Antibody</th>
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<td>-</td>
<td>NRA</td>
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<tr>
<td>K1735</td>
<td>-</td>
<td>α-IFN-γ</td>
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<td>N23</td>
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Table 2 Induction of K1735 apoptosis in vitro by IFN-γ

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<th>Tumor cell type</th>
<th>Hypoxia</th>
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EF5 binding is correlated with decreased blood flow (27), thus providing a link between reduced tumor perfusion and hypoxia.

Our results support the validity of this approach. Both rmIL-12 angiogenesis inhibition (measured in Matrigel neovascularization assays) and induction of tumor cell hypoxia were dependent on IFN-γ and required tumor cells to respond to IFN-γ. This latter requirement is likely related to IFN-γ-induced tumor cell IP-10 production because K1735 cells produce IP-10 in response to IFN-γ stimulation, whereas N23 cells do not (data not shown). The observed regions of tumor cell hypoxia were localized to relatively avascular areas of the tumor. The only difference between the two parameters is that, whereas angiogenesis inhibition was seen in the first week of rmIL-12 treatment, the appearance of severe tumor cell hypoxia was delayed. However, we believe that this delay can be explained physiologically and is consistent with a causal relationship. In a well-oxygenated tumor such as K1735, there are no hypoxic regions, indicating that oxygen delivery to tumor cells is uniformly adequate. Curtailment of new vessel development will likely reduce tumor perfusion gradually, so that there will be a temporal lag before oxygen delivery is reduced to a critical level and becomes manifest as severe hypoxia. In support of this model, median K1735 tumor cell oxygenation decreased after the first week of rmIL-12 treatment (Fig. 4A), although severe hypoxia was not yet apparent. Whereas we are confident from Matrigel neo-vascularization assays that rmIL-12 inhibits new blood vessel development, we do not know at this time whether it also has effects on existing vessels.

On the basis of the results with rmIL-12, we propose that inhibition of tumor angiogenesis produces severe tumor hypoxia (or exacerbates it if tumor hypoxia exists prior to therapy) and that hypoxia is largely responsible for the tumor cell apoptosis and tumor growth arrest seen with angiogenesis inhibitor therapy. This does not preclude a contribution from other apoptosis stimuli to tumor stasis, but hypoxia-dependent apoptosis may be an inevitable consequence of tumor ischemia from angiogenesis inhibition, provided that the tumor cells are susceptible. Of course, ischemia from angiogenesis inhibition brings about physiological changes in addition to hypoxia, such as acidosis or hypoglycemia, that may also contribute to tumor cell apoptosis. For example, acidosis has been demonstrated to induce apoptosis in a hypoxia-independent manner in vitro (28). In our tumor...
model, however, acidosis seems to contribute little to tumor cell apoptosis induced by hypoxia.

rmIL-12 controls K1735 tumor growth from the outset of therapy, unlike agents such as angiotatin and endostatin, which are suspected to act purely as angiogenesis inhibitors and which control tumor growth only after 1 or 2 weeks of therapy (29, 30). This was a clue that another antitumor mechanism is responsible for the initial phase of rmIL-12 tumor control, which we suggest is direct induction of tumor cell apoptosis by IFN-γ. An earlier study had demonstrated this to be an effect of high-dose IFN-γ stimulation in vitro (25), but the studies presented here suggest a role in suppressing tumor growth in vivo. IFN-γ has pleiotropic effects on cells, many of which are indirect and mediated by other factors, making it difficult to prove that hypoxia-independent, IFN-γ-dependent tumor cell apoptosis observed in vivo results from the apoptosis-inducing activity of IFN-γ. However, the absence of this type of apoptosis in N23 tumor cells, which should resist direct effects of IFN-γ but not those of intermediary factors induced by IFN-γ, argues that the apoptosis results from the direct action of IFN-γ on tumor cells. We do not know whether the concentrations of IFN-γ necessary to stimulate apoptosis in vitro are present in the local tumor environment in vivo. However, we do know that other proinflammatory cytokines likely to be induced by rmIL-12 in vivo (e.g., tumor necrosis factor-α) act synergistically in vitro with IFN-γ to induce K1735 tumor cell apoptosis (data not shown). The presence of these other cytokines reduces the concentration of IFN-γ needed to induce apoptosis, increasing the likelihood that effective induction of tumor cell apoptosis by IFN-γ occurs with cytokine concentrations that are achieved in vivo. This mechanism explains the immediate suppression of K1735 tumor growth seen with rmIL-12 administration and warrants consideration as a mechanism underlying the rapid response of other tumors to rmIL-12 therapy.

A question that arises is which host cells are responsible for the IFN-γ produced during the period of rmIL-12 administration. We believe that the best candidates for primary acute mediators of rmIL-12 antitumor activity and producers of IFN-γ are NK cells. These have been shown to be the first cells to infiltrate tumors in response to IL-12 therapy, where they appear to mediate both tumor cell killing and angiogenesis inhibition (31, 32). In addition, administration of antibodies directed against NK cells during the first week of rmIL-12 therapy is associated with a reduction in systemic levels of IFN-γ and a loss of angiogenesis inhibitory activity (data not shown). Finally, rmIL-12 used in the manner that generally produces the greatest acute antitumor effect (high doses administered frequently) has been shown to transiently but profoundly inhibit T-cell mitogenesis and antigen-specific responses (8, 9), making T cells unlikely mediators of rmIL-12 acute antitumor effects. However, further studies are required to define the role of different effector cell populations during IL-12 therapy more completely.

In summary, examination of the time course of rmIL-12 effects on the physiology of therapeutically responsive K1735 melanoma tumors reveals that two distinct antitumor mechanisms suppress tumor growth at different periods during treatment. IFN-γ-induced apoptosis is initially active but subsequently declines. This decline is accompanied by a corresponding rise in hypoxia-induced apoptosis, which results in a relatively stable level of tumor cell apoptosis that suppresses tumor expansion throughout treatment. Beyond rmIL-12 therapy, these studies suggest that hypoxia-induced tumor cell apoptosis may be a general mechanistic pathway whereby treatment with antiangiogenic agents leads to tumor stasis or regression. The involvement of hypoxia carries potentially important consequences and implications for this form of therapy. For example, hypoxia is known to induce a variety of cellular changes that can be adaptive for tumors, such as alterations in glucose metabolism and increased vascularity (33). In addition, hypoxia confers resistance to the cytotoxic effects of ionizing radiation (34), suggesting that care is warranted if angiogenesis inhibitors are to be used in combination with radiation therapy. Hypoxia has also been shown to select for cells with diminished apoptotic potential and p53 mutation (35), which could lead to expansion of tumor cell populations that are more therapeutically refractory (36, 37). Although these considerations may only be applicable with chronic hypoxia, long-term maintenance therapy with angiogenesis inhibitors has been proposed (30, 38). Additionally, tumors may vary in their susceptibility to the apoptotic effects of hypoxia, which may be a basis for variation in tumor response to antiangiogenesis therapy. Finally, if severe tumor hypoxia results from effective inhibition of tumor angiogenesis and mediates its antitumor effect, measurements of tumor hypoxia may be a sensitive and meaningful way to monitor clinical therapy with antiangiogenic agents.

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Hypoxia-mediated Apoptosis from Angiogenesis Inhibition Underlies Tumor Control by Recombinant Interleukin 12

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