Interleukin-12 Inhibits Angiogenesis and Growth of Transplanted but not in Situ Mouse Mammary Tumor Virus-induced Mammary Carcinomas


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ABSTRACT

We examined the ability of recombinant murine interleukin-12 (rmIL-12) to inhibit the vasculature and growth of mammary carcinomas arising in situ in mouse mammary tumor virus (MMTV)-infected female C3H/HeN mice. Although it is a potent antiangiogenic and antitumor agent in many transplanted murine tumor models, rmIL-12 failed to inhibit the vascularity, reduce the perfusion, or alter the growth of these autochthonous carcinomas. Factors intrinsic to these tumor cells were unlikely to be responsible for therapy failure. This is because primary cells derived from these carcinomas responded to IFN-γ, and rmIL-12 was effective against transplanted tumors arising from MmSMT cells, a line established from a MMTV-induced mammary carcinoma in C3H mice. Factors intrinsic to the mice that host the autochthonous mammary carcinomas were also not responsible for failure, because they sponsored rmIL-12 antiangiogenic and antitumor effects against transplanted K1735 murine melanoma tumors. Instead, the autochthonous nature of the mammary carcinomas and their possession of a high percentage of mature, pericyte-covered vessels that are resistant to therapeutic regression may be responsible. This is supported by the observation that transplanted MmSMT tumors had a lower proportion of pericyte-covered vessels and responded to rmIL-12 therapy. These results point to significant differences between the vasculature of transplanted and autochthonous murine tumors and indicate that their susceptibility to antivascular therapy may differ substantially.

INTRODUCTION

Evidence that tumor growth beyond a very small size is angiogenesis-dependent (1) has spurred the development of numerous angiogenesis inhibitors as potential antitumor agents (2). Several inhibitors have been shown to control tumor growth in mouse models of cancer (3–7), and, some of these have progressed into clinical trials. Most of the published studies that evaluate these agents in vivo have used model assays of neovascularization [e.g., chick chorioallantoic membrane (8), corneal micropocket (9), Matrigel pellet (10) assays] to demonstrate antiangiogenic efficacy and transplanted mouse tumor models to demonstrate antitumor efficacy. Use of transplanted mouse tumors has many advantages. They derive from a relatively homogeneous, fully transformed cell population so that tumors usually arise rapidly, and time-to-tumor appearance after transplantation and rates of tumor growth in a given model tend to be predictable and consistent. This permits sizeable cohorts of tumor-bearing mice to be set up for contemporaneous, matched treatment and control groups. Tumors transplanted heterotopically in s.c. locations have the additional advantage of being readily accessible for frequent monitoring of tumor appearance, growth, and therapeutic response. Although these tumor models are undeniably convenient and useful, they do not reproduce cancer, which is usually a more chronic disease that arises by gradual transformation of cells and evolutionary expansion of cell populations in situ. Autochthonous murine tumors arising after carcinogen administration, viral carcinogenesis, or transgenic manipulations better reproduce many of the features of human cancers but are experimentally more cumbersome.

Tumors are believed to develop their vasculature mainly by angiogenesis, a process whereby new vessels develop from preexisting ones (11). During the early phase of tumor formation, malignant cells have been observed to coopt normal vessels of the organ in which the tumor arises, prior to new vessel formation (12). Recently, endothelial cells arising from cells of bone marrow origin have also been shown to participate in tumor vessel formation (13). Tumor vessels are not all alike and probably undergo maturation like normal vessels. The transformation from immature to mature vessels is characterized by the association of vascular endothelial cells with periendothelial mesenchymal cells or pericytes (14). Investment by pericytes confers endothelial cell resistance to apoptosis and is associated with vessel survival when they are deprived of vascular growth and survival factors like vascular endothelial growth factor (15) or exposed to antiangiogenic agents like IL-12.4 The relative immaturity of vessels in transplanted tumors compared with those in normal organs may help explain the selective activity of antiangiogenic agents against vessels in these tumors and raises the question of whether the vasculature arising in transplanted murine tumors accurately represents the vasculature in authentic cancers.

In this study, we examined the ability of rmIL-12, a potent immunomodulatory cytokine, angiogenesis inhibitor and antitumor agent (16, 17), to control the growth of murine tumors that more closely resemble human cancers in their pathogenesis and formation than the transplanted tumors usually used in mouse experiments. We studied mammary carcinomas arising in MMTV-infected female C3H/HeN mice (18). Virtually all multiparous, infected mice develop tumors by 1 year of age. Carcinogenesis is initiated by the insertion of MMTV provirus adjacent to, and transcriptional activation of, one of a number of proto-oncogenes in mammary epithelial cells (usually Wnt-1/int-1 and int-2 in C3H/HeN mice; Refs. 19, 20). Mammary tumors arise in individual C3H/HeN mice usually between the age of 5–12 months, have different growth rates, and are clonally distinct. The majority of mice develop only one tumor with most of the rest developing only two, and their uninvolved mammary glands appear normal. These

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4 M. S. Gee, unpublished observations.

5 The abbreviations used are: IL-12, interleukin-12; rmIL-12, recombinant murine IL-12; vWF, von Willebrand factor; MVD, microvessel density; CWFA, color-weighted fractional average; hpf, high-powered field; MMTV, mouse mammary tumor virus; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; SMA, smooth muscle actin; FACS, fluorescence-activated cell sorting/sorter.

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lymphocytes. Flow cytometric analysis of MHC expression and apoptosis was exhibit detectably higher forward and side scattering than did erythrocytes and MMTV-induced mammary carcinomas a good model of human breast cancer.

MATERIALS AND METHODS

Mice. All mice were obtained from the National Cancer Institute (Frederick, MD) and were maintained in microisolator cages. For autochthonous mammary tumor studies, female MMTV+ C3H/HeN retired breeders, 24–30 weeks old at the time of shipment, were obtained in small cohorts over a period of 6 months. Mice were determined to be free of tumors by careful inspection and palpation on arrival and were monitored twice weekly for tumor appearance. When tumors appeared (~20 weeks after receipt) and their size became reliably measurable (1–2 mm diameters), mice were randomly assigned to receive either PBS or rmIL-12 (0.125 μg in 0.1 ml PBS; Genetics Institute, Andover, MA) i.p. on a daily, 5 days/week schedule (five daily injections followed by 2 days of rest). Sixteen mice hosting 21 discrete tumors were assigned to receive PBS, and 20 mice hosting 30 discrete tumors received rmIL-12 (see Table).

In the transplanted tumor studies involving older mice, female MMTV+C3H/HeN retired breeders were used as hosts for K1735 tumors. Tumor-free mice were inoculated s.c. with 10⁶ trypan blue-excluding, early-passage-cultured K1735 tumor cells in 0.1 ml PBS. For implanted Mm5MT and K1735 tumor studies involving younger mice, 3–4-week-old and 6–8-week-old MMTV+ female C3H/HeN mice were used, respectively. These mice were inoculated s.c. with 10⁶ K1735 cells or 5 × 10⁶ Mm5MT cells to initiate tumors. Mice with implanted tumors were treated with PBS or rmIL-12 in the manner described for mice with autochthonous mammary tumors. To monitor the effects of therapy, tumor size was measured every other day with calipers, and treatment was continued until tumors reached a size requiring euthanasia by the standards of the Institutional Animal Care and Use Committee.

Cells. K1735 murine melanoma (21) and Mm5MT (MMTV-induced) murine mammary carcinoma (22) cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin and maintained in a 5% CO₂ atmosphere. To establish cultures of freshly excised MMTV-induced mammary tumor cells, the tumor was mechanically disrupted using sterile scissors and dounce homogenization and placed in medium with 10% Dispase (Collaborative Biomedical, Cambridge, MA). After 30-min incubation with shaking at 37°C, the suspension was centrifuged at 1000 x g for 5 min. The cell pellet was washed twice with PBS, resuspended in complete media, plated on 6-cm tissue culture plates and maintained at 37°C and 5% CO₂. These cultures generally did not proliferate past the second passage.

In Vitro Cell Studies. For IFN-γ incubation studies, cells at 30–40% confluency on a 10-cm plate were treated with IFN-γ (R&D Systems, Minneapolis, MN) at 10 units/ml medium for 48 h. For both MHC and Apoptag staining, cells were harvested using 1 mm EDTA in PBS. To determine surface expression of murine MHC class I molecules, Mm5MT- and MMTV-derived tumor cells were stained with primary monoclonal mouse anti-H-2Kk antibody expression of murine MHC class I molecules, Mm5MT- and MMTV-derived with a Krypton-Argon (488 nm) laser to excite fluorescein fluorescence. Isolation with a polyclonal rabbit anti-vWF antibody (Dako, Carpinteria, CA) diluted 1:1500 for endothelial cells with anti-vWF antibody and for pericytes with mouse antimouse immunoglobulin antibody (Molecular Probes) at 1:200 dilution for cell studies, cells at 30–40% confluency on a 10-cm plate were treated with IFN-γ (R&D Systems, Minneapolis, MN) at 10 units/ml medium for 48 h. For both MHC and Apoptag staining, cells were harvested using 1 mm EDTA in PBS. To determine surface expression of murine MHC class I molecules, Mm5MT- and MMTV-derived tumor cells were stained with primary monoclonal mouse anti-H-2Kk antibody (clone 36-7-5, PharMingen, Inc.) and secondary goat antirat IgG fluorescein conjugated antibody [F(ab')₂], fragment-specific; Jackson ImmunoResearch Laboratories, Inc.). Apoptosis was measured by the TUNEL staining method according to the Apoptag manufacturer’s instructions (Intergen, Purchase, NY).

Flow Cytometry. Flow cytometry was performed on a Becton Dickinson Immunocytometry systems FACS Calibur. The FACS Calibur was equipped with a Krypton-Argon (488 nm) laser to excite fluorescein fluorescence. Standard collection optics was used to collect emitted fluorescence. Isolation of tumor cells in cell suspensions was achieved by establishing gate parameters around in vitro cultured Mm5MT- or MMTV-derived tumor cells, which exhibit detectably higher forward and side scattering than did erythrocytes and lymphocytes. Flow cytometric analysis of MHC expression and apoptosis was performed using CellQuest (Becton Dickinson, Mountain View, CA).

Doppler Ultrasound Imaging of Tumors. Power Doppler ultrasound imaging of tumors was performed as described previously (23). Briefly, tumor-bearing mice were anesthetized with ketamine and xylazine. The skin overlying the tumor was shaved, the mice were placed in sternal recumbency to facilitate tumor alignment with the ultrasound transducer. Imaging was performed using an Ultramark 9 HDI ultrasound machine (Advanced Technology Laboratories, Inc., Bothell, WA) with an L10–5 MHz transducer. A 5-mm acoustic standoff between transducer face and tumor was achieved by generous application of acoustic gel. The images were recorded on videotape and analyzed for vascularity using computer software (University of Pennsylvania, Philadelphia, PA). Ultrasound image analysis was performed as described previously to obtain values for CWFA (23), which is an overall measurement of tumor perfusion.

Confocal Microscopy. Tumor-bearing mice received i.v. injections of 150 μl of 1 mg/ml FITC-conjugated tomato (Lycopersicon esculentum) lectin (Vector Labs, Burlingame, CA) in PBS into the tail vein 15 min prior to tumor excision. After excision, the tumors were sectioned manually into thick (0.5–1.0 mm) slices that were mounted onto microscope slides with 50% glycerol in PBS and covered with a coverslip. Slides were examined using an upright Nikon (Augusta, GA) E-600 Eclipse microscope equipped with a Bio-Rad (Hercules, CA) 1024-ES confocal system. FITC fluorescence was detected by a three-line, 15-mW Argon-Krypton laser system (American Laser, Fraser, MI). Images were viewed by ×10 objective lens with field dimensions of 1004.5 × 1004.5 μm. For each slide, serial images were acquired at 2.5-μm intervals over a standard 100-μm depth, using Bio-Rad Lasersharp Acquisition software, and integrated to create a composite maximum intensity projection of tumor vasculature imaged in three dimensions. Projection images were analyzed using ImageTool software (University of Texas, San Antonio, TX) for vessel density, luminal diameter, and arborization. The vessel density of each image was defined as the number of vessel intersections with a four-axis grid (vertical, horizontal, and two diagonal axes through the center of the image) superimposed over the image. Lumen cross-sectional diameters were determined for vessels at the point at which they intersected the grid.

Immunohistological Staining. For conventional MVD determination, thin (4-μm) sections from formalin-fixed, paraffin-embedded tumors were stained for vWF to detect endothelial cells. Tissue slides were deparaffinized and incubated in 0.3% hydrogen peroxide for 10 min at 4°C. Antigen retrieval was performed by incubation in 0.12% Pronase (Boehringer Mannheim; Indianapolis, IN) for 15 min at 37°C followed by blocking with PBS containing 0.1% BSA and 5% goat serum for 20 min at 37°C. The tissue was then stained with a polyclonal rabbit anti-vWF antibody (Dako, Carpinteria, CA) diluted 1:1500 in blocking solution for 2 h at room temperature. Slides were then incubated with biotinylated goat antirabbit immunoglobulin antibody (Vector Labs) diluted 1:200 in blocking solution for 1 h at room temperature. Slides were incubated in streptavidin-horseradish peroxidase (Research Genetics, Huntsville, AL) for 1 h at room temperature and subsequently developed using amino ethyl carbazole substrate (Vector Labs). Slides were then counterstained with hematoxylin.

To determine pericyte coverage of vessels, thin sections were dually stained for endothelial cells with anti-vWF antibody and for pericytes with mouse anti-SMA antibody (Dako), respectively. Deparaffinized sections underwent antigen retrieval by incubation in 0.07% Pronase for 15 min at 37°C, followed by 30-min incubation with shaking at 37°C, the suspension was centrifuged at 1000 × g for 5 min. The cell pellet was washed twice with PBS, resuspended in complete media, plated on 6-cm tissue culture plates and maintained at 37°C and 5% CO₂. These cultures generally did not proliferate past the second passage.

Histology Image Acquisition and Analysis. All histological specimens were viewed using a Nikon light microscope equipped with a Hamamatsu digital camera and Nikon ImagePro acquisition software, and images were analyzed using ImageTool software. For MVD measurements, slides were scanned at low power (x4) to identify areas of highest vascularity. hps (x20) were then selected randomly within these areas, and MVDs were calculated based on the number of vWF-positive structures. In addition, vessel lumen cross-sectional areas were determined for all counted vessels automatically based on spatial calibration parameters established with a slide micrometer. Microvessels were counted by multiple blinded observers. An average of 17 sections per vehicle-treated tumor and 14 sections per rmIL-12-treated tumor were analyzed from six and eight tumors within each group, respectively.
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RESULTS

rmIL-12 Does Not Inhibit the Growth of MMTV-induced Mammary Tumors. We assessed the antitumor and antivascular effects of rmIL-12 in an autochthonous murine model of mammary tumorigenesis. Retired MMTV-infected (MMTV+/-) C3H/HeN female breeders were randomly assigned to receive either vehicle or rmIL-12 treatment soon after they developed mammary tumors. Sixteen mice received PBS, and 20 mice received rmIL-12 at the maximum tolerated dose (0.125 µg/day i.p., 5 days/week), and growth of their tumors was monitored. As shown in Table 1, five (31%) of the control mice and seven (35%) of the treated mice had multiple mammary tumors; both of the mice with more than two tumors were in the treated group. In total, we monitored the growth of 21 tumors in control mice and 30 tumors in rmIL-12-treated mice. We could only estimate the age at which these mice developed their tumors from the fact that they were 24–30 weeks old at the time of shipment, but there was no obvious age difference at the time of tumor appearance in mice of the two groups. Mammary tumors in individual MMTV-infected mice are clonally distinct and grow at highly variable rates, unlike the more predictable growth rates of implanted tumors from established cell lines. Nevertheless, the growth rates of rmIL-12-treated tumors was well within the range of growth rates of control tumors (Fig. 1A). To quantify tumor growth, we compared the time taken for tumors to grow to 5-mm diameter (Fig. 1B; Table 1). The median and mean times taken were not significantly different for tumors in the two groups. We also compared the estimated doubling time of control and treated tumors (Table); among tumors for which we could derive this value, neither the range nor the mean differed significantly. The rmIL-12 used during the course of this study was shown in other experiments to be biologically active (data not shown). Thus, whereas growth inhibition of individual MMTV-induced mammary carcinomas cannot be excluded, rmIL-12 treatment did not inhibit growth of these tumors as a group.

Table 1 Characteristics of mice and tumors treated and untreated with rmIL-12

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>rmIL-12-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>No. of tumors</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
<td>1 (11 mice)</td>
<td>1 (13 mice)</td>
</tr>
<tr>
<td></td>
<td>2 (5 mice)</td>
<td>2 (5 mice)</td>
</tr>
<tr>
<td></td>
<td>3 (1 mouse)</td>
<td>4 (1 mouse)</td>
</tr>
<tr>
<td>Median time to reach 5-mm diameter</td>
<td>6 days</td>
<td>6 days</td>
</tr>
<tr>
<td>Doubling time (mean)</td>
<td>3.06 ± 1.96 days</td>
<td>2.90 ± 1.42 days</td>
</tr>
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* Growth of 18 control and 23 rmIL-12-treated tumors reached 5-mm diameter before the host died or had to be euthanized.

** Growth of 19 control and 25 rmIL-12-treated tumors fitted to exponential curves with correlation coefficients > 0.8; doubling times were derived from these.

A

![MTTV-induced Mammary Tumors](#) PBS Treated

![MTTV-induced Mammary Tumors](#) rmIL-12 Treated

B

![Days for tumors to reach 5mm diameter](#)

Fig. 1. Growth of tumors with and without rmIL-12 treatment. Mammary tumors arising autochthonously in C3H/HeN retired female breeders infected with MMTV were randomly assigned to receive treatment with PBS or rmIL-12 as soon as tumors became reliably measurable. A, tumor growth was monitored every other day, and estimated tumor volume (mm³) is shown plotted against time (days) after the initiation of therapy. Each line, growth of an individual tumor (21 tumors in control mice and 30 tumors in rmIL-12-treated mice). B, time taken for tumors to reach 5-mm diameter or about 65-mm³ volume (18 control and 23 rmIL-12-treated tumors). Right-facing arrows, the median time for each group; left-facing arrows, the mean time for each group. Two control tumors took 25 and 32 days, and one rmIL-12-treated tumor took 24 days to reach a 5-mm diameter.

To better evaluate tumor vascularity, vessels were also visualized in three dimensions using confocal microscopy of thick (0.5–1-mm) sections in which functional vessels had been labeled by fluorescein-conjugated tomato (L. esculentum) lectin injected i.v. prior to excision (29). Although rmIL-12 treatment induced a visible change in both the caliber and branching pattern of K1735 tumor vessels, it did not do so in the mammary tumors (Fig. 2A). Quantitation of vessel density, branching, and diameter in the latter confirmed the absence of significant differences in these vessel parameters between control and rmIL-12-treated tumors (data not shown).

We also looked for evidence of tumor vascular inhibition on a functional level using power Doppler ultrasound to image blood flow through MMTV-induced tumors before and after initiation of rmIL-12 therapy (23). The pattern and magnitude of blood flow through mammary tumors appeared to change little as a consequence of treatment, in contrast to K1735 tumors, which showed a decrease in blood flow as a result of rmIL-12 treatment (Fig. 2B). This was confirmed by the CWFA, a quantitative blood flow parameter derived from the power Doppler ultrasound studies (23). CWFA, which re-
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rmIL-12 therapy.

factors are not responsible for the lack of response of these tumors to the type that host MMTV-induced mammary tumors and that host rmIL-12 can induce antitumor and antivascular effects in mice of breeders confirmed an antivascular effect of therapy that was as marked as in younger hosts (data not shown). These results indicate of the vasculature of untreated and treated K1735 tumors in retired CWFA decreased in rmIL-12-treated K1735 tumors as growth was slowed by therapy (Fig. 2, right panel, □ connected by solid lines), and increased in IFN-γ-unresponsive K1735.N23 tumors that grew normally despite rmIL-12 therapy (○ connected by dashed lines; Ref. 30). The change in CWFA from the first to the last measurement among the four responding K1735 tumors was −47% ± 8%, whereas the change among the five nonresponding K1735.N23 tumors was +99% ± 33%. Thus, perfusion in the mammary tumors, detected by power Doppler ultrasound, was unaffected by rmIL-12 therapy, which supports the apparent lack of effect on the vasculature of these tumors observed by immunohistochemistry and confocal microscopy. In sum, all of our studies that examined tumor vasculature and perfusion led us to conclude that rmIL-12 did not exert an antivascular effect on MMTV-induced mammary tumors.

Lack of Tumor Control by rmIL-12 of MMTV-induced Tumors Is Not Caused by Host Differences. One potential explanation for the lack of rmIL-12 antitumor and antivascular effect against MMTV-induced mammary tumors was the host. The MMTV＋ C3H/HeN retired breeders used here differed from the C3H/HeN mice that we usually use for K1735 tumors: in age (6–12 months versus 6–8 weeks); hormonal status (multiparous versus virgin); and having been infected by MMTV. These differences may have precluded or attenuated the response to rmIL-12 or affected other mechanisms needed for an antivascular or antitumor effect (31, 32). We, therefore, measured serum IFN-γ levels in tumor-bearing retired breeders receiving rmIL-12 and found that their induced levels were similar to those seen in rmIL-12-treated C3H/HeN mice bearing K1735 tumors (data not shown). This indicated a similar proximal host response to rmIL-12 administration. We also generated s.c. K1735 tumors in MMTV＋ C3H/HeN retired breeders that had not yet developed mammary tumors and treated these with rmIL-12. Therapy controlled K1735 tumor growth in these mice as effectively as in 6–8-week-old C3H/HeN hosts (Fig. 3). Confocal microscopy analysis of the vasculature of untreated and treated K1735 tumors in retired breeders confirmed an antivascular effect of therapy that was as marked as in younger hosts (data not shown). These results indicate that rmIL-12 can induce antitumor and antivascular effects in mice of the type that host MMTV-induced mammary tumors and that host factors are not responsible for the lack of response of these tumors to rmIL-12 therapy.

Fig. 2. Vasculature and perfusion of tumors with and without rmIL-12 treatment. A, mice bearing size-matched untreated or rmIL-12-treated K1735 melanoma tumors, autotiochthonous MMTV-induced mammary tumors, or transplanted MnSMT mammary tumors, were given injections of FITC-conjugated tomato lectin i.v. prior to tumor excision. Thick sections of these tumors were imaged by confocal microscopy. Shown are representative projections of serial confocal tumor images obtained at 4-μm steps along 100 μm of Z axis (depth; ×10). B, power Doppler ultrasound images of a representative K1735 melanoma and an MMTV-induced mammary tumor acquired before and after 2–3 weeks of rmIL-12 therapy. Red pixels, regions of detectable blood flow.; boxed area, the total field in which ultrasound data were acquired. C, CWFA, a quantitative parameter that reflects overall tumor blood flow, was derived from serial weekly power Doppler ultrasound studies of several untreated and rmIL-12 treated tumors. Left panel, serial CWFA measurements from individual MMTV-induced mammary tumors; □ connected by solid lines, control tumors; ○ connected by dashed lines, rmIL-12-treated tumors; Ref. 30). The change in CWFA scale between the two graphs should be noted.

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MMTV-induced Mammary Tumor Cells Can Respond to rmIL-12 Therapy. Having eliminated the host as an explanation for the lack of response to rmIL-12, we next examined whether MMTV-induced mammary tumor cells might be responsible for therapeutic refractoriness. Previously, we showed that IFN-γ mediates the antiangiogenic effects of IL-12 and that tumor cell responsiveness to IFN-γ is important (30). Therefore, we assessed IFN-γ-responsiveness in short-term cultures of tumor cells freshly dissociated from MMTV-induced mammary tumors and in immortal Mm5MT cells that were established from a MMTV-induced mammary tumor in a C3H mouse (22). MHC class I was significantly induced on both primary mammary tumor cells and on Mm5MT cells after in vitro IFN-γ incubation (Fig. 4A). IFN-γ incubation also induced apoptosis (33) in these cells detected by FACS analysis after TUNEL staining (Fig. 4B). Thus, IFN-γ responses are intact in MMTV-induced mammary tumor cells, and unresponsiveness to this critical mediator of rmIL-12 efficacy does not explain the latter’s lack of antitumor effect.

Next we examined whether tumors derived from MMTV-induced mammary tumor cells could respond therapeutically to rmIL-12. We generated s.c. Mm5MT tumors in young MMTV+ C3H/HeN mice and treated them with rmIL-12. As shown in Fig. 5, growth of control Mm5MT tumors is usually progressive but, in some cases, tends to plateau after reaching a certain size (usually 4–6 mm in diameter). In contrast, rmIL-12-treated tumors never progressed, and many regressed, indicating that implanted Mm5MT tumors responded to rmIL-12 therapy. To determine whether rmIL-12 control of Mm5MT tumor growth was caused by an antivascular effect, the vasculature of untreated and treated Mm5MT tumors was studied by confocal microscopy. A significant reduction in overall tumor vascularity was seen in treated Mm5MT tumors (Fig. 2A), which indicated that rmIL-12 had an antivascular effect in these tumors.

Pericyte coverage is a sign of vessel maturity and resistance to regression on antiangiogenic therapy (15). In tumors that respond to rmIL-12 treatment, the percentage of pericyte-positive vessels is relatively low prior to therapy and significantly higher after therapy because of preferential loss of pericyte-negative vessels. To explain why rmIL-12 did not inhibit angiogenesis and growth in MMTV-induced autochthonous mammary tumors, we stained histological sections of treated and untreated tumors for endothelial cells with anti-υ-WF antibody and for pericytes with anti-SMA antibody. Representative pericyte-negative and pericyte-positive vessels after dual staining are shown in Fig. 6, A and B, and sections from tumors with a predominance of pericyte-negative and pericyte-positive vessels are shown in Fig. 6, C and D, respectively. In K1735 tumors, 37% ± 4% and 66% ± 7% of vessels were pericyte-positive in control and treated tumors, respectively, which is significantly different (P < 0.01, Student t test; Fig. 6E). Similarly, transplanted Mm5MT tumors had 36% ± 4% and 58% ± 5% pericyte-positive vessels in control and treated tumors, respectively (P < 0.01). In contrast, 55% ± 8% and 46% ± 7% of vessels were pericyte-positive in control and in treated autochthonous MMTV-induced mammary carcinomas, which is not significantly different (P > 0.05). The difference in baseline pericyte coverage between MMTV tumors and K1735 and Mm5MT tumors was significant (P < 0.01). Thus, despite their common background,
autochthonous MMTV-induced tumors have a much higher percentage of their vessels covered by pericytes than transplanted Mm5MT tumors. Seeing this difference was not altogether surprising, considering how developmentally and morphologically different the two types of tumors are. The malignant cell population in the two are very similar, consisting of intermediate to large cells with fine to slightly clumped nuclear chromatin and low mitotic activity (Fig. 7). Architecturally, however, the two are very different. Transplanted Mm5MT tumors showed spindle cell morphology without evidence of glandular, papillary, or other features of epithelial differentiation, whereas autochthonous MMTV tumors showed areas of infiltrative growth arranged in solid sheets, cribriform plates, and focal papillary growth with associated desmoplastic response along the infiltrative edges. No significant inflammatory infiltrate was seen in either. Overall, the morphology of the autochthonous tumors appears similar to many human ductal carcinomas, whereas the transplanted tumors bear little resemblance.

DISCUSSION

We examined the ability of rmIL-12 to inhibit the growth of MMTV-induced mammary tumors arising in older, multiparous C3H/HeN mice and found that this therapy had no discernible effect. This unresponsiveness stands in striking contrast to the ability of rmIL-12 to inhibit tumor growth in many other murine models (23–26). We personally have observed rmIL-12 inhibit the growth of tumors produced by six different established murine tumor cell lines (K1735 and B16F10 melanoma, RENCA renal carcinoma, CT26 colon carcinoma and SCK and Mm5MT mammary carcinoma cells; Refs. 23, 30; data not shown). IL-12 was only shown to be ineffective when host IFN-γ responses were impaired (31) or when tumors arose from cells that were unresponsive to IFN-γ (30). Our studies excluded both of these considerations in the case of rmIL-12-unresponsive MMTV-induced mammary carcinomas. Additionally, the studies showed that rmIL-12 inhibited the growth of transplanted tumors in these older, multiparous hosts and the growth of transplanted tumors arising from Mm5MT cells, a MMTV-induced C3H mammary carcinoma cell line. Thus, the available evidence indicates that neither the host nor anything intrinsic to MMTV-induced tumor cells is responsible for the lack of efficacy.
of rmIL-12 against autochthonous MMTV-induced mammary carcinomas.

IL-12 is a multifaceted antitumor agent that promotes cellular immune responses (16, 17) and, through IFN-γ and other mediators, induces tumor cell apoptosis (26, 33) and inhibits tumor angiogenesis (34). These diverse activities make it difficult to attribute its ineffectiveness against autochthonous MMTV-induced mammary tumors to the failure of any one mechanism. However, when recombinant IL-12 successfully controls the growth of established tumors, evidence favors antivascular activity as the major mechanism. When rmIL-12 is given in frequent high doses, as in this study, T-cell-mediated immune responses are profoundly suppressed during the period of tumor growth control (35, 36); therefore, this mechanism is unlikely to account for rmIL-12 antitumor effect. Another potential mechanism, IFN-γ induction of tumor cell apoptosis, is maximal in the first week of rmIL-12 administration (when IFN-γ induction is highest) and wanes thereafter (26). In contrast, rmIL-12 antiangiogenic and antivascular activity is tightly correlated with its antitumor efficacy (26, 30), as was the case in this study. These considerations led us to conclude that rmIL-12 failed to control the growth of autochthonous MMTV-induced mammary carcinomas because it failed to engender an antivascular effect.

Why should rmIL-12 fail to produce an antivascular effect in MMTV-induced mammary tumors when it succeeds in transplanted K1735 melanoma tumors, Mm5MT mammary tumors, and many others? Because the mechanisms underlying rmIL-12 antivascular effect are complex, this question is difficult to answer, but after excluding tumor cell and host factors to the extent possible, one is faced with the autochthonous nature of the unresponsive tumors, the heterotopic transplanted nature of the responsive tumors, and what this may mean in terms of tumor vasculature development and susceptibility to inhibition. In autochthonous tumorigenesis, initiation and formation of tumor vasculature occurs in organ parenchyma over a lengthy timeframe during which cells are transforming, cell populations are evolving, and tumor cells may be coopting normal vessels for their blood supply (12). In contrast, during tumorigenesis by tumor cells implanted in heterotopic s.c. “space,” the vasculature develops rapidly, is induced by fully transformed cells, and is primarily or exclusively neovascular. Mouse mammary carcinomas resulting from these differences differ markedly in morphology and architecture, and there is a significant difference in the extent of pericyte coverage of their tumor vessels. Pericyte coverage of microvessels is a sign of vessel maturity and confers relative resistance to regression in the face of vascular growth factor withdrawal and other antiangiogenic stimuli (14, 15). With these insults, the percentage of tumor vessels covered with pericytes increases. In transplanted mouse tumors responding to treatment with rmIL-12, pericyte coverage increases as MVD decreases because of endothelial cell apoptosis and preferential loss of pericyte-negative vessels.

Fig. 7. Histopathology of autochthonous MMTV-induced and transplanted Mm5MT mammary carcinomas. Sections of untreated autochthonous MMTV-induced and transplanted Mm5MT mammary tumors were stained with H&E. A–C, sections from MMTV-induced mammary tumors at ×200 (A, B) and ×400 (C). D–F, sections from Mm5MT mammary tumors at ×200 (D, E) and ×400 (F). The inset boxes in B and E, the tumor regions shown at higher magnification (in C and F, respectively).
some of the vascular differences that we have noted between autochthonous and transplanted carcinomas may be attributable to the s.c. localization of the latter.

Additional evidence that the vasculature of autochthonous MMTV tumors develops differently may be found in the unchanged to slightly decreased level of perfusion, measured by Doppler ultrasound, as these tumors grow compared with the increased perfusion as s.c. tumors grow. What this means in terms of tumor vascular anatomy is not certain, because the determinants of a functional parameter, such as perfusion, are complex and involve more than just vessel number or density. However, it may mean that tumor vascular expansion relative to tumor growth is less in autochthonous tumors than in transplanted tumors. Beyond this, there is evidence from study of transgenic MMTV-neu mice that neoplastic angiogenic activity varies during the course of autochthonous tumorigenesis (38). Evidence of vigorous angiogenesis was present early during mammary tumorigenesis in atypical hyperplasia lesions, and angiogenesis appeared to abate in later carcinoma in situ lesions and palpable tumors. Interestingly, “early” initiation of rmIL-12 prophylaxis, when the transgenic mice were young and only atypical ductal hyperplasia was present in mammary glands, delayed mammary tumor appearance, reduced tumor multiplicity, and appeared to inhibit the angiogenesis accompanying atypical hyperplasia. In contrast, “late” initiation of preventative treatment, when carcinoma in situ was already present in the mammary glands, resulted in little or no clinical benefit and produced no evidence of angiogenesis inhibition (38). Thus, during autochthonous tumorigenesis, angiogenic activity may fluctuate. Tumor cells may not have much difficulty dealing with a discontinuous pattern of neovascularization, because they have been shown to be heterogeneous with regard to vascular dependence; and cells with decreased dependence have been shown to be selected during tumorigenesis (39).

A limitation of this study is its testing and analysis of one angiogenesis inhibitor. Although this needs to be extended, the results already suggest that one should be cautious about setting expectations of antiangiogenic therapies against authentic tumors based on the results of these therapies against transplanted murine tumors. The substantial differences between the two categories of tumors may carry over to their vessels, and this means that the targets and outcomes of therapy may be different. Of course, authentic murine and human tumors are highly heterogeneous, and this implies that the vulnerability of their vasculature is likely to vary. It would be immensely useful if the susceptibility of a tumor’s vasculature to regression could be determined or predicted. Currently, the only candidate association is an inverse correlation with vessel pericyte coverage; and, to date, there are few reports on this vascular feature in human tumors (15, 40). Pericyte coverage of human tumor vessels appears to be highly variable between tumor types; breast and colon cancers are tumors (15, 40). Pericyte coverage of human tumor vessels appears to association is an inverse correlation with vessel pericyte coverage; and this means that the targets and division could be determined or predicted. Currently, the only candidate carry over to their vessels, and this means that the targets and effects of therapies that target tumor vessels, is probably not reproduced during the growth of transplanted tumors.

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### References


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