Inhibition of Angiogenesis by Interferons: Effects on Tumor- and Lymphocyte-induced Vascular Responses

Younan A. Sidky and Ernest C. Borden

ABSTRACT

Interferons (IFNs) have established antitumor action; the mechanism underlying this effect is, however, not yet clear. To prove the possible contribution of inhibition of angiogenesis, we have assessed angiogenesis in the mouse initiated by either human or murine tumor cell lines. Whether test cells were inoculated in the dermis or tumor fragments were grafted onto the cornea, tumor-induced angiogenesis (TIA) was inhibited by IFNs. TIA was also inhibited by the potent IFN inducer polyriboinosinic-polyribocytidylic acid. The effect of IFN was species specific; human IFNs inhibited human tumors and mouse IFNs inhibited murine tumors. This effect suggested that in contrast to other angiogenesis inhibitors, IFNs modulated the signal for angiogenesis produced by the tumor cells. Tumor cells treated in vitro with homologous IFNs were significantly more (P < 0.005) leste competent to initiate angiogenesis than were untreated cells. Inhibition of angiogenesis was achieved whether vascular response was assessed 1 or 3 days after tumor cell inoculation, suggesting that antiangiogenesis activity was independent of the antiproliferative effects of IFNs. To further substantiate this, L1210 leukemia cells, resistant to the antiproliferative effects of IFNs, were treated with 500 units/ml IFN-β. IFN had no effect on their proliferation, but in four separate experiments, L1210R cells were impaired in their ability to induce angiogenesis. Thus, inhibition of TIA by IFNs was species specific, occurred at least partly by modulation of the signal inducing angiogenesis, and was expressed in the absence of antiproliferative effects.

IFNs also inhibited immunologically induced angiogenesis, whether initiated by allogeneic lymphocytes (LIA) or by the mouse’s own T-cells in response to an exogenous antigen (sheep RBC). LIA was markedly suppressed by treatment of host mice with homologous IFN-β. For example, mean vessel counts induced by allogeneic mouse lymphocytes were decreased from 22.8 ± 1.4 (SE) to 12.5 ± 0.8 (P < 0.0001); mouse IFN-β had no corresponding effect on xenogeneic human lymphocytes (mean vessel counts decreased to 21.7 ± 2.6 from 22.7 ± 2.0). Treatment with human IFN-α, -β, or -γ in vitro or host mice in vivo reduced the ability of inoculated human peripheral blood lymphocytes to initiate xenogeneic LIA. Injection of LIA required a lower dose and/or a shorter incubation period than that needed to modulate TIA. Treatment of the donor of the allogeneic spleen cells in vivo with murine IFN or inducers also resulted in lesser LIA. Polyriboinosinic-polyribocytidylic acid almost completely abolished the early vascular response to sheep RBC when injected before primary or secondary challenge. For example, 48 h after rechallenge, mean vessel counts decreased from an estimated 290 in controls to 10 in polyriboinosinic-polyribocytidylic acid–treated mice. Angiogenesis may thus be another facet of neoplastic growth and immunological responses subject to inhibition by IFNs or their inducers.

INTRODUCTION

IFNs are regulatory proteins with protean biological activities. Effects on cell function include promotion of differentiation, inhibition of cell proliferation, and augmentation of immune effector cell function (1–3). Each of these has been postulated to result in the antitumor activity of IFNs for human malignancy and for spontaneous, induced, and transplantable murine tumors. Which, if any, of these effects on cell function contribute to tumor growth inhibition remains, however, unclear (4, 5). In most animal tumors, IFNs have inhibited tumor growth rather than decreased tumor size (6–8). Furthermore, in tumor-bearing mice treated with IFNs, an identified histopathological change has been a pattern characteristic of ischemic necrosis (9). We have, therefore, considered another component of neoplastic growth, angiogenesis, as a possible site of IFN action.

Tumors require not only proliferation of malignant cells but also continuous expansion or proliferation of vascular components. Transplanted tumors grow rapidly only after they develop a vascular supply from the host (10). Neoplastic cells induce a complex network of vessels and capillaries through production of tumor angiogenesis factors (11–13). Angiogenesis can also be initiated by allogeneic lymphocytes as an expression of local graft-versus-host reaction (14, 15). Subsequently, media from secondary mixed leukocyte cultures were shown to contain lymphokine(s) which stimulated endothelial cell migration and proliferation (16).

In this study, we assessed effects of IFNs on vascularization initiated by implantation of tumor cell lines in mouse skin and cornea. Tumor cells were treated with IFNs, either in vivo after inoculation or in vitro prior to implantation. To assess effects in other biological settings, LIA as well as vascular activity occurring during DTH was also evaluated.

MATERIALS AND METHODS

Mice

Five- to 6-week-old female C3H/He, DBA/2, and outbred Swiss mice were purchased from Sprague-Dawley (Indianapolis, IN) and Madison, WI. They were housed in suspended metal cages with no more than 5 mice/cage. They were fed Wayne Rodent Blox (Continental Grain Co., Chicago, IL) and HCl-acidified (pH 2.7–3) tap water ad libitum and were placed in rooms with controlled temperature (22.2–24.4°C), humidity (40%), and 12-h light-dark cycles. Mice were only used 1 or more weeks after arrival from the supplier.

Tumors

Sarcoma 180 used for corneal grafts was a gift from the Frederick Cancer Research Facility, National Cancer Institute. It was passaged s.c. by a trochar once every 2 weeks in outbred Swiss mice. L1210R murine leukemia, originated by Dr. I. Gresser (17) and resistant to the antiproliferative effects of IFN, was received from Dr. H. Ankel of the Medical College of Wisconsin, Milwaukee, WI. Cells were grown in RPMI 1640 (Gibco) supplemented with 20% heat-inactivated fetal bovine serum, 10 μM 4-2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and 50 μg/ml gentamycin in 25-cm² flasks (Costar). L1210R cells were passaged twice per week. Mouse bladder tumor (MBT-2) was kindly provided by Dr. Mark Soloway (University of Tennessee, Memphis, TN).

Human transitional cell carcinoma of the bladder RT-4, human Chang hepatoma cells, and murine transitional cell carcinoma of the...
bladder MBT-2 were grown as monolayers in 75-cm² polystyrene flasks (Corning Glass Works, Corning, NY). Origin and growth in vitro has been previously described (18, 19). For implantation, cells were harvested by treatment with trypsin-EDTA for 1-5 min to allow for cell detachment, washed twice with PBS, and suspended in PBS so that the required number of viable cells was delivered by injecting 0.1 ml.

Drugs

Human IFN-α (20) (Hoffmann LaRoche, Nutley, NJ), human IFN-β (21) (Trition/Cetus, Alameda, CA), and human IFN-γ (22) (Genentech, San Francisco, CA) were produced by recombinant technology and purified to homogeneity. They were diluted with medium to the desired concentration. Murine IFN-β was derived from several sources. In experiments involving in vivo treatment of host mice, murine IFN-β with specific activity of 3 × 10^6 units/mg, a gift from Dr. P. Lengyel of Yale University, was used (23). For experiments involving treatment of donors of spleen cell suspensions, we used murine Type I IFN with a specific activity of 6.4 × 10^6 units/mg protein, a gift from Dr. I. Gresser of the Institut de Recherches Scientifique sur le Cancer, Villejuif, France (24). Murine IFN-β (Lot 83011, Lee Biomolecular, San Diego, CA) with specific activity of 2.0 × 10^6 units/mg protein was used in the remaining experiments. It was diluted with glycine buffer except in experiments involving injections in the anterior eye chamber when it was diluted with 1% heat-inactivated fetal bovine serum in minimal essential medium to avoid excessive acidity for the cornea.

Poly I:poly C (Lot 124723) and poly A:poly U (Lot 84211) were purchased from PL Biochemical, Milwaukee, WI. Each was weighed and allowed to dissolve in PBS at 4°C overnight for a final concentration of 1 mg/ml. Poly I:poly C was reannealed by heating in a 70°C water bath for 10 min; contents were mixed and returned to a 70°C bath for 5 min. The water bath was then allowed to cool gradually overnight. An aliquot of the solution was prepared and frozen.

Vascularization in the Dermis

TIA. MBT-2 cells were originally inoculated into syngeneic C3H hosts. Outbred Swiss mice were also evaluated in parallel with C3H mice. Results were identical in both strains; thus MBT-2, L1210, human RT-4, and Chang hepatoma cells were inoculated in Swiss mice since their skin usually has a background pattern of vasculature which interferes less with assessment of angiogenesis. Mice were anesthetized with Avertin (tribromoethanol). Tumor cells of human or murine origin were inoculated in the volume of 0.1 ml intracutaneously in the midlateroventral trunk region. A drop of 0.2% trypan blue was added to the cell suspensions to help identify the injection sites. Within each experiment, tumor cell lines were inoculated at the same body location with control and treated cells injected in exactly corresponding locations of the abdomen to avoid complications arising from regional differences in tumor growth (25). Day of inoculation was considered day 0.

On the day of assay, mice were killed with ether and the skin carefully separated to expose injection sites which were easily visible because of the color of trypan blue added to the cell suspension. Fat and other tissues covering vessels were carefully removed exposing a network of blood vessels associated with the scar region. Vessels were counted under a unified magnification of a dissecting binocular microscope. Any vessel in the scar area which contrasted with the background was included (14). Since the reaction site was usually three-dimensional, several planes of focus were evaluated. The extra vessels were straight when the counts were made soon after cell inoculation. As more time elapsed between time of inoculation and time of assay, extra vessels became more easily detectable since they tended to be more looped and winding. The mode of vessel counts has been described in detail (14). If the skin had a generalized increased background (<10% of Swiss mice), the mouse was discarded. Tumor cells and treatment were coded so that the individual performing quantitation of the vessels (Y. A. S.) was unaware of the treatment utilized.

LIA. Since irradiation increases angiogenic response to foreign lymphocytes (14–15), mice received 6 Gy from a cesium source at a dose rate of 0.2 Gy/min 1 h before lymphocyte inoculation. Allogeneic C3H mouse spleen cells were prepared by gently teasing the spleen in PBS and passing the resulting suspension in a Pasteur pipet to break the clumps. Spleen cells were then washed twice in PBS and adjusted to the required concentration. Human PBLs were obtained by layering diluted freshly drawn heparinized blood from volunteers on Ficoll-Hypaque. After centrifugation for 30 min at 400 × g, the rich in PBL was harvested, washed twice with PBS, and adjusted to 10^6 viable cells/ml. Vascular activity was then evaluated as in the case of TIA.

Corneal Grafts

Necrotic peripheral portions of Sarcoma 180 were cut in small explants about 0.1–0.2 mm³. Matched fragments were grafted in the corneas of female 6- to 7-week-old anesthetized Swiss mice according to the method described by Mathukurappan and Auerbach (26). Three–4 days later the eyes were examined for vascular activity at the limbus. Eyes with equal size grafts at equal distance from the limbus and eliciting equal initial vascular response were paired. Sometimes grafts in both eyes of the same mouse matched. More often eyes from different mice were considered to be more closely matched.

Murine IFN-β (Lee Biomolecular) and vehicle as the control were coded for corneal injection. Each of the paired eyes was given an injection in the anterior eye chamber of either IFN or control media. Injection in the anterior eye chamber of the mouse was carried out by introducing a 30-gauge needle at the rim of the cornea and parallel to it (27). Some of the aqueous humor was allowed to leak to make room for the IFN or vehicle. After about 5000 units of IFN (0.005 ml) were injected, the needle was withdrawn. The membranous iris formed a plug which prevented or slowed back leakage of the injected material. The eyes were then treated with PBS supplemented with penicillin and streptomycin (28). Eyes of anesthetized mice were examined every 2 or 3 days after treatment. The number of vessels directed from the limbus to the graft were counted. Vessel counts in the cornea were easier than in the skin since the cornea has no background vasculature. Since mice were not killed at the time of assay as was the case with the cutaneous reaction, response in the eye was followed over a longer period.

DTH

Swiss mice were sensitized by i.p. injections of 1 ml 0.025% SRBC on day 0. On day 4, 0.05 ml of 50% SRBC was injected intracutaneously. On successive days, mice were killed by ether, induration, vascular activity, and extent of decrease in heme color at the injection sites (as a measure of antigen clearance) were evaluated.

Statistical Methods

Student's 2-tailed t test was used to assess the statistical significance of difference between pairs of means.

RESULTS

Effect of IFNs on TIA in Mouse Dermis. Irradiated Swiss mice were inoculated intracutaneously with murine transitional cell carcinoma of the bladder MBT-2, human transitional cell carcinoma of the bladder RT-4, or human Chang hepatoma cells. Mice were treated on days 1 and 2 after tumor cell inoculation with 2.5 × 10^6 units of human IFN-αA or -βA. Vessel counts were assessed on day 3. Both human IFN-αA and -βA were effective in inhibiting TIA stimulated by human neoplastic cells (Table 1). Neither human IFN was effective in inhibiting angiogenesis induced by the murine tumor cells (Table 1). The observed inhibition of TIA may have resulted from the well-documented antiproliferative effects of IFN on inoculated tumor cells (2); fewer tumor cells as a result of IFN treatment might initiate less angiogenesis.

To focus more specifically on effects of IFNs on angiogenesis per se, vessel counts were assessed within 24 rather than 72 h
of implantation. At that time, the impact of increase in tumor cell number by proliferation, which might further stimulate angiogenesis in the control and magnify any difference from the treated group, should be minimal (28). Mice were treated twice, 1 and 20 h after cell implantation, with human IFN-β. Still a significant decrease in vessel count occurred with homologous IFNs (Table 2). Administration of mouse IFN-β did not inhibit vascular activity induced by human tumor cells (Table 2). Conversely, mouse IFN-β was effective in inhibiting the number of blood vessels induced by the murine tumor cells. Since only interferons from the species homologous to the implanted tumor cells were effective in inhibiting vessel counts, the results suggested a direct effect on the signal for angiogenesis.

To further determine whether inhibition by IFN of TIA was mediated by modulation of the signal to initiate angiogenesis, MBT-2 cells were treated in vitro with 200 units/ml of murine IFN-β for 3 days, then injected as coded samples into C3H mice. Vessel infiltration was markedly reduced at 24 h after implantation (Table 3). Tumor dimensions resulting from IFN-treated cells were generally bigger than those caused by untreated cells, suggesting increased size of cells treated with IFN (29). Similarly, treatment of Chang cells with human IFN-β was effective in reducing vessel counts. There was no effect of mouse IFN-β on human tumor cells or human IFN-β on mouse tumor cells (Table 3).

Incubation of MBT-2 cells in 200 or 2000 units/ml for only 24 h was insufficient to inhibit induction of angiogenesis (Table 4). Incubation in vitro with 5,000, 20,000 units/ml for 24 h or with 200 units/ml for 3 days was needed to result in a decrease of angiogenesis (Table 4). Treatment of tumor cells with 20,000 units/ml IFN had the same effect as with 5,000 units, inhibiting angiogenesis to about 50% of the control. Thus, escalation of the dose of IFN beyond 5,000 units did not achieve further reduction of angiogenesis.

Although results suggested an inhibition in production or release of an angiogenic factor, it was still possible that a decrease in tumor mass from the antiproliferative effects of IFN was resulting in the decrease of vessel counts. To eliminate this possibility, L1210R cells were incubated with or without 500 units/ml murine IFN-β for 3 days. As expected, IFN had no effect on cell counts (Table 5). Treated cells, when assayed 24 h after coded inoculation, induced significantly less vascular activity than did untreated cells in all experiments (Table 5). The antiproliferative potency of IFN-β was thus independent of the anti-angiogenesis effects.

Effects of IFNs on Tumor Corneal Grafts. Soon after grafting, tumor fragments spread to form a thin, slightly opaque sheet within the cornea. At the beginning, vessels from the limbus to the graft were quite distinct and formed a broad front (Fig. 1). As the tumor grew, vessels became interiorized within the tumor mass. Some of the responses were so vigorous that no effort was made to count individual vessels and the reaction was expressed as >50.

In 4 of 23 of the paired eyes, angiogenesis spontaneously stopped or regressed in both eyes. These eyes could not be assessed for the effects of IFN and were not included in the
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Fig. 1. Effects of treatment with murine IFN injected in the anterior eye chamber on angiogenesis initiated by intracorneal grafts of matched Sarcoma 180 tumor fragments. Left, vehicle; right, 5000 units IFN-β.

Table 6 Effects of IFN injected on day 3 or 4 in the anterior eye chamber on angiogenesis initiated by matched intracorneal grafts of Sarcoma 180 explants in Swiss mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Day</th>
<th>Vehicle (cohort pair no.)</th>
<th>IFNβ (cohort pair no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>&gt;50</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>50</td>
<td>&gt;50 50</td>
</tr>
<tr>
<td>11</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>10 10 50</td>
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<tr>
<td>13</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>7 0</td>
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<tr>
<td>3</td>
<td>6</td>
<td>20</td>
<td>25 15 8 15</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>&gt;50 5 25 17</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&gt;50</td>
<td>&gt;50 35 20</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>38</td>
<td>14 10 10 10 10 25 7 6 5 25 25</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>6 40 0 8 30 8 8 6 15 15</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>&gt;50</td>
<td>15 25 0p</td>
<td></td>
</tr>
</tbody>
</table>

* Five thousand units on day 3 or 4 after intracorneal graft. Eyes were paired earlier for equal vascular activity at the limbus.
* IFN-treated eye showed more vascular activity than the vehicle-treated eye.

Effects of IFNs on LIA. To further study the effects of IFNs on vascular response, angiogenesis induced by allogeneic and xenogeneic lymphocytes was determined (14, 15). LIA was markedly suppressed on both days 1 and 3 after mononuclear cell implantation in host mice treated twice in vivo with IFN (Tables 7–9). Similar to effects on TIA, greatest inhibition occurred in mice treated with IFN of the species homologous to the implanted lymphocytes. A slight but significant (P = 0.05) effect of human IFN-β on angiogenesis induced by murine lymphocytes was observed (Table 7). Murine IFN-β was, however, much more inhibitory. Conversely, there was no effect of murine IFN-β on vascular activity induced by human lymphocytes.

Murine IFN-β at 0.2 or 0.4 × 10^6 units injected twice in host mice inhibited angiogenesis caused by the inoculation of allogeneic spleen cells (C3H) in irradiated Swiss mice (Table 8). Angiogenesis initiated by MBT-2 cells was not affected by the lower dose of 0.2 × 10^6 units of IFN-β but was inhibited by the higher dose of 0.4 × 10^6 units (Table 8), indicating that more IFN was needed to inhibit TIA than to inhibit LIA.

analysis (Table 6). In fewer pairs (2 of 23), grafts in both eyes grew vigorously and were associated with strong angiogenesis. In 13 of 23 of the examined pairs, vascular proliferation in the eye treated with IFN was halted. Eventually in these eyes treated with IFN-β, tumor regressed, while the tumor grew very quickly in the control eye and was associated with marked angiogenesis (Fig. 1). In 4 of 23 pairs, the opposite occurred (Table 6). Thus, of 17 evaluable pairs of eyes, the coded injection of IFN-β inhibited angiogenesis in 13 (P < 0.049 by MacNemar’s test for discordant pairs).

In one experiment, effect of the potent IFN inducer poly I:poly C was assessed. Four mice were paired so that eyes had equivalent initial activity. One mouse of each of the 2 pairs received 10 mg/kg poly I:poly C i.p. on days 7, 9, and 11 after grafting. The other mouse of the pair received saline. In the saline-treated mice, intracorneal grafts continued to grow causing progressively severe angiogenesis. In both mice treated with poly I:poly C, progression of angiogenesis was halted (data not shown).

Table 7 Effects of human or mouse IFNs on LIA initiated by allogeneic mouse cells or xenogeneic human PBLs

<table>
<thead>
<tr>
<th>Cells</th>
<th>Vessel counts (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNβ</td>
</tr>
<tr>
<td>Human PBL</td>
<td>Human-β</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine C3H spleen</td>
<td>Human-β</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Murine-β</td>
<td></td>
</tr>
</tbody>
</table>

* Swiss hosts received 6.0 Gy 1 h before cell inoculation, and mice were assayed at 24 h; n = 6.
* Number of lymphocytes inoculated: human PBL, 1 × 10^6; C3H spleen, 0.75 × 10^6.
* One h after lymphocyte inoculation, 0.25 × 10^6 units IFN were given i.v. followed by the same dose i.p. after 20 h.

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were killed was consistently effective in inhibiting the ability of tumor cells and foreign lymphocytes, it was also inhibitory, resulting in more than 50% reduction in vessel counts (data not shown).

Table 9 Effects of in vivo treatment of donors of spleen cells with murine IFN or polyribonucleotides on the ability of their spleen cells to induce LIA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>n</th>
<th>Vessel counts (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IFN (-24 h)</td>
<td>4</td>
<td>43.1 ± 2.3</td>
</tr>
<tr>
<td>2</td>
<td>IFN (-24 h)</td>
<td>6</td>
<td>21.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>IFN (-3 h)</td>
<td>5</td>
<td>15.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Poly I:poly C (-24 h)</td>
<td>6</td>
<td>13.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Poly A:poly U (-24 h)</td>
<td>6</td>
<td>5.8 ± 0.8</td>
</tr>
</tbody>
</table>

* IFN-β at 0.15 x 10⁶ units i.p.; poly I:poly C or poly A:poly U at 10 mg/kg i.p.
* Swiss hosts were irradiated with 6 Gy 1 h before cell inoculation; assay on day 3.

LIA could also be suppressed by treatment of donors of allogeneic lymphocytes in vivo with either IFN or poly I:poly C. Injection of murine IFN 3 h before tumor cell proliferation was consistently effective in inhibiting the ability of their lymphocytes to induce LIA (Table 9). Treatment of the donor with 10 mg/kg poly I:poly C 24 h before they were killed caused an even more marked reduction in LIA (Table 9). Treatment of human PBLs with 100 units/ml IFN and 20 h after cell inoculation. Vascular response was assayed on day 3.

DISCUSSION

Neovascularization is essential for tumors to become established and to grow (10). IFNs inhibited angiogenesis elicited by murine or human tumor cells in both mouse skin and mouse cornea. Since the cornea is normally devoid of blood vessels, the vascular activity and induration were diminishing in the saline-treated mice while these parameters were increasing in mice treated with poly I:poly C. Treatment with poly I:poly C before primary or secondary challenge thus delayed and inhibited expression of DTH to SRBC, similar to the inhibition resulting from a primary antigen challenge with lymphocytes.

Table 10 Effects of in vitro treatment of human peripheral blood lymphocytes (0.75 x 10⁶) with human IFN on their ability to induce xenogeneic LIA in irradiated Swiss hosts

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>n</th>
<th>Vessel counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>(in vitro)</td>
<td></td>
<td>Untreated IFN-βα</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>18.3 ± 2.0†</td>
</tr>
<tr>
<td>48</td>
<td>8</td>
<td>17.3 ± 2.9</td>
</tr>
</tbody>
</table>

* Mean ± SE.
† Numbers in parentheses, P compared to untreated.

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effects, we tested the influence of IFNs on angiogenesis initiated by L1210R cells which are resistant to the antiproliferative effects of IFNs. In vitro treatment of these cells did not alter their proliferation but modulated their ability to induce angiogenesis.

IFNs also inhibited angiogenesis initiated by allogeneic or xenogeneic lymphocytes. Treatment of the donor of the allogeneic lymphocytes with murine IFN or the potent IFN inducer poly I:poly C reduced the ability of implanted lymphocytes to induce angiogenesis. The weak IFN inducer, poly A:poly U, was ineffective. In vitro treatment of lymphocytes with IFNs was also effective. Similar to the case of TIA, LIA was only affected by homologous IFNs. Inhibition of the vascular response induced by DTH suggests that IFNs may modulate angiogenic lymphokine production by T-cells responding to antigen. Thus IFNs or inducers inhibited the vascular response to both allogeneic and activated autologous lymphocytes.

Comparison of Effects of LIA and TIA Suggests That IFNs Inhibit LIA More Effectively Than TIA. In vitro treatment of PBL with 100 units/ml IFN- \( \beta \) for only 4 h was sufficient to significantly reduce induction of angiogenesis. Incubation with 5000 units/ml of IFN for 24 h or with 200 units/ml for 72 h was required to inhibit TIA. This result was supported by in vivo studies in which a high dose of IFN (0.4 \( \times 10^6 \) units) inhibited both LIA and TIA while a lower dose (0.2 \( \times 10^6 \) units) inhibited only LIA and had no effect on TIA. Since TIA was more difficult to inhibit than LIA, the signal for TIA may be more continuously produced and more potent than that of LIA (31).

We were unable to achieve more than a 50% inhibition of vascular activity. Escalating the dose in vitro above 5000 units/ml (Table 4) did not result in further reduction of vascular activity. More than one angiogenic factor produced by tumor cells has been described (13, 32). Conceivably, one factor may be sensitive to IFN action and the other(s) may not. Extracts from cartilage (33, 34), vitreous humor (35), antibodies to tumor angiogenic factor (36), vitamin A (37), protamine (38), and heparin in combination with hydrocortisone or other steroids (39, 40) have inhibited angiogenesis and suppressed tumor growth. All these inhibitors of angiogenesis do not affect factor production but primarily inhibit endothelial cell response (33–39). IFNs can inhibit angiogenesis not only by affecting endothelial cells (41) but also by suppression of the signal initiated by tumor cells or allogeneic lymphocytes. Thus, it is possible that IFNs might enhance the effectiveness of other inhibitors of angiogenesis.

As early as 1969, Levy et al. (42) suggested that poly I:poly C acts on blood vessels affecting tumor blood supply. It was suggested that the effect was primarily on vascular endothelium. Another cytokine, tumor necrosis factor, was also recently reported to modulate endothelial cell function in vitro (43, 44). Treatment with IFN has resulted in “ischemic necrosis” after Friend erythroleukemia cells were inoculated s.c. in DBA/2 mice (9), implying effects on the blood supply. Furthermore, treatment of glioblastoma patients with human leucocyte IFN-\( \alpha \) interfered with the “normal excessive proliferation of endothelial cells in the capillaries of the tumor and its surroundings” (45). We have now directly confirmed the inhibitory effects of IFNs on the process of angiogenesis. Inhibition of vascular proliferation is an additional mechanism by which IFNs may inhibit in vivo immunological response and tumor growth.

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