Inhibition of Angiogenesis by Interleukin-12 Is Mediated by the Interferon-Inducible Protein 10

By Cecilia Sgadari, Anne L. Angiolillo, and Giovanna Tosato

Interleukin 12 (IL-12), a multifunctional cytokine produced by macrophages and B-cell lines, induces interferon-γ (IFN-γ) production, stimulates growth of both T and natural killer cells, promotes Th1-type helper T-cell responses, and inhibits neovascularization. Because the human interferon-inducible protein 10 (IP-10) can also inhibit neovascularization, we tested whether IP-10, induced by IL-12 through the intermediate IFN-γ, might be a mediator of IL-12 angiogenesis inhibition. We report here that murine IL-12 profoundly inhibited basic fibroblast growth factor (bFGF)-induced Matrigel neovascularization in vivo, and that this effect of IL-12 was neutralized by systemic administration of antibodies to either murine IFN-γ or IP-10. Murine IL-12 induced murine IP-10 expression in mouse splenocytes, and human IFN-γ induced human IP-10 expression in purified human endothelial cells, suggesting that IL-12 can induce IP-10 expression in certain cells. These results document the important role of IP-10 as a mediator of angiogenesis inhibition by IL-12, and raise the possibility that IP-10 may also contribute to the antitumor effect of IL-12. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Mice, cells, reagents, and cytokines. Four- to six-week-old female BALB/c nu/nu mice (National Cancer Institute, National Institutes of Health [NIH], Frederick, MD or Taconic, Germantown, NY) maintained in pathogen-limited conditions were used throughout. Matrigel was prepared by H.K. Kleinman from the Engelbreth-Holm-Swarm tumor as previously described. Recombinant murine IL-12 was a kind gift of Genetics Institute, Inc (Cambridge, MA). Rabbit antiamineurine IP-10 antibody was a gift of J.M. Farber (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). A control rabbit serum was obtained from animals immunized with a human IL-6 peptide. A mouse monoclonal antibody (MoAb) to murine IFN-γ was a gift of David Finbloom (Center for Biologics Evaluation and Research [EBER], Bethesda, MD). A control mouse MoAb (specific for human IgG) was purchased from Cappel (Organon Technika Co, Durham, NC). bFGF was purchased from R&D Systems (Minneapolis, MN). Human umbilical vein endothelial cells (HUVEC), obtained from the American Type Culture Collection (Rockville, MD), were maintained in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD), 15% heat-inactivated fetal bovine serum (Intergen Co, Purchase, NY), 20 U/mL porcine preservative-free heparin (Squibb-Marsham, Inc, Cherry Hill, NJ), and 100 μg/mL endothelial cell growth supplement (ECGS; a crude extract of bovine neural tissue containing bFGF and acidic FGF; Calbiochem-Novabiochem Corp, La Jolla, CA).

In vivo matrigel assay. This assay was performed as described. Briefly, Matrigel (liquid at 4°C) was mixed with 150 ng/mL bFGF alone or in combination with either IP-10 or IL-12 at a final concentration of 400 ng/mL and 100 ng/mL, respectively. Matrigel alone, with bFGF, or with bFGF plus the test cytokine (total volume 0.5 mL), was injected subcutaneously into the midabdominal region of BALB/c nude mice. After injection, the Matrigel polymerized to form a plug. After 2 days, the animals were killed, and the Matrigel plugs were removed together with the abstract epidermis and dermis, fixed in 10% neutral buffered formalin solution (Sigma Chemical Co, St Louis, MO), paraffin-embedded, cut, and stained with hematoxylin and eosin.

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followed by treatment of the mice with intraperitoneal (i.p.) injection of CsCl centrifugation method, as described.43 Levels of cytokine from murine splenocytes or HUVEC by the guanidine thiocyanate/mercaptoethanol method were added individually to the Matrigel injection mixture (0.5 mL). Mice were also injected individually ip (0.3 mg/mouse) on days 1, 3, and 5 after subcutaneous Matrigel injection. The neutralizing murine IgG MoAb to murine IFN-γ and the control murine IgG MoAb (Cappel) were added individually to the Matrigel injection mixture (0.5 mL). Matrigel plus 150 ng/mL bFGF plus 100 ng/mL murine IL-12 (1 μg/mouse) at a final concentration of 1 mg/mL; they were also injected individually ip at a dose of 1 mg/mouse in 0.3 mL of a formulation buffer (saline solution containing 50 mg/mL human serum albumin and 5 mg/mL mannitol; a gift of Dr J. Henkin, Abbott Laboratories, Abbott Park, IL). The neutralizing rabbit antisera to murine IP-10 and the control rabbit antisera (antihuman IL-6 peptide) were added individually to the Matrigel injection mixture at a dilution of 1 μg/mL. Together with the Matrigel plus bFGF plus IL-12 injection mixture, the control rabbit antiserum (antihuman IL-6 peptide) were added individually to the Matrigel injection mixture at a dilution of 1 μg/mL. Matrigel plugs impregnated with bFGF (150 ng/mL), Matrigel plus bFGF (150 ng/mL) plus human IP-10 (400 ng/mL), or Matrigel plus bFGF (150 ng/mL) plus murine IL-12 (100 ng/mL), all in a final volume of 0.5 mL. Matrigel plugs, removed from the mice 6 days after injection and processed for histology, were analyzed for cell invasion. Previous studies have established that the cells invading bFGF-impregnated Matrigel plugs are mostly endothelial cells.44 In a representative experiment (Fig 1), plugs of Matrigel alone contained few infiltrating cells, accounting for a mean surface area of 104 μm²/field (4 × 10⁴ μm²). Matrigel plugs impregnated with bFGF contained 12.4-fold more infiltrating cells compared with plugs of Matrigel alone (mean surface area 1290 μm²/field), indicative of bFGF-induced neovascularization. Matrigel plugs with IL-12 added together with bFGF contained significantly fewer cells compared with Matrigel plus bFGF plugs (mean surface area 269 μm²/field, P < .001). These results are indicative of IL-12–induced inhibition of neovascularization. As reported previously, IP-10 suppressed bFGF–induced neovascularization of Matrigel plugs (mean surface area 412 μm²/field). These experiments confirm, in another model system, that IL-12 can act as a potent inhibitor of neovascularization in vivo. Because these studies used athymic mice, the results further indicate that the antiangiogenic effect of IL-12 is not dependent on the participation of immune T cells.

To assess whether the antiangiogenic activity of IL-12 could be caused by IP-10 induction, we first looked for IFN-γ.

<table>
<thead>
<tr>
<th>RNA Detected</th>
<th>Genbank Accession no.</th>
<th>Primer Pair (upper strand: lower strand)</th>
<th>Size (bp)</th>
<th>Temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>m-IP-10</td>
<td>M86829</td>
<td>ACCATGAAACCCAAGTGCTGCGCGTC; GCTTCACCTGAGTATAAGGACCTCT</td>
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<td>84</td>
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<tr>
<td>m-IFN-γ</td>
<td>M28621</td>
<td>TGGGGCTGCTGCTGAAACTAGA; TGAGGGCTCCGCTGTCGACCCTGTG</td>
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<td>64</td>
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<tr>
<td>m-G3PDH</td>
<td>M32599</td>
<td>GCCCCCAAGAGACTGTTGATGTCG; CATGGGGTAAACGTTGCAACGAC</td>
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<tr>
<td>h-IP-10</td>
<td>X02630</td>
<td>GGACCTCCAGCTCAGGCCAC; CAGCCCTCTTGTGTTGACATCC</td>
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<td>55</td>
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<tr>
<td>h-IFN-γ</td>
<td>M29383</td>
<td>TGGGTCTCTTGGCTGTTACTGCC; TACTGGAGATGCTCTTGACCTCGA</td>
<td>453</td>
<td>64</td>
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</table>

Abbreviations: m, murine; h, human.
INHIBITION OF ANGIOGENESIS BY IL-12

**Fig 1.** In vivo effects of IL-12 and IP-10 on endothelial cell invasion of Matrigel. Female BALB/c nu/nu mice were injected subcutaneously with either Matrigel alone, Matrigel plus bFGF, Matrigel plus bFGF and murine IL-12, or Matrigel plus bFGF and human IP-10. Plugs were removed 6 days after injection and processed for histology, and angiogenesis was quantified. The results are expressed as mean Matrigel surface area (μm²) occupied by cells/field (4 x 10⁵ μm²). Each data point reflects the mean (±SD) surface area in groups of three mice; six readings on nonoverlapping Matrigel fields were quantitated per plug.

γ and IP-10 gene expression in athymic mouse splenocyte cultures induced with murine IL-12. To this end, splenocytes (3 x 10⁶ cells/mL) from two 6-week-old BALB/c nu/nu mice were incubated for 5 to 30 hours in either medium alone, LPS (100 ng/mL), or IL-12 (10 ng/mL). At the end of culture, the presence of IFN-γ and IP-10 mRNAs was assessed through reverse transcription and PCR amplification using specific primers followed by gel electrophoresis of the PCR products. The results show that murine IL-12 induces both murine IFN-γ and murine IP-10 gene expression in splenocytes after 5, 24, and 30 hours of exposure (Fig 2A). Because both IFN-γ and IP-10 mRNAs were detectable at the earliest time point (5 hours) tested after IL-12 stimulation, a temporal relationship between IFN-γ and IP-10 induction by IL-12 could not be established. However, primary cultures of human umbilical cord endothelial cells (HUVEC) were incubated in medium alone, or in medium supplemented with either human IL-12 (10 ng/mL) or human IFN-γ (100 ng/mL) to assess whether IFN-γ is required for IP-10 gene expression. Cultures were obtained after a 6- or 24-hours incubation, RNA was extracted, and expression of human IP-10 was examined after reverse transcription and PCR amplification using specific primers. IFN-γ induced IP-10 expression in HUVEC at both time points whereas IL-12 did not (Fig 2B). Consistent with the notion that endothelial cells are not a source of IFN-γ, IL-12 did not induce IFN-γ mRNA expression in HUVEC (not shown). This failure of IL-12 to induce IP-10 expression in HUVEC could not be attributed to IL-12 being inactive because in parallel assays human IL-12 stimulated appropriately phytohemagglutinin (PHA) preactivated T cells (not shown). These findings show that IL-12 can stimulate IP-10 and IFN-γ expression in splenocyte cultures, and suggest that IL-12 cannot induce IP-10 expression in endothelial cells directly, but rather through the intermediate IFN-γ.

The observation that murine IL-12 is an inducer of murine IP-10 in vitro suggested the possibility that IL-12, present within the Matrigel, might also induce IFN-γ and IP-10 production locally. If so, the antiangiogenic effect of IL-12 could be indirect, and result from secretion of IP-10, a known inhibitor of angiogenesis in vivo. Therefore, we assessed the effects of murine IL-12 on angiogenesis in vivo in mice simultaneously treated with neutralizing antibodies to either murine IFN-γ or IP-10. As shown (Table 2), in each of three mice, a neutralizing mouse MoAb to murine IFN-γ, but not a control antibody, abolished IL-12–induced inhibition of neovascularization (results with the MoAb to IFN-γ are significantly different from those without antibody or with the control MoAb; P < .0002 in each case). This finding shows that IL-12 requires IFN-γ to inhibit angiogenesis in this system. In addition, a rabbit antiserum to murine IP-10 reduced IL-12–induced inhibition of neovascularization from 82.8% to only 21.2%, indicative of a prominent role played by IP-10 in this process (results with the antiserum to IP-10 are significantly different from those without the antibody, P = .003, or with the control antiserum, P = .03). Consistent with our previous findings, IP-10 at 400 ng/mL inhibited bFGF-inhibited neovascularization of Matrigel plugs by 52%. These results confirm that the antiangiogenic effects of IL-12 are entirely mediated by IFN-γ, and further establish the important role played by IP-10 as a downstream mediator of inhibition of neovascularization by IL-12.

**DISCUSSION**

The present experiments show that IP-10 is the downstream molecule primarily responsible for inhibition of angiogenesis by IL-12. In addition, these experiments confirm that inhibition of angiogenesis by IL-12 is mediated by IFN-γ, an inducer of IP-10. In these experiments, the antibody to murine IFN-γ completely neutralized the effects of IL-12, whereas the antiserum to murine IP-10 substantially reduced, but not completely removed, IL-12 inhibition. Also, IL-12 inhibited angiogenesis to a somewhat greater extent than IP-10. One interpretation of these experiments is that IL-12 is a more potent inhibitor of angiogenesis than IP-10, capable of inducing IP-10 along with other IFN-γ–inducible antiangiogenic compounds, and perhaps IFN-γ itself. If so, it is interesting to note that the angiogenesis inhibitor PF4, a member of the α chemokine subfamily like IP-10, is not induced by IFN-γ, and that the IFN-γ–inducible chemokines Mig and IL-8 did not display antiangiogenic activity in preliminary in vivo assays. In addition, results with IFN-γ have been variable, and when IFN-γ treatment caused inhibition of angiogenesis, IP-10, induced by IFN-γ in endothelial cells, could have been involved. However, it is still possible that compounds other than IP-10, induced directly
Effects of IL-12 on IFN-γ and IP-10 gene expression. (A) Splenocytes (3 x 10^6/mL) were cultured for 5, 24, and 30 hours in either medium alone or medium supplemented with murine IL-12 (10 ng/mL) or lipopolysaccharide (100 ng/mL). At the end of the culture, RNA was extracted and analyzed by RT-PCR. Amplification cycles used were as follows: murine IP-10, 22 cycles; murine IFN-γ, 30 cycles; and G3PDH, 21 cycles. (B) Confluent monolayers of HUVEC were cultured for 6 or 24 hours in either medium alone or medium supplemented with either human IL-12 (10 ng/mL) or human IFN-7 (100 ng/mL). After culture, RNA was extracted and analyzed by RT-PCR. Amplification cycles were as follows: human IP-10, 22 cycles; and G3PDH, 21 cycles.

Table 2. Effects of Neutralizing Antibodies to Murine IFN-7 or IP-10 on IL-12-Induced Inhibition of Endothelial Cell Invasion of Matrigel In Vivo

<table>
<thead>
<tr>
<th>Additions to Matrigel</th>
<th>Mean Surface Area (µm^2)</th>
<th>Occupied by Cells/Field (± SD)</th>
<th>% Inhibition of Neovascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>456 (252)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF</td>
<td>2,795 (546)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF + IL-12</td>
<td>858 (288)</td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td>bFGF + IP-10</td>
<td>1,673 (242)</td>
<td>52.0</td>
<td></td>
</tr>
<tr>
<td>bFGF + IL-12 + anti–IFN-γ IgG</td>
<td>3,309 (308)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>bFGF + IL-12 + mouse IgG</td>
<td>682 (177)</td>
<td>90.3</td>
<td></td>
</tr>
<tr>
<td>bFGF + IL-12 + anti-IP-10</td>
<td>2,299 (555)</td>
<td>21.2</td>
<td></td>
</tr>
<tr>
<td>bFGF + IL-12 + rabbit serum</td>
<td>1,179 (212)</td>
<td>69.1</td>
<td></td>
</tr>
</tbody>
</table>

BALB/c nu/nu female mice (3 mice per condition) were injected subcutaneously with either Matrigel alone, Matrigel plus bFGF (150 ng/mL), Matrigel plus bFGF (150 ng/mL) and murine IL-12 (100 ng/mL) alone or together with control or neutralizing antibodies to either IFN-γ or IP-10, or Matrigel plus bFGF (150 ng/mL) and human IL-10 (400 ng/mL). The mice injected subcutaneously with Matrigel plus IL-12 plus antibodies were also injected intraperitoneally with the appropriate antibodies on day 1 and again 3 and 5 days later. The Matrigel plugs were removed 6 days after inoculation and processed for histology. The results reflect the mean (± SD) surface area (µm^2) occupied by cells/field (4 x 10^4 µm^2) for each group of mice.

or indirectly by IL-12, contribute to inhibition of angiogenesis by IL-12. An alternative possibility is that the IP-10 used here may not express its full biologic potential, and/or that the antiserum to murine IP-10 may not display optimal neutralization capacity for murine IP-10, at least in comparison to the anti IFN-γ antibody used in parallel.

Only recently has inhibition of angiogenesis been identified as a biologic property of IP-10. In two distinct in vivo models, the rat corneal micropocket and the mouse Matrigel models, IP-10 in nanogram quantities inhibited neovascularization induced by either IL-8 or bFGF. Although the mechanisms underlying this biologic property of IP-10 have not been investigated in detail, in vitro data have shown that in nanogram concentrations IP-10 can inhibit endothelial cell chemotaxis and differentiation into tubelike structures, but does not affect cell proliferation. Microgram concentrations of IP-10 were required for endothelial cells to be growth inhibited, suggesting that unlike other inhibitors of angiogenesis, IP-10 may not suppress neovascularization primarily through regulation of endothelial cell proliferation.

Other biologic properties attributed to IP-10 include inhibition of colony formation by human bone marrow hematopoietic cells, chemoattraction of human monocyte and activated T cells, stimulation of T-cell adhesion to endothelial cells, suppression of tumor growth in vivo, and calcium flux in activated T lymphocytes. Because a variety of cell types, including mononuclear cells, fibroblasts, keratinocytes, endothelial cells, and T cells, are known to express the IP-10 gene in response to IFN-γ or other signals, one could expect IL-12 to express all the biologic properties of IP-10 because of its induction of IFN-γ. The antitumor activity is shared by IL-12 and IP-10, but other properties are either not shared or not known to be shared. In addition to underlying the complexities of regulation of cytokine ef-
fects, particularly those derived from pleiotropic cytokines such as IL-12, this observation raises the possibility that the antitumor activity of IL-12 might be secondary, in part, to its antiangiogenic activity mediated by IFN-γ and, downstream, by IP-10.

Tumor cells need to attract new vessels to grow locally and to produce distant metastasis.23 Consistent with this notion, a number of angiogenesis inhibitors, including PF429 and angiostatin,30 have proved effective in reducing tumor cell growth in experimental tumor models. The antitumor effect of IL-12 was characterized as associated with elevations of systemic IFN-γ levels, and was markedly reduced or eliminated by administration of antibodies to IFN-γ. This suggested that IFN-γ is essential to the antitumor effects of IL-12.23 Independently, IP-10 has also shown antitumor activity.51 Although IP-10 expression was not assessed during IL-12 treatment of tumor-bearing animals, we would suspect that the antiangiogenic effects of IP-10 contribute to the antitumor effects of both IP-10 and IL-12. Although this needs experimental confirmation, additional mechanisms may also play a role, as suggested by the observation that, in general, the antitumor response induced by IL-12 or by IP-10 was T-lymphocyte-dependent.14,51 It was noted that total body irradiation or T-cell depletion inhibited the antitumor effects of IL-12, and that tumor growth in euthymic but not in nude mice was inhibited by IP-10.

Although further studies will be needed to characterize the mechanisms underlying the antitumor effects of IL-12, the present studies establish the important role played by IP-10 as a downstream mediator of the antiangiogenic effects of IL-12. A more complete understanding of cytokine networks will lead to more rational approaches to therapy.

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Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10

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