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

By Cecilia Sgadari, Anne L. Angiulliolo, 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.

INTERLEUKIN-12 (IL-12), a disulfide-linked heterodimer composed of two subunits with molecular masses of 35 kD and 40 kD, is a multifunctional cytokine produced by macrophages, B-cell lines, and other cells. IL-12 was shown to stimulate the proliferation of activated T and natural killer (NK) cells, to enhance specific and nonspecific cytolytic lymphocyte responses, to induce interferon-γ (IFN-γ) production by NK and T cells, and to promote the development of Th1-type helper T-cell responses. In murine models, IL-12 demonstrates antitumor activity toward a variety of experimental malignancies and exerts antimicrobial and antifungal activity toward various infectious agents.

Recently, IL-12 was reported to inhibit angiogenesis in an in vivo mouse model of corneal neovascularization induced by basic fibroblast growth factor (bFGF). Because this inhibition was neutralized by antibodies to IFN-γ, it was concluded that the antiangiogenic activity of IL-12 was attributable to IFN-γ induced by IL-12. But the effects of IFN-γ on angiogenesis have been variable, and both stimulation and inhibition of angiogenesis have been reported in different experimental systems.

Several other compounds have been reported previously to inhibit angiogenesis in vitro and/or in vivo, including thrombospondin, platelet factor (PF) 4, transforming growth factor β (TGF-β), IL-1α, IL-1β, interleukin-17, angiostatin, the tissue inhibitor of metalloproteinase, and the human pregnancy hormone HCG. Recently, we and others have reported that the interferon inducible protein 10 (IP-10), a member of the chemokine family, is an inhibitor of angiogenesis in vivo. Because IP-10 is induced by IFN-γ, and IL-12 is a potent inducer of IFN-γ, we have tested the possibility that IL-12 might inhibit angiogenesis indirectly, through induction of IFN-γ and, secondarily, IP-10.

MATERIALS AND METHODS

Mice, cells, reagents, and cytokines. Four- to six-week-old female BALB/c nu/nu mice (National Cancer Institute, National Institute 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 human IP-10 (0.06 endotoxin U/mg; Pepro Tech, Inc, Rocky Hill, NJ) was either purchased or provided by the National Cancer Institute. Recombinant murine IL-12 was a kind gift of Genetics Institute, Inc (Cambridge, MA). Rabbit antimurine 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 Teknika 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 preservive-free heparin (Squibb-Marsham, Inc, Cherry Hill, NJ), and 100 µg/mL endothelial cell growth supplement (ECGS; a crude extract of bovine endothelial cells, 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 6 days, the animals were killed, and the Matrigel plugs were removed together with the abstract epidermis and its use.

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followed by treatment of the mice with intraperitoneal (i.p.) injections of the same neutralizing or control antibody on days 1, 3, and 5 after subcutaneous Matrigel injection. The neutralizing murine IgG MoAb to murine IFN-Y 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) at a final concentration of 1 mg/mL; they were also injected individually i.p. 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 antiserum to murine IP-10 and the control rabbit antiserum (antihuman IL-6 peptide) were added individually to the Matrigel injection mixture at a dilution of 1:10; they were also injected individually i.p (0.3 mL/mouse) on days 1, 3, and 5 after subcutaneous Matrigel injection.

**Neutralization experiments.** In vivo neutralization experiments were performed by coinjection of a neutralizing or control antibody together with the Matrigel plus bFGF plus IL-12 injection mixture, followed by treatment of the mice with intraperitoneal (i.p.) injections of the same neutralizing or control antibody on days 1, 3, and 5 after subcutaneous Matrigel injection. The neutralizing murine IgG MoAb to murine IFN-Y 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) at a final concentration of 1 mg/mL; they were also injected individually i.p. 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 antiserum to murine IP-10 and the control rabbit antiserum (antihuman IL-6 peptide) were added individually to the Matrigel injection mixture at a dilution of 1:10; they were also injected individually i.p (0.3 mL/mouse) on days 1, 3, and 5 after subcutaneous Matrigel injection.

**Isolation of RNA and reverse transcriptase-mediated polymerase chain reaction (RT-PCR).** Total cellular RNA was isolated from either murine splenocytes or HUVEC by the guanidine thiocyanate/Tris-chloroacetic acid method, as described. Levels of cytokine mRNA were assessed by a semiquantitative RT-PCR as described. Briefly, RNA (4 μg) was reverse transcribed, using an Rnase H-<sup>−</sup> cDNA synthesis kit (Perkin Elmer Cetus, Norwalk, CT) in a reaction mixture (50 μL) containing cDNA, 200 μmol/L each dNTP, 1 μCi α-[<sup>32</sup>P]dCTP (3,000 Ci/mmol; Du Pont-NEN, Boston, MA), 1 μmol/L each primer, 5% dimethyl sulfoxide (DMSO; Sigma Chemical Co.), 1.5 mmol/L MgCl<sub>2</sub>, and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus) in reaction buffer supplied by the manufacturer. Primers are listed in Table 1. The number of amplification cycles chosen empirically for each primer pair was such that the maximum signal intensity for a set of samples was within the linear portion of a product versus template amplification curve. Amplifications were performed in a thermocycler (Gene Amp PCR System 9,600; Perkin Elmer Cetus) as follows: 94°C, 45 seconds; primer annealing temperature as specified in Table 1, 45 seconds; 72°C, 45 seconds); and maintenance at 4°C until analysis. Aliquots (50 μL) of each amplification reaction were analyzed by electrophoresis on 7% acrylamide (Long Ranger; AT Biochem, Malvern, PA) Tris-borate EDTA gels, followed by autoradiography and quantitation by Phosphorimage analysis (Molecular Dynamics, Sunnyvale, CA). DNA sizes were determined using mobility standards derived by T4 DNA polymerase end-labeling of Gel Marker DNA (Research Genetics, Huntsville, AL).

**Statistical analysis.** Arithmetic means, standard deviations, and Student's t-test were calculated by conventional formulas using Systat for the Macintosh (Systat Inc, Evanston, IL).

**RESULTS**

In initial experiments, we tested the effects of IL-12 on neovascularization using a mouse model in which subcutaneous injection of basement membrane (Matrigel) impregnated with bFGF induces new vessel formation. Athymic mice, selected to exclude potential contributions by immune T cells, were injected subcutaneously with Matrigel alone, Matrigel plus 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. In a representative experiment (Fig 1), plugs of Matrigel alone contained few infiltrating cells, accounting for a mean surface area of 104 μm<sup>2</sup>/field (4 × 10<sup>4</sup> μm<sup>2</sup>). Matrigel plugs impregnated with bFGF contained 12.4-fold more infiltrating cells compared with plugs of Matrigel alone (mean surface area 1,290 μm<sup>2</sup>/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 μm<sup>2</sup>/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 μm<sup>2</sup>/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-

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**Table 1. PCR Primers, Product Sizes, and Annealing Temperatures**

<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>
</tr>
</thead>
<tbody>
<tr>
<td>m-IP-10</td>
<td>M88629</td>
<td>ACCATGAAACCACAGTGCGCCGTC; GCTCTACCTCAAGTTGAAGGCCCT</td>
<td>311</td>
<td>64</td>
</tr>
<tr>
<td>m-IFN-γ</td>
<td>M28621</td>
<td>TGCGGCTATCTGACAGCAATGA; GAGAGCTGTCGGCTGACCTGTG</td>
<td>413</td>
<td>64</td>
</tr>
<tr>
<td>m-G3PDH</td>
<td>M32599</td>
<td>GCCACCCAGAGACTTGATGATGC; CATGTAGGCCATAGGCTCACAC</td>
<td>446</td>
<td>56.5</td>
</tr>
<tr>
<td>h-IP-10</td>
<td>X02630</td>
<td>GGAACCTCAGCTCTGACGACC; CAGCCTCTGTGTTGCTCACC</td>
<td>375</td>
<td>55</td>
</tr>
<tr>
<td>h-IFN-γ</td>
<td>M29383</td>
<td>TGGGTCTTCTTTGCTGTGATGCC; TACTGGAGATCCTTCGACCTGA</td>
<td>453</td>
<td>64</td>
</tr>
</tbody>
</table>

**Abbreviations:** m, murine; h, human.
Inhibition of angiogenesis by IL-12

γ and IP-10 gene expression in athymic mouse splenocyte cultures induced with murine IL-12. To this end, splenocyte cultures (3 × 10^6 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...
Fig 2. Effects of IL-12 on IFN-γ and IP-10 gene expression. (A) Splenocytes (3 x 10⁶/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-γ (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.

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 neutralizing 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 to 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. Consistent with this notion, a number of angiogenesis inhibitors, including PF4 and angioatin, have proved effective in reducing tumor cell growth in experimental tumor models. The antitumor effect of IL-12 was characterizedly 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. Independently, IP-10 has also shown antitumor activity. 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. 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 most nu/nu 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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