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

By Cecilia Sgadari, Anne L. Angioliollo, 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. Kleiman from the Engelbreth-Holm-Swarm tumor as previously described. 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 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), and 100 U/mL 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 GGF; 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 dermis and dermis, fixed in 10% neutral buffered formalin solution (Sigma Chemical...
followed by treatment of the mice with intraperitoneal (i.p.) injection of CsCl centrifugation method, as described. Levels of cytokine were also injected individually i.p. on days 1, 3, and 5 after subcutaneous Matrigel injection. The neutralizing murine IgG MoAb to murine IFN-γ was 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 dilution of 1:10, 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. 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 control rabbit antiserum (anti-human 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/cesium chloride purification 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–reverse transcriptase (Superscript; Life Technologies, Inc, Gaithersburg, MD) according to the manufacturer’s conditions. The resultant cDNA was immediately diluted with H2O to a final volume of 200 μL, without heating or RNase H treatment. PCR was performed in thin-wall reaction tubes (Perkin Elmer Cetus, Norwalk, CT) in a reaction mixture (50 μL) containing cDNA, 200 μmol/L each dNTP, 1 μCi α-[32P]dCTP (3,000 Ci/mmol; Dupont-NEN, Boston, MA), 1 μmol/L each primer, 5% dimethyl sulfoxide (DMSO; Sigma Chemical Co), 1.5 mmol/L MgCl2, 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).

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

<table>
<thead>
<tr>
<th>RNA Detected</th>
<th>Genbank Accesion 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>M86829</td>
<td>ACCATGAAACCAAGTGCGCCGTC; GCTTCCTCGAGGAGCGGCTT</td>
<td>311</td>
<td>64</td>
</tr>
<tr>
<td>m-IFN-γ</td>
<td>M52821</td>
<td>TGGGCTGCTCGGAGAGGCTA; CGAACAGCTGGGAGGCTT</td>
<td>413</td>
<td>64</td>
</tr>
<tr>
<td>m-G3PDH</td>
<td>M32599</td>
<td>GCACCCCAAGAAGCTGGTGGTGGC; CATGTAGGCCATGGCTACAC</td>
<td>446</td>
<td>56.5</td>
</tr>
<tr>
<td>h-IP-10</td>
<td>X02630</td>
<td>GGAACCTCAGCTCTGACAC; CAGCAGGTTGCTGTTGACCC</td>
<td>375</td>
<td>55</td>
</tr>
<tr>
<td>h-IFN-γ</td>
<td>M29383</td>
<td>TGGTGTCTTCTGCTGTATGCC; TACTTTAGATGCTCTGACCTGA</td>
<td>453</td>
<td>64</td>
</tr>
</tbody>
</table>

Abbreviations: m, murine; h, human.

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 μm²/field (4 × 10⁴ μm²). Matrigel plugs impregnated with BFGF contained 12.4-fold more infiltrating cells compared with Matrigel alone (mean surface area 1,290 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-
INHIBITION OF ANGIOGENESIS 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 × 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.

Table 2. Effects of Neutralizing Antibodies to Murine IFN-γ 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 (µm²) Occupied by Cells/Field (±SD)</th>
<th>% Inhibition of Neovascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>456 (252)</td>
<td>—</td>
</tr>
<tr>
<td>bFGF</td>
<td>2,795 (546)</td>
<td>—</td>
</tr>
<tr>
<td>bFGF + IL-12</td>
<td>858 (288)</td>
<td>82.8</td>
</tr>
<tr>
<td>bFGF + IP-10</td>
<td>1,673 (242)</td>
<td>52.0</td>
</tr>
<tr>
<td>bFGF + IL-12 + anti-IFN-γ IgG</td>
<td>3,309 (308)</td>
<td>0</td>
</tr>
<tr>
<td>bFGF + IL-12 + mouse IgG</td>
<td>682 (177)</td>
<td>90.3</td>
</tr>
<tr>
<td>bFGF + IL-12 + anti-IP-10</td>
<td>2,299 (555)</td>
<td>21.2</td>
</tr>
<tr>
<td>bFGF + IL-12 + rabbit serum</td>
<td>1,179 (212)</td>
<td>69.1</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 IP-10 (400 ng/mL). The mice injected subcutaneously with Matrigel plus IL-12 plus antibodies were also injected intraperitoneally with the appropriate antibodies on days 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 (µm²) occupied by cells/field (4 × 10⁴ µm²) for each group of mice.
effects, 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 angiostatin, have proved effective in reducing tumor cell growth in experimental tumor models. The antitumor effect of IL-12 was characterizedly associated with elevations of specific 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 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.

ACKNOWLEDGMENT

The authors thank Drs J. Farber and D. Finlbloom for generously donating the antibodies; Dr G. Gupta for performing statistical analyses; Drs H. Klinman and J. Farber for critically reviewing the manuscript; and Dr P. Burd, M. Letzing, and H. Downing for their support.

REFERENCES


13. Seder RA, Gazzinelli A, Sher A, Paul WE: IL-12 acts directly on CD4⁺ T cells to enhance priming for IFN-γ production and diminishes IL-4 inhibition of such priming. Proc Natl Acad Sci USA 90:10188, 1993


18. Tripp CS, Wolf SF, Unane ER: Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc Natl Acad Sci USA 90:3725, 1993


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

C Sgadari, AL Angiolillo and G Tosato