Direct in Vitro Evidence and in Vivo Analysis of the Antiangiogenesis Effects of Interleukin 12

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ABSTRACT

As an antitumor agent, interleukin-12 (IL-12) has been revealed to be a key regulator of the immune response, particularly that involving CTL and natural killer (NK) cells. We report herein the antiangiogenesis effect of IL-12 on human as well as murine tumors in NK-depleted severe-combined immunodeficient mice using fibroblasts genetically engineered to secrete this cytokine. Although the in vitro growth of tumor cells was not affected by the presence of IL-12, coinoculation of IL-12-secreting fibroblasts strongly inhibited tumor growth in immunodeficient mice. The neovascularization surrounding the tumor was remarkably inhibited in the area in which the IL-12-secreting fibroblasts were implanted, resulting in the suppression of tumor growth. Lectin staining in tumor sample sections also showed a significant reduction in the number of vessels. The RNA expression of IFN-γ and its inducible antiangiogenic chemokine IFN-γ-inducible protein 10 was stimulated in endothelial cells cultured with IL-12. It was also found that IL-12 down-regulated the expression of the endothelial cell mitogens vascular endothelial growth factor and basic fibroblast growth factor. The antitumor effects of IL-12 were accompanied by interesting histological changes consisting of a high degree of keratinization and apoptosis and a decrease in the proliferation rate of human tumors and extensive necrosis in the murine ones.

INTRODUCTION

IL-12 is a disulfide-linked heterodimeric cytokine composed of a light chain (p35) and a heavy chain (p40; Ref. 1). IL-12 is produced in vitro. However, it is not easy to demonstrate that the antiangiogenesis effect of this cytokine on the tumor vasculature. We analyzed the process of human and murine tumor angiogenesis in a skinfold chamber installed in SCID mice pretreated with anti-asialo GM-1 antibody to block the strong nonspecific immune response induced by IL-12 administration (9). NK depletion was tested by FACScan analysis. To assess the involvement of IFN-γ in the effects of IL-12, we administered anti-IFN-γ antibody in mice inoculated with IL-12/3T3. By RT-PCR, a very useful semiquantitative method for RNA detection, we investigated the cytokine network in the tumor microenvironment both in vivo and in vitro. Several lines of evidence, including the histopathological features in tumor tissue sections, indicated that IL-12 exerts its antiangiogenesis effect through the expression of IFN-γ in endothelial cells and downstream of IP-10, but also by the down-regulation of the endothelial cell mitogens VEGF and bFGF. In addition, using a vital microscopy system, we demonstrated the antiangiogenesis effect induced by paracrine delivery of IL-12 on an established tumor.

MATERIALS AND METHODS

Mice. Male, 6- to 8-week-old SCID mice (Fox Chase B-17/Icr-SCID Jcl), were kept in pathogen-reduced conditions, and skinfold chamber bearing mice were housed individually. We followed the NIH guidelines for the care and use of the research animals.

Cells and Culture Conditions. PK-1 human adenocarcinoma, established in our department (10), and C26 murine colon carcinoma cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 5% CO2 atmosphere at 37°C. Wild-type fibroblasts (NIH3T3) were retrovirally transfected with IL-12 (IL-12/3T3). The construction and characterization of the retroviral vector have been described by Zitvogel et al. (11). The infection of NIH3T3 and the selection of high producing clones were performed as described previously (12). KOP 2.16 endothelial cells, described elsewhere (13), were used for the in vitro experiments. Fibroblast and endothelial cells were cultured in DMEM low glucose, 10% FCS, and antibiotics were maintained in the same conditions as the tumor cells.

Cytokine Level Measurement. IL-12—secreted in vitro by fibroblasts, endothelial, or tumor cells—was measured by enzyme-linked immunosorbent assay using an ELISA kit for murine IL-12 (Endogen Inc., Woburn, MA).

Antibodies. Rabbit anti-asialo GM-1 antibody (Wako, Tokyo, Japan) was administered via the tail vein (100 μg/mouse) twice a week, the first time 3 days before the PK-1 inoculation. Anti-IFN-γ antibody (α-mIFN-γ R4-GA2), provided by Dr. Yagita (Juntendo University, Tokyo), was administered i.v. (200 μg/mouse) every 2 days.

In Vivo Tumor Growth. Five × 106 PK-1 cells were inoculated s.c. in the SCID mice. In another group, the same number of tumor cells were inoculated admixed with IL-12/3T3, and anti-IFN-γ antibody was administered to some of the mice (n = 5 in all three of the groups) The tumor volume was measured twice a week, in a blind fashion. Using a caliper, the diameters of the tumors were measured, and the tumor volume was determined by the formula: \( V = D \times d^2 \times 0.4 \), where \( V \) = tumor volume, \( D \) = biggest dimension, and \( d \) = smallest dimension.
Table 1  RT-PCR conditions

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primers</th>
<th>No. of cycles</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIP-10*</td>
<td>5’-GCGGTCATTTCTGCTCAT-3’ 3’-GGAAGTAGGGTTAAGTC-5’</td>
<td>30</td>
<td>57°C</td>
</tr>
<tr>
<td>VEGF</td>
<td>5’-AGGATTCTCGTCTCGTGG-3’ 3’-CACTTCGACAGTCTGCT-5’</td>
<td>30</td>
<td>55°C</td>
</tr>
<tr>
<td>bFGF</td>
<td>5’-GAAAAGGGGGCCTTTCCCT-3’ 3’-TCCGCTTCTCTTCTCCGC-5’</td>
<td>30</td>
<td>55°C</td>
</tr>
<tr>
<td>mIFN-γ</td>
<td>5’-CTCAGGTTTCTCTAGTGCTGA-T3’ 3’-ACATTCTGCTACCGCTTGGAA-5’</td>
<td>30</td>
<td>59°C</td>
</tr>
<tr>
<td>GSPDH</td>
<td>5’-GCCACATCGTAAAGGACCATGGG-3’ 3’-CTCTGTTTCTAGCGCATA-5’</td>
<td>30</td>
<td>57°C</td>
</tr>
</tbody>
</table>

* mIP-10, murine IP-10; mIFN-γ, murine IFN-γ.

In Vitro Growth Measurement. PK-1 cells were suspended in RPMI 1640 and 0, 1, 2, or 3 ml of IL-12/3T3 48-h culture supernatant were added. Also, we cocultured PK-1 (1 x 10⁵ cells) with either IL-12/3T3 or NIH3T3 using BIOCOAT Cell Culture inserts in 24-wells plates (Becton Dickinson Labware, Bedford, MA). The PK-1 cells were counted after 2 days.

In Vivo Microscopy. A simplified version of the dorsal skinfold chamber previously described (14) was designed in our laboratory and manufactured by Aoba Science Ltd. (Sendai, Japan). Chamber implantation, cell inoculation, tumor observation, and off-line analysis were performed as described previously (15). PK-1 or C26 (1 x 10⁶ cells) were implanted on the skin muscular layer. After 11 and 8 days, respectively, 5 x 10⁶ IL-12/3T3 and/or NIH3T3 were also implanted on the tumor. We also used FITC-dextran staining for vessel contrast enhancement.

FACScan Analysis. NK depletion after antiasialo GM-1 antibody administration was assessed using FACScan analysis for hepatocytes harvested from treated SCID mice.

RT-PCR. PK-1 (1 x 10⁵ cells) and KOP 2.16 (5 x 10⁵ cells) were cocultured in 6-cm dishes, and 1 ml of 48-h culture supernatant of 1 x 10⁶ IL-12/3T3 was added. Cells were harvested after 30 min, 120 min, and 24 h. Total cellular RNA was extracted from these cells and also from PK-1 tumor tissue samples using Rneasy Mini Kit (Qiagen KK, Tokyo, Japan). Ten-μl aliquots were reversed transcribed using a Rneasy H Minus RT (TOYOBO Co., Tokyo, Japan) and 100 ng of the resultant cDNA was amplified using primers with the nucleotide sequences, annealing temperatures, and number of amplification cycles shown in Table 1. Glycerol-3 phosphate dehydrogenase was used as an internal control. Ten-μl aliquots from the amplification reactions were electrophoresed on 3% agarose (FMC BioProducts, Rockland, ME) and Tris-acetate EDTA gels and were autoradiographed. We recovered the PCR products from the gel using SpinBind System (FMC BioProducts) and then inserted them into plasmid vectors using TA Cloning kit (Invitrogen Corporation, Carlsbad, CA). Plasmids were purified using Wizard MiniPreps Plus DNA Recovery System, and fragment sequencing was performed using the ALFexpress II DNA Analysis System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Histology. Tumor samples were fixed in phospho-lysine-paraformaldehyde overnight, embedded in OCT compound (Sakura Finetechno, Ltd., Tokyo, Japan) and stored at -80°C. We also used tumor samples fixed in 10% formalin and then embedded in paraffin. Standard H&E staining was performed on 3-5-μm sections from frozen and paraffin-embedded samples. Furthermore, lectin staining of endothelial cells on frozen and paraffin-embedded sample cuts was performed using biotinylated wheat germ agglutinin-WGA-1 (Vector Laboratories, Burlingame, CA), as previously described (16). The proliferation index was assessed by staining for human ki-67, an antigen expressed throughout the cell proliferation cycle but absent in quiescent (G0) cells. The staining protocol using mouse anti-ki-67 monoclonal antibody (Immunotech, Marseille, France) was as previously described (17). Sections from lymph nodes with reactive germinal centers were used as positive controls. Quantification of the staining results was made by determining the percentage of stained cells in at least 10 high-power fields.

TUNEL. Tdt-labeling (Apoptosis Detection Kit, Wako, Tokyo, Japan) of DNA fragmentation in frozen cut-tumor samples was used for the staining of the apoptotic cells within the tumor sections.

Statistical Analysis. All of the experiments were performed in duplicate or triplicate. Two-tailed Student’s t test was used for the statistical analysis of data. Values of P ≤ 0.05 were considered significant.

RESULTS

In Vitro Cytokine Production by Genetically Engineered Fibroblasts. IL-12/3T3s were able to secrete 12.06 ng/10⁶ cells/48 h, measured by ELISA in the culture supernatant, a level that was stable after four passages. No IL-12 expression was detectable in the culture supernatant of NIH3T3, C26, PK-1 or KOP.

NK Depletion Test. FACScan was performed for hepatocytes harvested from SCID mice 3 days after antiasialo GM-1 antibody administration. The NK cell population was depleted from 5.38% in the control group to 0.89% in the treated group.

In Vitro Effect of IL-12 on PK-1 Growth. IL-12 released by IL-12/3T3 had no effect in vitro on the proliferation of endothelial cells or tumor cells, irrespective of the IL-12 dose (Fig. 1). Moreover, PK-1 cells were inoculated s.c. after coculture for 4 days using inserts with either IL-12/3T3 or NIH3T3. Both of them were able to form...
tumors in SCID mice, as did the wild-type PK-1. No differences could be observed in the histological examinations of the resultant tumor sections (data not shown).

**In Vivo Tumor Growth of PK-1 Admixed with IL-12/3T3 in SCID Mice.** Tumor growth was strongly inhibited in NK-depleted SCID mice receiving s.c. inoculation of PK-1 admixed with IL-12 transduced fibroblasts; in contrast, growing tumors were observed in mice inoculated with wild-type PK-1 cells. Anti-IFN-γ antibody (i.p. 200 μl every 2 days) completely nullified the antitumor effect of IL-12 (Fig. 2).

**Tumor Angiogenesis Inhibition by IL-12 Observed in Skinfold Chamber.** The transparent-chamber model, together with the in vivo microscopy system, allowed accurate observation of the process of tumor angiogenesis. Tumor angiogenesis of PK-1 and C26 was observed in the implanted chambers, whereas vessel disruption was observed and no tumor was detected after the admixed inoculation of PK-1 cells with IL-12/3T3 (Fig. 3A-F). To clarify the antiangiogenesis effect of IL-12, we examined the interaction between tumor vessels and IL-12/3T3. Wild-type fibroblasts or IL-12-transfected fibroblasts were coinoculated with tumor cells or implanted in established tumors (day 8 in C26 tumors and day 11 in PK-1 tumors). An increase in the vessel disruption was detected in the admixed implantation group and at the IL-12/3T3 implantation site in the established tumors in SCID mice, as did the wild-type PK-1. No differences could be observed in the histological examinations of the resultant tumor sections (data not shown).
tumors (Fig. 3, G and H and Fig. 4, A-C). On the other hand, tumor angiogenesis was promoted in the tumor cell implantation groups with or without wild type.

Molecular Biology. The cytokine network of the tumor microenvironment was assessed by RT-PCR. The IP-10 RNA expression of cultured tumor and endothelial cells was induced by the presence of IL-12 as early as 30 min after stimulation, with a peak at 2 h and detectable secretion after 24 h (Fig. 5A). A decrease in the endothelial cell mitogens VEGF and bFGF, but not in acidic FGF RNA levels, was detected in the tumor and endothelial cells (Fig. 5, C, D, and F). The elevated RNA expression of IFN-γ and the expression of tumor necrosis factor α RNA in the presence of IL-12 were confirmed in vitro as well as in vivo (Fig. 5B and data not shown). Of particular interest, the IFN-γ expression in endothelial cells after IL-12 stimulation could be detected in vitro (Fig. 5B). IL-12 failed to change the expression of α, β, or FasL in vivo and in vitro (data not shown). G3PDH amplification was used as internal control (Fig. 5E).

Band DNA recovery from gel after electrophoresis followed by TA cloning, and plasmid sequencing confirmed the PCR findings.

Histopathological Aspects. PK-1 tumors from the IL-12/3T3 coinoculation group presented completely different histological features compared with the PK-1 tumors. IL-12/3T3 induced the increase of keratinization (Fig. 6, A-C) and apoptosis in PK-1 tumors, assessed by Tdt-labeling in frozen tumor sections (Fig. 6, H and I). In C26 tumors treated with IL-12/3T3, there were larger areas of necrosis as compared with the wild-type tumor. These effects were diminished in mice treated with anti-IFN-γ antibody. These features were paralleled by a diminished proliferation index in the tumor and endothelial cells. ki-67 staining (Fig. 6, J-L) revealed a significant decrease (P < 0.01) in the percentage of stained cells in PK-1 and IL-12/3T3 tumor sections (1.54 ± 0.68%) as compared with PK-1 tumors (6.60 ± 0.87%). However, the percentage was increased to 7.65 ± 0.99% in coinoculation tumors treated with anti-IFN-γ antibody.

Lectin (biotinylated WGA-1) staining of PK-1 tumor sample sections demonstrated that IL-12 diminished the numbers and the diameters of the tumor vessels (Fig. 6, D and F). In C26 tumors treated with IL-12/3T3, there were large areas of necrosis, and fewer vessels were observed by WGA-1 staining as compared with the wild-type tumor (Fig. 6, E and G).

DISCUSSION

IL-12 is well known as a key regulator of the immune system and has received much attention as an antitumor and antimetastasis agent. Recent reports regarding the involvement of IL-12 in the antiangiogenesis process have drawn attention to its potential for cancer therapy as tumor growth and metastasis are dependent on aberrant neovascularization (18).

Fibroblasts genetically engineered to secrete IL-12 are able to express a relatively high and stable level of the cytokine. Moreover, culturing, selection, and transduction of the fibroblasts are easy, and the probability of migration of these cells compared with tumor or lymphoid cells is low. We used a paracrine delivery system to prove the effect induced by IL-12 on the growth of a human adenocarcinoma, a poorly vascularized tumor in immune-deficient mice. The same setting was applied for a murine tumor, C26. The vital microscope system, together with the skinfold chamber model and genetically engineered fibroblasts, enabled us to precisely analyze the inhibitory effect of IL-12 on the tumor growth. Moreover, by using this system, we could analyze the antiangiogenesis effect of IL-12 on the established neovascularature that supplies blood to the growing tumor.

Despite the efficient blocking of the IL-12-induced nonspecific immune response by the administration of anti-asialo GM-1 antibody, IL-12 was able to inhibit the tumor growth. Our precise analysis of the neovascularization has revealed that this antitumor effect could be attributed to the antiangiogenesis effect of IL-12. Different ratios between the tumor cells and IL-12/3T3 fibroblasts were used, and this effect was confirmed at the lowest dose used (5 × 10^5 cells). However, wild-type fibroblasts did not exhibit any antitumor effects. Our data showed that the antiangiogenic effect of IL-12, which is secreted
Fig. 6. Histology and immunohistochemistry examination of tumors grown in SCID mice. H&E staining (×4) shows an increased degree of keratinization in tumors from the coinoculation (PK-1 + IL-12/3T3) group (A) compared with the control (PK-1) group (B) but not in tumors from the coinoculation group, which had received anti-IFN-γ antibody treatment (C). Lectin staining of tumor samples revealed an increase in the numbers and diameters of vessels in the coinoculation groups [PK-1 + IL-12/3T3 (D, ×20) and C26 + IL-12/3T3 (E, ×10)] sections as compared with wild-type tumors [PK-1 (F) and C26 (G)]. TUNEL for apoptosis shows an increased apoptotic rate in samples from the coinoculation group (H) compared with wild-type PK-1 tumor samples (I, ×40). Proliferation index using Ki-67 staining (×10) revealed that the presence of IL-12 decreased significantly the tumor proliferation rate in mice from the coinoculation group (J) but not in tumors from the mice treated with anti-IFN-γ antibody (K). L, control PK-1 tumor sample staining.
continuously by IL-12/3T3, occurred at levels that can also induce a strong immune response in immune competent mice (6, 19).

IL-12 has no effect on endothelial cell proliferation and migration (6). The inhibition of the tumor neovascularature development in our system was promoted by the local delivery of IL-12, which caused IFN-γ release by endothelial cells. Although the antiangiogenesis effect induced by IL-12 is IFN-γ-dependent, IFN-γ administration could not induce the same effect (20). After IL-12 stimulation, the levels of VEGF and matrix metalloproteinases showed a decrease in contrast to the increase of their tissue inhibitor, tissue inhibitor of metalloproteinase (TIMP) (7). IFN-γ was reported to induce the release of two chemokines, IP-10 and MIG (21), which exert their effect induced by IL-12 is IFN-γ.

Antiangiogenic properties by inhibiting endothelial cell chemotaxis release of two chemokines, IP-10 and MIG (21), which exert their effect induced by IL-12 is IFN-γ.

IL-12 was able to decrease significantly the proliferation of IL-12/3T3, occurred at levels that can also induce a strong immune response in immune competent mice (6, 19).

Emiko Shibuya, Hiroko Fujimura, and Keiko Inabe.

ACKNOWLEDGMENTS

We thank Drs. S. Haruo Ohtani and Hirofumi Hamada for useful discussions and helpful advice. We also appreciate the expert technical assistance of Emiko Shibuya, Hiroko Fujimura, and Keiko Inabe.

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*Cancer Res* 2000;60:1111-1116.

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