Amount of Interleukin 12 Available at the Tumor Site Is Critical for Tumor Regression

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Abstract

The C26 colon carcinoma is resistant to systemic recombinant interleukin 12 (rIL-12) therapy. Transduction of C26 with genes encoding the two subunits of murine IL-12 resulted in delayed tumor onset after injection of 5 x 10⁴ cells into syngeneic BALB/c mice and in 40% tumor regression after injection into CD4-depleted mice. Here, we analyzed the activity of rIL-12 (1 μg/day) against C26 grown into CD4-depleted mice. Like in mice given injections of interleukin-12 (II-12) gene-transduced C26 cells, depletion of CD4⁺ cells led to tumor regression in 6 of 14 mice, and immunocytochemical characterization of tumor-infiltrating leukocytes showed abundant infiltration by CD8⁺ T cells and asialoGM1⁺ natural killer cells, which were scanty in tumors from non-depleted mice. On the basis of the percentage of tumor regression and leukocyte infiltration, we can conclude that, in the C26 system, systemic rIL-12 (1 μg/day) produces the same results as 30–80 pg/ml IL-12 released at the tumor site.

A new polyclonistic retroviral vector was then used to increase the amount of IL-12 produced by C26-transduced cells. C26 cells releasing 5 ng/ml IL-12, nearly 100 times more than the above-mentioned transduced cells, were tumorigenic in less than 50% of the mice given injections of 5 x 10⁴ cells. In mice given injections of 5 x 10⁵ cells, an initial tumor take of 100% followed by a complete tumor regression. Tumor regression was associated with infiltration of CD8⁺ and asialoGM1⁺ cells, which remained tumor free because they were challenging to a rechallenge of nontransduced C26 cells. The results indicate that the amount of IL-12 made available at the tumor site may determine both the type and number of infiltrating leukocytes and the events leading to tumor regression as well as it may overcome immunosuppression.

Introduction

IL-12 is a heterodimeric cytokine produced by antigen-presenting cells, phagocytes, and granulocytes (1). Despite few recognized direct effects on T and NK cells where it acts as a growth factor, an enhancer of cytotoxicity and activator of other cytokines, its in vivo activity is mediated through IFN-γ (1). An application of IL-12, already tested in clinical trials, is the treatment for neoplasias; with few exceptions, IL-12 was found to cure or improve the survival of mice bearing a great variety of tumors (2–6). The antitumoral activity of IL-12 is likely mediated by the IFN-γ released at the tumor site through stimulation of macrophages with release of other cytokines including tumor necrosis factor α and inducible nitric oxide, up-regulation of MHC on tumor cells, induction of IFN-inducible protein 10 by tumor cells (6), and by infiltrating T cells with consequent inhibition of angiogenesis (7, 8). Exogenous systemic injection of IFN-γ cannot replace IL-12 in mediating responses of such magnitude (9), simply because, in addition to a different half-life, the IFN-γ receptor is ubiquitously expressed whereas IL-12 receptor expression is restricted to NK cells and activated lymphocytes (1). This clearly implies that the IL-12 effect may depend on the amount of NK cells and activated T cells which infiltrate the tumor. In fact, in the C26 carcinoma model IL-12 is weakly effective unless the amount and the activation of infiltrating leukocytes is increased by IL-2 gene transfer that alone is insufficient to inhibit tumor growth (10). Accordingly, IL-12 exacerbates some autoimmune diseases characterized by local accumulation and activation of T cells (11). Local release of IL-12 has also been tested by direct transduction of tumor cells or fibroblasts with the IL-12 genes, and the derived antitumor effect was dose dependent (12, 13). These observations prompted our investigation of the effect of local versus systemic IL-12 delivery as well as the dose effect and the role of host CD4⁺ cells. We previously observed that C26/IL-12-transduced cells were tumorigenic, although with delayed tumors onset, and that depletion of CD4⁺ cells before C26/IL-12 injection allowed tumor regression in 40% of the mice (4). We tested here whether (a) CD4⁺ cell depletion may ameliorate the effect of systemic IL-12 treatment in C26-injected mice and (b) increased IL-12 production by transduced cells may induce complete tumor inhibition in the C26 system.

Materials and Methods

Tumors and Mice. C-26 is a murine colon adenocarcinoma cell line derived from BALB/c mouse treated with N-nitroso-N-methylurethane (14). Cells were maintained as tumors in vivo by s.c. transplantation in syngeneic mice and adapted to culture in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% FCS (Life Technologies, Inc.). BALB/c mice were purchased from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. Tumorigenic activity of control and virus-transduced C26 cells was assayed in mice given s.c. injections in the left flank of cells in 0.2 ml. Recombinant murine IL-12, kindly provided by Dr. Maurice Gately (Hoffmann LaRoche, Nutley, NJ) was injected (i.p., 1 μg in saline per day) daily from days 7 to 11 and from days 14 to 18 (2). Control animals received saline only. Some mice were given injections i.p., weekly, of 0.2 ml of HBSS containing 100 μg anti-CD4 (GK1.5 hybridoma, L3T4) mAb obtained from American Type Culture Collection (Rockville, Md).

Vector Construction and Retroviral Infection. cDNA for both p35 and p40 subunits of IL-12 were cloned using reverse RT-PCR poly(A)⁺ RNA obtained from spleen cells of lipopolysaccharide-treated mice. Primers for p35 were: 5’-CGGTCCAGATGTGCAATCAC-3’ (direct) and 5’-TT-GAACCTTACGCAGGAG-3’ (reverse) corresponding to bases 117–139 and 766–783, respectively, of published cDNA sequences (15). Nucleotides necessary to generate the restriction sites Hpal and Xhol were added 5’ to the direct and reverse primers, respectively. The resulting p35 amplified product was ligated into the Hpal and Xhol sites of L5SN (16) to give Lp35SN. Primers for p40 were: 5’-GATGTCCTCAGAAAGCTA-3’ (direct) and 5’-TTTGAGAACCTTACGCAGGAG-3’ (reverse) corresponding to bases 130–152 and 766–783, respectively, of published cDNA sequences (15). Nucleotides necessary to generate the restriction sites Hpal and Xhol were added 5’ to the direct and reverse primers, respectively. The resulting p40 amplified product was ligated into the Hpal and Xhol sites of L5SN (16) to give Lp35SN. Primers for p40 were: 5’-GATGTCCTCAGAAAGCTA-3’ (direct)
and 5′-TTGCATCCTAGGATCGGACC-3′ (reverse) corresponding to bases 35-53 and 1031-1050, respectively. Sites for NcoI and BamHI endonucleases were generated 5′ and 3′ of the direct and reverse primers, respectively. To fuse the p40 subunits downstream to the IRES, the plasmid pOS6 (also referred to as pTEMCAT) plasmid, kindly donated by B. Moss (17), was modified by substituting the CAT gene with the p35 DNA at the NcoI and BamHI sites. The NcoI site within the IRES sequences contains the ATG corresponding to the translation start site. An in-frame NcoI site was generated upstream of the p40 starting codon to avoid mutagenesis of the following triplet which encodes for cysteine that is necessary for the correct protein folding. To excise the IRES/p40 fusion product, pOS6 was linearized by ClaI digestion, blunt-ended with T4 DNA polymerase, and then digested with BamHI. The IRES/p40 fusion was cloned blunt—BamHI into the Lp35SN vector previously cut with XhoI, was cloned blunt—BamHI into the Lp35SN vector previously cut with XhoI, then digested with BamHI to blunt-ended with T4 DNA polymerase, and then digested with BamHI to generate the correct orientation. The plasmid vector was transfected into the gp+E86 packaging cell line (19) by standard calcium phosphate coprecipitation, and the supernatant of 48-h cultures was used to infect the amphotropic gp+AM12 packaging line. Infected gp+AM12 cells were selected with 0.8 mg/ml G418 and used to generate helper-free virus-containing supernatant. C26 target cells (106) were infected by three cycles of exposure to undiluted supernatant for 3 hr in the presence of 8 μg/ml polybrene. At 48 hr after infection, cells were diluted and selected in 0.5 mg/ml G418. Single G418-resistant colonies were isolated, expanded into cell lines, and injected into mice or subjected to further analysis. G418-resistant C26 cells infected with the LXSN vector alone were used as controls.

The IL-12 concentration was determined by a two-site sandwich ELISA using 9A5 mAb to p70 and peroxidase conjugate mAb to p40, 5C3-POD, kindly provided by Dr. Luciano Adorini (Roche Milano Ricerche, Milan, Italy).

Morphological Analysis and Immunocytochemistry. Tumor fragments and draining lymph nodes were embedded in OCT compound (Miles Laboratories, Elkart, IN), snap frozen in liquid nitrogen, and stored at -80°C. Analysis was performed as described (19). Briefly, 5-μm cryostat sections were fixed in acetone and immunostained with rat anti-mouse mAb against CD4 (GK1.5 hybridoma, L3T4), Mac-1 (M1/70.15.1H5 hybridoma, Ly6G), CD45 (M1/9.3.4.HL2 hybridoma, T200), CD8 (53.6.72 hybridoma, Lyt2), CD4 (GK1.5 hybridoma, L3T4), Mac-1 (M1/70.15.11.5 HL hybridoma), Mac-3 (M3/84.6.34 hybridoma) (all from American Type Culture Collection), CD3 (Boehringer Mannheim, Mannheim Germany), and antimonious granulocyte mAb (RB6—8C5 hybridoma; PharMingen, San Diego, CA). Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit anti-rat IgG (Zymed Laboratories, Inc., San Francisco, CA), and rat peroxidase antiperoxidase (Abbott Laboratories, North Chicago, IL). Each incubation step lasted 30 min and was followed by a 10-min wash in Tris-buffered saline. Sections were then incubated with 0.03% H2O2 and 0.06% 3,3′-diaminobenzidine (BDH Chemicals, Poole, England) for 2 to 5 min, washed in tap water, and counterstained with methylxyl. The number of immunostained cells was determined by light microscopy at X400 magnification in five fields on a 1-mm2 grid and is given as cells/mm2 (mean ± SD).

Results

Antitumoral Effect of rIL-12 on C26 Tumor Is Modulated by Host CD4+ Cells. We have previously shown that (a) systemic rIL-12 injection had a negligible effect on C26 tumor growth, whereas the same treatment reduced the size of B16 tumors (4); (b) C26 cells transduced with a polycistronic vector encoding p40 and p35 IL-12 subunits and producing 30–80 pg/ml IL-12 (C26/IL-1250°) were tumorigenic, although tumor onset was delayed; and (c) removal of CD4+ cells before injection of IL-12-transduced C26 cells resulted in further delay of tumor appearance and was associated with complete tumor regression in about 40% of mice (4).

Here, we tested whether depletion of CD4+ cells may also influence the effect of rIL-12 on C26 cells injected s.c. As for the above described experiments, murine rIL-12 (1 μg/day) was injected i.p. from days 7 to 11 and days 14 to 18. In BALB/c naive mice, injection of either IL-12 or diluent was ineffective on C26 outgrowth, whereas in mice depleted of CD4+ cells IL-12 induced C26 regression in 6 of 14 mice (Fig. 1). Roughly, we can estimate that in CD4-depleted mice, 1 μg rIL-12 given systemically over a period of 10 days gave the same results as 30–80 pg/ml secretion of IL-12 at the tumor site. Like C26/IL-1250° tumors (4), C26 tumors from mice treated with rIL-12 were characterized by a paucity of infiltrating leukocytes, mostly CD3+ whereas tumors from CD4-depleted mice were predominantly infiltrated by asialoGM1+ and CD3−/CD8− leukocytes (Table 1). The histological and immunocytochemical analysis further confirmed that a systemic injection of a high dose of IL-12 produces an effect similar to that of local pg release of cytokine.

Tumorigenicity and Antigenicity of C26 Cells Engineered to Release IL-12. A repeated dose of IL-12 (1 μg/day) is close to toxicity and a 2-day interruption over a 10-day schedule was suggested (2). This observation indicates that a dose increment of rIL-12, given systemically, to improve C26 therapy is impracticable, whereas it might be worth an attempt of increasing the amount of IL-12 released at the tumor site. To this aim a new polycistronic retroviral vector was prepared. Transduced C26 cells were grown to secrete about 5000 pg/ml/106 cells/48 h (thereafter named C26/IL-125000), nearly 100 times more than C26 transduced with the previous vector (C26/IL-1250°). Mice were given s.c. injections of increasing doses of transduced C26 cells starting from the minimum lethal dose (5 × 103 cell) of parental C26. As shown in Fig. 2, C26/IL-125000 is clearly less tumorigenic than C26/IL-1250°. However, the most interesting result is the dose-dependent behavior of C26/IL-125000 tumors. After injection of 5 × 103 or 103 cells, only 40% of the mice developed tumors with onset further delayed. All of these tumors grew progressively, and mice were sacrificed when tumor diameter...
Table 1 Characterization of leukocytes infiltrating C26 tumors from mice treated with rIL-12 and with cells which were or were not CD4⁺-depleted, and comparison with the leukocytes infiltrating IL-12 gene-transduced C26 tumors (C26/IL-125000).

Tumor nodules were obtained between days 10 and 15 after tumor injection. Frozen sections were stained with mAb by immunocytochemistry (peroxidase antiperoxidase method).

<table>
<thead>
<tr>
<th>Leukocyte Type</th>
<th>C26</th>
<th>C26 + rIL-12</th>
<th>C26 + rIL-12 in CD4-depleted mice</th>
<th>C26/IL-125000</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45⁺</td>
<td>171 ± 61</td>
<td>234 ± 89</td>
<td>841 ± 135</td>
<td>1058 ± 149</td>
</tr>
<tr>
<td>Macrophages</td>
<td>98 ± 28</td>
<td>118 ± 31</td>
<td>121 ± 35</td>
<td>74 ± 13</td>
</tr>
<tr>
<td>(Mac3⁺)</td>
<td>(57%)</td>
<td>(50%)</td>
<td>(14%)</td>
<td>(7%)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>45 ± 7</td>
<td>64 ± 23</td>
<td>165 ± 51</td>
<td>62 ± 25</td>
</tr>
<tr>
<td>(RB68CS⁺)</td>
<td>(26%)</td>
<td>(27%)</td>
<td>(19%)</td>
<td>(6%)</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>7 ± 7</td>
<td>21 ± 11</td>
<td>383 ± 91</td>
<td>693 ± 73</td>
</tr>
<tr>
<td>(4%)</td>
<td>(9%)</td>
<td>(33%)</td>
<td>(65%)</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>15 ± 5</td>
<td>54 ± 8</td>
<td>8 ± 6</td>
<td>264 ± 79</td>
</tr>
<tr>
<td>(9%)</td>
<td>(23%)</td>
<td>(21%)</td>
<td>(25%)</td>
<td></td>
</tr>
<tr>
<td>CD3⁺</td>
<td>2 ± 2</td>
<td>9 ± 4</td>
<td>400 ± 149</td>
<td>516 ± 98</td>
</tr>
<tr>
<td>(1%)</td>
<td>(4%)</td>
<td>(47%)</td>
<td>(49%)</td>
<td></td>
</tr>
<tr>
<td>NK</td>
<td>0</td>
<td>13 ± 11</td>
<td>139 ± 51</td>
<td>147 ± 35</td>
</tr>
<tr>
<td>(AsialoGM1⁺)</td>
<td>(5%)</td>
<td>(16%)</td>
<td>(14%)</td>
<td></td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>5 × 6</td>
<td>3 × 5</td>
<td>5 × 5</td>
<td>2 × 3</td>
</tr>
</tbody>
</table>

*Mean number of cells/mm² ± SD positive for immunostaining.

Discussion

Bringing cytokines to a tumor site allowed better efficacy and reduced toxicity. Engineering of tumor cells with the gene of a particular cytokine is an efficient way of ensuring that this cytokine will be present at the tumor site. Actually, the amount of cytokine released increases as the engineered tumor expands, then its release decreases in function of the magnitude of the inflammatory reaction provoked. The debulking efficacy of this action is determined by the type of cytokine, its quantity and activity, the histotype of the tumor and the molecules it releases, and its extracellular matrix (20).

IL-12 is a cytokine which, when injected systemically, provides antitumor activity at doses below those causing toxicity (2). Although most murine tumors are susceptible to IL-12 therapy at doses of 0.1–1 µg/day, others, like C26, appear to be resistant. We observed that with
established than incipient tumors, a situation that may be explained by the dependence on the number of infiltrating T and NK cells. Accordingly, the efficacy of IL-12 was improved on tumors transduced with the IL-2 gene (10) and characterized by an increased number of CD3+ and asialoGM1−-infiltrating cells (22). Here, we show that the efficacy of IL-12 depends on the presence of CD8 and NK cells at the tumor site. It remains to be established how and why CD4+ cells suppress CD8 and NK infiltration at low IL-12 concentrations. This occurs when tumors release 30—80 pg/ml IL-12 or when 1 μg/day rIL-12 is given systemically for 10 days. IL-12 in the range of ng/ml could be detected in serum of mice given injections of μg IL-12 (23); rIL-12 is given systemically for 10 days. IL-12 in the range of ng/ml could be detected in serum of mice given injections of μg IL-12 (23); on the basis of antitumor effect and leukocyte infiltration, we estimate that only pg IL-12 could accumulate at the tumor site. There, IL-12 is likely to stimulate IFN-γ production by infiltrating cells bearing the IL-12 receptor. In the case of C26, this infiltration is lacking unless (a) CD4+ cells are removed (4); (b) IL-2 is provided locally (10); or (c) IL-12 concentration is increased to the level of ng/ml. In addition, mounting of an efficient immune response characterized by long-lasting memory requires an initial growth of C26-transduced cells or at least a prolonged supply of both IL-12 and tumor antigen(s).

Systemic administration and local secretion of IL-12 might well produce the same effects if a comparable amount of IL-12 is available at the tumor site. The success of IL-12 treatments seems to depend on the number of NK and activated T lymphocytes which infiltrate the tumor (10) and on its ability to overcome tumor-induced immunosuppression (4, 5).

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References

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