Injection of Complementary DNA Encoding Interleukin-12 Inhibits Tumor Establishment at a Distant Site in a Murine Renal Carcinoma Model

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Abstract

Interleukin (IL-12) protein has been shown to elicit diverse immunological responses and potent antitumor activity. We demonstrate here that intradermal injection of IL-12 cDNA induces systemic biological effects characteristic of the cytokine in vivo. Intradermal injection of IL-12 cDNA resulted in local expression of IL-12 mRNA, which correlated with a 10-fold increase in natural killer activity and a 3-4-fold increase in anti-CD3-induced IFN-γ production in cultured splenocytes. Furthermore, when challenged with Renca tumor cells at a distant site, the day of tumor emergence was significantly delayed, and tumor growth was reduced in mice that received IL-12 cDNA, compared to mice given injections of plasmid vector alone. A number of the mice receiving IL-12 cDNA injections remained tumor free months after tumor challenge. In contrast to mice receiving recombinant IL-12 protein, no splenomegaly was detected when natural killer activity was significantly induced in mice receiving injections of IL-12 cDNA. Because purified plasmid DNA is more economical to prepare and has a longer shelf-life than recombinant proteins, and intradermal administration of cDNA encoding IL-12 did not cause splenomegaly, our findings suggest that the in vivo injection of cDNA encoding IL-12 may be a less toxic and more cost-effective alternative to IL-12 protein therapy in some clinical or experimental therapeutic applications.

Introduction

IL-12 exhibits a wide range of important immunological activities. These functions include its abilities to increase the lytic activities of NK cells; to enhance specific CTL responses; to induce IFN-γ production by both resting and activated NK cells, CD4+ T cells, and CD8+ T cells; and to promote differentiation of uncommitted T cells to Th1 cells that are associated with cellular immunity (1). IL-12 has been demonstrated to be a potent anti-viral and anti-bacterial agent, either alone or in conjunction with relevant antigens. Antitumor effects of IL-12 have also been well documented in studies using systemic or peritumoral administration of r-mIL-12 protein (2, 3), by injection of fibroblasts or tumor cells genetically engineered to secrete mIL-12 (4-6), and more recently by recombinant vaccinia virus encoding IL-12 (7). Recombinant mIL-12 protein has also been shown to be effective as a therapeutic cancer vaccine adjuvant (8).

Despite the mounting evidence that r-mIL-12 protein can exert strong immunological effects on viral infection and tumor establishment and regression, studies of its application to humans have been very limited. Recombinant IL-12 protein, like many other cytokines, has a relatively short half-life. In preclinical animal studies, frequent administration of high doses of r-mIL-12 protein, which often leads to side effects such as splenomegaly (9), is required to effectively induce antitumor activities. In addition, localized IL-12 gene therapy using retroviral vector-based gene transfer, although resulting in constitutive secretion of the cytokine at low levels, requires ex vivo cell culture manipulation.

Inoculation of DNA directly into animals using a variety of delivery methods has been shown to generate host immunity against various infectious agents (10-12) and tumor-associated-antigens (13, 14). The advantages of direct DNA injection are the ease with which high-purity plasmid DNA can be routinely prepared, the prolonged expression of the gene of interest, and no involvement of infectious agents or ex vivo tissue manipulations. We report here that intradermal injection of an IL-12 cDNA expression vector induced systemic immunological effects that are characteristic of the biological activities of rIL-12 cytokine protein, including antitumor effects. These results indicate that certain physiological and clinical effects of IL-12 can be achieved with the significant advantages of direct DNA injection.

Materials and Methods

Plasmid DNAs. The expression vector pWRG mIL12 (pIL12) was constructed and obtained from Agracetus, Inc. It contains cDNA sequences encoding the murine IL-12 p35 and p40 subunits, each of which was constructed to be driven by the cytomegalovirus immediate-early promoter, and linked in a tandem, unidirectional arrangement in the pBluescript SK(+) plasmid vector, as described recently (15). Plasmid DNAs were purified by twice banding in cesium chloride density gradients. DNA concentrations were determined by absorption at 260 nm and confirmed by agarose gel electrophoresis with DNA concentration standards. Plasmid DNA was stored at −20°C in 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Before injection, the plasmid DNAs were precipitated in ethanol, washed with 70% (v/v) ethanol, and redissolved in normal saline.

Animals. Female BALB/c mice were purchased from Harlan-Sprague-Dawley, Inc. (Madison, WI) and the National Cancer Institute (Frederick, MD) and were housed in the animal facility at the University of South Florida Health Sciences Center. Cohorts of 6-9-week-old mice were used for these experiments.

Cells. The Renca renal carcinoma cell line (kindly provided by R. Wiltrout, National Cancer Institute) was maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 1% minimal Eagle’s medium nonessential amino acids, 100 IU/ml penicillin, and 50 μg/ml gentamicin.

DNA Injection. BALB/c mice were injected intradermally with plasmid DNA in 80 μl of normal saline at 1-2 cm distal from the tail base, using a 1-ml syringe and a 30-gauge needle (12). The dosages of plasmid DNA and the number of injections varied with experiments, as indicated in the figure legends.
RT-PCR Analyses. Expression of mRNA encoding p40 subunit in tails injected with pIL12 was confirmed using RT-PCR. Total RNA was extracted from tails injected with either pIL12 or pBlue using a single-step RNA extraction method. DNase treatment was carried out in 50 µl total volume for 30 min at 25°C with one unit of DNase I. cDNA was synthesized with a cDNA Cycle Kit (Invitrogen, Inc.). PCR was performed for 30 cycles, and each cycle consisted of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, with 100 pmol of each primer (coding strand, 5'-GTGTTCCCTGAAGGCTAAC; noncoding strand, 5'-CACATGGCCCACTTGCGCT). The primers were designed to produce a 980-bp DNA fragment from the mRNA encoding murine IL-12 p40. In addition to the primers described above to detect IL-12 mRNA, another pair of primers specific for γ-actin (coding strand, 5'-CCGGTGCTTCTTAGGAGGCC; noncoding strand, 5'-CAGACTGAGTACTGCGCTC) kindly provided by T. Antonia, Moffitt Cancer Center), which distinguishes γ-actin cDNA from genomic DNA by size, was used to ensure no false positive IL-12 mRNA signals from DNA contamination. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

IFN-γ Assays. Single-cell suspensions of splenocytes from individual mice injected with pIL12 or pBlue DNA were prepared, washed, and resuspended in 10% FCS-RPMI medium containing penicillin-streptomycin. Cells (3 x 10^5/ml) were cultured in 24-well plates with either anti-CD3 antibody (1 µg/ml) or ConA (5 µg/ml) for 24 h. Supernatants were collected and assayed by a sandwich ELISA using antibody pairs obtained from PharMingen (San Diego, CA). Medium blank ELISA plates (Costar) were coated with anti-IFN-γ antibody (200 ng/well) overnight and blocked for 30 min; serial dilutions of IFN-γ (500 units/µl) and samples were incubated for 2 h, followed by biotinylated anti-IFN-γ antibody (100 ng/ml) for 1 h and streptavidin-alkaline phosphatase (1:1000) for 30 min. After addition of p-nitrophenylphosphate substrate, plates were developed for 15 to 45 min, and absorbance was measured at 405 nm.

NK Cell Cytolytic Assays. Single-cell suspensions of splenocytes from individual mice injected with pIL12 or pBlue DNA or individual normal mice were prepared, washed, and resuspended in the 10% FCS-RPMI medium at 10^7/ml. PBLs from these mice were individually collected using heparinized syringes, diluted 10-fold in PBS, and passed over Lympholyte-M (Accurate, Westbury, NY). The interphase was collected, washed, and adjusted to the desired cell density in 10% FCS-RPMI. Cytolytic assays using 51Cr-labeled YAC-1 target cells were performed essentially as described (9). E:T ratios used were: 40:1, 20:1, and 10:1; or 200:1, 100:1, and 50:1, in PBL or splenocyte experiments, respectively. The percentage of cytotoxicity and lytic units were calculated as described (9).

Tumor Challenge and Measurement. Mice, 5-10/group, were inoculated s.c. at the left abdomen in a blinded fashion with 1 x 10^5 or 2 x 10^5 syngeneic Renca cells in sterile HBSS. Mice were checked for tumor emergence every 1-2 days. Tumor size was measured with a caliper in two dimensions. Mice were euthanized when tumors were abscessed through the skin or reached a predetermined size (≥100 mm^2) to minimize discomfort.

Statistical Analysis. The experimental and control groups were compared with regard to the percentage without tumors and the size of the tumors at specific dates using the Wilcoxon Rank Sum test in representative experiments. Data for IFN-γ ELISA assays and NK cytolytic assays were analyzed by Student’s t test for comparing two groups.

Results

Direct Injection of mIL-12 Vector DNA Leads to Expression of IL-12 mRNA. To determine whether the intradermally injected pIL12 cDNA construct was transcriptionally expressed, RT-PCR analyses were performed with total cellular RNAs isolated from the tail tissues of mice injected once with 20 µg of either pBlue or pIL12 DNA vectors. Fig. 1 shows an agarose gel electrophoresis analysis of the RT-PCR products. The expected 1-kb fragment was detected as early as 2 days and up to 15 days, but not at 30 days, after injection of pIL12. In contrast, no 1-kb fragment was detected in the samples prepared from the tails of pBlue-injected mice 2 days after the injection. DNA contamination in the RNA preparation was not detectable, as indicated by the absence of γ-actin genomic DNA.

Induction of IFN-γ Production by IL-12 cDNA Injection. To evaluate whether IL-12 cDNA administration induces in vivo effects that are characteristic of IL-12 cytokine, we determined IFN-γ levels in both serum and cultured splenocytes from mice treated with 50 µg of pIL12 or pBlue at days 0 and 2. No detectable IFN-γ levels were observed in the sera of mice injected with either pIL12 or pBlue. Splenocytes from mice receiving IL-12 cDNA injections did not spontaneously secrete detectable levels of IFN-γ. However, when splenocytes were cultured in the presence of either anti-CD3 antibody or ConA, secretion of IFN-γ was noticeably increased at day 4 (3-4-fold over normal and control; Fig. 2) and declined at days 6 and 8 after the first injection. Of the spleens we examined, neither splenomegaly nor an increase in the number of cells recovered from the spleens of pIL12-treated mice was observed (data not shown).

Enhancement of NK Cell Activity in Vivo by Direct Injection of mIL-12 cDNA. Recombinant mIL-12 protein has been shown to augment NK cell lytic activity in vivo. To determine whether injection of IL-12 cDNA could have similar effects on NK cell activity, 51Cr-release assays using YAC-1 target cells were performed on mice treated with 50 µg of pIL12 or pBlue at days 0 and 2. Kinetic studies of PBL-derived NK cell activity showed that PBLs from mice treated with pIL12 exhibited detectable, but low, levels of NK induction by day 2 (data not shown), and the highest induction (more than 10-fold over control) was detected at day 4 (Fig. 3, A and B). 51Cr-Release
were administered into mice. Fig. 4D indicates that antitumor effects induced by injecting 100 μg had no benefits over 50 μg. Twenty-five % of the mice receiving injections of 50 μg pIL12 still remained tumor free after 4 months. In addition, the size of the tumors in mice receiving pIL12 injections were again smaller than control mice (data not shown). In this experiment, only two pIL12 injections were performed (days 0 and 6), and tumor challenge was carried out on day 10 by s.c. injection into the left abdomen. In an independent experiment, IL-12 cDNA was injected intradermally into mice that had already received tumor cells the day before. The size of the tumor in mice receiving IL-12 injections at day 18 (n = 10, mean = 19.8 ± 15 mm²) was significantly smaller than that observed in mice injected with pBlue (n = 10, mean = 89.2 ± 23 mm²; P < 0.00004). In addition, 1 of the 10 mice receiving IL-12 injections still remained tumor free after 3 months. This experiment was repeated again, and similar results were obtained (data not shown).

assays of lymphoid cells harvested from spleens at day 4 indicated that splenic NK cells were also markedly activated (Fig. 3C).

Local Injection of IL-12 cDNA Results in Inhibition of Renca Tumor Establishment and Growth at a Distant Site. Since intradermal injection of IL-12 cDNA led to enhancement of NK cell activity and mitogen-induced IFN-γ production in cultured splenocytes, we evaluated whether administration of IL-12 cDNA could also inhibit tumor growth. In an initial experiment, 30 μg of pIL12 were injected weekly for 4 weeks into each mouse. Although all of the control mice developed tumors by day 5 after challenge with 2 × 10⁵ Renca cells, 37.5% (three of eight) of mice receiving pIL12 injections remained tumor free at day 10, and 12.5% were still tumor free 8 months after tumor challenge when sacrificed (Fig. 4A). To determine an optimal dosage of DNA for antitumor effects, 10, 20, or 40 μg were injected weekly for four times, followed by tumor cell challenge (1 × 10⁵ Renca cells). As shown in Fig. 4B, 100% of mice receiving pBlue DNA (20 μg) injections developed tumors by day 10. Injection with 10 μg of pIL12 caused only a slight delay in tumor emergence. In contrast, both 20 μg and 40 μg of pIL12 DNA-treated mice showed significant delay in tumor emergence (n = 5, P ≤ 0.008). At day 20, 40% of the mice that received 40 μg of pIL12 were still tumor free, and one-half of these mice still remained tumor free after 6 months when sacrificed. On day 14 after tumor challenge, a majority (80%) of the tumors in the control mice had either erupted the skin or reached the predetermined size (≥100 mm²) for sacrificing. A final measurement of the tumor size of all the mice is presented in Fig. 4C. Tumor sizes in all groups that had received pIL12 were significantly smaller (P ≤ 0.008) than in control mice.

To determine whether injection with higher doses of pIL12 would result in a better antitumor effect, 50 and 100 μg of pIL12 or pBlue

Fig. 2. IFN-γ induction in splenocytes from mice injected with pIL12 DNA. Cultured splenocytes were stimulated in vitro for 24 h with anti-CD3 or ConA, from normal mice receiving injections of control vector, and mice receiving injections of pIL12 DNA at days 4, 6, and 8. The plasmid DNAs were injected at days 0 and 2. Data are presented as the means of six to nine mice from three experiments; bars, SE. *, P < 0.05 compared to control vector.

Fig. 3. Stimulation of NK activity of pL12 DNA-injected mice. A. lytic units of activity in PBLs at days 4, 6, and 8. B, percentage of cytotoxicity in the PBLs at day 4. C, lytic units of activity in the spleen at day 4. Control mice were injected with pBlue vector alone. Data are presented as the means of six to nine mice from three experiments; bars, SE. *, P ≤ 0.05 compared to control vector.
IL-12 cDNA INJECTION INHIBITS TUMOR ESTABLISHMENT

Fig. 4. Antitumor effect of intradermal injection of IL-12 cDNA expression vector. A, mice were injected once weekly with 30 μg of either control vector (pBlue) or IL-12 cDNA vector (pILI2) for 4 weeks. Two × 10⁶ Renca cells were used for tumor challenge on day 28 after the first DNA injection. In B and C, the schedules for DNA injections and tumor challenge are the same as in A. The mice were challenged with 1 × 10⁶ Renca cells. In D, DNA injections were done at days 0 and 4, and tumor challenge was at day 10 (1 × 10⁶ cells). Fifty μg of pILI2 and pBlue treatment were repeated once with similar results.

Discussion

Cytokine immunotherapy by direct gene transfer in vivo is relatively new, and only a few reports have been published. Raz et al. (16) have shown that skeletal muscle injections of cDNAs encoding IL-2, IL-4, or transforming growth factor β induce some systemic effects characteristic of these cytokines. In this study, they demonstrated that circulating levels of transforming growth factor β activity remained detectable for at least 4 weeks after the last injection (16). Sun et al. (17) demonstrated that bombardments by a gene gun of cytokine genes coated onto gold beads at sites where tumor cells had been implanted significantly inhibited tumor growth. More recently, Rakhmilevich et al. (15) extended the gene gun approach to IL-12 gene therapy and demonstrated tumor regression following bombardment at the tumor site.

In this report, we demonstrate that intradermal injection of IL-12 cDNA induced systemic immunological responses characteristic of IL-12 protein, including antitumor effects. Our results indicate that the persistence of IL-12 expression as a result of a single intradermal injection of IL-12 cDNA was at least 15 days but less than 30 days (Fig. 1). Compared to recombinant IL-12 protein, which has a half-life of less than 24 h, 15 days of expression should greatly reduce the frequency of administration. Furthermore, the fact that skin cells turn over rapidly should allow only transient gene expression and very likely only short-term presence of the injected DNA, thus causing less of a safety concern.

Induction of IFN-γ production and secretion is one of the hallmarks of IL-12 immunological responses (1). Uncommitted T cells that produce high levels of IFN-γ and other type 1 cytokines are associated with the development of strong cellular immunity (1). Neutralization of IFN-γ has been shown to affect IL-12-mediated antitumor effects (3). Induction of IFN-γ by IL-12 has also been shown to correlate with nitric oxide production (3), the expression of CXC chemokine IP-10 (17), and tumor regression. As was also reported by others using immunization by implanting IL-12-secreting tumor cells (5), in our present study no IFN-γ was detectable in serum of experimental animals after IL-12 cDNA administration. Since there is no detectable systemic activation of IFN-γ, less toxicity generated by the presence of IL-12 is expected. Detection of mitogen-induced IFN-γ production in cultured splenocytes, however, strongly supports an in vivo IL-12 transgenic activity resulting from IL-12 cDNA injection.

It has been demonstrated that r-mIL-12 protein enhances NK activity both in vitro and in vivo. Gately et al. (9) showed that i.p. administration of r-mIL-12 protein to normal C57BL/6 mice augments NK activities. In both spleen and liver lymphoid cells, NK activities were highest (6—7-fold over control) at days 1 and day after the last injection of r-mIL-12 protein. On day 4, however, NK activity was only about 2—3-fold higher than that of control mice (9). In our system, 4 days after the first DNA injection, an average of 10-fold increase in NK cytolytic activity was observed in splenic lymphoid and PBL cells, compared to control mice. Gately et al. (9) indicated that at least 1 ng/injection of r-mIL-12 protein was needed to generate a detectable but small increase in splenic NK cytolytic activity in C57BL/6 mice. Injection with 10⁻²⁻¹ μg of r-mIL-12 protein resulted in up to a 10-fold elevation of NK lytic activity (9). Although NK activities in different strains of mice vary, the magnitude of NK activity induction we observed in BALB/c mice after IL-12 cDNA injection is comparable to that induced by 10⁻²⁻¹ μg of r-mIL-12 protein in C57BL/6 mice. Because no splenomegaly was observed in our studies, it is likely that the levels of transgenic IL-12 protein expressed as a result of IL-12 cDNA injections were lower than the
r-mIL-12 protein injections. The fact that NK lytic activity was significantly induced, despite the apparent low level of IL-12 present, suggests that injection of IL-12 cDNA into skin, which is rich in lymphoid cells and antigen-presenting cells, may be more effective for the activation of NK than i.p. injections of r-mIL-12 protein.

Tahara et al. (5) showed that when both CD4+ and CD8+ T cells were depleted, IL-12-secreting tumor cells were eventually able to grow but at significantly reduced rates, and the size of tumors was smaller compared to control tumors that did not secrete IL-12 (5). Only when NK cells and both of the two predominant T-cell populations were depleted could the IL-12 antitumor effects be abrogated. NK cells, they concluded, are important for the IL-12 antitumor reactivity, especially during the early phase (5). Activation of NK cells in mice receiving IL-12 cDNA injections mice may also play a major role in mediating antitumor activities. However, since in most of our experiments (Fig. 4) tumor challenge occurred after the decline of NK activation (Fig. 3), it is unlikely that NK lytic activity directly inhibits tumor growth in these experiments. More likely, cytokines (e.g., IFN-γ) released as a result of NK cell activation, through a network of cytokines and transcriptional regulators, can facilitate the functional maturation of CD8+ and CD4+ T cells that are requisite for the IL-12-mediated antitumor effect.

Our experiments clearly demonstrate that intradermal injection of IL-12 cDNA results in systemic immunomodulation of the host with the ability to reject a tumor challenge. The degree of this protective effect is similar to vaccination with allogeneic 3T3 cells genetically engineered to secrete IL-12 and admixed with irradiated tumor cells (4). The observation that the in vivo administration of IL-12 cDNA in the skin can result in cytokine expression offers an opportunity to develop vaccines using defined antigens in the therapy of micrometastatic diseases. We have not examined the ability of intradermal inoculation of IL-12 cDNA to induce regression of an established tumor to date. Other investigators have demonstrated that IL-12 protein administered peritumorally, systemically, or via transduced tumor cells and fibroblasts, and recombinant vaccinia virus (2, 3, 5–7) can mediate the regression of macroscopic established tumors. The amount of protein made in vivo by direct injection of cDNAs is most likely lower than that of other methods; however, coinjection of a plasmid expressing the glycoprotein of rabies virus with granulocytemacrophage-colony-stimulating factor cDNA resulted in enhancement of the B and T helper cell activity against rabies virus (19). Furthermore, a recent report indicates that the presence of r-mIL-12 protein at a dose that lacks antitumor effect by itself greatly enhances p53 peptide vaccination against established tumors (8). Although the relative efficacy of IL-12 cDNA compared with r-mIL-12 protein remains to be determined, our results demonstrate that immune modulation is achieved by injection of naked DNA encoding IL-12. Direct injection of IL-12 cDNA has the potential to be used as a safe, effective, and widely applicable adjuvant for various vaccines, including DNA-based vaccines, for prevention or treatment of infectious diseases and micrometastatic cancers where rIL-12 protein has been shown to be effective.

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References

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