Interleukin 12 Gene Transfer into Skin Distant from the Tumor Site Elicits Antimetastatic Effects Equivalent to Local Gene Transfer

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ABSTRACT

We have reported that particle-mediated interleukin 12 (IL-12) gene transfer into the skin overlying the local tumor inhibits systemic metastases. To further characterize this effect, we compared the antitumor and antimetastatic effects of IL-12 cDNA delivered at the local tumor site versus at a site distant from the primary tumor, in a spontaneous metastasis model of LLC-F5 tumor. Local IL-12 gene delivery into the skin overlying the intradermal tumor (local IL-12 treatment) on days 7, 9, and 11 after tumor implantation resulted in the most suppression of the growth of the primary LLC-F5 tumor, whereas IL-12 gene transfer into the skin distant from the tumor (distant IL-12 treatment) was less effective. In contrast, both local IL-12 and distant IL-12 treatment, followed by tumor excision, inhibited lung metastases to a similar extent, resulting in significantly extended survival of test mice. The results of in vivo studies using depleting anti-asialo GM1 antibody and anti-CD4/anti-CD8 monoclonal antibodies, or neutralizing anti-interferon γ (IFN-γ) monoclonal antibody demonstrated that natural killer (NK) cells, CD8+ T cells, and IFN-γ contributed to the antimetastatic effects in both treatment groups. Furthermore, the levels of mRNA expression of vascular endothelial growth factor and matrix metalloproteinase 9 at the tumor microenvironment were suppressed after both local and distant IL-12 treatment. These results suggest that the current particle-mediated IL-12 gene delivery in the spontaneous LLC-F5 metastasis model can confer antimetastatic activities, irrespective of the gene transfection site, via a combination of several mechanisms involving CD8+ T cells, NK cells, IFN-γ, and antiangiogenesis.

OVERVIEW SUMMARY

We report here that a particle-mediated IL-12 gene therapy protocol can elicit antimetastatic effects in a spontaneous LLC-F5 metastasis model, even when the skin tissue is treated at a distant site from the primary tumor. Both local and distant IL-12 treatments, followed by a tumor excision, inhibited lung metastases to a similar extent. In vivo depletion studies demonstrated that natural killer (NK) cells, CD8+ T cells, and IFN-γ contributed to the antimetastatic effects in both treatment groups. Furthermore, mRNA expression of VEGF and MMP-9 at the tumor microenvironment were suppressed after both local and distant IL-12 treatment. These results suggest that the current particle-mediated IL-12 gene delivery in the spontaneous LLC-F5 metastasis model can confer antimetastatic activities, irrespective of the gene transfection site, via CD8+ T cells, NK cells, IFN-γ, and antiangiogenesis. Such a strategy may offer potential application for gene therapy for spontaneous cancer metastasis.

INTRODUCTION

INTERLEUKIN 12 (IL-12) is a heterodimeric protein consisting of two subunits (p35 and p40) that facilitates helper T type 1 (Th1) immune responses (Kobayashi et al., 1989; Hsieh et al., 1993; Manetti et al., 1993). IL-12 enhances T cell and nat-

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ural killer (NK) cell cytotoxicity (Chehimi et al., 1992; Robert-son et al., 1992; Mehrrota et al., 1993), induces cytokines such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) (Chan et al., 1991; Wu et al., 1993), and enhances proliferation of activated T cells (Gately et al., 1991; Perussia et al., 1992). IL-12 conferred a potent antitumor efficacy in a variety of murine tumor models (Brunda et al., 1993; Nastala et al., 1994; Tahara et al., 1994; Rakhmilevich et al., 1996; Kodama et al., 1999). Local or systemic treatment with recombinant IL-12 protein (rIL-12) has been shown to inhibit the growth of established subcutaneous tumor and tumor metastases (Brunda et al., 1993; Nastala et al., 1994; Mu et al., 1995; Takeda et al., 1996; Cavallo et al., 1999). Systemic administration of rIL-12, however, caused severe dose-dependent toxicity and led to an interruption of the first human trial (Marshall, 1995). In contrast, the local transfer of cytokine genes as a means for gene therapy can circumvent such systemic toxicity and provide effective, persistent local cytokine levels for immune cell activation (Tahara et al., 1994; Zitvogel et al., 1995; Rakhmilevich et al., 1996; Tan et al., 1996; Shi et al., 1999; Weber et al., 1999; Oshikawa et al., 1999). Several IL-12 gene therapy approaches have shown efficacy in inhibition of local tumor growth. These include the use of fibroblasts (Tahara et al., 1994; Zitvogel et al., 1995) or tumor cells (Colombo et al., 1996) transduced in vitro with IL-12 cDNA by a retroviral vector, the direct intratumoral injection of a recombinant vaccinia viral vector (Meko et al., 1995) or an adenoviral vector encoding a transgenic IL-12 protein (Chen et al., 1997; Bramson et al., 1996), and the direct intradermal or intramuscular injection of plasmid DNA expression vector encoding the IL-12 subunits (Rakhmilevich et al., 1996; Tan et al., 1996). In addition to the local effect of IL-12 cDNA, some reports have demonstrated antitumor and antitumor and antimetastatic potential of IL-12 plasmid DNA delivered at the local tumor site versus a distant site from the tumor. If particle-mediated transfer of the IL-12 gene into a nontumor site elicited a remote antitumor effect, such as therapeutic strategy would be potentially applicable for the treatment of metastatic tumors or tumors of internal organs, which are hard to approach directly. Considering the clinical setting for cancer gene therapy, we have chosen an aggressive, spontaneously metastasizing, poorly immunogenic LLC-F5 murine lung carcinoma as the tumor model.

MATERIALS AND METHODS

Mice and cell lines

C57BL/6 female mice between 6 and 8 weeks of age were purchased from Harlan Sprague Dawley (Indianapolis, IN), housed in an AAALAC-accredited facility under isothermal conditions, and allowed access to food and water ad libitum. The Lewis lung carcinoma (LLC) cell line was obtained from the American Type Culture Collection (Rockville, MD). A cloned cell line with highly metastatic properties was isolated from the parental line as follows: mice were implanted intradermally with 1 × 10^6 LLC cells, and tumor was resected on day 14 after tumor implantation. On day 28, mice were killed and metastatic nodules of the lung were aseptically removed and minced. The recovered cells were cultured for 2 weeks, and mice were then injected intradermally again with 1 × 10^6 of these recovered LLC cells (obtained from lung metastasis). After five of these in vivo passages, we generated a highly metastatic line of LLC, designated LLC-F5, and utilized it in this experiment. We confirmed that the metastatic potential of LLC-F5 was higher than that of parental LLC cells. The number of metastatic pulmonary nodules of LLC and LLC-F5 on day 35 after tumor implantation was 6.75 ± 2.72 and 23.5 ± 5.72, respectively (p < 0.05). Cell cultures were maintained in complete medium (RPMI 1640 [BioWhittaker, Walkersville, MD] supplemented with 10% heat-inactivated fetal bovine serum [Sigma, St. Louis, MO], 2 mM l-glutamine, 1 mM sodium pyruvate, 1% minimal Eagle’s medium nonessential amino acids, penicillin [100 U/ml], and streptomycin [100 μg/ml] [BioWhittaker]) in a humidified atmosphere of 5% CO_2 at 37°C.

cDNA expression plasmid construction

The murine IL-12 expression plasmid pNGVL3-mIL-12 was constructed in a backbone plasmid containing a cytomegalovirus (CMV) immediate-early enhancer promoter, intron A, and a kanamycin selection gene. The p35 and p40 subunits, separated by an internal ribosomal entry site, were subcloned into the multiple cloning site of pNGVL3-3 (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI). The backbone plasmid, pNGVL-3, was used as a control vector.

DNA–gold particle preparation

We used a helium-pulse PowderJect XR gene delivery device (kindly provided by PowderJect, Madison, WI). Plasmid DNA was precipitated onto 1.5 to 2.0-μm gold diameter particles as previously described (Rakhmilevich et al., 1996). Each...
pulse of helium expels the DNA-coated gold beads from a single 0.5-inch segment of gold bead-coated tubing, resulting in a delivery of 0.5 mg of gold and 1.25 μg of plasmid DNA per transfection.

**In vivo particle-mediated gene treatment protocol**

Mice were shaved in the abdominal area and injected intradermally with 1 × 10^6 tumor cells in 50 μl of phosphate-buffered saline. Mouse skin overlying and surrounding the target tumor was transected *in vivo* with a 400-psi blast with IL-12 cDNA expression vectors (pNGVL3-mIL-12) and control plasmid (pNGVL-3) on days 7, 9, and 11 after tumor implantation. Each treatment consisted of four transfections. We classified test mice into two groups according to the site of transfection as follows: (1) “local treatment group,” in which one transfection was directly over the target tumor site, and three additional transfections were then evenly spaced around the circumference of the tumor in a triangle pattern as previously described (Rakhmilevich et al., 1996); and (2) “distant treatment group,” in which four transfections were carried out adjacent to one another in a box pattern into normal skin on the back of the test mouse. Therefore in this study, there were four groups of treatments, which included (1) local IL-12 treatment; (2) distant IL-12 treatment; (3) local control treatment; and (4) distant control treatment. Tumor growth was monitored two or three times per week by caliper measurement of two perpendicular tumor diameters. Tumor area (mm^2) was presented as the product of the perpendicular tumor diameters.

**Spontaneous lung metastasis model**

Mice bearing LLC-F5 tumor was anesthetized with ketamine hydrochloride (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (XYLA-JECT; Phoenix Scientific, St. Joseph, MO), and tumor was removed by surgical resection 14 days after tumor cell implantation. The few mice that eventually displayed local tumor relapse (indicating incomplete primary tumor resection) were excluded from the experiment. Spontaneous metastases grew in the lungs of mice that were successfully treated surgically. Some animals were killed on day 35 to check lung metastases, and others were observed for survival time. To enumerate pulmonary metastases, lungs were insufflated with 15% India ink and were bleached in Fekete’s solution (Wexler, 1966). Without surgical resection of a primary tumor, all untreated or control vector treated tumor-bearing mice died within 6 weeks after tumor cell implantation.

**mIL-12 transgene expression**

To analyze IL-12 transgene expression after *in vivo* transfer of mIL-12 cDNA, samples of blood, gene-treated skin, splenocytes, and draining lymph node cells were obtained 24 hr after the last gene treatment. The serum was frozen at −80°C until assayed. Tissue extract was prepared as described in the preceding section. Splenocytes and lymph node cells freshly isolated from test mice were cultured for 24 hr in the absence of stimuli, and the supernatants were also frozen at −80°C until assayed. The quantification of mIFN-γ production in the gene gun-treated skin tissues and serum of test mice and in conditioned medium of cultured splenocytes and lymph node cells was performed by ELISA, using commercially available reagents as purified and biotinylated antibody pairs (PharMingen, San Diego, CA), streptavidin–horseradish peroxidase conjugate (Zymed, South San Francisco, CA), and tetramethylbenzidine substrate (Dako, Carpinteria, CA). The lower limit of detection sensitivity was 78 pg/ml. All samples were assayed in duplicate.

**Cytotoxic assay**

Spleen cells and draining (axillary and inguinal) lymph node cells (8 × 10^6), derived from C57BL/6 mice in the IL-12 treatment group and control group on day 12 after tumor implantation, were unstimulated or were stimulated with irradiated LLC-F5 cells (1 × 10^5) for 5 days *in vitro* and processed for the NK or cytotoxic T lymphocyte (CTL) assay, respectively. YAC-1 cells (for the NK assay) and LLC-F5 cells (for the CTL assay) were labeled with 100 μCi of Na_2^{13}CrO_4 for 2 hr at 37°C in complete medium, washed twice with the same medium, and subjected to a cytotoxic assay. Labeled target cells (5 × 10^3/well) were cultured in a total volume of 200 μl with effector cells in a round-bottomed 96-well plate. After 4 hr of incubation, the supernatant was harvested and counted in a γ counter. Specific lysis was calculated as follows: percent specific lysis = 100 × (mean experimental cpm − mean spontaneous cpm) / (mean maximum cpm − mean spontaneous cpm).

**In vivo depletion of NK cells, CD4^+ and CD8^+ T cell subsets, and in vivo neutralization of IFN-γ**

The relative contribution of NK cells, T cell subsets, and IFN-γ was evaluated by *in vivo* antibody inhibition. Twenty microliters of anti-asialo GM1 antiserum (ASGM1; Wako Bio-products, Tokyo, Japan) or normal rabbit serum was administered intraperitoneally 5 days after tumor implantation, and subsequently once every 5 days afterward for an additional 25 days (six times in total). We confirmed that this dose was effective in suppressing more than 95% of the lytic activity against YAC-1 cells mediated by spleen cells harvested from the animals receiving only two injections. The depletion schedule of CD4/CD8 and IFN-γ was based on our previous report (Oshikawa et al., 1999). Anti-CD4 monoclonal antibody (Mab) (clone GK1.5, rat IgG2b) and anti-CD8 Mab (clone 2.43, rat IgG2b) were administered intraperitoneally at 0.3 mg per injection per mouse on days 5, 9, 13, 17, 22, and 29 after tumor implantation. Anti-IFN-γ Mab (clone R4-6A2) was injected in-
traperitoneally at 0.5 mg per mouse on days 5, 7, 9, 11, 13, 15, 22, and 29. As a control antibody, rat IgG (Sigma) was injected at 0.5 mg per mouse with the same schedule as for the anti-IFN-γ MAb.

Analysis of VEGF and MMP-9 mRNA expression in tumors by RT-PCR

Tumor at the size of 15 mm in diameter and its surrounding skin tissue were excised 2 days after the last gene treatment, and total cellular RNA was extracted from the excised specimens, using an RNAeasy minikit (Qiagen, Valencia, CA). Three micrograms of total RNA was digested by RQ1 RNase-free DNase (Promega, Madison, WI) and reverse transcribed with a Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase (Promega). Five hundred nanograms of RNA-equivalent cDNA was amplified by polymerase chain reaction (PCR) under the following conditions: 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min for 29 cycles. The following sequences were used as primers: VEGF (vascular endothelial growth factor): (sense) 5'-CACCACCAACTGAACTCC-3' and (antisense) 5'-GGTCTGTCTTCTCTTCTTCTG-3'; MMP-9 (matrix metalloproteinase-9): (sense) 5'-ACTGACCCCTGCTTACTG-3' and (antisense) 5'-ACCCAACTTATCCAGACTCC-3'; β-actin: (sense) 5'-TGTCCCTGTATGCCTCTGGT-3' and (anti-sense) 5'-TGCTCCTGTATGCTTCTG-3'. The PCR products of VEGF, MMP-9, and β-actin are DNA fragments 384, 507, and 482 bp in length, respectively. The amplified products were electrophoresed on 2.0% agarose gels and the intensity of DNA bands was quantified by densitometry (National Institutes of Health Image 1.61).

Statistical analysis

Data were expressed as means ± SEM. The data were analyzed for significance by the Mann–Whitney U test. Survival rates were calculated according to the Kaplan–Meier method, and survival distributions were compared by the log-rank test. *p < 0.05 was considered significant.

RESULTS

Expression of mIL-12 transgene in the treated skin tissue

The IL-12 cDNA expression vector was effectively transduced by particle-mediated gene transfer into the skin tissue. The levels of transgene IL-12 protein in the treated skin tissue lysate 1 day after treatment with IL-12 cDNA and control plasmid were 1140 ± 280 and 165 ± 21 pg/ml in the local treatment group, and 1097 ± 33 and 87 ± 24 pg/ml in the distant treatment group, respectively (Fig. 1A). In contrast, the levels of IL-12 in skin tissue overlying the tumor in the group receiving distant treatment with IL-12 cDNA vector or control plasmid were 122 ± 9 and 94 ± 13 pg/ml, respectively, and similar to the “background” levels obtained in the skin transfected with the control pNGVL-3 plasmid. This result suggests that IL-12 gene expression is basically confined to the skin site of treatment, irrespective of tumor site. The levels of IL-12 in serum of test mice for all groups were not detectable within the sensitivity limit of this ELISA ($< 2$ pg/ml).

Tumor growth of Lewis lung carcinoma after mIL-12 gene therapy

We evaluated if particle-mediated mIL-12 gene transfer could elicit an antitumor effect against the established 7-day LLC-F5 tumor. Since the primary tumor of test mouse was resected on day 14 in our spontaneous metastasis protocol, tumor growth could be monitored up to 14 days after tumor implantation (data not shown). The most suppression of tumor growth

![FIG. 1. IL-12 transgene expression (A) and IFN-γ production (B) in the skin tissue after gene treatment. Means ± SD were calculated from triplicates. (a) Skin transfected with pNGVL3-mIL-12 in the local tumor site (local IL-12 treatment); (b) skin transfected with pNGVL-3 (control plasmid) in the local tumor site (local control treatment); (c) skin transfected with pNGVL3-mIL-12 in the distant site from the tumor (distant IL-12 treatment); (d) skin transfected with pNGVL-3 in the distant site (distant control treatment); (e) nontreated skin overlaying the tumor in group c (the distant IL-12 treatment group); (f) nontreated skin overlaying the tumor in group d (the distant control treatment group). (A) *p < 0.001 for groups a and c versus groups b, d, e, and f. (B) †p < 0.001 for groups a and c versus groups b, d, e, and f.](image-url)
was observed on day 14 in the local IL-12 treatment group, which showed a significant difference, compared with other groups \((p < 0.01)\). Tumor growth continued in all groups. Less substantial, but still significant, was the difference found between the IL-12- and control-distant treatment groups \((p < 0.05)\). All other comparisons are not significant between groups measured on day 14.

**Spontaneous lung metastases after gene treatment with IL-12 cDNA**

We compared the number of metastatic nodules of LLC-F5 in the lung between IL-12-treated and control plasmid-treated groups (Fig. 2). The number of metastatic nodules in both local and distant IL-12 treatment groups 35 days after tumor implantation was significantly lower than in local and distant control vector treatment groups (IL-12 vs. control vector in local treatment, \(3.3 \pm 3.0\) vs. \(19.2 \pm 5.2\) lung metastases, respectively, \(p < 0.01\); and IL-12 vs. control vector in distant treatment, \(5.2 \pm 3.6\) vs. \(17.2 \pm 6.8\) lung metastases, respectively, \(p < 0.01\)). Thus, when the skin distant from the primary tumor was transfected with IL-12 cDNA, the extent of antimetastatic effect measured in the lungs was similar to that induced by the local gene transfer of IL-12 into the tumor site.

**Survival time after IL-12 gene therapy in the spontaneous metastasis model of LLC-F5**

When treated mice were observed up to 50 days after implantation of the highly metastatic LLC-F5 tumor, 31.6 and 17.6% of test mice were found to effectively survive in the groups of local and distant IL-12 gene treatment, respectively (Fig. 3). There was a significant difference between IL-12 (both local and distant) treatment and control (both local and distant) treatment groups (e.g., local IL-12 treatment vs. local control vector treatment, \(\chi^2 = 19.04, p < 0.0001\); distant IL-12 treatment vs. distant control vector treatment, \(\chi^2 = 23.39, p < 0.0005\)). Survival time in the local IL-12 treatment group did not differ significantly from that in the distant IL-12 group \((p = 0.31)\).

**IFN-γ induction after mIL-12 gene transfer**

IFN-γ production in the gene-treated skin tissues and serum of test mice and in conditioned medium of cultured splenocytes and draining (axillary and inguinal) lymph node cells was compared among all test groups of local and distant treatment with IL-12 cDNA vector or control plasmid. The IFN-γ levels produced from the skin transfected with IL-12 cDNA and control plasmid were \(1054 \pm 346\) and \(91 \pm 21\) pg/ml in the local treatment group, and \(964 \pm 69\) and \(62 \pm 19\) pg/ml in the distant treatment group, respectively (Fig. 1B). In contrast, the levels of IFN-γ in skin tissue overlying the tumor in the group of distant treatment with IL-12 cDNA or control plasmid were \(168 \pm 25\) and \(94 \pm 21\) pg/ml, respectively (Fig. 1B). The level of IFN-γ in serum of test mice for all groups was not detectable within the sensitivity limit of our ELISA (\(\geq 78\) pg/ml). Thus, IL-12 gene delivery into the skin induces measurable IFN-γ at the skin site of IL-12 DNA delivery, but not at the distant skin sites or in the blood. When splenocytes and draining lymph node cells freshly isolated from test mice were cultured for 24 hr in the absence of stimuli, levels of IFN-γ secretion from these cells were considerably greater in the groups that were previously treated with IL-12 cDNA than in control vector treatment groups (Fig. 4).

**NK and CTL activities induced by delivery of IL-12 cDNA**

To evaluate whether the mice were able to develop antitumor immunity after *in vivo* transfer of IL-12 gene expression
vector, splenocytes and draining lymph node cells collected from mice 24 hr after the last transfection in each treatment group were assayed for NK and CTL activities. Both splenocytes and lymph node cells from mice that received local or distant IL-12 treatment generated similar trends of increased NK activities. In splenocytes, the NK activity was higher for the IL-12-treated groups, and a significant difference was observed between distant IL-12 and control treatment groups (p < 0.05), but not between local IL-12 and control treatment groups (p = 0.12; Fig. 5A). In draining lymph node cells, the NK activities were 4- to 6-fold higher for the IL-12-treated groups than those from control (local and distant) treatment groups (p < 0.05; Fig. 5A). Likewise, the results of CTL activities showed the same tendency as those of NK activities. Similar levels of high CTL activities in both splenocytes and lymph node cells were induced after both local and distant treatments with IL-12 gene. In both splenocytes and lymph node cells, CTL activities were significantly higher in IL-12 treatment groups as compared with those of their counterpart control groups (p < 0.05; Fig. 5B).

**Effect of in vivo depletion of NK cells or CD4+ and CD8+ subsets of T cells, and IFN-γ neutralization on antimetastatic efficacy of IL-12 gene therapy**

Although the NK and CTL activities in immune cells were higher for the IL-12-treated groups, the cytotoxicity levels were relatively low (Fig. 5). Therefore, to determine the role of NK cells and CD4+ and CD8+ subsets of T cells in the antimetastatic effect of both local and distant IL-12 treatments, in vivo depletion experiments were performed by injecting LLC-F5 tumor-bearing mice with anti-asialo GM1 Ab and anti-CD4+ or anti-CD8+ MAb (Fig. 6). In both local and distant treatments, mice receiving IL-12 gene therapy and anti-asialo GM1 Ab were found to develop significantly more lung metastases than the mice given a local or distant IL-12 treatment alone (Fig. 6, local IL-12 treatment [b] vs. local IL-12 treatment with anti-asialo GM1 Ab [c], 10.1 ± 2.1 vs. 18.7 ± 2.8 lung metastases, respectively, p < 0.05; and distant IL-12 treatment [j] vs. distant IL-12 treatment with anti-asialo GM1 Ab [k], 14.1 ± 1.9 vs. 21.3 ± 2.8 lung metastases, respectively, p < 0.05). In vivo depletion of CD8+ T cells, but not of CD4+ T cells, inhibited the antimetastatic effect of both local and distant IL-12 treatments (Fig. 6, local IL-12 treatment [b] vs. local IL-12 treatment with anti-CD8+ MAb [e], 10.1 ± 2.1 vs. 24.2 ± 2.2 lung metastases, respectively, p < 0.05; and distant IL-12 treatment [j] vs. distant IL-12 treatment with anti-CD8+ MAb [m], 14.1 ± 1.9 vs. 19.2 ± 2.1 lung metastases, respectively, p < 0.05).

The results in Fig. 6 also show that the inhibitory effect of anti-asialo GM1 Ab, anti-CD8+ MAb, or anti-IFN-γ MAb alone on antimetastatic efficacy of IL-12 gene therapy was not complete. Thus, lung metastasis, after local or distant IL-12 gene therapy and blocking of NK cells, CD8+ T cells, or IFN-γ, was still suppressed, as compared with that induced by local or distant control plasmid treatment, respectively (Fig. 6, local control treatment [a] vs. local IL-12 treatment with anti-asialo GM1 Ab [c], local IL-12 treatment with anti-CD8+ MAb [e], or local IL-12 treatment with anti-IFN-γ MAb [g], p < 0.005; and distant control treatment [i] vs. distant IL-12 treatment with anti-asialo GM1 Ab [k] or distant IL-12 treatment with anti-CD8+ MAb [m], p < 0.005; and distant control treatment [i] vs. distant IL-12 treatment with anti-IFN-γ MAb [o], p < 0.05).

When LLC-F5 tumor-bearing mice were treated with IL-12 cDNA and both anti-CD8+ and anti-IFN-γ MAb, lung metastases developed much more than with IL-12 cDNA and anti-CD8+ or anti-IFN-γ MAb alone in local IL-12 as well as distant IL-12 treatment groups (Fig. 7, [e] vs. [c] or [d]; [k] vs. [i].

**FIG. 4.** IFN-γ production from splenocytes (A) and lymph node cells (B) after gene treatment. Splenocytes (2 × 10⁶) and lymph node cells (2 × 10⁶) were isolated 24 hr after the third gene treatment, and were cultured in 2 ml of complete medium for 24 hr. Means ± SD were shown for four mice per group. (a) Local IL-12 treatment; (b) local control treatment; (c) distant IL-12 treatment; (d) distant control treatment. (A) IL-12 (local/distant) versus control (local/distant), 125 ± 78/134 ± 74 versus 30 ± 1/29 ± 2 pg/ml, respectively. *p < 0.05; **p < 0.05 for groups a and c versus groups b and d. (B) IL-12 (local/distant) versus control (local/distant), 136 ± 49/107 ± 30 versus 36 ± 9/24 ± 10 pg/ml, respectively. †p < 0.05 for groups a and c versus groups b and d.
Furthermore, simultaneous in vivo blocking of NK cells, CD8+ T cells, and IFN-γ was found to completely abrogate the antimetastatic effects of local IL-12 and distant IL-12 treatments (Fig. 7, [f] and [l]).

mRNA expression of VEGF and MMP-9 at the tumor environment after IL-12 gene therapy

To evaluate the possible role of antiangiogenesis in the antimetastatic effect induced by local or distant IL-12 treatment in our spontaneous lung metastasis model of LLC-F5, we analyzed the levels of mRNA expression of VEGF and MMP-9 in the tumor microenvironment (Fig. 8). The tumor size (mm²) 2 days after the last gene treatment (on day 13 after tumor implantation) with IL-12 cDNA and control plasmid was 57.4 ± 8.3 and 84.5 ± 5.8 in the local treatment group, and 77.1 ± 7.3 and 96.8 ± 4.3 in the distant treatment group, respectively. The expression of VEGF mRNA decreased after local and distant IL-12 treatment in comparison with their control counterparts. Furthermore, both local and distant IL-12 treatment resulted in approximately one-half of a decrease in MMP-9 mRNA expression in the tumor and its adjacent skin tissue, as compared with their control counterparts. As a positive control, the RNA sample extracted from a 13-day tumor without treatment was used in these assays (Fig. 8 [e]), and the mouse skin had minimal levels of mRNA expression for VEGF and MMP-9 (Fig. 8 [f]). We also examined MMP-2 mRNA expression in the tumor and its surrounding skin tissue 2 days after the last gene treatment with IL-12 cDNA and control plasmid. The MMP-2 mRNA expression after local and distant IL-12 treatment exhibited levels similar to those of control counterparts (data not shown).

DISCUSSION

The present study evaluated the effect of IL-12 gene transfer protocol for skin transfection on growth of primary tumors as well as on the outgrowth of spontaneous lung metastases, using the mouse LLC-F5 model. Specifically, we compared the antitumor and antimetastatic effect of IL-12 gene transfer into the skin overlying the primary tumor (local treatment) versus the skin distant from the tumor site (distant treatment). Our re-
Results demonstrate that the distant IL-12 cDNA treatment resulted in an inhibition of the growth of metastatic tumor in the lung, and that the antimetastatic efficacy was almost equivalent to that of the local IL-12 treatment.

In the current spontaneous metastasis protocol, treatment with IL-12 cDNA was performed, followed by tumor resection. Because IL-12 gene therapy resulted in effective suppression of local tumor growth, it is possible that tumor mass reduction by IL-12 gene therapy resulted in an inhibition of the growth of metastatic tumor in the lung, and that the antimetastatic efficacy was almost equivalent to that of the local IL-12 treatment.

![Graph showing number of metastatic nodules in the lung](image1)

**FIG. 6.** Effect of *in vivo* depletion of NK cells, CD4+ and/or CD8+ T cells, or neutralization of IFN-γ on the antimetastatic effect induced by IL-12 gene therapy. Anti-asialo GM1 Ab, anti-CD4 and/or CD8 MAb, and anti-IFN-γ MAb were injected intraperitoneally as described in Materials and Methods. Means ± SEM are shown for cumulative data from two independent experiments. (a) Local control treatment and no Ab (n = 9); (b) local IL-12 treatment and no Ab (n = 9); (c) local IL-12 treatment and anti-asialo GM1 Ab (n = 12); (d) local IL-12 treatment and anti-CD4+ MAb (n = 11); (e) local IL-12 treatment and anti-CD8+ MAb (n = 11); (f) local IL-12 treatment and both anti-CD4+ and anti-CD8+ MAb (n = 11); (g) local IL-12 treatment and anti-IFN-γ MAb (n = 11); (h) local IL-12 treatment and either normal rabbit serum (n = 10) or rat IgG (n = 11); (i) distant control treatment and no Ab (n = 9); (j) distant IL-12 treatment and no Ab (n = 9); (k) distant IL-12 treatment and anti-asialo GM1 Ab (n = 12); (l) distant IL-12 treatment and anti-CD8+ MAb (n = 12); (m) distant IL-12 treatment and both anti-CD4+ and anti-CD8+ MAb (n = 12); (n) distant IL-12 treatment and anti-IFN-γ MAb (n = 12); (o) distant IL-12 treatment and either normal rabbit serum (n = 11) or rat IgG (n = 11). *p < 0.05 versus groups c, e, and g. †p < 0.005 versus groups c, e, and g. ‡p < 0.05 versus groups k, m, and o. §p < 0.005 versus groups k and m. ¶p < 0.005 versus group o.

**FIG. 7.** Effects of a combination of *in vivo* depletion of NK cells and/or CD8+ T cells and *in vivo* neutralization of IFN-γ in antimetastatic effect induced by IL-12 gene therapy. Mixtures of anti-asialo GM1 Ab, anti-CD8+ MAb, and/or anti-IFN-γ MAb were injected intraperitoneally as described in Materials and Methods. Means ± SEM are shown for cumulative data from two independent experiments. (a) Local control treatment and no Ab (n = 10); (b) local IL-12 treatment and no Ab (n = 11); (c) local IL-12 treatment and anti-IFN-γ MAb (n = 10); (d) local IL-12 treatment and anti-CD8+ MAb (n = 10); (e) local IL-12 treatment and both anti-CD8+ and anti-IFN-γ MAb (n = 11); (f) local IL-12 treatment and mixture of anti-asialo GM1 Ab, anti-CD8+ and anti-IFN-γ MAb (n = 10); (g) distant control treatment and no Ab (n = 10); (h) distant IL-12 treatment and no Ab (n = 12); (i) distant IL-12 treatment and anti-IFN-γ MAb (n = 11); (j) distant IL-12 treatment and anti-CD8+ MAb (n = 12); (k) distant IL-12 treatment and both anti-CD8+ and anti-IFN-γ MAb (n = 11); (l) distant IL-12 treatment and mixture of anti-asialo GM1 Ab, anti-CD8+ and anti-IFN-γ MAb (n = 12). *p < 0.0001 versus groups e and f; †not significant versus groups e (p = 0.076) and f (p = 0.91); ‡p < 0.001 versus groups k and l; §not significant versus groups k (p = 0.060) and l (p = 0.499).
12 gene therapy can decrease the number of cancer cells metastasizing to target organs via the blood stream. Thus, the inhibitory effects of the IL-12 treatment on the primary tumor. Therefore, to clearly determine whether IL-12 gene therapy can inhibit any component of a distant metastatic process, further experiments were needed. One experimental approach could have been to test the IL-12 treatment performed only after the removal of primary tumors (Mu et al., 1995; Popovic et al., 1998). However, our preliminary data have already shown that the LLC-F5 tumor masses formed as early as 7 days after tumor cell implantation were well vascularized by the self-induced neoformed vessels, and micrometastases were already present in the lungs (our unpublished data). Thus, a treatment with IL-12 gene delivery started on day 14 would have been most likely too late to influence the pulmonary metastases (after primary tumor resection in this model). Instead, we have chosen to evaluate the antimetastatic effect of IL-12 gene therapy by delivering the gene at a site distant from the primary tumor. Even though distant IL-12 treatment induced no (or minimal) IL-12 or IFN-γ protein production at the primary tumor site, it induced a profound antimetastatic effect measured in the lungs.

Previous studies have shown that the antitumor effects of IL-12 are mediated by activation of cytotoxic T lymphocytes (CTLs) as well as NK cells (Martino et al., 1995; Tsung et al., 1997), and by the induction of IFN-γ production by T cells and NK cells (Nastala et al., 1994; Brunda et al., 1995). Although IFN-γ alone is not sufficient to induce tumor regression, and treatment with antibodies against IFN-γ does not completely block the antitumor effect of IL-12 (Nastala et al., 1994; Brunda et al., 1995), IFN-γ has been shown to be involved in the spectrum of antitumor effects of IL-12 (Brunda et al., 1995). For instance, IFN-γ induced by IL-12 was shown to upregulate MHC class I and II expression on tumor cells, to activate NK cells and macrophages, and to help generate CD8+ cytotoxic T cells. In the present study, splenocytes and draining lymph node cells obtained from test mice receiving either local or distant IL-12 cDNA treatment showed activation of NK cells and cytotoxic T cells, as compared with their control counterparts. However, their cytotoxicity levels were low. Therefore, to analyze further the mechanisms of the antimetastatic effect of IL-12 gene therapy in the spontaneous metastasis model of LLC-F5, antibody depletion experiments were performed. The results demonstrated that, by blocking either the activities of NK cells, CD8+ T cells, or IFN-γ, a partial inhibition of the antimetastatic effects induced by local and distant treatments with IL-12 cDNA was readily observed. Furthermore, a triple-antibody treatment, resulting in the depletion of CD8+ T cells and NK cells, and the neutralization of IFN-γ, effectively abrogated the antimetastatic effect, suggesting that CD8+ T cells, NK cells, and IFN-γ in combination can apparently contribute to the antimetastatic effect induced by IL-12 gene therapy.

Our results also suggest that the protocol of particle-mediated skin transfection with IL-12 cDNA, irrespective of the transfection site, is apparently capable of stimulating immunopotent cells systemically, resulting in eradication or inhibition of tumor cells. In fact, both local and distant IL-12 treatments elicited a suppressive effect on the growth of primary tumor as well as on the development of lung metastases when compared with their control counterparts. However, by comparing the antitumor activities between local and distant IL-12 treatment groups, we found that IL-12 distant treatment was less effective in suppressing the primary tumor growth than the local IL-12 treatment. In contrast, the inhibitory effect of distant IL-12 treatment on lung metastases was comparable to that of local IL-12 treatment. This discrepancy may be explained, in part, by the different mechanisms that are responsible for the inhibition of the growth of primary tumors versus those for the development of lung metastases. For regression of local tumor, the importance of increased production of IL-12 available at the local tumor site has been reported (Colombo et al., 1996). In addition, results of the study using genetically engineered fibroblasts expressing IL-12 demonstrated that the antitumor effect against the local tumor was obtained more effectively when transgenic fibroblasts were administered at the local tumor site, as compared with the effect obtained by administration of fibroblasts at a site distant from the primary tumor. These results indicate the advantage of having the IL-12 expression augmented locally at the tumor microenvironment (Zitvogel et al., 1995). In the current study, detectable levels of transgenic IL-
12 and IL-12-induced IFN-γ after particle-mediated treatment were found to be confined to the transfection site of the skin; the levels of these cytokines present in skin tissue of the primary tumor site were far higher after local IL-12 gene transfer into this tumor site than after IL-12 gene transfer into a skin site distant from the primary tumor (Fig. 1A, [a] versus [e]; Fig. 1B, [a] versus [e]). Thus, local IL-12 treatment is superior to distant IL-12 treatment in suppression of local primary tumor growth. In contrast, for inhibition of spontaneous metastases, both local and distant IL-12 treatment exhibited strong and similar antimetastatic effects. Together, these results suggest that a combination of NK cell and CTL activities and IFN-γ production, induced by transfection with IL-12 cDNA at the distant site, can be sufficient for an inhibition of spontaneous metastasis, but not enough for controlling the local tumor growth. This antimetastatic effect of distant IL-12 DNA therapy is consistent with the findings of Popovic et al., who have used autologous tumor cells transduced with IL-12 DNA (Popovic et al., 1998). We suggest here that during the early stage of metastasis, the number of cancer cells that can metastasize to the distant organs may be significantly less than that required to form a relatively large established tumor, and therefore may be more controllable by the antitumor immunity activated by distant IL-12 treatment.

It has been reported that the inhibition of tumor angiogenesis is an important feature of the systemic antitumor effect induced by IL-12 treatment (Voest et al., 1995; Tannenbaum et al., 1998). The antiangiogenic activities of IL-12 are thought to be largely due to the local IFN-γ production and its subsequent induction of angiostatic chemokines, such as interferon-inducible protein 10 (IP-10) and monokine induced by IFN-γ (MIG) (Tannenbaum et al., 1998). IL-12 can also regulate the levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) in the tumor microenvironment (Dias et al., 1998). In this respect, we show that our IL-12 gene therapy protocol results in a downregulation of gene expression of VEGF and MMP-9 in the tumor and its surrounding tissues (Fig. 8), which in concert with activation of NK cells and CTLs may contribute to the observed antimetastatic effect. Some experimental studies have shown that MMP inhibitors can block cancer metastasis, probably by inhibition of invasive growth at the tumor margins or inhibition of tumor-induced angiogenesis, while they are far less effective in reducing the growth rate of relatively large-size, established tumors (Brown, 1998). Accordingly, regulation of MMPs by IL-12 may also account for the observed antimetastatic activity induced by treatment with IL-12 CDNA at a site distant from the primary tumor, as observed in the present experiments.

Importantly, we showed in this study that potent antimetastatic effects induced by either local or distant IL-12 treatment were reflected by the prolongation of the survival time of treated animals. In the groups of mice receiving local IL-12 or distant IL-12 gene treatment, 31.6 and 17.6% of test mice, respectively, were found to survive by day 50, whereas all mice treated with control plasmid died from lung metastases before day 42. Metastasis rather than the primary tumor growth constitutes the major cause of morbidity and mortality in human malignancies. In our preliminary data, all of the control mice, with or without surgical resection of a primary LLC-F5 tumor, died within 6 weeks of tumor cell implantation (data not shown). Our data suggest that a combinational therapy of particle-mediated IL-12 gene transfer and surgical resection may serve as a treatment protocol improving the survival of tumor-bearing animals. In this regard, our results establish the therapeutic efficacy and clinical relevance of this particle-mediated IL-12 gene transfer method, using a murine spontaneous metastasis model.

In summary, particle-mediated transfer of a transgene expression vector for IL-12, even when performed at a site distant from the primary tumor, can result in the induction of a systemic antitumor immune response against poorly immunogenic and spontaneously metastatic tumors. This antitumor effect was apparently mediated via the activation in combination of NK cells, CD8+ T cells, and IFN-γ production, and via the downregulation of angiogenic factors. These findings suggest that the current particle-mediated delivery approach with the IL-12 gene may provide a potential clinical application for cancer gene immunotherapy for metastatic tumors at an early stage or at locations difficult to treat repeatedly with local gene delivery.

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