Direct Intratumoral Injection of an Adenovirus Expressing Interleukin-12 Induces Regression and Long-Lasting Immunity That Is Associated with Highly Localized Expression of Interleukin-12

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ABSTRACT

Mice bearing breast tumors were treated with a single dose of an adenovirus expressing interleukin-12 (AdmIL-12.1) injected intratumorally, which produced regressions in greater than 75% of the treated tumors; approximately one-third of the animals remained tumor free. Complete regression was associated with immunity to secondary challenge with fresh tumor cells. Analysis of local cytokine expression demonstrated maximum expression of IL-12 within the tumor between 24 and 72 hr post-injection, reaching 600–800 ng per tumor, with elevated local levels of IL-12 detectable for at least 9 days. This expression was highly localized as serum IL-12 peaked at 40–60 ng/ml at 24 hr and was less than 10 ng/ml from day 3 onward. Interferon-γ (IFN-γ) concentrations were markedly increased within the tumor following AdmIL-12.1 administration, demonstrating that IL-12 was acting locally. Tumor-draining lymph node cells spontaneously produced IFN-γ following AdmIL-12.1 treatment, suggesting these cells were activated by IL-12. These data demonstrate that AdmIL-12.1 can be used to deliver very high levels of localized cytokine production. Moreover, we have confirmed that the IL-12 produced from our vector actually affects the local cytokine environment of the tumor and activates responder cells present within the tumor.

OVERVIEW SUMMARY

The systemic toxicities associated with many recombinant cytokines can limit their use in vivo for cancer immunotherapy. We have directly infected murine tumors in vivo with an adenoviral vector expressing interleukin-12 (IL-12) to generate high intratumoral cytokine levels. This treatment lead to tumor regression and long-term immunity in those animals whose tumors regressed completely. The expression of IL-12 was localized primarily to the tumor, resulted in an increase in interferon-γ (IFN-γ) levels within the tumor, and promoted IFN-γ expression in the cells from the draining lymph node. These data demonstrate that adenoviral vectors can be used to produce very high levels of cytokine locally. Moreover, we have confirmed that intratumoral IL-12 production can alter the local cytokine environment of the tumor and activate responder cells present within the tumor.

INTRODUCTION

A MAJOR LIMITATION in the effective treatment of cancer is the presence of residual disease and metastases following standard treatment protocols involving surgical resection, chemotherapy or radiation. Many novel therapies are being developed in an attempt to overcome this hurdle, including immunologic and genetic approaches. Immunologic intervention represents one of the more promising strategies, because the immune system is uniquely suited to seek out and destroy residual tumor cells that may otherwise persist, resulting in a recurrence of the malignancy. The development of tumor immunity is inhibited in many ways by the malignant cells during tumor progression (Abbas et al., 1991). One mechanism by which these barriers may be overcome is through stimulation of anergic T cells by the addition of exogenous cytokines. Systemic administration of cytokines can produce substantial antitumor activity but the severe toxicity associated with this
fector cells draining from the tumor. causes local increases in IFN-γ production and activation of expression of IL-12 within the tumor appears to be localized and rechallenge with fresh tumor cells. Moreover, the expression of Ad vector has been shown to be quite immunogenic (Yang et al., 1994), which, although a disadvantage for some gene therapies, may be beneficial for immunotherapy because this will limit the duration of cytokine expression and could provide adjuvant stimulus for the development of antitumor immunity. These viruses have been used by several groups, including our own, to transfer successfully a variety of genes into tumors in vivo (Bramson et al., 1995).

Interleukin-12 (IL-12) is a multifunctional cytokine that has attracted much attention for its antitumor properties. IL-12 has been shown to enhance the cytolytic activity of natural killer (NK) and cytotoxic T lymphocyte (CTL) cells, is a very strong inducer of interferon-γ (IFN-γ), and appears to be a major determinant in the development of a Th1/Th2 response (cell-mediated immunity) (reviewed by Hendrzak and Brunda, 1995). In mouse models, systemic treatment using recombinant IL-12 revealed no serious side effects at doses that lead to significant antitumor responses (Brunda et al., 1993; Nastala et al., 1994; Zou et al., 1995; Tannenbaum et al., 1996). Systemic administration of IL-12 in mice and squirrel monkeys can result in a reversible, dose-dependent suppression of peripheral blood counts (anemia, leukopenia, thrombocytopenia) and splenomegaly associated with enhanced splenetic extramedullary hematopoiesis (Gatley et al., 1994; Sarmiento et al., 1994; Tare et al., 1995). Phase I trials of systemic IL-12 treatment in renal cell carcinoma have been completed and although phase II trials were temporarily suspended due to apparent toxicity, the toxicity has been overcome and the trials have been resumed (Cohen, 1995). As mentioned above, direct delivery of IL-12 to the tumor mass should reduce associated systemic effects. A number of gene transfer vectors have been developed that express this cytokine, including vectors based on Ad, retroviruses, and vaccinia virus (Bramson et al., 1996; Zitvogel et al., 1994; Meko et al., 1995).

In the studies described here, we have investigated the outcome of direct injection of the Ad expressing IL-12 (AdmIL12.1) into subcutaneous murine tumors using a transgenic mouse model of metastatic breast cancer (Guy et al., 1992). We show that a single dose of AdmIL-12.1 can effectively induce tumor regression, resulting in subsequent immunity to rechallenge with fresh tumor cells. Moreover, the expression of IL-12 within the tumor appears to be localized and causes local increases in IFN-γ production and activation of effector cells draining from the tumor.
used, the antiserum was delivered 4 hr prior to virus injection on day 1 and again on days 3 and 5 as described by Chensue et al. (1995) (the antibody half-life in serum is 50 hr). Tumors were measured biweekly using calipers and the tumor volume as calculated from the longest diameter and average width, assuming a prolate spheroid. The animals were sacrificed when the longest diameter was greater than 20 mm or when any two measurements were greater than 10 mm.

Cytokine ELISA

At specified times, blood was drawn from the retro-orbital sinus of randomly selected mice and tumors were removed from euthanized mice and snap-frozen in liquid nitrogen. Frozen tissue was homogenized in PBS (0.5–1 ml, depending on tumor size) containing 100 μM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin. The homogenate was then sonicated twice for 10 sec and cleared by centrifugation in a microfuge for 5 min at room temperature. Animal sera and tumor homogenates were stored at −20°C.

The IL-12 and IFN-γ ELISAs were performed as described previously (Chensue et al., 1995). Briefly, flat-bottomed 96-well microtiter plates (NUNC Maxisorp) were coated overnight with polyclonal rabbit antiserum (18 μg/ml anti-IL-12; 10 μg/ml anti-IFN-γ) at 4°C. The day of the assay, the wells were washed three times and nonspecific binding was blocked using 2% bovine serum albumin in PBS for 90 min at 37°C. The blocking buffer was removed and the wells washed three times. The samples were then added and incubated at 37°C for 60 min. The wells were washed five times and biotinylated antibody was added (18 μg/ml anti-IL-12; 4 μg/ml anti-IFN-γ) followed by a 45-min incubation at 37°C. The plates were washed three times and avidin-conjugated horseradish peroxidase (Bio-Rad) was added and incubated 30 min at 37°C. The plates were again washed three times, the peroxidase substrate OPD (Pierce) was added, and the reaction was terminated after 5 min by the addition of 3 N H₂SO₄. Recombinant proteins were included in each analysis and the ELISAs were consistently sensitive to 50 pg/ml.

Statistical analysis

All data are expressed as mean ± SEM and compared using an unpaired, two-tailed Student’s t-test. The statistics were calculated using Statview 512+ on a Macintosh Iic (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Antitumoral activity of AdmIL-12.1

Previous studies using Ad vectors expressing IL-2 and IL-4 to treat PyMT tumors demonstrated that direct injection of 5 × 10⁶ plaque-forming units (pfu) was most efficacious (Addison et al., 1995a,b). Therefore, we used this dose of AdmIL-12.1 initially and compared it to the effects of a control virus, d70-3, which lacks the E1 region making it replication deficient. The tumors were injected with virus on day 21 following implantation of tumor cells on the right hind flank and animals were subsequently monitored on a regular basis to follow tumor progression. Injection of the control virus did not prevent tumor growth (Addison et al., 1995a) (Fig. 1A, open circles). In contrast, AdmIL-12.1 treatment resulted in substantial reduction in tumor volume (Fig. 1B, closed squares). In the experiment shown in Fig. 1, the mean tumor volume at the time of injection for the AdmIL-12.1 treated group was 76.8 ± 17.9 mm³ and by day 20 the mean volume had diminished to 32.2 ± 6.4 mm³ (p = 0.573). This effect was cytokine dependent because when neutralizing antibody to IL-12 was delivered at the same time as the virus, the antitumor effects were abrogated (Fig. 1A, open squares). Normal rabbit serum had no effect on the outcome of AdmIL-12.1 therapy (data not shown). Treatment with AdmIL-12.1 produced regression in 28/36 tumors (78%), and 11/36 (32%) of the animals have remained tumor free for the duration of the experiment, and in some cases greater than 1 year (Fig. 2; Table 1). In the remaining 8 animals, five tumors remained static for a period of 2–3 weeks, at which point they resume growth, whereas the other three tumors displayed no response. The results from six experiments (36 animals treated with AdmIL-12.1) are summarized in Table
FIG. 2. Long-term survival following treatment with AdmIL-12.1. The survival of the experimental animals is plotted as a function of time. (○) d70-3-treated tumors (n = 34); (■) AdmIL12.1-treated tumors (n = 36).

The overall response rate was 92%, clearly demonstrating the potency of this therapy. None of the animals in the study treated with d70-3 (n = 34) showed evidence of a response. Even among those animals whose tumors did not completely regress, mean survival following virus injection was increased approximately two-fold [28 ± 3 days (d70-3) vs. 52 ± 4 days (AdmIL-12.1); p = 0.0001]. We observed similar results using 2.5 × 10^8 pfu AdmIL-12.1 per treatment, but increasing the viral load to 2 × 10^9 pfu AdmIL-12.1 per tumor did not improve the outcome (data not shown). We also found that using as low as 5 × 10^7 pfu AdmIL-12.1, we could achieve complete regression in 1 of 4 treated animals. Thus, the AdmIL-12.1 treatment seems to have a wide window of activity. We did not observe any gross side effects of the treatment, although 1 animal out of 37 died after treatment with AdmIL-12.1. It was not clear, however, that the death was cytokine related.

Table 1. Summary of Responses Following Intratumoral Injection of AdmIL-12.1

<table>
<thead>
<tr>
<th>Complete regression</th>
<th>Partial regression</th>
<th>Growth delay</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>17</td>
<td>5</td>
<td>3</td>
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Complete response = 11/36 (31%); overall response = 33/36 (92%).

aAnimals with established tumors were treated intratumorally with 5 × 10^8 pfu AdmIL-12.1 and the resultant changes in tumor volume were measured regularly. The table summarizes the results of six experiments involving 3–9 animals each.

bTumors regressed completely and did not return for at least 2 months.

cTumors initially regressed to less than 50% of the initial size but were not eliminated and eventually returned.

dTumors displayed no change in tumor volume for at least 2 weeks following treatment.

Tumor regression is associated with long-term immunity

One of the objectives of cytokine immunotherapy is to induce immunity against the treated tumor and, thus, protect against recurrence and metastatic spread. To test whether AdmIL12.1 treatment could produce long-term immunity, the 11 animals that had been cured were rechallenged with fresh tumor cells on the opposite flank 2–3 months following disappearance of the primary tumor. Ten of the 11 animals were resistant to rechallenge, whereas all control mice developed tumors at the usual time (approximately 2–3 weeks post-challenge). Only 1 animal was not completely resistant to rechallenge; however this mouse did demonstrate a delay in tumor appearance by greater than 3 weeks compared to the controls.

Expression of IL-12 following intratumoral injection is localized to the tumor

To determine the extent of localization of IL-12 within the tumor mass, a time course of IL-12 expression following intratumoral injection of either AdmIL12.1 or d70-3 was performed across a period of 2 weeks. Animals were injected with 5 × 10^8 pfu of either AdmIL-12.1 or d70-3 and 2 animals from each group were sacrificed at various times to quantify the IL-12 levels in the serum and within the tumor (Fig. 3). We compared intratumoral expression of IL-12 to serum IL-12 in Fig. 3a by approximating total IL-12 in the serum, assuming a total blood volume of 2 ml per animal, which would yield 1 ml of serum. The peak expression of IL-12 reached approximately 800 ng within the tumor mass, yet never exceeded 60 ng/ml in the serum. Moreover, IL-12 expression in the tumor peaked between 24 and 72 hr whereas serum IL-12 was maximal at 24 hr post injection (60 ng/ml) and by 72 hr serum IL-12 was reduced to less than 10 ng/ml. Thus, IL-12 expression appeared to be primarily localized to the tumor with little dissemination in the circulation. Both intratumoral and serum IL-12 were detectable by ELISA throughout the 14-day experiment. IL-12 expression was also slightly elevated in the d70-3-treated tumors (Fig. 3b), most likely due to the host response to Ad infection. Over the course of the experiment, the tumors in the control group increased in size, whereas those of the AdmIL-12.1 group began to regress. Therefore, the data in Fig. 3b were normalized to tumor mass to take into account the changes in tumor volume.

Expression of IL-12 within the tumor causes increases in local IFN-γ production

Induction of IFN-γ production is a well-defined characteristic of IL-12 treatment and it has been shown that IFN-γ is required for at least part of the antitumor properties of IL-12 (Nastala et al., 1994; Brunda et al., 1995; Zou et al., 1995). We have examined the expression of IFN-γ within the tumor as an indicator of local changes within the cytokine environment caused by AdmIL-12.1. In the tumors injected with AdmIL-12.1, we observed a very rapid increase in the local concentration of IFN-γ (Fig. 4; the data was normalized to tumor volume as described in the previous paragraph). It is notable that IFN-γ production reached maximum levels from days 6 to 9,
a comparison of intratumoral expression of IL-12 and serum IL-12. Following intratumoral treatment with AdmIL-12.1 (■) or d70-3 (●), 2 animals were sacrificed on days 1, 3, 6, 9, and 14, and the IL-12 concentration was measured in both their tumors and serum. IL-12 expression in tumor homogenates and sera was measured using an ELISA as described in Materials and Methods. These results represent one of two experiments with similar results. A. Total IL-12 in the tumor (■) following intratumoral injection of 5 × 10⁶ pfu AdmIL-12.1 is compared to total IL-12 in the serum (□). Total serum IL-12 was approximated by assuming a total blood volume per mouse of 2 ml yielding 1 ml of serum. B. Intratumoral expression of IL-12 following injection of 5 × 10⁶ pfu AdmIL-12.1 (■) or d70-3 (●). The IL-12 expression was normalized to the tumor mass as an indication of local “concentration.”

after the IL-12 expression had begun to decline (compare Fig. 4 vs. Fig. 3b). The levels of IFN-γ were three- to five-fold higher in the AdmIL-12.1-treated tumors than the control group at day 6. The control virus also induced an increase in IFN-γ expression, but this did not reach the same level as the AdmIL-12.1 treatment and was probably a consequence of an antiviral response.

AdmIL-12.1 treatment induces IFN-γ expression in draining lymph node cells

To demonstrate a direct effect of AdmIL-12.1 treatment on the effector cells present within the tumor, we removed the lymph nodes draining the tumors (TDLN) and determined the IFN-γ production from the TD LN cells after 24 hr in culture (Fig. 5). The TD LN cells from all the AdmIL-12.1-treated animals secreted high levels of IFN-γ, whereas only half of the lymph nodes from the d70-3-treated animals expressed any detectable IFN-γ. Taken together, these data indicate that intratumoral injection of AdmIL-12.1 produces tumor regression that is associated with local changes in the cytokine environment of the tumor.

FIG. 4. Increased local IFN-γ expression following treatment with AdmIL-12.1. The AdmIL-12.1-treated tumors (■; two samples per day) were screened for the expression of IFN-γ and compared to d70-3-treated tumors (●; two samples per day). The IFN-γ expression was measured in tumor homogenates using an ELISA as described in Material and Methods. IFN-γ expression was then normalized to the mass of the tumor as an indication of “concentration.”

FIG. 5. IFN-γ production by draining lymph node cells. Cells were harvested from the draining lymph nodes of mice (two per day) treated with d70-3 (open bars) or AdmIL-12.1 (cross-hatched bars). The cells were incubated for 24 hr in vitro at a density of 10⁶ cells/ml, and the IFN-γ in the medium was determined by ELISA. IFN-γ production in the culture supernatants was normalized to 10⁶ cells.
DISCUSSION

Systemic toxicities associated with immunotherapy might be overcome if cytokine expression were localized to the tumor site. Our approach to this problem is to use Ad vectors to modify tumor cells genetically with cytokine cDNAs so that the tumor cells can supply the cytokine of interest in a paracrine fashion to the antitumor responder cells present within the tumor. Using an Ad vector expressing mIL-12, we have shown that a single injection of the virus can lead to complete tumor regression in >30% of the cases with an overall response rate of >90%. This response was similar to that observed with other Ad vectors expressing IL-2 or IL-4 (Addison et al., 1995a,b; Cordier et al., 1995), whereas vectors expressing IL-5 or IL-6 had little or no effect (T. Braciak, unpublished data). In contrast to the AdmIL-12.1 treatment, the IL-2- and IL-4-expressing vectors exhibited toxicity at doses needed to induce regression (Addison et al., 1995a, 1995b), whereas there were no obvious toxic side effects associated with AdmIL-12.1 treatment. We have observed one death following treatment of 37 animals with AdmIL-12.1, but it is not clear that the vector alone was responsible for that death. We found that similar results could be achieved using 2.5 x 10^{6} and 5 x 10^{6} pfu AdmIL-12.1 and that complete regression could be achieved in 1 out of 4 animals using as little as 5 x 10^{5} pfu AdmIL-12.1. This vector has a relatively broad window of therapeutic activity. Increasing the viral load to 2 x 10^{9} pfu did not improve the outcome, so it does not appear that the failure to induce complete regression can be explained by the quantity of AdmIL-12.1 being limiting, although the duration of expression may play a factor.

Zitvogel et al. (1995) have used fibroblasts transduced with a retroviral vector expressing murine IL-12 to successfully treat pre-established tumors. An antitumor response was observed whether the fibroblasts were implanted at the tumor site or at a distant site, indicating that circulating levels of IL-12 played an important role in the antitumor activity of IL-12 in that model. The authors do state that the "antitumor effects were observed more rapidly" when the cells were injected at the tumor site, suggesting that local expression was more effective. It is not clear in our model what role circulating IL-12 plays in the antitumor response and it is quite possible that the low level of serum IL-12 we have detected may be beneficial in our therapy. The advantage of AdmIL-12.1 over the retrovirus is that the tumor cells could be transduced directly, without the need to culture autologous fibroblasts, although it may be possible that retroviruses can also be used in vivo. A vaccinia virus expressing mIL-12 has been shown to suppress tumor growth (Meko et al., 1995) but the effect of this vector in a model of pre-established tumors has not yet been reported.

In the present study, 10 of 11 animals that were rechallenged following regression of the initial tumor demonstrated long-term immunity, whereas 1 animal succumbed to the challenge. Nastala et al. (1994) also found that a fraction of animals cured of tumors by IL-12 did not possess long-term immunity, suggesting that the antitumor effects of IL-12 may also include non-T cell-mediated events. Voest et al. (1995) have demonstrated that IL-12 exhibits antiangiogenic activities that can account for some antitumor activity. It is also interesting to note that at least 2 animals have maintained antitumor immunity for as long as 8 months following tumor regression. However, we have also observed 1 animal that was immune 3 months after treatment but lost this immunity by 8 months.

Very high levels of IL-12 (400–800 ng) within the tumor could be detected within the first 3 days following AdmIL-12.1 administration. Interestingly, these levels were similar between the various tumor samples, indicating that viral delivery to the tumor was relatively consistent. Although it is difficult to determine what fraction of the IL-12 produced within the tumor is intracellular, we do know that in vitro, tumor cells infected with AdmIL-12.1 at an moi of 30 will secrete 5,000 ng of bioactive IL-12/10^{6} cells per 24 hr (Branson et al., 1996). Therefore, we would expect that the tumors would express similarly high levels of IL-12 in vivo. It is notable that the serum IL-12 declined five-fold from day 1 to day 3 whereas the intratumoral IL-12 only began to decline around day 6. One would expect that changes in the levels of serum IL-12 would parallel those in the tumor, unless structural alterations occur within the tumor during that time period that limit the access of IL-12 to the circulation. IL-12 is known to have angiostatic effects (Voest et al., 1995) and thus the reduced serum levels compared to intratumoral levels at days 3 and 6 may reflect destruction or alteration of the tumor vasculature, preventing IL-12 from leaking into the serum. This is a desirable effect because it would further localize IL-12 expression to the tumor. We are currently investigating the role of antiangiogenesis/vascular destruction in IL-12-mediated killing in our model. Increased IL-12 was also observed within the control tumors treated with dU70-3, consistent with the role IL-12 plays in the antiviral response (Orange and Biron, 1996). Moreover, we have observed adenoviral CTL in the tumor draining lymph nodes of mice treated with the control vector alone (J. Bramson, unpublished data). The development of cellular immunity against adenovirus is consistent with the modest antitumoral effects of the control vector seen in previous studies (slightly slower growth as compared to PBS; Addison et al., 1995a).

The induction of IFN-γ production by activated NK and T cells is a hallmark of IL-12 stimulation in vitro and in vivo (Seder et al., 1993; Gately et al., 1994; Manetti et al., 1994) and IL-12-mediated tumor regression has been shown to be partially dependent on IFN-γ (Nastala et al., 1994; Brunda et al., 1995; Zou et al., 1995). The expression of IFN-γ mRNA increases dramatically in tumors and spleens following systemic IL-12 treatment (Zou et al., 1995; Tannenbaum et al., 1996). We have demonstrated that intratumoral IFN-γ levels rise very rapidly following intratumoral injection of AdmIL-12.1, peaking at 6–9 days, compared to maximal expression of IL-12 at days 1–3, indicating that the local cytokine environment of the tumor has been altered. Most importantly, cells isolated from the tumor-draining lymph node (TDLN) express IFN-γ in culture without additional stimulation, suggesting that these cells had been activated within the tumor as would be expected following exposure to IL-12. These cells may also have been activated within the circulation; however, the ele-
vation in IFN-γ expression as early as day 3 indicates that activation probably occurs within the vicinity of the tumor. Fallarino et al. (1996) demonstrated that high-level production of IFN-γ by TDLN cells correlated with tumor rejection in an IL-12-dependent model of tumor rejection. Similarly, we have observed that TDLN cells from AdmIL-12.1-treated animals produce varying levels of INF-γ, suggesting that IFN-γ production by TDLN cells in our model may also reflect which tumors will go on to be completely rejected. The effects of intratumoral AdmIL-12.1 injection on IFN-γ expression support the hypothesis that Ad-mediated cytokine gene delivery to tumors can affect the local cytokine environment and activate the local effector population with the potential to overcome anergy induced by the tumor.

The results of these initial studies are very promising and we have been experimenting with treatment regimens that may improve the therapeutic value of AdmIL-12.1 treatment. The observation that >75% of the tumors initially regress following treatment indicates that we should be able to increase the number of complete regressions with some modifications of the treatment protocol. We have found that the outcome can be improved by combining AdmIL-12.1 with a vector expressing IL-2 and that repeated injections may be better than a single injection in certain circumstances (J. Bramson, unpublished data). These studies support the use of AdmIL-12.1 as a safe and efficacious treatment for tumor immunotherapy. The virally expressed cytokine is produced at high levels within the tumor and remains localized to this site, indicating that this approach will likely diminish systemic complications due to excess IL-12 in the serum. Thus, continued preclinical experimentation should yield improved treatment strategies that can be applied effectively in a clinical setting.

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