Systemic Vector Leakage and Transgene Expression by Intratumorally Injected Recombinant Adenovirus Vectors

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ABSTRACT

Interleukin 12 is a heterodimeric cytokine that exhibits potent immunostimulatory effects. It has shown some promise in preclinical and clinical studies but was accompanied by serious systemic toxicity such as flu-like syndromes, a rapid transient leukopenia, elevated liver transaminases, gastrointestinal toxicity, and/or liver dysfunction. Gene therapy with intratumorally injected recombinant adenoviral vectors offers the potential to restrict therapeutic gene expression in the tumor. Here we show that a substantial amount of adenoviral vectors disseminates into the systemic circulation and infects parenchymal organs. We further show that this results in high systemic levels of potentially toxic transgene products. To reduce potential toxicity, we tested an inducible promoter based on the heat shock proteins (hsp70B) and present evidence that high intratumoral levels of a therapeutic transgene can be obtained while systemic expression is reduced to a minimum, increasing the safety of adenovirus-based tumor gene therapy.

INTRODUCTION

Therapeutic use of cytokines with antitumoral activities, such as IL-12, has shown some promise in preclinical and clinical anticancer studies. IL-12 is produced by monocytes, macrophages, and dendritic cells. The antitumor activity of IL-12 has been demonstrated in animal models (1, 2). In human trials, however, only limited efficacy was achieved because of serious dose-limiting systemic toxicities. These toxicities include a flu-like syndrome, rapid transient leukopenia, elevated liver transaminases, gastrointestinal toxicity, and/or liver dysfunction (3, 4). Varying degrees of toxicity have been observed in a variety of other animal species that included chimpanzee (5), squirrel monkey (6), cynomolgus macaques (7), and mouse (4). Many of these toxicities overlap with those observed for IFN-γ up-regulation (8). Experiments in animal models clearly demonstrate the dose-dependent nature of the antitumor efficacy of IL-12, and there is a clear need for novel therapeutic strategies that facilitate an increase in its intratumoral cytokine concentration and a reduction in its systemic levels. Gene therapy with intratumorally injected recombinant adenoviral vectors is a promising approach because it offers the potential to restrict therapeutic gene expression in the tumor. Indeed, several groups have obtained encouraging results (9–11). However, there is no consensus on how “localized” intratumorally injected adenovirus vectors are. Potential virus leakage has not been considered a major problem in human trials thus far because the adenovirus infection itself does not pose any problems unless exceedingly high doses are given systemically. It could, however, be a serious impediment for vectors that encode cytokine genes such as IL-12 that are not only expressed in situ but that are secreted into the system and have considerable normal tissue toxicity. Therefore, approaches that can target therapeutic gene expression to tumors are necessary to facilitate the human application of the gene transfer-based immunotherapy of cancer.

In this study, we investigated two important issues:

(a) We sought to characterize potential systemic leakage of locally injected adenoviral vectors by use of murine tumor models and an adenovirus vector containing a reporter GFP gene under the control of a constitutively active CMV promoter (AdGFP).

(b) We investigated whether therapeutic gene (IL-12) expression can be restricted to the tumor by use of a heat-inducible promoter. For this purpose, we used two adenoviral vectors: a control vector containing the mIL12 gene with the constitutively active CMV promoter (AdCMVIL12); and one containing the mIL12 gene with a heat-inducible hsp70B gene promoter (AdhspIL12).

MATERIALS AND METHODS

Tumor Model and Viral Vectors. B16.F10 (American Type Culture Collection, Manassas, Virginia) is a spontaneously arising subline of the B16 melanoma syngeneic with the C57BL/6, and 4T1 is a mouse mammary cancer cell line obtained from Dr. Fred Miller (Michigan Cancer Foundation, Detroit, MI). Both lines were maintained in DMEM (Life Technologies, Inc., Bethesda, MD).

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5 The abbreviations used are: IL, interleukin; GFP, green fluorescent protein; CMV, cytomegalovirus; mIL12, murine IL-12; pfu, plaque-forming unit(s); hsp, heat shock protein.
When transplanted at $10^6$ cells/animal in the respective syngeneic animals, both lines induced tumors in 100% of mice. The 293 cells (American Type Culture Collection, Manassas, Virginia) for virus propagation were maintained in DMEM. All cell culture media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). AdGFP is an adenovector that encodes enhanced green fluorescence protein under the control of the CMV promoter in the E1 region and was kindly provided by Dr. Peter Corry (William Beaumont Hospital, Detroit, MI). AdCMVIL12 (kindly provided by Dr. Frank L. Graham, McMaster University, Hamilton, Ontario, Canada; Ref. 12) contains both IL-12 subunits under the control of the constitutive CMV promoter. The gene expression cassette is inserted in the E1 region. Construction of AdhspIL12 was conducted according to an established protocol using a set of commercially available adenovirus plasmids (Microbix, Toronto, Ontario, Canada). The p40 and p35 subunits of IL-12 were expressed under the control of the hsp70B promoter. Recombinant adenovirus was then obtained by transfection of pΔEsp1A and pBHIG10 into 293 cells (14). After plaque purification, viruses were propagated in 293 cells and purified by CsCl banding according to a standard protocol (15), resulting in a particle/pfu ratio of ≈100:1.

Systemic Dissemination of Reporter Gene Virus (AdGFP). All animal procedures were performed in accordance with institutional guidelines. About $10^6$ melanoma cells were transplanted s.c. into the right hind leg of C57BL/6 mice. Seven days after transplantation (tumor diameter, 5–7 mm), $3 \times 10^8$ pfu (in 50 µl of PBS) of AdGFP were injected intratumorally by use of a Hamilton 100-µl microsyringe with a 30-gauge needle. The dosage was derived from previous experiments carried out in this laboratory to give good therapeutic efficacy (11). Each tumor received just one injection into the center of the tumor. Care was taken to ensure that the viruses were injected into the tumors. After 24 h, tumors and organs of animals that received injections of AdGFP and untreated control animals were harvested, cut, and mounted in aqueous solution for fluorescence microscopy. To visualize GFP, a Xenon arc lamp and a FITC filter were used on a Zeiss Axioskop. Images were acquired with a color CCD camera and framegrabbing equipment at identical magnification, light intensity, and amplification for each sample pair of organs or tumor from treated and untreated animals, respectively.

Intratumoral and Systemic Expression of mIL-12 after Intratumoral Vector Injection. Intratumoral and systemic expression of mIL-12 was studied after intratumoral injection of either control adenovirus, adenovirus constitutively expressing mIL-12 (AdCMVIL12), or adenovirus expressing mIL-12 controlled by a heat-inducible promoter (AdhspIL12) combined with heat treatment in s.c. B16.F10 melanomas and 4T1 mammary adenocarcinomas. About $10^6$ melanoma or mammary adenocarcinoma cells were transplanted s.c. into the right hind leg of C57BL/6 or Balb/c mice, respectively. Seven days after transplantation (tumor diameter, 5–7 mm), equal amounts ($3 \times 10^8$ pfu for B16.F10 and $1 \times 10^8$ pfu for Balb/c in 50 µl) of control adenovirus (AdGFP), AdCMVIL12, or AdhspIL12 were injected intratumorally (to the center of the tumor in one shot). Animals that received injections of control virus or AdCMVIL12 were sacrificed 24 h after virus injection. Animals injected with AdhspIL12 were treated with local hyperthermia to the right hind leg (by immersion in a water bath at 42.5°C for 40 min) 24 h after virus injection under anesthesia with pentobarbital (1.5 mg/20-g mouse i.p.). The animals were sacrificed 24 h after heating. Serum samples (tail vein blood) of all animals were acquired immediately before euthanasia. Tumors and organs (liver, spleen, and lung) of sacrificed animals were disaggregated in PBS (1 ml/100 mg tumor) with Complete protease inhibitor (Boehringer Mannheim, Mannheim, Germany) with a tissue homogenizer for 1 min on ice. After centrifugation, the supernatant was harvested. IL-12 concentrations in tumor and organ extracts and serum samples were detected with a mIL-12 ELISA kit detecting the p70 heterodimer (R&D Systems, Minneapolis, MN) with a detection level of 2.5 pg/ml. The results are plotted as mean ± range of two to four animals/data point.

RESULTS AND DISCUSSION

In the first series of experiments, 3 $\times 10^8$ pfu (a dose used in previous studies to have optimal therapeutic efficacy; Ref. 16) of AdGFP in 50 µl of PBS were intratumorally injected when s.c. B16.F10 melanoma tumors reached a diameter of 5–7 mm. The mice were sacrificed 24 h later. The 24-h time point was chosen because it was determined to be optimal for observing adenovirus-mediated gene expression in vivo in previous experiments (16). Fluorescence microscopy of harvested tumors and organs (Fig. 1) demonstrated high numbers of GFP-expressing cells in the liver and spleen and to a lesser extent in the lungs of C57BL/6 mice bearing B16 melanoma at 24 h after virus injection. The GFP-expressing area in the tumor was limited to a small volume around the presumed needletrack. GFP expression was seen in most areas of the liver. It was also obvious throughout the spleen. No GFP expression was detected in kidneys or brain of animals receiving AdGFP injections or in any organs of the control animals. Therefore, there is significant systematic leakage of adenoviral particles despite local injection of the vectors. This finding is consistent with some earlier reports (17–19). Similar observations were also made in Balb/c mice that were implanted with 4T1 tumors. An intratumoral injection of 1 $\times 10^8$ pfu (maximum tolerated dose in this strain) readily generated liver and spleen infections of the virus (as evidenced by GFP expression). These results point to the need to establish approaches to reduce leakage-mediated systemic gene expression.

Because adenovirus vectors do possess significant advantages (e.g., high-titer production and high infectivity in a wide spectrum of tissues) that are not easily replaced by other vectors, new strategies that involve the use of adenovirus with reduction in systemic gene expression are highly desirable. There are two strategies that can potentially reduce unwanted systemic gene expression. The first is to use adenovirus vectors that infect tumor cells selectively by modification of viral surface molecules (20, 21). The second approach is to use promoters that can be selectively activated in the tumor. Promising results have been obtained with the radiation inducible EGR-1 promoter (22). We chose to study promoters that can be induced in a spatially controlled fashion by use of the promoter of hsp70B, which is strongly heat inducible in vitro (>13,000 × for IL-12) but maintains a low constitutive background expression (23–26). An adenovirus vector containing mu-
rine IL-12 under the control of this promoter (AdhspIL12) was built. We then tested the local and systemic gene expression profiles of this vector and two control vectors (AdGFP and AdCMVhspIL12) in B16.F10 melanoma models. For AdGFP and constitutively expressing AdCMVIL12, the intratumoral and systemic IL-12 levels 24 h (peak time of intratumoral and serum expression as determined previously) were compared. For AdhspIL12, the cytokine levels were measured 24 h after local hyperthermia. AdhspIL12 was administered 24 h prior to heating (2–4 mice/data point). Gene expression in several organs, such as the liver, spleen, and lung, was also measured. As shown in Fig. 2, the heat-inducible construct produced substantial tumor levels of IL-12, comparable with those achieved with the constitutive CMV promoter. The difference in IL-12 expression was negligible between AdhspIL12-injected tumors treated at the control temperature (34°C) and tumors injected with the control virus (AdGFP). This illustrates the very tight thermal control of the promoter. In addition, the cytokine levels detected in parenchymal organs and serum were reduced to those observed for control adenoviruses (AdGFP).

When similar experiments were conducted in 4T1 tumors using AdhspIL12, the levels of intratumoral IL-12 after heating were marginally higher in the 4T1 tumors (4–6 ng/g tumor tissue in 4T1 versus 2–4 ng/g tumor tissue in B16). Systemic levels were again negligible. AdCMVIL12 mediated higher intratumoral IL-12 levels (12–14 ng/g) at 1 × 10⁶ pfu/tumor. However, it also induced very high serum IL-12 levels (20 ng/ml). Therefore, data in the 4T1 model again confirms the high inducibility of AdhspIL12 and low systemic level. However, these results were initially puzzling, given the two tumor cell lines’ significantly different susceptibility to adenovirus infection in vitro (11). A potential explanation may be the widespread infiltration of the B16 tumors by normal host C57/BL6 mouse cells. The host cells may compensate for the lack of infection to the B16 cells because they are almost equally susceptible to adenovirus-mediated infection in both mouse strains. Indeed, when the two types of tumors were infected with AdCMVGFp and sectioned 24 h later, the two types of tumors show similar levels of GFP, indicating a similar extent of GFP infection. It can therefore be concluded that high intratumoral levels of a therapeutic transgene can be obtained when the heat-inducible promoter hsp70B is used. In addition, better results are obtained in comparison to a radiation-inducible EGR-1 promoter as reported previously. The EGR-1 promoter mediates a 9-fold in vitro induction when irradiated with a high dose (20 Gy). Furthermore, the EGR promoter has high background (22, 27), whereas the hsp70B promoter mediates >13,000 fold in vitro induction (23) and negligible background. An additional advantage is that systemic expression is reduced to a minimum when the hsp70B promoter is used, thereby increasing the safety of this adenovirus-based tumor gene therapy.

With the completion of the human genome and the dawn of the era of rational drug design, more and more therapeutics are being developed for cancer treatment. A key to the success of these new therapeutics is the ability to target tumor cells so that a wider therapeutic window can be obtained than traditional chemotherapy. This is especially true for gene therapy approaches that use thera-
peutic genes that are toxic to normal tissue/organisms. For these strategies to be practical, it has to demonstrate selective or preferential tumor killing. Otherwise, it would be difficult to justify the use of gene therapy because of difficulties in delivering the therapeutic genes. On the other hand, the availability of different genetic elements that can be regulated by various chemical, biological, and physical agents offers ample opportunities for fine tuning therapeutic gene expression so that it is targeted to the tumor cells. Our hsp promoter data demonstrated significant promise in this direction.

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