Effects of rate, volume, and dose of intratumoral infusion on virus dissemination in local gene delivery

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Abstract

Recent studies have shown that up to 90% of viral vectors could disseminate to normal organs following intratumoral infusion. The amount of dissemination might be dependent on the infusion conditions. Therefore, we investigated the effects of infusion rate, volume, and dose on transgene expression in liver and tumor tissues after intratumoral infusion of an adenoviral vector encoding luciferase. Luciferase expression was determined through bioluminescence intensity measurement. We observed that the luciferase expression in the liver was independent of the infusion rate but increased with the infusion dose, whereas the luciferase expression in the tumor was a bell-shaped function of the infusion rate. The latter observation was consistent with the distribution pattern of Evans blue–labeled albumin after its solution was infused into tumors at the same infusion rates. We also observed that the infusion volume could affect luciferase expression in the tumor but not in the liver. These observations implied that virus dissemination was determined mainly by the infusion dose, whereas the amount of transgene expression in the tumor depended on the distribution volume of viral vectors in the tumor as well as the infusion dose. [Mol Cancer Ther 2006;5(2):362–6]

Introduction

Intratumoral infusion is currently the most common method for viral gene delivery in cancer treatment because it can circumvent transvascular barriers and enhance interstitial transport in tumors (1–5). In addition, it has been expected that the infused viral vectors and transgene products are confined to solid tumors, thereby causing minimal toxicity in normal tissues (6–9). However, data in the literature has shown that a fraction of infused viral vectors could disseminate from tumors during and after the infusion (10–16). In a mouse study, up to 90% of adenoviral vectors are observed in the liver 10 minutes after intratumoral infusion (15). The dissemination reduces transgene expression in the tumor and leads to the accumulation of viruses and transgene products in normal tissues.

To increase transgene expression in tumor and simultaneously reduce it in normal tissues, various strategies have been developed to control transgene expression at transductional and/or transcriptional levels (16–18). These strategies are based on the modification of viral surface ligands that can specifically bind to molecular markers in tumors, or the integration of unique regulatory elements into viral genomes that can be activated by specific endogenous or exogenous stimuli (16). However, investigators may not be able to solve the problem of virus dissemination via these strategies because the mechanism of dissemination is likely to be infusion-induced convective transport of viral vectors into leaky tumor microvessels near the infusion site (13–16). To directly show the effects of convection on virus dissemination, we investigated the dependence of adenovirus dissemination on infusion rate, volume, and dose. Meanwhile, we quantified transgene expressions in tumor and liver tissues under these infusion conditions. Our data indicated that virus dissemination was determined mainly by the infusion dose and that the amount of transgene expression in the tumor depended on the distribution volume of viral vectors in the tumor as well as the infusion dose.

Materials and Methods

Tumor Model

A mouse mammary carcinoma cell line (4T1) was used as the tumor model in this study (13). One million 4T1 cells in 50 µL PBS were s.c. injected into the right hind leg of 4- to 6-week-old syngeneic female BALB/c mice (Charles River Laboratory, Wilmington, MA) after the animals were anesthetized with an i.p. injection of a cocktail of ketamine and xylazine (80 mg ketamine and 10 mg xylazine per kg body weight). The s.c. tumors were used in experiments when they reached 5 to 8 mm in diameter. To minimize the effects of tumor size on experimental data, we randomly separated tumors into different experimental groups. The animal protocol was approved by the Duke University Institutional Animal Care and Use Committee.

Adenoviral Vector

The Ad5-based recombinant system was used to produce an adenoviral vector, AdCMVLuc, encoding
Luciferase (15). Adenoviruses were propagated in 293 cells (American Type Culture Collection, Manassas, VA), harvested at 48 hours after infection, and purified by cesium chloride gradient centrifugation according to a standard protocol (19).

**Quantification of Luciferase Expression**

AdCMVLuc suspension was infused into 4T1 tumors after mice were anesthetized using the protocol described above. The infusion was done via a 30-gauge needle mounted on a syringe pump (model 22, Harvard Apparatus Co., Cambridge, MA; ref. 15). Luciferase expressions in the liver and tumor were quantified at different time points after intratumoral infusion of AdCMVLuc, using the Xenogen *In vivo* Imaging System (Xenogen Corp., Alameda, CA; ref. 15).

**Distribution of Evans Blue–Labeled Albumin**

Evans blue–labeled albumin solution was prepared by mixing 0.04% Evans blue and 0.1% albumin in 0.9% saline. The solution was infused into 4T1 tumors, following the same procedure as that for viral vector infusion. After the infusion, the mice were immediately sacrificed by cervical dislocation. The tumors were harvested, mounted on a specimen block, transferred to the stage of the Vibratome (model 3000, Technical Products International, St. Louis, MO), and sectioned into 300 μm slices. The slices were mounted on glass slides and scanned into a computer with a Plustek Optic Pro document scanner (model 12000P).

**Statistical Analyses**

The Mann-Whitney *U* test was used to compare the difference between two unpaired groups. *P* < 0.05 was considered to be significant.

**Results and Discussion**

Data in the literature have shown that >95% of adenoviruses in the mouse systemic circulation will eventually accumulate in the liver (16). As a result, adenoviral vectors accumulate mainly in the liver after they disseminate from the tumor following intratumoral infusion, and the amount of luciferase (a nonsecreted transgene...
product) in the liver should correlate with the number of disseminated adenoviruses (13, 15). Meanwhile, luciferase expression in other tissues was negligible and thus not examined in our study.

Transgene expression in the liver and tumor is generally time-dependent. Luciferase expression in the liver decreased initially and then reached a plateau at 3 days after the intratumoral infusion (Fig. 1A). There was no significant change in the luciferase expression in the liver between 3 and 21 days ($P > 0.05$). Luciferase expression in the tumor increased during the first 2 to 3 days and then decreased exponentially (Fig. 1A). It could not be detected at 21 days after the infusion. At this time point, we observed bioluminescence in the liver and other normal organs, such as ears, nose, feet, and tail (Fig. 1B), which could not be detected during the first 7 days after the intratumoral infusion. The source(s) of the bioluminescence in these tissues remains to be determined.

Based on the data shown in Fig. 1A, we chose to quantify luciferase expression only at 2 days after intratumoral infusion in the following three experiments. First, we investigated the effects of infusion rate on luciferase expression. In this experiment, adenoviral vectors were infused into the tumor at a rate between 0.25 and 5.0 µL/s. This range of infusion rates covered the values used in previous studies. The infusion dose and volume per tumor were fixed at $2.0 \times 10^8$ plaque-forming units (pfu) and 50 µL, respectively. As a result, the time period of infusion varied according to the infusion rate. We observed that the bioluminescence intensity in the liver was independent of the infusion rate at 2 days after intratumoral infusion (Fig. 2A), indicating that the infusion rate had minimal effects on virus dissemination. The mean bioluminescence intensity in the tumor was a bell-shaped function of the infusion rate (Fig. 2A), but the difference between any pairs of data in the tumor or liver were not statistically significant ($P > 0.05$). The lack of significance was presumably due to the heterogeneity in tumor tissues which has also been observed in previous studies (2). To understand why the intensity was low at the infusion rate of 5.0 µL/s, we infused the Evans blue–labeled albumin into tumors at different rates and examined its distribution in tumors after the infusion. The results are shown in Fig. 2B. We observed that albumin concentration was high near the needle track and low far away from the track in all experimental groups. We also observed that the Evans blue–labeled albumin accumulated only in the periphery of tumors if the infusion rate was maintained at 5.0 µL/s, indicating that albumin leaked out through some cracks in these tumors. Crack formation has been reported in previous studies of intratumoral infusion (2, 20, 21). It is likely to be due to the presence of necrotic regions and blood pools as well as abnormal assembly of extracellular matrix (22). At both macroscopic (i.e., necrotic regions and blood pools) and microscopic (i.e., abnormal assembly of extracellular matrix) levels, these defects form weak structures that can be ruptured to form macroscopic cracks during the infusion. Qualitatively, the images shown in Fig. 2B also showed that the pattern of albumin

**Figure 3.** Effects of infusion volume on luciferase expression in the liver and tumor. The bioluminescence intensity was determined at 2 d after intratumoral infusion of AdCMVLuc. The infusion rate and volume were fixed at 1.0 µL/s and 50 µL/tumor, respectively. Columns, means from five animals; bars, SE; *, $P < 0.05$.

**Figure 4.** Effects of infusion dose on luciferase expression in the liver and tumor. A, the bioluminescence intensity was determined at 2 d after intratumoral infusion of AdCMVLuc. The infusion rate and volume were fixed at 1.0 µL/s and 50 µL/tumor, respectively. Columns, means from three to five animals; bars, SE; *, $P < 0.05$.

B, the bioluminescence intensities in (A) normalized by the corresponding doses of infusion. The unit of the vertical axis is $(10^5 p/s) / (10^8 pfu/tumor)$.
distribution correlated with the bioluminescence intensity in the tumor shown in Fig. 2A. Although infusion-induced convection of albumin differed quantitatively from that of viral vectors, the results shown in Fig. 2 suggest that transgene expression depends on the distribution volume of adenoviral vectors in the tumor. Second, we fixed the infusion dose and rate at 2.0 × 10^8 pfu/tumor and 1.0 µL/s, respectively, and varied the infusion volume from 20 to 50 µL. As a result, the time period of infusion and the virus concentration in the infusate were varied according to the infusion volume. Again, we observed that the bioluminescence intensity in the liver was independent of the infusion volume (Fig. 3), indicating that the infusion volume had minimal effects on the virus dissemination. Luciferase expression in tumors increased with increasing infusion volumes (P < 0.05; Fig. 3). We also infused different volumes (i.e., 20 and 50 µL) of the Evans blue–labeled albumin solution into tumors. The distribution volume of Evans blue–labeled albumin was consistent with the luciferase expression in tumors (data not shown). These results again suggested that transgene expression in the tumor was determined by the distribution volume of adenoviral vectors.

Finally, we varied the dose of viral vectors from 0.1 × 10^8 to 5.0 × 10^8 pfu/tumor by changing the virus concentration in the infusate and fixing the infusion rate as well as the volume at 1.0 µL/s and 50 µL, respectively. We observed that the bioluminescence intensity in both tumor and liver tissues increased with increasing doses of the infusion (Fig. 4A). The mechanism of the dose–dependence is likely to be that transgene expression is a sigmoidal function of the local concentration of viral vectors (23), which in turn depends on the dose of infusion. However, the bioluminescence intensities normalized by the dose of infection in both tissues were independent of the dose (Fig. 4B), except that the normalized intensity in the liver at the dose of 5.0 × 10^8 pfu/tumor was significantly higher than the value of the same variable at a dose of 0.1 × 10^8 pfu/tumor (P = 0.03). Furthermore, we observed that four out of nine mice in the dose group of 5.0 × 10^8 pfu/tumor died within 30 minutes after intratumoral infusion, whereas no mice died in the other dose groups.

The dose–dependence of virus dissemination has also been observed in previous studies (12, 14, 24, 25). No adenoviruses could be detected in normal tissues when the dose was <1 × 10^8 pfu/tumor (9). On the other hand, virus dissemination was observed clearly when the dose was >1 × 10^8 pfu/tumor (10–12). It has also been reported that some animals die within a short period after either systemic or intratumoral infusion of high-dose adenoviral vectors (12, 14, 24, 25); and the death is likely to be due to acute immune response to adenoviruses in the systemic circulation and normal tissues (25–28).

In summary, we showed that the virus dissemination was determined mainly by the dose of intratumoral infusion, whereas transgene expression in the tumor depended on not only the infusion dose but also the infusion volume. These data provided further evidence to support the conclusion that virus dissemination was caused by infusion-induced convective transport in tumors (14, 15). This information will be useful in the optimization of intratumoral infusion of viral vectors in cancer gene therapy.

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