A Novel Method for Viral Gene Delivery in Solid Tumors

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Abstract

Intratumoral infusion is the most commonly used method for viral gene delivery in clinical trials for cancer treatment. However, a potential problem in this approach is that viral vectors may disseminate from tumor to normal tissues during and after the infusion. To reduce the dissemination, we developed a novel method based on a biocompatible polymer, poloxamer 407, which could significantly increase the viscosity of virus suspension when the temperature was changed from 4°C to 37°C. With this method, we could significantly increase transgene expression in solid tumors and reduce virus dissemination by 2 orders of magnitude after intratumoral infusion of adenoviral vectors. The mechanism of reduction was likely to be that the viscous poloxamer solution blocked convection of viral vectors in the interstitial space and the lumen of microvessels in the vicinity of the infusion site. This method has a potential to be used in the clinic for enhancing efficacy and reducing toxicity in viral gene therapy.

Introduction

Virus-based gene therapy has shown promising results in preclinical cancer treatment (1, 2). However, its clinical applications are currently limited by the efficacy/toxicity ratio (1, 3). To increase the ratio, scientists have developed various strategies that may control transgene expression in target cells at transcriptional and translational levels (4, 5). A prerequisite for the success of these strategies is that viral vectors must reach most tumor cells, which is unlikely to happen in systemic gene delivery. The obstacle is primarily due to (a) physiologic barriers to virus transport in tumors and (b) rapid clearance of viral vectors in systemic circulation (3, 6, 7). To circumvent these problems, researchers have directly infused viral vectors into solid tumors. Intratumoral infusion can bypass the initial filtering events in normal tissues, which are inevitable in systemic gene delivery, and facilitate virus transport in tumor tissues (7–9). At present, intratumoral infusion is the most commonly used method for viral gene delivery in clinical trials. However, a potential problem in this approach is that viral vectors can disseminate from tumor to normal tissues during and after the infusion (10–15). The amount of disseminated viruses can be 1 order of magnitude higher than that retained in the tumor. Virus dissemination decreases the efficacy/toxicity ratio in gene therapy. To this end, we developed a new delivery method for reducing virus dissemination. The rationale is as follows. Virus dissemination is due to the convective transport of viral vectors into the systemic circulation (14, 15); and the rate of convection is inversely proportional to the viscosity of fluids. Therefore, virus dissemination will be reduced if the viscosity of virus suspension is increased. On the other hand, merely increasing the viscosity may make the infusion too difficult to be done in the clinic. To avoid this problem and yet increase the viscosity of virus suspension in tumor tissues, we used poloxamer, a polymer consisting of poly(ethylene oxide) and poly(propylene oxide) units (16, 17), as a delivery vehicle. Poloxamer is biocompatible and has been widely used for wound healing, drug and gene delivery, and tissue engineering. Its viscosity can increase by 2 to 3 orders of magnitude when the temperature was increased from 4°C to 37°C. To test the feasibility of the new method, we prepared a poloxamer 407 solution at 4°C, mixed it with the suspension of adenoviral vectors, and infused the mixture into tumors. We observed that the poloxamer solution could be easily injected and after the intratumoral infusion, the new vehicle could reduce virus dissemination by 2 orders of magnitude and significantly increase transgene expression in solid tumors.

Materials and Methods

Tumor model. A mouse mammary carcinoma cell line (4T1) was used as the tumor model (14). 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 i.p. injection of a cocktail of ketamine and xylazine (80 mg ketamine and 10 mg xylazine per kilogram of body weight). The s.c. tumors were used in experiments when they reached 5 to 8 mm in diameter. The animal protocol has been approved by the Duke University Institutional Animal Care and Use Committee.

Adenoviral vectors. The Ad5-based recombinant system was used to produce the adenoviral vectors, AdCMVEGFP and AdCMVLuc, encoding enhanced green fluorescence protein (EGFP) and luciferase, respectively (14). All adenoviral vectors were propagated in 293 cells (American Type Culture Collection, Manassas, VA) and stored in 10% glycerol at −80°C.

Poloxamer solution. Poloxamer 407 was purchased from BASF Corporation (Mount Olive, NJ). The poloxamer solution was prepared by dissolving poloxamer powder into Opti-med solution at 4°C. Brookfield DV-III Rheometer (Brookfield, MA) was used to measure the viscosity of solutions at different shear rates and temperatures. The measurement at each shear rate and temperature was repeated thrice.

Intratumoral infusion. Fifty microliters of viral vector suspension were infused into 4T1 tumors at 1 μL/s via a 30-gauge needle mounted on a Model 22 Harvard syringe pump (Harvard Apparatus Co., Cambridge, MA; ref. 15). The dose of infusion was 3.0 × 10^8 plaque-forming units (pfu)/tumor for AdCMVEGFP and 2.0 × 10^8 pfu/tumor for AdCMVLuc.

Analysis of adenoviruses in the blood. Blood samples were collected from anesthetized mice through the orbital sinus with a heparinized capillary (80 mg ketamine and 10 mg xylazine per kilogram of body weight). The s.c. tumors were used in experiments when they reached 5 to 8 mm in diameter. The animal protocol has been approved by the Duke University Institutional Animal Care and Use Committee.

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Analysis of adenoviruses in the blood. Blood samples were collected from anesthetized mice through the orbital sinus with a heparinized capillary immediately after the intratumoral infusion of AdCMVEGFP (15). They were centrifuged at 14,000 rpm for 3 minutes to separate the plasma from cells. Then, 1 μL of plasma was added into each well in 96-well plates with 70% to 80% confluent 293 cells. After 24 hours, the number of EGFP-positive cells, which could be used as a measure of the amount of adenoviruses in the plasma, was examined under a fluorescence microscope (15).

Quantification of adenovirus copy numbers in liver and tumor tissues. The details of the quantification procedures have been described in the literature (15). In brief, mice were sacrificed at 10 minutes after the
intratumoral infusion of AdCMVEGFP. The livers and tumors were harvested, frozen in liquid nitrogen, and ground into fine tissue powder in a mortar. Viral DNAs were isolated from the tissue powder using a DNeasy Tissue kit (QIAGEN, Clarita, CA) and its copy number was determined using real-time PCR (15).

**Analysis of transgene expression following intratumoral infusion.**
For the analysis of EGFP expression, samples of liver and tumor were harvested at 24 hours after the infusion of AdCMVEGFP. The tissue samples were sectioned into 300 μm slices with a Vibratome (model 3000, Technical Products International, St. Louis, MO) and examined under a confocal laser-scanning microscope (LSM 510, Carl Zeiss, Thornwood, NY). This experiment was repeated thrice and the typical results are shown. For the analysis of luciferase expression, bioluminescence images of mice were acquired at 24 hours after the infusion of AdCMVLuc using the Xenogen In vivo Imaging System (Xenogen Corp., Alameda, CA; ref. 15).

**Tail vein injection of nanosphere or AdCMVLuc suspension.**
Suspension of rhodamine-labeled polystyrene nanosphere (114 ± 1.8 nm in diameter; Polysciences, Inc., Warrington, PA) or AdCMVLuc was mixed with either PBS or poloxamer solution and stored at 4°C before use. In experiments, the tail vein of anesthetized BALB/c mice was cannulated with a 30-gauge needle connected to a tubing of ~10 cm long. The tubing was connected to a syringe. After the cannulation, a droplet of super glue was used to secure the position of the needle on the tail. In the nanosphere experiment, a 50 μL suspension of nanospheres in PBS was injected over an ~5-second period. At 5 minutes after the first injection, we replaced the syringe with a new one containing the nanospheres suspended in poloxamer solution and injected the suspension into the same tail vein. During and after both injections, the video of nanosphere movement in the tail was recorded continuously under an intravital fluorescence microscope (MPS, Carl Zeiss, Hanover, MD) equipped with a rhodamine filter set and a color video camera (Carl Zeiss ZVS-3C75DE), which was connected to a videocassette recorder (Sony S-HVS). After the experiment, individual images at different time points were captured from the video using the Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). In the AdCMVLuc experiment, the procedure for the tail vein injection was the same as that described above. The dose of injection was 1.0 × 10^9 pfu/mouse. At 24 hours after the injection of AdCMVLuc suspension with or without poloxamer, we examined luciferase expression in mice using the Xenogen In vivo Imaging System. The nanosphere and AdCMVLuc experiments were repeated thrice and the typical results are shown.

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

**Results and Discussion.**
The viscosity of poloxamer solution (21%, w/w) depended on temperature and shear rate. When the temperature was changed from 4°C to 37°C, the viscosity could be increased by several orders of magnitude, depending on the shear rate. At the shear rate of 7.5 second⁻¹, the viscosity was increased from 41 to 4,200 cp within a few seconds.

The rapid increase in the viscosity would allow the poloxamer solution to retain viral vectors in tumors during and after intratumoral infusion. To directly show this, we infused AdCMVEGFP suspended in poloxamer solution into 4T1 tumors. Immediately after the infusion, we observed that the poloxamer solution could significantly reduce the number of viral vectors, isolated from the blood, which could transfect 293 cells in vitro (see Fig. 1A). Quantitatively, we observed that the poloxamer solution could significantly reduce the number of viral vectors, isolated from the blood, which could transfect 293 cells in vitro (see Fig. 1A). In Fig. 1B, we observed that the poloxamer solution could significantly reduce the number of viral vectors, isolated from the blood, which could transfect 293 cells in vitro (see Fig. 1A). In Fig. 1B, the lowest ratio in the poloxamer group was 28 whereas the highest ratio in the control group was only 1.6. The median ratios were 35.1 and 0.35 in the poloxamer and control groups, respectively.

We also observed that two mice in the control group died within half an hour after the intratumoral infusion of AdCMVEGFP but...
mice in the poloxamer group did not show any problem after the infusion. The death was likely due to acute immune response to viral vectors that escaped from the tumor during and after the intratumoral infusion (18, 19). These results provided direct evidence showing that the poloxamer solution could reduce the systemic toxicity caused by virus dissemination.

In addition to the effects of poloxamer solution on virus dissemination, we examined the effects of the same solution on transgene expression in normal and tumor tissues at 24 hours after the intratumoral infusion of AdCMVEGFP or AdCMVLuc. We observed that EGFP expression in the liver was minimal in the poloxamer group but strong in the control group (Fig. 2A). In the tumor, the opposite was observed: EGFP expression in the poloxamer group was stronger than that in the control group (Fig. 2A). Luciferase expression, indicated by the bioluminescence, is shown in Fig. 2B to D. Its distribution in mice indicated that the poloxamer solution could significantly reduce transgene expression in the liver and increase transgene expression in the tumor (Fig. 2B and C). Consequently, the poloxamer solution could increase the tumor/liver ratio of transgene expressions by 12- to 275-fold (Fig. 2D). These results were consistent with those in the EGFP experiment shown in Fig. 2A.

The mechanism of reduction in virus dissemination was likely to be that the poloxamer solution blocked the convection of viral...
vectors in the interstitial space and the lumen of microvessels in the vicinity of the infusion site because we have shown that infusion-induced convective transport is the mechanism of dissemination (14, 15). To directly show that the poloxamer solution could block convective transport in blood vessels, we injected the suspension of polystyrene nanospheres into the mouse tail vein. Without poloxamer, we observed that the nanospheres disappeared within a few seconds from the tail vein after the injection (Fig. 3A). Although the nanospheres could circulate in mice, they were significantly diluted by the blood and most of them were likely to be rapidly taken up by normal organs. Thus, the nanospheres could be observed only during the first pass through the tail vein. We also noticed that the width of the fluorescence distribution in the tail was larger than the diameter of the vein. It was likely to be caused by the light scattering in tissues and unlikely to be due to the accumulation of nanospheres in the perivascular regions because if the latter happened, the nanospheres would be cleared slowly from the tail after the injection. In contrast, nanospheres suspended in poloxamer solution stayed in the tail after the injection (Fig. 3B). They could accumulate both within the lumen of the tail vein and in the surrounding interstitial space. The reason is as follows. The flow resistance in the tail vein increased rapidly with time during the injection due to the in situ gelation of poloxamer. The gel blocked the flow and led to a rapid increase in microvascular pressure when the injection was continued. The pressure increase could cause rupture of the vein, which allowed the nanospheres to leak out and accumulate in the interstitial space. Therefore, the fluorescence distribution in the poloxamer group was much wider than that in the control group. It was unlikely that the rupture occurred during the tail vein cannulation because the same vessel was used for both injections and we did not observe any significant leak of nanospheres when the suspension without poloxamer was injected (see Fig. 3A). During the intratumoral infusion, the pressure-induced rupture of tumor microvessels was not an issue because the interstitial fluid pressure was always higher than or equal to the microvascular pressure. In this case, tumor microvessels could only be compressed. Vessel compression might be another mechanism of reduction in virus dissemination.

In addition to the nanospheres, we injected the suspension of AdCMVLuc into the tail vein. Without poloxamer, we observed that luciferase expression was high in the liver and minimal in the tail at 24 hours after the injection (Fig. 3C). With poloxamer, we observed the opposite: luciferase expression was high in the tail and minimal in the liver (Fig. 3D). These results were consistent with the distribution of nanospheres in the tail shown in Fig. 3B, indicating that the viral vectors accumulated both within the lumen of the tail vein and in the surrounding interstitial space. As a result, the viral vectors might have transected vascular endothelial cells and cells in perivascular regions (Fig. 3D).

Figure 3. Effects of the poloxamer solution on the convective transport in the tail vein of BALB/c mice. Suspension of rhodamine-labeled nanospheres or AdCMVLuc with or without poloxamer was injected into the tail vein over a 5-second period. The volumes of all injections were 50 μL and the dose of AdCMVLuc was 1.0 × 10⁸ pfu/mouse. The concentration of poloxamer was 21%. A and B, fluorescence images of the tail at different time points (0, 2, 5, and 10 seconds) after the injection of the nanospheres suspended in PBS and poloxamer solution, respectively. C and D, bioluminescence distribution in mice at 24 hours after the injection of AdCMVLuc suspended in PBS and poloxamer solution, respectively.
One question in the study was whether poloxamer would chemically change the ability of adenoviral vectors to transfect cells. To answer this question, we mixed AdCMVEGFP with PBS or a diluted poloxamer solution (0.1% w/w) and used the mixture to treat cultured 4T1 cells. We observed that the percentage of EGFP-positive cells was not affected by the presence of poloxamer (data not shown).

A potential adverse effect of the poloxamer solution is that microclots could escape from the tumor and accumulate in normal organs. However, this effect is likely to be insignificant because Poloxamer 407 has been used as a temporary vascular occlusion agent (20). Although small fragments of clots may detach and escape from the occluded vessels, especially the large veins (i.e., iliac veins and vena cava), they will accumulate in the lungs and be rapidly dissolved there (20). The rate of dissolution is much faster than that of large clots formed in the lung after a direct injection into the pulmonary artery because the rate of dissolution decreases with increasing size of clots. In our study, poloxamer 407 was infused into tumor tissues. Thus, clots could be formed only in the tumor microvessels and the interstitial space so that they were much smaller than those formed in the large vessels mentioned above and should be rapidly dissolved if any fragments escaped from the tumor. Another related issue was the leak of poloxamer solution from the tumor before the gel formation. In this case, the polymer solution would be significantly diluted by the blood and hence unlikely to form gels in normal tissues.

In summary, we developed a new delivery method that could significantly increase virus copy number and transgene expression in the tumor and decrease them in the liver in intratumoral infusion-mediated gene delivery. This improvement is critical for enhancing the efficacy/toxicity ratio in viral gene therapy. Although this method was tested only for adenoviral vectors, it could also be used to deliver other therapeutic agents in solid tumors because the mechanisms of passive transport are the same for all agents. Furthermore, the same method may be useful for delivering viral vectors in other tissues where the dissemination is a significant problem.

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References

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