Systemic dissemination of viral vectors during intratumoral injection

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Abstract

Intratumoral injection is a routine method for local viral gene delivery that may improve interstitial transport of viral vectors in tumor tissues and reduce systemic toxicity. However, the concentration of transgene products in normal organs, such as in the liver, may still exceed normal tissue tolerance if the products are highly toxic. The elevated concentration in normal tissues is likely to be caused by the dissemination of viral vectors from the tumor. Therefore, we investigated transgene expression in the liver, the serum, and a mouse mammary carcinoma (4T1) in mice after intratumoral injection of adenoviral vectors for mouse interleukin-12, luciferase, enhanced green fluorescence protein, or β-galactosidase. We also performed numerical simulations of virus transport in tumors after intratumoral injection, based on the Krogh cylinder model. Our experimental data and numerical simulations demonstrated that virus dissemination was significant in mice and it occurred mainly during the intratumoral injection. To reduce virus dissemination, we mixed these vectors with a viscous alginate solution and injected the mixture into the tumors. Our data showed that the alginate solution could significantly reduce virus dissemination while having minimal effects on transgene expression in tumors and on interleukin-12-induced tumor growth delay. These data suggest that virus dissemination is a potential problem in local viral gene therapy of cancer and that the dissemination could be significantly reduced by the alginate solution without compromising the efficacy of gene therapy. (Mol Cancer Ther. 2003;2:1233–1242)

Introduction

Discoveries in molecular and cell biology have led to a significant development in novel strategies for cancer gene therapy (1, 2). However, the efficacy of gene therapy is still limited by the delivery of therapeutic genes into target cells. Systemic delivery of viral vectors is inadequate, primarily due to the poor interstitial penetration in solid tumors (3, 4) and normal tissue toxicity caused by viral vectors and/or gene products (5–15).

Different approaches have been developed for reducing the toxicity in normal tissues. One is to switch to non-viral vectors, such as cationic liposomes or polymers (16, 17). Non-viral vectors are less toxic and may have similar transfection efficiency *in vitro* as viral vectors. However, non-viral vectors are in general less efficient *in vivo*. The second approach is to use tissue targeting viral vectors (18–23), which can be achieved through at least two mechanisms. One is to incorporate specific molecular structures on the vector surface that can bind to unique markers on the plasma membrane of cells or extracellular matrix in tumors; another is to incorporate specific transcriptional promoters in viral vectors that can be triggered by either endogenous factors or exogenous interventions. In all these cases, transgene expression is restricted in target cells or tissues. However, targeted gene delivery requires identification of unique markers in cells and tissues that can capture the vectors or identification of specific transcriptional mechanisms that can control gene expression. Both requirements cannot be always achieved.

The two approaches mentioned above can reduce the toxicity in normal tissues, but they cannot solve the problem of poor interstitial penetration if the vectors are delivered systemically. To simultaneously improve interstitial transport and reduce normal tissue toxicity, the optimal approach is to locally inject viral vectors into tumors (24, 25). Intratumoral injection can improve interstitial transport through at least three mechanisms. One is to establish a pressure gradient for enhancing convection, which is critical for delivery of macromolecules and nanoparticles (3, 4). The second is to increase the pore size in tumor tissues due to pressure-induced tissue deformation (26, 27). Tissue deformation will also, as the third mechanism, improve the connectedness of interstitial space (28). When injected directly into tumors, viral vectors are expected to infect only cells near the injection site and hence cause minimal toxicity in normal tissues. However, data in the literature have shown that the normal tissue cytotoxicity has been a limiting factor for achieving an optimal dose of viral vectors in tumors (29, 30). The concentration of transgene products in the liver can be on the same order of magnitude as that in tumor tissues (31, 32).

The normal tissue toxicity is likely to be caused by viruses disseminated from tumors. Virus dissemination has been suggested in previous studies (29–34), although the significance of this problem and mechanisms of the dissemination remain to be determined. There exist at least two possible mechanisms of dissemination: (*a*) direct

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injection of viral vectors into blood vessels that are damaged by the injection needle; and (*b*) diffusion of viral vectors into microvessels after intratumoral injection. To demonstrate virus dissemination, we injected adenoviral vectors encoding either mouse interleukin-12 (IL-12), luciferase, or enhanced green fluorescence protein (EGFP) into tumors, measured IL-12 concentrations in the liver, serum, and tumors, and compared qualitatively the expressions of luciferase and EGFP in the liver and tumors, respectively. In addition, we developed a mathematical model of virus transport for determining which of the two mechanisms mentioned above was dominant in virus dissemination. Our experimental data and numerical simulations demonstrated that virus dissemination was a potential problem in local viral gene therapy of cancer, and that the dissemination occurred mainly during the intratumoral injection.

Based on the mechanistic studies mentioned above, we further developed a method for reducing the systemic dissemination of viral vectors. The method was based on the biological and physical properties of alginate solutions, which are biocompatible and biodegradable and are highly viscous. The alginate solution, when mixed with viral vectors before intratumoral injection, can reduce both the rates of convection and diffusion of viral vectors. In addition, intratumoral injection of this solution requires a higher pressure gradient which may cause a transient compression of blood vessels in tumors. As a result, the virus dissemination was reduced significantly when we injected into tumors with the mixture of viral vectors and the alginate solution rather than the free suspension of viral vectors.

Materials and Methods

Tumor Models

4T1 mouse mammary carcinoma cells were obtained from Dr. Fred Miller's lab (Michigan Cancer Foundation, Detroit, MI). They were maintained in DMEM supplemented with 10% newborn bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY) at 37°C with 5% CO_2. One million 4T1 cells in 50 μl PBS were s.c. injected into the right hind leg of 4- to 6-week-old syngeneic Balb/c female mice (Charles River Laboratory, Wilmington, MA). The s.c. tumors were ready for our experiments when they reached 5–8 mm in diameter.

Adenoviral Vectors

The Ad5-based recombinant system was used to produce the adenoviral vectors in this study. The cDNAs for both mouse IL-12 subunits (p35 and 40) were inserted into the E1 region of the adenoviral vector, AdCMVIL-12. Expression of IL-12 cDNAs was driven by the cytomegalovirus (CMV) promoter. The EGFP, luciferase, and β-galactosidase expressing adenoviral vectors, AdCMVEGFP, AdCMVLUC, and AdCMVLacZ, respectively, were also under the control of the CMV promoter. All adenoviral vectors were propagated in 293 cells (American Type Culture Collection, Manassas, VA), harvested at 48 h after infecting cells, and purified through cesium chloride gradient centrifugation according to a standard protocol (35). Viral vectors were stored in 10% glycerol at −80°C. The virus suspensions were mixed with the solution of alginate (Sigma Chemical Co., MO) or PBS before intratumoral injection. The final concentration of the alginate was 4% w/w concentration.

Measurement of Solution Viscosity

Brookfield DV-III Rheometer (Brookfield, MA) was used to measure the viscosity of solutions. The temperature of water bath was set at 37°C. The samples were put into the center of the cup and preheated for 10 min before the viscosity measurement. The measurement was repeated three times and the average value is reported in this paper.

EGFP Expression in Different Tissues

Fifty microliters of AdCMVEGFP in PBS or alginate solution were injected into 4T1 tumors via a 30-gauge needle. The dose of injection was either 1.0×10^8 or 5.0×10^8 plaque-forming units (pfu)/tumor. The samples of the blood, the spleen, the lung, the kidney, the heart, the liver, and tumors were harvested at 2 days after virus injection. Then, the samples were glued onto a specimen block and transferred to the stage of a Vibratome (Model 3000; Technical Products International, St. Louis, MO) maintained at 4°C. The tissues were then sectioned into 300-μm slices. EGFP expression in tissues was examined under a confocal laser scanning microscope (LSM 510, Carl Zeiss, Thornwood, NY).

Luciferase Expression in Different Tissues

Fifty microliters of AdCMVLUC in PBS or alginate solution were injected into 4T1 tumors via a 30-gauge needle. The dose of injection was 2.0×10^8 pfu/tumor. At 2 days after virus injection, mice were anesthetized with i.p. injection of 80 mg ketamine and 10 mg xylazine/kg body weight. Then, 50 μl of aqueous D-luciferin solution (28.6 mg/ml) were injected into the peritoneal cavity. Twenty minutes later, the mice were placed in the Xenogen In Vivo Imaging System (IVIS) (Xenogen, Alameda, CA), and a grey scale reference image was obtained under a low-level illumination. Then, the reference image was overlaid with the bioluminescence image which was acquired by integrating photons emitted from the animals for 30 s. The amount of bioluminescence was quantified as the number of photons emitted per second per square centimeter per steridian (sr) and is shown in the pseudo-color indicated by the color bar.

IL-12 Expression in Different Tissues

Fifty microliters of AdCMVIL-12 in PBS or alginate solution were injected into 4T1 tumors via a 30-gauge needle. The dose of injection was 1.0×10^8 pfu/tumor. The samples of the blood, the liver, and tumors were harvested at 1, 3, or 5 days after virus injection. Tumor and liver samples were homogenized in PBS containing the Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). All samples were then centrifuged at 10,000 × *g*

Table 1. Baseline values of model constants[a]

R	0.01 cm	S	0.27 cm^2
a	5.0×10^{-4} cm	C_0	6.15×10^{-11} M
D^v	2.0×10^{-9} cm^2/s	C_{R0}	8.74×10^{-9} M
P^v_{eff}	2.0×10^{-8} cm/s	k_{on}	3.0×10^5 $(M \cdot s)^{-1}$
ϕ^v	0.05	k_{off}	2.0×10^{-4} s^{-1}
V_p	1 ml	k^v_d	5.8×10^{-6} s^{-1}

[a] The determination of baseline values is discussed in the "Materials and Methods" section.

for 10 min. The supernatants were extracted and stored at –80°C. IL-12 concentration in different supernatants was quantified by an ELISA kit with a detection sensitivity of 2.5 pg/ml (R&D Systems, Minneapolis, MN). The results were then converted to the concentrations of IL-12 in different tissues based on the sample volume or weight.

β-Galactosidase Expression in Tumor Tissues

Fifty microliters of AdCMVLacZ in PBS or alginate solution were injected into 4T1 tumors via a 30-gauge needle. The dose of injection was 2.0×10^8 pfu/tumor. Tumor samples were harvested at 2 days after virus injection. Tissue samples were fixed in 4% paraformaldehyde in PBS for 24 h and rinsed with 100 mM sodium-phosphate buffer (pH 7.3, 2 mM $MgCl_2$, 0.1% Triton X-100) twice for 1 h. Tissues were then mounted on the Vibratome and sectioned into 300-μm slices. The tissue slices were then incubated at 25°C overnight in a staining solution with 1 mg/ml X-gal (Life Technologies), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 0.1% Triton X-100. The stained slices were scanned into a computer, using a Plustek Optic Pro document scanner (Model 12000P).

Tumor Size Measurement

Tumor size was measured at different time points after virus injection. A caliper was used to measure the longest (L) and the shortest (W) dimensions of the tumors. The tumor was assumed as an ellipsoid. Thus, its volume could be calculated as: $V_t = (\pi/6)LW^2$. The relative volume of tumors, V_r, was defined as: $V_r = V_t/V_0$, where V_t was measured at different time points after virus injection and V_0 was the tumor volume on the day of virus injection.

Transfection of Cells *in Vitro*

Alginate was dissolved in cell culture medium at concentrations of 0.1% and 4% w/w, respectively. 4T1 cells were cultured in the 24-well plates until they reached 80–90% confluence. Two hundred-microliter virus suspensions in the culture medium or alginate solutions were first added into the wells. The number ratio of viral particles *versus* cells was on the order of 10. After virus suspension was placed in the well, 600 μl culture medium was slowly added into the well to avoid the mixing with the virus suspension. After 48 h, the cells were examined under a fluorescence microscope. The same experiment was repeated three times in different days and the typical results are reported in the "Results" section.

Statistical Analysis

The Mann-Whitney U test was used to compare the difference between two unpaired groups. The tests were considered significant if the P values were less than 0.05.

Mathematical Model of Virus Dissemination after Intratumoral Injection

To understand the mechanisms of virus dissemination, we developed a mathematical model of virus transport in tumor tissues after intratumoral injection. The model was based on the Krogh cylinder geometry and considered the interstitial diffusion of viruses, binding of viruses to their receptors on the cell membrane, internalization of viruses into cells, and inactivation of viruses in tumors. In general, convection should also be considered in transport analysis of viruses. However, convection was negligible in solid tumors (3). The diffusion in this model was assumed to be time-dependent and only in the radial direction, and the rate of virus inactivation was assumed to be proportional to the local concentrations of viruses. With these considerations and assumptions, the governing equation for free virus transport was derived as

$$\frac{\partial C_i^v}{\partial t} = \frac{D^v}{r}\frac{\partial}{\partial r}\left(r\frac{\partial C_i^v}{\partial r}\right) - k_{on}C_i^v C_R^v/\phi^v + k_{off}C_B^v - k_d^v C_i^v \quad (A)$$

where $\boldsymbol{C}^v_i$, $\boldsymbol{C}^v_B$, $\boldsymbol{C}^v_R$ were the concentrations of free viruses, bound viruses, and available virus receptors in tumors, respectively; $\boldsymbol{t}$ was time; $\boldsymbol{r}$ was the radial distance; $\boldsymbol{D}^v$ was the diffusion coefficient of viruses; $\boldsymbol{k}^v_d$ was the rate constant of free virus inactivation; and $\boldsymbol{\phi}^v$ was the available volume fraction of viruses in tumors. The overall association and dissociation rate constants were approximately equal to the intrinsic rate constants, $\boldsymbol{k}_{on}$ and $\boldsymbol{k}_{off}$, respectively, because $4\pi \boldsymbol{a}_c \boldsymbol{D}^v >> \boldsymbol{k}_{on}$ if $\boldsymbol{a}_c$ was the radius of a cell (36) (see also Table 1). $\boldsymbol{C}^v_R$ was related with the total concentration of virus receptor, $\boldsymbol{C}_{R0}$, by

$$C_R^v = C_{R0} - C_B^v \quad (B)$$

The governing equation for bound viruses was derived as

$$\frac{\partial C_B^v}{\partial t} = k_{on}C_i^v C_R^v/\phi^v - k_{off}C_B^v - k_d^v C_B^v - R_{in}^v \quad (C)$$

where we assumed that the rate constant of bound virus inactivation was the same as that of free viruses. Furthermore, we assumed that the rate of internalization of bound viruses, $\boldsymbol{R}^v_{in}$, was much smaller than the rate of inactivation, $\boldsymbol{k}^v_d \boldsymbol{C}^v_B$. Thus, $\boldsymbol{R}^v_{in}$ in Eq. C was neglected in our numerical simulations.

The amount of intravasated viruses per unit volume of the plasma, $\boldsymbol{C}^v_p$, could be calculated as

$$\frac{dC_p^v}{dt} = \frac{P_{eff}^v S}{V_p} C_i^v/\phi^v \quad (D)$$

where we assumed that the concentration of viruses in the plasma was much smaller than that in the tumor. This

assumption was verified later by the numerical simulations as shown in Fig. 5. In Eq. D, $\boldsymbol{P}_{\text{eff}}^{\text{v}}$ was the effective microvascular permeability of viruses, $\boldsymbol{S}$ was the total surface area of tumor vessels exposed to viruses, and $\boldsymbol{V}_{\text{p}}$ was the plasma volume in the body.

The initial conditions were:

$$C_i^v = C_0 \qquad (E1)$$

$$C_B^v = 0 \qquad (E2)$$

$$C_p^v = 0 \qquad (E3)$$

where $\boldsymbol{C}_0$ was the initial virus concentration in the tumor. It was assumed to be equal to the amount of viruses left in the tumor immediately after intratumoral injection divided by the distribution volume of virus in tumors. The boundary conditions were

$$\frac{\partial C_i^v}{\partial r} = 0 \qquad at\ r = R \qquad (F1)$$

$$D^v \frac{\partial C_i^v}{\partial r} = P_{eff}^v C_i^v / \phi^v \qquad at\ r = a \qquad (F2)$$

where $\boldsymbol{R}$ was the radius of the Krogh cylinder and $\boldsymbol{a}$ was the radius of blood vessel.

Baseline Values of Model Constants

The baseline values of model constants are shown in Table 1. We assumed that the radii of the microvessel, a, and the Krogh cylinder, R, were 5 and 100 μm, respectively, and the plasma volume, V_{p}, in a mouse was 1 ml. The distribution volume of viruses in tumors, V_{d}, could be approximated by $V_{\text{inj}} f^3/(1 - \varepsilon_{\text{c}})$, where V_{inj} was the volume of injection, f was the retardation coefficient of convective transport (26), and ε_{c} was the volume fraction of cells. In our experiment, V_{inj} = 50 μl. We assumed that f = 0.3 and ε_{c} = 0.5 (37). Thus, V_{d} = 2.7 μl. Within this volume, the total surface area of tumor microvessels, S, could be calculated as $\varepsilon_{\text{ves}} V_{\text{d}}(S/V)$, where ε_{ves} was the volume fraction of tumor vessels and S/V was the ratio of the surface area *versus* the volume of tumor microvessels. We assumed that ε_{ves} = 5% (38) and S/V = 2000 cm^2/cm^3 (39). Thus, S = 0.27 cm^2. The initial concentration of the viruses, C_0, was calculated as the number of viruses divided by V_{d} and the Avogadro's number. The number of viruses in tumors immediately after injection was assumed to be 10^8 pfu. Thus, $C_0 = 6.15 \times 10^{-11}$ M. The number of adenovirus receptors is approximately 10,000 per cell (40) and the volume of a cell is approximately 950 μm^3 (37). Thus, the total concentration of the receptor, C_{R0}, was assumed to be 8.74×10^{-9} M. The diffusion coefficient, D^{v}, the effective microvascular permeability, $P_{\text{eff}}^{\text{v}}$, and the available volume fractions, ϕ^{v}, of adenoviruses in tumors are still unknown. Their values were assumed in our study (see Table 1), based on the size of viruses and the data of macromolecules and liposomes in tumor tissues (37, 41–44). The intrinsic rate constants of association and dissociation between adenoviruses and their receptors, k_{on} and k_{off}, were 3.0×10^5 $(\text{M}\cdot\text{s})^{-1}$ and 2.0×10^{-4} s^{-1}, respectively (45). Our experimental data showed that the peak of IL-12 concentration in mice was reached in less than 1 day after virus injection. Thus, the half-life of adenoviruses in mice was less than 1 day. Based on this information, we assumed that the rate constant of virus inactivation, k_{d}^{v}, was 0.5 day^{-1} or 5.8×10^{-6} s^{-1}.

Results

Dissemination of Adenoviral Vectors for EGFP and Luciferase

EGFP is an exogenous and non-secretable protein. It should not be detected in normal tissues after intratumoral injection of AdCMVEGFP unless the viral vectors escaped from tumors. In our experiment, we observed EGFP expression in both liver (Fig. 1A) and tumors (Fig. 1B) at 2 days after intratumoral injection of 5.0×10^8 pfu AdCMVEGFP. These results indicated that AdCMVEGFP could disseminate into the liver. EGFP expression in the liver could not be detected when the dose of injection was reduced to 1.0×10^8 pfu/tumor.

To reduce AdCMVEGFP dissemination, we mixed the vectors with the alginate solution before the injection. The final concentration of alginate in the mixture was 4%. The viscosity of pure alginate solution at the same concentration was 1130 cp, which was three orders of magnitude higher than that of PBS. Mixing viral vectors with the alginate solution significantly reduced EGFP expression in the liver after intratumoral injection of 5.0×10^8 pfu AdCMVEGFP (Fig. 1C), but had minimal effects on EGFP expression in tumors (Fig. 1D). These results demonstrated that virus dissemination could be significantly reduced by the alginate solution.

We could not detect EGFP, under a regular fluorescence microscope, in tissue samples from the serum, the spleen, the lung, the kidney, and the heart after intratumoral injection of 5.0×10^8 pfu AdCMVEGFP. These results were consistent with the data in the literature, indicating that the liver was the main target organ of disseminated viruses (29, 31, 32).

We also injected AdCMVLUC into tumors. The pattern of luciferase expression was similar to that of EGFP (Fig. 2A). It was strong in the tumor and the liver, but could not be detected in other organs. When the viral vectors were mixed with the alginate solution, luciferase expression in the liver was significantly reduced (Fig. 2B). These results confirmed the observations, shown in Fig. 1, in the EGFP experiment.

Dissemination of an Adenoviral Vector for IL-12

Results shown in Figs. 1 and 2 are qualitative, since we could not quantify the concentration of EGFP and luciferase in tissues. To quantify transgene expression, we investigated the dissemination of an adenoviral vector for IL-12,

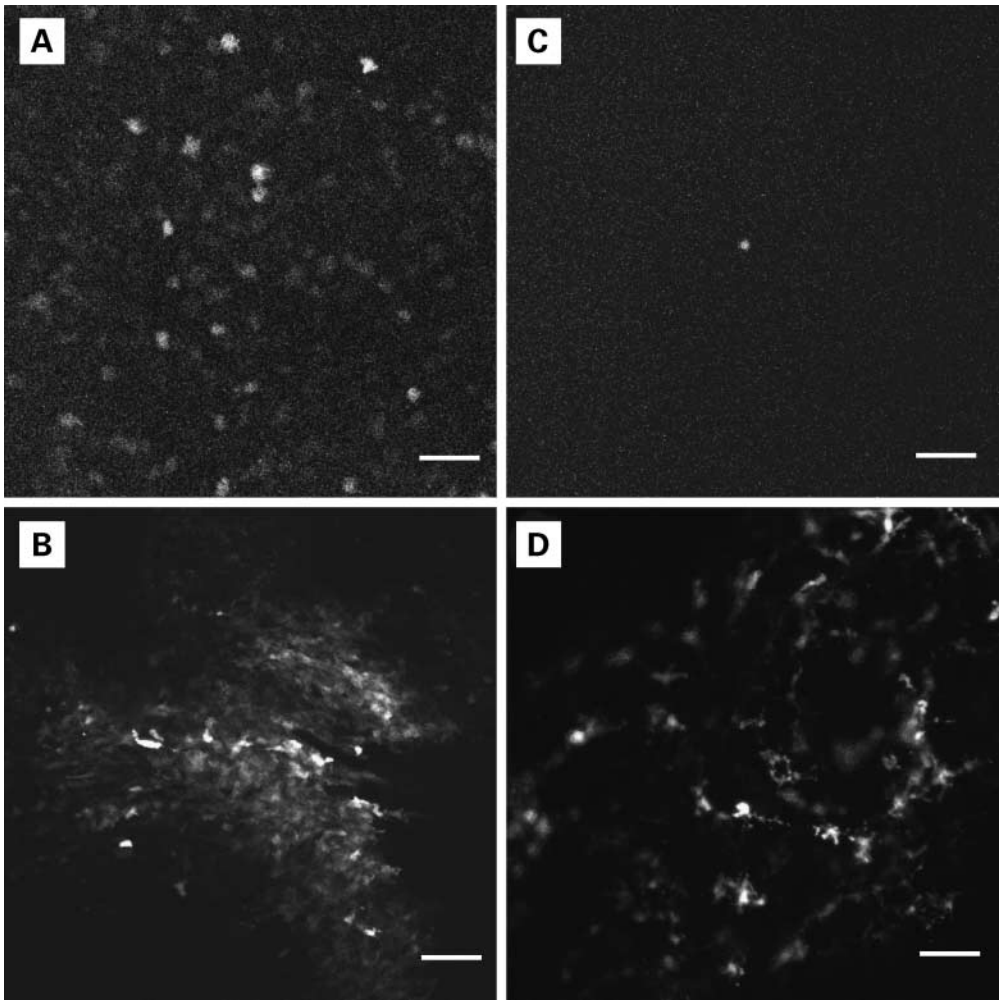

Figure 1. Comparison of EGFP expression in the livers (**A**, **C**) and the tumors (**B**, **D**) after intratumoral injection of AdCMVEGFP at the dose of 5.0×10^8 pfu/tumor. In **A** and **B**, the fluorescence images show EGFP distributions in the liver and the tumor, respectively, treated with AdCMVEGFP in PBS. In **C** and **D**, the fluorescence images show EGFP distributions in the liver and the tumor, respectively, treated with AdCMVEGFP in the alginate solution. *Scale bars,* 100 μm.

AdCMVIL-12. In this experiment, we injected AdCMVIL-12 into 4T1 tumors at the dose of 1.0×10^8 pfu/tumor and quantified IL-12 concentrations in the serum, liver, and tumors after intratumoral injection. IL-12 expression in other tissues was not determined, since the liver was the main target organ of the disseminated viruses as discussed above. The concentration of IL-12 was time-dependent (Fig. 3). The peak concentrations of IL-12 in all tissues were reached on day 1 post-intratumoral injection (Fig. 3).

The concentration of IL-12 in the liver was reduced approximately 8-fold, if the viral vectors were mixed with the alginate solution before injection (Fig. 3A). The reduction was statistically significant ($P < 0.05$). On days 3 and 5, IL-12 concentration in the liver was between 0.3 and 0.4 ng/g, and there was no significant difference in the IL-12 concentrations between PBS and alginate groups (Fig. 3A).

The concentration profile of IL-12 in the serum was similar to that in the liver and consistent with the results in a previous report (33). The peak concentration was reduced approximately 4-fold, if the viral vectors were mixed with the alginate solution before injection (Fig. 3B). Again, the reduction was statistically significant ($P < 0.05$). The serum concentrations of IL-12 in both groups decreased with time; and the differences between the two groups could not be detected on days 3 and 5. On day 5, the concentration of IL-12 ml in the serum was less than 0.1 ng/g in both groups.

The time dependence of IL-12 concentration in tumor tissues was similar to those in the liver and serum, respectively (Fig. 3). However, there was no significant difference in the IL-12 concentration between PBS and alginate groups at all time points (Fig. 3C) ($P > 0.05$). These results indicated that the net effect of alginate solution on transgene expression in tumors was negligible. Possible explanations of these results will be discussed later.

IL-12 is a cytokine that has a potential to treat human infectious and malignant diseases. Thus, we also quantified the effects of AdCMVIL-12 treatment on tumor growth. We observed that the growth of tumors treated with AdCMVIL-12 was slower than that treated with AdCMVEGFP (Fig. 4). The difference in tumor size was statistically significant from day 6 to day 21 after intratumoral injection of viral vectors. Quantitatively, the tumor doubling time

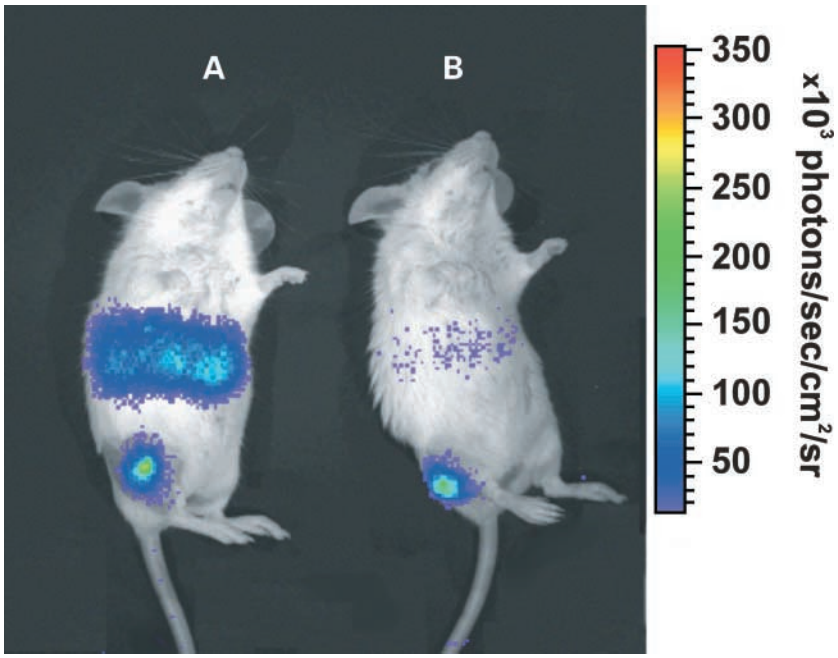

Figure 2. Comparison of luciferase expression in the livers and the tumors treated with AdCMV-LUC in PBS (**A**) and the alginate solution (**B**). The dose of AdCMVLUC injection was 2.0×10^8 pfu/tumor. The amount of bioluminescence, which is related to the concentration of luciferase, was quantified as the number of photons emitted per second per square centimeter per steridian (*sr*) and is shown in pseudo-color indicated by the *color bar*.

was increased from 3 to 6 days in the control group to 18–21 days in AdCMVIL-12-treated groups (Fig. 4). Mixing AdCMVIL-12 with the alginate solution had minimal effects on the efficacy of AdCMVIL-12 treatment ($P > 0.05$) (Fig. 4), presumably because it did not change IL-12 concentration in tumors (see Fig. 3C). We also quantified IL-12 concentration in tumors at 1 day after AdCMVEGFP injection, and observed that it was three orders of magnitude lower than that in AdCMVIL-12-treated tumors.

Virus Intravasation after Intratumoral Injection

Results shown in Figs. 1–3 demonstrated clearly that viral vectors could disseminate into the systemic circulation during and/or after intratumoral injection. To understand the mechanisms of virus dissemination, we performed numerical simulations of virus diffusion into microvessels (*i.e.*, the intravasation process), based on the Krogh cylinder model. Results from numerical simulations indicated that the concentration of free viruses in the tumor decreased rapidly with time and became negligible, compared with that of bound viruses, during the first few hours after the virus injection (data not shown). The concentration of bound viruses decreased gradually with time, except that near the microvessel wall (Fig. 5A). In this region, the spatial gradient of the concentration of bound viruses was also large (Fig. 5A). When the average concentration of bound viruses in tumors was calculated, we observed that it decreased gradually with time (Fig. 5B). The amount of intravasated viruses per unit volume of the plasma increased gradually with time (Fig. 5B). Quantitatively, the data shown in Fig. 5B indicated that less than one viral particle could escape from the tumor during a 3-day period. Therefore, virus dissemination after intratumoral injection was negligible; it could not explain the transgene expression in the liver shown in Figs. 1–3.

The concentrations of bound and intravasated viruses shown in Fig. 5B depend also on the concentration and the distribution of virus receptors on the cell membrane; and the receptor distribution in a tumor can be heterogeneous. To understand the effects of receptor concentration and distribution on virus dissemination, we varied the receptor concentration by three orders of magnitude in our numerical simulations. The simulation results demonstrated that the amount of bound viruses was decreased and the amount of intravasated viruses was increased when the receptor concentration was decreased. However, the amount of intravasated viruses was always much less than that of bound viruses. Therefore, the heterogeneous distribution of virus receptors within a tumor or among different tumors should have minimal effects on virus dissemination.

Effects of Alginate Solution on Virus Distribution and Transfection Efficiency

To investigate the effects of alginate solution on virus distribution and its ability to transfect tumor cells, we performed two additional experiments. In the first experiment, we divided tumors into three groups. In the first and second groups, adenoviral vectors for β-galactosidase in PBS and alginate solution, respectively, were injected into tumors. In the third group, we injected Evans Blue dye into tumors. At 48 h after adenovirus injection or immediately after Evans Blue injection, we observed that both β-galactosidase and Evans Blue dye accumulated at the periphery of tumors in all three groups (images not shown). The similarity in these spatial distributions suggested that the alginate solution had insignificant effects on viral vector distribution in tumor tissues, and that the distribution of transgene expression was determined mainly by the distribution of viral vectors immediately after injection.

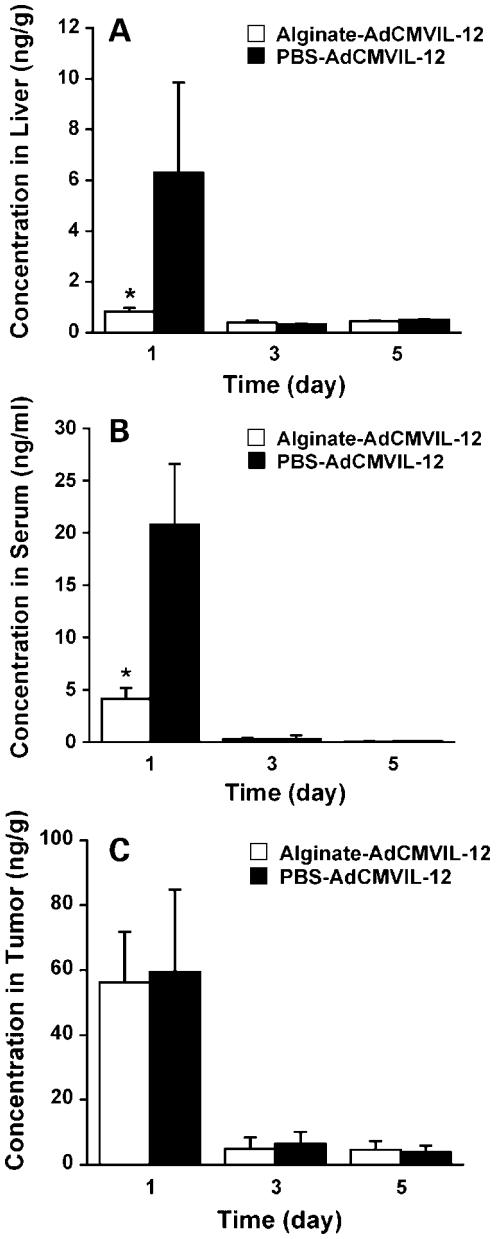

Figure 3. IL-12 concentrations in the liver (**A**), the serum (**B**), and the tumor (**C**) at different time points after intratumoral injection of AdCMVIL-12 in the alginate solution or PBS. The dose of injection was 1.0×10^8 pfu/tumor. *Error bars,* SE in four tumors. The *asterisk* indicates that the difference in IL-12 concentrations between alginate and PBS groups is statistically significant ($P < 0.05$).

In the second experiment, we treated 4T1 cells cultured in 24-well plates with the adenoviral vector for EGFP for 48 h. The vectors were suspended in either PBS or alginate solutions with two different concentrations: 0.1% and 4%. At the end of treatment, we observed that the alginate solution at the concentration of 4% reduced the number of transfected cells (Fig. 6A), compared with that in the PBS group (Fig. 6B). The reduction was unlikely to be due to alginate-induced changes in the bioactivity of viral vectors since the alginate solution at the concentration of 0.1% had no significant effects on the number of transfected cells (Fig. 6C). These results suggested that the alginate solution could reduce the rate of virus transport in solutions near the surface of cells, and thus decrease the number of viral vectors that could infect tumor cells.

Discussion

We investigated systemic dissemination of viral vectors in local viral gene delivery. Our experimental data showed that the viral vectors injected into tumors could disseminate into normal tissues and the amount of dissemination was significant. Meanwhile, our numerical simulations indicated that interstitial diffusion of viral vectors after intratumoral injection could not account for the amount of disseminated viral vectors observed in our experiments. Therefore, the virus dissemination might occur during the intratumoral injection. In the second part of the study, we developed a method to reduce the virus dissemination through mixing the virus suspension with the alginate solution before the injection. The alginate solution significantly reduced the virus dissemination but had minimal effects on the concentration of transgene products in tumors. Two issues in our study need to be discussed to understand these experimental data. One is the mechanisms of the alginate-mediated reduction in virus dissemination. Another is why the reduction in virus dissemination did not lead to an increase in the transgene expression in tumors.

The mechanisms of the reduction in virus dissemination might be 2-fold. As discussed above, the virus dissemination occurred during the intratumoral injection. More

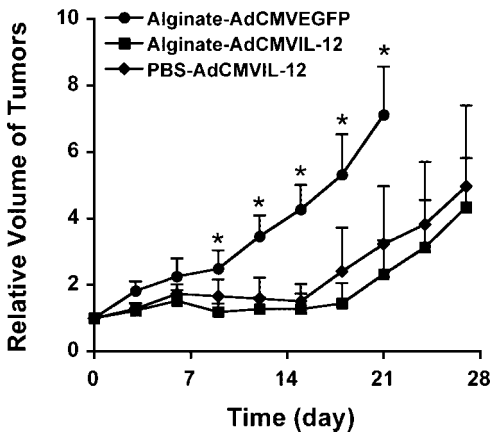

Figure 4. Tumor growth in mice treated with either AdCMVEGFP in the alginate solution (*i.e.*, the control), AdCMVIL-12 in PBS, or AdCMVIL-12 in the alginate solution. The data are reported as the volume of tumors relative to that in the same animals immediately before the treatment. The injection doses of AdCMVEGFP and AdCMVIL-12 were all equal to 1.0×10^8 pfu/tumor. Symbols, mean of the relative volume of six tumors; *error bars,* SE. The *asterisks* indicate that the differences in size between AdCMVIL-12- and AdCMVEGFP-treated tumors at the same time points are statistically significant ($P < 0.05$).

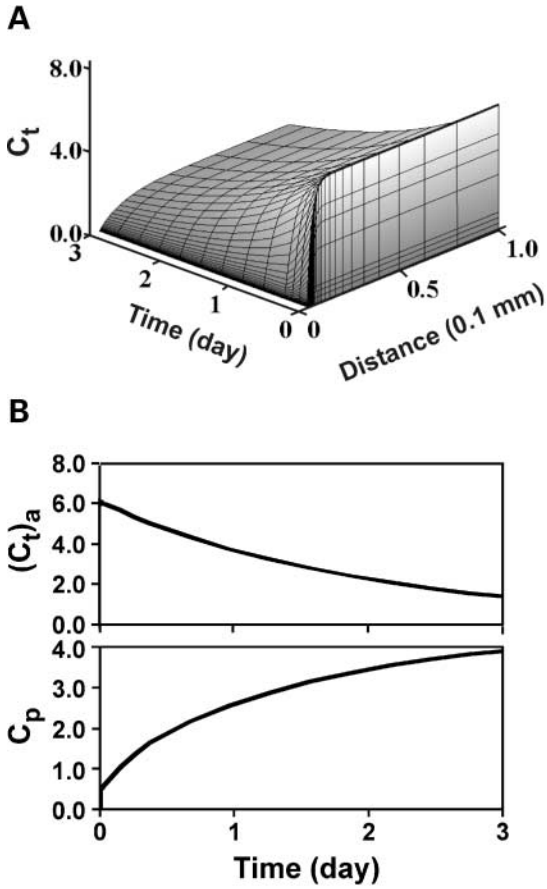

Figure 5. Numerical simulations of virus transport after intratumoral injection: (**A**) spatial and temporal distribution of bound virus concentration in tumors, C_t, (10^{-14} mol/ml) and (**B**) spatial average of the concentration of bound viruses in tumors, $(C_t)_a$, (10^{-14} mol/ml) and the amount of intravasated viruses per unit volume of the plasma, C_p, (10^{-25} mol/ml). The time indicates the period after intratumoral injection of viral vectors and the distance indicates the location from the axis of the blood vessel in the Krogh cylinder model.

specifically, the dissemination could result from a direct injection of the viral vectors into the blood vessels that were damaged by the injection needle. When the viral vectors were mixed with the alginate solution, the blood vessels near the injection site could be compressed by the high interstitial fluid pressure caused by the injection. This was because the viscosity of the alginate solution was three orders of magnitude higher than that of PBS. For injecting the same amount of viral suspension over the same time period, we had to apply a much higher pressure for the alginate solution than for the PBS. When the blood vessels were compressed, the pathways for virus dissemination were transiently blocked. After virus injection, the interstitial fluid pressure was reduced but the damaged vessels could still be sealed by the alginate solution. In fact, we had observed small pieces of alginate gels near the injection site in tumor tissues. Even if a small amount of alginate solution was injected into the blood vessels, the flow of the solution was negligible under a normal blood pressure gradient. This was because the viscosity of the alginate solution was one to two orders of magnitude higher than that of the blood. Therefore, both the compression and the sealing of damaged vessels could be potential mechanisms of the alginate-mediated reduction in virus dissemination.

To address the second issue, we need to consider three possible scenarios. First, alginate molecules might react chemically with the viral particles. The reaction would reduce the bioactivity of viral vectors in tumors. However, we argued that this possibility was very small because alginate is a biocompatible and biodegradable polysaccharide. It has been used for three-dimensional cell culture, tissue engineering, and virus encapsulation (46–51). Alginate does not affect the infectivity or bioactivity of viruses based on the plaque assay, even though the viruses have been heated and vortexed during the immobilization

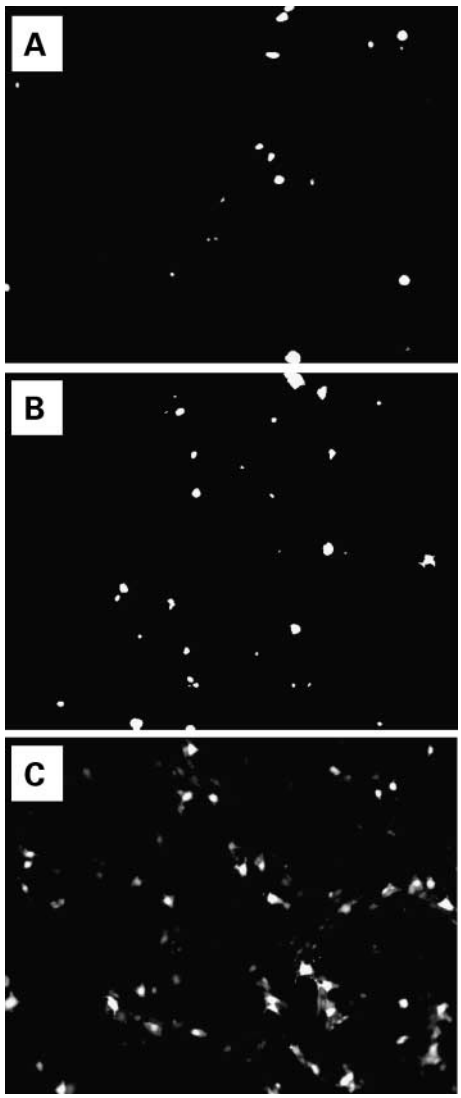

Figure 6. Comparison of EGFP expression in cultured 4T1 cells treated with AdCMVEGFP for 48 h in 4% alginate solution (**A**), cell culture medium (**B**), and 0.1% alginate solution (**C**). The images of cells with EGFP were acquired at the end of the treatment.

and lyophilization processes (50, 51). In our study, viral vectors were only gently mixed with the alginate solution at the room temperature immediately before the intratumoral injection. Thus, the bioactivity of viral vectors in our experiments was unlikely to be reduced by alginate molecules. This conclusion was also supported by the *in vitro* data shown in Fig. 6 when the concentration of alginate was low (0.1% w/w).

Second, alginate molecules might interact physically with the viral particles. The interaction would significantly decrease the diffusion coefficient of viral particles since the diffusion coefficient is inversely proportional to the viscosity of alginate solutions. Data shown in Fig. 6 were consistent with this scenario. However, the amount of viral particles that can diffuse from the middle of a solution to the surface of tumor cells over a fixed time period is also proportional to the square of the diffusion distance which was very small (< 1 μm) in porous tumor tissues but large (~1 mm) in cell culture medium. Therefore, the total amount of viral particles that could infect tumor cells *in vivo* was less affected by the alginate solution in the experiments than that *in vitro*. On the other hand, alginate could trap viral vectors since it could exist as a gel in the tumor. The trapped viral particles could not reach tumor cells. Taken together, the alginate-mediated reduction in virus transport might explain partly why the transgene expression in tumors was not increased when the virus dissemination was decreased by the alginate solution.

Third, it can be shown mathematically that the percentage of alginate-mediated reduction in virus dissemination is always larger than the percent increase in the number of viral particles retained in the tumor if the total amount of disseminated viruses is less than 50% of the injected dose. For example, we may consider a case in which mixing the viruses with the alginate solution leads to a reduction in virus dissemination by 8-fold (see Fig. 3A). In this case, the virus concentration in the tumor is increased by only 58% if we assume the amount of disseminated viruses in PBS to be 40% of the injected dose. If the amount of disseminated viruses in PBS is 20% of the injected dose, then mixing the viruses with the alginate solution only results in an increase in virus concentration in the tumor by 23%. This analysis suggests that the alginate solution is more effective in reducing virus dissemination than increasing virus concentration in the tumor.

Taken together, the reduced transport of viral vectors and the way of calculation were potential mechanisms for explaining why there was no increase in the transgene expression in tumors when the virus dissemination was reduced significantly by the alginate solution. These effects of the alginate solution could not be achieved through decreasing the dose of injection, because the dose decrease would proportionally reduce the total number of viral particles in tumors. Consequently, transgene expression in tumors would be decreased as well.

In summary, data in this study suggest that virus dissemination is a potential problem in local viral gene therapy and that virus dissemination can be reduced significantly by the alginate solution without compromising the therapeutic efficacy of the therapy. This approach is not limited to the delivery of adenoviral vectors; it may be more useful for intratumoral injection of highly potent and self-replicating viral vectors, because systemic toxicity is higher for these vectors once they leak out into normal tissues.

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References

1. Verma, I. M. and Somia, N. Gene therapy—promises, problems and prospects. Nature, *389:* 239 – 342, 1997.

2. Anderson, W. F. Human gene therapy. Nature, *392:* 25 – 30, 1998.

3. Jain, R. K. Delivery of molecular and cellular medicine to solid tumors. Microcirculation, *4:* 1 – 23, 1997.

4. McGuire, S., Zaharoff, D., and Yuan, F. Interstitial transport of macromolecules: implication for nucleic acid delivery in solid tumors. *In:* R. I. Mahato and S. W. Kim (eds.), Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics, pp. 434 – 454. London: Taylor & Francis Books, 2002.

5. Crystal, R. G. Transfer of genes to humans: early lessons and obstacles to success. Science, *270:* 404 – 410, 1995.

6. Ledley, F. D. Pharmaceutical approach to somatic gene therapy. Pharm. Res., *13:* 1595 – 1614, 1996.

7. Lozier, J. N., Metzger, M. E., Donahue, R. E., and Morgan, R. A. Adenovirus-mediated expression of human coagulation factor IX in the rhesus macaque is associated with dose-limiting toxicity. Blood, *94:* 3968 – 3975, 1999.

8. Lehrman, S. Virus treatment questioned after gene therapy death. Nature, *401:* 517 – 518, 1999.

9. Marshall, E. Gene therapy death prompts review of adenovirus vector. Science, *286:* 2244 – 2245, 1999.

10. Stone, D., David, A., Bolognani, F., Lowenstein, P. R., and Castro, M. G. Viral vectors for gene delivery and gene therapy within the endocrine system. J. Endocrinol., *164:* 103 – 118, 2000.

11. Brand, K., Arnold, W., Bartels, T., Lieber, A., Kay, M. A., Strauss, M., and Dorken, B. Liver-associated toxicity of the HSV-tk/GCV approach and adenoviral vectors. Cancer Gene Ther., *4:* 9 – 16, 1997.

12. van der Eb, M. M., Cramer, S. J., Vergouwe, Y., Schagen, F. H., van Krieken, J. H., van der Eb, A. J., Rinkes, I. H., van de Velde, C. J., and Hoeben, R. C. Severe hepatic dysfunction after adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene and ganciclovir administration. Gene Ther., *5:* 451 – 458, 1998.

13. Bustos, M., Sangro, B., Alzuguren, P., Gil, A. G., Ruiz, J., Beraza, N., Qian, C., Garcia-Pardo, A., and Prieto, J. Liver damage using suicide genes. A model for oval cell activation. Am. J. Pathol., *157:* 549 – 559, 2000.

14. Siegel, J. P. and Puri, R. K. Interleukin-2 toxicity. J. Clin. Oncol., *9:* 694 – 704, 1991.

15. Golab, J. and Zagozdzon, R. Antitumor effects of interleukin-12 in preclinical and early clinical studies. Int. J. Mol. Med., *3:* 537 – 544, 1999.

16. Luo, D. and Saltzman, W. M. Synthetic DNA delivery systems. Nat. Biotechnol., *18:* 33 – 37, 2000.

17. Putnam, D., Gentry, C. A., Pack, D. W., and Langer, R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc. Natl. Acad. Sci. USA, *98:* 1200 – 1205, 2001.

18. Datta, R., Rubin, E., Sukhatme, V., Qureshi, S., Hallahan, D., Weichselbaum, R. R., and Kufe, D. W. Ionizing radiation activates transcription of the EGR1 gene via CArG elements. Proc. Natl. Acad. Sci. USA, *89:* 10149 – 10153, 1992.

19. Weichselbaum, R. R., Hallahan, D. E., Beckett, M. A., Mauceri, H. J., Lee, H., Sukhatme, V. P., and Kufe, D. W. Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. Cancer Res., *54:* 4266 – 4269, 1994.

20. Richards, C. A., Austin, E. A., and Huber, B. E. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. Hum. Gene Ther., *6:* 881 – 893, 1995.

21. Spitzweg, C., Zhang, S., Bergert, E. R., Castro, M. R., McIver, B., Heufelder, A. E., Tindall, D. J., Young, C. Y., and Morris, J. C. Prostate-specific antigen (PSA) promoter-driven androgen-inducible expression of sodium iodide symporter in prostate cancer cell lines. Cancer Res., *59:* 2136 – 2141, 1999.

22. Huang, Q., Hu, J. K., Lohr, F., Zhang, L., Braun, R., Lanzen, J., Little, J. B., Dewhirst, M. W., and Li, C. Y. Heat-induced gene expression as a novel targeted cancer gene therapy strategy. Cancer Res., *60:* 3435 – 3439, 2000.

23. Yamamoto, M., Alemany, R., Adachi, Y., Grizzle, W. E., and Curiel, D. T. Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers. Mol. Ther., *3:* 385 – 394, 2001.

24. Lu, Y., Carraher, J., Zhang, Y., Armstrong, J., Lerner, J., Rogers, W. P., and Steiner, M. S. Delivery of adenoviral vectors to the prostate for gene therapy. Cancer Gene Ther., *6:* 64 – 72, 1999.

25. Paielli, D. L., Wing, M. S., Rogulski, K. R., Gilbert, J. D., Kolozsvary, A., Kim, J. H., Hughes, J., Schnell, M., Thompson, T., and Freytag, S. O. Evaluation of the biodistribution, persistence, toxicity, and potential of germ-line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. Mol. Ther., *1:* 263 – 274, 2000.

26. McGuire, S. and Yuan, F. Quantitative analysis of intratumoral infusion of color molecules. Am. J. Physiol., *281:* H715 – H721, 2001.

27. Zhang, X-Y., Luck, J., Dewhirst, M. W., and Yuan, F. Interstitial hydraulic conductivity in a fibrosarcoma. Am. J. Physiol., *279:* H2726 – H2734, 2000.

28. Yuan, F., Krol, A., and Tong, S. Available space and extracellular transport of macromolecules: effects of pore size and connectedness. Ann. Biomed. Eng., *29:* 1150 – 1158, 2001.

29. Toloza, E. M., Hunt, K., Swisher, S., McBride, W., Lau, R., Pang, S., Rhoades, K., Drake, T., Belldegrun, A., Glaspy, J., and Economou, J. S. *In vivo* cancer gene therapy with a recombinant interleukin-2 adenovirus vector. Cancer Gene Ther., *3:* 11 – 17, 1996.

30. Tjuvajev, J. G., Chen, S. H., Joshi, A., Joshi, R., Guo, Z. S., Balatoni, J., Ballon, D., Koutcher, J., Finn, R., Woo, S. L., and Blasberg, R. G. Imaging adenoviral-mediated herpes virus thymidine kinase gene transfer and expression *in vivo*. Cancer Res., *59:* 5186 – 5193, 1999.

31. Bramson, J. L., Hitt, M., Gauldie, J., and Graham, F. L. Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. Gene Ther., *4:* 1069 – 1076, 1997.

32. Zhang, R., Straus, F. H., and DeGroot, L. J. Effective genetic therapy of established medullary thyroid carcinomas with murine interleukin-2: dissemination and cytotoxicity studies in a rat tumor model. Endocrinology, *140:* 2152 – 2158, 1999.

33. Nasu, Y., Bangma, C. H., Hull, G. W., Lee, H. M., Hu, J., Wang, J., McCurdy, M. A., Shimura, S., Yang, G., Timme, T. L., and Thompson, T. C. Adenovirus-mediated interleukin-12 gene therapy for prostate cancer: suppression of orthotopic tumor growth and pre-established lung metastases in an orthotopic model. Gene Ther., *6:* 338 – 349, 1999.

34. Lohr, F., Hu, K., Huang, Q., Zhang, L., Samulski, T. V., Dewhirst, M. W., and Li, C. Enhancement of radiotherapy by hyperthermia-regulated gene therapy. Int. J. Radiat. Oncol. Biol. Phys., *48:* 1513 – 1518, 2000.

35. Graham, F. L. and Prevec, L. Manipulation of adenovirus vectors. *In:* E. J. Murray (ed.), Gene Transfer and Expression Protocols, pp. 109 – 128. Clifton, NJ: The Humana Press Inc., 1991.

36. DeLisi, C. The biophysics of ligand-receptor interactions. Q. Rev. Biophys., *13:* 201 – 230, 1980.

37. Krol, A., Maresca, J., Dewhirst, M. W., and Yuan, F. Available volume fraction of macromolecules in a fibrosarcoma: implications for drug delivery. Cancer Res., *59:* 4136 – 4141, 1999.

38. Jain, R. K. Transport of molecules in the tumor interstitium: a review. Cancer Res., *47:* 3039 – 3051, 1987.

39. Yuan, F., Baxter, L. T., and Jain, R. K. Pharmacokinetic analysis in two-step approaches using bifunctional and enzyme-conjugated antibodies. Cancer Res., *51:* 3119 – 3130, 1991.

40. Wickham, T. J., Shuler, M. L., Hammer, D. A., Granados, R. R., and Wood, H. A. Equilibrium and kinetic analysis of *Autographa californica* nuclear polyhedrosis virus attachment to different insect cell lines. J. Gen. Virol., *73:* 3185 – 3194, 1992.

41. Berk, D. A., Yuan, F., Leunig, M., and Jain, R. K. Direct *in vivo* measurement of targeted binding in a human tumor xenograft. Proc. Natl. Acad. Sci. USA, *94:* 1785 – 1790, 1997.

42. Pluen, A., Boucher, Y., Ramanujan, S., McKee, T. D., Gohongi, T., di Tomaso, E., Brown, E. B., Izumi, Y., Campbell, R. B., Berk, D. A., and Jain, R. K. Role of tumor-host interactions in interstitial diffusion of macromolecules: cranial *vs.* subcutaneous tumors. Proc. Natl. Acad. Sci. USA, *98:* 4628 – 4633, 2001.

43. Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D., and Jain, R. K. Microvascular permeability and interstitial penetration of sterically stabilized (Stealth) liposomes in a human tumor xenograft. Cancer Res., *54:* 3352 – 3356, 1994.

44. Yuan, F. Transvascular drug delivery in solid tumors. Semin. Radiat. Oncol., *8:* 164 – 175, 1998.

45. Lortat-Jacob, H., Chouin, E., Cusack, S., and van Raaij, M. J. Kinetic analysis of adenovirus fiber binding to its receptor reveals an avidity mechanism for trimeric receptor-ligand interactions. J. Biol. Chem., *276:* 9009 – 9015, 2001.

46. Smidsrod, O. and Skjak-Braek, G. Alginate as immobilization matrix for cells. Trends Biotechnol., *8:* 71 – 78, 1990.

47. Klock, G., Frank, H., Houben, R., Zekorn, T., Horcher, A., Siebers, U., Wohrle, M., Federlin, K., and Zimmermann, U. Production of purified alginates suitable for use in immunoisolated transplantation. Appl. Microbiol. Biotechnol., *40:* 638 – 643, 1994.

48. Becker, T. A., Kipke, D. R., and Brandon, T. Calcium alginate gel: a biocompatible and mechanically stable polymer for endovascular embolization. J. Biomed. Mater. Res., *54:* 76 – 86, 2001.

49. Langer, R. and Vacanti, J. P. Tissue engineering. Science, *260:* 920 – 926, 1993.

50. Offit, P. A., Khoury, C. A., Moser, C. A., Clark, H. F., Kim, J. E., and Speaker, T. J. Enhancement of rotavirus immunogenicity by microencapsulation. Virology, *203:* 134 – 143, 1994.

51. Kalyanasundaram, S., Feinstein, S., Nicholson, J. P., Leong, K. W., and Garver, R. I., Jr. Coacervate microspheres as carriers of recombinant adenoviruses. Cancer Gene Ther., *6:* 107 – 112, 1999.

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