Pharmaceutical Research, Vol. 24, No. 4, April 2007 (© 2007)
DOI: 10.1007/s11095-006-9200-5

Research Paper

Adenoviral Gene Delivery to Solid Tumors by Recombinant Silk–Elastinlike Protein Polymers

A. Hatefi,[1,3] J. Cappello,[2] and H. Ghandehari[1,4]

Received October 21, 2006; accepted November 29, 2006; published online February 17, 2007

Purpose. The purpose of this study was to investigate the potential of silk–elastinlike protein polymers (SELPs) in controlling the release rate of adenoviruses *in vitro* and *in vivo* while preserving their bioactivity.
Materials and Methods. A hydrogel system composed of SELP/adenovirus mixture was prepared. The release of the adenovirus particles from the hydrogels was quantified by Real Time-PCR and the bioactivity of the released viruses was evaluated using confocal microscopy and β-galactosidase assay. To demonstrate the ability of SELP in entrapping virus cargo and releasing it over a prolonged period of time *in vivo*, a SELP/adenovirus mixture was prepared and injected directly into xenograft tumor models of breast and head and neck cancer in mice. At various time points mice were sacrificed, tumors dissected, and tissue sections studied under confocal microscope.
Results. *In vitro* studies demonstrated that SELP hydrogels release viruses over a period of 4 weeks while preserving their bioactivity. After intratumoral injection, a prolonged and localized expression of adenoviruses was observed.
Conclusions. These results suggest the potential of SELPs in local adenoviral delivery to solid tumors as an alternative approach to intratumoral virus infusion.

KEY WORDS: adenovirus; intratumoral; silk–elastinlike; 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 ratio of efficacy to toxicity (3,4). For maximum efficiency, viral vectors must reach most tumor cells. Physiologic barriers to virus transport in tumors and rapid clearance in systemic circulation by liver limits the efficacy of viral vectors (5,6). To circumvent rapid clearance problems, researchers have directly infused viral vectors into solid tumors (7–9). Intratumoral infusion can bypass the initial filtering events in normal tissues and facilitate virus transport in tumors (6). However, a potential problem in this approach is that most of the viral vectors disseminate from tumor to normal tissues during and after infusion (8). The concentration of transgene products in normal organs such as liver, may still exceed normal tissue tolerance if the products are highly toxic (10).

One viable approach to control the spatial and temporal release of adenoviruses is to use polymeric matrices. It has been shown that localized delivery of adenoviral particles encoding growth factors by collagen matrices result in efficient transgene expression *in vitro* and *in vivo*, with potential to overcome some of the safety and efficacy limita tions of current gene therapy strategies for tissue repair (11). However, natural polymers such as collagen used in adenoviral delivery suffer from batch to batch variations to allow control over the release profile. More recently, it was demonstrated that localized adenoviral delivery by Poloxomers can block the convection of viral vectors in the interstitial space and the lumen of microvessels in the vicinity of the infusion site (12). Poloxomers upon injection *in vivo* form viscous materials that can entrap viruses but are prone to migration from the injection site. Other studies using poly lactic-co-glycolic acids (PLGA) also demonstrate the potential utility of controlled viral delivery using polymeric biomaterials. However, release profile from particulate delivery systems such as PLGA are poorly controlled (13). Furthermore, chemically synthesized polymers may contain solvents and monomer residues resulting in long-term complications in humans, and/or adversely influencing the bioactivity of the therapeutic viruses.

Molecular biology techniques have provided the tools to genetically engineer large molecular weight polymers containing repeating blocks of amino acids with precise composition,

Electronic supplementary material. The online version of this article (doi:10.1007/s11095-006-9200-5) contains supplementary material, which is available to authorized users.

[1] Center for Nanomedicine and Cellular Delivery, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, HSFII Room 625, Baltimore, Maryland 21201-1075, USA.
[2] Protein Polymer Technologies, Inc., San Diego, California 92121, USA.
[3] Present Address: Department of Pharmaceutical Sciences, Washington State University, Pullman, Washington 99164, USA.
[4] To whom correspondence should be addressed. (e-mail: hghandeh@rx.umaryland.edu)

sequence and length (14–16). One unique class of genetically engineered biomaterials is the family of silk–elastinlike protein polymers (SELPs) (14). Structurally, SELPs are composed of amino acid sequence motifs from silk (Gly-Ala-Gly-Ala-Gly-Ser) and mammalian elastin (Gly-Val-Gly-Val-Pro). By combining the silk-like and elastin-like blocks in various ratios and sequences, it is possible to precisely control solubility, gelation, stimuli-sensitivity, material strength, biorecognition, and biodegradation profiles of SELPs (17,18). SELP copolymers, with the appropriate sequence and composition undergo an irreversible sol-to-gel transition. The gelation process is accelerated at body temperature to form mechanically robust hydrogels (19,20); hence, reduced possibility of migration from the injection site.

Previously, we demonstrated that SELPs can be used to prolong expression of plasmid DNA in solid tumors up to 21 days (21). We further demonstrated that by controlling polymer composition, it is possible to control release of reporter adenoviruses from SELP hydrogels up to 22 days (21). These initial encouraging results led us to hypothesize that SELPs are suitable hydrogel matrices for localized adenoviral delivery for the treatment of solid tumors where transduction is prolonged at the site and migration to distant sites is minimized.

In this study the *in vitro* release, bioactivity, and intratumoral infection of cancer cells by the released adenoviruses from SELP hydrogels are reported.

MATERIALS AND METHODS

Preparation of SELP-47K Hydrogels Containing Adenoviruses for *In Vitro* Studies

An *in vivo* grade silk–elastinlike polymer namely SELP-47K with the structure: $[(GVGVP)_4\ GKGVP(GVGVP)_3(GAGAGS)_4]_{12}$ (head and tail residues are not shown), was provided by Protein Polymer Technologies, Inc. (San Diego, CA) as 12 wt% solutions in 3 ml syringes and stored at −80°C. The frozen SELP was thawed for 5 min at room temperature and gently mixed with adenovirus (Ad.GFP or Ad.CMV.LacZ, Qbiogene, Montreal, CA) of appropriate concentrations. The volume of the mixture was adjusted by addition of PBS. The polymer/virus suspensions in 50 and 100 μl aliquots were transferred to disposable syringes, incubated at 37°C for 4 h, and allowed to gel. Using a razor blade, 50 and 100 μl gel discs containing 1×10^9 adenoviruses were prepared, respectively.

Cycle 1:	(1X)		
	Step 1:	95.0 ºC	for 03:00
Cycle 2:	(40X)		
	Step 1:	95.0 ºC	for 00:20
	Step 2:	60.0 ºC	for 00:45
Cycle 3:	(1X)		
	Step 1:	95.0 ºC	for 01:00
Cycle 4:	(1X)		
	Step 1:	55.0 ºC	for 01:00
Cycle 5:	(80X)		
	Step 1:	55.0 ºC	for 00:10

Fig. 1. PCR program parameters used to amplify the extracted viral DNA from the samples and subsequent measurement of the viral copy number.

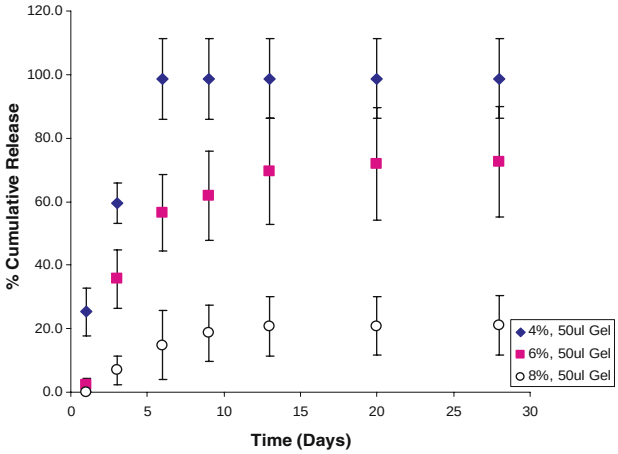

Fig. 2. Adenovirus release profile from a 50 μl 4% gel (*filled diamond*), 50 μl 6% gel (*filled square*), and 50 μl 8% gel (*open circle*) over the period of 28 days. The release media (PBS) was collected at various time points and the number of virus particles was determined by Real Time-PCR. Data are shown as Mean ± standard deviation, $n=3$.

Release of Viruses from Hydrogels

Hydrogels were placed in 1.5 ml cryogenic vials containing 800 μl of sterile PBS or complete culture media (DMEM supplemented with serum) and incubated at 37°C in a shaking incubator for 28 days. Sampling and replacement of the release media was carried out at predetermined time points. At each time point, all the sample volume (i.e., 800 μl) was taken out; vials were washed with 500 μl media, and replaced with 800 μl of fresh media. The number of released viruses was measured using Real Time-PCR and a calibration curve ranging from 10^2 to 10^{10} virus particles.

Virus Quantification by Real Time-PCR

The viral DNA was extracted from the released viruses in the media using Qiagen's QIAamp DNA Blood Mini Kit and protocol, page 27–29. In brief, 20 μl proteinase K, 200 μl sample containing adenoviruses, and 10 μg poly dA (DNA carrier) were added into a microfuge tube and mixed thoroughly. Lysis buffers were added, samples were loaded onto columns, and the protein contaminants were washed away. Viral DNA was eluted using 2×200 μl of distilled water (DNase, RNase Free). The purity of extracted DNA was determined by measuring the absorbance at 260/280 nm. The number of extracted viral DNA was quantified using Biorad's IQ SYBR Green Supermix kit and protocol (Hercules, CA), Biorad iCycler Real Time-PCR (forward

primer: 5′–aaaatctcctgcacgtctcctgag–3′: 10,850–10,873; and reverse primer: 5′–cggagatctccaggaggaagaga–3′: 10,911–10,933). The PCR parameters used to amplify the viral DNA copy number are shown in Fig. 1.

In Vitro Virus Bioactivity

Hydrogels containing Ad.GFP or Ad.CMV.LacZ were prepared as described in "Preparation of SELP-47K Hydrogels Containing Adenoviruses for *In vitro Studies*," and incubated in complete growth media (DMEM plus serum) for 28 days. At predetermined time points, samples were taken and used to evaluate the transduction efficiency of the viruses performed on HeLa cells (ATCC, Manassas, VA) either by direct visualization using a confocal microscope or by β-galctosidase enzyme assays (Promega, Madison, WI). The HeLa cells were transduced using Qbiogene's cell transduction protocol for Ad.GFP. Transduction efficiencies were evaluated 48 h after cell infection by viruses (n=3).

In Vivo Virus Bioactivity

Five million of Fadu and MDA-MB-435 cell lines (ATCC, Manassas, VA) in 50 ul DMEM (without phenol red) was s.c. injected into the right and left hind leg of 4–6 week-old female nu/nu mice (Harlan, Indianapolis, IN) using a 1 ml syringe and 28 gauge needle. The s.c. tumors were used in experiments when they reached 4 to 6 mm in diameter. MB-435 and Fadu tumors were injected directly with 4×10^9 pfu Ad.GFP. The tip of a 28-gauge needle was carefully

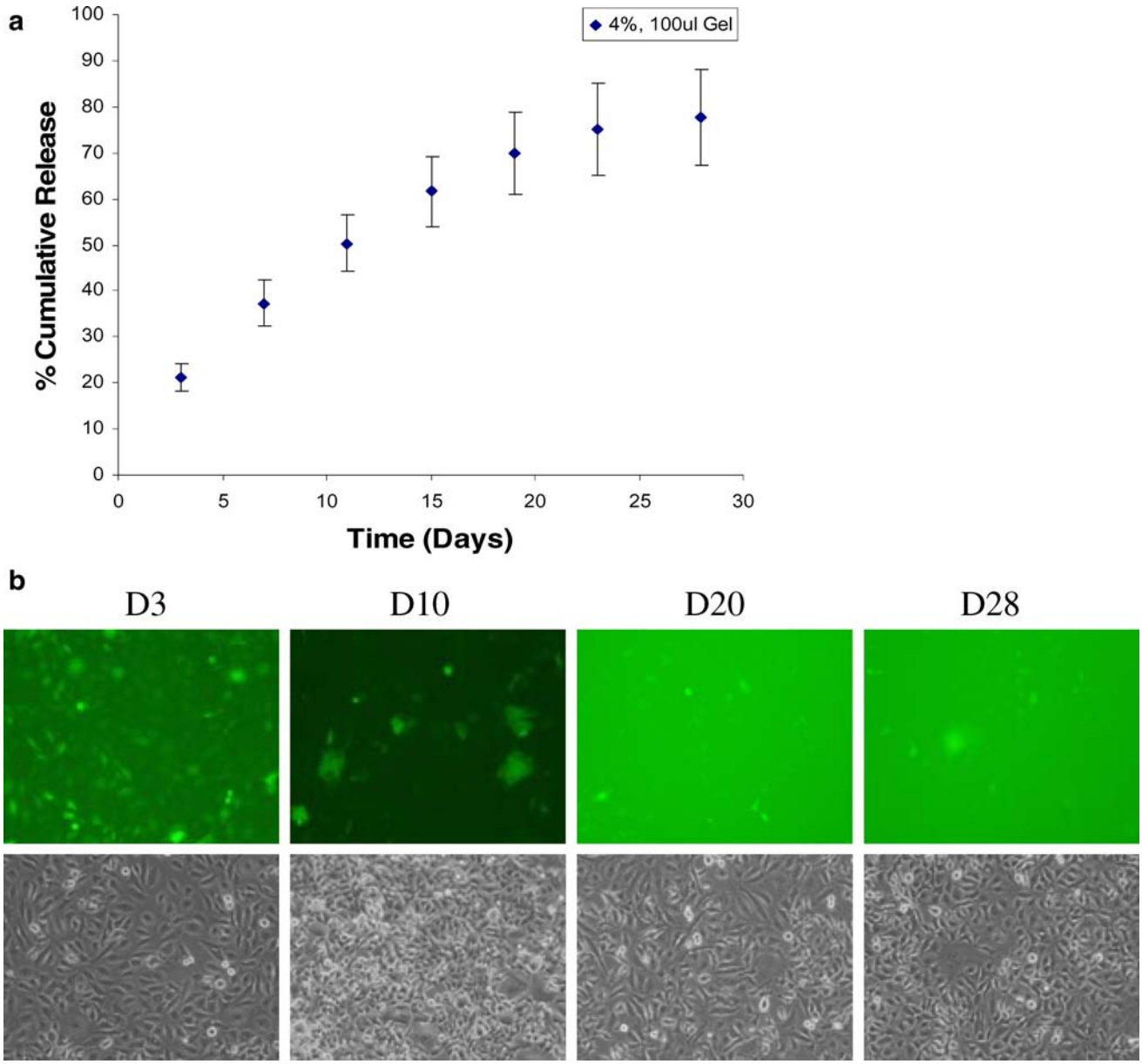

Fig. 3. (**a**) Adenovirus release profile from 100 μl 4% hydrogel in complete growth media in the presence of serum. (**b**) Corresponding transduction of the HeLa cells by viruses released in the media followed by visualization by a confocal microscope (×40 objective). Samples were taken from the release media on days 3, 10, 20, 28 and used to transfect HeLa cells to demonstrate the ability of the hydrogel system in preserving virus infectivity over the period 28 days. Data are shown as Mean ± standard deviation, $n=3$.

placed (90° angle) near the top–centre of tumors by controlling the depth of needle insertion relative to the tumor size measured by a caliper. At different time points, mice were sacrificed and tumors were dissected followed by rapid freezing and storage at −80°C. Six frozen sections were prepared from each tumor at different levels (500–800 μm apart) with 7–10 μm thickness. Sections were stained with ProLong Gold antifade reagent with Dapi (Invitrogen, Carlsbad, Ca) and visualized using a Zeiss confocal microscope.

RESULTS AND DISCUSSION

Limitations of the existing adenoviral vectors in cancer gene therapy include the inability to achieve: a) targeted delivery of the adenoviruses to tumors with high efficiency and low toxicity by systemic administration; b) local and sustained delivery of the adenovirus to tumors with less frequent dosing, and c) transduction of a large fraction of cells comprising tumor masses. To study the potential of SELP hydrogels in prolonging the release of the adenoviruses, preserving their bioactivity and transfecting a large fraction of tumor cells, several *in vitro* and *in vivo* experiments were conducted. *In vitro* virus release studies demonstrated that by changing polymer concentration it is possible to control virus delivery up to 28 days. The 4% gel with 50 ul size released 100% of the loaded viruses in one week, followed by more than 70% release from 6% gel, and up to 20% from 8% v/v hydrogels (Fig. 2). The release profiles of adenoviruses from 4, 6 and 8% gels were significantly different ($p<0.05$) from each other. An increase in polymer concentration leads to increased hydrogel crosslinking density resulting in decreased rate of virus release.

To determine the infectivity of the released viruses, SELP discs (100 μl) containing Ad.GFP were incubated in complete growth media. At predetermined time points samples were drawn to measure the number of released viruses (Fig. 3a) and the corresponding green fluorescence activity as a qualitative measure of virus infectivity (Fig. 3b). Results demonstrate that SELP hydrogels can sustain the release of the adenoviral particles over the 28 day period of study and preserve their infectivity. After a series of experiments in various release media (i.e., PBS, DMEM, DMEM/Serum), different gel sizes (i.e., 50 μl and 100 μl) and number of loaded viruses (10^8–10^9), we obtained sustained release of infectious adenoviruses over the period of 28 days. The bioactivity of the released adenoviruses was also measured quantitatively. SELP discs containing Ad.CMV.LacZ were incubated in complete growth media and the number of released viruses was measured at each time point (Fig. 4a). Under the same conditions but in a separate set of samples, at each time point samples were withdrawn and used to transduce HeLa cells followed by the β-gal activity measurements [Fig. 4b(a)]. The β-gal activity of the released viruses from the gels [Fig. 4b(a)] was compared with the β-gal activity of the same number of viruses with 100% infectivity [Fig. 4b(b)] ($n=3$, $p<0.05$). It was observed that the decline in transduction efficiency of the viruses is due to the combination of decrease in release rate and virus inactivation during incubation in complete growth media over the 28 days. The results also show 30–40% loss of virus activity in this period. It is noteworthy that in our studies, after the incubation of the

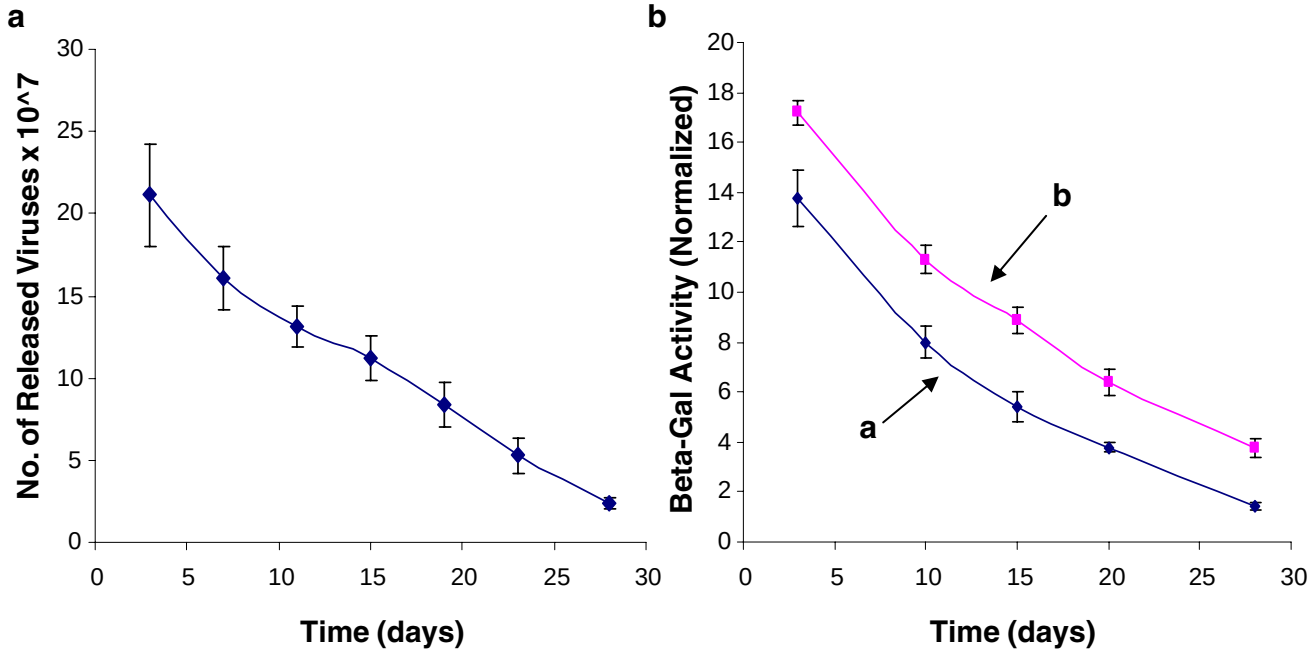

Fig. 4. Panel **a**: The decrease in Ad.LacZ release rate from 100 ul gels in complete growth media. The number of released viruses was measured using Real Time-PCR. Panel **b**: HeLa cells were seeded 24 h before transduction, transduced by Ad.CMV.LacZ and the β-gal activity was measured using Promega's β-galactosidase enzyme assay system; (**a**) 3 gel discs with the specifications mentioned in Panel **a** were prepared. The released viruses were used to transduce HeLa cells followed by β-gal enzyme assay (*observed activity*). (**b**) A serial dilution of 100% infectious virus particles corresponding to the number of released viruses shown in Panel **a** were prepared. The infectious viruses were used to transduce HeLa cells and β-gal activity was measured (*expected activity*). Data are shown as Mean ± standard deviation, $n=3$.

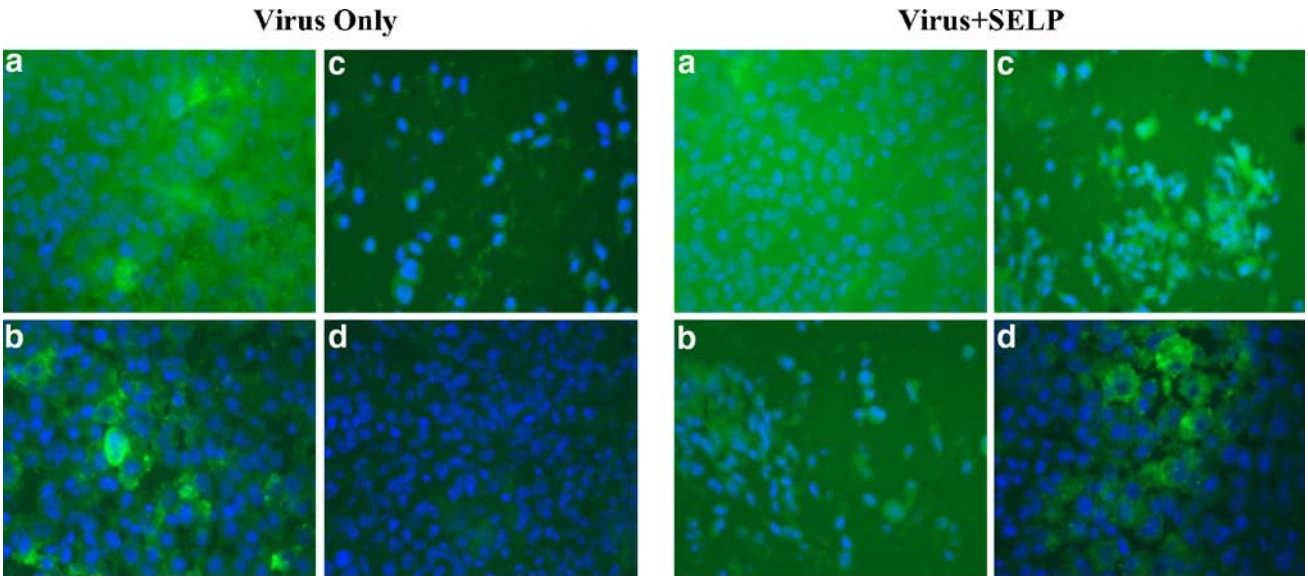

Fig. 5. The cell nucleus of MB-435 breast cancer cells is shown in blue and GFP expression in green (×40 objective). Panels **a**–**d** show GFP expression of the tumor cells at days 3, 7, 11 and 15, respectively. The GFP expression level after day 11 was significantly decreased in tumors injected with virus only whereas sustained GFP expression was observed in tumors injected with virus/SELP mixture up to day 15.

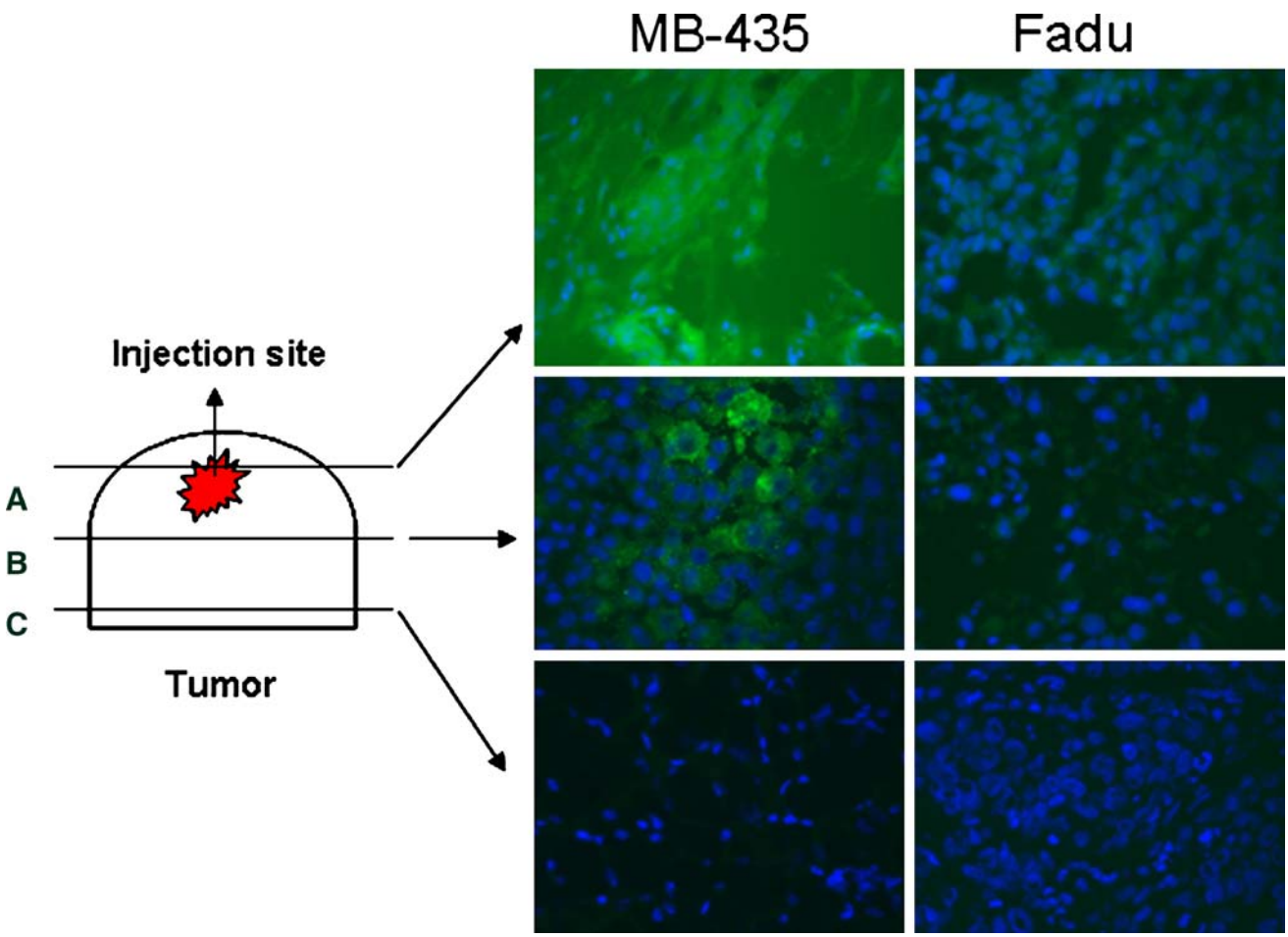

Fig. 6. GFP expression levels from MB-435 tumor tissue sections at different levels (×40 objective). Section A which was close to the SELP+virus injection site had higher GFP expression than section C. Section C was approximately 4–5 mm apart from section A. GFP expression was localized to the cells in proximity of the injection site.

viruses (no SELP) in the complete growth media in the presence of serum, virus infectivity was detectable only for 9±3 days (Mean ± S.E., n=9). The preservation of virus infectivity in the SELP matrix could be the result of the stabilization of the virus envelop by the silk or elastin units, although this needs to be explored more in depth.

To examine the ability of SELP matrices in localizing and prolonging viral transduction *in vivo*, murine models of breast (MDA-MB-435) and head and neck (Fadu) tumor xenografts were used. The animal protocol in this study has been approved by the University of Maryland Institutional Animal Care and Use Committee. The control group received 4×10^9 plaque forming units of Ad.GFP per tumor while the test group received 50 μl of SELP mixed with 4×10^9 plaque forming units. Our previous studies have shown that SELP by itself does not have any green fluorescent activity (21). The results of the MB-435 and Fadu control group injected with viruses (no SELP), revealed that the tumors infected with Ad.GFP could express GFP up to 15 days with expression levels decreasing on days 11 and 15. The significant reduction in GFP expression at later days could be the result of the virus inactivation by the mouse immune system, leakage of viruses to the blood stream and clearance by liver, or combination thereof. In the first week of the study, the distribution of GFP expression in MB-435 tumors was uniform at all levels of the tumor sections suggesting the free convection of viruses throughout the tumor tissue whereas patchy in Fadu tumors. This could be related to the existing barriers within Fadu tumors preventing uniform viral spread. Overall, the level of GFP expression in MB-435 tumors was much higher than Fadu tumors which could be due to the higher expression level of CAR (Coxsackie and Adenovirus Receptor) on the surface of MB-435 tumor cells. The number of adenovirus receptor on the surface of the tumor cells can play a significant role in the outcome of the adenoviral cancer gene therapy (22,23). The test group infected with the mixture of SELP+virus showed persistent GFP expression up to 15 days confirming the compatibility of SELP with adenoviruses in preserving their activity (Fig. 5). Overall, the GFP expression level up to day 15 was much higher in SELP+virus group than virus only, suggesting the possible utility of these polymers for localized and sustained adenoviral delivery.

Although in both control and test groups high transduction levels were observed up to day 11, in test group (SELP+virus) at day 15 GFP expression was mostly observed in the proximity of the injection site and minimum to no apparent cell transduction in the tumor tissue farther from the injection site (Fig. 6). This could be the result of virus entrapment in the gel matrix leading to reduced rate of dissemination to distant sites within tumors. This finding is important as it suggests SELP is capable of entrapping viruses and of forming a virus depot to localize its effect. These results are in agreement with previously reported studies (12) and corroborate the potential of virus laden hydrogel systems as an alternative to direct intratumoral virus infusion.

CONCLUSION

This study is the first report of the intratumoral delivery of adnoviruses using hydrogels made from genetically engineered silk–elastinlike copolymers. The in vitro infectivity of viruses released from silk–elastinlike copolymers over 28 days was evaluated. *In vivo* data demonstrates prolonged and localized expression of adenoviruses. These results suggest the potential of SELPs in adenoviral delivery for cancer treatment. The next steps are to quantify and optimize gene expression, measure virus spread in liver and neighboring tissues, optimize polymer composition and structure and evaluate the utility of these matrices for the delivery of therapeutic adenoviral gene carriers.

ACKNOWLEDGMENTS

Financial support was provided by grants from the National Institutes of Health (R01CA107621-01) and Department of Defense (DAMD17-03-1-0237).

REFERENCES

1. L. Thorrez, T. Vanden Driessche, D. Collen, and M. K. Chuah. Preclinical gene therapy studies for hemophilia using adenoviral vectors. *Semin. Thromb. Hemost.* **30**:173–183 (2004).
2. T. VandenDriessche, L. Naldini, D. Collen, and M. K. Chuah. Oncoretroviral and lentiviral vector-mediated gene therapy. *Methods Enzymol.* **346**:573–589 (2002).
3. Y. Kawashita, H. Fujioka, A. Ohtsuru, Y. Kaneda, Y. Kamohara, Y. Kawazoe, S. Yamashita, and T. Kanematsu. The efficacy and safety of gene transfer into the porcine liver *in vivo* by HVJ (Sendai virus) liposome. *Transplantation* **80**:1623–1629 (2005).
4. M. Guan, J. R. Rodriguez-Madoz, P. Alzuguren, C. Gomar, M. G. Kramer, S. Kochanek, J. Prieto, C. Smerdou, and C. Qian. Increased efficacy and safety in the treatment of experimental liver cancer with a novel adenovirus-alphavirus hybrid vector. *Cancer Res.* **66**:1620–1629 (2006).
5. S. Worgall, G. Wolff, E. Falck-Pedersen, and R. G. Crystal. Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. *Hum. Gene Ther.* **8**:37–44 (1997).
6. F. Yuan. Transvascular drug delivery in solid tumors. *Semin. Radiat. Oncol.* **8**:164–175 (1998).
7. H. Ren, T. Boulikas, K. Lundstrom, A. Soling, P. C. Warnke, and N. G. Rainov. Immunogene therapy of recurrent glioblastoma multiforme with a liposomally encapsulated replication-incompetent Semliki forest virus vector carrying the human interleukin-12 gene-a phase I/II clinical protocol. *J. Neuro-oncol.* **64**:147–154 (2003).
8. Y. Wang, H. Wang, C. Y. Li, and F. Yuan. Effects of rate, volume, and dose of intratumoral infusion on virus dissemination in local gene delivery. *Mol. Cancer. Ther.* **5**:362–366 (2006).
9. H. Sauthoff, J. Hu, C. Maca, M. Goldman, S. Heitner, H. Yee, T. Pipiya, W. N. Rom, and J. G. Hay. Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: virus persists and spreads systemically at late time points. *Hum. Gene Ther.* **14**:425–433 (2003).
10. Y. Wang, J. K. Hu, A. Krol, Y. P. Li, C. Y. Li, and F. Yuan. Systemic dissemination of viral vectors during intratumoral injection. *Mol. Cancer Ther.* **2**:1233–1242 (2003).
11. L. A. Chandler, J. Doukas, A. M. Gonzalez, D. K. Hoganson, D. L. Gu, C. Ma, M. Nesbit, T. M. Crombleholme, M. Herlyn, B. A. Sosnowski, and G. F. Pierce. FGF2-Targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. *Mol. Ther.* **2**:153–160 (2000).

12. Y. Wang, S. Liu, C. Y. Li, and F. Yuan. A novel method for viral gene delivery in solid tumors. *Cancer Res.* **65**:7541–7545 (2005).
13. S. Kalyanasundaram, S. Feinstein, J. P. Nicholson, K. W. Leong, and R. I. Garver Jr. Coacervate microspheres as carriers of recombinant adenoviruses. *Cancer Gene Ther.* **6**:107–112 (1999).
14. J. Cappello, J. Crissman, M. Dorman, M. Mikolajczak, G. Textor, M. Marquet, and F. Ferrari. Genetic engineering of structural protein polymers. *Biotechnol. Prog.* **6**:198–202 (1990).
15. M. Haider, A. Hatefi, and H. Ghandehari. Recombinant polymers for cancer gene therapy: a minireview. *J. Control. Release* **109**:108–119 (2005).
16. D. W. Urry, C. M. Harris, C. X. Luan, C.-H. Luan, D. Channe-Gowda, T. M. Parker, S. Q. Peng, and J. Xu. Transductional protein-based polymers as new controlled-release vehicles. In K. Park (ed)., *Controlled Drug Delivery: Challenges and Strategies*, American Chemical Society, Washington, DC, 1997, pp. 405–438.
17. Z. Megeed, J. Cappello, and H. Ghandehari. Genetically engineered silk–elastinlike protein polymers for controlled drug delivery. *Adv. Drug Deliv. Rev.* **54**:1075–1091 (2002).
18. Z. Megeed and H. Ghandehari. Genetically engineered protein-based polymers: potential in gene delivery. In M. Amiji (ed)., *Polymeric gene delivery: Principles and applications*, CRC, Boca Raton, FL, USA, 2005, pp. 489–507.
19. A. A. Dinerman, J. Cappello, H. Ghandehari, and S. W. Hoag. Swelling behavior of a genetically engineered silk–elastinlike protein polymer hydrogel. *Biomaterials* **23**:4203–4210 (2002).
20. Z. Megeed, J. Cappello, and H. Ghandehari. Controlled release of plasmid DNA from a genetically engineered silk–elastinlike hydrogel. *Pharm. Res.* **19**:954–959 (2002).
21. Z. Megeed, M. Haider, D. Li, B. W. O'Malley Jr, J. Cappello, and H. Ghandehari. *In vitro* and *in vivo* evaluation of recombinant silk–elastinlike hydrogels for cancer gene therapy. *J. Control. Release* **94**:433–445 (2004).
22. J. F. Zhang, C. Hu, Y. Geng, J. Selm, S. B. Klein, A. Orazi, and M. W. Taylor. Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy. *Proc. Natl. Acad. Sci. USA* **93**:4513–4518 (1996).
23. D. Li, L. Duan, P. Freimuth, and B. W. O'Malley Jr. Variability of adenovirus receptor density influences gene transfer efficiency and therapeutic response in head and neck cancer. *Clin. Cancer Res.* **5**:4175–4181 (1999).