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ORIGINAL ARTICLE

Local sustained delivery of oncolytic adenovirus with injectable alginate gel for cancer virotherapy

J-W Choi[1,5], E Kang[2,5], O-J Kwon[2], TJ Yun[2], H-K Park[3], P-H Kim[2], SW Kim[2,4], JH Kim[3] and C-O Yun[2]

Adenoviruses (Ad) have been investigated for their efficacy in reducing primary tumors after local intratumoral administration. Despite high Ad concentrations and repetitive administration, the therapeutic efficacy of Ad has been limited because of rapid dissemination of the Ad into the surrounding normal tissues and short maintenance of Ad biological activity *in vivo*. To maximize the therapeutic potential of Ad-mediated gene therapeutics, we investigated the efficacy of local, sustained Ad delivery, using an injectable alginate gel matrix system. The biological activity of Ad loaded in alginate gel was prolonged compared with naked Ad, as evidenced by the high green fluorescent protein gene transduction efficiency over an extended time period. Moreover, oncolytic Ad encapsulated in alginate gel elicited 1.9- to 2.4-fold greater antitumor activity than naked Ad in both C33A and U343 human tumor xenograft models. Histological and quantitative PCR analysis confirmed that the oncolytic Ad/alginate gel matrix system significantly increased preferential replication and dissemination of oncolytic Ad in a larger area of tumor tissue *in vivo*. Taken together, these results show that local sustained delivery of oncolytic Ad in alginate gel augments therapeutic effect through selective infection of tumor cells, sustained release and prolonged maintenance of Ad activity.

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INTRODUCTION

The efficacy of adenovirus (Ad) vectors in cancer gene therapy has been heavily investigated. Several characteristics make Ad attractive gene therapy candidates, including high gene-transfer efficiency in both dividing and non-dividing cells, easy production of high-titer Ad stocks, low risk of insertional mutagenesis and induction of oncolysis by viral replication. Among the 1600 clinical trials studying gene therapy since 1990, investigators have chosen Ad as a delivery vector in over 390 clinical trials because of these characteristics http://www.wiley.co.uk/genmed/clinical/. A significant advance in the second generation of oncolytic Ad is the capability of replicating selectively in cancer cells.[2–10] After the lysis of the infected cancer cells, progenies of oncolytic Ad disseminate locally and infect neighboring cancer cells. This sequential replication and lysis of cancer cells makes oncolytic Ad a promising candidate for cancer gene therapy, overcoming the modest efficacy of non-replicating virus-mediated cancer gene therapy.[11–17] Cancer cell-specific replication of oncolytic Ad also reduces toxicity and maximizes safety by not replicating in normal healthy cells[18–20] Moreover, the combination of therapeutic gene expression and lytic ability of oncolytic Ad further increase the likely impact of Ad-mediated cancer gene therapy. Combining virotherapy and expression of genes with anti-cancer roles, such as decorin, relaxin, cytokines and vascular endothelial growth factor- or interleukin-8-specific short hairpin RNA, have greatly increased the efficacy of cancer gene therapy.[11,13,14,21–27]

Despite these therapeutically advantageous characteristics, the use of oncolytic Ads has thus far had low efficacy in clinical settings because of induction of immune responses against Ad and the short duration of therapeutic gene expression.[28] ONYX-015 oncolytic Ad administered intratumorally into patients with head and neck cancer demonstrated limited local viral replication as a result of robust innate immune responses.[29,30] The limited efficacy of this oncolytic Ad is a result of viruses being engulfed by Kupffer cells and hepatocytes.[31] Effective treatment of large tumors requires maintenance of the therapeutic dose of the virus at targeted disease site. To increase the effectiveness of Ad virotherapy, experiments have been conducted involving high Ad titer and repeated injections, but efficacy was hampered by fast clearance of the virus from tumor tissue to the blood microvasculature and normal tissues.[32–34]

Efficacy is further diminished by the fact that Ad is slow to diffuse through the extracellular matrix of a very large tumor. The limited viral infection of neighboring cells far from the initial injection site greatly limits the ability of virus-mediated cancer gene therapy to be a successful form of cancer gene therapy. In a phase I clinical trial using p53-expressing Ad to treat recurrent glioma, the zone of transduced cells did not extend beyond 5 mm from the injection site.[35] Moreover, tumor biopsies taken from subjects in a phase II clinical trial studying head and neck cancer demonstrated that ONYX-015 oncolytic Ad are present in tumor tissues, but the pattern of viral dissemination was very focal in

[1]Graduate Program for Nanomedical Science, Yonsei University, Seoul, Republic of Korea; [2]Department of Bioengineering, College of Engineering, Hanyang University, Seoul, Republic of Korea; [3]Department of Chemical and Biomolecular Engineering, Yonsei University, Seoul, Korea and [4]Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT, USA. Correspondence: Dr JH Kim, Department of Chemical and Biomolecular Engineering, Yonsei University, 134 Shinchon-Dong, Seodaemoon-Gu, Seoul, Korea or Dr C-O Yun, Department of Bioengineering, College of Engineering, Hanyang University, 17 Haengdang-dong, Seongdong-gu, Seoul 133 791, Korea.
E-mail: jayhkim@yonsei.ac.kr or chaeok@hanyang.ac.kr
[5]These authors contributed equally to this study.
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nature, indicating that the virus did not distribute evenly throughout the tumor tissue.[30] With the aim of improving oncolytic viral spread and tumor penetration to enhance cancer gene therapy, we recently demonstrated the importance and utility of the peptide hormone relaxin. Relaxin has been shown to be a potent inhibitor of collagen expression when collagen is overexpressed, but does not markedly alter basal levels of collagen expression, in contrast to other collagen-modulatory cytokines, such as interferon-γ[36] and transforming growth factor-β.[37] DWP418 is an oncolytic Ad that expresses relaxin and demonstrates markedly improved viral distribution in large tumors, resulting in higher transduction efficiency and enhanced antitumor efficacy.[2,22]

The development of optimized Ad and an effective Ad delivery system would further advance Ad vector therapy by maximizing safety, efficacy and duration of transgene expression. Recently, a locally injectable virus delivery system has been used for bone morphogenic protein-2-expressing Ad gene therapy of osteogenesis using a collagen carrier,[38] antibody complexation with Ad in a collagen carrier,[39] lentivirus entrapment in hyalulonic acid/collagen matrix[40] and mixture of silk elastin-like protein polymers/Ad for breast and head and neck cancer xenograft tumor models.[34] The use of a matrix-based Ad transduction system assisted widespread and uniform virus transduction.[34,41] In addition, the local injection of a matrix-based Ad delivery system prevented rapid viral dissemination and infusion from tumor to normal organ during intratumoral injection, which results in low normal cell transduction efficiency.[42,43] However, no previous studies have used localized and sustained oncolytic Ad delivery to clearly demonstrate the therapeutic effects of this Ad *in vivo*. Moreover, it is as yet unknown whether spatial Ad delivery using a matrix-based sustained delivery retains sufficient Ad biological activity to facilitate long-lasting oncolytic Ad replication and cancer cell killing.

In this study, we tested a locally injectable Ad in a matrix-based depot system involving a biodegradable alginate gel, a natural polymer that is frequently used in biomedical applications.[44] Alginate has been previously used as an injectable gel carrier for protein[45,46] and cells[47] because of its biocompatibility, low toxicity, relatively low cost and mild gelation behavior with divalent cations. Here we report the *in vitro* characterization of Ad entrapped in alginate gel, prolonged gene expression as a result of the sustained Ad release and protective microenvironment within the gel, and enhanced oncolytic Ad-mediated cancer cell killing. We also demonstrate that local injection of oncolytic Ad-loaded gel resulted in sustained release of Ad with prolonged activity *in vivo*, leading to enhanced antitumor therapeutic effects. Enhanced local preferential replication of oncolytic Ad mediated by sustained release in the protective gel was determined by immunohistochemical analysis and quantitative PCR (Q–PCR).

RESULTS

The effect of calcium chloride and gel dissolution solution on the biological activity of Ad

We first examined the effects of calcium chloride and gel dissolution solution that were used for gel formation and dissociation, respectively, on Ad biological activity. Ads were incubated with various concentrations of $CaCl_2$ (20–200 mM) for 5 h at various temperatures (4 °C, 25 °C and 37 °C). A549 cells (5×10^4) were then transduced with 5×10^2 viral particle (VP) of dE1/green fluorescent protein (GFP) for 48 h at 37 °C. The fluorescence intensity of GFP expression was analyzed as a function of $CaCl_2$ concentration to determine if the biological activity of Ad was affected by the $CaCl_2$ solution. As shown by the consistent GFP expression in Figure 1a, treating Ad with concentrations of $CaCl_2$ up to 50 mM did not negatively affect the biological activity of Ad. However, treating Ad with $CaCl_2$ at 100 or 200 mM resulted in markedly reduced GFP expression at 37 °C, although GFP expression was maintained at room temperature (25 °C) or 4 °C. These results indicate that concentrations of $CaCl_2$ greater than 50 mM can impair Ad's biological activity at 37 °C, but it is maintained at 25 °C and 4 °C at $CaCl_2$ concentrations up to 200 mM. Therefore, we chose 50 mM as the optimal concentration of $CaCl_2$ to generate the Ad-loaded alginate for all experiments described below.

We next investigated the effect of gel dissolution solution on Ad activity by exposing Ad-loaded alginate gels (500 µl) to an equal volume of gel dissolution solution (55 mM sodium citrate, 0.15 M sodium chloride and 30 mM EDTA, pH 6.8). After incubating Ad-loaded gels with gel dissolution solution for 10 min, A549 cells were treated with 5×10^2 VP of gel dissolution solution-treated Ad, and the resulting GFP fluorescence intensity was compared with that of naked Ad (Figure 1b). The addition of gel dissolution solution onto Ad/alginate gel did not affect Ad's biological activity, as demonstrated by a level of GFP expression equivalent to that of naked Ad. These results show that exposure of Ad to gel dissolution solution containing 30 mM EDTA had no negative effect on its biological activity as evidenced by normal transgene expression. To visualize the released Ad from Ad/alginate gel, GFP expression was observed at varying distances from the Ad-loaded alginate gel. The Ad/alginate gel ($\sim$1.3 mm in diameter) was placed gently on the cell bed and GFP expression was determined after 48 h of incubation at 37 °C. The fluorescence intensity of GFP expression was of high intensity near the gel and was progressively weakened at distances further from the gel (Figure 2). These results show the spatial dissemination of Ad based on fluorescence intensity as a function of distance from the Ad/alginate gel, indicating that Ad is continuously released from the alginate gel for a considerable time period and that the Ad/alginate gel system is potentially useful for the local treatment of tumors in an *in vivo* system.

Long-term protection of viable Ad by alginate gel encapsulation

We have evaluated the biological activity of Ad that were loaded in alginate gel (Figure 3). To determine if gel encapsulation prolongs Ad biological activity, gene expression of Ad in alginate gel was examined as a function of time. GFP-expressing Ad (1×10^{10} VP) was loaded into a 5% alginate gel with 50 mM $CaCl_2$. The Ad/alginate gel was then incubated in cell culture media at 37 °C for 1, 3, 5, 7, 9 or 11 days. At the indicated time points, medium containing the released Ad was then sampled (gel-released Ad) and the remaining Ad/alginate gel was dissolved (Ad in dissolved gel). The transduction efficiency of naked Ad, gel-released Ad or Ad in dissolved gel was then evaluated using SK-Hep1 cells. GFP expression of naked Ad was markedly reduced at 3 days after incubation at 37 °C, and progressively decreased through 7 days of incubation; expression was completely absent at 9 and 11 days of incubation (Figure 3a). These results indicate that the biological activity of naked Ad is significantly impaired over time at 37 °C. In marked contrast, cells treated with gel-released Ad demonstrated strong, sustained GFP expression over a prolonged time period through day 11. The level of GFP expression was quantified by fluorescence-activated cell sorting analysis, and the results demonstrated that cells transduced with gel-released Ad at 7 days of incubation expressed GFP at a 13-fold greater intensity than that of naked Ad incubated for the same number of days (Figure 3b). Moreover, the level of GFP expression in the cells treated with gel-released Ad at 11 days of incubation remained 64% of that of naked Ad at day 1, whereas the GFP expression of naked Ad was almost completely absent at day 11.

The transduction efficiency of Ad in dissolved gel was also examined after treatment with gel dissolution solution to demonstrate that Ad biological activity can be maintained in

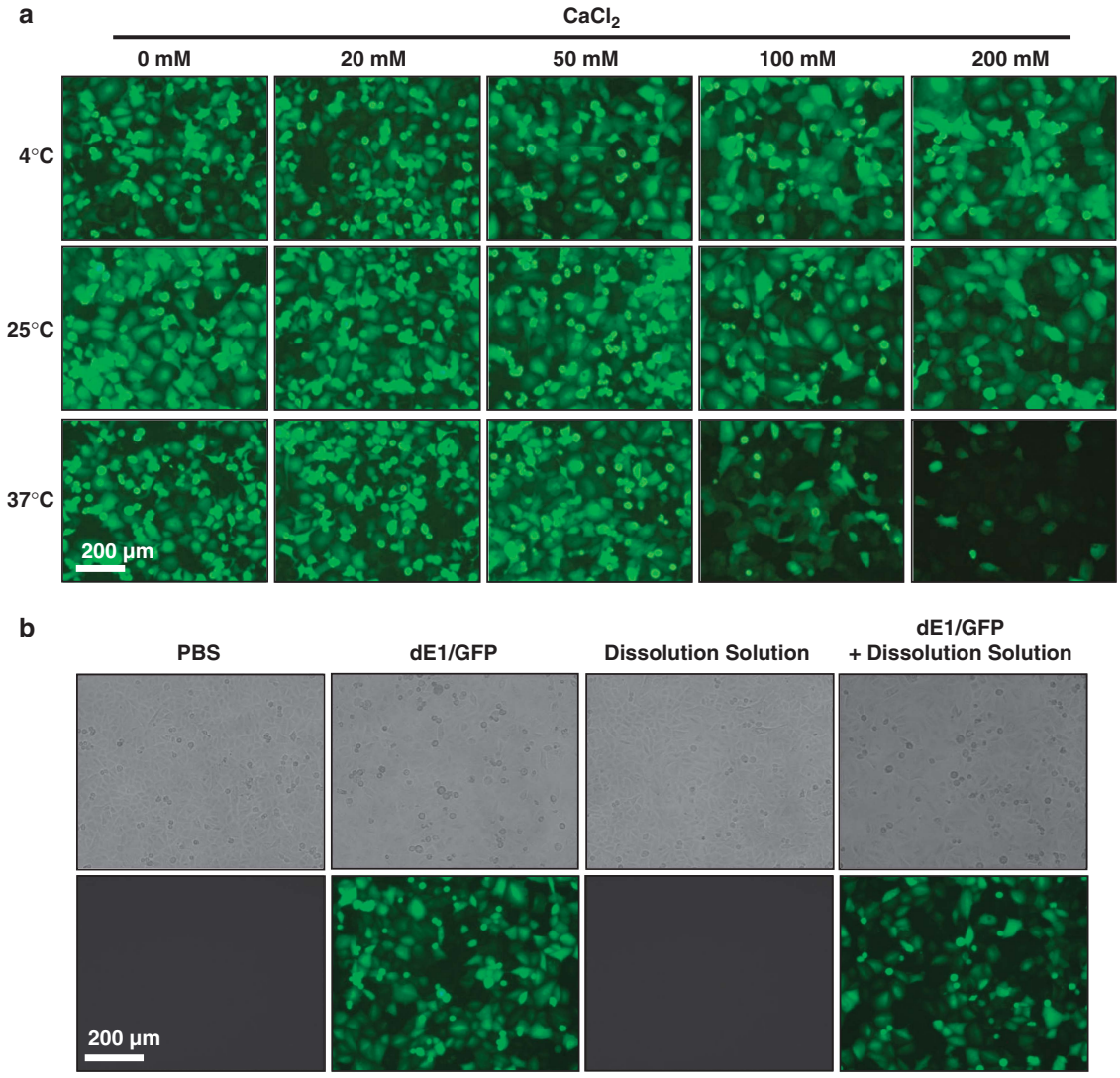

Figure 1. The effect of $CaCl_2$ and dissolution solution on the biological activity of Ad. (**a**) Fluorescent micrographs demonstrating the effect of $CaCl_2$ on the biological activity of Ad. Ad expressing GFP (dE1/GFP) was incubated with 20, 50, 100 and 200 mM of $CaCl_2$ for 5 h at 4 °C, 25 °C or at 37 °C. At 48 h after transduction into A549 cells, GFP expression was observed under fluorescence microscope. Original magnification: × 100. (**b**) Light and fluorescent micrographs demonstrating the effect of gel dissolution solution on the biological activity of Ad. After dE1/GFP Ad was incubated with dissolution solution for 10 min at 37 °C, A549 cells were transduced with dissolution-treated Ad. At 48 h after transduction, GFP expression was observed under fluorescence microscope. Original magnification: × 100.

alginate gel. Like gel-released Ad, the transduction efficiency of Ad from the dissolved gel was higher than naked Ad over a prolonged time period (Figure 3b). Specifically, the level of GFP expression in the cells treated with Ad in dissolved gel was 8.64 times higher than that of naked Ad at 7 days, and strong and persistent GFP expression was observed through 11 days of incubation. More importantly, the total level of GFP expression from gel-released Ad and Ad in dissolved gel was much higher than naked Ad throughout the course of this experiment (Figure 3b), indicating the potential utility of Ad/alginate gel for *in vivo* applications, for which long-term and efficient gene-transfer efficiency is needed. Collectively, these results demonstrate that the biological activity of Ad is preserved in alginate gel for an extended time period, and alginate gel-encapsulated Ad may provide a biocompatible environment for Ad to maintain viral activity.

To quantitatively assess how much Ad is released from the gel over time, the total number of viral genomes was quantified by Q–PCR (Figure 3c). Ad-loaded gels were incubated at 37 °C and each sample for Q–PCR analysis was taken at 1, 3, 5, 7, 9 or 11 days of incubation, similar to the transduction experiment assessing GFP expression. The total number of viral genomes of dE1/GFP in phosphate-buffered saline (PBS) was maintained over time at an average of $1.49 \times 10^{10} \pm 0.2 \times 10^{10}$ throughout the experiment (Figure 3c). As Q–PCR analysis detects viral genomes regardless of viral infectivity, we can measure the total number of existing active and inactive Ad. Together with transduction efficiency analysis presented in Figures 3a and b, these data demonstrate that viability of Ad rapidly decreases at 37 °C, even though viral genomes are not significantly degraded, unless Ad are protected by the alginate gel. Conversely, the accumulated amount of released Ad gradually increased to a maximum of 21-fold at day 11 ($2.57 \times 10^9 \pm 1.00 \times 10^5$) compared with day 1 ($1.24 \times 10^8 \pm 7.22 \times 10^4$), demonstrating that Ad in alginate gel underwent sustained release over time. A large quantity of viral genomes was detectable in the gel at day 11 ($4.79 \times 10^9 \pm 1.27 \times 10^6$); about 35% of the Ad present at day 1 ($1.35 \times 10^{10} \pm 3.69 \times 10^5$) was still encapsulated in the gel at day 11. Considering that long-term

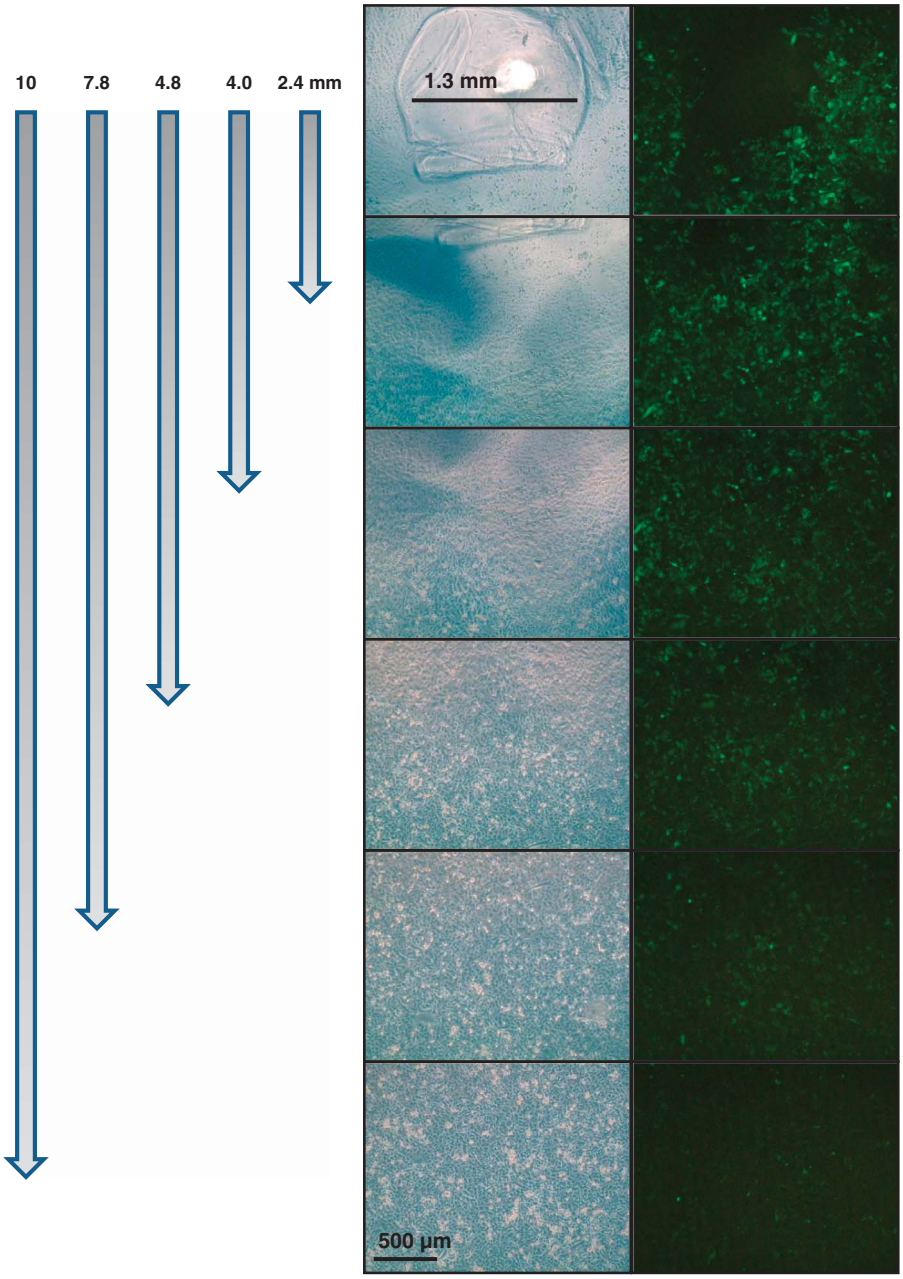

Figure 2. Visualization of released Ad from Ad/alginate gel. Fluorescent and light micrographs showing dE1/GFP Ad incubated with SK-Hep1 cells. Ad (3.5×10^4 VP) was encapsulated in the alginate gel ($\sim$1.3 mm in diameter), which was then placed on SK-Hep1 cells at 37 °C. After 48 h, GFP expression surrounding the gel was observed under fluorescence microscope. The arrow indicates the direction moving away from the gel; that is, images in the top row represent cells near the gel and images on the bottom depict cells that are furthest from the gel. Note the strong GFP fluorescence intensity in the vicinity of the gel and how the intensity weakens in a gradient-dependent manner due to local Ad release. Original magnification: $\times$40.

transduction is needed for *in vivo* applications, using Ad/alginate gel as a depot system may be very useful by acting as a reservoir that releases Ad in a sustained manner, while at the same time maintaining Ad's biological activity.

Long-term cancer cell killing efficacy of oncolytic Ad/gel

We then examined the antitumor therapeutic efficacy of locally injectable alginate gel loaded with oncolytic Ad (DWP418). DWP418 is a modified telomerase promoter-regulated replicating Ad, which actively but selectively replicates in cancer cells and not normal cells, thus inducing a cancer cell-specific killing effect (Figure 4a).[2] In addition, DWP418 expresses the peptide hormone relaxin, which facilitates spreading of the virus throughout the tumor bed, thus overcoming extracellular barriers and resulting in even and wide distribution of Ad in solid tissues. U343 brain cancer cells were infected with either naked DWP418 or DWP418 released from alginate gel sampled at various times of incubation at 37 °C, and the cell-killing effect of this Ad was examined. The oncolytic ability of naked DWP418 rapidly decreased over time, with $\sim$0% (300 VP) and 17.6% (500 VP) cancer cell-killing ability remaining at 9 days of incubation at 37 °C (Figure 4b). In marked contrast, the oncolytic ability of gel-released DWP418 was maintained up to an incubation period of 9 days, with a

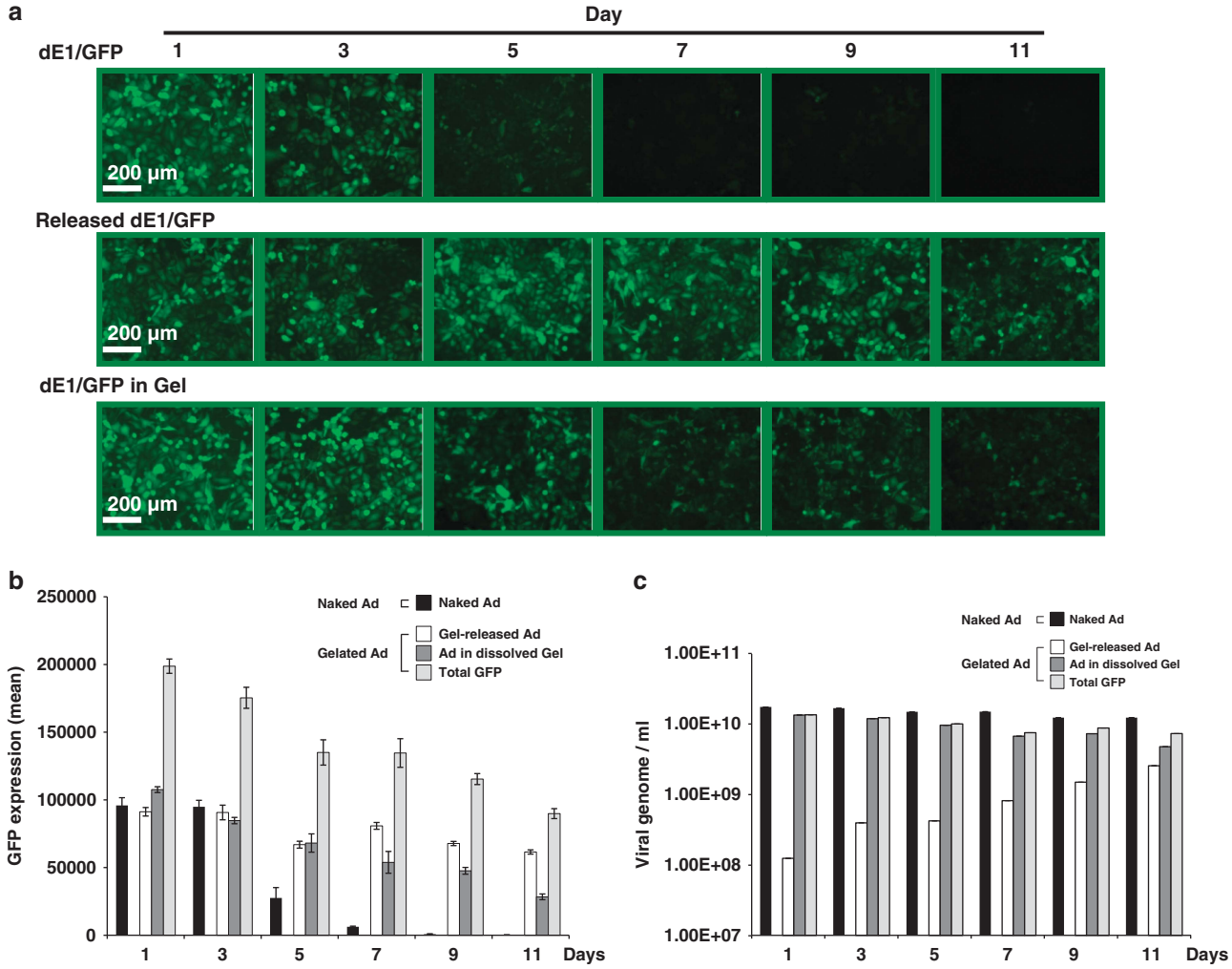

Figure 3. Sustained release of Ad from alginate gel. (**a**) Fluorescent micrographs showing SK-Hep1 cells incubated with Ad in different forms. Ad (1×10^{10} VP of dE1/GFP) was encapsulated in 5% alginate gel, and incubated with Dulbecco's modified Eagle's medium at 37 °C for 1, 3, 5, 7, 9 and 11 days. SK-Hep1 cells transduced with naked Ad (top row), released Ad from 5% gel (second row), and dissolved Ad-gel (third row). Original magnification: × 100. (**b**) Bar graphs showing quantitative GFP expression as measured by fluorescence-activated cell sorting analysis. The GFP expression of naked Ad-transduced cells decreased drastically over time, whereas those of other groups demonstrated a more gradual release. Each data point represents the mean ± s.e.m. of triplicate cultures transduced with each Ad entity. (**c**) Bar graph of Q-PCR results showing accumulated Ad released from alginate gel by Q-PCR. The accumulated amounts of released Ad gradually increased to an amount 21-fold greater on day 11 versus day 1, demonstrating that Ad in alginate gel is released over time in a sustained manner. The quantitative Ad release over time is presented as the viral genome number (mean ± s.e.m.).

cell-killing efficacy of 60.9% (300 VP) and 80.6% (500 VP), demonstrating that alginate gel mediates long-term protection of oncolytic Ad's cancer cell-killing ability.

Assessment of antitumor efficacy of DWP418 in tumor tissue

To validate the antitumor therapeutic efficacy of locally injectable alginate gel loaded with DWP418, the growth of C33A and U343 xenograft tumors was examined. Once tumors of each type grew in nude mice, they were divided into four experimental groups: mice were treated with (1) PBS; (2) 5% alginate gel; (3) naked DWP418; or (4) DWP418/alginate gel. Intratumoral injection with either naked DWP418 or DWP418/alginate gel resulted in significantly reduced tumor growth, indicating oncolysis-mediated effective antitumor activity (Figure 5). With regards to the C33A xenograft tumor, the volumes of tumors treated with PBS, 5% alginate gel, DWP418 or DWP418/gel were 1585.3 ± 374.9, 2254.9 ± 435.4, 978.8 ± 142.4 and 408.2 ± 147.4 mm^3, respectively, at 41 days post treatment (Figure 5a). The tumor volumes of mice treated with DWP418 or DWP418/gel were 61.7% and 25.7%, respectively, of those in mice treated with PBS, demonstrating a significant reduction in tumor growth ($P < 0.01$). Treating mice with gel-encapsulated DWP418 resulted in tumors that were 2.4-fold smaller compared with those in mice treated with naked DWP418 at day 41 after treatment ($P < 0.05$). Interestingly, the tumors of control mice treated with 5% gel alone grew larger than those treated with PBS. One explanation for this effect is that the gel may serve as an extracellular matrix supporting the growth of cancer cells. Further investigation is now under way in our laboratory to more rigorously address this observation. In contrast, alginate gel containing DWP418 may serve as the platform for sustained release of and shielding microenvironment for the Ad, which may protect the Ad from degradation and/or attack by immune cells, ultimately resulting in greater therapeutic efficacy. Experiments with the U343 xenograft tumor model demonstrated results similar to that of the C33A tumor model (Figure 5c). Tumor volumes of mice treated with PBS, 5% gel, DWP418 or DWP418/gel were 3165.4 ± 286.7, 2775.3 ± 221.3, 773 ± 212.3 and

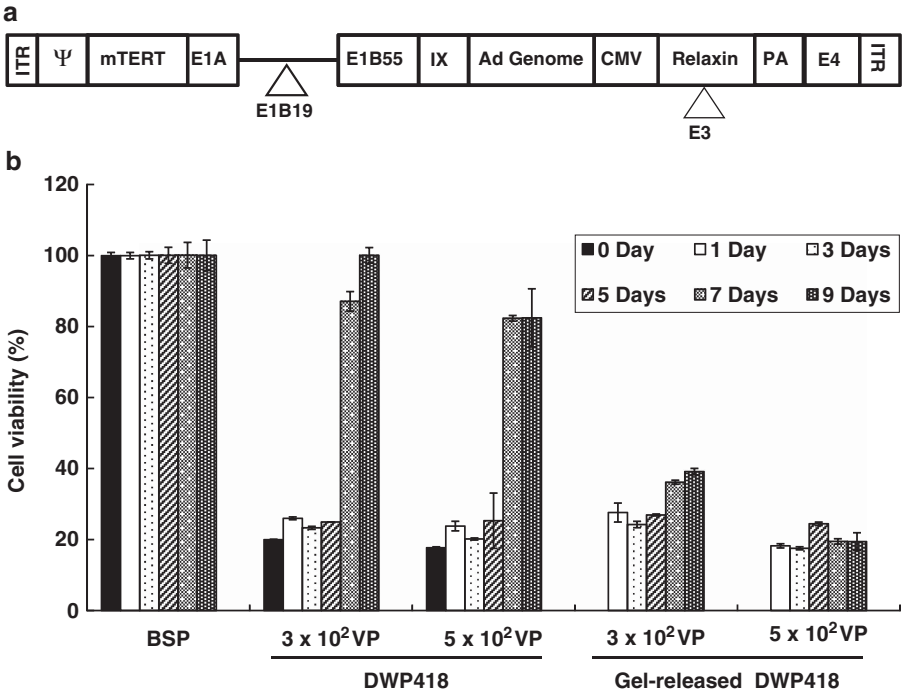

Figure 4. Long-term cancer cell-killing effect of oncolytic Ad released from the gel over time. (**a**) Schematic diagram of oncolytic Ad (DWP418). DWP418 elicits a cancer cell-specific killing effect as evidenced by the mTERT promoter controlling viral replication. (**b**) Bar graph depicting cancer cell viability. U343 cancer cells were incubated with naked or gel-released oncolytic Ads sampled at 1, 3, 5, 7 and 9 day (3×10^2 or 5×10^2 VP). The oncolytic ability of naked DWP418 rapidly decreased over time. In marked contrast, the oncolytic ability of gel-released DWP418 was preserved over time up to 9 days of incubation, indicating that oncolytic Ad activity was maintained by entrapment in gel. Each data point represents the mean ± s.e.m. of triplicate cultures infected with each Ad entity.

$399.6 \pm 103.2\,mm^3$, respectively, at 41 days after treatment. In mice treated with either naked DWP418 or DWP418/gel, tumor volume was 24.4% and 12.6%, respectively, of that in mice treated with PBS ($P<0.05$). Moreover, treating tumor-bearing mice with DWP418/gel elicited a 1.9-fold greater antitumor effect compared with the antitumor effect of naked DWP418 at 41 days ($P<0.05$), demonstrating that local injection of Ad within alginate gel enhances the antitumor activity of oncolytic Ad. To quantitatively assess the amount of Ad in tumor tissue, Q–PCR to detect the Ad genome was conducted. Tumor tissues were collected at 22 days after treatment with Ad in the C33A tumor xenograft model, and the number of viral genome in tumor tissue was measured for mice treated with PBS, alginate gel, DWP418 or DWP418/gel (Figure 5b). The viral genome copy numbers were $2.42 \times 10^3 \pm 1.69 \times 10^2$ for PBS, $1.03 \times 10^3 \pm 3 \times 10^2$ for gel, $1.85 \times 10^5 \pm 3.61 \times 10^4$ for DWP418 and $8.36 \times 10^5 \pm 2.91 \times 10^4$ for DWP418/gel. The viral genome copy number in the tumor tissue injected with DWP418/gel was 4.51-fold higher than those treated with naked DWP418 ($P<0.05$), indicating that local injection of Ad encapsulated within alginate gel is much more effective for long-term maintenance of Ad in tumor tissue. Tumors were also collected 3, 7, 15 or 24 days after intratumoral injection of DWP418 or DWP418/alginate gel in U343 xenograft tumor model to determine the amount of Ad in tumor tissue as time progressed (Figure 5d). At early time points post treatment (3 and 7 days), the viral genome copy numbers in tumor tissues injected with naked DWP418 or DWP418/alginate gel was not significantly different, whereas treatment with DWP418/alginate gel resulted in significantly higher viral genome copy numbers compared with treatment with naked DWP418 at later time points post treatment (15 and 24 days; $P<0.01$). The total viral genome copy number was $5.46 \pm 1.03 \times 10^8$ for DWP418/gel-treated tumor tissue and $1.30 \pm 0.02 \times 10^7$ for DWP418 treatment group at 15 day, demonstrating a 42-fold greater accumulation of Ad in tumor tissue treated with DWP418/alginate gel. The maximum viral genome copy numbers in tumor tissue was observed in the DWP418/alginate gel treatment group at 15 days, whereas Ad accumulation in tumor tissues treated with naked DWP418 diminished over time. Even though the total viral genome copy number in tumor tissue treated with DWP418/alginate gel was less at day 24 than at day 15, this copy number was still 7.6-fold greater than that of the naked DWP418 treatment. Taken together, these results indicate that local administration of Ad in 5% alginate gel by intratumoral injection enhances oncolytic Ad-mediated antitumor efficacy. The greater Ad accumulation in tumor tissue is likely due to the protective Ad microenvironment the gel provides and the sustained release of Ad into tumor tissue over time.

The enhanced antitumor effect of oncolytic Ad administered by local injection of alginate gel was further assessed by hematoxylin and eosin staining and immunohistochemical analysis. At 22 days post treatment, C33A tumors treated with PBS or alginate gel had normal and proliferating cancer cells, whereas tumor tissues treated with DWP418/alginate gel demonstrated necrosis in a larger area compared with naked DWP418 (Figure 6a). The presence of DWP418 in tumor tissue was confirmed with the binding of Ad E1A-specific antibody, and tumors treated with DWP418/alginate gel had a broader distribution of Ad particles compared with tumors treated with naked DWP418. Interestingly, extensive necrosis and widespread pattern of Ad particles were observed near the gel in tumor tissue treated with DWP418/alginate gel (Figure 6b).

Similarly, U343 tumors treated with naked DWP418 or DWP418/alginate gel were collected sequentially and sections were stained with hematoxylin and eosin, Ad E1A antigen-specific antibody and Ad hexon antigen-specific antibody (Figure 7a). Tumor tissue treated with DWP418/gel demonstrated a larger spread of Ad in tumor tissue at 15 and 24 days after

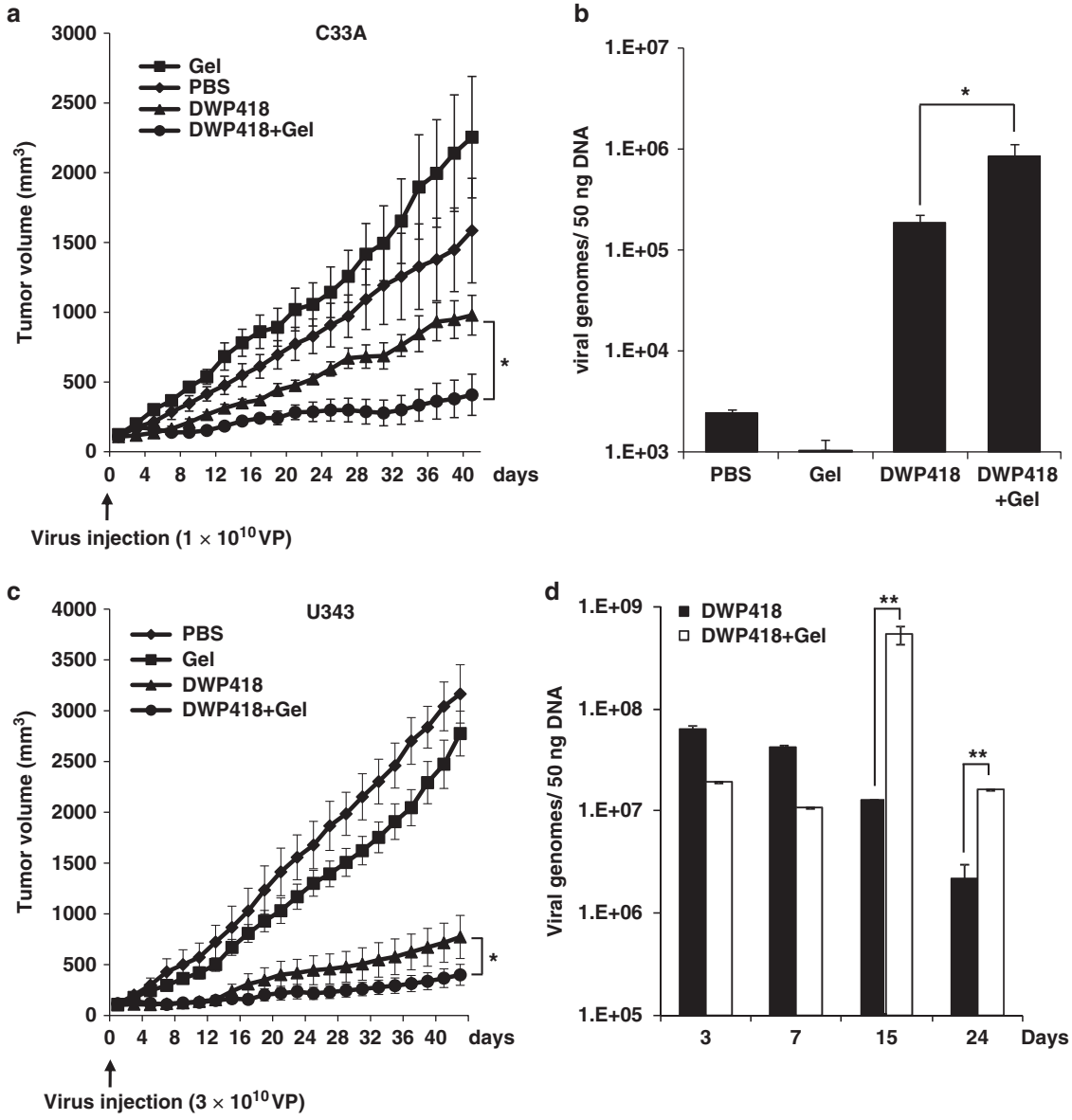

Figure 5. Antitumor efficacy of naked versus gelated Ads. (**a** and **c**) Graphs depicting days post-viral injection versus tumor volume (mm^3). C33A ($n = 6$ mice per each group) and U343 ($n = 8$) xenografts were established in immune-deficient nude mice. Once the tumor size grew to 100–120 mm^3, PBS, alginate gel, naked DWP418 or DWP418/gel was administered intratumorally. Data represent mean ± s.e.m. $^*P < 0.05$ versus naked DWP418-treated tumors. (**b** and **d**) Bar graphs depicting amount of viral genome present as determined by Q-PCR analysis. (**b**) C33A tumor tissues from mice treated with naked DWP418 or DWP418/gel were collected at day 22, and the number of viral genomes in tumor tissue was measured. (**d**) U343 tumor tissues were collected at 3, 7, 15 and 24 days after viral injection, and total number of viral genomes in tumor tissue over indicated time period was measured. Data represent mean ± s.e.m. from three mice in each group. $^{**}P < 0.01$ versus naked DWP418-treated tumors.

treatment, compared with tumors treated with naked DWP418. These results indicate that the Ad in gel was preserved for an extended time, which is consistent with the *in vitro* results. A magnified image (× 200) of Ad E1A antigen-specific antibody staining is shown in Figure 7b, for which tumor tissue was collected at 24 days to observe Ad and the neighboring gel in detail. This image shows that Ad particles were dispersed near the alginate gel. Dark staining of Ad E1A antigen in tumor tissue indicates that oncolytic Ad actively replicated in cancer cells. Taken together, these results demonstrate that the sustained release of viable oncolytic Ad from the alginate gel amplifies the therapeutic effect of oncolytic Ad administered locally.

Biodistribution of alginate gel-encapsulated oncolytic Ad in tumor-bearing mice. To evaluate the benefits of gelated Ad system for efficient *in vivo* shielding and preferential accumulation in the tumor, *in vivo* biodistribution study was carried out. When U343 tumor volume reached ~100–150 mm^3 in size, tumor-bearing mice were injected intratumorally with 3×10^{10} VP of naked DWP418 or DWP418/alginate gel in 50 μl of volume. The level of accumulated viral genome in normal tissues such as the heart, kidney, lung, stomach, liver, spleen and blood were lower in mice treated with DW418/alginate gel in comparison with those treated with naked DWP418 ($^{**}P < 0.01$, $^*P < 0.05$). In particular, the level of viral genome was significantly reduced by 56-fold for the kidney, 21-fold for the stomach and 12.8-fold for the spleen,

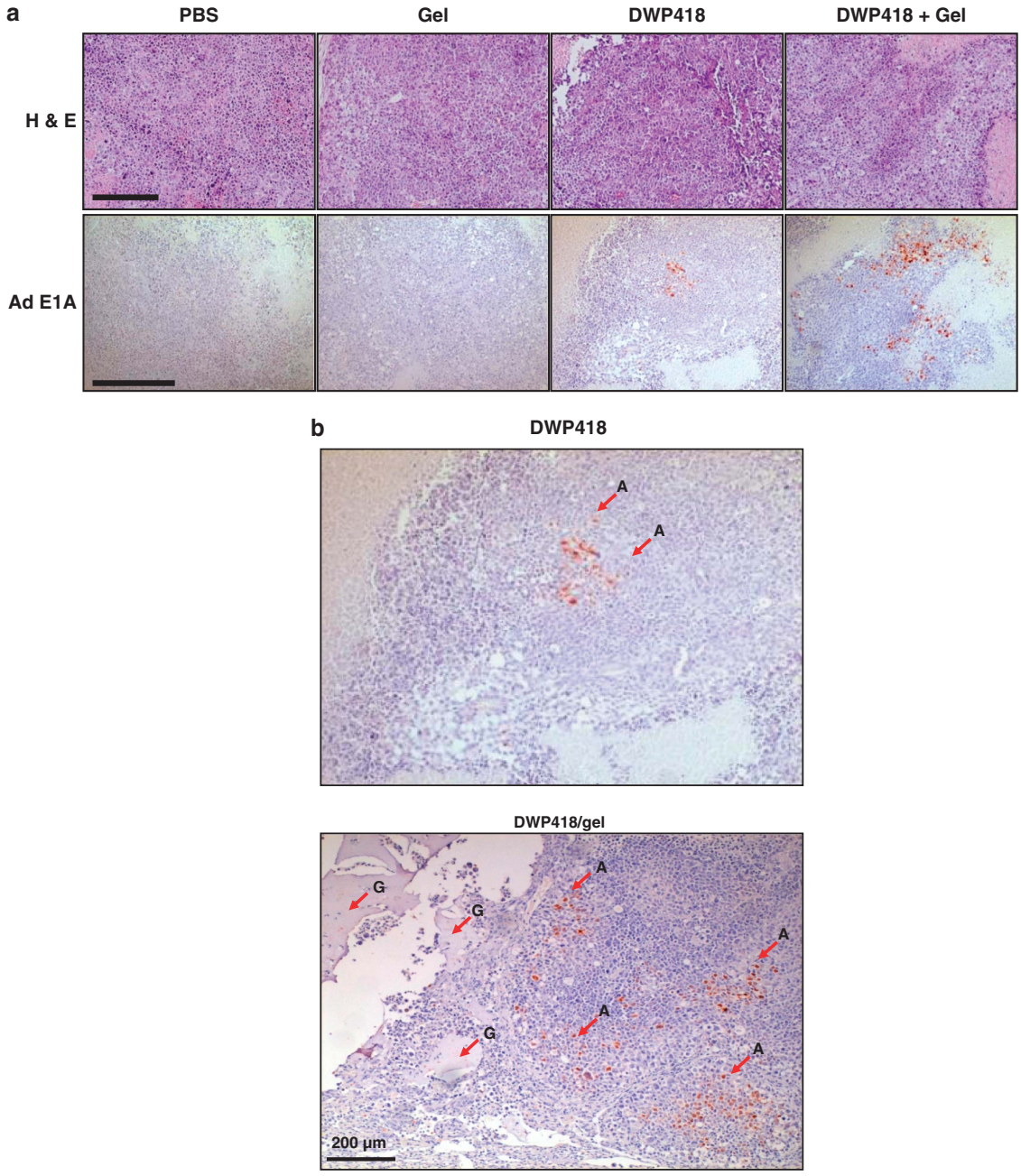

Figure 6. Histological and immunohistochemical assessment of tumor tissues treated with naked DWP418 or DWP418/gel. (**a**) Light micrographs depicting tumor tissues stained with hematoxylin and eosin (H&E; top row) or Ad E1A-specific antibody (bottom row). C33A tumor tissues were collected at 22 days post viral treatment before staining. Treatment with DWP418/gel induced more extensive tumor necrosis and larger distribution of Ad particles compared with naked DWP418 treatment. Original magnification: H&E, × 200; Ad E1A, × 100. (**b**) Light micrograph of Ad E1A-specific antibody immunohistochemical staining. Local necrosis and accumulated Ad particles were present in the tumor tissue treated with DWP418 (upper). Extensive necrosis and widespread Ad particles were present near the gel in the tumor tissue treated with DWP418/gel (bottom). The red arrows indicate the following: G, gel; A, adenovirus (× 100).

compared with the naked DW418-treated group (Figure 8). In addition, DWP418/alginate gel showed a 13-fold decrease in the blood compared with DWP418. In marked contrast, the amount of Ad genome accumulation in the tumor tissues treated with DWP418/alginate gel was 2.28-fold higher than that of naked DWP418 ($^*P < 0.05$). These results suggest that alginate gel system not only has a role as depot system for sustained release at local tumor site, but also minimize non-specific accumulation of Ad in normal tissues.

Evaluation on potential toxicity from systemic leakage of alginate encapsulated oncolytic Ad. To assess the potential toxicity caused by systemic leakage of the oncolytic Ad and Ad/gel, we analyzed complete blood count test and serum chemistry profile in U343 tumor-bearing mice at 72 h after intratumoral injection of PBS, gel only, DWP418 or DWP418-loaded alginate gel. In Table 1, the DWP418-administered mice at a dose of 3×10^{10} VP showed elevated alanine transferase and aspartate transferase levels than PBS-treated control ($P < 0.05$). In marked contrast, both alanine

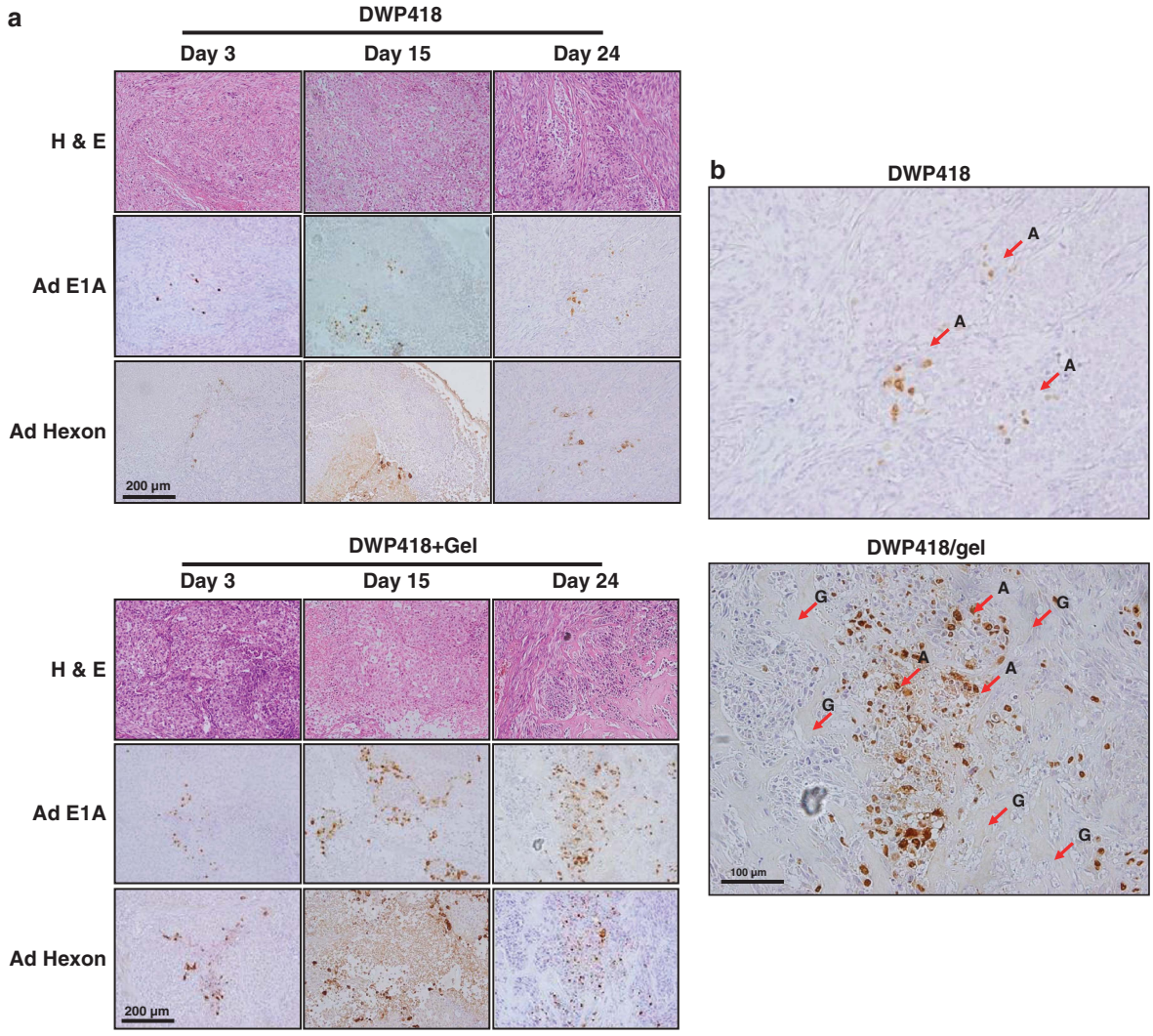

Figure 7. Histological and immunohistochemical analysis of tumor tissues collected at various time points. (**a**) Representative light micrographs of hematoxylin and eosin and immunohistochemical of tumor tissues incubated with various Ad preparations over time. U343 tumors were injected with either naked DWP418 or DWP418/gel, and tumor tissues were collected at 3, 15 and 24 days post viral injection. As shown in the representative photograph of Ad E1A- or Ad hexon-stained tissues, increased distribution of Ad particles in the tumors treated with DWP418/gel was observed on days 15 and 24. Original magnification: $\times$ 200. (**b**) Magnified image of tumors treated with DWP418 and DWP418/gel depicting area near alginate gel ($\times$ 200). The red arrows indicate the following: G, gel; A, adenovirus.

transferase and aspartate transferase levels were not significantly increased for the DWP418-loaded alginate gel-injected mice in relation to PBS-treated control mice ($P > 0.05$), suggesting that alginate gel prevent viral dissemination into blood circulation, resulting in less liver toxicity. Likewise, blood urea nitrogen, creatinine, T-bilirubin for kidney function, and blood chemistry and cell count was not affected for DWP418-loaded alginate gel-injected mice.

DISCUSSION

Local delivery of Ad by direct intratumoral injection into exterior primary tumors is a promising antitumor therapy. However, low therapeutic efficacy has led to treatment with high Ad concentrations and repetitive administration to elicit the desired antitumor effect. Regional administration of a high concentration of Ad in tumor tissues can cause a potent innate immune response and rapidly disseminate into neighboring normal cells, thus impairing the full potential therapeutic efficacy and overall safety of the injected Ad.[48,49] To develop effective virotherapy, an advanced delivery system is needed that provides sustained Ad infection of tumor cells and protects the virus from rapid clearance. This system would also enable the use of a lower dose that would avoid toxicity. We have tested the efficacy of one such delivery system in this study, and have demonstrated that the virus can be loaded in sodium alginate-based hydrogel and delivered by local injection at the target disease site. Our results showed that local injection of alginate gel loaded with Ad maintained the biological activity of this virus for an extended period of time and released Ad in a sustained manner, allowing for effective local virotherapy.

Encapsulating Ad in alginate gel resulted in sustained Ad biological activity and release of the Ad into the tumor tissue. The microenvironment around the gel-encapsulated Ad may provide protection from clearance by innate immune systems such as macrophages, dendritic cells and natural killer cells that have infiltrated into the tumor tissue, resulting in sustained Ad activity over an extended time period.[50–52] Quantitative assessment by Q–PCR analysis of accumulated Ad released from gel demonstrated that the Ad was gradually released from gel in a sustained manner over 24 days, suggesting that alginate gel

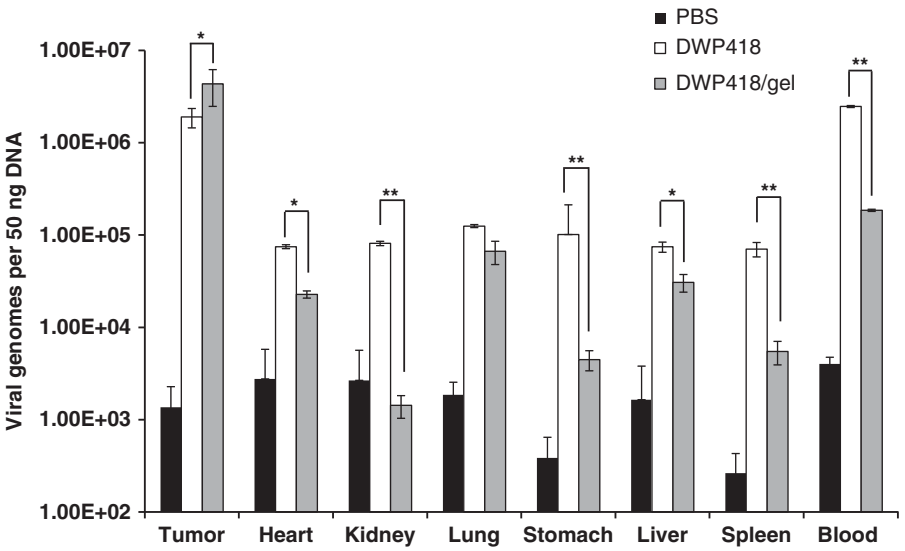

Figure 8. Biodistribution of alginated gel encapsulated oncolytic Ad in tumor-bearing mice. U343 tumor-bearing mice were intratumorally injected with 3×10^{10} VP of naked DWP418 or DWP418/gel, along with PBS as control. The tumor, heart, kidney, lung, stomach, spleen and liver were collected at 24 h after injection, and blood samples were collected at 5 min after injection. Real-time Q-PCR was then performed to detect Ad genomes. Data are expressed as copy number of Ad E1A gene. Data represent means ± s.e. and $n = 3$ for each experimental condition. $^*P < 0.05$, $^{**}P < 0.01$ versus DWP418 treated mice.

Table 1. Evaluation of the toxicological profile after administration of PBS, DWP418 or DWP418/gel

Description	*Test (unit)*	*PBS*	*DWP418*	*DWP418/gel*
Liver	AST ($U\,l^{-1}$)	100 ± 35	244 ± 67	153 ± 12
	ALT ($U\,l^{-1}$)	25 ± 1.1	76 ± 36	31 ± 6.3
	T-bilirubin ($mg\,dl^{-1}$)	< 0.1	< 0.1	< 0.1
Kidney	BUN ($mg\,dl^{-1}$)	24 ± 1	27 ± 6.5	23 ± 1
	CREA ($mg\,dl^{-1}$)	0.25 ± 0.023	0.335 ± 0.08	0.25 ± 0.04
CBC	RBC ($mEq\,l^{-1}$)	7.7 ± 0.6	8.4 ± 1	7.6 ± 1.4
	Hemoglobin ($mg\,dl^{-1}$)	11.85 ± 1	12.8 ± 1.8	12.6 ± 0.96
	Hematocrit (%)	38.4 ± 2.3	39.5 ± 4.4	35.9 ± 7.4
	MCV (fl)	49.7 ± 1	46.7 ± 0.73	47.2 ± 1.5
	MCH (pg)	15.3 ± 0.21	15.2 ± 0.2	16.9 ± 2.4
	MCHC (%)	30.8 ± 1	32.5 ± 0.95	32.6 ± 6.3

Abbreviations: ALT, alanine transferase; AST, aspartate transferase; BUN, blood urea nitrogen; CBC, complete blood cell count; CREA, creatinine; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PBS, phosphate-buffered saline; RBC, red blood cell count; VP, viral particle. U343 tumor-bearing mice were injected with 3×10^{10} VP of either DWP418 or DWP418/gel, along with PBS as a control. Blood was sampled on day 3, and AST, ALT, T-bilirubin, CREA, BUN, RBC count, hemoglobin, hematocrit, MCV, MCH and MCHC were determined for each mouse.

functions as a depot system for Ad preservation (Figure 5). Moreover, the level of GFP expression in the cells treated with gel-released Ad was also prolonged compared with that of naked Ad (Figure 3a), indicating that the biological activity of Ad released from alginate gel was preserved. On the other hand, the loss of naked Ad's activity continued to decrease, losing over 93% of ability to express GFP at 7 days. Ad protein structure depends on hydrogen bonds, hydrophobic forces and electrostatic interactions similar to most proteins, to maintain the secondary and tertiary structures of the structural components. These non-covalent interactions are sensitive to changes in pH, temperature and composition of the aqueous environment. In accordance with our results, previous reports have shown that incubation of naked Ad at various temperatures resulted in a time-dependent decrease in the virus infectivity.[25,53] Taken together, these results suggest that alginate gel is a functional delivery system for the sustained release of Ad and provides a protective matrix for the Ad, making alginate gel a feasible carrier for local injectable virotherapy.

Comparing the antitumor activity of oncolytic Ad encapsulated in alginate gel with that of the naked oncolytic Ad supports the usefulness of this system; treatment with DWP418/alginate gel had 1.9- (U343) and 2.4-fold (C33A) enhanced antitumor activity compared with that of naked Ad treatment (Figures 5a and c). These *in vivo* results are consistent with the results of the GFP expression study using replication-incompetent Ad expressing GFP (Figure 3), indicating that alginate gel can be used as a local delivery system for Ad with both replication-incompetent Ad expressing transgenes and replication-competent oncolytic Ad. Furthermore, histological analysis confirmed that DWP418/gel treatment resulted in dissemination of oncolytic Ad in a broader area of the tumor bed and with a higher density compared with that of naked DWP418 treatment. Importantly, the accumulation and spreading of Ad through the tumor tissue was sustained over time (Figures 5d and 7). These synergistic effects are likely achieved by the sustained release of Ad, preserved biological activity and propagation of Ad progenies by viral replication of released oncolytic Ad.

The results of this study support the significance of local Ad delivery using a biocompatible alginate gel. Importantly, the activity of gelated Ad was maintained over an extended period of time, maximizing the full potential of Ad-mediated cancer gene therapy. Collectively, these data show that encapsulating Ad in

alginate gel may provide an effective delivery system for the sustained release of Ad, enhancing the therapeutic effects of cancer treatment. In addition, these results may lead to the development of a novel therapeutic gene delivery system for cancer virotherapy.

CONCLUSION

We successfully combined biocompatible alginate gel and Ad to create a delivery system that maximized the full therapeutic potential of oncolytic Ad. The biological activity of Ad loaded in alginate gel was preserved for an extended period of time, as evidenced by the high GFP gene transduction efficiency over a prolonged time period compared with naked Ad. In addition, together with preserved biological activity, sustained release of oncolytic Ad from the locally administered alginate gel resulted in the continued delivery of Ad in tumor tissue, ultimately leading to enhanced antitumor therapeutic efficacy. To our knowledge, this is the first study to demonstrate the efficacy of a locally injectable delivery system for oncolytic Ad, demonstrating that the microenvironment in a matrix-based delivery system for Ad allows sustained Ad release and biological activity, thus enhancing the gene transduction efficacy as well as the antitumor therapeutic effect. The use of this Ad delivery system could help to maximize the therapeutic effects of oncolytic Ad.

MATERIALS AND METHODS

Cell lines and cell culture

All cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 1% penicillin–streptomycin (100 U ml^{-1}). Human embryonic kidney cell line expressing the Ad E1 region (HEK293), liver cancer cell line (SK-Hep1), non-small lung cancer line (A549), brain cancer cell line (U343) and cervical cancer cell line (C33A) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2.

Preparation of Ads

A replication-incompetent Ad expressing GFP (dE1/GFP) and an oncolytic Ad, DWP418 [2,22], were used in this study. DWP418 expresses the E1A gene under the control of modified hTERT promoter for cancer cell-specific viral replication and expresses the relaxin gene for efficient viral spreading throughout tumor tissues. All Ads were propagated in HEK293 cells, followed by CsCl (Sigma, St Louis, MO, USA) density purification. VP number was calculated from measurements of optical density at 260 nm (OD_{260}), for which an absorbance value of 1 is equivalent to 10^{12} VP per ml.

Preparation of alginate gel

Alginate (alginic acid sodium salt, Sigma) solution of 5 wt.% was prepared with 0.5 g of alginate and 0.09 g of NaCl (Sigma) in 10 ml of PBS. The alginate solution was stirred for 24 h at room temperature, and then gelated at various concentrations of $CaCl_2$ (Sigma), ranging from 20 to 200 mM, to determine the optimal condition for Ad transduction.

Preparation of Ad-loaded alginate gel and quantitative release study

Ad (1×10^{10} VP; 2 μl of 5×10^{12} VP per ml) was mixed with 5% sodium alginate/PBS solution (248 μl). The 50 mM $CaCl_2$ solution was prepared in PBS (volume 250 μl). The Ad/alginate mixture and $CaCl_2$ (250 μl each) were loaded into a 1-ml KOVAX-syringe (Korea Vaccine Co., Seoul, Korea). The Ad/alginate mixture and $CaCl_2$ solution in the syringe were co-injected to form a gel in the 24-well plate. After allowing gelation for 20 min, 1 ml of PBS was added over the gel. For non-gelated naked Ad control, Ad (1×10^{10} VP) was dispersed in 1 ml of PBS. After 1, 3, 5, 7, 9 and 11 days, Ad released from the gel was obtained from the supernatant in the gel-containing wells. To retrieve Ad trapped in the gel, alginate gel was dissolved by the addition of 500 μl of gel dissolution solution (sodium citrate 55 mM, sodium chloride 0.15 M, EDTA 30 mM, pH 6.8). Naked Ad in PBS was also sampled at the same time period.

For real-time Q–PCR analysis of Ad, each Ad sample was diluted 1- to 1000-fold, and 5 μl of each sample was analyzed by Q–PCR (TaqMan PCR detection; Applied Biosystems, Foster City, CA, USA). A fluorescence probe (FAM-5′-CCGCCGCTTCAGCC-3′-NFQ) was designed to anneal the sense primer (5′-GGAACGCCGTTGGAGACT-3′) and the antisense primer (5′-GGAAAGCAAAGTCAGTCACAATCC-3′) in the IX protein region of the Ad. Each sample was amplified for 40 cycles in an ABI 7500 sequence detection system (Applied Biosystems). The quantitation data, in terms of the crossing point value (C_t; which is expressed as the fractional cycle number and is the intersection of the log-linear fluorescent curve with threshold crossing line), were determined using the second derivative method of the ABi 7500 software package (Applied Biosystems). A reference dye, ROX, was included in all assays to normalize data for non-PCR-related signal variation. All experiments were performed in triplicate, and data were analyzed by the 7500 software package. The quantitative Ad release over time was presented as the number of viral genome.

Cellular uptake of released Ad from the gel

Cellular transduction efficiency of naked Ad, Ad released from the gel and Ad trapped in gel were measured by GFP expression. Ad-encapsulated alginate gel was prepared by co-injection of 1-ml KOVAX-syringe loaded with 250 μl of Ad (1×10^{10} VP)/5% alginate mixture and 250 μl of 50 mM $CaCl_2$. Ad/alginate gel mixture was then incubated in 2 ml of cell culture media at room temperature or 37 °C. The media containing released Ad (2 ml) was collected at 1, 3, 5, 7, 9 and 11 days. After removal of media, gel was dissolved by adding 400 μl of gel dissolution solution. At 10 min following addition of gel dissolution solution, 2 ml of cell culture media was added to gel dissolution solution-treated gel. Naked Ad, released Ad from the gel, and gel-encapsulated Ad were then used to transduce SK-Hep1 cells that were seeded 24 h before transduction at a density of 1×10^5 cells in a six-well plate. After 12 h of culture at 37 °C, Ad-containing media were replaced with 3 ml of fresh cell culture media. At 72 h post transduction, GFP-expressing cells were identified by fluorescence microscopy (Olympus BX51; Olympus Optical, Tokyo, Japan).

Fluorescence-activated cell-sorting analysis

GFP expression was measured by flow cytometry (LSR2, Becton Dickinson, San Jose, CA, USA) in the SK-Hep1 cells transduced with naked Ad, Ad released from gel or gel-encapsulated Ad from various time periods (1, 3, 5, 7, 9 or 11 days). At 72 h after transduction with 1×10^5 VP of each Ad preparation, cells were detached with the cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) and washed with PBS, and 400 μL of PBS was added for flow cytometric analysis with FACScan (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson).

MTT assay

The cancer cell-killing effect of oncolytic Ad was determined by measuring the conversion of the tetrazolium salt MTT to formazan as a function of time. Naked Ad or Ad released from alginate gel was sampled after incubation for 1, 3, 5, 7 or 9 days at 37 °C. U343 cells (4×10^4) were seeded to ~40–60% confluence in a 24-well plate for 24 h. Cells were then treated with naked or gel-released DWP418 oncolytic Ad (3×10^2 VP or 5×10^2 VP) for 2 days at 37 °C. MTT in PBS (250 μl; 2 mg ml^{-1}) was then added to each well for 4 h at 37 °C. The supernatant was then discarded, and the precipitate was dissolved in 1 ml of dimethylsulfoxide. Plates were then read on a microplate reader at 540 nm.

Characterization of sustained release of Ad *in vivo*

The sustained release of oncolytic Ad from alginate gel and resulting enhanced antitumor effect was evaluated in xenograft tumors established from C33A (cervical cancer cell line) and U343 (brain cancer cell line). C33A or U343 cells (1×10^7) were inoculated subcutaneously into the abdomen of 6- to 8-week-old female and male nude mice, respectively (Orientbio Inc., Kyunggi-do, Korea). Tumor growth was monitored, and when tumors had grown to ~100 mm^3 in volume, mice were randomly assigned to one of the following four groups: (1) DWP418-loaded alginate gel (1×10^{10} VP for C33A and 3×10^{10} VP for U343) in 25 μl of DWP418 in alginate solution (alginic acid sodium salt, from brown algae; Sigma) and 25 μl of $CaCl_2$, totally 50 μl; (2) naked DWP418 at equivalent viral doses in 50 μl of PBS; (3) PBS; and (4) alginate gel alone used as negative controls. When the size of tumor reached 150 mm^3, samples were injected directly into tumors at a single time with insulin syringe (needle gauge: 31). Every other day, tumor

growth was assessed by measuring tumor volume ($v = Lw^2 \times 0.523$). Tumors of each group were collected at 3, 7, 15 and 24 days after naked DWP418 or alginate/DWP418 injection and whole tumors were homogenized for Q–PCR analysis or sliced for histological and immunohistochemical evaluation.

Histological and immunohistochemical analysis

U343 tumor xenografts were collected on days 3, 15 and 24 after intratumoral injection of naked DWP418 or DWP418/alginate gel mixture for histological and immunohistochemical analysis. C33A tumor xenografts were collected on day 22 for histological and immunohistochemical analysis. Collected tumor tissues were fixed in 10% formalin, processed for paraffin embedment, and cut into 5-μm sections. Sections were stained with hematoxylin (nucleus) and eosin (cytoplasm) and examined by light microscopy at $\times 200$ magnification. To detect Ad particles, paraffin-embedded tumor tissues were incubated in xylene for 10 min, and then incubated sequentially with 100%, 90% and 70% ethanol for 5 min each. Deparaffinized tissues were blocked with 3% bovine serum albumin for 2 h at room temperature, and Ad E1A protein and Ad hexon protein were probed with Ad-2/5 E1A-specific antibody (SC-430; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ad hexon protein antibody (AB1056; Chemicon, Billerica, MA, USA). Sections were counterstained with Mayer's hematoxylin.

Ad biodistribution assessment by real-time Q–PCR

When U343 tumor volume had reached $\sim$100–150 mm^3 in size, tumor-bearing mice were injected intratumorally with 3×10^{10} VP of naked DWP418 or DWP418/alginate gel, along with PBS as control. The tumor, heart, kidney, lung, stomach, spleen and liver were collected at 24 h after injection, and blood samples were collected at 5 min after injection. DNA was then extracted from the tissues and blood using the QIAamp DNA blood mini kit (Germany) according to the manufacturer's instructions. The number of viral genomes in each sample was assessed by real-time Q–PCR, as described above.

Toxicology studies

For blood analysis, U343 tumor xenograft mice received a single intratumoral injection of 3×10^{10} VPs of DWP418 or DWP418/alginate gel. After 72 h, blood samples were obtained by cardiac puncture and assayed for the serum levels of aspartate transferase, alanine transferase, T-bilirubin for liver function, and blood urea nitrogen and creatinine for kidney function. In addition, red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, were further evaluated.

Statistical analysis

Data are expressed as mean ± s.e.m. Data were compared with the Mann–Whitney test (non-parametric method) using Stat View software (Abacus Concepts, Inc., Berkeley, CA, USA). Survival was assessed with the Kaplan–Meier method, and results were compared with the log-rank test (SPSS software, version 13.0, SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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