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Tumor Vascular Targeted Delivery of Polymer-conjugated Adenovirus Vector for Cancer Gene Therapy

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Previously, we generated a cancer-specific gene therapy system using adenovirus vectors (Adv) conjugated to polyethylene glycol (Adv-PEG). Here, we developed a novel Adv that targets both tumor tissues and tumor vasculatures after systemic administration by conjugating CGKRK tumor vasculature homing peptide to the end of a 20-kDa PEG chain (Adv-PEG_{CGKRK}). In a primary tumor model, systemic administration of Adv-PEG_{CGKRK} resulted in ~500- and 100-fold higher transgene expression in tumor than that of unmodified Adv and Adv-PEG, respectively. In contrast, the transgene expression of Adv-PEG_{CGKRK} in liver was about 400-fold lower than that of unmodified Adv, and was almost the same as that of Adv-PEG. We also demonstrated that transgene expression with Adv-PEG_{CGKRK} was enhanced in tumor vessels. Systemic administration of Adv-PEG_{CGKRK} expressing the herpes simplex virus thymidine kinase (*HSVtk*) gene (Adv-PEG_{CGKRK}-HSVtk) showed superior antitumor effects against primary tumors and metastases with negligible side effects by both direct cytotoxic effects and inhibition of tumor angiogenesis. These results indicate that Adv-PEG_{CGKRK} has potential as a prototype Adv with suitable efficacy and safety for systemic cancer gene therapy against both primary tumors and metastases.

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INTRODUCTION

The adenovirus vector (Adv) is the most used vectors in cancer gene therapy.[1,2] In several clinical trials, intratumoral injection of Adv resulted in therapeutic efficacy against primary tumors.[3–6] However, it is difficult to treat metastases with Adv because of low accumulation and poor transgene expression in tumors after systemic administration.[7,8] Moreover, systemic administration of Adv leads to accumulation and transgene expression in the liver, which may cause considerable toxicity.[9] For this reason, clinical application of systemically administered Adv as gene therapeutic vectors has been limited.

Covalent conjugation of polyethylene glycol (PEG) to the Adv surface, called "PEGylation," is a promising strategy to overcome the limitations of Adv.[2,10–14] PEGylation can prolong the plasma half-life, and alter the tissue distribution of the conjugates compared with the native form.[15,16] The extended circulating lifetime in blood induces the enhanced permeability and retention effect, which is based on the leaky nature of the tumor blood vessels, resulting in increased delivery of the conjugates to tumor tissue.[17] Previously, we showed that systemic administration of Adv PEGylated with 20-kDa PEG at a 45% modification ratio (Adv-PEG) resulted in higher tumor-selective transgene expression than unmodified Adv.[14] In addition, we showed that Adv-PEG, expressing the herpes simplex virus thymidine kinase (*HSVtk*) gene as the therapeutic gene, induced strong antitumor effects against metastases after systemic administration.[14]

Attaching targeting ligands to the ends of PEG chains is considered as a promising approach for achieving tissue-specific gene transfer using PEGylated Adv.[18–25] However, there are no reports that demonstrate efficient tumor-specific transgene expression and anticancer efficacy against primary and metastatic cancer through systemic administration of PEGylated Adv-containing targeting ligands *in vivo*.

Tumor angiogenesis is widely recognized to be crucial for the progression and metastasis of tumors.[26,27] Angiogenesis and tumor vasculature have received increased attention as targets for potential anticancer therapies. For example, *in vivo* screening of peptide-phage libraries has proven useful for the discovery of novel peptide ligands that selectively home to tumor vessels.[28,29] One such ligand, the CGKRK peptide, accumulates both on the surface of tumor vessels and within tumor tissues after intravenous injection, suggesting that CGKRK peptide has potential as an active targeting ligand.[30,31]

We here report a novel vector system that can be systemically administered and that targets both tumor tissues and tumor

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vasculatures. We constructed a targeting Adv that has the CGKRK peptide at the end of the 20-kDa PEG chain conjugated with Adv (Adv-PEG$_{\text{CGKRK}}$).

RESULTS

Construction of Adv-PEG$_{\text{CGKRK}}$

To conjugate the CGKRK peptide with Adv-PEG, we used a heterobifunctional-activated PEG with NHS and maleimide. We constructed Adv-PEG by using 50-fold molar heterobifunctional-activated PEG and 150-fold molar monofunctional-activated PEG for the modification of adenoviral lysine residues. The modification ratio of Adv by PEG was ~45% in all experiments, which was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described (data not shown).[14] In addition, fluorescamine assay[32] also showed that ~45% of free amines on capsid of Advs were modified by PEG (data not shown). To construct Adv-PEG$_{\text{CGKRK}}$, cysteine of the CGKRK peptide was reacted with maleimide of the heterobifunctional PEG chain conjugated with Adv-PEG, resulting in attachment of the CGKRK peptide to the end of the PEG chain. We could not measure the number of CGKRK peptide on Adv-PEG$_{\text{CGKRK}}$.

Transgene expression of Adv-PEG$_{\text{CGKRK}}$

It has been reported that CGKRK peptides bind to and accumulate within certain tumor cells such as MDA-MB-435S and tumor endothelial cells *in vitro* and *in vivo*.[30] We selected MDA-MB-435S cells for *in vitro* evaluation of transgene expression of Adv-PEG$_{\text{CGKRK}}$ encoding the luciferase gene. B16BL6 cells were selected for *in vitro* and *in vivo* evaluation of transgene expression of Adv-PEG$_{\text{CGKRK}}$ because it is known that sufficient gene expression by Adv is difficult to obtain in these cells[33] due to a lack of Ad primary receptor expression. Luciferase expression from Adv-PEG was lower than that of unmodified Adv in MDA-MB-435S cells (**Figure 1a**) and B16BL6 cells (**Figure 1b**). In contrast, luciferase expression from Adv-PEG$_{\text{CGKRK}}$ was 7-fold and 20-fold higher than the expression from Adv-PEG in MDA-MB-435S and B16BL6 cells, respectively (**Figure 1a,b**). Next, we evaluated *in vivo* transgene expression of Adv-PEG$_{\text{CGKRK}}$ in tumor and liver tissue after intravenous injection into B16BL6 primary tumor model mice (**Figure 1c**). Luciferase expression from Adv-PEG in tumor tissue was fourfold higher than that from unmodified Adv and in liver tissue >600-fold lower than that from unmodified Adv (**Figure 1c**). Furthermore, luciferase expression in tumor tissue with administration of Adv-PEG$_{\text{CGKRK}}$ was 480-fold and 110-fold higher than those with administration of unmodified Adv and Adv-PEG, respectively (**Figure 1c**). In liver tissue, luciferase expression after Adv-PEG$_{\text{CGKRK}}$ administration was 370-fold less than that after unmodified Adv administration, and was almost the same as that after Adv-PEG administration (**Figure 1c**). In addition, we examined the dose dependency of *in vivo* transgene expression of each Adv (**Figure 1d**). In tumor tissue, luciferase expression at 1×10^{10} virus particles (vp) and 2×10^{9} vp Adv-PEG$_{\text{CGKRK}}$ was 100-fold and 20-fold, respectively, higher than that with unmodified Adv at the same doses (**Figure 1d**). Furthermore, luciferase expression of Adv-PEG$_{\text{CGKRK}}$ at 1×10^{10} vp was the same as that of Adv-PEG at 5×10^{10} vp, and ninefold higher than that of unmodified Adv at 5×10^{10} vp (**Figure 1d**). These results indicate that administration of Adv-PEG$_{\text{CGKRK}}$ results in significant tumor-specific transgene expression compared with Adv-PEG and unmodified Adv.

Next, we evaluated the transgene expression of Adv-PEG$_{\text{CGKRK}}$ at 5×10^{10} vp in an established pulmonary metastasis mouse model (**Figure 1e**).[34] In normal mice, the luciferase expression in lung of both Adv-PEG and Adv-PEG$_{\text{CGKRK}}$ were several-fold lower than that of unmodified Adv (**Figure 1e**). In contrast, in B16BL6-metastatic mice, luciferase expression in lung with Adv-PEG$_{\text{CGKRK}}$ was 27-fold and 12-fold higher than those with unmodified Adv and Adv-PEG, respectively (**Figure 1e**). These results suggest that Adv-PEG$_{\text{CGKRK}}$ not only reduces expression in liver, but also that it actively targets both primary tumor and metastases.

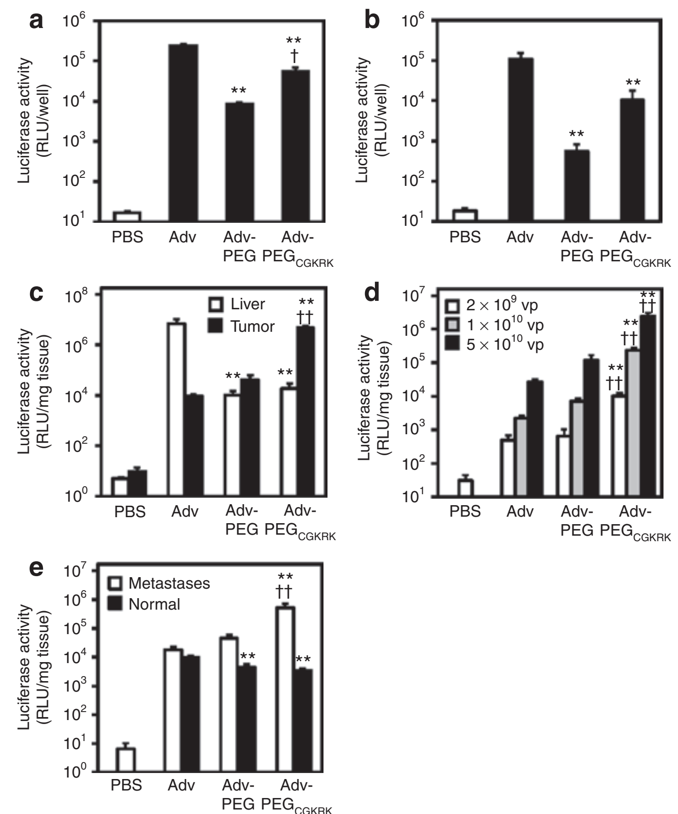

Figure 1 Transgene expression of Adv-PEG$_{\text{CGKRK}}$. (**a**) MDA-MB-435S cells or (**b**) B16BL6 cells (2×10^4 cells/well) were transduced with the indicated adenovirus vectors (Advs). After culturing for 24 hours, luciferase expression was measured ($n = 3$). (**c**) *In vivo* transgene expression in a primary tumor model. B16BL6-bearing mice were intravenously administered with 5×10^{10} virus particles (vp) of the indicated Advs. After 48 hours, the luciferase expression in tumors and livers was measured ($n = 4$). (**d**) Gene transduction at various doses in a primary tumor model. B16BL6-bearing mice were intravenously administered with several doses of the indicated Advs. After 48 hours, the luciferase expression in tumors was measured ($n = 4$). (**e**) *In vivo* transgene expression in a pulmonary metastasis model. B16BL6 cells were injected into C57BL6 mice *via* the tail vein. After 12 days, 5×10^{10} vp of the indicated Advs were intravenously injected into metastatic mice or normal mice. Forty-eight hours later, the luciferase expression in lungs was measured ($n = 5$). All data are represented as the means ± (**a**,**b**) SD or (**c**–**e**) SEM ($^{**}P < 0.01$ versus value for unmodified Adv-treated group by analysis of variance (ANOVA); $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus value for Adv-PEG-treated group by ANOVA). PEG, polyethylene glycol; RLU, relative light units.

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Blood clearance, tissue distribution, and localization of Adv-PEG$_{CGKRK}$

We assessed blood clearance by using normal C57BL6 mice (**Figure 2a**) and tissue distribution by using B16BL6-bearing mice (**Figure 2b**) of each Adv after intravenous administration into mice by using real-time quantitative PCR. The plasma level of Adv-PEG$_{CGKRK}$ was higher than the plasma level of unmodified Adv and was almost the same as that of Adv-PEG (**Figure 2a**). At 6 hours after intravenous administration into B16BL6-bearing mice, the amount of Adv-PEG$_{CGKRK}$ in tumor tissue was 60-fold and 3-fold higher than that of unmodified Adv and Adv-PEG, respectively (**Figure 2b**). Moreover, the amount of Adv-PEG$_{CGKRK}$ in liver tissue was 15-fold less than that of unmodified Adv, and almost the same as that of Adv-PEG (**Figure 2b**). These results indicate that systemic administration of Adv-PEG$_{CGKRK}$ enabled tumor targeting due to active targeting and the enhanced permeability and retention effect, and decreased distribution in the liver. Next, we investigated the localization of the transgene expression of Adv-PEG$_{CGKRK}$ in tumor tissues. B16Bl6-bearing mice were intravenously administered with 5 × 10^{10} vp of Advs encoding the green fluorescent protein (*GFP*) gene and we visualized the localization by using histological procedures (**Figure 3**). GFP expression could not be detected after intravenous administration of unmodified Adv or Adv-PEG (**Figure 3**). In contrast, intravenous administration of Adv-PEG$_{CGKRK}$ resulted in strong GFP expression in endothelial cells and colocalization with CD31$^+$ endothelial cells (**Figure 3**). These results indicate that transgene expression of Adv-PEG$_{CGKRK}$ is localized at tumor endothelial cells.

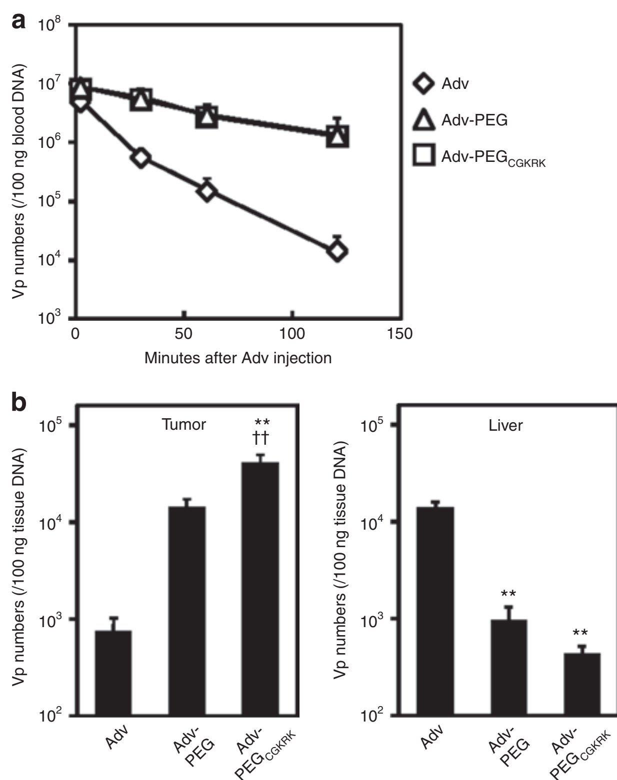

Figure 2 Blood clearance and tissue distribution of adenovirus vectors (Advs). (**a**) Circulation time of Adv-PEG$_{CGKRK}$. Normal C57BL6 mice were intravenously administered with 5 × 10^{10} virus particles (vp) of the indicated Advs. Blood samples were collected at 2, 30, 60, and 120 minutes after intravenous administration of each Adv. The total amount of DNA in the blood at various time points was determined using real-time quantitative PCR. (**b**) Tissue distribution of Adv-PEG$_{CGKRK}$. Once the tumor diameter was ~8 mm, B16BL6-bearing mice were intravenously administered with 5 × 10^{10} vp of the indicated Advs. Six hours after the administration, the number of viral genomes in tumors and livers was measured by real-time quantitative PCR. Data are presented as means ± SEM (n = 5; $^{**}P < 0.01$ versus value for unmodified Adv-treated group by analysis of variance (ANOVA); $^{††}P < 0.01$ versus value for Adv-PEG-treated group by ANOVA).

Therapeutic effect of Adv-PEG$_{CGKRK}$

To evaluate the therapeutic potential of Adv-PEG$_{CGKRK}$, we intravenously administered each Adv encoding the *HSVtk* gene to B16BL6-bearing mice (**Figure 4a**). Administration of 5 × 10^{10} vp unmodified Adv induced a marked reduction in body weight, and within 7 days of administration all treated mice had died from toxicity *via* HSVtk expression in the liver (data not shown). Administration of 1 × 10^{10} vp unmodified Adv did not inhibit tumor growth (**Figure 4a**). Administration of 5 × 10^{10} vp Adv-PEG reduced tumor volumes by 35% compared with that of phosphate-buffered saline (PBS) (**Figure 4a**). However, a dose of 1 × 10^{10} vp, Adv-PEG did not show any antitumor effects (**Figure 4a**). In contrast, Adv-PEG$_{CGKRK}$ showed strong antitumor effects with administration of 1 × 10^{10} and 2 × 10^9 vp Adv-PEG$_{CGKRK}$ reducing tumor volume by 48 and 42%, respectively (**Figure 4a**). Next, we evaluated the therapeutic effect of Adv-PEG$_{CGKRK}$ against metastases (**Figure 4b,c**). Consistent with the results of the primary tumor experiment, systemic administration of unmodified Adv lacked therapeutic efficacy (**Figure 4b,c**). Administration of 5 × 10^{10} vp Adv-PEG reduced the number of metastatic colonies, but 1 × 10^{10} vp Adv-PEG did not (**Figure 4b,c**). In contrast, compared with untreated mice, administration of 1 × 10^{10} and 2 × 10^9 vp Adv-PEG$_{CGKRK}$ significantly suppressed metastases in lung (**Figure 4b,c**). Furthermore, to evaluate the side effects of intravenous administration of Adv-PEG$_{CGKRK}$, we measured the blood levels of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) on day 7 after Advs administration (**Figure 4d**). Administration of 5 × 10^{10} vp Adv-PEG

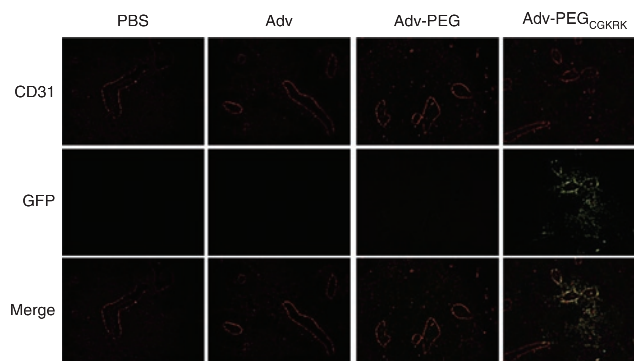

Figure 3 Tumor tissue and vascular localization of intravenously injected Adv-PEG$_{CGKRK}$. Once the tumor diameter was ~8 mm, B16BL6-bearing mice were intravenously administered with 5 × 10^{10} virus particles (vp) of the indicated adenovirus vectors- (Advs) expressing green fluorescent protein (GFP). The mice were sacrificed 2 days later and GFP expression was visualized in tumor tissue sections with fluorescein (green). Blood vessels were stained with rat anti-mouse CD31 (red; Alexa568). Original magnification: ×200.

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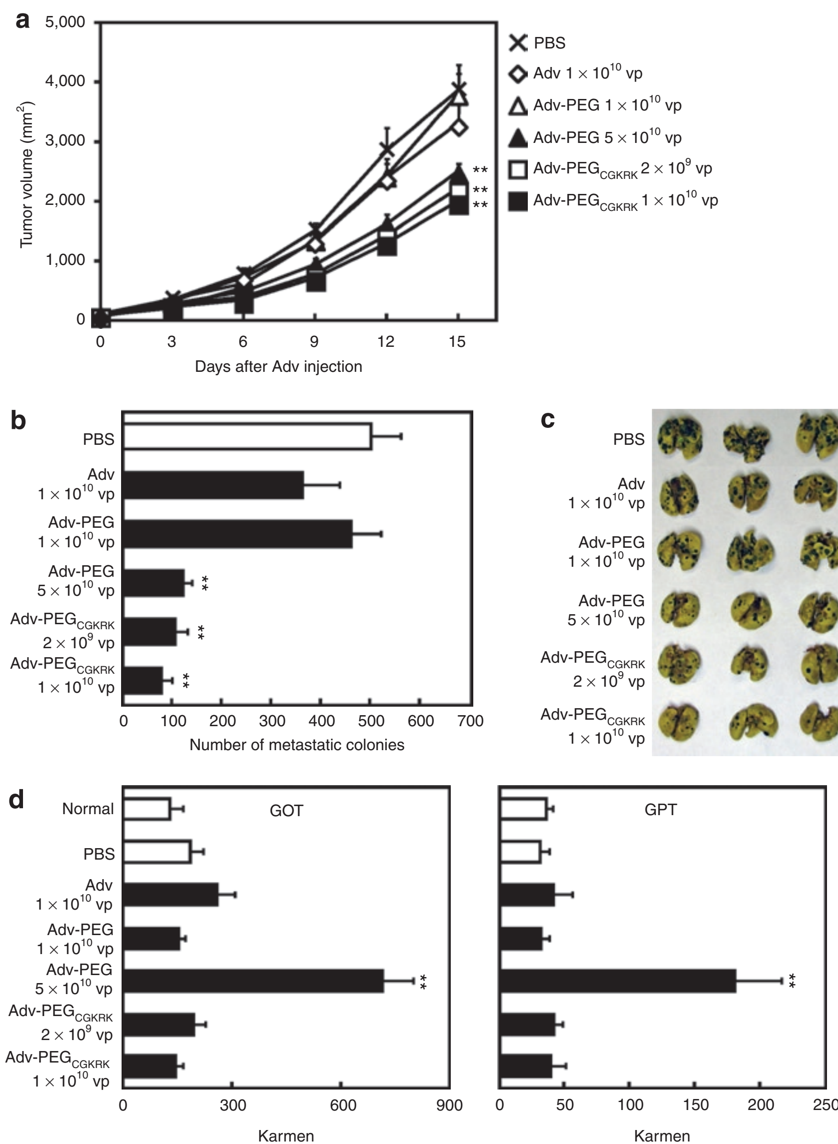

Figure 4 Therapeutic effect of Adv-PEG$_{CGKRK}$ against primary tumors and metastases. (**a**) Therapeutic effect of intravenously injected Adv-PEG$_{CGKRK}$ against primary tumors. Once the tumor diameter was ~8 mm, phosphate-buffered saline (PBS) or the indicated adenovirus vectors (Advs) encoding the herpes simplex virus thymidine kinase (*HSVtk*) gene was intravenously administered at the indicated doses to B16BL6-bearing mice. The mice then received daily intraperitoneal administrations of ganciclovir (GCV) (50 mg/kg) for 10 days. Tumor sizes were calculated every 3 days. (**b**,**c**) Therapeutic effect of intravenously injected Adv-PEG$_{CGKRK}$ against metastases. B16BL6 cells were injected intravenously into C57BL/6 mice *via* the tail vein. PBS or the indicated Advs encoding the *HSVtk* gene at the indicated doses was injected intravenously on day 7 after the B16BL6 administration. The mice then received daily intraperitoneal administrations of GCV (50 mg/kg) for 7 days. On day 14 after the B16BL6 administration, therapeutic effects were assessed by (**b**) counting the number of metastatic colonies in the lungs and by (**c**) examining photographs of the lungs. (**d**) Side effects were assessed using glutamic-oxaloacetic transaminase (GOT)/glutamic-pyruvic transaminase (GPT) activity in serum. Data are presented as means ± SEM [$n = 6$; $^{**}P < 0.01$ versus value for phosphate-buffered saline (PBS)-treated group by analysis of variance (ANOVA)].

induced a substantial elevation in the levels of GOT and GPT compared with that of normal mice (**Figure 4d**). In contrast, administration of 1×10^{10} and 2×10^9 vp Adv-PEG$_{CGKRK}$ did not induce any elevation of GOT or GPT levels (**Figure 4d**), although 5×10^{10} vp Adv-PEG$_{CGKRK}$ induced a elevation in the levels of GOT and GPT as similar as 5×10^{10} vp Adv-PEG (data not shown). Taken together, these data suggest that Adv-PEG did not induce the therapeutic effect without severe side effects and intravenous administration of Adv-PEG$_{CGKRK}$ could be therapeutically effective at therapeutic doses 5–25-fold lower than that of Adv-PEG.

Delayed wound healing by systemic administration of Adv-PEG$_{CGKRK}$

Angiogenesis is essential not only for tumor growth but also for wound repair.[35] To investigate whether the therapeutic effect of Adv-PEG$_{CGKRK}$ is related to damage of neovascular vessels, we examined the wound repair effects of Adv-PEG$_{CGKRK}$ after injury in mice. Wounds were created on the abdominal area of mice and 1×10^{10} vp of each Adv encoding the *HSVtk* gene were intravenously injected into the mice 1 day after injury (**Figure 5**). The wound was completely closed by day 9 after PBS administration

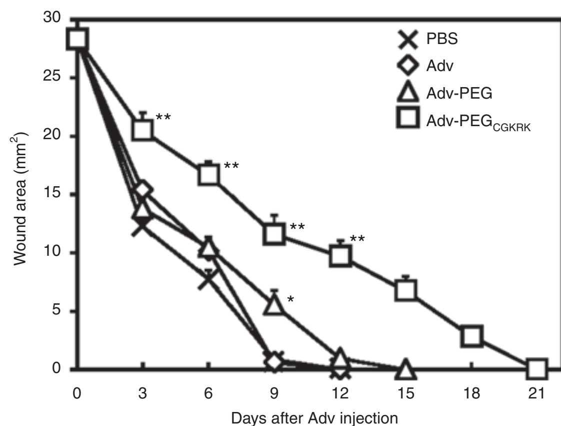

Figure 5 Inhibition of wound healing by Adv-PEG$_{CGKRK}$. Wounds of 6-mm diameter were created on the abdominal area of BALB/c mice and phosphate-buffered saline (PBS) or 1 × 10^{10} virus particles (vp) of the indicated adenovirus vectors (Advs) encoding the herpes simplex virus thymidine kinase (*HSVtk*) gene were intravenously injected into mice 1 day after injury. The mice then received daily intraperitoneal administrations of ganciclovir (GCV) (50 mg/kg) for 10 days. Data are represented as means ± SEM [$n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$ versus value for PBS-treated group by analysis of variance (ANOVA)].

(**Figure 5**). No obvious differences in the wound-healing process were observed in the mice injected with unmodified Adv or Adv-PEG compared with that of the mice injected with PBS (**Figure 5**). In contrast, the wound areas of the mice injected with Adv-PEG$_{CGKRK}$ were still recovering until day 21 after administration (**Figure 5**), indicating that the wound-healing process was markedly delayed by Adv-PEG$_{CGKRK}$. These findings suggest that intravenous administration of Adv-PEG$_{CGKRK}$ might destroy neovascular vessels, thereby producing a therapeutic synergy.

DISCUSSION

Various molecular weights of PEGs have been used in the PEGylation of Adv because PEGs with different molecular weights result in different body distribution patterns and retention times in blood.[11,12,36,37] Previously, we examined the correlation between PEG modification and gene expression patterns, and we showed that PEGylation with 20-kDa PEG at 45% modification ratio was optimal for tumor-selective gene expression after systemic administration.[14] Therefore, we used 20-kDa PEG at 45% in this study, with the CGKRK peptide attached to the end of the PEG chain. Although the modification ratio of the CGKRK peptide was not evaluated, the peptide was used in excess quantity. A quantitative analysis of the modification ratio is needed in a future study.

Compared with Adv-PEG, the transgene expression of Adv-PEG$_{CGKRK}$ was higher in tumor tissue and the same in liver tissue (**Figure 1c–e**). We found that after intravenous administration, Adv-PEG$_{CGKRK}$ was present in plasma at the same level as Adv-PEG, but accumulated to higher levels in tumor tissue (**Figure 2**). In addition, we confirmed that transgene expression of Adv-PEG$_{CGKRK}$ occurs at tumor endothelial cells mainly (**Figure 3**). Taken together, our findings suggest that PEGylation results in greater suppression of the transition of Adv-PEG and Adv-PEG$_{CGKRK}$ into the liver, thereby prolonging the circulating lifetime. Adv-PEG$_{CGKRK}$'s long-retention time in the blood increases the chances of contact with tumor vasculature, leading to increased gene transduction in tumor endothelial cells. In addition, the tumor endothelium-targeting ligand CGKRK peptide may actively lead Adv-PEG$_{CGKRK}$ to tumor tissue, resulting in increased accumulation and gene transduction in tumor cells.

In both primary and metastatic tumor models, intravenous administration of Adv-PEG$_{CGKRK}$ showed remarkable therapeutic effect with negligible side effects (**Figure 4**). Administration of unmodified Adv lacked a therapeutic window and administration of Adv-PEG only induced a therapeutic effect at 5 × 10^{10} vp and was associated with substantial side effects. In contrast, a therapeutic effect comparable with 5 × 10^{10} vp Adv-PEG was induced by 2 × 10^{9} vp Adv-PEG$_{CGKRK}$ with negligible side effects. However, transgene expression with 2 × 10^{9} vp of Adv-PEG$_{CGKRK}$ was lower than that with 5 × 10^{10} vp Adv-PEG (**Figure 1d**). In addition, we demonstrated that Adv-PEG$_{CGKRK}$-HSVtk inhibits wound healing, indicating that Adv-PEG$_{CGKRK}$-HSVtk might destroy angiogenic vessels (**Figure 5**). Thus, we speculated that the therapeutic effects of Adv-PEG$_{CGKRK}$ might be caused not only by a direct antitumor effect, but also by an antiangiogenesis effect *via* transgene expression in tumor endothelial cells. In general, it is known that destroying one vascular endothelial cell in tumor tissue vessels results in the death of many more tumor cells because a single vessel supports the survival of many tumor cells through the provision of oxygen and nutrients.[38] Therefore, the remarkable therapeutic antitumor effect of Adv-PEG$_{CGKRK}$ might be considered to be a combination of a direct tumor killing effect and an antiangiogenesis effect.

One of the hurdles confronting Adv-mediated gene transfer is that infection with Adv is dependent on the presence of the coxackie-adenovirus receptor on the target cells.[39] Many tumor cells that are targets for gene therapy, such as melanoma cells, express little or no coxsackie-adenovirus receptor, making it difficult to achieve sufficient gene expression and therapeutic effect.[39,40] We demonstrated here that Adv-PEG$_{CGKRK}$ induces a high level of transgene expression in coxackie-adenovirus receptor-negative B16BL6 cells *in vivo* compared with unmodified Adv and Adv-PEG. In addition, because angiogenesis is a known hallmark of most tumors[41] and the CGKRK peptide targets several tumor types[30], it is possible that Adv-PEG$_{CGKRK}$ might be effective across a range of tumors. Adv-PEG$_{CGKRK}$ has the potential to be a wide-ranging tumor-targeting vector that overcomes the problem of conventional Adv.

In this study, we showed that Adv-PEG conjugated with the targeting ligand CGKRK peptide targeted both tumor tissues and tumor vasculatures after systemic administration. We propose that Adv-PEG$_{CGKRK}$ has potential for use as a prototype vector with suitable efficacy and safety for systemic cancer gene therapy against primary tumors and metastases.

MATERIALS AND METHODS

Mice and cell lines. Five-week-old female C57BL/6 mice and BALB/c mice were purchased from SLC (Hamamatsu, Japan). All of the animal experimental procedures were performed in accordance with the Osaka University guidelines for the welfare of animals. B16BL6 (mouse melanoma) cells were obtained from RIKEN Cell Bank (Tsukuba, Japan).

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MDA-MB-435S (human breast carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA). The B16BL6 cells were cultured in Minimal Essential Medium (Sigma-Aldrich, St Louis, MO) containing 7.5% fetal bovine serum and antibiotics. The MDA-MB-435S cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal bovine serum and antibiotics.

Plasmids and vectors. An improved *in vitro* ligation method[14,33,42,43] was used to construct E1/E3-deleted adenovirus type 5 expressing firefly luciferase, GFP, or HSVtk, all under control of the cytomegalovirus promoter. Each Adv was generated using established methods.[10] The vps and biological titer were determined as described.[10,44] The ratio of the particle-to-biological titer was between 10 and 30.

Preparation of Adv-PEG$_{CGKRK}$. A heterobifunctional PEG (SUNBRIGHT MA-200TS; molecular weight, 20,000; NOF, Tokyo, Japan), and methoxy-PEG-succinimidyl propionate, a monofunctional PEG (mPEG-SPA; molecular weight, 20,000; Nektar, San Carlos, CA), were used for PEGylation. To confirm the attachment of SUNBRIGHT MA-200TS to Adv, we used two-step procedure. To construct Adv-PEG, Adv was incubated with SUNBRIGHT MA-200TS at 50-fold molar excess to viral lysine residues at 37 °C for 45 minutes and then incubated with mPEG-SPA at 150-fold molar excess to viral lysine residues at 37 °C for 45 minutes. The excess NHS group of free PEG was blocked by incubation with 6-aminocaproic acid (Sigma-Aldrich) at 37 °C for 30 minutes. Adv-PEG$_{CGKRK}$ was prepared by adding CGKRK peptide at fivefold molar excess to SUNBRIGHT MA-200TS to Adv-PEG and incubating at room temperature for 1 hour. Unreacted maleimide was blocked by incubation with 2-mercaptoethanol at room temperature for 1 hour. To purify Adv-PEG and Adv-PEG$_{CGKRK}$, free peptides and free PEG were removed by dialysis using 1,000-kDa molecular weight cutoff membranes (Spectrum Laboratories, Rancho Dominguez, CA). The concentration of Adv was measured by picogreen assay (Invitrogen, Carlsbad, CA). The modification ratio of Adv-PEG and Adv-PEG$_{CGKRK}$ was 45%, which was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis as described.[10]

In vitro *gene transduction.* MDA-MB-435S and B16BL6 cells (2×10^4 cells/well) were seeded into separate 48-well plates. The following day, the cells were transduced with 10^4 vp/cell each Adv, encoding the luciferase gene. After 24 hours luciferase activity was determined using the luciferase assay system (Promega, Madison, WI) and a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) in accordance with the manufacturers' instructions. Luciferase activity was calculated as relative light units/well.

In vivo *gene transduction.* C57BL/6 mice were intradermally inoculated in the flank with 3×10^5 B16BL6 cells. After the tumor diameter had reached ~8 mm, the mice were intravenously administered 5×10^{10} vp each Adv encoding the luciferase gene. The tumors and livers were harvested 48 hours after administration and luciferase activity was measured. To evaluate transgene expression in a pulmonary metastasis model *in vivo*, 3×10^5 B16BL6 cells were injected into the tail veins of C57BL/6 mice. After 12 days, the mice were intravenously administered 5×10^{10} vp each Adv encoding the luciferase gene. The lungs were harvested 48 hours after injection and luciferase activity was measured.

Blood clearance of Adv-PEG$_{CGKRK}$. Blood clearance was determined using real-time quantitative PCR, as described.[14] In brief, blood samples were collected at 2, 30, 60, and 120 minutes after intravenous administration of 5×10^{10} vp each Adv in normal C57BL6 mice. Total DNA, including Adv DNA, from whole blood was extracted. The number of viral genomes was counted using real-time quantitative PCR. Standard samples were generated using known amounts of viral DNA with control whole blood DNA.

Tissue distribution of Adv-PEG$_{CGKRK}$. Once the tumor diameter was ~8 mm, B16BL6-bearing mice were intravenously administered 5×10^{10} vp each Adv. The tumors and livers were harvested 6 hours after administration and the DNA was extracted. The number of viral genomes in each sample was counted using real-time quantitative PCR as described.[10]

Localization of Adv-PEG$_{CGKRK}$. Once the tumor diameter was ~8 mm, B16BL6-bearing mice were intravenously administered 5×10^{10} vp each Adv encoding the *GFP* gene. Two days later, the tumors were collected and fixed in 4% paraformaldehyde. Frozen sections (6 µm) were prepared and subjected to double-label immunohistochemical staining with rat anti-mouse CD31 antibody (BD Biosciences, San Diego, CA) and goat anti-rat IgG Alexa 568 antibody (Invitrogen).

Antitumor effect of Adv-PEG$_{CGKRK}$ against primary tumors. Once the tumor diameter was ~8 mm, B16BL6-bearing mice were intravenously administered PBS or Advs encoding the *HSVtk* gene. After Adv injection, the mice received daily intraperitoneal administrations of ganciclovir (50 mg/kg) for 10 days. Every 3 days, the major and minor axes of the tumor were measured with microcalipers; tumor volume was calculated using the following formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; mm)^2 × 0.5. The mice were euthanized when tumor volume exceeded 4,000 mm^3.

Antitumor effect of Adv-PEGCGKRK against metastases. Into the tail vein of C57BL/6 mice, we injected 3×10^5 B16BL6 cells. After 7 days, the mice were intravenously administered PBS or Advs encoding the *HSVtk* gene. The mice then received daily intraperitoneal administrations of ganciclovir (50 mg/kg) for 7 days. On day 7 after Adv administration, serum activities of GOT and GPT, used as indicators of hepatotoxicity, were measured using the Transaminase CII test (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. On the same day, the lungs were harvested and fixed in Bouin's solution for 24 hours; photographs of lung sections were taken, and the numbers of metastatic colonies were counted.

Wound healing by Adv-PEG$_{CGKRK}$. Six-mm diameter wounds were created on the abdominal area of BALB/c mice by using sterile disposable biopsy punch (Natsume Seisakusho, Tokyo, Japan) and PBS or 1×10^{10} vp each Adv encoding the *HSVtk* gene were intravenously injected into the mice 1 day after injury. After Adv administration, ganciclovir was intraperitoneally administered to the mice (50 mg/kg) daily for 10 days. Every 3 days, the major and minor axes of the wound were measured with microcalipers by a single investigator who was blinded to the treatment group; wound area was calculated using the following formula: (wound area; mm^2) = (major axis; mm) × (minor axis; mm) × 3.14 × 0.25.

Statistical analysis. All results are expressed as mean ± SD or SEM. Differences were compared using Bonferroni's method after analysis of variance.

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