Adenovirus Vector Covalently Conjugated to Polyethylene Glycol with a Cancer-Specific Promoter Suppresses the Tumor Growth through Systemic Administration

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Cancer gene therapy with adenovirus vectors (Adv) is limited to local administration because systemic administration of Adv produces a weak therapeutic effect and severe side effects. Previously, we generated a dual cancer-specific Adv system by using Adv covalently conjugated to polyethylene glycol (PEG) for transductional targeting and the telomere reverse transcriptase (TERT) promoter as a cancer-specific promoter for transcriptional targeting (PEG-Ad-TERT). We demonstrated that systemic administration of PEG-Ad-TERT showed superior antitumor effects against lung metastastic cancer with negligible side effects. Here, we investigated the therapeutic efficacy of systemic administration of PEG-Ad-TERT for the treatment of primary tumors. We first evaluated the transgene expression of PEG-Ad-TERT containing the luciferase gene (PEG-Ad-TERT/Luc) in primary tumors. Systemic administration of PEG-Ad-TERT/Luc resulted in high transgene expression, similar to that observed in tumors for the conventional cytomegalovirus (CMV) promoter-driven Adv containing the luciferase gene (Ad-CMV/Luc). By comparison, transgene expression was 2500-fold lower than that of Ad-CMV/Luc in liver. We then examined the therapeutic effect of systemic administration of PEG-Ad-TERT containing the herpes simplex virus thymidine kinase (HSVtk) gene (PEG-Ad-TERT/HSVtk) for the treatment of primary tumors. We showed that PEG-Ad-TERT/HSVtk produced a notable antitumor effect against primary tumors with negligible side effects. These results demonstrated that PEG-Ad-TERT can be regarded as a prototype Adv with suitable efficacy and safety for systemic cancer gene therapy against both metastatic and primary tumors.

Key words: biocorjugation; gene therapy; polymer

Gene therapy is expected to play a key role in next-generation cancer therapy together with the conventional modalities for the treatment of cancer, such as surgery, chemotherapy, and radiotherapy. Use of adenovirus vectors (Adv) in gene therapy has been developed in diverse animal models and clinical trials because Adv can be propagated to high yields and has the capacity to efficiently transduce a wide variety of cell types and tissues regardless of the mitotic status of the cell. In several clinical trials, intratumoral injection of Adv has demonstrated therapeutic efficacy against primary tumors. However, successful cancer gene therapy also requires treatment of unselectable primary cancers and distant metastases, which are the major cause of cancer mortality. To treat unselectable primary and metastatic cancers, Adv must be administered systemically, but its effects must be restricted to tumor cells. The major disadvantage in treating cancer with systemically administered conventional Adv is related to its accumulation in the liver immediately after systemic injection, which can cause severe liver toxicity. Therefore, to enable the application of Adv to the treatment of both primary and metastatic cancers, it is necessary to develop Adv with the capacity for tumor-selective distribution and gene expression after systemic administration.

In a previous study, we generated a dual cancer-specific targeting vector system by using Adv covalently conjugated to polyethylene glycol (PEG), for transductional targeting, and a cancer-specific promoter, for transcriptional targeting. Covalent conjugation to PEG (PEGylation) can prolong the plasma half-life of Adv, prevent hepatic uptake, and alter the tissue distribution of Adv because of the steric hindrance of the PEG. We showed that systemic administration of PEGylated Adv (PEG-Adv) with 20-kDa PEG at a 45% modification ratio resulted in higher tumor-selective transgene expression than unmodified Adv. In addition, we showed that the telomere reverse transcriptase (TERT) promoter-driven PEG-Adv (PEG-Ad-TERT), which contained the herpes simplex virus thymidine kinase (HSVtk) gene (PEG-Ad-TERT/HSVtk) for the treatment of primary tumors. We showed that PEG-Ad-TERT/HSVtk produced a notable antitumor effect against primary tumors with negligible side effects. Therefore, PEG-Ad-TERT can be regarded as a prototype Adv with suitable efficacy and safety for systemic cancer gene therapy against both metastatic and primary tumors.

MATERIALS AND METHODS

Mice and Cell Lines Female 5-week-old C57BL6 mice were purchased from SLC Inc. (Hamamatsu, Japan). All procedures involving animal experimentation were performed in...
accordance with the Osaka University guidelines for the welfare of animals. HEK293 cells were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). CT26 cells (murine colon carcinoma cells) were kindly provided by Prof. N. P. Restifo (National Cancer Institute, Bethesda, MD, U.S.A.). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum (FBS) and antibiotics. CT26 cells were cultured in RPMI (Sigma-Aldrich) containing 10% FBS and antibiotics.

**Construction of Adv** An improved in vitro ligation method\(^6\) was used to construct the E1/E3-deleted adenovirus type 5 expressing firefly luciferase or HSVtk under the control of the CMV or TERT promoter (Ad-CMV/Luc, Ad-CMV/HSVtk, Ad-TERT/Luc, and Ad-TERT/HSVtk) as described previously.\(^7\) Each Adv was amplified in HEK293 cells using established methods, purified from cell lysates through CsCl gradients, dialyzed, and stored at 80°C.\(^11\)

The virus particle (vp) titer and biological titer were determined by using a spectrophotometric method\(^2\) and the Adeno-X Rapid Titer protocol (Clontech Laboratories, Mountain View, CA, U.S.A.), respectively. The ratio of the particle-to-biological titer of each Adv was between 10 and 40.

**PEGylation of Adv** Activated methoxy(polyethylene glycol)-succinimidyl propionate (mPEG-SPA; molecular weight 20000; Nektar, San Carlos, CA, U.S.A.) was used for PEGylation. For modification of viral lysine residues Adv was incubated with 200-fold excess mPEG-SPA at 37°C for 45 min with gentle stirring. The modification ratio of PEG to Adv was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE was carried out in a gradient gel containing 2 to 15% polyacrylamide (PAGE Mini 4/20, Daiichi Pure Chemicals, Tokyo, Japan) as previously reported.\(^8,13\) The gel was stained with Coomassie blue. The PEGylated hexon band was separated from the unmodified hexon band. The signal intensity of each band was measured using NIH Image software, and the PEG modification ratio was calculated as: [signal intensity of PEGylated hexon/(signal intensity of unmodified hexon)] \(\times 100\).

**Adv-Mediated Transduction in Vivo** To evaluate transgene expression in vivo, mice bearing CT26 cells were intravenously administered \(10^{10}\) vp of Ad-CMV/Luc, Ad-TERT/Luc, or PEG-Ad-TERT/Luc after the tumor diameter had reached approximately 7 mm. The tumor and liver were harvested 48 h after the injection, and luciferase activity was measured. Luciferase activity in the homogenates was determined by use of a commercial assay system (Promega, Madison, WI, U.S.A.) and a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) according to the manufacturer’s instructions. Luciferase activity was reported as relative light units (RLU)/mg tissue.

**Gene Therapy to Tumor-Bearing Mice** Female 5-week-old BALB/c mice were inoculated with CT26 cells. Once the tumor diameter reached 7 mm, each mouse was injected intravenously with Ad-CMV/HSVtk, Ad-TERT/HSVtk, or PEG-Ad-TERT/HSVtk at \(1 \times 10^{10}\) or \(5 \times 10^{10}\) vp. Beginning the day after Adv injection, the mice received daily intraperitoneal injections of ganciclovir (GCV) (50 mg/kg) for 10 d. Body weight was monitored every 2 or 3 d and the major and minor axes of the tumor were measured with microcalipers. Tumor volume was calculated by the following formula: (tumor volume; mm\(^3\)) = (major axis; mm) \(\times\) (minor axis; mm) \(\times\) 0.5. The mice were euthanized when tumor volume exceeded 4000 mm\(^3\). Serum levels of glutamic oxaloacetic transaminase and glutamic-pyruvic transaminase (GOT and GPT, used in liver function tests as indicators of hepatotoxicity) were measured on day 10 after Adv injection by using the Transaminase CII test (Wako Pure Chemical, Osaka, Japan) according to the manufacturer’s instructions.

**Statistical Analysis** Results were expressed as mean± S.D. or S.E.M. Differences were compared using Mann-Whitney U test or Bonferroni’s method after analysis of variance (ANOVA).

**RESULTS AND DISCUSSION**

In a previous study we generated a dual cancer-specific targeting Adv system by using PEGylation and the TERT promoter.\(^7\) We showed that PEG-Ad-TERT/HSVtk with 20-kDa PEG at a 45% modification ratio had an antitumor effect against metastasis when administered systemically. Here, we examined the therapeutic effect of PEG-Ad-TERT/HSVtk against primary cancers.

We assessed the effect of using PEGylation and the TERT promoter in combination by constructing PEG-Ad-TERT/HSVtk using 20-kDa PEG with a 45% modification ratio and examining luciferase expression in tumors and liver after intravenous injection into mice bearing CT26 primary colon tumor cells. Luciferase expression was 6-fold lower in tumors from Ad-TERT/Luc than from Ad-CMV/Luc, but was 450-fold lower in liver. By comparison, luciferase expression was 4-fold higher in tumors from PEG-Ad-TERT/Luc than from Ad-TERT/Luc, but was 5-fold lower in liver (Fig. 1). Thus, PEG-Ad-TERT/Luc gave almost the same level of luciferase expression in tumors but 2500-fold lower luciferase expression in liver compared to Ad-CMV/Luc. These results suggest that PEGylation together with the TERT promoter could restrict Adv-expressed genes to tumor tissue in a primary tumor model.

Next, to evaluate the effectiveness of combining PEGylation and the TERT promoter for cancer therapy, we assessed

![Fig. 1. Luciferase Expression of Systemically Administered PEG-Ad-TERT/Luc in Tumors and Liver](image-url)
the therapeutic efficacy of systemic administration of PEG-Ad-TERT to treat primary tumors (Fig. 2). We used Adv expressing HSVtk as the therapeutic gene; HSVtk is an enzyme that can convert the nontoxic, clinically used antiviral drug GCV into a highly cytotoxic phosphorylated form. Previ-
ously we showed that PEG-Ad-CMV/HSVtk induced un-
wanted side effects after systemic administration. Therefore, we did not examine the therapeutic effects of PEG-Ad-
TERT/HSVtk. We constructed PEG-Ad-TERT/HSVtk using 20-kDa PEG with a 45% modification ratio and examined its antitumor effects in the mouse CT26 primary colon tumor model. Each mouse was injected intravenously with $5 \times 10^{10}$ vp or $1 \times 10^{10}$ vp of unmodified Ad-CMV/HSVtk or $5 \times 10^{10}$ vp of Ad-TERT/HSVtk or PEG-Ad-TERT/HSVtk. Ad-CMV/HSVtk at $5 \times 10^{10}$ vp induced a marked reduction in body weight, and all treated mice had died by 5 d after Adv injection (Fig. 2). By comparison, Ad-CMV/HSVtk at $1 \times 10^{10}$ vp did not induce GPT or GPT activity, an indicator of hepatoto-
xicity (data not shown), but had no antitumor activity (Fig. 2). We considered that the side effects of Ad-CMV/HSVtk at $5 \times 10^{10}$ vp were due to toxicity resulting from HSVtk expres-
sion in the liver, because Ad-CMV/Luc showed high luc-
iferase expression in liver (Fig. 1). Therefore, systemic ad-
ministration of Ad-CMV/HSVtk appeared to lack therapeutic efficacy because of the side effects, although Ad-CMV/HSVtk at $5 \times 10^{10}$ vp showed transgene expression in tumor as same as PEG-Ad-TERT/HSVtk. Ad-TERT/HSVtk at $5 \times 10^{10}$ vp had no antitumor activity (Fig. 2) and did not in-
duce GPT or GPT activity (data not shown). In contrast, PEG-Ad-TERT/HSVtk at $5 \times 10^{10}$ vp inhibited tumor growth by approximately 63% when compared with administration of PBS (control). At this titer PEG-Ad-TERT/HSVtk did not in-
duce GPT or GPT activity (data not shown). Previously we showed that the modification of Adv with 20-kDa PEG strongly suppresses the transition into the liver, thereby prolonging the circulating time and leading to the accumulation of Adv in the tumor because of the enhanced permeability and retention effect.7)

Recent reports revealed that modification of Adv with 20-
kDa PEG decreased liver transduction in vivo when com-
pared with modification with 5-kDa PEG.15–17) However, these groups used a low modification ratio for PEGylation and did not show the maximal effect of PEGylated Adv for tumor targeting. In a previous study, we examined the corre-
lation between PEG modification and gene expression pat-
terns and showed that PEGylation with 20-kDa PEG at a modification ratio of 45% was optimal for PEGylation of Adv for tumor-selective gene expression.7) Under these optim-
ized modification conditions, PEG-Ad strongly suppresses

The increased therapeutic effects of proteins, liposomes, and Adv conjugated to polymers has been attributed to the pharmacokinetics of the polymer.18,19) Therefore, it is neces-
sary to select the optimum polymer by considering the dispo-
sition of drugs and objectives such as targeting or controlled release. Previously, we showed that the therapeutic antitumor effects were stronger when tumor necrosis factor-α was con-
jugated with polyvinylpyrrolidone (PVP) than with PEG; this is because PVP is better than PEG for prolonging plasma half-life and increasing stability in blood.20,21) Therefore, conjugation of PVP to Adv could produce a more potent an-
titumor therapeutic agent. We are now investigating the use-
fulness of PVP-Ad for cancer gene therapy.

In summary, we showed that PEG-Ad-TERT/HSVtk with 20-kDa PEG at a 45% modification ratio had antitumor ef-
fector against primary tumor when administered systemically. Thus, PEG-Ad-TERT/HSVtk would be useful as a platform vector for cancer gene therapy.

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