Combination gene therapy of lung cancer with conditionally replicating adenovirus and adenovirus-herpes simplex virus thymidine kinase

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Abstract. A major obstacle to the success of gene therapy strategies that directly target cancer cells is the low gene transfer rate. To address this problem, we had previously proposed a combination adenoviral gene therapy containing a conditionally replicating adenovirus (CRAD) expressing mutant E1 (Δ24RGD), and a replication-defective E1-deleted adenovirus to enhance the efficiency of gene transfer. Suicide/pro-drug gene therapy has an important additional benefit to the therapy of cancer. This relates to the transfer and expression of non-mammalian genes encoding enzymes that convert non-toxic pro-drugs into cellular toxins. We investigated the interaction between CRAD (Δ24RGD) and a replication-defective E1-deleted adenovirus (ad-HSTK) containing a suicide gene (HSTK: *herpes simplex virus thymidine kinase* gene) with respect to therapeutic gene production and tumor cell killing efficacy. Combined transduction of CRAD and ad-HSTK increased the transduction efficiency of HSTK and increased its sensitivity to ganciclovir (GCV) more efficiently than ad-HSTK alone. Transfer of medium of CRAD and ad-HSTK co-transduced cells induced the transfer of HSTK (media transferable bystander effect), and enhanced its sensitivity to GCV. In an animal tumor model, combined intratumoral injection of CRAD and ad-HSTK followed by GCV administration induced prolonged expression of HSTK and stronger growth suppression of established lung cancer xenografts than single injections. These data demonstrate that the selective replication of ad-HSTK due to the presence of mutant E1, produced by a Δ24RGD and HSTK/GCV suicide gene system, resulted in a striking improvement in anti-tumor effects *in vitro* and *in vivo*.

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Introduction

Gene therapy of cancer has made remarkable advancements in animal tumor models and has been rapidly tested in many clinical trials. However, the practical impact of gene therapy in clinics is still disappointing. Low gene transfer rate is a major obstacle to the success of gene therapy strategies that directly target cancer cells. In a past clinical trial, when intratumoral implantation of the *herpes simplex virus-thymidine kinase* (HSTK) gene and ganciclovir (GCV) administration was carried out in 15 patients with progressive growth of recurrent malignant brain tumors, antitumor activity was detected in five of the smaller tumors and usually restricted to the injection site (1). This suggests that the delivery and distribution of the therapeutic gene was confined to the injection site in a human tumor mass.

A conditionally replicating adenovirus (CRAD, previously known as an oncolytic adenovirus) that can selectively replicate in tumor cells represents a novel and promising approach for treating malignant diseases (2,3). The Ad mutant *dl*1520 (ONYX-015) is the first CRAD that can replicate only in p53 mutated cells (4). The use of CRADs offers two advantages over conventional gene therapy. First, CRADs have an intrinsic amplification capacity that allows extensive tumor

infection, leading to expansive oncolysis by the actual cytopathic effect of the virus and spreading to adjacent tumor. Second, the restriction of viral replication to tumors avoids damage to normal host tissues and improves the therapeutic index. However, clinical studies with CRAD have failed to produce convincing results although some positive responses have been reported (5,6). Most of these viruses cannot carry a therapeutic gene. Furthermore, the genetic heterogeneity of tumors poses a problem as typically CRAD can only replicate in p53-mutated or pRb/p16 pathway-inactivated cells, or in cells expressing a specific protein (i.e., CRAD containing cancer-specific promoters such as telomerase or cyclooxygenase 2 promoter).

Another strategy is the use of CRAD engineered to also express a therapeutic gene inserted into the E1 or E3 region of the adenovirus (an 'armed' therapeutic virus) (7-10). This approach takes advantages of the viruses ability to selectively replicate and spread in the tumor mass, thus safely and efficiently delivering therapeutic genes to target tissues where their therapeutic products can accumulate to levels that afford maximal effect. While packaging of therapeutic genes is generally not an issue for large viruses such as HSV (herpes simplex virus) (nearly 50% of HSV genes are non-essential for viral replication) and vaccinia (where it is estimated that the virus may be able to package ~50 kb of foreign DNA), this is a considerable hurdle for smaller viruses like adenovirus (10).

Another new CRAD strategy is to combine two adenoviruses, CRAD and E1-deleted, replication-defective adenovirus containing a therapeutic gene. We have previously shown that a conventional replication-defective adenovirus containing an E1 deletion can become replication competent. However, it must be cotransduced with a CRAD (Δ24RGD) capable of supplying mutant E1 protein *in trans* as this can act as normal E1 protein in pRB/p16 inactivated cancer cells. The resulting selective production of large numbers of the therapeutic adenovirus within a tumor mass *in situ* could infect adjacent tumor cells and spread laterally through the tumor mass, and finally increase overall transduction efficiency (11,12). Habib *et al* have already reported this basic idea by demonstrating that coinfection with a replication-defective adenovirus, expressing a green fluorescent protein, and a replication-competent adenovirus enhanced the expression of the green fluorescent protein throughout human lung tumors in immunodeficient nude mice (13).

In our previous studies (11,12), we used a CRAD designated Δ24RGD, which produces a mutant E1 protein without the ability to bind retinoblastoma (Rb), but which retains viral replication competence. This E1 can theoretically permit viral replication only in cancer cells with a defective pRb/p16 pathway. In addition, this Δ24RGD contains an Arg-Gly-Asp (RGD) sequence, known to interact with α_v integrins, in adenoviral fibers to enhance tumor infection (14). The addition of RGD-integrin interactions on primary CAR (Coxsackie adenoviral receptor) binding confers an expanded tropic range to the fiber-modified adenoviruses.

As a replication-defective therapeutic adenovirus with an E1 deletion, we used E1 deleted adenoviral vectors expressing HSTK (ad-HSTK). HSTK is the most widely used suicide gene. It confers sensitivity to ganciclovir (GCV) (15). The cytotoxic effect of GCV results from its incorporation into DNA by a process involving several steps, starting with its phosphorylation to form a monophosphate (GCV monophosphate). Since its affinity for eukaryotic thymidine kinase is 1000 times lower, GCV is essentially phosphorylated only by the viral enzyme HSTK. The ultimate product of further phosphorylation by cellular kinases, GCV-triphosphate, competitively inhibits incorporation of the endogenous DNA precursor dGTP into DNA, and induces apoptotic cell death (16).

We hypothesized that combination of Δ24RGD and ad-HSTK enhance the expression of HSTK, and following GCV administration can induce a more potent antitumor effect by the enhanced expression of HSTK *in vivo* and *in vitro*.

Initially, we compared the effectiveness of an armed therapeutic adenovirus that contains the luciferase gene (Δ24-luc) and our current strategy [CRAd(Δ24) + adenovirus-luciferase] by luciferase assay. Then we compared the effectiveness of Δ24RGD + ad-HSTK and Δ24RGD alone or ad-HSTK alone in the lung cancer xenograft model in nude mice.

Materials and methods

Recombinant adenoviruses. Δ24RGD (provided by Dr David Curiel, Gene Therapy Center at the University of Alabama at Birmingham) contains E1A with a 24-bp deletion in the CR2 region and E3 with an RGD-4C modification of the fiber gene. A deletion in the CR2 domain in Δ24RGD that is responsible for binding pRb that allows the adenovirus-infected cell to enter S phase, can make this virus replicate only in the cells with defects in the pRb/p16 pathway where this binding is unnecessary. Furthermore, the RGD-4C motif in the E3 region enables Δ24RGD to infect cells without binding to CAR (14).

Δ24 (no RGD motif) and Δ24-luc, Δ24 expressing the luciferase gene, were kindly provided by Dr Victor van Beuschechem (VU University, The Netherlands). Ad-HSTK is a replication-defective E1-deleted adenovirus where the E1 region of the virus was replaced with the HSTK gene with a human CMVie promoter. Adenovirus-luciferase (ad-luc) is a replication defective E1-deleted adenovirus which carries the luciferase gene and a CMVie promoter. Ad-HSTK, ad-luc and ad-null (E1-deleted adenovirus without any therapeutic gene), were constructed in our laboratory (17). The adenoviruses were concentrated and purified with a BD Adeno X™ virus purification kit (BD Bioscience Clontech, Palo Alto, CA, USA). The titers of each adenovirus were determined by the tissue culture infectious dose 50 (TCID50) method.

Cancer cell lines. NCI H460 (human lung large cell carcinoma cell line) and A549 (human lung adenocarcinoma cell line) were purchased from American Type Culture Collection (Manasas, VA, USA).

Comparison of luciferase expression by Δ24-luc and Δ24 + ad-luc transduced lung cancer cell lines. To compare the effectiveness of our two strategies [armed therapeutic virus (Δ24-luc) versus combination of CRAD (Δ24) and E1-deleted adenovirus (ad-luc)], we measured the luciferase expression (Luciferase assay system: E4030, Promega, Madison, MI, USA) in NCI H460 transduced with ad-luc (10 moi:

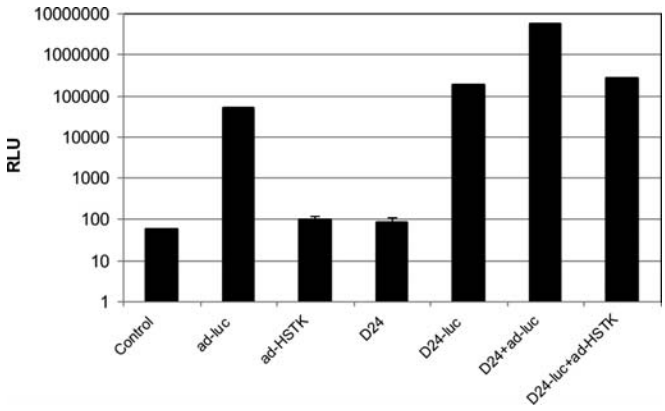

Figure 1. Transduction with CRAD (Δ24) and E1 deleted replication defective adenovirus (ad-luc) was more effective than CRAD with the transgene (Δ24-luc) in the expression of the transgene. Δ24 (10 moi) + ad-luc (10 moi) transduction in the lung cancer cell line (NCI H460) produced an almost 30 times higher luciferase concentration than Δ24-luc (10 moi) and 115 times more than ad-luc alone (10 moi). This finding demonstrated that cotransduction with ad-transgene + CRAD was more effective in expression of the transgene than CRAD-transgene (so called armed therapeutic adenovirus) or ad-transgene.

multiplicity of infection), ad-HSTK (10 moi), Δ24 (10 moi), Δ24-luc (10 moi), Δ24 (10 moi) + ad-luc (10 moi), and Δ24 (10 moi) + ad-HSTK (10 moi) at 48 h after transduction.

Effect of Δ24 on luciferase expression in ad-luc transduced lung cancer cell line. We investigated the effect of Δ24 on luciferase expression in NCI H460 transduced with ad-luc. Cancer cells were transduced with a fixed dose of ad-luc (10 moi) and increasing doses of Δ24 (from 0.01 to 100 moi) for 48 h. Luciferase expression was measured in cell lysates with Luciferase Cell Culture Lysis Reagent (Promega).

Effect of E1 deleted adenovirus on luciferase expression in Δ24-luc transduced lung cancer cell line. We also investigated the effect of E1-deleted adenovirus dose on proliferation of Δ24-luc by measuring luciferase expression. NCI H460 was transduced with Δ24-luc (10 moi) and increasing doses of ad-null (E1-deleted adenovirus without any therapeutic gene) from 0.01 to 100 moi. Luciferase expression was measured in cell lysates 24 h after transduction.

Change of in vitro transduction efficiency of ad-HSTK by cotransduction of Δ24RGD. To assess the change in transduction efficiency of ad-HSTK in combination with Δ24RGD *in vitro*, we transduced NCI H460 (human lung large cell carcinoma) with Δ24RGD (1 moi) alone, ad-HSTK (1 moi) alone, ad-HSTK (10 moi) alone, ad-HSTK (1 moi) and Δ24RGD (1 moi) together, or ad-HSTK (10 moi) and Δ24RGD (1 moi) together. After 24 h of transduction, we measured the efficiencies of HSTK gene transfer by performing Western blot assay for the HSTK protein.

Growth suppression after combined transduction with Δ24RGD and ad-HSTK. NCI H460 cells were plated into 96-well plates ($5x10^3$ cells/well). After 24 h they were transduced with Δ24RGD (1 moi) alone, ad-HSTK (1 moi) alone, ad-HSTK (5 moi) alone, Δ24RGD (1 moi) + ad-HSTK (1 moi), and Δ24RGD (1 moi) + ad-HSTK (5 moi). After 24 h of transduction, the NCI H460 cells were treated with 0, 1, 5, or 10 μM of GCV, respectively. A further 24 h after GCV treatment tumor cell viability was assessed by MTT assay.

Presence of intact ad-HSTK in the media of ad-HSTK and Δ24RGD-cotransduced lung cancer cell lines and sensitivity to GCV. After combined transduction with ad-HSTK (10 moi) ± Δ24RGD (10 moi), or Δ24RGD (10 moi) alone, in A549 and NCI H460 cells in 6-well plates, we washed the cells with PBS to remove the viruses remaining in solution. After 24 h of transduction, we collected and concentrated the media by Centriplus-50 (Millipore, Billerica, MA, USA) and then transferred this medium to uninfected A549 and NCI H460 cells. After 24 h of the transfer, we performed Western blot assays for HSTK protein so as to confirm the release of ad-HSTK into medium. Chemosensitivity to GCV was also examined after media transfer. Briefly, media from ad-HSTK (10 moi) and Δ24RGD (1 moi) was transferred to an untransduced lung cancer cell line (NCI H460). GCV was then added (1-50 μM) after 24 h. The viability of the lung cancer cells was assessed by MTT assay after 72 h and compared with cells transferred from a media containing untransduced cells or ad-HSTK transduced cells.

Combined treatment with Δ24RGD and ad-HSTK in an animal tumor model. We investigated the antitumor effect of Δ24RGD and ad-HSTK on lung cancer xenografts established by injecting A549 ($2x10^6$ cells per mouse) into the subcutaneous tissue of BALB/c nude mouse. Ten days later, ad-luciferase ($1.0x10^8$ plaque forming unit: pfu), Δ24RGD ($1.0x10^7$ pfu), ad-HSTK ($1.0x10^8$ pfu), and Δ24RGD ($1.0x10^7$ pfu) + ad-HSTK ($1.0x10^8$ pfu) were injected intratumorally and this was repeated 3 times from day 1 to day 3. GCV (100 mg/kg) was injected into the peritoneum in 1 ml of PBS daily for 5 days beginning on day 4. Tumor sizes were measured using the formula (0.5 x length x width^2). Tumor growth was analyzed using the repeated ANOVA test. At day 34, all mice were sacrificed because of large tumor mass and tumor masses were excised. Immunohistochemical staining of tumor mass for HSTK protein with anti-rabbit polyclonal anti-HSTK antibody (provided by Dr William Summers, Yale University, New Haven, CT) was performed. Animal care and all exeriments were performed in accordance with the institutional ethics guidelines.

Results

Lung cancer cells transduced with Δ24 and ad-luc expressed higher luciferase than cells transduced with Δ24-luc. We compared CRAD + E1 deleted adenovirus with target gene and CRAD containing the therapeutic gene by measuring luciferase expression in cells (NCI H460) transduced with Δ24 + ad-luc or Δ24-luc alone. The RLU (relative light unit) from cells transduced with Δ24-luc (10 moi) or ad-luc (10 moi) were 183,073 and 47,045 respectively. However, the RLU from Δ24 + ad-luc (10 moi) was the highest at 5,431,441. Δ24 + ad-luc transduction produced almost a 30 times higher luciferase expression compared to Δ24-luc (Fig. 1).

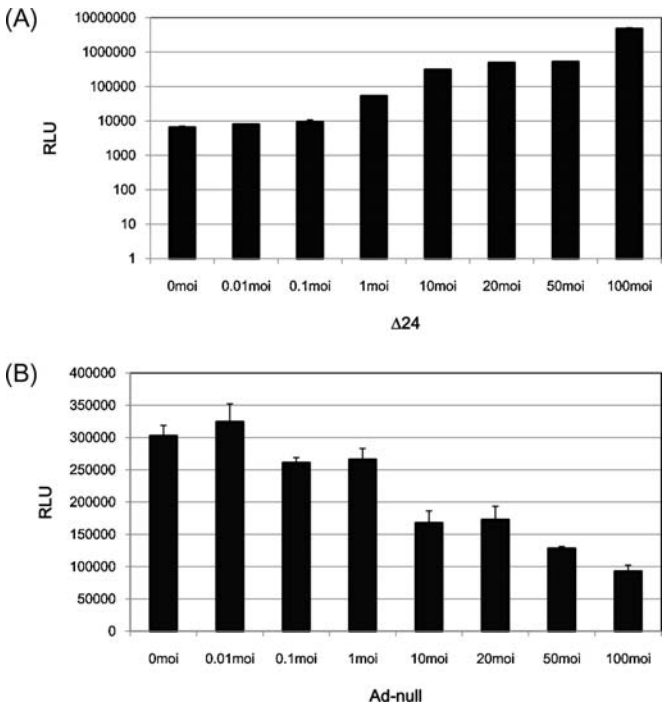

Figure 2. (A) Luciferase expression in ad-luc increased in a dose-dependent manner with CRAD (Δ24). We measured luciferase expression from lung cancer cell line (NCI H460) cotransduced with ad-luc (10 moi) and various doses of Δ24 (from 0.01 to 100 moi). Luciferase expression in ad-luc transduced cell line dramatically increased up to a dose of 100 moi of Δ24. The luciferase expression from cells transduced with ad-luc + Δ24 (100 moi) was 90 times higher than for cells with ad-luc + Δ24 (1 moi). (B) Luciferase expression in cells transduced with Δ24-luc decreased in a dose-dependent manner with E1-deleted adenovirus (ad-null). Cotransduction with ad-null (100 moi) in lung cancer cells decreased luciferase expression compared with cells transduced with Δ24-luc alone (30%) or cotransduction with ad-null (1 moi) (34%). However, the suppressive effect of the E1-deleted adenovirus on Δ24-luc was very weak compared with the enhancing effect of Δ24 on ad-luc transduced cells.

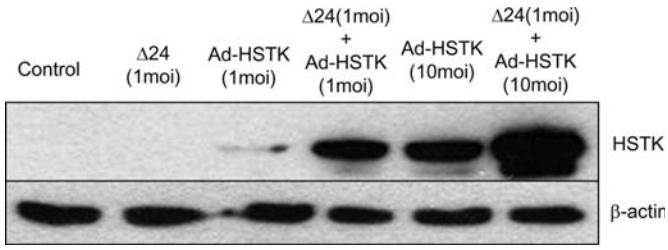

Figure 3. Combined transduction with ad-HSTK and Δ24RGD dramatically increased the expression of HSTK. NCI H460 cells were infected with Δ24RGD (1 moi) alone, ad-HSTK (1 moi) alone, ad-HSTK (10 moi) alone, ad-HSTK (1 moi) + Δ24RGD (1 moi), or ad-HSTK (10 moi) + Δ24RGD (1 moi). Western blot analysis was carried out after 24 h. The expression of HSTK in 10 moi ad-HSTK alone showed no appreciable difference to 1 moi ad-HSTK (1 moi) + Δ24RGD (1 moi). This strongly suggested the occurrence of replication of the E1-deleted ad-HSTK by the mutant E1 produced by Δ24RGD in cancer cells.

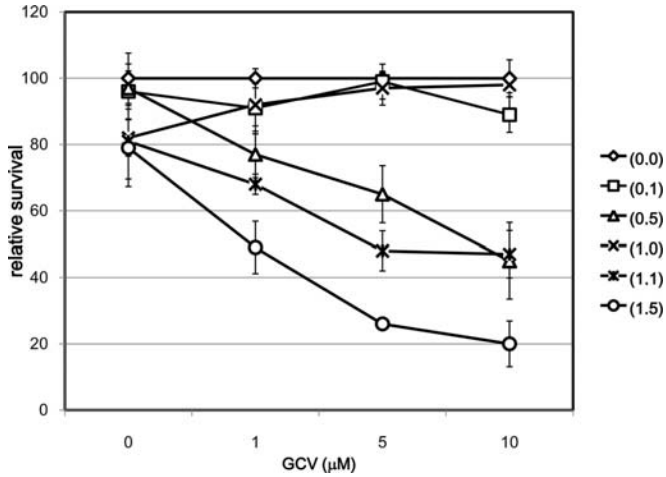

Figure 4. Enhanced sensitivity of lung cancer cell line to GCV by combined transduction with Δ24RGD and ad-HSTK. We transduced NCI H460 cells with Δ24RGD (1 moi) alone, ad-HSTK (1 moi) alone, ad-HSTK (5 moi) alone, Δ24RGD (1 moi) + ad-HSTK (1 moi), and Δ24RGD (1 moi) + ad-HSTK (5 moi). After 24 h of transduction, we treated the NCI H460 cells with 0, 1, 5, or 10 μM GCV, respectively. Tumor cell survival was measured by MTT assay and compared with an untransduced control (relative survival). Cells transduced with Δ24RGD (1 moi) and ad-HSTK (5 moi) were more sensitive to GCV-induced cytotoxicity than cells transduced with Δ24RGD (1 moi) or ad-HSTK (5 moi) alone. This means the increased production of HSTK from cotransduced cells confers a strong sensitivity to GCV (moi of Δ24RGD: moi of ad-HSTK).

Expression of luciferase from ad-luc transduced lung cancer cells increased in a dose-dependent manner with Δ24. We investigated the dose-effect of Δ24 on ad-luc transduced lung cancer cells (NCI H460). Luciferase expression in ad-luc (10 moi) transduced NCI H460 was 6,465 RLU. The luciferase expression in ad-luc (10 moi) + Δ24 transduced cells increased according to increasing doses of Δ24. The luciferase expression in cells transduced with ad-luc + Δ24 (1 moi) was 52,411 RLU and in cells with ad-luc + Δ24 (100 moi) was 4,721,087 (Fig. 2A).

Expression of luciferase from Δ24-luc transduced cells decreased in a dose-dependent manner with E1 deleted adenovirus (ad-null). We also investigated the dose-effect of ad-null on Δ24-luc transduced lung cancer cells. The luciferase expression in Δ24-luc (10 moi) transduced NCI H460 was 302,424 RLU. The luciferase expression in Δ24-luc (10 moi) + ad-null transduced cells decreased according to increasing doses of ad-null. The luciferase expression in cells transduced with Δ24-luc (10 moi) + ad-null (1 moi) was 265,694 RLU and in cells with Δ24-luc + ad-null (100 moi) was 92,444 (Fig. 2B). Though a high dose of ad-null suppressed luciferase expression in Δ24-luc, this suppressive effect was very weak compared to the enhancing effect of Δ24 on ad-luc transduced cells.

Enhanced in vitro transduction efficiency of ad-HSTK by cotransduction with Δ24RGD. We tested our hypothesis by measuring the gene transfer efficiency of ad-HSTK in combination with Δ24RGD. Coinfection with Δ24RGD increased the transduction rate of ad-HSTK to a greater extent than ad-HSTK alone (Fig. 3). The expression of HSTK in 10 moi ad-HSTK alone was similar to the expression of HSTK in 1 moi ad-HSTK (1 moi) + Δ24RGD (1 moi). This provides indirect evidence of replication of the E1-deleted ad-HSTK by the mutant E1 produced by cotransduced Δ24RGD in

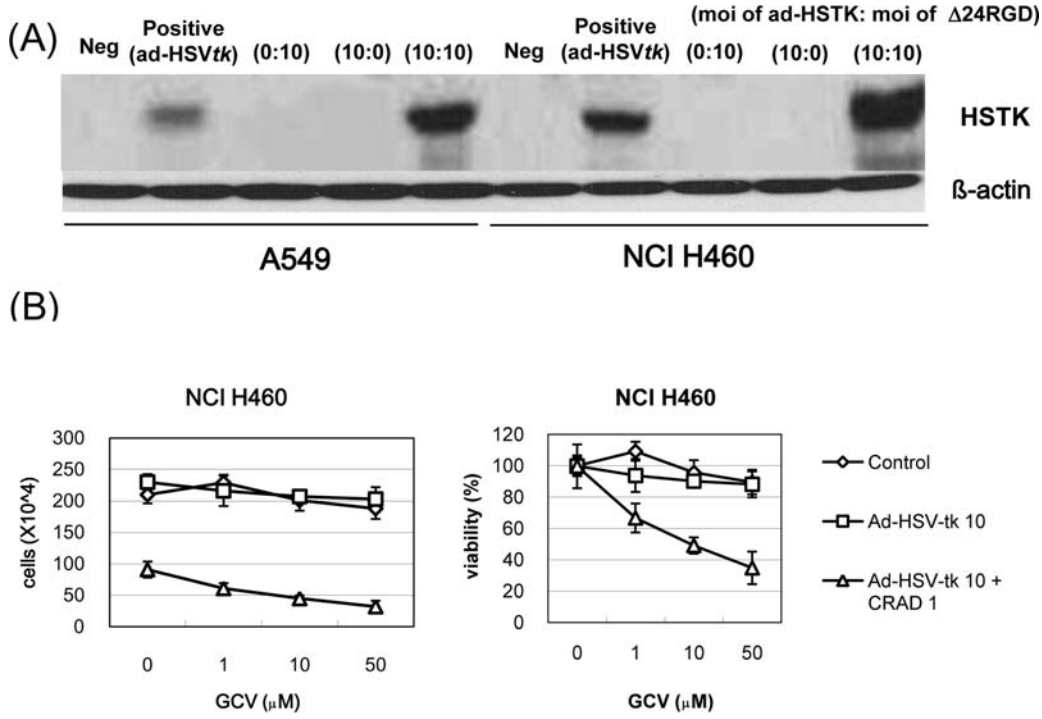

Figure 5. (A) Media transferable bystander effect. A549 and NCI H460 cells were transduced with Δ24RGD (10 moi) alone, ad-HSTK (10 moi) alone, or ad-HSTK (10 moi) and Δ24RGD (10 moi). After 24 h, the media was collected and concentrated by Centriplus-30 and transferred to uninfected A549 and NCI H460 cells in culture. Western blot for HSTK was done after 24 h. HSTK was found to be expressed only in cells treated with the media from ad-HSTK and Δ24RGD cotransduced cells. This demonstrates the presence of ad-HSTK in the media and supports the bystander effect. (B) Media transfer from Δ24RGD and ad-HSTK cotransduced cells conferred the sensitivity to GCV. After media transfer, NCI H460 cells were treated with GCV for 72 h and cell numbers were measured by MTT assay. Only the NCI H460 cells treated with media from Δ24RGD and HSTK showed sensitivity to GCV (left, absolute cell number; right, relative viability).

cancer cells. This is agreement with the results of some of our previous studies (11,12).

Enhanced in vitro growth suppression by combining Δ24RGD and ad-HSTK. A single transduction with ad-HSTK (1 moi) showed little growth suppression after GCV treatment. A single transduction with Δ24RGD (1 moi) showed minimal cytotoxic effects. No additional antitumor effect in the GCV administrated cells was detected. Ad-HSTK at a 5-moi dose showed a modest cytotoxic effect with the addition of GCV. Addition of GCV to cells cotransduced with Δ24RGD and ad-HSTK induced a strong cytotoxic effect. Combined transduction with Δ24RGD (1 moi) and ad-HSTK (5 moi) induced a stronger sensitivity to GCV than ad-HSTK (5 moi) (Fig. 4).

The presence of intact ad-HSTK in the media of ad-HSTK and Δ24RGD-cotransduced cells. We confirmed the propagation and release of ad-HSTK from ad-HSTK and Δ24RGD cotransduced cells by transferring media to untransduced cells. Media from A549 and NCI H460 cells transduced with ad-HSTK (10 moi) alone did not transfer HSTK into cancer cells. However, medium from ad-HSTK and Δ24RGD-cotransduced cells transferred HSTK to cancer cells (Fig. 5A). This finding confirmed the presence of intact ad-HSTK in the media, demonstrating the propagation and release of ad-HSTK from the cotransduced cells (media transferable bystander effect). Furthermore, media transferred lung cancer cells (NCI H460) were treated with GCV. Only cells with transferred media from Δ24RGD and ad-HSTK cotransduced cells showed a sensitivity to GCV (Fig. 5B).

Strong antitumor effect of combined treatment with Δ24RGD and ad-HSTK on established lung cancer xenografts. Two weeks following the intratumoral injection of eight mice in each group with ad-luciferase ($1.0x10^8$ pfu), Δ24RGD ($1.0x10^7$ pfu), ad-HSTK ($1.0x10^8$ pfu), and Δ24RGD ($1.0x10^7$ pfu) + ad-HSTK ($1.0x10^8$ pfu), the difference of tumor volume among each group increased. On day 34 (the last day of the experiment), all mice were alive. The mean tumor volume of A549 xenografted mice treated with ad-luciferase ($1.0x10^8$ pfu) followed by GCV administration was 1730.4 mm^3, which was 23 times larger than the initial tumor volume (75.1 mm^3). Intratumoral injection with Δ24RGD ($1.0x10^7$ pfu) alone resulted in a mean tumor volume of 367.8 mm^3 on day 34, which was ~5.2 times larger than the initial tumor volume (70.3 mm^3). Intratumoral injection with ad-HSTK ($1.0x10^8$ pfu) alone resulted in a mean tumor volume of 351.1 mm^3 on day 34, which was ~5.0 times larger than initial tumor volume (70.0 mm^3). The ad-HSTK group showed similar tumor suppression effect with the Δ24RGD group. In these single treatment groups, the initial tumor mass grew ~5 times after 34 days. However, Δ24RGD + ad-HSTK treatment induced stronger growth suppression than ad-HSTK alone treatment ($p<0.05$) or Δ24RGD alone treatment ($p<0.05$). The initial tumor volume (58.5 mm^3)

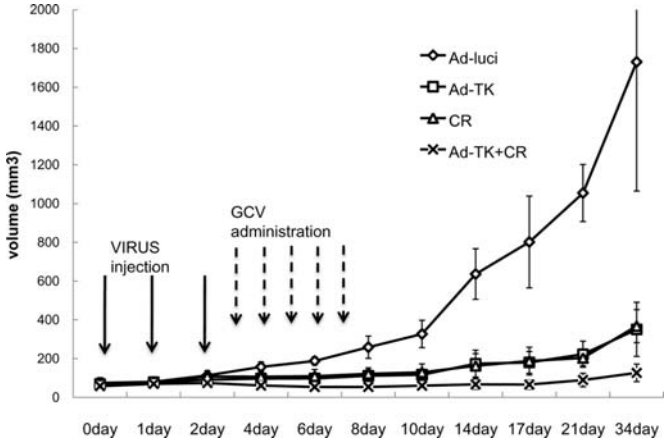

Figure 6. Enhanced *in vivo* tumor suppression by combining Δ24RGD and ad-HSTK. Ten days after subcutaneous injection of A549 cells into BALB/c mouse ($2x10^6$ cells per mouse), ad-luc ($1.0x10^8$ pfu), Δ24RGD ($1.0x10^7$ pfu), ad-HSTK ($1.0x10^8$ pfu), and Δ24RGD ($1.0x10^7$ pfu) + ad-HSTK ($1.0x10^8$ pfu) (8 mice per group) were injected intratumorally for 3 days. On the day following the final intratumoral injection of each virus, ganciclovir (100 mg/kg) was injected into the peritoneum once for 5 days. The Δ24RGD injected group demonstrated modest tumor growth suppression. The Ad-HSTK + GCV group showed a similar tumor suppression effect to Δ24RGD group. The Δ24RGD + ad-HSTK treatment induced stronger growth suppression than ad-HSTK ($p<0.05$) or Δ24RGD ($p<0.05$) (Ad-TK, ad-HSTK; CR, Δ24RGD).

showed growth up to ~2 times (126.8 mm^3) after 34 days in the mice treated with Δ24RGD + ad-HSTK followed by GCV administration (Fig. 6).

Immunohistochemical staining for HSTK revealed that little HSTK expression was found in tumor mass treated with ad-HSTK (Fig. 7A and B). In contrast, strong expression of HSTK was found in the cytoplasm of tumor cells treated with ad-HSTK and Δ24RGD (Fig. 7C and D). This finding reconfirmed the continued replication of ad-HSTK in tumor mass by the aid of Δ24RGD.

Discussion

Conventional replication-defective adenoviruses have low transduction efficiency because of limited infectivity and the probability of dose-rated vector toxicities in clinical trials. However, CRAD represent a promising new option for the treatment of malignant diseases, including non-small cell lung cancer. The virus replicates selectively in the infected tumor cells after initial infection, and kills the cells by oncolysis. The progeny virions then infect a new population of surrounding target cells, replicating again and eradicating the infected tumor cells without affecting normal cells. However, two main limitations remain to successful clinical application of these CRAD agents, poor infectivity and poor tumor specificity.

An armed therapeutic CRAD designed to express a therapeutic gene inserted into the E1 or E3 region of the adenovirus would appear ideal due to the oncolytic potential and antitumor effect of its therapeutic gene. However, armed therapeutic CRADs are limited in their therapeutic gene packing and low gene transfer potentials. In addition, it has been suggested that the presence of E3 augments the oncolytic

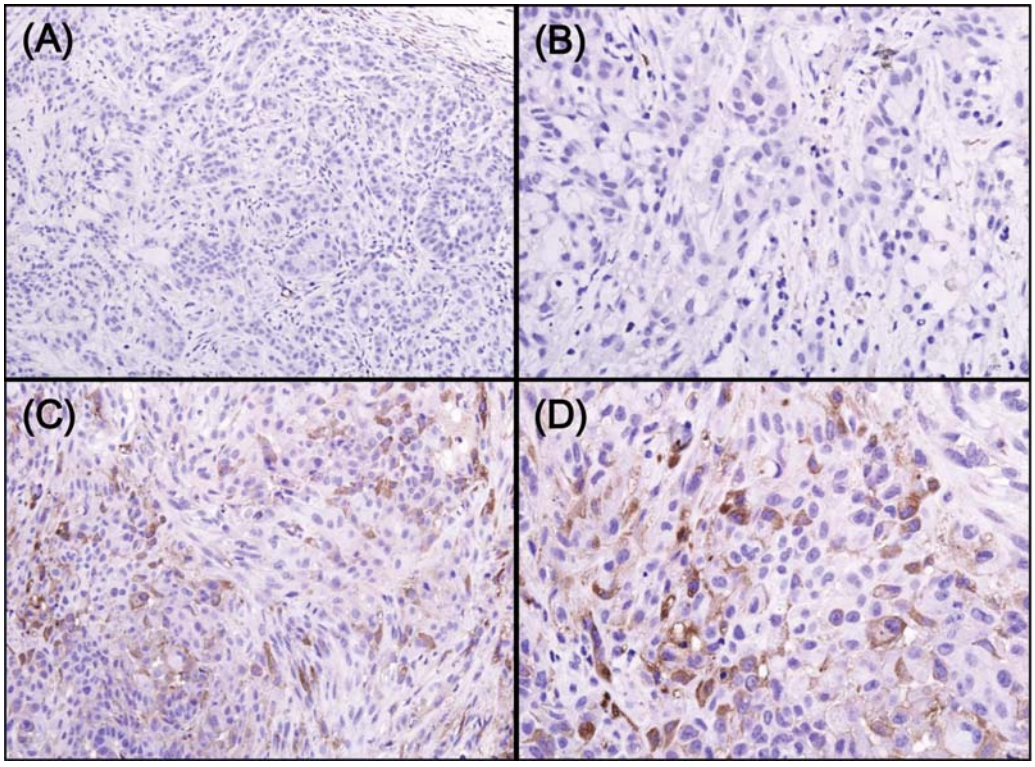

Figure 7. Immunohistochemical staining for HSTK in tumor mass treated with ad-HSTK or ad-HSTK + Δ24RGD. Tumor masses from the mice of treatment experiment were excised at day 34. Immunohistochemical staining for HSTK by anti-rabbit polyclonal antibody to HSTK were performed. (A and B) HSTK was not detected in the tumor cells in tumor mass treated with ad-HSTK only (A: x200, B: x400). (C and D) Positive immunoreactivity of HSTK in the cytoplasm of tumor cells in tumor mass treated with ad-HSTK and Δ24RGD (C: x200, D: x400). No HSTK expression was found in tumor masses treated with ad-luc or Δ24RGD (data not shown).

potency of CRAD *in vitro* and *in vivo* (18). Therefore, they reasoned that the conservation of E3 will certainly induce a significant increase in the antitumor efficacy of CRADs. Early death of infected cells by therapeutic gene products may inhibit the replication of CRAD and the problem of low gene transfer rate remains unsolved.

In this study, we used the gene therapy strategy of combining CRAD and a replication-defective adenovirus with a therapeutic gene to enhance tumor transduction by *in situ* amplification, allowing the initial viral inoculum to spread within tumor cells. The theoretical background of this strategy is that the propagation of replication-defective adenoviruses carrying a therapeutic gene within tumor cells can be induced by using the E1 protein from the cotransduced CRAD. In our previous studies (11,12), we demonstrated the improved gene transduction efficiency gained by combining CRAD and replication-defective adenovirus with a therapeutic gene.

We compared the two CRAD strategies: armed therapeutic adenovirus (Δ24-luc) versus combination CRAD (Δ24) and E1-deleted adenovirus (ad-luc) by measuring luciferase expression in cancer cells. As shown in Fig. 1, the luciferase expression in Δ24 + ad-luc was ~100 times higher than that in Δ24-luc. This could be taken as indirect evidence that a combination of CRAD + E1-deleted adenovirus with the therapeutic gene will be more effective than an armed therapeutic adenovirus, at least where therapeutic gene production is concerned.

We attempted to reconfirm the usefulness of combination gene therapy utilizing a suicide gene/GCV system. We used Δ24RGD as a CRAD and ad-HSTK as a replication defective adenovirus carrying the therapeutic gene.

We reconfirmed this effect by demonstrating an increase in the transduction of lung cancer cells with ad-HSTK and Δ24RGD coadministered *in vitro* (Fig. 3). The expression of HSTK in 10 moi ad-HSTK alone was not appreciably different than the expression of HSTK in 1 moi ad-HSTK + 1 moi Δ24RGD. In other words, in combination with CRAD, small amounts of ad-HSTK administration showed a similar effect to large amounts of ad-HSTK administration. This is indirect evidence of ad-HSTK replication aided by CRAD. Increased production of HSTK by cells enhanced the sensitivity to GCV (Fig. 4), and supported the therapeutic advantage of this combination over a single transduction.

The presence of intact ad-HSTK in the media from the cells transduced with Δ24RGD was confirmed by media transfer experiment (Fig. 5A) (11). This phenomenon implies the release of ad-HSTK from the lysis of CRAD and ad-HSTK cotransduced cells and its lateral spread to adjacent cells. This feature forms the theoretical basis of the media transferable bystander effect observed.

The Ad-HSTK/GCV system had already showed the bystander effect which involved gap junctional intercellular communication (GJIC). GJIC is directly involved in the transfer of the toxic metabolites of ganciclovir, which pass directly from the herpes simplex virus thymidine kinase expressing cells to adjacent cells that do not express it (16,19-21). In other words, GJIC is considered as a mediator of the bystander effect in the ad-HSTK/GCV system. The level of GJIC *in situ* in tumors is predictive of the efficacy of the bystander effect (22). However, many cancer cell lines are defective for GJIC. The bystander effect witnessed in our study employed a different mode of action compared to the bystander effect through GJIC. Rather than passage of toxic metabolites through GJIC being involved, the efflux of intact virus from cell to media accounted for our effect. Ad-HSTK alone did not express any HSTK in media transferred uninfected cancer cells. This means that ad-HSTK replicated only with coadministration of CRAD, and was released from initially infected cells.

The effectiveness of our strategy was confirmed again *in vivo*. Combined injection of CRAD and ad-HSTK followed by GCV almost completely eradicated pre-existing lung cancer xenografts (Fig. 6). Especially, strong expression of HSTK was still found in tumor masses treated with ad-HSTK + Δ24RGD, however, almost no expression of HSTK was found in tumor masses treated with ad-HSTK only at the end of treatment experiment (day 34) (Fig. 7). This phenomenon was consistent with our hypothesis that ad-HSTK continued to replicate in tumor mass by the help of Δ24RGD and produced HSTK for a long time. This would be the mechanism of the superiority of combination strategy. If we injected GCV for a long time, we would get better antitumor effect in ad-HSTK + Δ24RGD treated mice.

In the previous studies (9,23), CRAD armed with HSTK resulted in a striking improvement in antitumor efficacy. In the present study, we did not compare the combined transduction of Δ24RGD and ad-HSTK with Δ24RGD expressing HSTK. A direct comparison study will be needed even though we demonstrated that Δ24 + ad-luc causes a greater increase in luciferase production than Δ24-luc.

In conclusion, we confirmed that the combination of a CRAD (Δ24RGD) and an E1-deleted adenovirus expressing a therapeutic gene (ad-HSTK) was an extremely effective inhibitor of tumor growth suppression of established lung cancer xenografts. These findings suggest that a combination therapy of Δ24RGD and an ad-HSTK/GCV suicide gene system may be useful for lung cancer therapy.

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