In vivo Imaging of Adenovirus Transduction and Enhanced Therapeutic Efficacy of Combination Therapy with Conditionally Replicating Adenovirus and Adenovirus-p27

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Abstract
Gene therapy is hampered by poor gene transfer to the tumor mass. We 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. Mutant E1 expressed by Δ24RGD enables the replication of replication-defective adenoviruses in tumors when cancer cells are co-infected with both viruses. In this study, gene transfer rates in xenografts tumors were monitored by bioluminescence in cells infected with the replication-defective adenovirus-luciferase (ad-luc). Tumor masses treated with CRAD + ad-luc showed dramatically stronger and more prolonged luciferase expression than ad-luc-treated tumors and this expression spread through the entire tumor mass without significant systemic spread. Transduction with CRAD + replication-defective adenovirus-p27 increased the expression of p27 by 24-fold versus transduction with ad-p27 alone. Treatment of a lung cancer cell line and of established lung cancer xenografts with CRAD + adenovirus-p27 also induced stronger growth suppression than treatment with either virus alone. These findings confirm the selective replication of E1-deleted adenovirus containing a therapeutic gene due to the presence of mutant E1 produced by Δ24RGD in tumors. Moreover, this replication increased the therapeutic gene transfer rate and enhanced its antitumor effects. (Cancer Res 2006; 66(1): 372-7)

Introduction
Low gene transfer rates in human cancers present a major obstacle to the efficacy of gene therapy. In human tumor masses, it is difficult to transfer therapeutic genes to tumor cells given the gene transfer vectors currently available. The intratumoral dispersion of replication-defective viral vectors is usually confined to the injection site and has produced impractical and ineffective clinical trial results.

A conditionally replicating adenovirus (CRAD) that can selectively replicate in tumor cells has been developed to overcome this problem (1–3). This antitumor effect of CRADs depends on the oncolysis effect of adenovirus replication in tumor cells and the dissemination of adenovirus through the tumor mass. However, CRAD-based clinical studies have failed to meet expectations although some positive responses have been recorded (4–6). It seems that the majority of these viruses cannot carry a therapeutic gene due to size constraints. Furthermore, the genetic heterogeneity of tumors presents a problem as typically CRAD can only replicate in p53-mutated (7) 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 (COX2) promoter].

The new CRAD strategy described here involves combining 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 when cotransduced with a CRAD capable of supplying E1 in trans. The resulting selective production of large numbers of the therapeutic adenovirus in situ within a tumor mass could transduce neighboring tumor cells and increase overall transduction efficiency (8).

This basic concept of trans-complementation was first reported with the demonstration that transduction with an E1-deleted adenovirus and transfection of plasmid containing E1 (pE1) in a cell induced the production of E1-deleted adenovirus and amplified viral transgene expression (9, 10).

Trans-complementation of two replication-defective adenoviral vectors with missing different genes was reported. Alemany et al. (11) showed that two replication-defective adenoviruses generated by splitting adenoviral genome complemented each other and replicated in certain tumor cells. Cotransduction of GT5610, an adenovirus containing minimal adenoviral genome for replication with E1, and AdH15, an adenovirus containing most adenoviral genome except E1, induced the coreplication of both and led to cell lysis. Wolkersdorfer et al. (12) combined AV.C2.TK, an E1-deleted nonreplicating adenovirus expressing herpes simplex thymidine kinase, and Ad5.d1014, an E4-deleted/E4orf4-only expressing adenovirus in malignant melanoma. Both E1-deleted virus and E4-deleted virus replicated in cancer cells by transcomplementing adenoviral gene products.

Habib et al. (13) showed that transduction with a replication competent adenovirus + replication-defective adenovirus containing reporter genes induced the replication of reporter viruses. In our previous study (8), we used a CRAD designated Δ24RGD, which...
produces a mutant E1 protein without the ability to bind Rb but which retains viral replication competence. Theoretically, this E1 can permit viral replication in cancer cells with a defective pRB/p16 pathway. To produce a replication-defective therapeutic adenovirus with an E1 deletion, we combined adenoviruses expressing a dominant negative insulin-like growth factor I receptor (IGF-IR; ad-IGF-IR dn) with a stop codon at 950 or 482 to block IGF-I expression (14) and found that combined transduction with Δ24RGD and a dominant negative IGF-IR adenovirus increased transduction efficiency and therapeutic efficacy versus either approach in isolation in lung cancer xenografts (8). The theoretical advantage of this combination is that E1-deleted adenovirus with any therapeutic gene can be applied with a CRAD.

In this study, we combined an adenovirus expressing p27 [a known cell cycle–dependent kinase (CDK) inhibitor] with Δ24RGD in a lung cancer xenografts model. In addition, we visualized the distribution of transduced gene expression by noninvasive bioluminescent optical imaging techniques in living animals (15).

Materials and Methods

Recombinant adenoviruses. Δ24RGD, kindly provided by David T. Curiel (Gene Therapy Center at the University of Alabama, Birmingham, AL), contains a 24-bp deletion in the CR2 region of E1A and an RGD-4C modification of the fiber gene of E3. The CR2 domain is responsible for binding pRB, which allows adenovirus-infected cells to enter the S phase. Therefore, adenoviruses with a deletion in this region can replicate only in the cells with defects in the pRB/p16 pathway where this binding is not necessary. Furthermore, the RGD-4C motif in the E3 region enables this virus to infect cells without binding to CAR (16).

We previously described the construction of adenoviruses expressing mutant p27 (ad-p27; ref. 17). Briefly, the cDNA of human p27 containing a mutation of Thr-187/Pro-188 (ACGCCC) to Met-187/Ile-188 (ATGATC) was subcloned into the KpnI and BamHI sites of the polylinker pAC CMV plpA (kindly provided by Robert Gerard, University of Texas Southwestern Medical Center, Dallas, TX). Adenovirus-p27 was generated by cotransfecting pAC CMV-p27mt and pJM17 into 293 cells. This ad-p27 was driven by CMVIE promoter and was replication-defective due to E1 deletion. Adenovirus-luciferase (ad-luc) and adenovirus without the therapeutic gene (ad-null) were also driven by CMVIE promoter and replication-defective due to E1 deletion.

Changes in the in vitro expression of luciferase by cotransduction with Δ24RGD. Human lung cancer cells (NCI H460) were seeded into a 24-well plate (3 × 10^3 per well). After incubation for 18 hours, cells were transduced with ad-luc [1 multiplicity of infection (m.o.i.)] from the first to the fifth lane. Cells from the second to fifth lane were cotransduced with 0.1, 0.5, 1, and 3 m.o.i. of Δ24RGD whereas cells in the first lane were transduced with ad-luc only and those in the sixth lane were transduced with Δ24RGD only. In vitro luciferase imaging was done by scanning (LAS-3000, 2-minute acquisition) 2 minutes after Bright Glo inoculation.

Changes in the transduction efficiency of ad-luc caused by Δ24RGD cotransduction by in vivo tumor imaging. Lung cancer xenografts were established by s.c. injecting human lung cancer cells (NCI H460, 1.0 × 10^6 per site) into nude mice. Four tumor masses were established in each animal (right and left shoulders and right and left thighs). Two weeks after injection, when tumor masses had reached 10 mm in diameter, tumor masses were treated with a combination of CRAD and AD-p27. The combination of CRAD with Δ24RGD caused a dose-dependent increase in luciferase expression in lung cancer xenografts models. In this study, we observed that the combination of CRAD and Δ24RGD caused a 2-fold increase in luciferase expression in lung cancer xenografts models. In addition, we visualized the distribution of transduced gene expression by noninvasive bioluminescent optical imaging techniques in living animals (15).

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diameter, adenoviruses were injected. PBS, Δ24RGD [1.0 × 10^7 plaque-forming units (pfu)], and ad-luc (1 × 10^7 pfu) were injected into tumor masses on the left shoulder, right shoulder, and left thigh, respectively, and a combination of Δ24RGD (1 × 10^7 pfu) and ad-luc (1 × 10^7 pfu) was injected into right thigh mass. On days 2, 5, and 14, D-luciferin was injected into the peritoneum, and 10 minutes after injection, tumor imaging was done using a charged-coupled device camera (LAS-3000) for 10 minutes (10-minute acquisition). Ratios of the luciferase activities of Δ24RGD + ad-luc injected tumors and ad-luc injected tumors in individual mice were calculated.

Changes of p27 expression in ad-p27 transduced lung cancer cell due to Δ24RGD cotransduction. NCI H460 (human lung cancer cell line) was transduced with ad-p27 alone, Δ24RGD, or Δ24RGD + ad-p27. Twenty-four hours after transduction, we compared the production of p27 protein in cells by Western blotting.

Changes in cell growth after cotransduction with ad-p27 and Δ24RGD. NCI H460 cells (a human lung cancer cell line) were plated into six-well plates and transduced with ad-p27 (1 m.o.i.), Δ24RGD (0.1 m.o.i.), or Δ24RGD (0.1 m.o.i.) + ad-p27 (1 m.o.i.). Cell numbers were counted daily using a hemocytometer and their survivals were compared against untransduced controls.

Combined treatment with Δ24RGD and ad-p27 in animal tumor models. Finally, we examined the antitumor effect of Δ24RGD + ad-p27 in lung cancer xenografts established by injecting NCI H460 (1 × 10^6 per mouse) into s.c. tissue. Seven days later, ad-null (1.0 × 10^7 pfu), Δ24RGD (1.0 × 10^7 pfu), ad-p27 (1.0 × 10^7 pfu), Δ24RGD (1.0 × 10^7 pfu) + ad-null (1.0 × 10^7 pfu), and Δ24RGD (1.0 × 10^7 pfu) + ad-p27 (1.0 × 10^7 pfu) were injected intratumorally and this was repeated for 5 days.

Tumor sizes were measured using the formula (0.5 × length × width^2). Tumor growths were analyzed using the repeated ANOVA test.

Results

Increased expression of luciferase in ad-luc transduced lung cancer cells due to Δ24RGD cotransduction. Luciferase expression from cells transduced with a low dose of ad-luc (1 m.o.i.) was low (lane 1) and almost the same as that of Δ24RGD transduced cells (containing no ad-luc; lane 6). However, cotransduction with Δ24RGD increased luciferase expression in a dose-dependent manner in the range 0.1 to 3 m.o.i. (Fig. 1).

Strong and prolonged expression of luciferase was observed in tumors coinfected with Δ24RGD and ad-luc. Figure 2 shows increased luciferase expression in vivo in tumor masses treated with Δ24RGD + ad-p27. No luciferase expression was observed in two tumor masses treated with PBS (mass on left shoulder) or Δ24RGD (mass on right shoulder). Injection with ad-luc caused very limited luciferase transfer into tumor masses (i.e., weak and focal signals). However, injection with ad-luc + Δ24RGD produced strong prolonged luciferase expression for up to 2 weeks. This expression was observed throughout the tumor masses but without evidence of systemic spread, a very important characteristic of this combination therapy with respect to potential clinical application. This was true up to 2 weeks after transduction when the animals needed to be sacrificed due to large tumor burdens.

Increased p27 expression in ad-p27 + Δ24RGD transduced cells. Western blots for p27 showed that cotransduction with Δ24RGD + ad-p27 markedly increased p27 production. The amount of p27 in Δ24RGD + ad-p27 cotransduced cells was 24-fold that of ad-27 transduced cells at the same ad-p27 dose (Fig. 3).

Enhanced growth suppression of lung cancer cells by ad-p27 + Δ24RGD cotransduction in vitro. A single transduction with ad-p27 (1 m.o.i.) failed to show significant growth suppression versus the control. A single transduction with Δ24RGD (0.1 m.o.i.) showed moderate growth suppression initially but induced complete oncolysis at day 7. Transduction with ad-p27 + Δ24RGD
produced more rapid and stronger growth suppression than \( \Delta 24 \text{RGD} \) alone and induced complete oncolysis at day 5 (Fig. 4).

\( \Delta 24 \text{RGD} + \text{ad-p27} \) had a more potent antitumor effect on established lung cancer xenografts \( \textit{in vivo} \). \( \Delta 24 \text{RGD} \) injected intratumorally induced weak growth suppression. The ad-p27-treated group showed moderate growth suppression, which was similar to that shown by the \( \Delta 24 \text{RGD} + \text{ad-null} \)–treated group. However, \( \Delta 24 \text{RGD} + \text{ad-p27} \) treatment induced stronger growth suppression than ad-p27 alone \( (P < 0.05) \) or \( \Delta 24 \text{RGD} + \text{ad-null} \) \( (P < 0.05) \). Forty percent of \( \Delta 24 \text{RGD} + \text{ad-p27} \)–treated mice showed complete regression of established tumors (Fig. 5).

Discussion

Disappointing gene therapy outcomes are primarily caused by poor gene transfer rates in human tumors. Vectors that are highly effective in culture result in minimal effects in tumors \( \textit{in vivo} \). CRAD was developed to selectively induce oncolysis and to induce adenoviral replication in tumors. However, although CRAD improves gene transfer in human tumors, its antitumor effects in human tumors have not been impressive.

To potentiate the antitumor effect of CRAD, several modifications have been introduced. CRAD carrying therapeutic genes such as herpes simplex thymidine kinase (18, 19), uracil phosphoribosyltransferase (20), and \( p53 \) (21) in its E1 or E3 region showed substantially enhanced antitumor effects. CRADs that can replicate in cells expressing specific proteins have also been introduced. For example, CRAD containing a telomerase-derived promoter selectively replicated and destroyed telomerase-positive cancer cells (22, 23) and CRAD containing a COX2 promoter produced a promising result in \( \textit{in vivo} \) tumor models (24, 25).

This study proposes a novel and straightforward way of increasing the therapeutic gene transfer of replication-defective adenovirus by combining with CRAD. As we previously showed, CRAD produces mutant E1 in pRb/p16–inactivated cancer cells enabling the replication of an E1-deleted adenovirus if both viruses were transduced in the same cancer cell again. E1-deleted adenovirus will selectively replicate in pRb/p16–inactivated cancer cells and spread through the tumor mass.

Gene transfer \( \textit{in vivo} \) is not easily monitored but advances in molecular imaging may enable real-time noninvasive monitoring (15). Several gene products have been used for tumor imaging \( \textit{in vivo} \) and firefly luciferase and green fluorescent protein are most commonly used for this purpose. Moreover, bioluminescence from cells transduced with luciferase can easily be monitored using a charged-coupled device camera after injecting D-luciferin, a luciferase substrate. Luciferase reacts with D-luciferin in the presence of ATP and oxygen to produce light that can be detected using a charged-coupled device camera (26). The \( \textit{in vivo} \) distribution of adenovirus achieved by mediated gene transfer
therapy has been previously monitored by bioluminescence using luciferase gene transduced cells (27).

We adopted this technique to show the replication of ad-luc in tumor masses and to monitor the intratumoral and systemic viral transduction distributions and the duration of therapeutic gene expression. In Fig. 2, a single injection of ad-luc into the tumor mass showed weak, localized, and transient luciferase expression and, 14 days later, little luciferase activity remained. This finding partly explains why current gene therapies have failed to show a significant response in clinical trials. In contrast, tumors injected with ad-luc + Δ24RGD showed strong long-term activity for up to at least 2 weeks. Furthermore, luciferase activity was found throughout the tumor mass, showing ad-luc spread. Another important finding was the absence of luciferase activity in other organs in a mouse model. These findings support the value and safety of this gene therapy combination.

The p27 is a CDK inhibitor of the Cip/Kip family and participates at many points within the cell cycle (28). The main action of p27 arises from its binding with cyclin E/Cdk2 complex and its induction of the dephosphorylation of pRb (29, 30). Most non-small cell lung cancer cell lines contain inactivated p16 and intact pRb and are effectively killed by ad-p27 (17, 31) and by armed CRAD. The intracellular level of p27 is mainly regulated by its phosphorylation at Thr-187, which targets p27 for ubiquitination and proteosomal degradation (32, 33). We previously reported the development of an adenovirus expressing p27 with a mutation at Thr-187, this adenovirus expressing a stable mutant p27 has a more potent antitumor effects (via cell cycle arrest and apoptosis) than an adenovirus expressing wild-type p27 (17). This effect was later confirmed in lung cancer (17, 31), pancreatic cancer (34), and malignant glioblastoma (35). Thus, this adenovirus was used in the present study.

Transduction of ad-p27 + Δ24RGD into lung cancer cells was found to up-regulate p27 versus ad-p27-only transduced cells infected with identical doses of ad-p27. Moreover, treatment of established lung cancer xenografts produced dramatically improved therapeutic efficacy. Treatment with CRAD + ad-p27 suppressed tumor growth significantly more than CRAD, ad-p27, or CRAD + ad-null.

The lack of direct comparison of a CRAD + E1-deleted adenovirus with a therapeutic gene with a CRAD containing therapeutic gene in E1 or E3 region (armed CRAD) is the limitation of this study. Armed therapeutic adenovirus is limited with respect to the size of the inserted gene. In addition, Suzuki et al. (36) suggested that deletion of E3 for therapeutic gene insertion may decrease the oncolytic potency of CRAD. However, further study will be needed to directly compare the potencies of a CRAD + E1-deleted adenovirus with a therapeutic gene with armed CRAD.

In spite of this limitation, this study reconfirms the value of combination strategies based on CRAD and E1-deleted adenovirus containing a therapeutic gene by in vivo tumor imaging techniques in a lung cancer model and suggests that this strategy may be useful for therapies based on any E1-deleted recombinant adenovirus.

Acknowledgments

Received 5/3/2005; revised 8/22/2005; accepted 10/12/2005.

Grant support: Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea, grant 03-P1-PG3-2000-0048 and M 10334040003-GN340-00310 by KOSEF (C-T. Lee), the Vanderbilt Specialized Program of Research Excellence in Lung Cancer grant CA90949 (D.P. Carbone), and NIH grants R01 CA088321 and R01 CA094084 (D.T. Curiel).

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