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PRINCIPAL INVESTIGATOR: David T. Curiel, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294

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David T. Curiel, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Alabama at Birmingham
Birmingham, Alabama 35294

E-Mail: david.curiel@ccc.uab.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have been developing conditionally replicative adenoviral agents (CRADs) for prostate cancer gene therapy. We have sought to improve these agents by increasing their infectivity for tumor targets and by enhancing the specificity of their replication exclusively within tumor targets.

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A. INTRODUCTION

Replicative adenoviral vectors (CRAD) represent a promising therapeutic approach which has been applied recently in context of cancer of the prostate. As for the non-replicative cancer gene therapy approaches, however, the efficacy of the replicative strategies is subservient to vector-mediated tumor transduction. In this regard, one of the attractive features of the CRAD approach is that it capitalizes on the unparallel efficiency of adenoviral vectors (Ad) in accomplishing *in vivo* transduction. Indeed, of all of the currently available vector approaches, Ad vectors possess the highest capacity to achieve *in situ* transduction of tumor. Despite this capacity, overall efficacy of Ad-based cancer gene therapy approaches remain limited by suboptimal vector efficiency. Of note, human trials carried out to date have demonstrated relatively inefficient gene transfer to tumor achieved by Ad vectors employed in *in vivo* delivery schemas. This has been understood to result from a relative paucity of the primary adenovirus receptor, coxsackie-adenovirus receptor (CAR), on tumor cells relative to their cell line counterparts. Indeed, a relative paucity of CAR has been shown to limit Ad vector efficacy in a number of tumor contexts, possibly representing a fundamental practical barrier to realizing the full benefit of this vector system for cancer gene therapy applications. On this basis, it has been proposed that gene delivery via "CAR-independent" pathways may be required to circumvent this key aspect of tumor biology. Especially noteworthy in this regard, has been the observation in the Onyx trials of transductional-barriers limiting overall efficacy of CRAD-based replicative approaches. Thus, it is clear that augmenting the gene transfer efficacy of Ad vectors is essential to deriving their full benefit in the context of the conceptually promising CRAD strategies. To this end, we have developed novel approaches to alter the tropism of Ad vectors such that CAR-independent gene transfer may be achieved. Such CAR-independent gene transfer allows dramatic augmentations of the gene transfer efficacy of the Ad vector, especially in context whereby CAR levels are limiting. We thus propose to develop such modifications in the context of CRAD to improve its infection efficacy for tumor cells. We hypothesize that such modifications will overcome biological limits of tumor noted in human clinical trials and thereby allow full realization of the potential benefits of the CRAD approach. The demonstration of this principle, in model systems, would allow a rapid translation of this strategy into the human clinical context for prostate cancer therapy.

B. BODY

Preliminary work in years 1-2 of this grant accomplished the key concepts that:
(1) Genetic capsid modification allowed trophism modification of adenovirus (Ad) (2) trophism modified Ad could accomplish gene transfer via cellular pathways distinct from the native Ad receptor coxackie-and adenovirus receptor (CAR) (3) CAR independent re-routing of Ad could enhance target cell infectivity (4) infectivity enhancement augmented the potency of conditionally replicative adenovirus agents (CRAds) and (5) tumor/tissue selective promoters with the desirable phenotype "tumor on /liver off" could be identified for possible utility in deriving a prostate cancer CRAd. These studies established the conceptual and practical basis of deriving a prostate cancer CRAd which embodied the key attributes of efficient tumor cell infection and tumor selective replicative capacity.

These earlier studies had established that infectivity enhancement could be achieved via either: (1) fiber knob serotype chimerism by replacing the type 5 knob with the fiber knob of serotype 3 and (2) incorporation of the integrin-binding peptide RGD4C within the HI loop of the fiber knob. In addition, promoters deriving from the cyclooxygenase-2 (cox-2) gene and the vascular endothelial growth factor (VEGF) gene were shown to embody useful inductivity/specificity characteristics. On this basis CRAd agents were derived whereby infectivity was enhanced via one of the two approaches noted above and replicative specificity controlled via the cox-2 or VEGF promoters. In this latter regard, replicative specificity was achieved via placing the adenovirus E1 gene under the transcriptional control of the aforementioned promoter.

The infectivity enhanced CRAd ("IE CRAds") were validated in *in vitro* and *in vivo* systems for efficacy and safety. In the former regard, IE CRAd of the cox-2 and VEGF type both exhibited tumor selective toxicity which correlated with target cell expression of the tumor selective marker. Specificity of target cell killing was validated with control cells which lacked such expression. Of note, infectivity enhancements of both types (3/5 fiber knob chimerism, fiber knob HI loop additions) enhanced the oncolytic potency of the CRAd agent.

In vivo studies in murine xenograft systems confirmed the anti-tumor efficacy of the CRAd agents. Again, studies in the *in vivo* context highlighted the efficacy gains accrued by virtue of the infectivity enhancement maneuvers. Toxicity studies confirmed that the CRAds were restricted with respect to viral gene expression in the liver. Further, an overall profile compatible with clinical safety was suggested by these studies.

Our studies have thus resulted in the realization of novel CRAd agents with utility parameters commensurate with prostate cancer therapy. Specifically, we have augmented CRAd potency via infectivity enhancement maneuvers and optimized selectivity via tumor specific promoters. We have validated these properties of our IE CRAds in *in vitro* and *in vivo* systems and we have established the safety profile our or new agent. In the aggregate, these findings have allowed the realization of an optimized CRAd agent for prostate cancer therapy.

C. KEY ACCOMPLISHMENTS

- We have shown that the receptor of Ad serotype 3 can be exploited to achieve infectivity enhancement of tumor targets.

- We have shown that CRAd agents which achieve infection via the receptor of Ad serotype 3 can achieve enhanced oncolytic potency.

- We have identified additional promoter elements with potential relevance to the derivation of prostate cancer CRAds.

- We have derived cox-2 and VEGF infectivity enhanced CRAds and demonstrated their utility in *in vitro* and *in vivo* model systems.

- These findings have established the basis of constructing an optimized CRAd agent for prostate cancer which embodies the desired properties of enhanced infectivity and replicative specificity.

D. REPORTABLE OUTCOMES

Haviv YS, Takayama K, Glasgow JN, Blackwell JL, Wang M, Lei X, Curiel DT. A model system for the design of armed replicating adenoviruses using p53 as a candidate transgene. Mol Cancer Ther. 2002 Mar;1(5):321-8.

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Gomez-Navarro J, Curiel DT. Conditionally replicative adenoviral vectors for cancer gene therapy. Lancet Oncol. 2000 Nov;1:148-58. Review.

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Yamamoto Y, Davydova J, Wang M, Siegal GP, Krasnykh V, Vickers SM, Curiel DT. Infectivity enhanced, cyclooxygenas-2 promoter based conditionally replicative adenovirus for pancreatic cancer. Submitted, 2002.

Takayama K, Reynolds PN, Adachi Y, Kaliberova L, Krasnykh V, Nakanishi Y, Curiel DT. VEGF promoter based conditionally replicative adenovirus for pan carcinoma application. Submitted 2002.

Adachi Y, Takayama K, Reynolds PN, Grizzle WE, Pereboeva L, Curiel DT. Augmentation of the efficacy of anti tumor replicating adenovirus via inhibitition of TGF-B signaling. Submitted, 2002.

Adachi Y, Wang M, Takayama K, **Curiel DT** and Reynolds PN. Deletion of E1B 19k improves the efficacy of a midkine-promoter based conditionally replicative adenovirus for neuroblastoma cells. Submitted, 2002.

E. PRESENTATIONS

American Association for Cancer Research, 93rd Annual Meeting, Forum, Cancer Gene Therapy, Session Moderator, San Francisco, California, April 6-10, 2002.

American Association for Cancer Research, 93rd Annual Meeting, Minisymposium, Gene Therapy, Session Chair and Invited Speaker, San Francisco, California, April 6-10, 2002.

American Association for Cancer Research, 93rd Annual Meeting, Symposium, Cell and Gene Therapy, Invited Speaker, San Francisco, California, April 6-10, 2002.

The American Society of Gene Therapy, 5th Annual Meeting, Scientific Symposium, Cancer-Oncolytic Vector and Replicative Systems, Chair and Invited Speaker, Boston, Massachusettes, June 5-8, 2002.

The American Society of Gene Therapy, 5th Annual Meeting, Meet-the-Expert Luncheon, "Targeting Strategies to Adapt Adenovirus for Clinical Applications", Boston, Massachusetttes, June 5-8, 2002.

10th SPORE Investigators' Workshop, Preclinical and Clinical Models for Therapy, Chantilly, Invited Speaker, Chantilly, Virginia, July 13-16, 2002.

10th SPORE Investigators' Workshop, Clinical Trial Design Challenges, Invited Speaker, Chantilly, Virginia, July 13-16, 2002.

F. PATENTS

None

E. CONCLUSION

Our findings have established key facets of CRAd biology which have direct implications on their design/utility. Specifically, infectivity-enhancement maneuvers substantially improve the potency of these agents and enhance their anti-tumor capacity. In addition, we have defined novel regulatory elements relevant to prostate cancer CRAd design. These elements exhibit an ideal inductivity/specificity profile for the current application. In the aggregate, these advancements have

allowed the derivation of a CRAd agent for prostate cancer with optimized biologic properties predicating anti-tumor efficacy.

APPENDIX A

A Model System for the Design of Armed Replicating Adenoviruses Using *p53* as a Candidate Transgene[1]

Yosef S. Haviv, Koichi Takayama, Joel N. Glasgow, Jerry L. Blackwell, Minghui Wang, Xiaosheng Lei, and David T. Curiel[2]

Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center, The University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract

Cancer gene therapy endeavors to overcome the low therapeutic index of currently available therapeutic modalities via the efficient and safe delivery of genetic material into tumor cells. However, despite promising preclinical results, replication-deficient viral vectors have demonstrated a limited efficacy in the clinical setting. To increase vector efficiency, replication-competent viruses have been proposed. Clinical trials have shown the safety of locally injected, conditionally replicative adenoviruses (Ads) but have underscored the need for improved potency. To further increase the therapeutic effect of replicating viral vectors, armed therapeutic viruses (ATVs) have recently been used for high-efficiency transgene expression. However, interference with cellular signaling and viral production by constitutive transgene expression may be counterproductive for ATV replication, thereby hindering the therapeutic outcome. Consequently, studies are equivocal with regard to the potential benefits of ATVs. To address this issue, we hypothesized that induction of replication of an Ad expressing *p53* may be a useful strategy in the context of ATV because *p53* does not interfere with Ad replication and may even increase its cytolytic effect. We show that in our *in vitro* ATV model system, *E1* transcomplementation of a replication-deficient Ad encoding *p53* resulted in dramatic augmentation of cell killing and circumvented resistance to apoptosis. Correlation was found between the degrees of cell killing and apoptosis induction, rather than with viral burst. Furthermore, both Ad5 *E1B 55kDa* and *E4 orf6* genes were required to enhance the cell killing. In conclusion, our *p53*-ATV model system demonstrates the potential utility of therapeutic transgene expression by a replicating Ad after a rational selection of a candidate transgene.

Introduction

Gene therapy has been suggested as a novel strategy to improve the therapeutic index of cancer therapy. Whereas replication-deficient viral vectors have demonstrated great promise as anticancer agents in preclinical studies, this has not been translated into patient benefit in the clinical setting (1). As a natural extension, replication-competent viruses have been suggested as a means to address the multidimensional biological aspects of tumors (2). To date, replication-competent viruses used in cancer clinical trials have included Ads[3] and, to a lesser extent, HSVs. Whereas replication-competent vectors have been shown to be safe and potentially beneficial for therapy of localized tumors, their potency clearly needs to be improved (3). Therefore, "armed" replicative viruses, incorporating therapeutic transgenes, have been introduced for cancer gene therapy (1, 4, 5). These ATVs embody two potential advantages. First, they exhibit a capacity for up to 3 orders of magnitude higher levels of transgene expression relative to their replication-defective vector counterparts, in selected instances (1) (6). Second, incorporated transgenes may provide a fail-safe mechanism to abolish viral replication by the induction of toxic cell death (6, 7). There are, however, potential limitations to the use of ATVs. In this regard, constitutive gene expression by Ad vectors may interfere with cellular signaling and result in premature cellular toxicity. Consequently, early apoptosis may impair viral replication (8), confounding the antitumor effects linked to oncolysis.

Accordingly, the utility of ATVs for cancer gene therapy is uncertain. In three studies, the inclusion of suicide/prodrug gene therapy with HSV*tk*/GCV in a replicating Ad did not augment antitumor efficacy *in vitro* or *in vivo* (9–11). In contrast, other studies have shown that combined oncolysis, caused by a replicating virus and suicide/prodrug gene therapy with HSV*tk*/GCV, is complementary in improving outcome *in vivo* (4, 5). Furthermore, a replicating Ad with double suicide gene therapy containing the cytosine deaminase/5-FC (*cd*/5FC) and HSV*tk* fusion gene markedly enhanced the CPE relative to the isolated viral effect (7, 12). Of note, the role of E1B 55kDa deletion in the context of ATV is also inconclusive (9, 12).

To address these inconsistencies, we have developed a strategy that induces replication of transgene-expressing, replication-deficient Ad vectors. As a proof of principle, we

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[2] To whom requests for reprints should be addressed, Division of Human Gene Therapy, University of Alabama at Birmingham, WTI 620, 1824 6th Avenue South, Birmingham, AL 35294. Phone: (205) 934-8627; Fax: (205) 975-7476; E-mail: david.curiel@ccc.uab.edu.

[3] The abbreviations used are: Ad, adenovirus; ATV, armed therapeutic virus; HSV, herpes simplex virus; tk, thymidine kinase; GCV, ganciclovir; CMV, cytomegalovirus; MOI, multiplicity of infection; CPE, cytopathic effect.

have selected a replication-deficient Ad vector encoding *p53*. Because the inhibition of viral replication by HSV*tk*/GCV or *cd*/5FC may counterbalance the therapeutic effect of ATV (13), *p53* may be a useful therapeutic transgene in the context of ATV because it does not interfere with Ad replication (14) and may even increase its cytolytic effect (15).

Furthermore, selection of *p53* to indirectly induce apoptosis may circumvent transgene effects that induce apoptosis downstream of *p53* and thus do not allow effective production and lateralization of the Ad vector (16, 17).

To this end, we induced replication of Ad vector encoding *p53* by a variety of Ad mutants and evaluated cell killing and viral kinetics. Our studies show that ATV has a potential for higher cancer cell killing rates *in vitro* in the context of a transgene that is not counterproductive for viral replication.

We further observed that the burst of replicating Ad did not fully correlate with cell killing in the context of ATV. Finally, we found that both the Ad *E1B 55kDa* and *E4 orf6* genes were essential for the enhanced therapeutic effect of ATV encoding *p53*. These findings are highly consequential for an understanding of the efficacy of replicating Ad agents and for a rational design of ATV.

Materials and Methods

Recombinant Ads. A replication-deficient Ad expression vector for the delivery of wild-type human *p53* cDNA has been reported previously (18). This vector expresses human wild-type *p53* under the transcriptional control of the CA promoter comprising a CMV enhancer and the chicken β-actin promoter (AdCA*p53*). Ad338 is an Ad mutant lacking 524 bp within the *E1B 55kDa* gene (19). Ad355 is deleted for the *E4 orf6* gene (20). As a control for AdCA*p53*, we used Ad5luc1, a replication-deficient Ad (*E1*/*E3* deleted) expressing the *luciferase* gene from the *E1* region. As a wild-type equivalent we used Ad5*luc*3, a replication-competent Ad (*E1* intact, *E3* deleted) expressing the *luciferase* gene from the *E3* region. Both these viruses were constructed and propagated in our laboratory.

Cells, Transfections, and Infections. A549 and H460, human lung cancer cell lines with intact *p53*, were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were grown at 37°C in RPMI 1640 with 2 mM L-glutamine, supplemented with 10% fetal bovine serum. Infections were performed 24 h after seeding 2×10^5 cells/well in 12-well plates. For infections, growth medium was replaced by serum-free medium with the index virus at the indicated MOI. An hour later, the infection medium was removed, cells were rinsed with PBS, and 5% fetal bovine serum growth medium was restored. The medium was not replaced thereafter during the experiment and was sampled daily for determination of Ad *E1* or *E4* gene copy numbers.

For transient transfections, cells were seeded on 12-well plates and transfected with the indicated plasmids (1 μg/well) at a confluence in the range of 70%, using the Superfect (Qiagen, Santa Clarita, CA) method, according to the instructions of the manufacturer. Expression vectors used for transfection were constructed as follows; pCMV*E1* was derived from the shuttle plasmid (pShuttle) of the "Adeasy" system (21) by cloning the consecutive Ad E1 region extending from position 489 to 5789 of the Ad genome into the multicloning site, thereby deleting the right arm of pShuttle. Next, the CMV promoter/enhancer was cloned into the *Xho*I and *Eco*RV restriction sites of the recombinant plasmid. pCMV*luc* was derived from cloning of the CMV promoter/enhancer into the mammalian expression vector pGL3 basic vector (Promega, Madison, WI) upstream of the *luciferase* gene.

Apoptosis Assay. To determine whether apoptosis or necrosis was the underlying mechanism of cellular death, we infected A549 cells with AdCA*p53* at a MOI of 20, and 48 h later, we coinfected the cells with either the replication-competent Ad5luc3 or the *E1B 55kDa*-deleted Ad338, at a MOI of 5. Control wells were initially infected with the replication-deficient control virus Ad5luc1 and coinfected 48 h later with Ad5luc3. Forty-eight h after the second infection, cells were harvested, washed in cold PBS, and adjusted for a cell density of $\sim 1 \times 10^6$ cells/ml in PBS.

To detect apoptosis or necrosis, cells were stained with the dyes of the Vybrant Apoptosis Assay (Molecular Probes, Eugene, OR). This assay allows the detection of three groups of cells under a fluorescence microscope equipped with the appropriate filters for fluorescein or rhodamine. Whereas live cells show only a low level of fluorescence, apoptotic cells show green fluorescence, and necrotic cells show both red and green fluorescence (and therefore show yellow fluorescence when merged).

TaqMan PCR Assay. The *E1a* copy number was determined for each medium sample obtained as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue Kit (Qiagen), following the instructions of the manufacturer. The concentration of isolated DNA was determined by spectophotometry. TaqMan primers and probe design, the forward primer, the reverse primer, and the 6-carboxyfluorescein-labeled probe to amplify the *E1a* and *E4* genes were designed by the Primer Express 1.0 software (Perkin-Elmer, Foster City, CA) following the recommendations of the manufacturer. The sequences of the forward and the reverse *E1a* primers were AACCAGTTGCCGTGAGAGTTG (anneals between residues 966 and 986) and CTCGTTAAGCAAGTCCTCGATACAT (anneals between residues 1033 and 1009), respectively, whereas the TaqMan probe was CACAGCCTGGCGACGCCA (anneals between residues 988 and 1006). The sequences of the forward and the reverse *E4* primers were GGAGTGCGCCGAGACAAC (anneals between residues 816 and 833 of the E4 orf6 open reading frame) and ACTACGTCCGGCGTTCCAT (anneals between residues 883 and 865), respectively.

The sequence of the TaqMan probe was TGGCATGACACTACGACCAACACGATCT (anneals between residues 836 and 863). With optimized concentration of primers and probe, the components of real-time PCR mixture were designed to result in a master mix with a final volume of 10 μl/reaction containing 1× Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1 nM probe, and 0.025% BSA. For the assay, 1 μl of extracted DNA sample was added to 10 μl of PCR mixture in each reaction capillary. A no-template control received 10 μl of reaction mixture with 1 μl of water. All capillaries were then sealed and centrifuged using LC Car-

ousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All PCR reactions were carried out using a LightCycler System (Roche Molecular Biochemicals). The thermal cycling conditions were 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

Statistical Analysis. Data were initially tested for normality by the Shapiro-Wilk test. All abnormal tests were further tested for significance by the Wilcoxon scores test. Results are expressed as a mean of at least three samples. Results were considered statistically significant for $P < 0.05$.

Results

Induction of Replication of AdCA*p53* by *E1* Transfection to Determine Cell Killing of ATV. Expression of *p53* has been reported to be detrimental for Ad replication and cellular transformation (22), suggesting that Ad *E1B 55kDa* mutants may not grow in normal tissues (23). In contrast, others have shown that *p53* may in fact be essential for productive Ad infection (15) and that *p53* overexpression does not interfere with Ad replication (14). Based on these considerations, we first determined the effect on viral replication and cell killing of heterologous *p53* expression. In a previous study, we have confirmed that AdCA*p53* expresses *p53*, induces apoptosis, and inhibits the growth of selected lung cancer cell lines *in vitro* and *in vivo* (18).

To evaluate the effect of *E1* transfection on AdCA*p53* replication and cell killing, we first confirmed that at a MOI of 20 plaque-forming units/cell, AdCA*p53* does not replicate or cause significant cell killing. Specifically, A549 cells infected with either the replication-defective Ad5*luc*1 or with AdCA*p53* remained viable for more than 12 days after infection. After this period, cells began to degrade but did not manifest any overt signs of viral CPE. Additionally, Ad *E1a* gene copy levels, as determined by quantitative PCR, were at the background level (data not shown). These results indicate that AdCA*p53* does not replicate and does not cause a significant CPE in A549 cells. Next, we induced replication of AdCA*p53* by transcomplementation with an intact *E1* gene. For this study, A549 cells were plated in each well of 12-well plates. After reaching 70% confluence, cells were transfected in triplicates with either pCMV*E1* or pCMV*luc*.

Twenty-four h later, cells were infected with either Ad5*luc*1 or AdCA*p53* at a MOI of 20. An advanced CPE was observed 3 days after infection only for the *E1*-transfected, AdCA*p53*-infected cohort (Fig. 1*A*). Transfection with pCMV*luc* followed by infection with AdCA*p53* did not induce any CPE, whereas transfection with pCMV*E1* followed by infection with Ad5*luc*1 resulted in delayed and low CPE relative to *E1*-transcomplemented AdCA*p53*. These data suggest that in our *p53*-ATV model, cell killing by *p53* overexpression after induction of replication of AdCA*p53* is more efficient relative to Ad-mediated oncolysis or relative to cell killing of nonreplicating AdCA*p53*. Because previous studies have shown that transgene expression may impair viral oncolysis and that *E1* has an independent apoptotic effect (24), we next evaluated the kinetics of viral replication and burst relative to CPE. To this end, we assayed Ad *E4* gene copies in the medium and found that kinetics of Ad DNA accumulation in the medium of *E1*-transfected, AdCA*p53*-infected cells indicated that viral replication was similar to the viral replication of *E1*-transfected, Ad5*luc*1-infected cells (Fig. 1*B*). Therefore, *p53* overexpression by AdCA*p53* does not significantly inhibit or support Ad replication and burst. These patterns were corroborated by the corresponding intracellular Ad DNA levels (data not shown). To account for enhanced cell killing, a synergistic effect of E1 and p53 proteins, independent of Ad replication, is unlikely because it would be counterproductive for viral replication. Thus, it appears that the enhanced killing effect of *E1*-transcomplemented AdCA*p53*

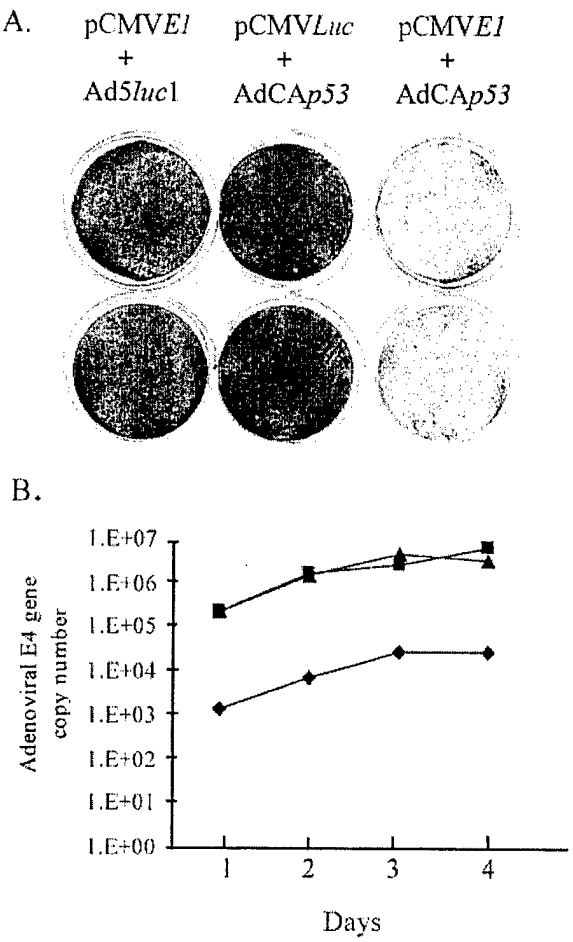

Fig. 1. Transfection of the adenoviral *E1* gene enhances the cell killing effect of AdCA*p53* in an oncolytic-independent fashion. *A*, the human lung adenocarcinoma cell line A549 was transfected in triplicates with the adenoviral *E1* expression vector pCMV*E1* or with a control plasmid expressing luciferase (pCMV*luc*). Twenty-four h later, cells were infected with *E1*-deleted, replication-incompetent adenoviral vectors at a MOI of 20 plaque-forming units/cell. Vectors studied included a control vector expressing the luciferase reporter gene (Ad5*luc*1) and a *p53*-expressing vector (AdCA*p53*). Cells were stained with crystal violet after observation of advanced CPE. *B*, analysis of replication of Ad vectors induced by *E1* transcomplementation. A549 cells were transfected with pCMV*E1* or pCMV*luc* and infected 24 h later with Ad5*luc*1 or AdCA*p53*, exactly as described in *A*. Media samples were collected in triplicates from the different cohorts and subjected to quantitative PCR analysis of Ad *E4* copy number as an index of Ad replication and burst. Experimental groups included pCMV*E1* + Ad5*luc*1 (▲), pCMV*E1* + AdCA*p53* (■), and PCMV*luc* + AdCA*p53* (◆).

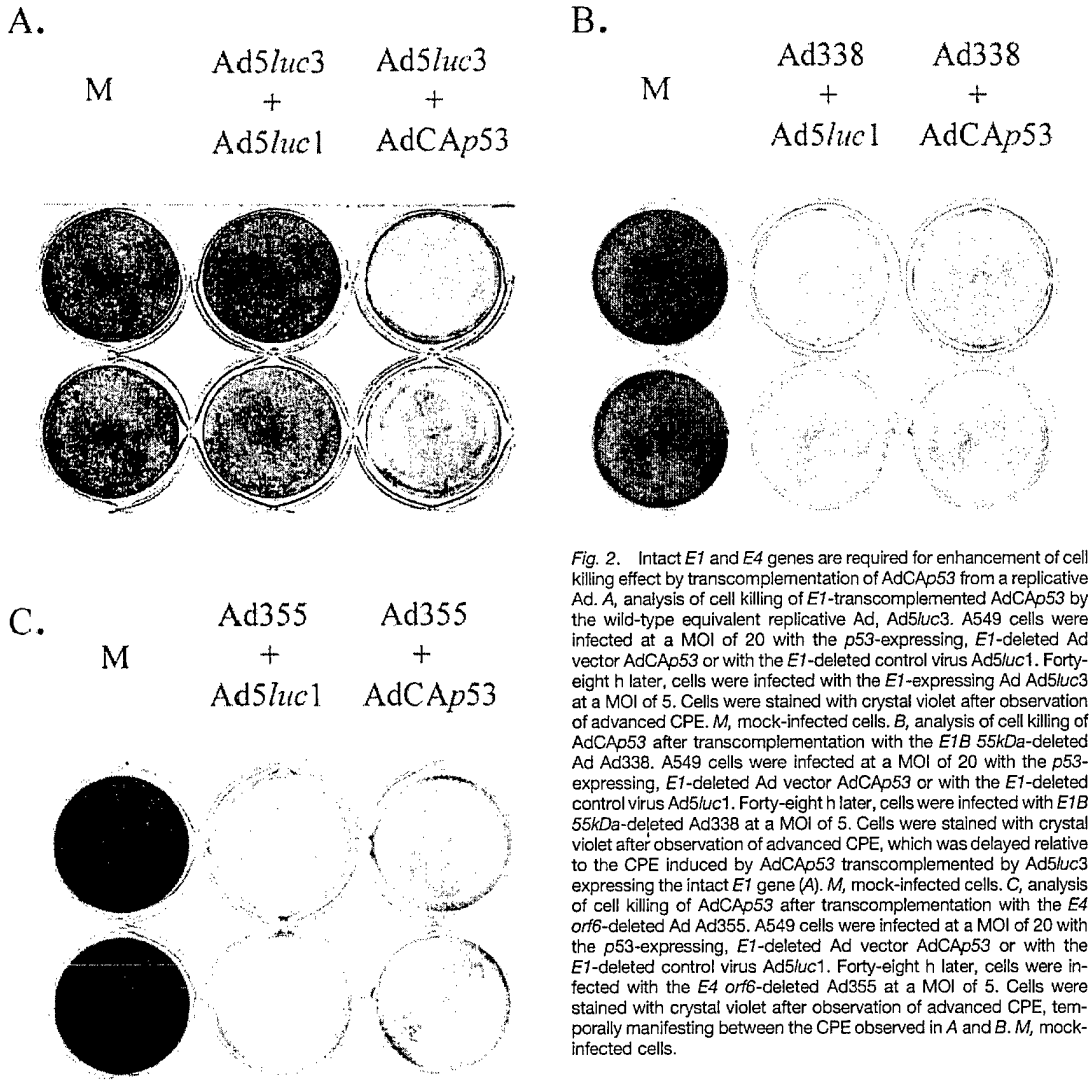

Fig. 2. Intact *E1* and *E4* genes are required for enhancement of cell killing effect by transcomplementation of AdCA*p53* from a replicative Ad. *A*, analysis of cell killing of *E1*-transcomplemented AdCA*p53* by the wild-type equivalent replicative Ad, Ad5*luc*3. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with the *E1*-expressing Ad Ad5*luc*3 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE. *M*, mock-infected cells. *B*, analysis of cell killing of AdCA*p53* after transcomplementation with the *E1B 55kDa*-deleted Ad Ad338. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with *E1B 55kDa*-deleted Ad338 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, which was delayed relative to the CPE induced by AdCA*p53* transcomplemented by Ad5*luc*3 expressing the intact *E1* gene (*A*). *M*, mock-infected cells. *C*, analysis of cell killing of AdCA*p53* after transcomplementation with the *E4 orf6*-deleted Ad Ad355. A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* or with the *E1*-deleted control virus Ad5*luc*1. Forty-eight h later, cells were infected with the *E4 orf6*-deleted Ad355 at a MOI of 5. Cells were stained with crystal violet after observation of advanced CPE, temporally manifesting between the CPE observed in *A* and *B*. *M*, mock-infected cells.

is caused by induction of AdCA*p53* replication and efficient transgene expression, rather than by the isolated effects of Ad oncolysis or the toxic protein effect of the combination of *E1* and *p53*.

Intact E1 and E4 Transcomplementation Are Required to Enhance the Cell Killing Effect of AdCAp53. To further evaluate the enhancement of cell killing by AdCA*p53* in the context of a replicating Ad, we transcomplemented AdCA*p53* or Ad5*luc*1 with a wild-type equivalent Ad vector (Ad5*luc*3) or with Ad vectors deficient in either the *E1B 55kDa* or the *E4 orf6* genes. We reasoned that evaluation of distinct deletions of the Ad genome would allow the identification of Ad genes that are essential to achieve enhanced cell killing in the context of ATV. First, we validated that the fidelity of *E1* transcomplementation of AdCA*p53* is maintained at the viral level and that it results in augmentation of cell killing. To this end, A549 cells were infected with AdCA*p53* or Ad5*luc*1 at a MOI of 20. Forty-eight h later, cells were infected with Ad5*luc*3 at a MOI of 5. Three days after infection with Ad5*luc*3, CPE was evident only for cells coinfected with AdCA*p53* and Ad5*luc*3, whereas CPE for cells coinfected with Ad5*luc*1 and Ad5*luc*3 was delayed (Fig. 2*A*). To confirm the enhanced cell killing potency of *E1* transcomplementation of AdCA*p53* in the context of a replicating virus, we performed the same experiment with the human lung adenocarcinoma H460 cell line. These cells have a significantly lower infectivity rate by Ad, and because they express wild-type *p53*, they are also relatively resistant to apoptosis induced by the replication-deficient AdCA*p53* (18). We infected H460 exactly as described above for Fig. 2*A*, and we found that AdCA*p53* transcomplemented by *E1* from Ad5*luc*3 induced cell killing efficiently. As in A549 cells, transcomplementation of Ad5*luc*1 by Ad5*luc*3 resulted in delayed CPE, whereas AdCA*p53* alone had no effect at this MOI (data not shown).

A plausible interpretation of the capacity of *E1*-transcomplemented AdCA*p53* to circumvent the resistance

of A549 and H460 to AdCA*p53* would be that the therapeutic effect of the replication-deficient AdCA*p53* is limited due to the low efficiency of infection of the initial viral inoculum and the inherent resistance of these *p53*-positive cells to heterologous *p53* expression. In contrast, the potential therapeutic advantages afforded by *E1* transcomplementation involve augmented transgene expression, viral replication, and spread of the viral progeny to infect neighboring tumor cells. Thus, *E1* transcomplementation with an Ad vector encoding *p53* results in highly efficient killing of resistant cancer cells.

To further evaluate the indispensability of candidate Ad genes for the enhancement of the therapeutic effect of AdCA*p53*, we evaluated the interaction of several Ad mutants with AdCA*p53*. To this end, we used Ad338 and Ad355, Ad vectors with deleted *E1B 55kDa* or *E4 orf6* genes, respectively. First, we infected A549 cells with AdCA*p53* or Ad5*luc*1, as described above for Fig. 2*A*. After 48 h, cells were coinfected with the *E1B 55kDa*-deleted Ad338 at a MOI of 5. Plates were stained with crystal violet after the observation of advanced CPE (Fig. 2*B*). In this instance, transcomplemenation of AdCA*p53* by Ad338 was distinct from transcomplementation by the *E1*-intact Ad5*luc*3 in two ways. First, CPE induced by Ad338 transcomplementation of AdCA*p53* was delayed in comparison with the efficient CPE after Ad5*luc*3 transcomplementation of AdCA*p53*. Second, there was no difference in the cell killing patterns of AdCA*p53* or Ad5*luc*1 after coinfection with Ad338. These findings were corroborated by coinfecting AdCA*p53* with another *E1B 55kDa*-deleted Ad vector constructed in our laboratory.

Thus, deletion of the *E1B 55 kDa* gene prevents the enhancement of cell killing observed for transcomplementation of AdCA*p53* by an intact *E1* gene. Furthermore, these results support previous reports regarding the significance of *E1B 55kDa-p53* interaction for efficient Ad-mediated cell killing (15). Because the E1B 55kDa protein functions in concert with the *E4 orf6* gene product during the late Ad infection phase, we hypothesized that the latter is also essential to augment the effect of AdCA*p53* in the context of ATV. To this end, we infected A549 cells with AdCA*p53* or Ad5*luc*1, as described above for Fig. 2*A*. Forty-eight h later, we coinfected cells with the *E4 orf6*-deleted Ad355 at a MOI of 5. As for Ad338, transcomplementation of AdCA*p53* with Ad355 did not increase cell killing to a level greater than that achieved by Ad oncolysis alone (Fig. 2*C*). However, cell killing induced by Ad355 was observed before CPE was detected in Ad338-infected cells. Of note, whereas *E4 orf6* is intact in AdCA*p53*, coinfection with Ad355 and AdCA*p53* was comparable with coinfection with Ad355 and Ad5*luc*1, implying that the primary *E4 orf6* mutation in the complementing Ad negated its capacity to significantly enhance the therapeutic effect of AdCA*p53*. These studies, taken together with the important role of the *E1B 55kDa* and *E4 orf6* genes in the replicative life cycle of Ad (25, 26), suggest that transformation of the replication-deficient AdCA*p53* into an ATV may depend on intact *E1B 55kDa* and *E4 orf6* genes.

The Cell Killing Mechanism of E1-transcomplemented AdCAp53 Involves Augmented Apoptosis. To further delineate the mechanism of the augmentation in cell killing after *E1* transcomplementation of AdCA*p53*, we evaluated parameters of apoptosis and necrosis. Ads have developed distinct strategies to counteract cellular responses to viral infection by blocking cellular apoptosis at critical junctions in the death-signaling cascade (27). On the other hand, wild-type *p53* may enhance the ability of Ad to induce cell death (28). In the absence of *E1B 55kDa*, cell death is delayed similar to that observed for *p53*-deficient cells infected with a wild-type Ad (15). Based on these considerations, we hypothesized that the combination of heterologous *p53* and intact *E1* expression, in the context of our ATV model, would be optimal in terms of apoptosis induction. To this end, we infected A549 cells with AdCA*p53* at a MOI of 20, and 48 h later, we coinfected the cells with either the replication-competent Ad5*luc*3 or Ad338 at a MOI of 5. Control wells were initially infected with the replication-deficient vector Ad5*luc*1 and coinfected 48 h later with Ad5*luc*3. Forty-eight h after the second infection, cells were stained to detect apoptosis or necrosis. Whereas necrosis was detected in all cohorts, significant apoptosis was detected only in cells coinfected with AdCA*p53* and Ad5*luc*3 (Fig. 3). The *E1B 55kDa* deletion at Ad338 abolished the apoptosis-enhancing potency of *E1*-transcomplemented AdCA*p53*. In addition, under these conditions, we could not detect significant apoptosis for the replicating Ad5*luc*3 alone. Thus, these data indicate that the augmented cell killing induced by intact *E1* transcomplementation of AdCA*p53* is mediated by efficient apoptosis induction.

Enhancement of AdCAp53-induced Cell Killing Is Related to the Replication and Burst Kinetics of the Transcomplementing Ad Vectors. One interpretation of the above-mentioned studies would be that in this model system of *p53*-ATV, AdCA*p53* transcomplementation by *E1* results in dramatically higher cell killing by virtue of increased transgene expression.

Alternatively, it could be suggested that *p53* overexpression supports the oncolytic effect of Ad5*luc*3, thereby inducing Ad burst as the primary cause of cell death. To address this issue, we assayed the burst kinetics of Ad5*luc*3, Ad338 and Ad355. We selected the measurement of the Ad *E1a* gene as a specific indicator of the burst kinetics of the complementing viruses because it is absent from AdCA*p53*. To this end, we sampled daily the media from wells infected as described above for Fig. 2 and determined the Ad *E1a* gene copies for the relevant combination of viruses (Fig. 4*A*). Whereas media sampling is a direct method to evaluate viral burst of replication-competent viruses (2), it indicates replication only indirectly. Therefore we also evaluated intracellular Ad DNA parameters (Fig. 4*B*). When evaluating the kinetics of the transcomplementing vectors Ad5*luc*3, Ad338, and Ad355, without the effect of heterologous *p53* overexpression, the gene copy levels of the wild-type equivalent Ad5*luc*3 in the media were the highest as of the first day after infection, indicating efficient primary replication and burst of this vector. In contrast, Ad355 levels were lower than those of Ad5*luc*3, and Ad338 levels were the lowest. These patterns are in accord with the major role of *E1B 55kDa* in the Ad life cycle (25), and its role in supporting AdCA*p53*-mediated cell killing in the context of ATV. Likewise, the impaired

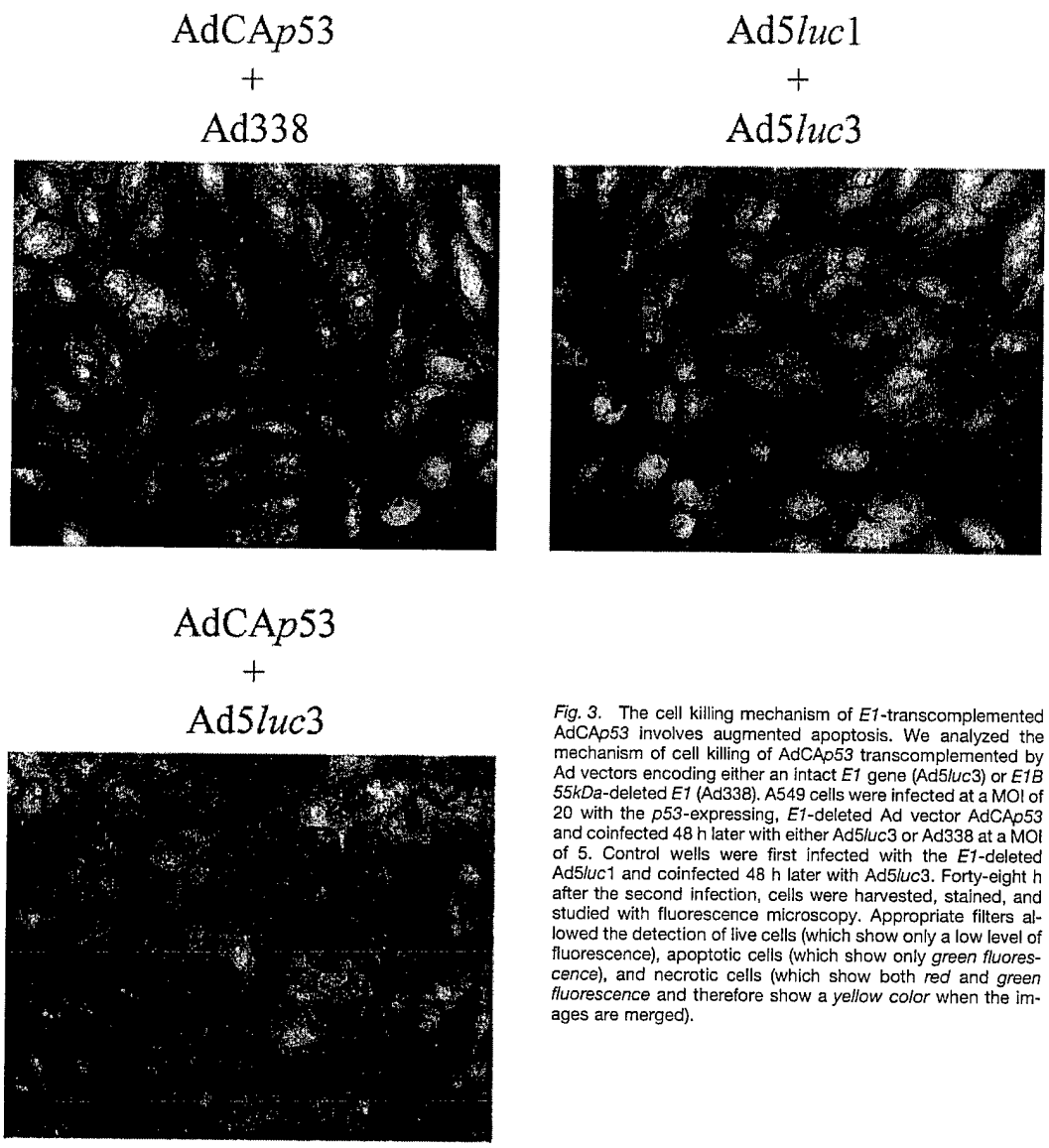

Fig. 3. The cell killing mechanism of *E1*-transcomplemented AdCA*p53* involves augmented apoptosis. We analyzed the mechanism of cell killing of AdCA*p53* transcomplemented by Ad vectors encoding either an intact *E1* gene (Ad5*luc*3) or *E1B 55kDa*-deleted *E1* (Ad338). A549 cells were infected at a MOI of 20 with the *p53*-expressing, *E1*-deleted Ad vector AdCA*p53* and coinfected 48 h later with either Ad5*luc*3 or Ad338 at a MOI of 5. Control wells were first infected with the *E1*-deleted Ad5*luc*1 and coinfected 48 h later with Ad5*luc*3. Forty-eight h after the second infection, cells were harvested, stained, and studied with fluorescence microscopy. Appropriate filters allowed the detection of live cells (which show only a low level of fluorescence), apoptotic cells (which show only *green fluorescence*), and necrotic cells (which show both *red* and *green fluorescence* and therefore show a *yellow color* when the images are merged).

replication and gene expression of *E4 orf6* mutants (26, 29) may have negated the effect of Ad355 on both AdCA*p53* replication and cell killing. Consequently, it appears that the functional *E4 orf6* from AdCA*p53* could not have complemented Ad355 to support viral replication and cell killing. Therefore, only the wild-type equivalent Ad5*luc*3 could achieve both high replication and burst rates and enhance the cell killing of AdCA*p53*.

A striking finding of the evaluation of Ad5*luc*3 burst after coinfection with AdCA*p53* was that despite the clear augmentation of cell killing upon coinfection with AdCA*p53*, the kinetics of the Ad5*luc*3 burst did not differ significantly from the burst kinetics assayed for coinfection of Ad5*luc*3 with Ad5*luc*1. A trend toward earlier burst of Ad5*luc*3 was observed after coinfection of Ad5*luc*3 with AdCA*p53*, possibly indicating the effect of *p53* overexpression and earlier cell death on viral burst. However, the slightly earlier burst is clearly distinct from the unequivocally higher cell killing effect induced by coinfection with these two viruses. Because the burst of the wild-type equivalent, Ad5*luc*3, was not significantly affected by coinfection with AdCAp53, an earlier Ad burst, possibly induced by transgene expression, cannot completely account for the enhanced cell killing potency after coinfection with a replicative Ad and the *p53*-expressing Ad. Rather, *E1* transcomplementation of AdCA*p53* may have resulted in an augmented effect of the toxic transgene, as has been recently demonstrated for HSV*tk*/GCV expressed by a replicative Ad (6). Thus, because differences in burst kinetics cannot explain the enhanced cell killing, timely and efficient transgene expression by the *E1*-transcomple-

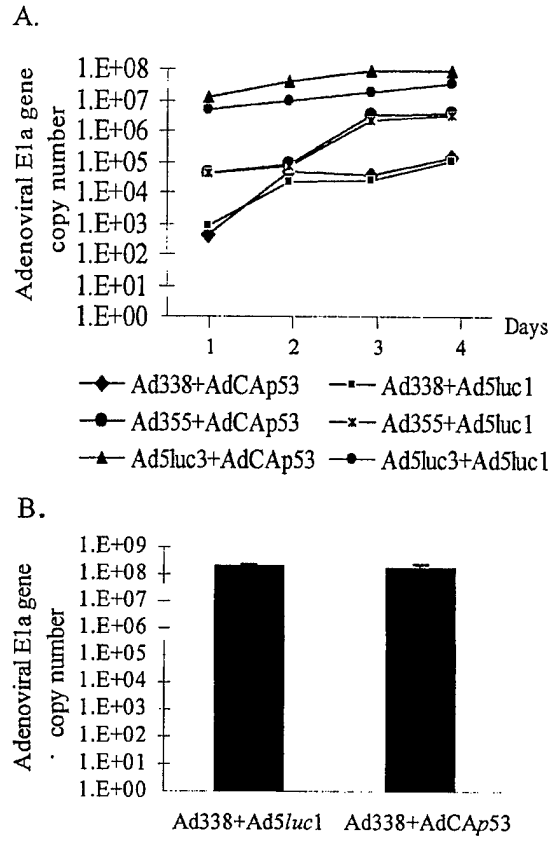

Fig. 4. Wild-type equivalent Ad and E1B and E4 mutant Ad vectors differ in their replication and burst kinetics. *A,* analysis of replication and burst of Ad vectors as a function of *E1* and *E4* status and as a function of *p53* overexpression. A549 cells were infected with AdCA*p53* or Ad5*luc*1 at a MOI of 20. Forty-eight h later, cells were infected with Ad5*luc*3, Ad338, or Ad355. Media samples were collected daily in triplicates from the different cohorts and analyzed with quantitative PCR for Ad *E1a* gene copy numbers. *B,* direct analysis of Ad338 DNA replication as a function of *p53* status. A549 cells were infected as described in Fig. 3*A* with AdCA*p53* or Ad5*luc*1 and coinfected 48 h later with Ad338. Seventy-two h after the second infection, the cellular fraction was harvested and analyzed for Ad DNA.

mented Ad vector AdCA*p53* may account for the augmented cell killing in this ATV model system.

Discussion

A major limitation of cancer gene therapy is the inadequacy of replication-deficient Ad vectors to efficiently infect tumors (30). To address this problem, replicating vectors have been suggested as a means to amplify an initial infection event (31). However, despite the safety of these viruses deriving from the tumor selectivity of replication dynamics, their efficacy is limited (3). Although incorporation of a therapeutic transgene into a replicating Ad to form an ATV has the potential to enhance the potency of replication-competent vectors, recent studies could not resolve this issue. Specifically, expression of incorporated transgenes may directly compromise the goal of viral replication and thus indirectly interfere with antitumor oncolysis (32).

In this study, we hypothesized that alleviation of the replication restriction on AdCA*p53* would form a platform for studying the therapeutic effects and viral kinetics of ATV. First, we validated that intact *E1* induces replication of AdCA*p53* and that Ad replication is not affected by *p53* overexpression. These findings validated the recent documentation of Ad replication despite *p53* overexpression (14). Next, we showed that induction of replication of AdCA*p53*, mimicking an ATV, results in a higher therapeutic effect than the oncolytic effect of replicative Ad alone. However, this effect depended on Ad transcomplementation by intact *E1B 55kDa* and *E4 orf6*. In this regard, although one might expect faster killing of cells infected with an *E1B 55kDa*-deleted Ad because of unbalanced up-regulation of *p53* by *E1a*, this is not the case (15, 33, 34). Rather, both *E1B 55kDa* and *E4 orf6* genes are important for productive Ad infection and viral oncolysis (24, 35). Furthermore, the cell killing potency of both *E1B 55kDa* and *E4 orf6* may in fact depend on cellular expression of *p53* (15, 28, 36).

Our findings of the enhanced cancer cell killing potency after *p53* overexpression, in the context of intact *E1B 55kDa* and *E4 orf6* genes, question the utility of ATV derived from *E1B 55kDa*-deleted or *E4 orf6*-deleted Ad genomes, at least for vectors encoding *p53*, and require additional studies. Because a number of previous studies have shown that ATVs with HSV*tk*/GCV are not more oncolytic than replicating Ad alone, stringent selection of the therapeutic transgene is clearly essential for the design of ATV. In this regard, our ATV model allows screening of therapeutic transgenes *in vitro*. Careful scrutiny of transgenes according to this model may select for ATV expressing transgenes that are not counterproductive for Ad replication.

A pertinent finding of this study involves the mechanism of the induction of cell death by adenoviral vectors. In this ATV model, it appears that earlier viral burst may have been secondary to the primary transgene therapeutic effect, as indicated by the selective and extensive apoptosis in cells coinfected with Ad5*luc3* and AdCA*p53* and by the borderline induction of an earlier burst of Ad5*luc*3 by AdCA*p53*. In this regard, the role of intact Ad genes in the context of an ATV has been recently demonstrated by augmented transgene expression and oncolytic effect *in vitro* and *in vivo* (6). However, in the context of cancer cell killing by an ATV, earlier viral burst may act in concert with transgene overexpression to expedite infection of neighboring cells. Therefore, means to support burst and lateralization of replicative Ad are also warranted to achieve efficient infection of all tumor cells.

In conclusion, we have developed an *in vitro* model for the evaluation of therapeutic transgenes in the context of ATV. A replication-deficient Ad vector encoding *p53* was induced to function as an ATV and shown to be superior to replicative Ad alone by virtue of its enhanced apoptotic cell killing effect. In view of the need to improve vector efficacy for cancer gene therapy, we suggest this model system to allow for a rational design of ATV.

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APPENDIX B

Advances in Brief

The Presence of the Adenovirus E3 Region Improves the Oncolytic Potency of Conditionally Replicative Adenoviruses[1]

Kaori Suzuki, Ramón Alemany, Masato Yamamoto, and David T. Curiel[2]

Division of Human Gene Therapy, Departments of Medicine, Surgery, and Pathology, and Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama 35294-2172 [K. S., M. Y., D. T. C.], and Gene Therapy Program, Institut Català d'Oncologia, Hospital Duran i Reynals, 08907 Barcelona, Spain [R. A.]

Abstract

Purpose: **The initial development of conditionally replicative adenoviruses (CRAds) for cancer treatment has aimed at achieving selective replication in and killing of malignant cells. Other aspects such as the potentiation of the cytolytic capacity have also been investigated but still require new endeavors. As an extension of our prior work, we analyzed the effect of the E3 region, which includes the adenovirus death protein, in the context of CRAd oncolytic potency.**

Experimental Design: **We constructed E3-positive (E3+) and E3-negative (E3−) variants of the previously characterized CRAd, Ad5-Δ24, and its infectivity enhanced version, Ad5-Δ24RGD, and compared their oncolytic effect in human cancer cell lines infected with 0.01 viral particle/cell and in s.c. xenografts of A549 human lung cancer cells injected intratumorally with a single dose of 10^7 adenoviral particles in immunodeficient mice.**

Results: **The *in vitro* experiments showed that the E3+ viruses kill tumor cells 1.6–20 times more effectively in different cell lines. As well, the *in vivo* study demonstrated that the administration of E3+ CRAds resulted in a more potent oncolytic effect compared with the same dose of their E3− counterparts 35 days after virus administration. Moreover, a time course study of virus replication within the tumor xenografts established a correlation between higher *in situ* propagation of E3+ CRAds and tumor growth inhibition compared with E3− viruses.**

Conclusions: **These results indicate that the presence of E3 can enhance the antitumor potency of CRAds over and above the levels conferred by the enhancement of infectivity via Arg-Gly-Asp (RGD).**

Introduction

In the last 5 years, virotherapy of cancer has made remarkable advancements and has been rapidly tested in several clinical trials. The variety of viruses designed to replicate selectively in malignant tissue is widening, and Ad[3] (1–4), herpes simplex virus (5), influenza virus (6), Newcastle disease virus (7, 8), poliovirus (9), reovirus (10, 11), vaccinia virus (12, 13), and vesicular stomatitis virus (14), among others, are being developed for this purpose. In the context of CRAds, two strategies have been used to achieve tumor or tissue selectivity (15). One is the generation of partial deletions of E1 genes that are necessary for a virus to replicate in normal cells but are dispensable in cancer cells. Ads deleted of the p53-binding protein E1B-55K or the retinoblastoma (pRB) binding site of protein E1A are examples of this group (1–4). The other is the introduction of tissue- or tumor-specific promoters to control transcription units in adenoviral genes essential for replication, namely, E1, E2, and/or E4 (2, 16–21). There are more than 10 clinical trials in Phases I to III (reviewed in Refs. 22 and 23) that demonstrated tolerability and safety of CRAd administration; however, the antitumor efficacy of the CRAds used as a single agent was found to be modest (22, 24, 25).

Efforts to improve oncolytic potency of CRAds include the infectivity enhancement of Ad by the incorporation of motifs like the integrin-binding RGD into the HI loop of the fiber knob (26) or the heparan sulfate-binding polylysine at the COOH-terminal of the fiber protein (27). Further strategies are the combination with chemotherapeutic agents and/or radiotherapy (28–31) or the generation of "armed" CRAds with pro-drug-activating genes (32, 33). Nevertheless, these strategies have to cope with the possibility that the action of the drugs themselves may operate at cross-purposes with the viral replication (34). Moreover, the strategy of "arming" the replicative virus with other genes has to be carefully evaluated against a possible detrimental effect of deleting viral genes to create space for the new transgenes (35, 36). Few expression cassettes can be incorporated into the adenoviral genome without deletions (35), and frequently these are introduced in the E3 region, especially if conservation of the replication capacity is the concern (37). Therefore, it is important to quantify the decrease in oncolytic

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[2] To whom requests for reprints should be addressed, at Division of Human Gene Therapy, Departments of Medicine, Surgery, and Pathology, and Gene Therapy Center, University of Alabama at Birmingham, 901 19th Street South BMR2-502, Birmingham, AL 35294-2172. Phone: (205) 934-8627; Fax: (205) 975-7476; E-mail: david.curiel@ccc.uab.edu.

[3] The abbreviations used are: Ad, adenovirus; CRAd, conditionally replicative adenovirus; ADP, adenovirus death protein; pRB, retinoblastoma protein; CAR, Coxsackie and adenovirus receptor; vp, viral particle(s); GFP, green fluorescent protein; CPE, cytopathic effect; FBS, fetal bovine serum; CMV, cytomegalovirus; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide.

potency that results from E3 deletions. The Ad E3 region encodes, among other proteins, a M_r 11,600 protein known as ADP (38). Low-level expression of ADP starts at early phases of the viral life cycle; however, this protein exerts its function at very late stages, provoking the cytolysis of the infected cell, the release of viral progeny, and the spread of the virus to the surrounding cells (39–41).

In a previous study, we demonstrated the superiority of the oncolytic effect of an infectivity-enhanced CRAd, Ad5-Δ24RGD, compared with the unmodified Ad5-Δ24 *in vitro* and *in vivo* (26). This mutant contains an RGD motif in the fiber knob that binds to α_v integrins and allows cell entry via a pathway independent from the CAR, the primary Ad receptor. At the initial stages of the development of Δ24 Ads, the role of the proteins encoded by the E3 region in the context of CRAd efficacy was not clear. Along with the realization of the importance of the E3 region, we performed subsequent studies. The results from these experiments indicate that the presence of E3 augments the oncolytic potency of both Ad5-Δ24 and Ad5-Δ24RGD *in vitro* and *in vivo*. These observations are explained by the occurrence of amplification loops consisting of earlier host cell disruption by E3-containing CRAds, lateral dispersion of the progeny, and viral replication that leads to further cytolysis. Therefore, we reason that the conservation of E3 will certainly induce a significant increase in the antitumor efficacy of CRAds.

Materials and Methods

Cell Lines. A549 human lung adenocarcinoma, Hs 766T human pancreatic cancer, and SKOV-3 human ovarian adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA; ATCC numbers CCL-185, HTB-77, and HTB-134, respectively). All of them are defective in p16 disrupting the pRB/p16 pathway of cell cycle control (42–44). Human embryonic kidney 293 cells were obtained from Microbix Biosystems Inc. (Toronto, Canada). Cells were cultured in DMEM supplemented with 5% heat-inactivated FBS in the absence of antibiotics and maintained at 37°C in a 5% CO_2 atmosphere.

Virus Construction. All of the CRAds used in this study contain a 24-bp deletion (Δ24), from Ad5 bp 923 to 946 (both included), corresponding to the E1A region necessary for pRB protein binding (45). Details of the tumor-specific replication of the Δ24 mutant virus are presented elsewhere (3). Viruses were grown in A549 lung cancer cells and purified by cesium chloride banding, and the physical titer was calculated by absorbance measurement at a wavelength of 260 nm ($A_{260\ nm}$; 1 A_{260} unit = 1.1×10^{12} vp/ml; Ref. 46). The vp:CPE unit ratio (vp:CPE units) of the Δ24 mutants ranged from 9 to 42. AdGFP is a complete E1-deleted virus that contains a GFP expression cassette driven by the CMV promoter (47).

Ad5-Δ24/GFP (E3−). The plasmid pE3-GFPzeo carries the expression cassette containing the CMV promoter and the GFP-Zeocin fusion gene (1,771 bp) from pTracer-SV40 (Invitrogen, Carlsbad, CA) flanked by Ad5 sequences (left, bp 27,040–28,045; right, bp 30,863–31,948; Ref. 48). An *Afl*II/*Sph*I fragment from this plasmid was inserted by homologous recombination into the E3 region of pKS4050 that contains the viral genome except the E1 and E3 genes generating pKS4050-GFPzeo. The plasmid pXC1-Δ24 has been described previously (3). In brief, this plasmid was constructed by site-directed mutagenesis of pXC1 (Microbix Biosystems Inc.) to loop out bp 923–946 from the E1A transcription unit. pKS4050-GFPzeo and pXC1-Δ24 were cotransfected into 293 cells using the calcium phosphate method to generate the virus.

Ad5-Δ24E3 (E3+). Two fragments from pXC1-Δ24 (*Bsr*GI/*Xba*I and *Xba*I/*Mfe*I) containing E1A with the 24-bp deletion and the E1B transcription unit were ligated with the *Bsr*GI/*Mfe*I fragment of pShuttle (Stratagene, La Jolla, CA) to generate pShuttle-Δ24. Plasmid pΔ24E3+ was generated by homologous recombination between a pShuttle-Δ24 linearized by *Pme*I/*Eco*RI digestion and pTG3602 linearized by *Pac*I digestion (49). The resulting adenoviral genome was transfected into 293 cells to rescue the virus.

Ad5-Δ24GFP/RGD (E3−). The *Afl*II/*Sph*I fragment from pE3-GFPzeo containing the CMV promoter-driven GFP-Zeocin gene flanked by the left and right arms of E3 was introduced into pVK526 (26) by homologous recombination, and the resulting adenoviral genome linearized with *Pac*I was transfected into 293 cells to rescue the virus.

Ad5-Δ24RGD (E3+). This virus was constructed by homologous recombination of the E1 fragment containing the 24-bp deletion that was isolated from the plasmid pXC1-Δ24 and the *Cla*I-digested plasmid pVK503 containing the RGD fiber (50) to generate pVK526. To rescue the virus, the resulting Ad genome was excised from the plasmid with *Pac*I and transfected in 293 cells (26).

Validation of Virus Constructs. The Δ24 deletion in all of the Ad mutants was analyzed by PCR with primers E1a-1 (5′-ATTACCGAAGAAATGGCCGC-3′) and E1a-2 (5′-CCATTTAACACGCCATGCA-3′) followed by *Bst*XI digestion. This deletion results in the loss of a *Bst*XI cleavage site yielding only one band of 1023 bp upon *Bst*XI digestion, as opposed to the two bands (725 and 322 bp) obtained from the wild-type E1A. The presence/absence of the RGD motif in all of the viruses was confirmed by PCR of the modified fiber region with the primers FiberUp (5′-CAAACGCTGTTGGATTTATG-3′) and FiberDown (5′-GTGTAAGAGGATGTGGCAAAT-3′). The expected sizes of the PCR products are 247 bp for the viruses with wild-type fiber and 274 bp for the RGD-containing mutants (26). Further verification was performed by sequence analysis (CEQ2000 Dye Terminator Cycle Sequencing kit; Beckman-Coulter, Fullerton, CA) with primers E1a-1 and Fiber-up and E3-Adp (5′-CAGCGACCCACCCTAACAGA-3′).

Virus Infection. Cells were infected at indicated concentrations (vp/cell) in DMEM supplemented with 2.5% FBS. After an absorption time of 2 h, cells were washed once with serum-free DMEM and maintained in DMEM supplemented with 2.5% or 5% FBS at 37°C in a 5% CO_2 atmosphere. Physical titers (vp) were used because the viruses contained different capsids.

Virus Spread Assay. A549 cells cultured in 2-well chamber slides (Nalge Nunc International Corp., Naperville, IL) were infected with 0.1 vp/cell Ad5-Δ24GFP or Ad5-Δ24E3 as described. On days 2, 3, 4, and 5 after infection, the slides were fixed with 3% formaldehyde, blocked, incubated with goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc., Temecula, CA) followed by Texas red-labeled donkey antigoat

IgG (10 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and counterstained with Hoechst 33342 (20 μg/ml; Molecular Probes, Eugene, OR). The slides were analyzed by fluorescence microscopy (Olympus 1X70; Olympus America, Inc., Melville, NY), and photographs were taken using the MagnaFire system (Optronics, Goleta, CA).

Plaque Development Assay. A549 cells cultured in 6-well plates were infected with 0.1 vp/cell Ad5-Δ24/GFP or Ad5-Δ24E3. After a 2-h adsorption time, they were washed once with serum-free DMEM and a 6-ml agar overlay consisting of a 1:1 mix of autoclaved 1.33% Bacto-agar (Difco, Detroit, MI), and 2× DMEM supplemented with 5% FBS was added. Formation of plaques was observed during a period of 12 days, and individual plaques were photographed using an inverted microscope (Olympus 1X70) and the MagnaFire imaging system.

Oncolysis Assay. A549, Hs 766T, and SKOV-3 cells were cultured at 80% confluence in 12-well plates in triplicate, infected with Ads at a dose of 0.01 vp/cell as described, and cultured until CPE was evident in 90% of the cells in any of the treatment groups. Cell viability was assessed using the methods described next.

***In Vitro* Cytotoxicity Assay (XTT).** Cell survival was determined using a colorimetric assay based on XTT (Sigma, St. Louis, MO) that quantifies the number of living cells based on mitochondrial activity. The media were carefully aspirated from the wells, and 300 μl/well DMEM without phenol red supplemented with 2.5% FBS and 20% XTT was added. After an incubation time of 2 h, the absorbance of the media from each well was measured at a wavelength of 450 nm. The percentage of viable cells/well was calculated considering the uninfected cells as 100% viable.

Crystal Violet Staining. After the XTT assay was performed, the cells were fixed with 10% buffered-formaldehyde for 10 min and stained with 2% crystal violet solution.

Spectrophotometric Titration of Ad. To analyze multiple samples originated from both *in vitro* (cell pellets and supernatants) and *in vivo* (tumor extracts) experiments, we used a modified version of the titration method proposed by O'Carroll *et al.* (51). Briefly, 293 cells were seeded in 96-well plates (4×10^4 cells/well) in DMEM containing 0.5% FBS. After an overnight attachment period, cells were infected with serial dilutions of each sample. Four days after the infection of 293 cells, media and detached cells were removed, and cell loss (CPE) was determined by measuring changes in total protein concentrations in each well. This concentration is obtained by adding 200 μl of BCA protein assay reagent (Pierce, Rockford, IL) to each well, shaking for 2 h at room temperature, and measuring the absorbance in a microtiter plate reader at a wavelength of 595 nm. The readings were expressed as a percentage of the respective uninfected controls, and graphs were plotted against the virus dilutions using the Origin scientific graphing and analysis software (OriginLab, Northampton, MA). The Ad titers (CPE units/ml) were calculated as the dilution that corresponds to CPE_{50}, multiplied by the number of cells killed (20,000), and divided by the inoculum (0.1 ml).

Dynamics of Production and Release of Ad Progeny. A549 cells seeded in 6-well plates were infected with 0.1 vp/cell Ad5-Δ24GFP or Ad5-Δ24E3 as described previously and maintained in DMEM with 2.5% FBS. Supernatants and cells were harvested separately on days 3, 6, and 9 and kept frozen until analysis. Cell pellets were resuspended in 1 ml of DMEM with 2.5% FBS and subjected to three freeze/thaw cycles before titration as described above.

s.c. Tumor Xenograft Model. Female 5–7-week-old athymic nu/nu mice (Frederick Cancer Research, Frederick, MD) were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Eight million A549 cells were xenografted s.c. into the right flank of the mice under anesthesia. When the nodules reached a volume of 70–110 mm^3, a single Ad dose (10^7 vp in 30 μl of PBS) or the same volume of PBS was administered intratumorally ($n = 5$). This administration scheme was based on previous studies by us that demonstrated that a single low Ad dose suffices to produce tumor cell destruction across the entire tumor mass (26). Tumor size was monitored twice a week, and fractional volume was calculated by the formula: (length × width × depth/2) (52). The mice were euthanized 35 days after the treatment to prevent excessive tumor growth in the control group. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Ad Hexon Immunodetection. The presence of Ad particles in the tumor xenografts was assessed by immunofluorescence detection of the hexon protein. A549 tumors were dissected aseptically, embedded in tissue-freezing media (Triangle Biomedical Sciences, Durham, NC), flash-frozen in liquid nitrogen, and stored at −80°C until sectioning. Tumor sections were fixed with 3% formaldehyde, blocked, incubated with goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc.) followed by Alexa Fluor 488-labeled donkey antigoat IgG (10 μg/ml; Molecular Probes), and counterstained with Hoechst 33342 (20 μg/ml; Molecular Probes). The slides were analyzed with an inverted fluorescence microscope (Olympus 1X70), and photographs were taken using the MagnaFire system.

Virus Replication in Tumor Xenografts. The *in vivo* replication of the viruses was assessed using the s.c. tumor xenograft model described in a previous paragraph. Similarly, the nodules were injected with a single dose of 10^7 vp of conditionally replicative viruses, a nonreplicative AdGFP, or PBS. On days 3, 6, and 9 after injection, whole tumors were harvested ($n = 3$), weighed, frozen, and stored at −80°C until analysis. To make the extracts, the tumors were thawed, minced using sterile scissors, and digested with Liberase Blendzyme 3 (0.1 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN) with constant agitation for 1.5 h at 37°C. The resulting cell suspensions were freeze/thawed three times and spun, and the supernatants were filtered through a low protein binding 0.2-μm-pore membrane (Pall Gelman Laboratory, Ann Arbor, MI). The concentration of virus progeny present in the extracts was titered by the spectrophotometric method.

Statistical Analysis. Determinations of significant differences among groups were assessed by calculating the value of Student's t using the Origin data analysis and graphing software (OriginLab).

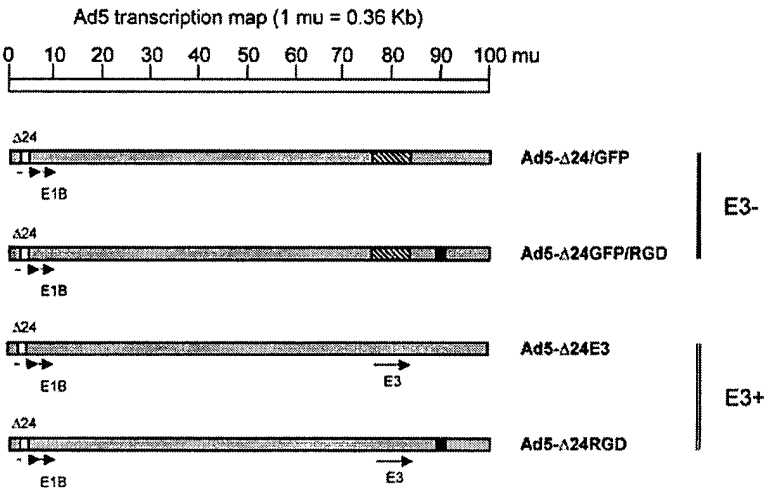

Fig. 1 Schematic representation of the CRAds. The *white boxes* indicate the 24-bp deletion in the CR2 region of the E1A gene, the *gray-shaded boxes* represent the GFP gene substituting the E3 gene, and the *black boxes* symbolize the 27-bp sequence encoding the RGD motif in the fiber knob region.

Results

Virus Structure. Fig. 1 shows the structure of the mutant Ads used in this study. All of the viruses contain a 24-bp deletion corresponding to the amino acid sequence L_{122}TCHEAGF$_{129}$ of the E1A protein known to be necessary for pRB protein binding (45). Ad5-Δ24/GFP and Ad5-Δ24GFP/RGD E3 regions (Ad5 bp 28,045–30,863) were substituted with a CMV promoter-driven GFP-Zeocin expression cassette, whereas Ad5-Δ24E3 and Ad5-Δ24RGD contain the wild-type E3 region (bp 27,858–30,828). The presence or absence of a GFP cassette in the deleted E3 region did not change the cytolytic capacity of Ad5-Δ24E3− (formerly named Ad5-Δ24; Ref. 26) *versus* Ad5-Δ24/GFP (data not shown), and we chose to use the viruses containing GFP as E3− viruses considering the possibility of direct observation of the virus presence. Additionally, Ad5-Δ24GFP/RGD and Ad5-Δ24RGD contain 27 bp in the HI loop of the fiber knob that code for the amino acid sequence CDCRGDCFC (50).

The Presence of E3 Enhances the Spread of CRAds. The ability of the E3− and E3+ CRAds to spread through a cell monolayer was analyzed using an assay based on the immunodetection of Ad hexon protein. A549 cells cultured in chamber slides were infected with 0.1 vp/cell Ad5-Δ24/GFP or Ad5-Δ24E3, and the number of cells positive for Ad presence was examined on days 2–5 after infection. When administered at low dose, the virus must go through several cycles of replication, cell lysis, and secondary infection to spread through the cell monolayer. We detected the presence of Ad in the cytoplasm of a similar number of cells in both groups 2 days after infection (Fig. 2, *a* and *e*). Afterward, the E3+ CRAd spread quickly. The number of virus-carrying cells increased considerably on day 3 when the virus could still be observed in the cytoplasm, but cell disruption, virus release, and spread took place on day 4, and the monolayer was completely destroyed on day 5 (Figs. 2, *f*–*h* and *n*–*p*). In contrast, the number of E3− CRAd-bearing cells showed only a slight increment starting on day 4, and the majority of the virus was still located inside the cells after 5 days (Fig. 2*d*). When an overlay of agar is added on top of similarly infected cells, the diffusion of Ad progeny to the media is prevented, thus allowing the observation of plaque formation by local spread and CPE of the virus. In this experiment, the growth of E3− CRAd plaques was slower compared with E3+ CRAds resulting in smaller plaques after 12 days of incubation (data not shown). These results indicate that the presence of the E3 region increases the cell spreading ability of Δ24 mutants.

E3+ CRAds Gain Oncolytic Potency *in Vitro*. The cell killing capacity of the different mutants was compared in three human cancer cell lines defective in the pRB/p16 pathway. For this purpose, we infected the cells with low doses (0.01 vp/cell) of each virus to allow multiple cycles of viral replication over the following days. Cell viability was assessed by XTT assay (Fig. 3*A*), and the live cells attached to the wells were stained with crystal violet on days 9 (A549), 10 (Hs 766T), and 17 (SKOV-3) after infection (Fig. 3*B*). Fig. 3*A* shows the percentage of cells killed by the different CRAd mutants in the respective experiments shown in Fig. 3*B*. In all three cell lines, the viruses containing the E3 gene exerted a more potent cell killing effect than the viruses lacking E3 (statistical differences ranging from at $P < 0.02$ to $P < 0.0000004$). The gain in oncolytic potency obtained by the E3+ viruses ranged from 1.6-fold in Hs 766T cells treated with Ad5-Δ24/GFP *versus* Ad5-Δ24E3 to 20-fold in SKOV-3 cells treated with Ad5-Δ24GFP/RGD *versus* Ad5-Δ24RGD. The enhancement in cell killing effect was more evident in the cell lines A549 and SKOV-3 that were more resistant to E3− CRAds. With regard to the RGD mutation, no differences were detected between the groups treated with E3− viruses in any cell type. In contrast, in the E3+ virus-treated groups, Ad5-Δ24RGD demonstrated higher oncolytic potency compared with Ad5-Δ24E3 (A549, $P < 0.01$; Hs 766T, $P < 0.02$; SKOV-3, $P < 0.02$).

The Presence of E3 in a CRAd Augments the Release of Ad Progeny. To study the effect of the E3 region on the release of CRAd progeny and its correlation with oncolysis, we infected A549 cells with Ad5-Δ24/GFP or Ad5-Δ24E3 to compare two titers of infectious virus: one liberated to the media and the other remaining inside the cells. Media and cells were

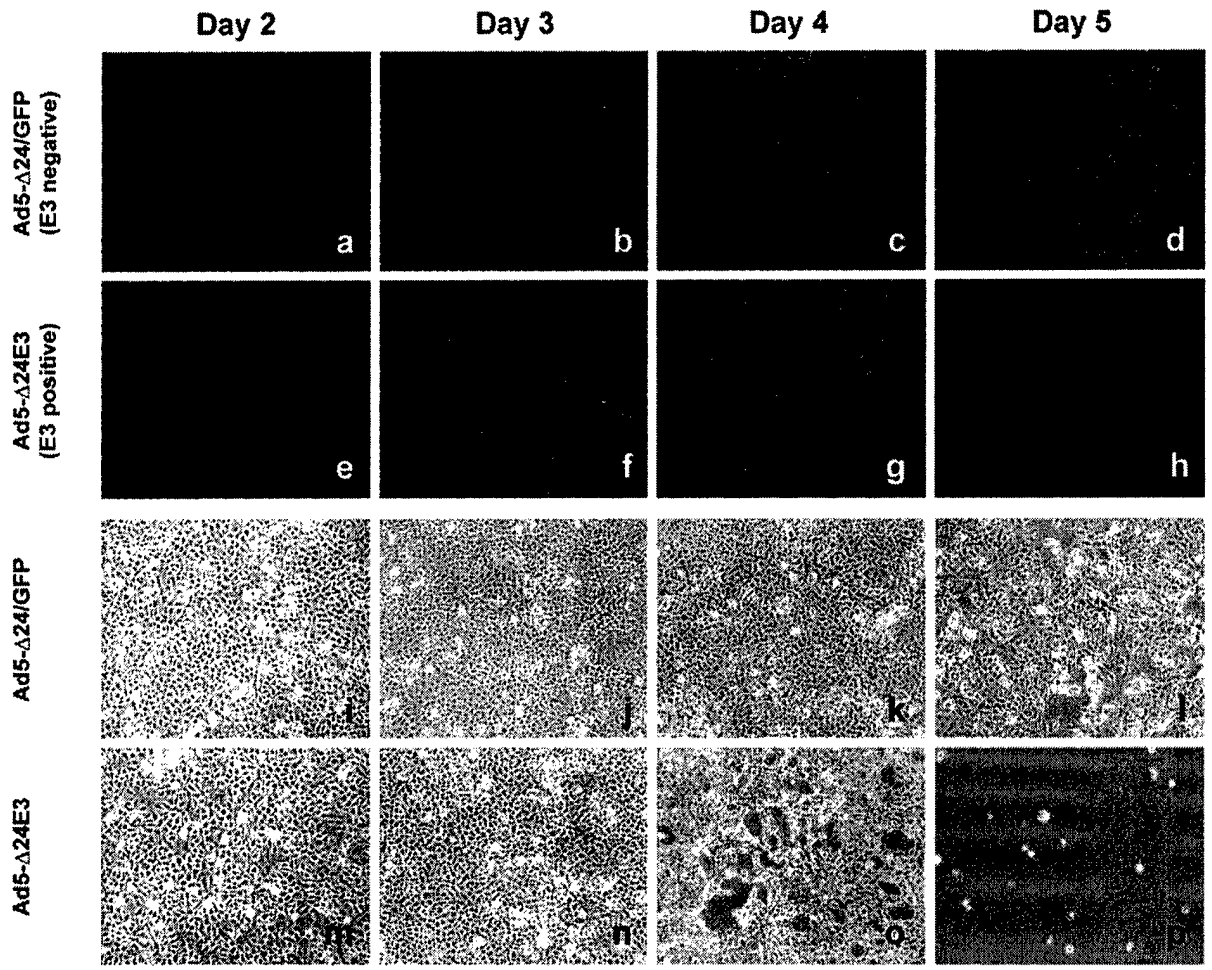

Fig. 2 Virus spread assay. A549 cells cultured in chamber slides were infected with 0.1 vp/cell Ad5-Δ24/GFP (*a*−*d* and *i*−*l*) or Ad5-Δ24E3 (*e*−*h* and *m*−*p*). On days 2, 3, 4, and 5 after infection, the cells were fixed, incubated with goat anti-hexon antibody followed by Texas red-conjugated donkey antigoat antibody, and counterstained with Hoechst 33342. The same optical field was photographed under fluorescence light to demonstrate the presence of Ad and under normal light to show the condition of the cell monolayer (Ad5-Δ24/GFP, *a*−*d* correspond to *i*−*l*; Ad5-Δ24E3, *e*−*h* correspond to *m*−*p*). The *blue fluorescence* corresponds to cell nuclei, and the *red fluorescence* corresponds to Ad hexon. Magnification, ×100.

harvested separately on days 3, 6, and 9 after infection and titrated using a spectrophotometric assay (Ref. 51; Fig. 4). Titers are expressed as total CPE units either in the supernatant (entire volume of media in the well) or in the cell pellet (totality of cells in the well). In the E3− Ad5-Δ24/GFP-infected group, the titer in the cell fraction increased 5-fold on day 6 compared with day 3, indicating that virus replication took place. However, viral progeny was released to the supernatant only after 9 days (3.1×10^6 total CPE units; Fig. 4*A*). At the end of the experiment, the media:cell virus titer ratio was 4.9:1. In contrast, in the group treated with the E3+ Ad5-Δ24E3, the release of virus progeny to the media started as early as day 3 (2.9×10^6 total CPE units), increasing constantly throughout the incubation period until the titer reached 1×10^9 CPE units on day 9. The titer of the cell fraction did not show a big increase, presumably because the new viruses were rapidly released from the cell (Fig. 4*B*). The final media:cell titer ratio was 37.7:1. These findings indicated that the E3-containing CRAd released its progeny 7.7 times more efficiently than the one lacking E3. The results closely paralleled the *in vitro* oncolysis experiment performed in A549 cells (Fig. 3*A, top panel*) with the 7-fold increase in oncolytic potency of the Ad5-Δ24E3 virus compared with its E3− counterpart.

E3+ CRAds Show More Potent Oncolytic Effect than E3− CRAds *in Vivo*. To analyze the *in vivo* lytic potency of our viruses, A549 human lung cancer cells xenografted into nude mice were treated with a single intratumoral injection of 10^7 vp of CRAds or PBS. As observed in Fig. 5*A*, tumor growth was partially (Ad5-Δ24E3) or totally (Ad5-Δ24RGD) arrested during the 35 days following the administration of E3+ CRAds. In contrast, the size of the tumors treated with either E3− CRAd, nonreplicative virus AdGFP, or vehicle progressively increased in the same period [fractional tumor volumes: Ad5-Δ24E3 (3.22 ± 0.77) *versus* Ad5-Δ24/GFP (7.95 ± 3.54);

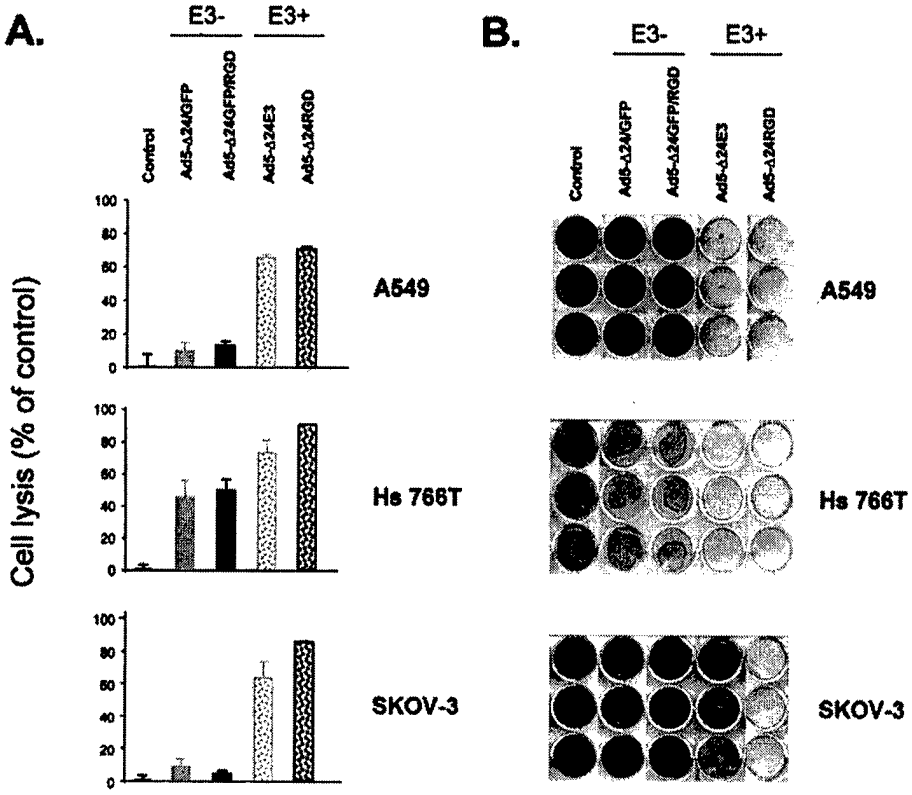

Fig. 3 Oncolysis assay *in vitro*. *A*, oncolytic potency of E3− and E3+ CRAds. Three cell lines were infected with 0.01 vp/cell, and viable cells after variable days (A549 = 9 days, Hs 766T = 10 days, and SKOV-3 = 17 days) from infection were quantified by XTT assay. Cell killing capacity was calculated by subtracting the number of viable cells from the total cell number/well found in the control group. The plot shows the amount of lysed cells expressed as percentages. The statistical differences between the groups were as follows: A549, $P < 0.00005$ (Ad5-Δ24/GFP *versus* Ad5-Δ24E3) and $P < 0.000005$ (Ad5-Δ24GFP/RGD *versus* Ad5-Δ24RGD); Hs 766T, $P < 0.02$ (Ad5-Δ24/GFP *versus* Ad5-Δ24E3) and $P < 0.0006$ (Ad5-Δ24GFP/RGD *versus* Ad5-Δ24RGD); and SKOV-3, $P < 0.001$ (Ad5Δ-24/GFP *versus* Ad5-Δ24E3) and $P < 0.0000004$ (Ad5-Δ24GFP/RGD *versus* Ad5-Δ24RGD). *B*, crystal violet staining of the viable cells attached to the wells corresponding to the quantitative results shown in Fig. 2*A*.

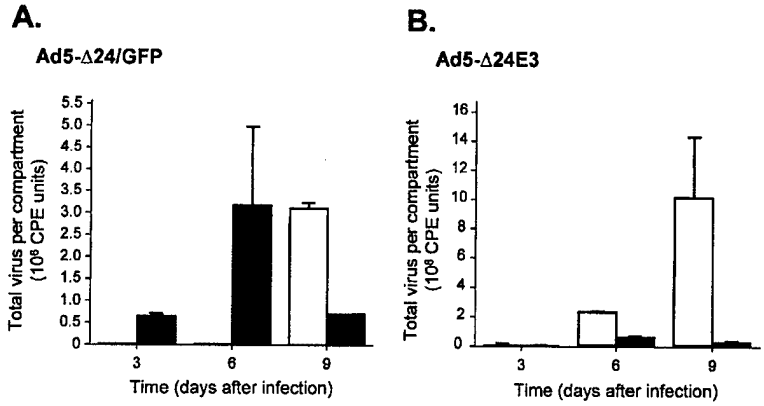

Fig. 4 Titration of total virus progeny in the supernatant *versus* cell pellet. A549 cells were infected with Ad5-Δ24 with and without E3. Supernatants and cells were harvested on days 3, 6, and 9 after infection, and titer was calculated in 293 cells using a spectrophotometric assay (see "Materials and Methods"). □, titer in the media; ■, titer in the cell fraction. *A*, in the Ad5-Δ24/GFP (E3−)-infected group, the titer of the virus found in the cell fraction increases 5-fold on day 6 compared with day 3. However, the progeny release starts only at day 9 after infection, reaching a titer of 3.1×10^6 total CPE units in the supernatant. *B*, in contrast, in the group treated with Ad5-Δ24E3 (E3+), the release of virus progeny to the media starts as early as day 3 (2.9×10^6 total CPE units), increasing constantly throughout the incubation period to reach 1×10^9 CPE units at the end of the study. The titer of the cell fraction does not show big increases because the new viruses are rapidly released from the cell. The titers are expressed as 10^6 (*A*) and 10^8 (*B*) total CPE units/compartment (supernatant or cells), and data are the mean ± SD ($n = 3$).

Ad5-Δ24RGD (1.87 ± 0.53) *versus* Ad5-Δ24GFP/RGD (7.83 ± 2.81)]. Also, the combination of E3 and the infectivity enhancement conferred by the RGD motif resulted in an additional increment in the oncolytic potency. The statistical significance of the tumor volume measurements compared with the vehicle-treated group was as follows: $P < 0.05$ for Ad5-Δ24E3 and $P < 0.01$ for Ad5-Δ24RGD. No significant difference was found for Ad5-Δ24/GFP and Ad5-Δ24GFP/RGD (Fig. 5*A*).

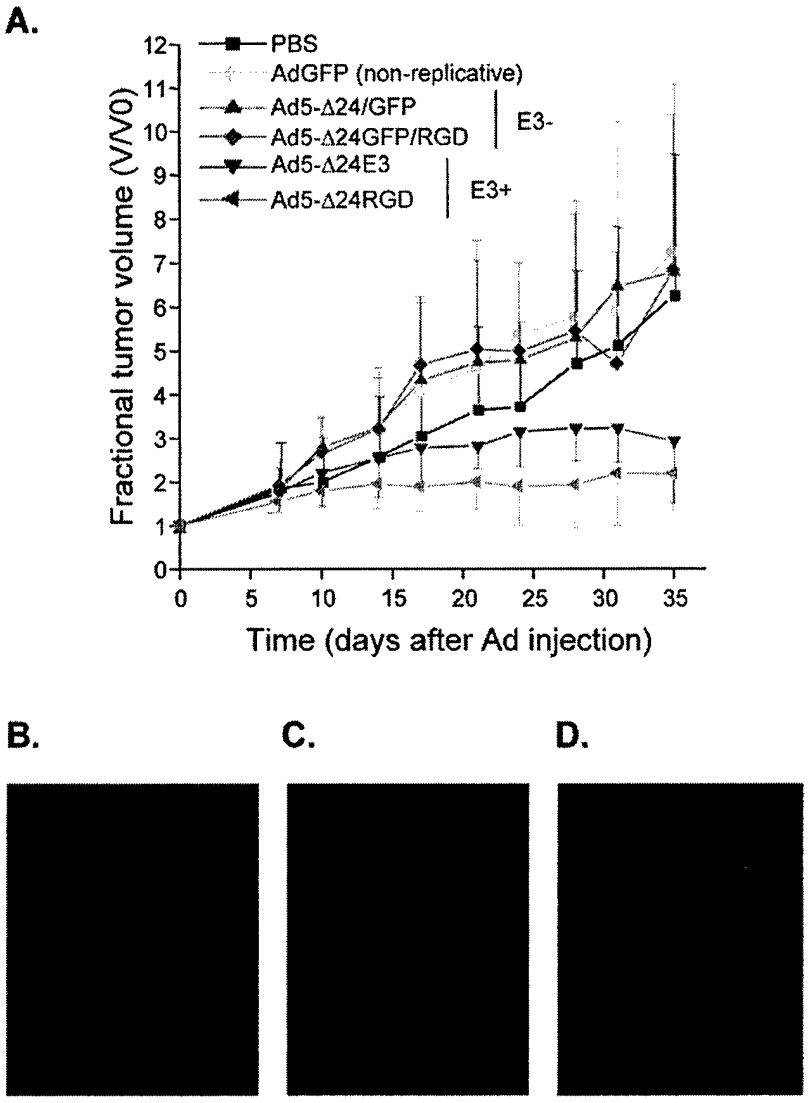

Fig. 5 Oncolysis study *in vivo*. *A*, s.c. A549 xenografts in nude mice were treated with a single intratumoral injection of 10^7 vp of E3− and E3+ versions of Ad5-Δ24 or Ad5-Δ24RGD or with PBS alone, and tumor size was measured twice a week. Results are shown as fractional tumor volumes (*V/V0*, where *V* = volume at each time point; *V0* = volume at Ad injection), and each *line* represents the mean ± SD of five tumors. Tumors treated with any of the E3− viruses or PBS show an ascending growth curve in contrast to the nodules treated with E3+ versions of the viruses that demonstrate an oncolytic effect (Ad5-Δ24/GFP *versus* Ad5-Δ24E3, $P = 0.019$; Ad5Δ24RGD/GFP *versus* Ad5Δ24RGD, $P = 0.0017$). *B–D*, detection of Ad hexon in tumor xenografts by immunofluorescence. Frozen sections of tumor specimens injected with PBS or any of the four viruses were treated with goat anti-hexon antibody and Alexa Fluor 488-labeled donkey antigoat antibody (*green*), and nuclei were counterstained with Hoechst 33342 (*blue*). Images were captured using an Olympus 1X70 microscope (×100 magnification) with a double filter. Samples taken from tumors treated with PBS exhibited no hexon signal (*B*), whereas all of the CRAd-treated ones showed *green fluorescence* displaying different patterns. The tumors treated with E3+ CRAds have clearly localized *green dots* (*C*), whereas the E3− counterparts have a scattered dusty image (*D*).

Also, the comparison between RGD *versus* non-RGD mutants of either E3− or E3+ CRAds did not show statistical differences. At the end of the experiment, we studied the presence of CRAd progeny in the tumor tissue by immunostaining of the Ad hexon (Fig. 5, *B–D*). All nodules were sampled and analyzed except for two E3+ Ad5-Δ24E3-treated tumors, two E3+ Ad5-Δ24RGD-treated tumors, and one E3− Ad5-Δ24GFP/RGD-treated tumor due to either the small volume or the pulpous consistency of the nodule (necrotic material). Whereas vehicle-treated nodules showed no green fluorescence (Fig. 5*B*), E3+ viruses were readily detected in the tumor nodules with a locally concentrated pattern (Fig. 5*C*). Similarly, 30% of the tumors treated with E3− viruses presented a scattered faint fluorescent signal (Fig. 5*D*). However, the rest of the E3− CRAd-treated tumors failed to show any signal. Altogether, the *in vivo* data indicate that CRAds replicate in the tumor tissue and that the inclusion of the E3 region enhances the oncolytic capacity of CRAds.

Titration of Virus Progeny Recovered from Tumor Xenografts. To correlate the *in situ* replication of the CRAds with the lytic effect inflicted on the tumors, we quantified the amount of infectious virus present in the tumors in a time course experiment. The extracts used for titration were obtained from the nodules by enzymatic dissociation. In a preliminary test performed to rule out possible alteration of structural and/or functional characteristics of the Ad due to protease exposure, we treated nonreplicative AdGFP with an enzyme solution at the same concentration that was used for tissue dissociation. Next, A549 cells were transduced with protease-treated or PBS-treated AdGFP virus, and the transduction efficiency was shown to be the same for both groups (data not shown). Titration of extracts from CRAd-treated tumors ranged from 3×10^8 CPE

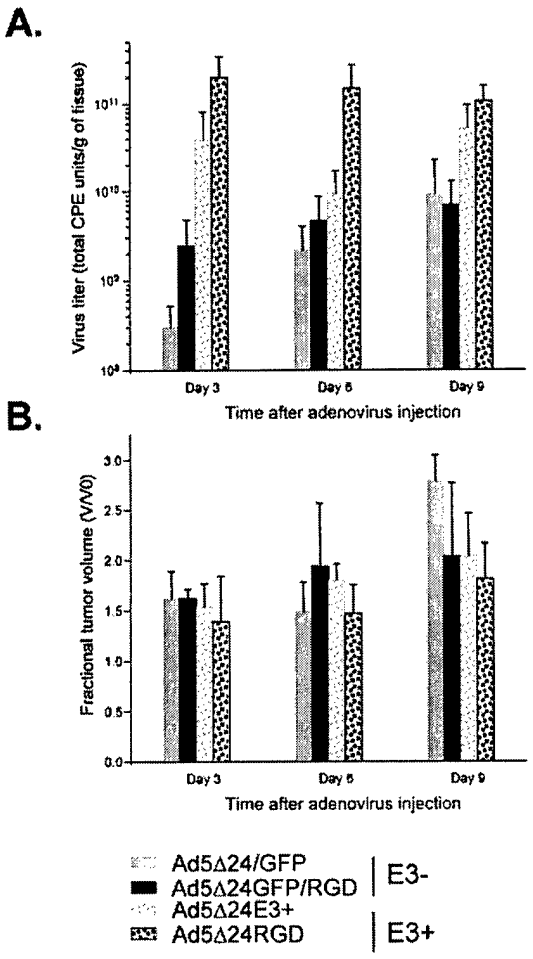

Fig. 6 Titration of virus progeny recovered from tumor xenografts. *A*, time course titration of the virus progeny present in tumors treated with CRAds. Extracts were prepared by enzymatic dissociation of the nodules and titrated using a spectrophotometric assay (see "Materials and Methods"). The titer was adjusted to the weight of each tumor and expressed as CPE units/g tissue. Each column represents the mean titer ± SD (n = 3). *B*, fractional tumor volumes of the corresponding tumors. The columns indicate the mean volume ± SD (n = 3). V = volume at harvest; $V0$ = volume at CRAd injection.

units/g tissue (Ad5-Δ24/GFP) to 2 × 10^{11} CPE units/g tissue (Ad5-Δ24RGD; Fig. 6*A*). At all time points, the mean virus titer of the nodules treated with E3+ CRAds tended to be higher compared with that of their E3− counterparts, although statistical significance was reached only on day 9 (Ad5-Δ24/GFP *versus* Ad5-Δ24E3, P = 0.057; Ad5-Δ24GFP/RGD *versus* Ad5-Δ24RGD, P = 0.036). Of note, Ad5-Δ24RGD-treated tumors show the highest mean amount of virus progeny, although Ad5-Δ24E3 attains a similar value at the end of the experiment. As expected, Ad was not present in any of the extracts derived from tumors injected with nonreplicative AdGFP. Tumor volumes were also measured at the moment of harvest (Fig. 6*B*), and there are no statistically significant differences among the treatment groups because the time points chosen for this experiment are included in the early phase of an *in vivo* study (Fig. 5*A*; see "Discussion"). However, Ad5-Δ24RGD-treated tumors clearly tend to have smaller volumes. Altogether, these data establish a correlation between virus replication and the oncolytic effect of the different CRAds *in vivo*.

Discussion

In this report, we analyzed the effect of the Ad E3 region on the oncolytic capacity of previously described CRAds, Ad5-Δ24 (3) and Ad5-Δ24RGD (26). ADP, one of the seven E3 proteins, mediates cell lysis and release of the progeny, leading to spread of Ad from cell to cell. Here, we compared the dispersion of E3− or E3+ CRAds throughout A549 cell monolayers after infection with 0.1 vp/cell by means of Ad hexon immunostaining. Our results indicate that the presence of the E3 region enhances the spreading ability of Ad5-Δ24E3, which started as early as 3 days after infection, peaked on day 4, and destroyed the cells in 5 days (Fig. 2, *e*−*h*). In contrast, Ad5-Δ24/GFP showed only a slight increase at the end of the experiment, indicative of its slower spread (Fig. 2*d*).

The higher capacity of E3+ CRAds to spread resulted in a superior oncolytic efficacy compared with E3− CRAds *in vitro*. Cytotoxicity assays indicated that the increment in oncolytic efficiency ranges from 1.6× to 20×, depending on the cell line (Fig. 3, *A* and *B*). Also, although modest, Ad5-Δ24RGD demonstrated a statistically significant oncolytic advantage over the non-RGD virus in all of the cell lines. In low CAR-expressing cell lines, such as Hs 766T (53) and SKOV-3 (54), the presence of α_v integrins led to infectivity enhancement via the interaction with RGD modified fiber increasing the oncolytic effect (P < 0.02 for both cell lines). Also in A549 cells, which express moderate levels of CAR, the notably high expression of $\alpha_v\beta_5$ integrin (Refs. 55 and 56; data not shown) contributed to enhance infectivity and oncolysis (Ad5-Δ24E3 *versus* Ad5-Δ24RGD, P < 0.01).

Recently, viral burst has been proposed as a more relevant measurement of oncolytic potency of CRAds compared with live-dead assays (57). Therefore, we conducted a burst titration experiment comparing Ad5-Δ24/GFP and Ad5-Δ24E3 *in vitro*. The resulting progeny release patterns revealed remarkable differences dependent on the E3 status when virus titers of the media *versus* those of the cell fraction were compared in A549 cells infected with the same dose of either virus. Of note, the results of the early time points correlate to the observations of the virus spread assay. The E3− Ad5-Δ24/GFP was not able to disrupt the host cell and escape until day 9, at which time the majority of the progeny is released into the media, leaving a small amount of virus in the remaining cells (Fig. 4*A*). In contrast, virus was detected in the E3+ Ad5-Δ24E3-infected cells and supernatants as early as day 3, indicating the onset of viral replication, early cell lysis, and progeny release. The virus titer of the cell fraction increased 62-fold from day 3 to day 6. Afterward, this number diminished due to the low number of live cells remaining that can support further viral replication after 9 days (Fig. 4, ■). The titer in the media showed a constant increment up to 1 × 10^9 total CPE units on day 9, and the total virus yield (sum of media and cells compartments) at the end of the experiment was 274 times higher in the E3+ virus than in

the E3− one. ADP mutants are affected only in the cell lysis capacity and release of the progeny, but the virus growth remains unchanged (40). Consistently, we do not think that this 274-fold increase reflects an advantage in the E3+ virus replication process itself, but rather that the faster virus release led to more free virus to continue further rounds of infection, replication, release, and logarithmic expansion. In conclusion, these results confirmed the existence of a strong correlation between a bigger viral progeny burst (Fig. 4*B*) and a greater oncolytic potency of the E3+ Ad5-Δ24E3 (Fig. 3*A*) *in vitro*.

The oncolysis study performed *in vivo* confirmed the *in vitro* findings and showed that a single dose of 10^7 vp injected intratumorally sufficed to demonstrate the superior antitumor effect of E3+ CRAds over E3− CRAds. Ad5-Δ24E3 and Ad5-Δ24RGD exerted, respectively, partial or total oncolytic effects 35 days after treatment in comparison with the nodules treated with the E3− viruses (Fig. 5*A*). This graph shows the strong role that E3 plays in the Ad5-Δ24 context. As mentioned, the Ad5-Δ24 used in previous reports does not contain E3 (26), corresponding to Ad5-Δ24/GFP in the present report. Accordingly, the differences between Ad5-Δ24 and Ad5-Δ24RGD reported previously (26) were more evident due to a contribution of both E3 and RGD. The present work dissects these effects and shows that both E3 and RGD contribute to enhance the oncolytic potency. The fact that we were able to detect viruses in cryosections of CRAd-treated tumors 35 days after injection suggested that virus progeny was produced. The growth curves of E3+ CRAd-treated tumors together with the immunodetection data indicated that cell lysis and lateral spread were strong and fast enough to abolish tumor growth, and although complete tumor eradication was rarely seen, some of these nodules (40%) were composed mostly of necrotic material. In contrast, the presence of E3− CRAds in the growing tumors suggested that their cell killing capacity is not potent or fast enough to oppose tumor growth (Fig. 5*A*). Nevertheless, it has to be considered that the interaction of each of the mutant viruses with the cells is dynamic. It is possible to have more inefficiently released virus (E3−) if this accumulates slowly during the 35 days of treatment. In contrast, the E3+ viruses that rupture cells and expand faster might face a lack of host cells after overcoming the balance between tumor cell growth and cell killing by the virus, and the number of virus might decrease together with the tumor lysis. From these results, we concluded that the presence of E3 confers the advantages of early cell disruption and progeny release that are required for an efficient oncolytic effect *in vivo* and that the combination with the infectivity-enhancing RGD motif results in additional enhancement of the antitumoral effect. In fact, work done in our group sustains the superiority of the RGD-containing viruses in the context of oncolysis.[4]

To further study the *in vivo* behavior of the CRAds, we recovered infectious virus particles from the tumors without compromising their infectivity by using an enzymatic dissociation of the tissue. As the method of titration, we chose the spectrophotometric assay reported by O'Carroll *et al.* (51). This is based on the biological activity of the virus, unlike other techniques such as quantitative PCR that are based on the DNA (or RNA) sequences in the sample. Considering that not all of the viral DNA is packaged and not all of the encapsidated viruses are capable of infection (58, 59), we wished to measure exclusively the bioactive progeny virus. The result showed that the tumors treated with E3+ CRAds have higher mean values compared with the E3− counterparts, and Ad5-Δ24RGD is highest among all of the treatment groups (Fig. 6*A*). The 293 cells used for the titration assay were not affected by exposure to either the extracts obtained from PBS-treated tumors or the nonreplicative AdGFP-treated tumor extracts. This assured that the CPE, on which the titration is based, was indeed provoked by the presence of CRAd progeny and not by toxic substances derived from the tumor. The side-by-side comparison with the corresponding tumor sizes indicated that higher virus titers corresponded to smaller tumor volumes. However, several factors should be taken into account when quantitative analyses of this type are done. It is important to include the entire tumor because the Ad is not homogeneously distributed throughout the tumor mass. As suggested by Harrison *et al.* (60), structural barriers inside the established tumor may preclude the spread of the CRAds, and, as a result, a sample taken from the tumor might not be representative of what is happening to the nodule as a whole. Another point is that the tumor is composed not only of cells but also of fibrous and necrotic tissue, making representative sampling a problem. As a matter of fact, previous experiments have shown that Ad5-Δ24RGD-treated nodules were composed mainly of necrotic material 35 days after the injection and showed lower titers than the nodules that still contained cellular components (data not shown). Necrotic tissue does not support virus production. This is the reason why early time points were chosen for the *in vivo* titration of virus growth (Fig. 6). As described in Fig. 5*A* and also in our previous work (26), it is not possible to demonstrate statistical difference in size at this initial phase, but this time frame allows the recovery of tumors without necrosis that lead to more real comparisons of virus replication capacity.

Similar to the study performed with promoter-based CRAds by Yu *et al.* (41), the E3+ mutants used in the present report contain the complete E3 region. For this reason, we cannot dissect the effect of the individual genes within this region. However, we speculate that ADP is the principal molecule eliciting cell lysis. It is known that the dynamics of plaque formation and cell lysis by mutants that lack other proteins encoded by E3 is normal *in vitro* (40). In addition, there is proof that transgene substitutions for ADP, but not for the other E3 genes, reduce the effectiveness of the virus cell killing *in vitro* (37, 61, 62). The role of ADP would therefore be manifested in preclinical antitumor assays and could have an important implication in therapy. Nevertheless, other E3 genes with immune suppressive function may also have an important role for oncolysis in the presence of an immune system. As mentioned previously, the E3 region encodes six other proteins, four of which are involved in immunoevasion, namely protein 14.7K, the RIDα/β complex (formerly 10.4K/14.5K), and gp-19K (63–

[4] M. Yamamoto, J. Davydova, M. Wang, G. P. Siegal, V. Krasnykh, S. M. Vickers, and D. T. Curiel. Infectivity enhanced, cyclooxygenase-2 promoter-based conditionally replicative adenovirus for pancreatic cancer. Manuscript in preparation.

65). The preservation of immunoregulatory E3 genes in a CRAd may thus be favorable because it enhances the immune evasion potential of the virus (37). On the other hand, it has been suggested that the deletion of the E3 region combined with an overexpression of the *ADP* gene in a CRAd may lead to a combined tumor cell killing effect elicited by the CRAd and the immune system (66, 67). The interplay of the other E3 proteins with the immune system and its influence on the oncolytic effect of CRAds requires further investigation in immune-competent models for Ad oncolysis.

In the present report, we have extended our studies on the enhancement of the oncolytic potency of Ad5-Δ24 and Ad5-Δ24RGD by the inclusion of the E3 region. We have demonstrated that the addition of the E3 gene enhances the antitumor effect of CRAds by promoting an earlier cell lysis, progeny release, and faster spread compared with E3− viruses. Improvements such as the incorporation of the RGD motif for infectivity enhancement and the E3 region for oncolysis enhancement will have a major impact on the clinical applications of CRAds.

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APPENDIX C

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ARTICLE

The Organotypic Multicellular Spheroid Is a Relevant Three-Dimensional Model to Study Adenovirus Replication and Penetration in Human Tumors *in Vitro*

Jacques Grill,[1,2] Martine L. M. Lamfers,[1,3,*] Victor W. van Beusechem,[1] Clemens M. Dirven,[1,3] D. Shareen Pherai,[1,3] G. Mathijs Kater,[1,3] Paul Van der Valk,[4] Ronald Vogels,[5] W. Peter Vandertop,[3] Herbert M. Pinedo,[1] David T. Curiel,[1,6] and Winald R. Gerritsen[1]

[1]*Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, 1007 MB Amsterdam , The Netherlands*
[2]*Department of Pediatric Oncology, Gustave Roussy Institute, 94805 Villejuif cedex, France*
[3]*Department of Neurosurgery, VU University Medical Center, 1007 MB Amsterdam, The Netherlands*
[4]*Department of Pathology, VU University Medical Center, 1007 MB Amsterdam, The Netherlands*
[5]*Crucell Holland BV, 2301 CA, Leiden, The Netherlands*
[6]*Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama, 35294, USA*

**To whom correspondence and reprint requests should be addressed. Fax: 31(0)20-444-8168. E-mail: m.lamfers@vumc.nl.*

The use of adenoviruses for gene transfer and as oncolytic agents is currently receiving widespread attention. As specific constraints to adenovirus distribution and spread cannot be studied in cell cultures, there is a need for an *in vitro* three-dimensional (3D) model mimicking the *in vivo* biology of tumors. We studied the interactions between tumor and adenoviruses using multicellular spheroids grown from primary brain tumor material. Using β-galactosidase and luciferase reporter genes expressed by replication-defective adenoviruses, we showed that infection was restricted to the first layer of cells. Using a replication-competent adenovirus expressing the luciferase gene, we showed that transgene expression in the spheroid was considerably enhanced and that viral spreading deep into the 3D structure took place. In addition, a tetrazolium salt–based metabolic assay could be used to compare the oncolytic activity of different concentrations of replication-competent adenoviruses. We can conclude that organotypic spheroids offer a versatile *in vitro* system for studying distribution, spread, and oncolysis by adenoviruses in a clinically relevant model.

Key Words: spheroid, adenovirus, replication, *in vitro* model, cancer gene therapy, oncolytic viruses

Introduction

Adenoviruses are increasingly used as vectors for gene transfer in tumor cells or as oncolytic viruses. In the development of these antitumoral agents, specific questions regarding the pathogenic steps involved in the therapeutic effect need to be addressed in relevant models. Certain aspects of the biology of interactions of human adenoviruses and human tumors (for example, distribution and lateral spread) can only be studied *in vivo*. Moreover, when studying adenoviral replication in human xenografts growing in rodents, one must take into account the nonhuman nature of the stroma (cells and extracellular components) of these tumors, knowing that rodent cells do not allow complete lytic cycle of human adenovirus. It is thus desirable to develop alternatives to animal experimentation with relevant *in vitro* models that are closer to the human tumor tissues and in which specific biological determinants of adenoviral-mediated gene therapy can be studied.

Since their introduction in 1957 by Moscona [1], multicellular tumor spheroids have gained experimental importance as an *in vitro* model of tumors [2,3]. Spheroids are intermediate in complexity between standard two-dimensional monolayer cultures *in vitro* and tumors *in vivo*. They can be grown from cell lines [3] or can be derived from primary tumors [4] of many different types including brain tumors, and lung and ovary carcinomas. Spheroids are grown in recipients with a nonadhesive surface, on which aggregates of isolated tumor cells or small explants derived from tumor material progressively round up and form spheres that can grow up to 1 mm in diameter. The complex tissue architecture present in the tumors (that is, tumor cells, connective tissue, immune cells, capillaries, and noncellular constituents) is present in the

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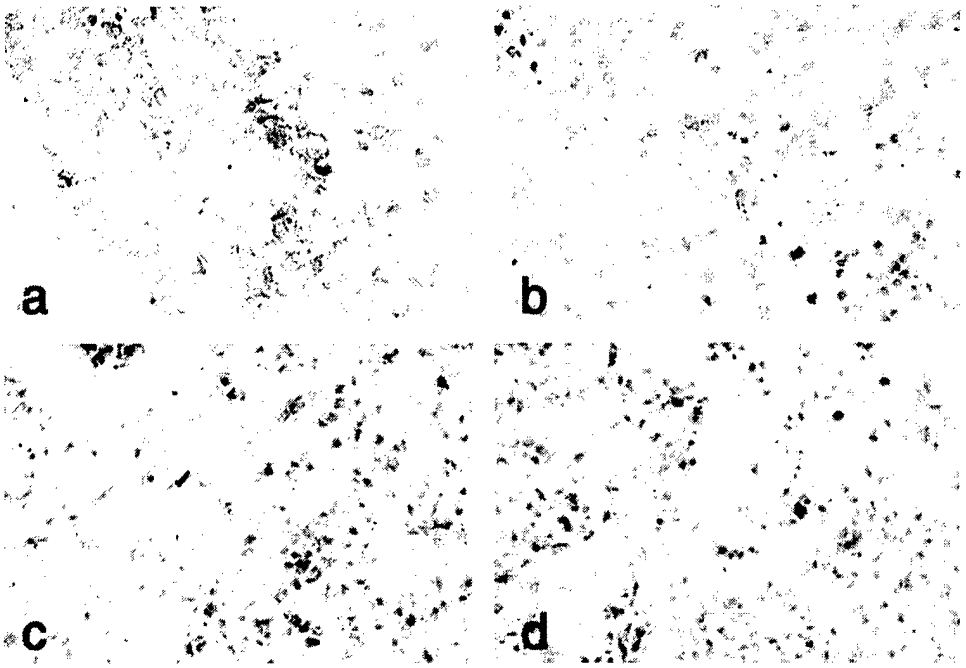

FIG. 1. Immunohistochemistry for coxsackievirus adenovirus receptor (CAR) on primary tumors and derived spheroids. Frozen sections were stained using an anti-CAR mouse monoclonal antibody raised against the CAR ectodomain and counterstained with H&E. Spheroids were processed after 2 weeks of culture, that is, at the time they are routinely used for experiments. (A) Glioma tumor VU-61. CAR expression is detected in > 50% of the tumor cells with a perinuclear and cytoplasmic distribution. Analysis of the whole-tumor sample showed clear heterogeneity in the intensity of the staining. (B) Glioma spheroid VU-61. A similar pattern of CAR staining is detected as in the primary tumor. (C) Meningioma tumor VU-59. CAR expression is detected in ~ 50% of the cells. Staining was mostly cytoplasmic. (D) Meningioma spheroid VU-59. Similar CAR expression is found in the spheroids. Note that spheroids conserved the architecture of the tumor they were derived from in both cases. Original magnifications: ×40.

organotypic spheroids grown from primary tumors. In this respect, organotypic multicellular spheroids (OMS) resemble the situation *in vivo* more closely than tumor cell lines do [5]. The histological characteristics of these spheroids, as well as the principal immunocytochemical ones, have proved to be unchanged compared with the primary tumor after prolonged culture periods up to 16 weeks [5]. In addition, cytogenetic characteristics of tumor cells in OMS of glioma are comparable to tumor cells of the original tumor, whereas they are lost when the same primary tumor cells are grown in monolayer cultures after a few passages [6]. Spheroids have been used in radiation biology and to study diffusion of drugs, or macromolecules such as antibodies, immunotoxins, or radiopharmaceuticals. These 3D structures have usually been shown to be more resistant to the therapeutics tested than monolayers of the same cell type, although the underlying mechanisms, such as the "contact effect," are not fully understood [7]. In addition, cell line spheroids have proved to predict more accurately the response of xenografts to irradiation than monolayers [8].

One of the major obstacles to successful delivery of large anticancer agents, such as adenoviruses, is the low uptake of these therapeutic agents in tumor nodules. Penetration of these types of agents by convection and diffusion is affected by the structure and composition of the tumor extracellular matrix (ECM) and the cellular contacts [9,10]. In this regard, the OMS closely resemble glioma tissue *in situ*, in contrast to monolayer cultures [11]. Investigators recently showed the negative impact of some ECM components on adenoviral infection [12]. This indicates that *in vitro* models with controlled microenvironment such as spheroids will allow for important investigations in this area.

Unlike monolayer cell cultures, in which all the cells are cycling, in the spheroid the mitotic cells are mostly found in the periphery while in the core of the spheroid most of the tumor cells are in G0 phase, resembling the *in vivo* situation of avascular stages of early tumor growth [2,5]. Replication-selective adenoviruses have been designed to replicate specifically in tumor cells lacking specific cell cycle checkpoints [13]. Further evaluation of these mutant adenoviruses has shown that S-phase cells were more susceptible to virus-induced cell kill than G1-phase cells [13]. We thus hypothesized that adenovirus replication in OMS will mimic the *in vivo* situation more closely than replication in monolayer cell cultures. Although several important issues related to *in vivo* administration of adenoviruses, such as efficient access to the tumor and the effect of interstitial pressure, cannot be addressed using OMS, we anticipated that the study of this model could help to answer pivotal questions regarding adenovirus-mediated gene therapy and/or oncolysis.

Results and Discussion

Expression of Adenoviral Receptors in Spheroids

While primary tumor cells derived from patient biopsy material exhibit considerable changes in expression of specific antigens after a few passages in monolayer cultures, OMS show a pattern of immunohistochemical staining that is similar to the tumor itself for a wide range of molecules, such as glial fibrillary acidic protein (GFAP), epidermal growth factor receptor (EGFR), or proliferating cell

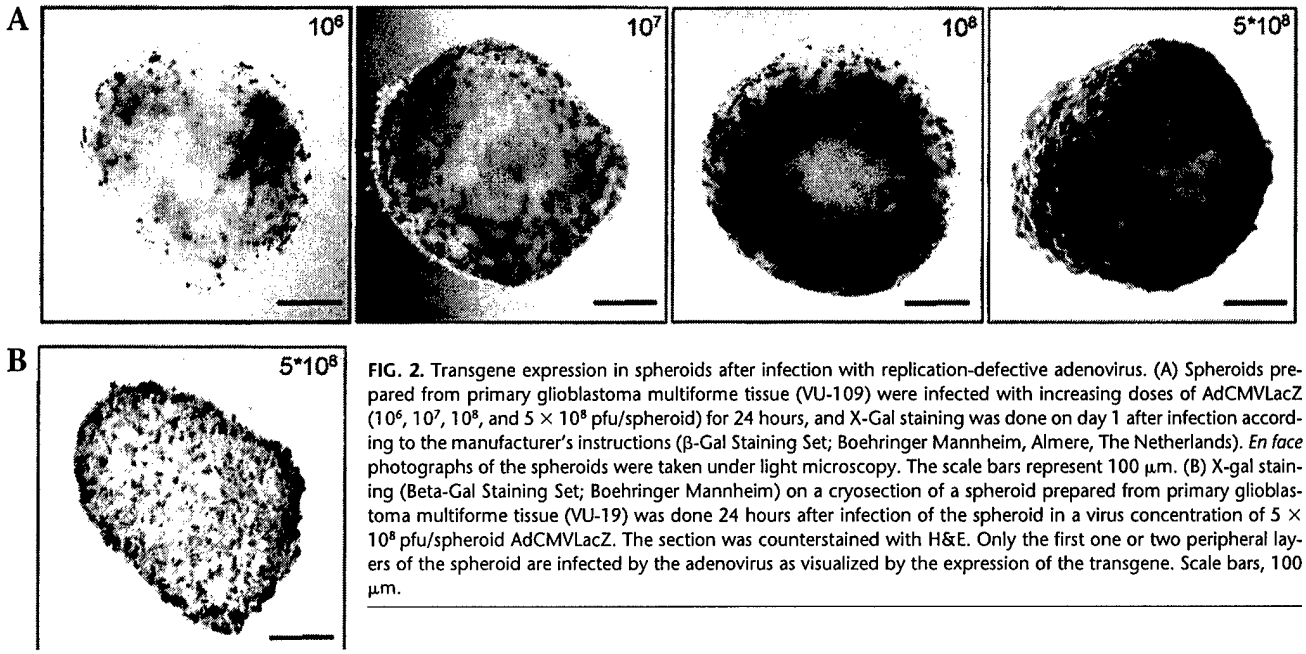

FIG. 2. Transgene expression in spheroids after infection with replication-defective adenovirus. (A) Spheroids prepared from primary glioblastoma multiforme tissue (VU-109) were infected with increasing doses of AdCMVLacZ (10^6, 10^7, 10^8, and 5×10^8 pfu/spheroid) for 24 hours, and X-Gal staining was done on day 1 after infection according to the manufacturer's instructions (β-Gal Staining Set; Boehringer Mannheim, Almere, The Netherlands). *En face* photographs of the spheroids were taken under light microscopy. The scale bars represent 100 μm. (B) X-gal staining (Beta-Gal Staining Set; Boehringer Mannheim) on a cryosection of a spheroid prepared from primary glioblastoma multiforme tissue (VU-19) was done 24 hours after infection of the spheroid in a virus concentration of 5×10^8 pfu/spheroid AdCMVLacZ. The section was counterstained with H&E. Only the first one or two peripheral layers of the spheroid are infected by the adenovirus as visualized by the expression of the transgene. Scale bars, 100 μm.

nuclear antigen (PCNA) [5,11,14]. Cellular uptake of most human adenovirus serotypes requires coxsackie–adenovirus receptor (CAR) for primary binding and αv-integrins for subsequent internalization. Therefore, the presence of these receptors was analyzed by immunohistochemistry on brain tumor spheroids and compared to the corresponding tumor from the same patient. Because recent reports have attributed to CAR the function of a cell adhesion molecule [15,16], it is possible that CAR expression may depend on the 3D organization of the cells. When spheroids were grown from tumors that were positive for CAR, they retained the expression of this receptor. Matched tumor tissues and the derived OMS usually exhibited a comparable staining pattern of CAR expression, as shown in Fig. 1 for a glioma and for a meningioma tumor sample. In primary OMS derived from

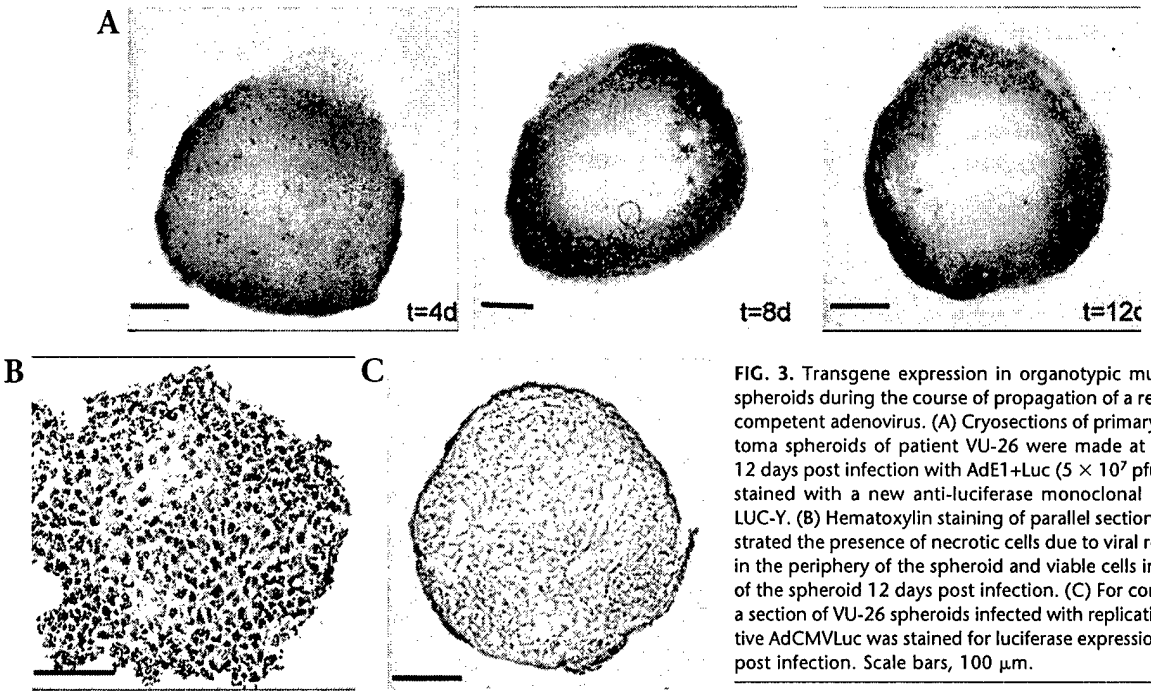

FIG. 3. Transgene expression in organotypic multicellular spheroids during the course of propagation of a replication-competent adenovirus. (A) Cryosections of primary glioblastoma spheroids of patient VU-26 were made at 4, 8, and 12 days post infection with AdE1+Luc (5×10^7 pfu/ml) and stained with a new anti-luciferase monoclonal antibody, LUC-Y. (B) Hematoxylin staining of parallel sections demonstrated the presence of necrotic cells due to viral replication in the periphery of the spheroid and viable cells in the core of the spheroid 12 days post infection. (C) For comparison, a section of VU-26 spheroids infected with replication-defective AdCMVLuc was stained for luciferase expression 12 days post infection. Scale bars, 100 μm.

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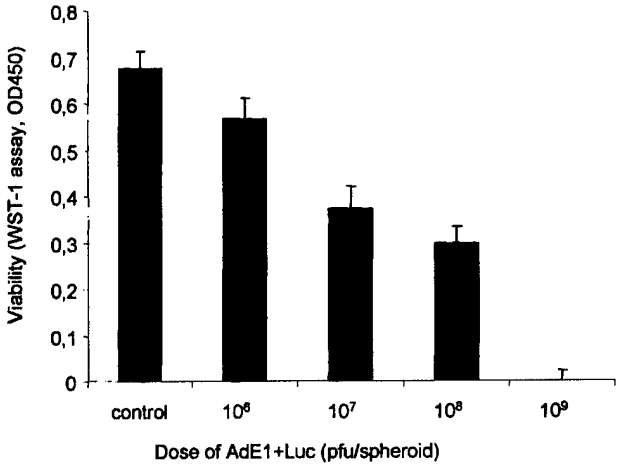

FIG. 4. Dose-dependent oncolysis of organotypic glioma spheroids with a replication-competent adenovirus. Spheroids (n = 7 per group) derived from the glioblastoma tumor of patient VU-65 were infected with increasing concentrations of AdE1+Luc, and viability was measured 10 days later with the WST-1 assay according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Data are presented as mean ± SEM of the OD_{450} after subtraction of the background. A significant dose-dependent oncolysis was observed 10 days after infection (P = 0.0001, ANOVA). Dose-dependent oncolysis could also be observed as early as day 6 (data not shown).

CAR-positive tumors, this receptor was still expressed after 3 months of culture.

Also, αv-integrin expression in OMS (data not shown) reflected the pattern of expression described for primary tumors [14]. Thus, also with regard to CAR and αv-integrin expression, brain tumor OMS resemble primary tumors more closely than monolayer cultures. This makes OMS very suitable for the *in vitro* study of adenovirus targeting toward tumor-specific receptors [14].

Replication-Defective Adenoviruses Do Not Penetrate into Spheroids

When cell monolayers are infected with adenoviruses, virtually all cells are in contact with the viruses. Substantial *in vitro* infection of cell lines can be obtained with adenoviruses even when CAR is not expressed, provided high vector concentrations are used. *In vivo*, however, diffusion of large molecules, in particular viruses, is hampered by several variables, including the hydrostatic pressure in the tumor and the structure or the composition of the tumor ECM [9,10].

In primary glioma spheroids infected with replication-defective adenovirus encoding β-galactosidase, we observed a semiquantitative dose response (Fig. 2A). However, cross-sectioning of spheroids demonstrated that expression of the reporter protein was limited to the first two layers of the spheroid (Fig. 2B). Additional increase of the adenovirus concentration up to 5×10^{10} pfu/spheroid or analyzing gene transfer at later time points did not show infection of tumor cells beyond the rim of the spheroid (data not shown). Using the same reporter gene, Grace and co-workers have shown similar limited penetration after adenoviral-mediated gene transfer in an animal model for peritoneal carcinomatosis [17]. Thus, poor penetration of adenoviruses in the tumor is a major obstacle for efficient gene therapy using these vectors. Spheroids represent a valuable *in vitro* model to study this "edge-in" barrier as well as strategies to overcome this problem, such as the use of replication-competent adenoviruses [13], diffusible toxins/enzymes, or proteinases that disrupt the ECM [12].

Propagation of Replication-Competent Adenoviruses in Spheroids

One of the approaches proposed to improve gene delivery into solid tumors is the use of replication-competent vectors that spread throughout the tumor. To study this strategy, we compared an E1-deleted replication-defective adenovirus (AdCMVLuc) and an E1-positive replication-competent adenovirus with the same reporter gene inserted in the E3 region (AdE1+Luc). Adenoviral spread in OMS was studied immunohistochemically using a new monoclonal antibody against luciferase. The replication-competent virus substantially improved the distribution of the transgene in the 3D structure of the OMS (Fig. 3). In the course of 12 days, several consecutive layers (more than eight layers on day 12) were infected with AdE1+Luc as demonstrated by the spreading of luciferase expression toward the core of the spheroid (Fig. 3A). Hematoxylin staining on parallel sections from day 12 demonstrated that the lack of gene expression in the core of the spheroid was not a result of necrosis, in that viable cells were detected in this area (Fig. 3B). We never observed transgene expression beyond the first two layers of the spheroid when the infection was carried out using the nonreplicating AdCMVLuc (Fig. 3C).

Accordingly, luciferase expression in AdE1+Luc-infected spheroids, as quantified by luciferase assay, increased 8.6-fold within 9 days, indicating continued viral replication and tumor cell infection. In contrast, luciferase expression in spheroids infected with nonreplicating AdCMVLuc decreased 4.1-fold within 9 days (data not shown). This may result from cytomegalovirus (CMV) promoter shutoff or from a loss of infected cells due to cell turnover in the spheroids. These results demonstrate that replication-competent vectors achieve amplification of transgene expression, which is consistent with earlier reports [18]. In addition we demonstrate that distribution of the transgene in solid tumors is improved.

At the same time, our findings demonstrate the limitations of replication-competent adenoviruses in cancer therapy. At day 12, the spread of the replication-competent virus had still not reached the core of the 400-mm

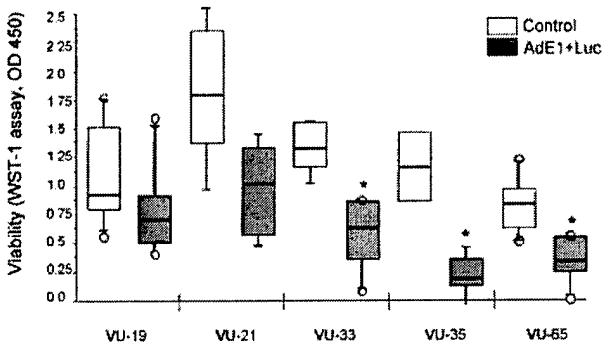

FIG. 5. Oncolysis of organotypic multicellular spheroids from five glioblastoma multiforme patients after infection with a replication-competent adenovirus. Spheroids from the tumor of five different patients were infected with 10^8 pfu/spheroid of AdE1+Luc, and viability was assessed 2 weeks after infection. Results are given as the OD_{450} value of the WST-1 assay, 24 hours after initiation of the reaction. The graph represents the distribution in percentiles of the values observed for each spheroid ($n = 5$ to 8 per group), according to Cleveland and Tukey using StatView software. The control groups are shown in white and the treated groups are shown in gray. The boxes represent 50% of the distribution. The horizontal lines represent the medians. The bars encompass 90% of the distribution and the circles represent the extreme values. The following differences in metabolic activity of untreated controls and treated spheroids compared by Student's *t*-test were observed: $P > 0.05$ for VU-19 and VU-21, $P = 0.02$ for VU-33, $P = 0.005$ for VU-35, and $P = 0.002$ for VU-65. $^*P < 0.05$.

organotypic spheroid (Fig. 3A). Conversely, in spheroids derived from glioma cell lines, we observed complete oncolysis due to viral replication within 1 week (data not shown). The limited penetration into OMS may be explained by such variables as deficiency of adenoviral receptors in the primary glioma cells or entrapment of the virus by components of the ECM. We estimated the speed of lateral spread of human adenovirus serotype 5 inside the OMS at ~ 100 μm per week at the best, which is 10 times below the estimated speed of the invading wave of the glioma [19]. It is thus understood that any modification of the adenovirus that increases the dissemination of the therapeutic effect would have a major impact on the potential efficacy of this agent.

Measurement of Therapeutic Effect of Replication-Competent Adenoviruses

To measure a therapeutic effect on spheroids, one can use morphological parameters such as relative volume change [20] or outgrowth of the spheroid [21]. However, in our hands, the former parameter is not reliable, especially not with spheroids derived from primary tumor material in which the dead cells often do not detach from the spheroid because of ECM cohesion. The spheroid outgrowth assay was found to be unsuitable for studying oncolysis in primary spheroids, because this assay requires extended culture periods (up to 21 days). During this period viral replication continues, thereby inducing cell lysis and cell detachment, which hampers spheroid outgrowth. Finally, as it is desirable to study as many organotypic spheroids as possible in view of the intrinsic heterogeneity of primary gliomas [11], the use of a convenient assay is advantageous. Therefore, we have directed our attention to the use of an MTT-derived cell viability assay (WST-1). In Fig. 4 we show the survival of VU-65 OMS treated with increasing doses of AdE1+Luc. A clear dose-dependent effect of the virus on spheroid viability was found ($P = 0.0001$, ANOVA); the post-hoc comparison of Scheffe showed that the differences were significant between control and viral doses > 10^6 pfu/spheroid and between the dose of 10^9 pfu/spheroid and all the other doses.

In Fig. 5 we demonstrate the oncolytic activity of AdE1+Luc in primary glioma spheroids isolated from five different patients. The oncolysis was highly variable, ranging from no significant effect to almost complete kill within the 2-week time frame of the experiment. As expected, the baseline metabolic activity of untreated spheroids varied among patients ($P = 0.0045$, ANOVA). There was, however, no correlation between the baseline metabolic activity and the therapeutic effect of the virus. Replication-competent adenovirus induced a significant killing effect in the spheroids of three of the five patients studied (Fig. 5). This suggests that brain tumor OMS allow *in vitro* monitoring of biological effects of replication-competent adenoviruses. Alternatively, these OMS can be used to define the parameters regulating oncolytic activity of replication-competent adenoviruses, for example CAR expression or ECM composition.

Intratumoral distribution of vectors, oncolytic viruses, and their products is a major issue in the design of new adenovirus-based gene therapy strategies for cancer. The spheroid model offers a versatile *in vitro* system to approach these specific questions that could otherwise only be tested *in vivo* using animal models. Moreover, spheroids derived directly from patients' tumor material, including glioma but also many other tumor types, offer the unique opportunity to test these new strategies on tumor cells in their original microenvironment without any process of *in vitro* selection. The role of connective tissue and extracellular matrix in viral spread, diffusion of secreted enzymes, and cellular responses to gene transfer can in this way be explored *in vitro* on clinically relevant specimens. The 3D structure of spheroids retains many characteristics of the tumor itself, including barriers to viral infection and penetration, thus allowing studies of modifications aimed to increase viral spread. Of particular interest, histological analysis of spheroids showed that replication-competent viruses spread inside the tumor, layer by layer, and that the speed of spreading is suboptimal even with wild-type viruses. In addition, we showed here that the oncolytic potency of replication-competent adenoviruses can be monitored in this model. We expect that rediscovery of this *in vitro* tumor model will stimulate research in the growing field of oncolytic adenoviruses and adenoviral-mediated cancer gene therapy.

Materials and Methods

Preparation and culture of spheroids. Fresh tumor tissue was obtained at surgery and processed in the laboratory within 2 hours. The brain tumor specimens used for spheroid preparation are denoted by "VU" numbers and were diagnosed as glioblastoma multiforme (VU-19, VU-26, VU-33, VU-35, VU-62, VU-65, VU-109) or meningioma (VU-21, VU-59). Blood and necrotic tissues were removed from the tissue, and fragments of 300–500 μm were dissected with sterile 21-gauge needles. These explants were plated individually in 2% agarose-coated 48-well plates and cultured in DMEM supplemented with L-glutamine, gentamicin, and 10% heat-inactivated FBS, at 37°C with 5% CO_2 and 100% humidity. Medium was renewed once every 2 weeks. Only fragments that became spherical (i.e., spheroids) and that were ~ 400 μm in diameter were used for experiments.

Before adenoviral infection, spheroids were transferred individually by gentle pipetting into a 96-well plate coated with 2% agarose in PBS. Wells were filled with 200 ml culture medium.

Adenoviruses. AdCMVLuc and AdCMVLacZ are replication-defective, E1- and E3-deleted adenoviruses encoding the firefly luciferase gene and the *Escherichia coli* β-galactosidase gene, respectively. Both transgenes are under control of the CMV promoter. AdE1+Luc is a replication-competent adenovirus with a wild-type E1 region and the luciferase gene replacing the gp19k coding sequence in the E3 region. Luciferase expression is therefore under the control of the E3 endogenous promoter.

WST assay. Quantification of cell proliferation and cell viability using the WST assay (Roche Diagnostics, Mannheim, Germany) is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial hydrogenases in viable cells. After addition of 10% WST-1 (vol/vol, in culture medium), color development by formazan dye formation is monitored spectrophotometrically (OD_{450}) in time. Incubation time with WST-1 was defined according to the manufacturer's instructions. Analysis of the kinetics of formazan formation demonstrated that incubation times between 12 and 24 hours are optimal for this particular experimental setup with OMS. In frozen sections of the spheroids prepared at various time points of the assay, we observed that *in situ* conversion of WST-1 throughout the whole diameter of the spheroid required 10 hours of incubation.

Histology and immunohistochemistry (IHC). Frozen sections of 5 to 8 μm were fixed by acetone. Nonspecific antibody binding was blocked by incubation with 2% normal rabbit serum. Slides were incubated for 1 hour at room temperature with the following mouse monoclonal antibodies diluted at 10 μg/ml in blocking buffer (1% BSA in PBS): Mouse anti-CAR monoclonal antibody (provided by Igor Dmitriev, University of Alabama at Birmingham), anti-$\alpha_v\beta_5$ integrin MoAb clone P1F6 (Life Technologies, Breda, The Netherlands), and anti-$\alpha_v\beta_3$ integrin MoAb clone LM609 (Chemicon, Temecula, CA). IHC staining for luciferase was done using a 2.5 μg/ml dilution of the novel monoclonal antibody LUC-Y (J.G., manuscript submitted). Two negative controls were included for each staining. These negative controls were treated either with blocking buffer only or with normal mouse IgG1-κ. Specimens were then washed in PBS and incubated with biotinylated rabbit anti-mouse IgG antibody (Dako A/S, Glostrup, Denmark) diluted 1/500 in PBS for 30 minutes. Second-antibody binding was visualized with a peroxidase-conjugated streptavidin ABC kit (Dako A/S) diluted 1/200 in PBS for 1 hour. The slides were counterstained with hematoxylin.

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APPENDIX D

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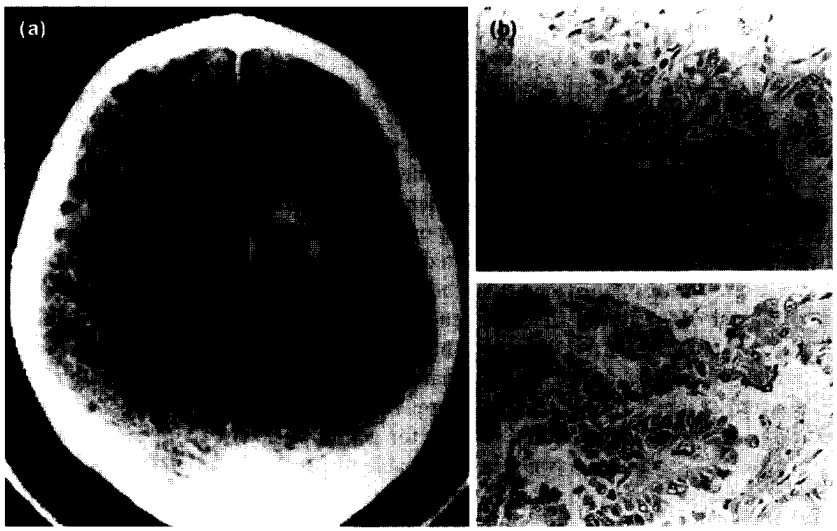
Clinical picture

A metastatic dormant tumour in the brain

An 78-year old woman was admitted with headache and progressive hemiparesis of 4 months duration. Eight years before admission, the patient underwent radical surgery for adenocarcinoma of the right lung and had no tumour recurrence. A CT scan revealed a brain tumour in the left caudate head (a). After 9 Gy of radiotherapy, the patient developed severe pneumonia and died. A post-mortem examination showed that the brain tumour was a metastatic adenocarcinoma and that she had no other malignancy in her body, including both lungs. Histopathology showed that the brain tumour (c) was very similar to those of the original lung adenocarcinoma (b).

We believe that the brain lesion was a manifestation of the metastasis, which had been in a dormant state for 8 years. The tumour cells remain in the G0 phase, in a dormant state, and escape the immunosurveillance system of the host. The dormant tumour cell microsphere can survive if it is only 1 – 2 mm in diameter, by diffusion of nutrients from the surrounding tissue. The tumour cells can be induced to start dividing again by certain events. In this case, the reduced immunosupression of the host due to normal ageing may have been responsible for the tumour reversing its dormant state.



N Tsuzuki, T Miyazawa, H Nawashiro, K Shima
Departments of Neurosurgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Tokorozawa, Japan.
Tel: +81 42 995 1656. Fax: +81 42 996 5207.
Email: tsuzuki@me.ndmc.ac.jp

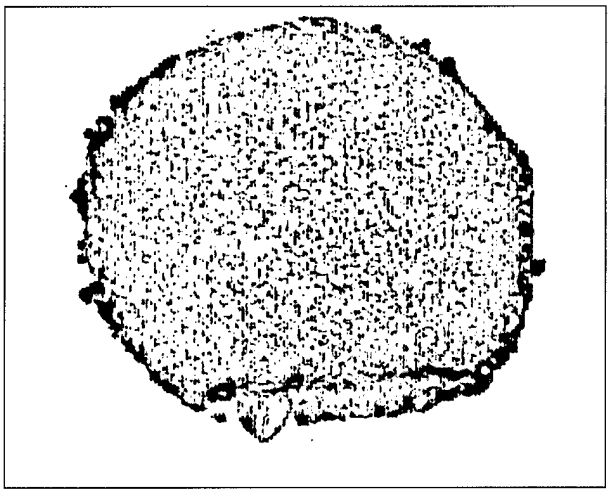
Conditionally replicative adenoviral vectors for cancer gene therapy

Jesús Gómez-Navarro and David T Curiel

During the past century, many attempts have been made to exploit the ability of some viruses to infect and destroy cancer cells. Crippled, non-replicative viruses have been used as vectors to transfer genes into tumours. Both strategies have serious limitations. The time is now ripe, however, for full convergence of these two research tracks. On the one hand, the intratumoral propagation of replicative viruses would overcome the low levels of gene transfer achieved by current viral vectors. On the other hand, the versatility provided by vectors encoding foreign genes, which are limited in their uses only by our ingenuity, would overcome the physiological barriers to robust propagation of the viral progeny in the tumour. This empowering synthesis will provide truly new opportunities that might realise the promises of gene transfer for the therapy of cancer.

Lancet Oncol 2000; **1**: 148–58

The genetic fingerprint associated with malignant transformation and progression, consisting of numerous acquired and inherited genetic lesions, is now being identified rapidly and precisely in a large variety of human cancers. With this knowledge of the molecular anatomy of the cancer cell, gene therapy has emerged as a rational method for therapeutic, and possibly preventive, intervention against cancer, targeted at cellular gene expression. Various approaches have been rapidly translated into almost 300 clinical trials of gene therapy in human beings. Several critical requirements, however, have become apparent in these initial studies that preclude the full realisation of the promise of gene therapy. Most notably, the efficiency of presently available systems for gene transfer is inadequate. Typically, it has not been possible to genetically modify a large enough number of tumour cells to achieve a clinically relevant tumoral response. This is true even after multiple intratumoral injections or when the vector is infused into closed compartments, such as the intrapleural and intraperitoneal cavities (Figure 1). Importantly, increasing the infused dose of the most efficient vectors available (recombinant viruses) to obtain transduction of a larger fraction of the tumour cells has been associated with limiting regional and systemic side-effects, both in animal models and in human beings. Thus, the small therapeutic index of currently available vectors is a critical limiting factor.[1] To overcome this obstacle, intensive research has aimed to expand and exploit the increasing molecular knowledge of viral biology, including the mechanisms of cellular entry, the viral genome and its replication, and host – virus relations. As a result, a novel generation of targeted vectors with greater infectivity and more selective tropism for tumour cells is emerging.



Courtesy of J Grill, Vrije Universiteit, Amsterdam

Figure 1. Vectors are limited in their capacity for penetrating tumours. A spheroid formed by glioma cells GLI-6 is depicted after infection with 10^7 viral particles of a recombinant adenovirus encoding the lacZ reporter gene. Gene transfer is revealed by X-gal staining 24 h after infection (H&E counterstaining). In contrast to the high efficiency for gene transfer typically shown by adenoviral vectors in vitro *in assays of cell monolayers, only a very small fraction of tumour cells were accessible to the viral vector in this three-dimensional aggregate. This pattern clearly indicates a major limitation in the* in vivo *situation of current non-replicative vectors.*

The crucial importance and difficulty of the need for efficient gene delivery was recognised early in the development of gene therapy. The observation of the low amounts of gene transfer achievable *in vivo* with plasmids and recombinant, non-replicative viruses, and of the difficulties that macromolecules such as monoclonal antibodies had for penetrating tumour deposits, led several researchers to propose the development of replicative vectors as a means to provide the necessary higher rates of tumour-cell transduction. According to this concept, genes delivered initially to a small number of cells would be copied and transferred to adjacent cells and beyond, thereby

JGN and DTC are at the Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center at the University of Alabama at Birmingham, Birmingham, Alabama 35294, USA

Correspondence: David T Curiel, MD, Director, Division of Human Gene Therapy, University of Alabama at Birmingham, 620 Lurleen B Wallace Tumor Institute, 1824 Sixth Avenue South, Birmingham, AL, 35294-3300, USA. Tel: +1 205 934 8627. Fax: +1 205 975 7476. Email: david.curiel@ccc.uab.edu

increasing the overall efficacy of gene delivery.[2] There is abundant evidence for the ability of certain viruses to replicate and spread selectively in tumour deposits,[3] and Webb and Smith suggested 30 years ago that viruses could be used to treat cancer.[4] Thus, the concept of developing replicative vectors has linked gene therapy with a long tradition of experimental therapy within oncology.

Both gene therapy and the use of viruses as oncolytic agents have severe limitations. However, their use together might well overcome these obstacles. Gene therapists are learning to engineer heterologous genes with therapeutic value within their vectors and to modify the vector tropism in ways that will overcome many of the obstacles of classic oncolytic viruses. In turn, replicating vectors may have the capacity for extensive and widespread gene delivery that current non-replicating vectors lack.

Recombinant adenovirus blends together several features that strongly position it as a vector of choice. Adenovirus readily infects both dividing and non-dividing cells, and it does not integrate into the host cell's genome, so the risks associated with insertional mutagenesis are avoided. In humans, it typically causes benign infections of the respiratory tract and eyes. Adenoviruses stimulate strong innate and immune responses that clear the vector from the body – although recognised as a barrier, this safety feature can arguably be also exploited therapeutically. Adenovirus can be easily produced in the laboratory, and it has been very extensively characterised. Through knowledge of its anatomy and genomic structure, many of the characteristics needed for clinical use can be engineered. Several academic and industrial organisations have already started development of adenoviruses as an agent for clinical use.

We review here some of the principles that are guiding the development of replicative adenoviral vectors, applying special emphasis to clinical considerations related to the treatment of cancer. We have also reviewed the data from the first reports of human clinical trials. For discussion of characteristics of adenovirus vectors, recent reviews are available.[5-7]

Concepts of conditional replication

Viruses evaluated during the past century to treat cancer had two distinguishing features, namely the capacity to propagate in permissive cancer cells, thereby killing the cells by inducing lysis (oncolysis), and a full, wild-type genome. A vector is an agent, such as a virus or a plasmid, which, in contrast, has been engineered in the laboratory and carries a modified or foreign gene. In the context of gene therapy, a vector delivers the desired gene to a target cell. A replicative viral vector is a recombinant virus in which the genome has been engineered to contain a heterologous gene, for instance a toxin gene, in addition to the structural and non-structural viral genes required to allow viral replication. Thus, it may also kill cells by oncolysis. In addition, the modifications introduced in the genome may consist of deletions or substitutions of viral genes that modify viral metabolism in the host cell, for instance crippling the viral replication machinery in a way that can be complemented only by a tumour cell or tissue trait. In this case, the vector is said to be conditionally replicative. Historically, dozens of replicative viruses have been tested, and many viral vectors are currently being evaluated. However, the number of these that are replicative is still small, and only a minority fully and rationally exploit the capacity to exert oncolysis and to bear a therapeutic transgene.

Mechanisms of action of replicative adenoviral vectors

The effects of a replicative vector in the host cell can be attributed to the viral metabolism or to the expression of the encoded transgene. Also, the immune system of the host may react against, and be modulated by, both the viral proteins and the product of the encoded transgene.

Adenoviral propagation releases cytotoxic proteins

Replicative vectors were conceived as a way to amplify the dose of delivered genes. Their intracellular propagation in tumour cells may, in addition, be associated with cell death or oncolysis. The mechanisms by which tumour cells die after viral intracellular propagation are not completely understood, although several contributory factors have been described. For instance, pronounced inhibition of host protein synthesis occurs early in the course of adenoviral infection, which is associated with a destruction of the intermediate filament network that maintains the cell's structural integrity.[8] Also, the early adenoviral E1A protein sensitises infected cells to the cytotoxic effects of tumour necrosis factor.[9] Later, the E3-11.6kDa protein, or adenoviral death protein, is required for efficient cell lysis and virus release,[10] and the E4orf4 protein contributes to cytotoxicity by promoting apoptosis involving a mechanism which is independent of P53 and caspase.[11] In general, it seems probable that the cells die mostly as a result of viral interference with several intracellular metabolic processes. If this view, first proposed by Southam and Moore in 1952,[12] is correct, the oncolytic effect mediated by viruses is not dissimilar to the oncolytic effect of metabolic antagonists, and the virus might be considered as "a highly complex and self-synthesizing antimetabolite".[12] The idea of replicative viruses as, perhaps more correctly, a 'cocktail of antimetabolites' has important practical implications, and is discussed later.

Delivered exogenous genes may modulate many cellular functions

A unique advantage of the replicative adenoviral vectors in current development in comparison with their historical wild-type predecessors is the possibility of delivering exogenous genes into tumour cells, and of doing so in a self-amplifying manner. This capacity allows modulation of the cellular and extracellular environment in which the viral replication takes place by inducing the expression of a protein of interest. As a result, a very diverse number of interventions can be conceived, including but not limited to: (i) increasing the yield and potency of viral replication and viral release from tumour cells; (ii) targeting additional cellular metabolic pathways; (iii) lowering the cellular threshold for apoptosis; and (iv) modulating the inflammatory and immune response in the tumour milieu. Although not extensively explored yet, recent laboratory studies confirm the possibility of successfully combining the effects of viral replication and expression of prodrug-

converting enzyme genes with therapeutic intent,[13,14] ie the virus does not entirely take over the transcription and translation machinery of the cell, which allows expression of the transgene. Current clinical trials are exploring this concept. Of note, the amplification of gene delivery and expression provided by a replicative adenovirus can be very substantial. In studies with adenovirus encoding a prodrug-converting enzyme, the increase in activity of the therapeutic protein was up to 2000-fold.[13,14]

Viral oncolysis might increase the antitumour immune response

The role, if any, of inflammatory and immune responses in mediating the antitumour effects associated with viral replication within tumours in human patients is not known. Intratumoral viral replication might induce or augment the development of antitumoral immunity,[4] and this could occur by attracting dendritic cells and T lymphocytes into the tumour and increasing the amount of tumour antigens available in an immunostimulatory environment. In animal models, there is confirmation of cell-mediated immunity to tumour cells infected with a replicating herpesvirus. There is no firm published evidence obtained with adenoviral vectors, however, that supports the concept. Interestingly, reports on the largest cohorts of cancer patients treated with various replicative viruses, including West Nile virus and adenovirus (34 and 40 patients, respectively), suggest that a humoral immune response was mounted against the injected viruses that ultimately contained and ablated viral spread. No evidence was obtained, however, of an acute or prolonged effect of the cellular immune system against the tumour.[12,15] Again, the opportunities of engineering, in replicative adenoviral vectors, genes that augment the antitumour immune response, such as interferon and interleukin 2, have been well recognised. After the initial studies in severe combined immunodeficient mice,[16,17] studies remain to be done in immunocompetent animals to prove this intriguing concept.

The definitive studies to clarify the respective roles of the viral and transgene proteins in determining an immune response would require a comparison in human subjects of 'empty' and 'loaded' vectors. To date, these studies have not been performed for lack of evidence supporting empty vectors as antineoplastic agents. However, the prospects for a higher therapeutic value in the case of conditionally replicative adenovirus (CRAd) should perhaps make it easier to propose such studies.

Types of conditionally replicative adenoviral vectors

From viruses to vectors

Viruses are by far the most efficient means currently available to introduce nucleic acids into tumour cells, and also to generate a self-amplifying cytotoxic agent able to spread within a tumour. Many viral types have been evaluated in laboratory and clinical studies,[3] including adenovirus, and the lessons learnt are of permanent value. Transient tumour regressions could be induced in a few patients, and localisation of virus in tumour tissue was frequently shown. However, after periodic bouts of excitement, there was a lack of consistent antitumour potency in large cohorts of patients, with occasional excessive toxic effects.[15,18-20] There was no understanding either of the basis for the tumour tropism of certain viruses, nor of the capacity for some, but not others, to multiply in tumours and cause cell destruction. Thus, the strategy was abandoned. Recently, viruses have been proposed again for their capacity to infect and sustain replication and spread selectively within tumours. This time, the molecular basis for selectivity can be better defined. It depends on either natural mechanisms of the wild-type virus life cycle, such as the dependence of reovirus on an activated *RAS* pathway or the requirement for cell replication of autonomous parvoviruses, or on engineered mutations, such as those introduced in recombinant herpesvirus and adenovirus vectors.

Adenoviral vectors

Several features of adenovirus recommend it for development as a conditionally replicative vector. First, its genome and replicative cycle are well characterised (Figure 2). Secondly, it has been extensively used for gene transfer in experimental gene therapy during the past decade. Thirdly, much work has already been done to modify features of the vector that are essential for the success also of replicative vectors, such as vector tropism and modulation of the immune response. Finally, adenoviruses (subgroup C) are benign in adults except in cases of severe immunodeficiency.

Knowledge of the replicative cycle of adenovirus has suggested strategies to engineer conditionally replicative strains (Table 1).[5] Two main approaches have been evaluated. First, genes that are essential for replication of the virus in tumour cells are mutated or deleted (Figure 3). In this way, the virus is crippled and its replication in non-target, normal cells is prevented, whereas the tumour cells provide a permissive environment for replication (Table 2). Alternatively, a second adenovirus may complement the transgene-encoding adenovirus by delivering the required replication-enabling viral sequence (Table 3). Second, viral genes critical for replication are put under control of promoters that are selectively active in the target tumour cells (Table 4). A third strategy, not yet implemented, would involve engineering an exquisitely targeted adenovirus with wild-type replicative capacities. In this way, the basis for the conditionality would depend on the virus being infectious only in tumour cells, and not necessarily on modifying its replicative cycle. It is not yet clear which of these two approaches, restricting viral replication or viral tropism, is preferable, and efforts to develop both are underway. Certainly, modifications of viral tropism to increase viral infectivity will be needed in any case (see below), suggesting that this could be a most efficient strategy.

Clinical results: back to the past?

Early attempts

The viruses chosen for early attempts at cancer treatment were those that inhibited the growth of certain transplanted tumours in mice, a property of relatively few of the many viruses tested. When tumour inhibition does occur, virus is invariably present in the tumour, and tumour inhibition never occurs if virus fails to multiply. These observations led to the conclusion that the virus particle itself, rather than a

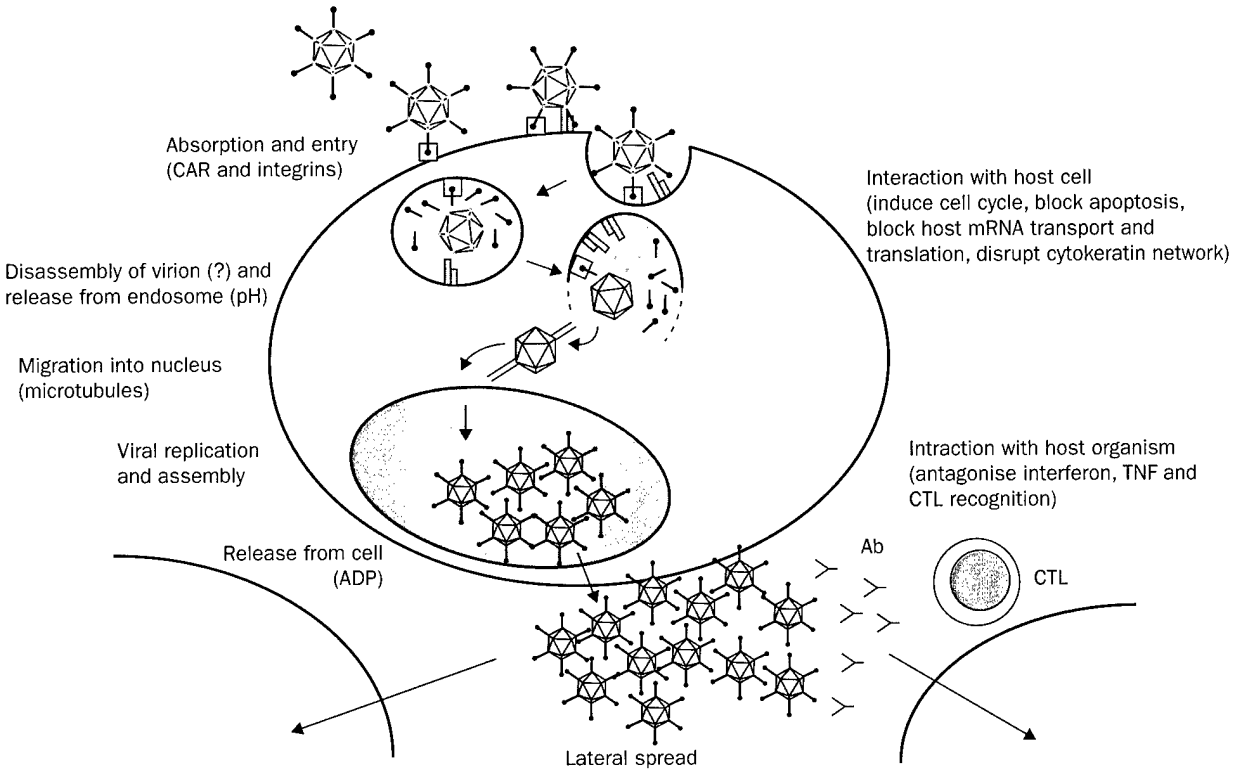


Figure 2. Life cycle of replicative adenovirus. The cycle includes three groups of events: viral replication, host – cell interactions, and host – organism interactions. For each, viral gene products and host cell components have been described, and many are being modified to increase the infectivity, yield, and spread of viral progeny, including those involved in the innate and immune response. Note that antineoplastic interventions applied simultaneously with replicative adenoviral vectors could adversely affect some of these processes. Ab: antibodies; ADP: adenoviral death protein; CAR: coxsackie and adenovirus receptor; CTL: cytotoxic T lymphocytes.

host reaction, causes the antitumour effect.[12] Clinical studies with several dozen patients were then undertaken with low-virulence viruses, including Newcastle disease, West Nile, Ilheus, Bunyamwera, Egypt 101, adenovirus, Langat, Kyasanur Forest disease, and mumps viruses in patients with advanced neoplastic disease.[12,15,19-21] Inocula consisted of bacteriologically sterile preparations of virus grown in live animals or in cell culture. Viruses were administered into the tumour, muscle, vein or artery, and by topical, oral, and inhalation routes. Some viruses administered systemically displayed a preferential tropism for tumour foci, as shown by comparison of viral titres in the tumour and other tissues.[12] Viruses occasionally multiplied in tumours and when that occurred, seemed to have an inhibitory effect on tumour growth, with some responses observed. However, no cancer cure was ever documented in these large studies. Virus-neutralising antibodies appeared quickly, which was typically accompanied by regrowth of the tumour. Serious toxicity was seldom a problem. Although these findings offer some hope, the observed limitations of the approach should clearly help to guide further developments of replicative vectors for cancer gene therapy.

Table 1. Types of CRAd vector systems

Basis of conditional replication	Example
Tumour traits complement mutations in Ad genome	E6 and E7 in HPV-positive cervical cancer cells allow replication of an E1a mutant Ad
A second conditional helper Ad enables replication	Simultaneous infection with a conditional E1a-expressing Ad and an E1a-deleted Ad allows replication of the second virus
Tumour-specific promoter drives replication-enabling Ad gene(s)	PSA promoter driving E1a allows replication of Ad in prostate cancer cells
Targeted Ad (wild-type or altered replication capacity) with tropism tightly restricted to target cells	Theoretical

Ad, adenovirus; E1a, early Ad gene that regulates replication; E6 and E7, oncoproteins encoded by HPV (human papillomavirus); PSA, prostate-specific antigen

Current clinical trials

The results from recent human clinical trials of the first CRAd generation (Table 5), although preliminary, support the conclusions derived from the historical experience. Most advanced is the study of dl1520, or ONYX-015. Frank McCormick's group at Onyx Pharmaceuticals proposed that this adenovirus, deleted of the gene encoding the E1b-55kDa protein, would replicate selectively in P53-deficient cells. This protein binds and inactivates P53 in normal cells to initiate virus replication. Therefore, in cells that have lost P53, this viral protein would become dispensable. To evaluate this concept, the assessment of this virus has proceeded in a safe 'staged development' approach. Thus,

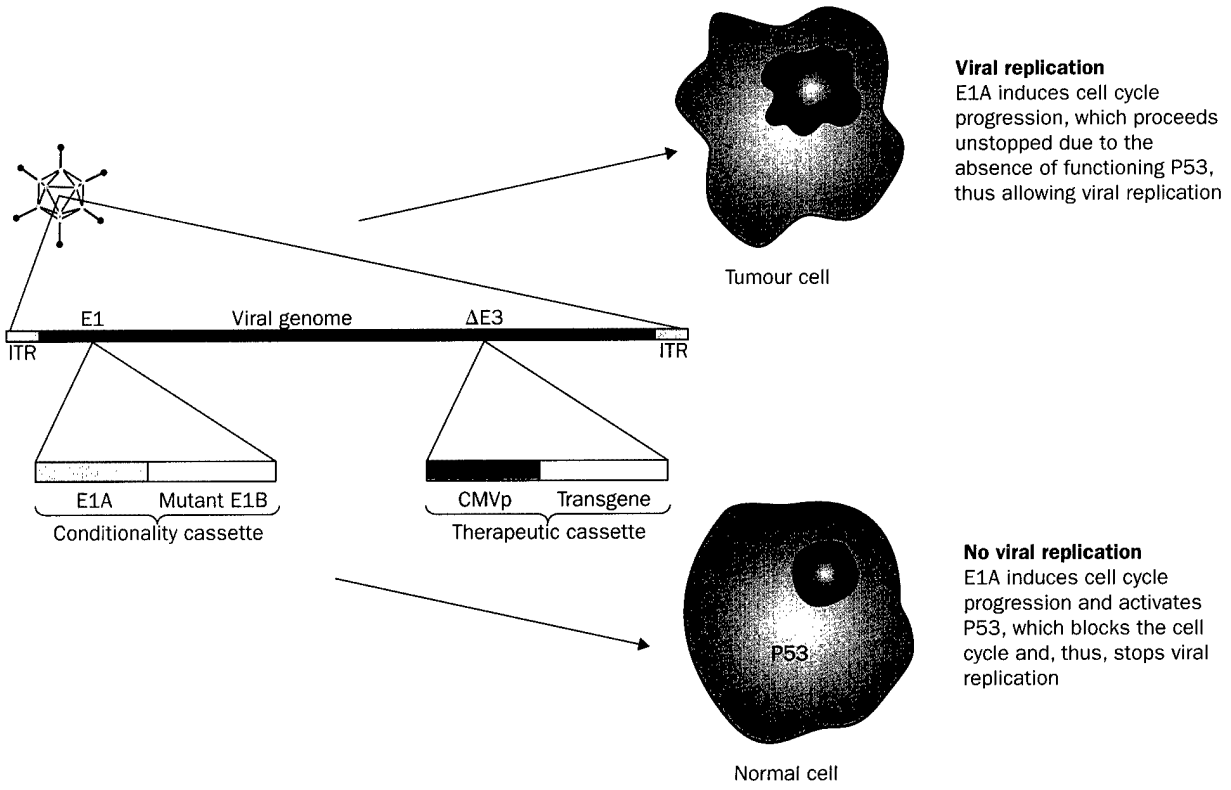


Figure 3. Genome modifications in vectors determine selective replication. In nature, viruses gain control of the cell cycle to favour their own replication. In tumour cells, the already disturbed cell cycle can provide the virus with factors required for replication, which makes some of the viral functions redundant. Dispensing of the corresponding viral genes cripples the virus for infection of normal cells, but still allows propagation in tumour cells. In the schema, a prototypical example is depicted, whereby the virus has attenuated the function of E1B, which impedes its replication in the presence of P53, but not in its absence.

the virus was first administered directly into the tumour in patients with head and neck cancer[22] or pancreatic tumours. Then, when safety was shown, intracavitary administration was initiated in ovarian cancer patients, followed by the intra-arterial route in patients with liver metastasis of colorectal cancer and by intravenous administration in lung cancer patients. In these trials, up to 1×10^{12} plaque-forming units have been administered systemically, but have resulted in no objective tumour responses by conventional standards, despite suggestions of tumour necrosis by magnetic resonance imaging at the site of viral injection and the decrease in tumour size in a small number of patients. Very interestingly, a trend toward a higher 'response' rate (these were responses of individual lesions) has been observed when ONYX-015 was administered in combination with chemotherapeutic drugs (Table 5).[23] Remarkably, none of the responding tumours had progressed after 6 months. Thus, despite a low antitumoral activity when used alone, the efficacy of ONYX-015 can be increased with chemotherapy. The other consistent finding has been its typically minor toxic effects, limited generally to an acute flu-like syndrome.[23]

At the molecular level, viral replication has been documented in the initial head and neck study by *in situ* hybridisation in four patients (of 22 studied), all of whom had mutant *P53* tumours. Most mutant *P53* tumours, however, did not show evidence of replication,[22] which was also the case in the wild-type *P53* tumours. As the researchers point out, the small tumour biopsy samples can hardly reach the exact area where the virus was injected, and a negative result in the assay may not rule out the possibility that replication has occurred. The negative results show more clearly, however, the limited intratumoral spread of the replicating virus, if any. In this regard, a neutralising antibody response was documented in all the patients. The role of this humoral response in limiting the efficacy of adenovirus spread is highly probable, although hard to prove in the clinical context. Thus, the results of this important study confirm what was already known: efficacy, and not toxicity, is the limiting factor in the use of replicative viruses as oncolytic agents.

The obstacles encountered dictate novel strategies

The list of failed attempts to exploit replicative viruses as therapeutic agents against cancer seems daunting. But the definition of the involved barriers at the organism, cellular, and molecular levels, and the tools to overcome them, seem more precise and powerful than ever. The real usefulness of oncolytic vectors, we contend, remains untapped. Major efforts are underway to improve delivery of adenoviral vectors into cells, augment the specificity and yield of viral replication, control the inflammatory and immune response, and establish the most effective combinations of oncolytic vectors with other drugs and radiotherapy to increase potency and minimise resistance to treatment (Table 6).[5]

Table 2. CRAds based on mutations that can be rescued by tumour traits

Name	Design features	Activity	Stage	Reference
ONYX-015 (dl1520)	E1b 55 kDa deletion abrogates P53 binding	Preferential oncolysis of P53-deficient cells	Phase I-III	22
Ad5-CD/TKrep or FGR	E1b 55 kDa-deletion abrogates P53 binding	Oncolysis + suicide gene therapy (CD+TK)	Phase I	14
AdTKRC	E1b 55 kDa-deletion abrogates P53 binding	Oncolysis + suicide gene therapy (TK)	Preclinical	13
AdvE1AdB-F/K20	E1b 55 kDa-deletion abrogates P53 binding	Oncolysis with enhanced infectivity	Preclinical	Shinoura N *et al.*, 1999*
AxE1AdB + AdCAhIL-2	E1b 55 kDa-deletion abrogates P53 binding	Oncolysis + immuno-stimulatory gene therapy	Preclinical	16
dl118	Complete deletion of E1b abrogates P53 binding. However E1a-induced apoptosis is not inhibited by E1b-19kD	Oncolysis, but viral yield potentially less powerful	Preclinical	Duque PM *et al.*, 1998*
AdD24	E1a deletion abrogates Rb binding	Oncolysis	Preclinical	Fueyo J *et al.*, 2000*
Ad5-D24RGD	E1a deletion abrogates Rb binding	Oncolysis with increased infectivity	Preclinical	Suzuki K *et al.*, 2000*
ONYX-838	E1a deletion abrogates Rb binding	More potent than ONYX-015 and wild-type	Preclinical	Heise CC *et al.*, 2000*
E1ACB016	Deletion of E1a domains CR1 and CR2 greatly reduces replication in normal cells; HPV proteins E6 and E7 complement those deletions allowing replication of adenovirus in HPV+ cancer cells	Oncolytic activity restricted to HPV positive (cervical and others such as some head and neck) cancer cell	Preclinical	Balague *et al.*, 2000*
E1Adl01/07	Double E1a mutation abrogates Rb and p300 binding	Higher selectivity of replication may improve therapeutic index	Preclinical	Howe *et al.*, 2000*
KD1 and KD3	Double E1a mutation abrogates Rb and p300 binding; *adp* gene inserted in E3 region substituting E3.	Expression of adp (adenovirus death protein) facilitates cell lysis; absence ofE3 should facilitate immune responseagainst infected cells	Preclinical	26
dl316	Complete deletion of E1a makes this mutant dependent on intrinsic or interleukin-6-induced E1a-like activity		Preclinical	Rancourt R *et al.*, 1999*

CD, cytosine deaminase; HSV-TK, herpes simplex virus thymidine kinase
*These references are available on *The Lancet Oncology* website: http://oncology.thelancet.com

Improving delivery and intratumoral spread requires targeted vectors

The use of CRAds has been considered not only for locoregional tumours, but also for disseminated disease.[24,25] In each context, the requirements for modifying the replicative vector tropism are very different.[5] The goal in both cases is common and inescapable: a very substantial increase in virus infectivity has to be obtained to achieve the necessary high levels of tumour-cell infection. A general principle of oncology is pertinent here. When cytotoxic interventions do not attack all the tumour cells initially, a positive selection of those cells that are not sensitive ensues and, therefore, the intervention unavoidably favours the ultimate selection of resistant tumour clones. Thus, it is vitally important that CRAds are given increased infectivity and capacity for lateral spread. *In situ* hybridisation studies in animal models and in the first clinical trial reported confirm the very limited capacity of CRAds to disseminate within tumours after intratumoral injection.[22,26]

Knowledge of the biology of adenoviral entry into cells has allowed a very intense research effort towards engineering the viral entry with the properties of selectivity, higher efficiency, and, in certain contexts, broadened tropism. To this end, surface proteins of non-replicative adenovirus vector involved in cellular binding have been genetically modified; alternatively, cellular ligands such as epidermal growth factor and fibroblast growth factor, and specific antibodies have been immunologically linked to the viral particle.[1] These advances will be rapidly applied to the development of the replicative versions of the adenoviral vectors. Another practical oncology principle is here in order – treatments based on molecularly targeted drugs should be tailored to the corresponding specific subset of patients. For instance, tamoxifen is given for maximum efficacy to patients whose breast tumours express oestrogen receptors. By analogy, viral vectors should be given to patients whose tumours express viral receptors. Indeed, low expression of coxsackie and adenovirus receptor (CAR) might determine tumour resistance to CRAds. Although not evaluated yet in patients, correlative studies to explore this possibility are needed. The levels of CAR, and of other receptors that retargeted vectors might use, can in this regard be easily determined by sensitive molecular techniques.

Vector replication should be selective but robust

The renewed interest in replicative viruses within gene therapy might arguably be attributed to the compelling design and promises of selectivity and efficacy in mutant P53

Table 3. Other types of replicative adenovirus vector systems

Name	Design features	Activity	Stage	Reference
A second conditional helper Ad enables replication				
dl1520 + E1a-deleted Ad	dl1520 contains an E1b mutation that restricts replication but provides E1a protein to the second Ad	Oncolysis and amplified expression of therapeutic transgene	Preclinical	Cook *et al.*, 2000*
GT5610 + AdHβ	AdHβ contains all Ad genes except E1 and complements GT5610,- a minimal virus with E1a under the α-fetoprotein promoter	These vectors complement each other, spread and lyse hepatocarcinomas	Preclinical	Alemany R *et al.*, 1999*
Non-conditionality				
Ad wild-type	None	Oncolysis	Preclinical and clinical observation	15; Khoobyarian N *et al.*, 1975*
Ad5/IFN	Insertion of interferon gene	Oncolysis + immunostimulatory gene therapy	Preclinical	17
dl337	Deletion of E1b 19 kDa	Increased oncolysis		Sauthoff H *et al.*, 2000

Ad = adenovirus

*These references are available on *The Lancet Oncology* website: http://oncology.thelancet.com

tumours raised by ONYX-015. Paradoxically, reports from several groups including Onyx itself have shown a lack of association between the status of P53 and the replicative capacity of the virus, and suggest that wild-type P53 might even be necessary for a productive adenovirus infection. Data from clinical trials do not yet resolve the controversy.[22] The pragmatic view is that the therapeutic value of the virus blurs the importance of an unequivocal explanation of its oncolytic specificity. However, it seems too early to accept either of these assumptions, and, thus, further exploration of the determinants of selective replication of this and other candidate CRAds seems sensible (Table 2).

Several vector modifications are being explored to increase the viral yield per infected tumour cell, and as a consequence the final local 'dose' of self-synthesising vector. A family of CRAds encoding an excess of the adenovirus death protein (ADP) that mediates cell lysis has been reported to lyse tumour cells and spread from cell to cell more rapidly than wild-type adenovirus.[27] Interestingly, plasmids encoding the E1b-55 kDa protein in lung cancer cell lines increase by 1 – 2 logs the production of E1-defective adenovirus when cotransfected with E1A-encoding plasmids.[28] Indeed, E1b is associated with the shutoff of host protein synthesis, and disruption of this function may diminish the efficiency of the viral replicative cycle. This observation shows the risks of potentially decreasing the robustness of replication when trying to modify the vector genome for the sake of selectivity.

The possible impact of high-throughput techniques for molecular profiling, including microarrays and serial analysis of gene expression, should not be underestimated. For instance, most transcripts in tumour and normal cells infected with varied efficiency, and sustaining different levels of viral replication, could be quantified and compared. Together with sensitive new methods to evaluate the viral yield, such as quantitative RT-PCR, these techniques promise to revolutionise the study and clinical development of CRAds.

Host inflammatory and immune response

As early as 1952, Chester Southam and Alice Moore identified the immune response as the first obstacle to practical clinical use of viruses for cancer therapy.[12] Both the innate defence mechanisms and the immune response proper should be considered as possible deterrents for viral efficacy. With respect to the acute response to viral infection, many studies have shown the capacity of non-specific cellular factors, such as the cells forming the mononuclear phagocytic system, to dispose very efficiently of the intravenously administered viral particles.

The humoral immune response has been most consistently observed and associated with resistance to viral spread and tumour regression. In an animal model, titres of antibody to adenovirus were related to a decreased toxicity of intravenously injected CRAd, and with a reduction in the antitumour efficacy of the vector. In an ONYX-015 trial, however, pre-existing antibody titres did not influence antitumoral efficacy or toxicity, although both have been rare events and make difficult its interpretation.[22] In any case, it is difficult to exaggerate the need to develop methods that allow modulating the effect of pre-existing neutralising antibodies in viral spread and the upsurge of production that follows infection. We observed that when ovarian cancer cells are exposed to an adenovirus vector in the presence of ascites containing neutralising antibodies, infection, as indicated by a reporter gene expression, was blocked almost completely. However, the block is reduced several orders of magnitude when genetically modified adenovirus, displaying the endogenous RGD peptide, was used. It seems that the use of this integrin-dependent heterologous pathway for cellular entry, by employing an endogenous peptide to which we are naturally tolerant, renders the adenovirus

Table 4. CRAds based on tumour-specific promoters driving heplication-enabling adenovirus genes

Name	Design features	Activity	Stage	Reference
CN706	Regulation of E1a under PSA promoter; Deletion of E3	Oncolysis of prostate tissue	Phase I	Rodriguez R *et al.*, 1997
CV763	Regulation of E1a under kallikrein 2 promoter	Oncolysis of prostate tissue	Preclinical	Yu DC *et al.*, 1999
CV764	Regulation of E1a under prostate-specific enhancer and E1b under kallikrein 2 promoter	Oncolysis of prostate tissue with higher specificity	Preclinical	Yu DC *et al.*, 1999
CV739	Regulation of E1a under rat probasin promoter and E1b under human PSA promoter	Oncolysis of prostate tissue	Preclinical	23
CV787	Regulation of E1a under rat probasin promoter and of E1b under enhancer of PSA; includes Ad5 E3 region	Oncolysis of prostate tissue; greater potency than CV739 due to the presence of E3	Phase I-II	23
AvE1a041	Regulation of E1a under AFP promoter	Oncolysis of hepatocarcinomas	Preclinical	Hallenbeck PL *et al.*, 1999
GT5610 + AdHB	Regulation of E1a under AFP promoter	Oncolysis of hepatocarcinomas	Preclinical	Alemany R *et al.*, 1999
KD1-SPB	Regulation of E4 under surfactant protein B	Oncolysis of pulmonary tumours of type II alveolar cells	Preclinical	Doronin *et al.*, 2000*
Ad460CEA	Regulation of E1a under CEA promoter	Oncolysis of CEA-expressing tumours	Preclinical	Toyoizumi *et al.*, 2000*
AdCEAp/Rep	Regulation of E1a under CEA promoter and deletion of E1b 55 kDa	Oncolysis of CEA-expressing tumours	Preclinical	Sagawa *et al.*, 2000*
Ad-Lp-E1A	Regulation of E1a under L-plastin promoter	Oncolysis of L-plastin-encoding tumours such as ovarian carcinoma	Preclinical	Zhang *et al.*, 2000*
Ad-OC-E1a	Regulation of E1a under osteocalcin promoter; partial deletion of E3	Oncolysis of osteocalcin-encoding tumours such as metastatic prostate carcinoma	Preclinical	Matsubara *et al.*, 2000*

AFP: α-fetoprotein;

*These references are available on *The Lancet Oncology* website: http://oncology.thelancet.com

impervious to the existence of a blocking humoral response.[29] This and other modifications of CRAds, in addition to transient drug interventions, should be evaluated to overcome this most important obstacle.

Later during infection, cytotoxic T lymphocytes and perhaps also natural killer cells limit adenovirus-mediated gene expression by destroying infected cells. This cellular immune response may also limit the production and spread of viral progeny by destroying the virus-producing tumour cells. The dynamics of these different processes has not been studied *in vivo*, given the current lack of a permissible animal model.[5] The use of drugs that inhibit the cellular component of the immune system, widely used in cancer therapy, such as dexamethasone, cyclophosphamide, and etoposide, should be used with caution until the possible interactions are further studied. In addition, local modulation of the immune response to viral replication could be achieved by inducing the expression of the appropriate cytokines by adenovirus-encoded transgenes. Recently, encouraging evidence has been obtained. The administration of an E1b attenuated CRAd together with adenovirus vectors encoding interleukin 2 or 12 has shown an increase of several hundred-fold in expression of the cytokines, and complete tumour regressions in a severe combined immunodeficiency mouse model of pancreatic cancer.[16]

Multi-modality treatments are required to avoid resistance

If replicative viruses are considered, as we proposed earlier, as complex antimetabolites, several well-recognised principles of anticancer treatment should be applied to their development. First, only drugs known to be partially effective against the same tumour when used alone should be selected for use in combination. This means that a combination cannot substitute for a lack of activity of one of its components. In other words, efficacy of CRAds should be fully evaluated, and only then combined with other drugs if the efficacy justifies it. Second, when several drugs are available, a drug should be selected on the basis of toxicity that does not overlap with the toxicity of the other drugs to be used in the combination. This is a practical reason to define the mechanisms of cytotoxicity of viral proteins. Third, drugs should be used in their optimum dose and schedule, which speaks, again, for full evaluation of CRAds given alone. Fourth, drug combinations should be given at consistent intervals, trying to keep the treatment-free interval as short as possible. One obstacle in the CRAd context is the immune response generated by the virus, and to pursue a multiple-dosing schedule will probably require concomitant modulation of the immune response.

To design optimum treatment combinations, the life cycle of the adenoviral vector needs to be considered, and judged compatible with the mechanism of action of the companion drugs. For instance, drugs such as paclitaxel, that disturb the functioning of microtubules, might inhibit the migration into the nucleus of infecting viral particles (Figure 2), and thus antagonise the virus-mediated propagation and oncolysis. As yet, several preclinical

Table 5. Clinical trials of conditionally replicative adenoviruses

Phase	Vector	Stage	Tumour	Dose (max)	Route	Toxicity	Complete and partial responses (total number of patients)	Notes	Reference
I	ONYX-015	Recurrent disease, radiotherapy refractory	Head and neck	$1x10^{11}$ pfu	IT	No DLT; frequent flu-like symptoms and injection site pain	0+3 (22)	Universal increase in neutralising Antibody to Ad; replication showed in 4 of 22 (all with mutant P53)	22
I	ONYX-015	Metastatic disease, CT refractory	Liver metastasis from GI origin	$1x10^{11}$ pfu every 3 weeks	IT	No DLT, and no MTD reached; frequent flu-like symptoms and PT/PTT elevations	None (16)	-	Bergsland E *et al.*, 1998*
I	ONYX-015	Unresectable	Pancreas	$1x10^{10}$ pfu every 4 weeks	IT	No MTD reached; DLT: hyperbilirubinaemia; frequent flu-like symptoms	None (16)	P53 mutant in 5 of 13 patients	Mulvihill SJ *et al.*, 1998*
I	ONYX-015		Pancreas	$1x10^{10}$ pfu every 4 weeks	IT	DLT: bacteriaemia	None (5)	EUS guidance, concomitant gemcitabine in some	Hecht JR *et al.*, 1999*
I/II	ONYX-015	Unresectable	Pancreas	$1x10^{10}$ pfu qwk x8	IT	DLT: bacteriaemia (2); duodenal perforation (2)	0+2 (18)	EUS guidance, concomitant gemcitabine in last 4 weeks	Hecht JR *et al.*, 2000*
I/II	ONYX-015	Premalignant	Oral dysplastic lesions	$1x10^{10}$ pfu x5 every 4 weeks	Mouthwash	None	-		Rudin CM *et al.*, 1999*
I	ONYX-015	Recurrent, refractory	Ovarian	$1x10^{11}$ pfu in 5 doses every 3 weeks	IP	Frequent emesis, flu-like symptoms	None (16)	-	Vasey PA *et al.*, 2000*
I	ONYX-015	Refractory	Various	$1x10^{12}$ pfu x3 per week	IV	Frequent flu-like . symptoms	None (9)	Carboplatin and taxol added if no response after 2 cycles; viral DNA detected (50%) in blood at 7 days after infusion when dose >$1x10^{11}$ pfu	Nemunaitis J *et al.*, 2000*
I/II	ONYX-015	Metastatic disease, CT refractory	Liver metastasis from GI origin	$1x10^{11}$ pfu, days 1, 8, 22, 50	IHA	No DLT, and no MTD reached; frequent flu-like symptoms	0+2 (13)	In combination with 5-FU/leucovorin	Reid T *et al.*, 2000*
II	ONYX-015	Recurrent, CT and/or RT refractory	Head and neck	$1x10^{10}$ pfu x5	IT	Frequent flu-like symptoms and injection site pain	2+2 (13)	-	Kirn D *et al.*, 1998*
II	ONYX-015	Recurrent, RT refractory	Head and neck	$1x10^{10}$ pfu x5	IT	That of chemotherapy	8+11 (37)	In combination with CDDP and 5-FU	23
III	ONYX-015	Metastatic, CT refractory	Head and neck	-	IT	-	-	Starting during 2000	-
I	CN706	Recurrent after RT	Prostate	$5x10^{13}$ vp	IT	No DLT; frequent fever and prostatitis (12)	0+1 (13)†	Stereotactic techniques used for infusion	Simons JW *et al.*, 2000*
I	Ad5-CD/ TKrep	Recurrent after RT	Prostate	$1x10^{12}$ vp	IT	Frequent pain at site of injection	None (2)†	In combination with 5-FC and GCV	Freytag S *et al.*, 2000*
I	E1B-attenuated	Concomitant with 5-FC and VCV	Penile	-	IT	-	-	-	RAC database
I-II	CV787	After RT	Prostate	-	IT	-	-	-	RAC database
I-II	CV787	Metastatic, hormone refractory	Prostate	-	IV	-	-	-	RAC database

5-FC, 5-fluorouracil; Ad, adenovirus; CT, chemotherapy; DLT, dose-limiting toxicity; EUS, endoscopic ultrasonography; GCV, ganciclovir; IHA, intrahepatic artery; IP, intraperitoneal; IT, intratumoral; MTD, maximally tolerated dose; PSA, prostate specific antigen; PT/PTT, prothrombin time of thromboplastin; RAC, Recombinant DNA Committee at http://www4.od.nih.gov/oba/protocol.pdf; RT, radiotherapy; VCV, valaciclovir; vp, viral particles; †Open and still accruing patients; *These references are available on *The Lancet Oncolgy* website: http://oncology.thelancet.com

Table 6: Obstacles encountered and strategies to overcome them

Obstacle	Strategy	Example
Inefficient delivery	Genetic and immunological targeting of Ad	RGD.Ad
Poor specifity and yield of replication	Further genetic modifications Ad genome	Double mutants in E1a rescued by HPV oncoproteins
Resistance of tumour cells to the induction of cytotoxicity	Rational combinations with drugs and radiotherapy targeted against multiple cellular metabolic pathways	Onyx-015 in combination with CDDP and 5-FU
Lack of intratumoral spread	Genetic targeting of Ad; adding genes that augment cell lysis	KD1 and KD3 (CRAds that overexpress ADP)
Inflammatory and immune responses	Stealth Ad; adding genes that locally modulate the inflammatory and immune response; drugs	RGD.Ad

5-FU: 5-fluorouracil; Ad: adenovirus; ADP: adenoviral death protein; CDDP: cisplatin; RGD.Ad: genetically modified adenovirus that displays the tripeptide RGD in the fibre knob

studies of multimodality intervention have shown the feasibility of exploiting therapeutically the replication of an adenovirus vector and expression of a prodrug-converting enzyme.[13,30] In one of the studies, the prodrug systems not only increased the tumour cell-specific cytopathic effects of the CRAd *in vitro*, but also sensitised cells to radiation *in vivo* with achievement of a 100% tumour cure.[30] These results are very encouraging, and emphasise the soundness of applying general principles of oncology, such as targeting multiple metabolic pathways, in the context of this new biological therapy based on replicative oncolytic adenoviruses.

Search strategy and selection criteria

Published and unpublished data for this review were identified by searches of databases of published reports (MEDLINE, CancerLit) and of clinical trials (National Institutes of Health Recombinant DNA Activities Register of Gene Therapy Clinical Trials and the UK Current Controlled Trials metaregister). Referenced papers were identified by searching MEDLINE from 1966 onwards. MESH terms used were: adenovirus AND ('replicative' or 'replication' or 'oncolytic' or 'oncolysis') and replication-competent [TI]. Additional papers were identified through searching of reference lists from retrieved papers. Abstracts communicated during the past 3 years (1998 – 2000) to the following societies were searched on the respective Society's websites: American Association of Cancer Research (www.aacr.org), American Society of Clinical Oncology (www.asco.org), and American Society of Gene Therapy (www.asgt.org). Search terms were: 'oncolytic' or 'oncolysis' or 'virotherapy', 'adenovirus' and '(replicative or replication)', 'virus and '(replicative or replication)'. Results of clinical trials were mostly extracted from abstracts containing preliminary information. Thus, no formal attempt was done to evaluate the quality of the methodology or data provided. Only English language papers were included.

Conclusions

"The fact that transient tumour regressions have occurred in a few patients and that localisation of virus in tumour tissue has frequently been demonstrated, may permit the hope that continued work might lead eventually to useful results." These words were written in 1952, but they seem perfectly appropriate, we are afraid, in the summary of results of many ongoing clinical trials. It should be apparent, however, that by applying the recent advances in genetic engineering, vector development, and gene therapy, the therapeutic exploitation of replicative virus in cancer patients might be feasible, and fully realised, at last. A delicate balance has to be reached between increasing the potency of viral replication for augmenting oncolysis, and inhibiting the many countermeasures that nature imposes into viral invasion of the body.

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A list of references for further reading appears on *The Lancet Oncology's* website: http://oncology.thelancet.com

APPENDIX E

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Review

Toward a New Generation of Conditionally Replicating Adenoviruses: Pairing Tumor Selectivity with Maximal Oncolysis

FRANK A.E. KRUYT[1] and DAVID T. CURIEL[2]

ABSTRACT

Conditionally replicating adenoviruses (CRADs) represent a promising new platform for the treatment of cancer. CRADs have been demonstrated to kill tumor cells when other therapies fail, indicating that their antitumor properties are complementary to, and distinct from, those of standard treatments such as chemotherapy and radiation. In clinic trials CRADs have shown encouraging results, demonstrating mild side effects when administered at high doses and via different routes, including intratumorally, intraperitoneally, and intravenously. Tumor-selective replication has been detected, although as a single agent the efficacy appears to be limited. Interestingly, combined treatment with radiation or chemotherapy has been found to enhance CRAD efficacy considerably. To date, the molecular mechanisms underlying adenovirus-mediated oncolysis, and the way in which chemotherapy enhances oncolysis, are not well understood. A fuller knowledge of these processes will open up new strategies to improve the cell-killing potential of CRADs. Here, we discuss several possibilities that may lead to CRADs with enhanced oncolytic activity. These approaches include strategies to functionally couple tumor targeting and optimal oncolytic activity, and ways to further increase tumor cell disruption at later stages of infection to facilitate the spreading of virus throughout the tumor mass. In addition, improved methods to evaluate the efficacy of these agents in animal models, and in the clinic, will be required to systematically test and optimize CRAD efficacy, also taking into account the influence of tumor characteristics and the administration route.

OVERVIEW SUMMARY

The recognition that genetic alterations are at the basis of the development of cancer has initiated the design of therapeutic approaches, known as cancer gene therapy, aimed at correcting the primary genetic defect in tumor cells. Both nonviral and viral strategies have been used for gene correction or the introduction of therapeutic genes into tumor cells. Viruses have been particularly popular because of their natural ability to infect cells. Initially, nonreplicating viral vectors were used; however, limitations related to their inability to infect all cancer cells, and to the relatively low levels of therapeutic gene expression obtained, have shifted attention to the use of replicating viruses as the treatment platform. In this respect, genetically modified adenoviruses that selectively replicate in cancer cells have been generated and tested in clinical trials. Although promising, several hurdles have been encountered that need to be addressed to expand their therapeutic potential.

CONDITIONALLY REPLICATING ADENOVIRUSES

THE CONCEPT OF USING replication-competent adenoviruses for the treatment of cancer, also known as adenoviral ther-

[1]Division of Gene Therapy, Department of Medical Oncology, VU University Medical Center, NL-1007 MB Amsterdam, The Netherlands.
[2]Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, University of Alabama at Birmingham, Birmingham, AL 35294.

apy, originated in the 1950s. The knowledge that adenoviruses could eliminate cancer cells *in vitro*, as a consequence of their reproductive cycle leading to cell lysis ("oncolysis"), resulted in clinical studies in which various wild-type adenoviral serotypes were examined for their effect on cervical cancer patients (Smith *et al.*, 1956). In the studies, no significant toxicity was reported after intratumoral injection or intravenous administration and a moderate tumor response was observed. It was not until 1996 that this concept regained attention via the use of a genetically engineered adenovirus with tumor-selective replication characteristics, leading to the first conditionally replicating adenovirus (CRAD) that was developed for cancer therapy (Bischoff *et al.*, 1996). It is now broadly recognized that these agents have beneficial properties when compared with their nonreplicating counterparts that were initially used for cancer gene therapy.

In this regard, cancer gene therapy with nonreplicating adenoviruses, although promising in preclinical models, has not resulted in successful treatments in the clinic. Armed with various therapeutic genes, including prodrug-converting enzymes and tumor suppressor genes, these agents have not demonstrated the expected beneficial effects *vis à vis* the eradication of cancer cells in the human clinical context. The main reason for the poor clinical therapeutic effect of these agents is related to the relatively small number of cancer cells in the tumor mass that are transduced by these vectors *in vivo*. As a consequence, the levels of expression and the dispersion of the therapeutic genes limit the clinical effect.

The new therapeutic platforms provided by CRADs are expected to overcome these limitations by their ability to increase the input dose of the therapeutic gene and, moreover, by the oncolysis and eradication of cancer cells during replication. In clinical trials, CRADs have been shown to be promising and safe agents. On the other hand, in clinical trials as single agents their antitumor effects have been somewhat disappointing. Thus, these clinical studies have been of great importance for defining the current limitations of the system. In this review, we address these limitations and focus on advancing several strategies that may improve the tumor-killing properties of CRADs, resulting in the development of agents with enhanced therapeutic potential.

CRADs IN HUMAN CLINICAL TRIALS

The most studied CRAD so far is the one originally generated in the laboratory of Arnold Berk (Barker and Berk, 1987), designated *dl*1520, and for the first time used by Frank McCormick as a selective vector (Bischoff *et al.*, 1996). In this CRAD, also known as ONYX-015 and more recently renamed CI-1042 (Pfizer, Groton, CT), the viral gene encoding E1B-55kD, which is required for binding and subsequent inactivation of the p53 protein, has been deleted. Being part of a cellular antiviral mechanism, the p53 protein would normally trigger a cellular response leading to cell cycle arrest and early death of the host cell, thereby preventing replication and spreading of the virus. In cancer cells that lack functional p53, E1B-55kD would be dispensable, thus resulting in selectivity, a concept that has been confirmed in preclinical studies (Heise *et al.*, 1997). However, on this basis reduced replication and cytopathogenicity have been reported for this CRAD when compared with wild-type virus (Bischoff *et al.*, 1996; Harada and Berk, 1999). The clinical tests of this oncolytic agent in various cancer types, including head and neck and pancreatic cancer, have been evaluated extensively in reviews in terms of safety and efficacy (Alemany *et al.*, 2000b; Kirn, 2001). The overall conclusion is that adenoviral therapy is a safe method when applied via various routes. Further, tumor-selective replication has been documented, thus validating the concept *in vivo*. Of note, no evidence was obtained for the expected reduced efficacy in patients with pre-existing antibodies against adenoviruses (Ganly *et al.*, 2000; Nemunaitis *et al.*, 2000). In fact the immune response is generally considered to be a factor that can increase the antitumor effect of the therapy. However, CRADs as a single agent have demonstrated limited efficacy, with an overall response rate of approximately 15 % in patients receiving the agent. Interestingly, the efficacy could be significantly enhanced by combined treatment with chemotherapy.

The results obtained in these studies have been helpful in determining the limitations of the current generation of CRADs, and in determining which aspects need to be addressed in order to develop a new generation of improved agents. In this respect, critical problems that have been encountered involve the following: (1) infectivity of cancer cells by adenovirus, (2) tumor selectivity of CRADs in relation to efficacy, (3) oncolytic activity or cell death-inducing ability of CRADs, (4) accessibility of tumor for virus internalization and spreading, and (5) methods to evaluate CRAD efficacy in animal models and in the clinic. In the following sections we elaborate in more detail on these aspects of CRAD efficacy.

ADENOVIRAL INFECTION OF CANCER CELLS

Apart from the favorable characteristics of CRADs compared with nonreplicating adenovirus vectors, some problems are common to both approaches. A major issue is the fact that cells can be resistant to adenoviral infection because of the lack of the primary receptor for viral entry, the coxsackievirus-adenovirus receptor (CAR) (Douglas *et al.*, 1996; Wickham *et al.*, 1996). It has been noted that primary tumor cells often express relatively low levels of the CAR, resulting in poor infectivity, which in the case of CRADs will also affect the lateral dispersion of the virus in tumor tissue. This has been demonstrated by analyses of the oncolytic activity of wild-type adenovirus in a pair of tumor cell lines that differed only in CAR expression levels, demonstrating that low CAR levels strongly reduced viral replication and oncolysis in monolayer cultures and murine tumor models (Douglas *et al.*, 2001). To circumvent this, CAR-independent entry pathways have been identified that can bypass this deficiency, such as the use of the RGD motif in the fiber knob of the virus, which facilitates binding and entry via integrin receptors that are abundantly expressed on tumor cells (Dmitriev *et al.*, 1998; Krasnykh *et al.*, 2000). CRADs have been generated to contain fiber knobs with intact CAR-entry capability, and an additional integrin-entry capability resulting in more effective antitumor characteristics by enhanced infectivity (Suzuki *et al.*, 2001). Additional strategies have been explored to obtain tumor-specific entry of adenoviruses, involv-

ing the modification of the viral coat or the use of secondary targeting moieties, approaches that have been reviewed in more detail elsewhere (Curiel, 1999; Wickham, 2000).

SELECTIVITY OF CRADs

Exploitation of replicating adenoviruses as a new modality for cancer gene therapy has led to the use of novel ways to obtain tumor selectivity. The so-called type 1 CRADs that are now available make use of the frequent inactivation of tumor suppressor genes in cancer that occurs as part of the process leading to malignant transformation (Curiel, 2000). Examples are mutations or deletions in the *p53* and retinoblastoma (*Rb*) genes; their protein products are known to interact with, and to be modulated by, adenoviral gene products as an essential step in virus propagation. However, these strategies to obtain selectivity often occur at the expense of efficacy, as the adenoviral reproductive cycle is a highly orchestrated process. At the molecular level, completion of the infectious cycle relies on the timely expression of a set of regulatory proteins that interact with essential endogenous cellular pathways that determine cell viability in order to facilitate viral DNA replication, expression of adenoviral genes, and, finally, disruption of the cell and the release of new viral particles (Yeh and Perricaudet, 1997). Specifically, the viral genome encodes eight transcriptional units that are activated in a timely way at different phases of infection, referred to as immediate-early (E1A), early (E1B, E2, E3, and E4), intermediate (IX and IVa2), and late genes encoding structural proteins for the capsid and the internal core. The early genes are mainly regulatory proteins that set the stage for viral DNA replication, thereby blocking cellular antiviral strategies such as the activation of cell death programs and the downregulation of immune response stimulatory proteins, strategies that are shared by other mammalian DNA viruses (for review see Wold *et al.*, 1999; Mahr and Gooding, 1999). At all stages of infection adenovirus proteins control various cellular processes by interacting with multiple host cellular proteins; many of these interactions are not yet completely understood or remain to be identified.

On infection, the immediate expression of E1A, and its binding to Rb, leads to the release of the transcription factor E2F, which forces the host cell to enter the S phase of the cell cycle in order to facilitate the coreplication of the viral genome (Flint and Shenk, 1997). Infection, E1A expression, and the unscheduled entry of the cell into the S phase inflict cellular stress signals leading to the activation of cell cycle checkpoints and the onset of suicide pathways or programmed cell death (PCD), including apoptotic cell death. Of note, the p53 protein plays an important role in the activation of apoptosis in the infected cell. In opposition to these processes, the adenoviral proteins E1B-55kD and E4orf6 work in concert to bind to p53, causing its degradation and thereby facilitating host cell survival (Steegenga *et al.*, 1998). The exploitation of the interaction between E1B-55kD and cellular p53 to obtain tumor selectivity with *dl*1520 has caused considerable controversy. It has been reported that the wild-type virus grows more efficiently in cells expressing wild-type p53, compared with *dl*1520 in p53-mutated cells, suggesting that either functional p53 is required for effective replication and/or that interaction between E1B-55kD and p53 has a favorable effect (Ridgway *et al.*, 1997; Hall *et al.*, 1998; Dix *et al.*, 2000). In addition, several groups have demonstrated that the host range specificity of *dl*1520 in *in vitro* models is independent of p53 status (Goodrum and Ornelles, 1998; Rothmann *et al.*, 1998; Harada and Berk, 1999; Turnell *et al.*, 1999). One reason for these discrepancies may be that in most studies cells derived from various cancer types were compared in examining the relationship between p53 status and *dl*1520 replication, rather than isogenic cell systems to exclude the involvement of other genetic factors. In this regard, Rogulski and co-workers (2000) showed that in an isogenic colorectal cancer cell model *in vivo* *dl*1520 replication occurred in both p53 wild-type and mutant cells, although with significantly higher antitumor activity in p53-deficient tumors. Another cause for these differences involves the multifunctional properties of p53 and the particular function being inactivated in the cancer cell studied. In a study of hepatocellular carcinoma cell lines, it was found that an intact transcription activator function of p53 in mutants leads to increased susceptibility for *dl*1520 when compared with p53 mutants in which this function was disrupted (Zhao *et al.*, 2001). In addition, other factors in the p53 pathway have been identified that affect the oncolytic properties of *dl*1520, such as deletions in the *INK4a/ARF* locus, which occur at high frequency in cancer cells (McCormick, 2000). This locus encodes two proteins, $p14^{ARF}$ and $p16^{INK4a}$, that are part of the pRb and p53 pathways, respectively. On transcriptional activation by E2F, $p14^{ARF}$ can promote the degradation of MDM-2 in a manner similar to E1B-55kD, leading to stabilization and activation of p53. In this way, $p14^{ARF}$ links the pRb with the p53 pathway and thus connects adenovirus-dependent activation of the pRb route with the p53 response. It has been shown that a functional $p14^{ARF}$–p53 pathway is required to inhibit ONYX-015 replication and that disrupted $p14^{ARF}$ function facilitates replication (Ries *et al.*, 2000). Also, in mesothelioma cells with wild-type p53, mutated $p14^{ARF}$ enabled ONYX-015 replication whereas restored expression of functional $p14^{ARF}$ significantly increased resistance to oncolysis (Yang *et al.*, 2001). These studies illustrate the relative lack of knowledge about the cellular mechanisms underlying the tumor selectivity of type 1 CRADs, such as ONYX-015, and the ongoing identification of relevant cellular factors.

Another promising CRAD, not yet tested in the clinic, is Ad-Δ24, which makes use of the function of the E1A protein to bind to Rb in order to trigger cell cycle progression into the S phase (Fueyo *et al.*, 2000). Independently, a similar CRAD has been developed, designated *dl*922-947 (Heise *et al.*, 2000a). These CRADs are designed for selective replication in tumor cells that have a deficiency in the Rb pathway, which is the case in the majority of cancers, by deleting from conserved region 2 (CR2) of E1A the sequence encoding eight amino acids that are required for binding to pRb and related pocket proteins (Dyson *et al.*, 1992). In addition, an infectivity-enhanced variant containing the RGD targeting motif in the fiber knob of the virus has been generated with even more favorable antitumor characteristics in lung and prostate cancer cells (Suzuki *et al.*, 2001). Both CRADs are effective in eradicating various types of cancer cells in preclinical studies, in most cases more effectively than *dl*1520 or wild-type virus (Heise *et al.*, 2000a). However, a more recent study employing an organotypic model, derived from human primary keratinocytes to examine the effect of various E1A mutants on replication and their potential to kill

cells, indicated that Ad-Δ24 is not as selective as anticipated (Balague *et al.*, 2001). While studying the complementary activity of human papillomavirus (HPV) E6 and E7 proteins toward the E1A deletion mutants, normal and E6,E7-expressing keratinocytes facilitated the replication of Ad-Δ24 as efficiently as wild-type adenovirus. In addition, a CRAD carrying an additional deletion in CR1, a region also known to be involved in binding to members of the Rb pocket protein family, demonstrated substantial selectivity for HPV protein-expressing cells with strongly reduced but not completely abrogated replication in normal cells.

The superior antitumor activity of Ad-Δ24 is likely to be due to the fact that only a small, but specific, mutation in E1A was introduced without altering other functions of E1A. In a similar way, an improved version of *dl*1520 may be generated by making more precise mutations in the E1B gene. Apart from binding to p53 and E4orf6, E1B-55kD is known to facilitate the transport of late viral mRNAs from the nucleus to the ribosomes and a nuclear export signal has been identified in this protein controlling nuclear/cytoplasmic export (Kratzer *et al.*, 2000). Impaired mRNA transport in the E1B-55kD-deleted CRAD *dl*1520 is probably the cause of its reduced potency when compared with wild-type adenovirus or Ad-Δ24 (Harada and Berk, 1999). A report describes the identification of an E1B-55kD mutant, R240A, that fails to degrade p53 but has retained its E4orf6-binding and mRNA-transporting potential (Shen *et al.*, 2001). According to expectation, this mutant in the context of a replicating adenovirus was demonstrated to enhance replication and to be effective in a broader range of cell types when compared with *dl*1520.

The above-described studies of the selectivity and efficacy of CRADs indicate that it is possible to generate CRADs with improved specificity and activity by introducing more precise mutations in adenoviral genes that affect only the critical and desired functions facilitating tumor preferentiality. To derive such CRADs, a better knowledge of the function of the viral genes will be required. On the other hand, it is also evident that the development of such CRADs may actually be at the expense of selectivity of these agents, thereby compromising safety issues for applications in the clinic. Additional safety measures will need to be incorporated if true selectivity cannot be obtained in this way, for example, by making use of tumor/tissue-specific promoters (TSPs). Indeed, apart from CRADs that make use of mutations in adenoviral genes that can be rescued in cancer cells, CRADs have been generated in which essential adenoviral genes are driven by TSPs, also known as type 2 CRADs (Curiel, 2000). The use of TSPs that direct the expression of genes essential for viral replication, leading to the transcriptional targeting of viruses to cancer cells, has been widely exploited (for review see Gomez-Navarro and Curiel, 2000; Kirn *et al.*, 2001). To drive E1A expression, multiple promoters have been used for specific cancers, such as the prostate-specific antigen (PSA) promoter for prostate cancer and the α-fetoprotein (AFP) promoter for hepatocarcinomas (Rodriguez *et al.*, 1997; Alemany *et al.*, 1999). An additional favorable feature of the use of TSPs is the prevention of potential replication at unwanted sites in the body, such as in the liver, where adenoviruses accumulate via specific and nonspecific interactions. There is an ongoing quest for promoters that display "tumor-on" and "liver-off" features. Several have been employed to mediate tumor-specific expression of suicide genes, including the cyclooxygenase 2 (Cox-2), midkine (Mk), and telomerase reverse transcriptase (hTert) promoters, which may also be suitable for use in the context of a CRAD (Adachi *et al.*, 2000; Majumdar *et al.*, 2001; Yamamoto *et al.*, 2001).

CRAD-INDUCED CELL DEATH

Intensive research has revealed that PCD is a genetically controlled process, with many cellular factors involved in sensing and balancing survival and death-inducing stimuli. This balance can be disturbed in various ways, including by cytotoxic agents, radiation, growth factor withdrawal, and virus infection. The molecular events triggering PCD, as well as the accompanying cellular characteristics, can vary greatly between different cell systems and in relation to the type of stimulus, classic apoptosis being one of them.

Several adenovirus-encoded gene products have been found either to block or activate cell death, and the coordinated and timely expression of these factors leads to optimal conditions for generating progeny viruses. Among the inducers of cell death are the E1A proteins, the E4 region-encoded proteins orf4 and orf6/7, and the E3-11.6kD protein, also known as the adenovirus death protein (ADP), whereas E1B-19kD, E1B-55kD, and E4orf6 suppress cell death (for review see Braithwaite and Russel, 2001). Except for ADP, the gene products encoded by the E3 region act to prevent cell death induced by external stimuli such as factors from the immune system (for review see Wold *et al.*, 1999). ADP appears to be the only adenovirus-encoded protein that directly affects cell lysis, whereas the other gene products modulate cell survival through existing pathways present in the host cell. The latter include the p53 pathway and the Bcl-2 family of pro- and antiapoptotic proteins that control mitochondria integrity, a crucial factor in the regulation of PCD (Kroemer, 1997; Green and Reed, 1998). In this regard, a window of opportunity for enhancing the antitumor potential of CRADs is at the final stage of the reproductive cycle, which involves the lysis of the host cell and spreading of viral progeny (see also Fig. 1). Oncolysis of cancer cells, when compared with lysis of the natural host cells of adenoviruses, that is, cells of the upper and lower respiratory tract, may be suboptimal because of cancer cell-specific genetic alterations. Cancer cells are often resistant to therapeutic treatment, either at the start or during the course of treatment, and one may argue that the molecular alterations conferring resistance will also lead to cancer cells being refractory to adenovirus-induced cell death. Thus far, only the enhancing effect of ADP on adenovirus-induced oncolysis has been established in this regard; ADP is transcribed at low levels at early stages from the E3 promoter, whereas at later stages the major late promoter facilitates high expression levels of ADP (Tollefson *et al.*, 1992). The mode of action of ADP, an integral membrane glycoprotein, is not well understood (Scaria *et al.*, 1992); however, adenoviruses lacking functional ADP have been shown to kill cells and release progeny virus more slowly than wild-type adenovirus (Tollefson *et al.*, 1996). Doronin and co-workers (2000) have shown that overexpression of ADP, in the context of a CRAD with small deletions in the E1A region, is more potent in eradicating tumor cells than versions lacking ADP.

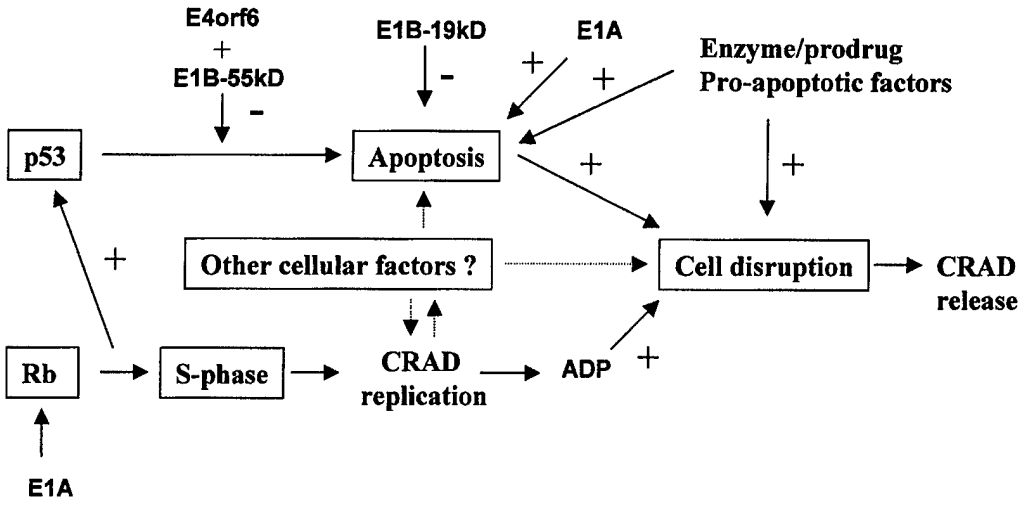

FIG. 1. Interactions between adenovirus-encoded proteins and cellular factors that facilitate CRAD replication and host cell disruption; possible enhancement of CRAD efficacy by stimulating cell disruption at late stages of infection. The currently identified major players are indicated, with cellular factors boxed. E1A expression induces forced entry into the S phase, resulting in activation of the p53 pathway leading to cell death. This is counteracted by E1B-55kD and E4orf6 that bind to, and inactivate, p53, whereas E1B-19kD provides an additional block to apoptosis by maintaining mitochondrial integrity through inhibition of proapoptotic Bcl-2 family members. Balancing of the cell death status of the host cell by the virus may involve other, yet unidentified factors, in the mean time allowing the virus to replicate. At later stages of replication the balance shifts toward an excess of cell death-inducing stimuli, either directly mediated by viral proteins (e.g., ADP) or through modulation of the cellular apoptotic machinery. In general, cancer cells have acquired genetic rearrangements or mutations during malignant transformation that make them cell death resistant. The inclusion of cell death-inducing factors in CRADs, such as genes encoding prodrug-converting enzymes or pro apoptotic factors, and on expression at later stages of infection may facilitate the disruption of cancer cells, thus enhancing viral release and dispersion. See text for additional details.

The notion that altering the cell death-inducing properties of adenoviruses may lead to improved oncolytic potential has also been tested by deleting adenoviral genes that have an anti-apoptotic function, such as that encoding the Bcl-2 homolog E1B-19kD. Realizing that cancer cells often have developed cell death-inhibitory mechanisms during malignant transformation, Sauthoff and co-workers (2000) have used a viral mutant in which E1B-19kD has been deleted; this mutant was shown to be more effective at tumor killing than wild-type virus. This variant displayed a more rapid release of viral particles from infected tumor cells in monolayer compared with the wild-type virus, likely due to enhanced PCD, and was more potent in a lung cancer xenograft model in mice (Harrison *et al.*, 2001).

Another strategy involves the use of cytotoxic genes or proapoptotic genes in the context of a CRAD. Combining the prodrug–enzyme suicide gene strategy, ganciclovir (GCV)–thymidine kinase (TK), with an E1B-55kD-deleted virus was found to be more effective in killing tumor cells than control virus without TK (Wildner *et al.*, 1999). Interestingly, GCV treatment failed to enhance the efficacy of a replicating adenovirus expressing functional E1B-55kD in combination with TK, whereas E1B-55kD-deleted viruses showed an increased cytopathic effect after GCV treatment, perhaps because of the already optimal conditions and enhanced baseline oncolytic activity of the previous agent (Wildner and Morris, 2000). In addition, the incorporation of the proapoptotic tumor necrosis factor (TNF) gene driven by the cytomegalovirus (CMV) promoter in a replicating adenovirus, rendered breast cancer specific by employing the *MUC1* promoter, showed enhanced oncolytic activity compared with the TNF-deleted version (Kurihara *et al.*, 2000).

Although these approaches in the models described above appeared to be of utility, it is realized that the optimal effect conferred by an incorporated cell death-inducing gene may depend on the timing of its onset of expression. Effective replication of the adenovirus is dependent on the coordinated and timely expression of adenoviral genes; inappropriate timing of expression of a death-inducing gene may be counterproductive to the cycles of infection. This is illustrated by the complex interactions observed between the oncolytic effect of a replicating vaccinia virus and the cytosine deaminase (CD)–5-fluorocytosine (5-FC) enzyme–produg system (McCart *et al.*, 2000). The investigators found that the tumor response-enhancing effect of 5-FC was virus dose dependent; at low multiplicities of infection (MOIs) the prodrug enhanced the response whereas at higher MOIs (>0.1) a decrease in efficacy was evident.

A likely hypothesis is that the induction of expression of cytotoxic genes in a replicating virus would be most effective at later stages of infection in order to enhance the outburst of viruses in cell death-resistant cancer cells. Papers from Hermiston and colleagues have dealt with this issue by inserting toxin genes in the E3 region in place of the native adenoviral genes, in consideration of the fact that the majority of genes in this region are not essential for viral replication *in vitro*. They found that the replacement of E3-6.7/gp19K-, ADP-, and E3B-encoding regions with toxin genes resulted in a timing of expression similar to that of the replaced viral genes, thereby maintaining normal expression of the resident adenoviral genes

(Hawkins and Hermiston, 2001; Hawkins *et al.*, 2001). Although the various viruses containing as replacements a cDNA encoding CD or TNF-α were not evaluated in detail for their oncolytic activity, the expression of these genes was stronger than that obtained with the CMV promoter, probably because of the high copy numbers of viral DNA at later stages. Moreover, deletion of ADP resulted in a longer survival of the infected cells accompanied by attenuated protein synthesis leading to increased production from the inserted gene (Hawkins and Hermiston, 2001). These studies appear to indicate that the E3 region is an attractive locus for inserting potent death-inducing genes in the CRAD genome, thereby not interfering with viral replication but facilitating the disruption of possibly death-resistant cancer cells to obtain optimal dispersion.

CRADs AND CHEMOTHERAPY

Several investigators of various *in vitro* and *in vivo* model systems, as well as in the clinic, have reported additive or synergistic effects between chemotherapy and CRADs. In a phase II trial for patients with recurrent head and neck cancer, *dl*1520 (ONYX-015), in combination with cisplatin and 5-fluorouracil, had the strongest antitumor effect when compared with the separate treatments (Khuri *et al.*, 2000). Studies to characterize this interaction in nude mouse–human tumor xenograft models indicated that this synergism was independent of the route of administration and p53 status. However, the order of administration of the agents appeared to be crucial; treatment with ONYX-015 first, or simultaneous exposure to cisplatin and the virus, is superior to cisplatin followed by ONYX-015 (Heise *et al.*, 2000b). Contrary to the lack of involvement of p53, You *et al.* (2000) found in monolayer lung cancer cultures that cells with nonfunctional p53 were at least 10 times more sensitive to ONYX-015 cytolysis than cells with wild-type p53, and chemotherapy with taxol and cisplatin was able to enhance oncolysis only in p53 mutant lung cancer cells. Synergistic effects have also been observed in prostate cancer cells, both *in vitro* and *in vivo*, on treatment with CV787, a prostate cancer-specific replicating adenovirus and the taxanes paclitaxel and docetaxel (Yu *et al.*, 1999). In addition, radiotherapy has also been found to enhance the antitumor activity of ONYX-015 in xenograft mouse models (Rogulski *et al.*, 2000). Using isogenic cell lines with wild-type or mutant p53, the authors observed no effect of irradiation on viral DNA replication in monolayer cultures. However, *in vivo* little oncolytic activity was found in p53 wild-type tumors, with radiation having no enhancing effect, whereas in mutant p53 tumors the already higher antitumor basal level was further increased after irradiation.

Classically, synergy is defined as greater than additive therapeutic effects when compared with the therapeutic efficacy of each drug alone. The molecular mechanisms underlying the synergy between replicating adenoviruses and chemotherapy are currently unknown. For the *in vivo* tumor context, this interaction may involve the enhanced activity of the immune system due to chemotherapy-dependent tumor cell damage or may perhaps be due to antiangiogenic effects elicited by these cytotoxic agents. In addition, it could be envisioned that chemotherapy may alter the structure of the tumor mass, thereby facilitating the penetration and spreading of virus throughout the tumor (see also Fig. 2). However, interaction between the two types of anticancer agents can also occur at the cellular level in the absence of an immune response and a structural context as, for example, illustrated by the synergy found between ONYX-015 and subtoxic concentrations of paclitaxel or cisplatin in lung cancer cell lines and primary lung cancer cells (You *et al.*, 2000) and CV787 in prostate cancer cells (Yu *et al.*, 1999).

Additive or synergistic interactions between drugs may depend on dissimilar mechanisms of action of the drugs, thereby targeting two independent pathways. In this respect, the molecular mechanism(s) underlying the enhancing effects of chemotherapy and irradiation on CRAD efficacy may be due to interactions between viral genes and stress-activated host cellular factors leading to enhancement of the tumor cell-killing effect of CRADs. For example, the two major mRNAs transcribed from the E1A region, E1A 12S and E1A 13S, encode proteins of 243 and 289 residues (243R and 289R) that can induce apoptosis via both p53-dependent and -independent mechanisms (White, 1998). Apoptosis induced by 243R was found to require the presence of functional p53 that correlated with deregulation of Bcl-2 and Bax, whereas the 289R variant could trigger apoptosis independent of p53. However, more recently, 243R has been reported to induce apoptosis also independent of p53 (Putzer *et al.*, 2000). E1A expression has also been found to enhance the sensitivity to apoptosis induced by ionizing radiation and various cytotoxic agents in murine embryonic fibroblasts, keratinocytes, and human ovarian cancer and leukemia cells (Stiewe *et al.*, 2000 and references therein). CRAD-mediated E1A expression may thus contribute to synergistic effects with chemotherapy or radiation, although also other adenovirus genes may be involved, which currently remains to be investigated. Results from such studies may be translated into the generation of CRADs with enhanced activity.

On the other hand, although much is known on the molecular basis of cancer cell resistance to chemotherapy-induced cell death, the potential effect on the oncolytic activity of CRADs has currently not been addressed. Interestingly, in a study with cisplatin-sensitive and -resistant p53 mutant-expressing ovarian cancer cell lines, ONYX-015 displayed preferential replication in cisplatin-resistant cells in *in vitro* and *in vivo* models (Ganly *et al.*, 2001). The restored expression of wild-type p53 in the sensitive cell line resulted in early onset of apoptosis that probably formed the basis for the observed reduction of viral production. These findings provide more evidence for the concept that early onset of cell death is detrimental for virus production whereas cell death resistance may delay or block viral release.

TUMOR CHARACTERISTICS AND OTHER BARRIERS TO CRAD EFFICACY

In the ideal situation, a CRAD should be applied intravenously and, on reaching the tumor site(s), infect tumor cells and spread throughout the tumor to eradicate all cancer cells, even when dealing with advanced metastatic disease. Although these features have been challenging to meet for all anticancer therapies, CRADs encounter several barriers that are particular to this class of agents. In this respect, apart from difficulties encountered by viral particles entering a tumor via the blood

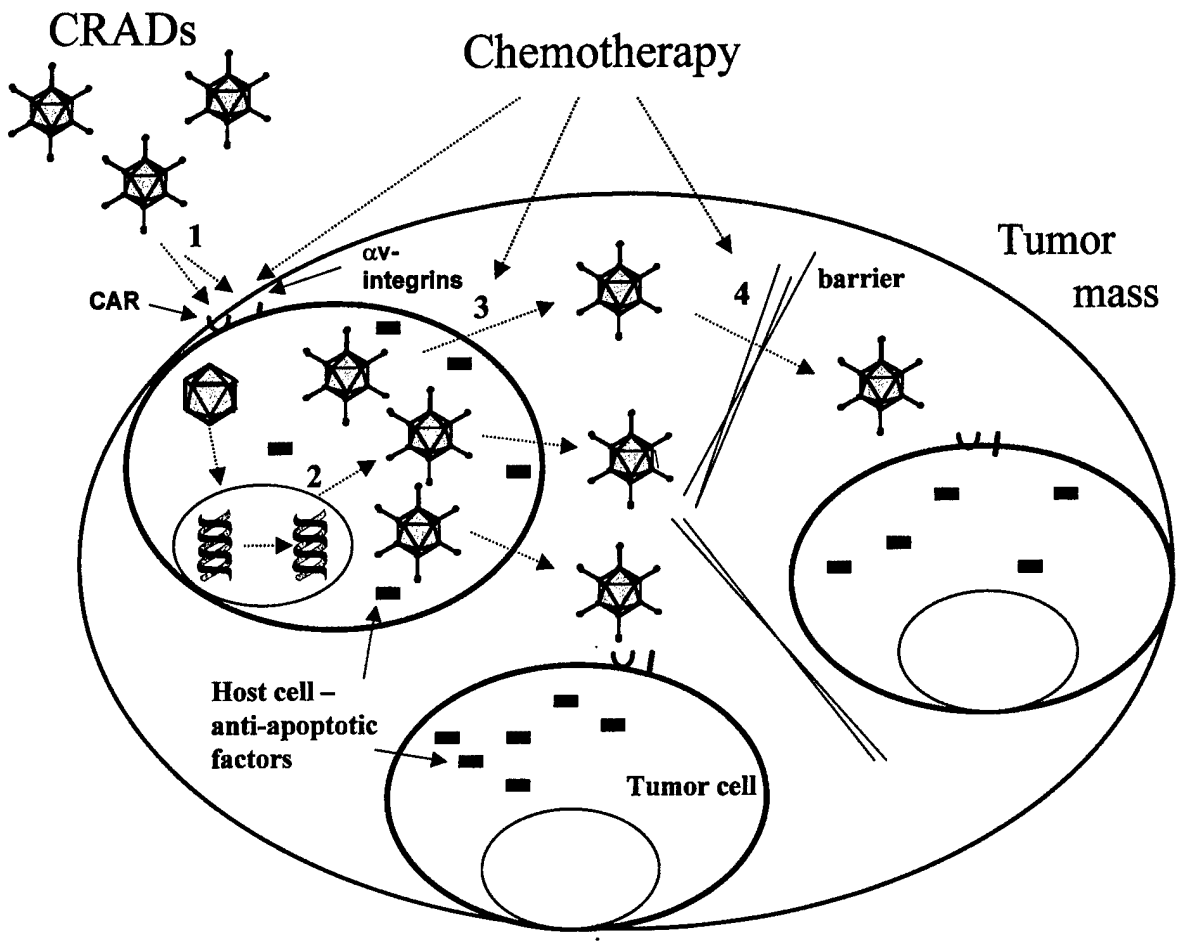

FIG. 2. Schematic representation of several obstacles for CRAD efficacy and the possible stages at which combined treatment with chemotherapy may sort its beneficial effect. On administration of CRADs, a fist hurdle may be difficulties to pass the extracellular matrix of the tumor or the lack of adenoviral receptors on tumor cells (1). Combined treatment with chemotherapy may loosen the tumor structure, thus facilitating CRAD penetration. The potential absence of the CAR may be circumvented by exploiting other virus entry routes, including allowing primary entry via the α_v-integrin pathway by incorporation of the RGD motif in the fiber knob of the virus. After infection, the replication of CRADs may be influenced by both viral and cellular factors, such as gene deletions in the CRAD genome, in order to obtain tumor selectivity and/or host cell factors such as p53 and additional, yet to be identified factors (2). After viral assembly, cellular mechanisms conferring cell death and/or chemotherapy resistance that are common to cancer cells may counteract cell death induced by CRADs and hamper the release of virus (3). At this stage chemotherapy may work in concert with CRADs to enhance tumor cell killing, resulting in improved dispersion of the virus; however, it may also act to disrupt possible existing intratumoral barriers (4). On the other hand, chemotherapy may increase the antitumor potential of CRADs via indirect phenomena such as a possible costimulatory effect on the immune response induced by dying tumor cells or potential antiangiogenic effects of the drugs (see text for more details).

stream, a process about which little is known, adenoviruses are known to be cleared rapidly by the liver on systemic administration, as illustrated by the 90% clearance rate detected within 24 hr in both immune-competent and -deficient mice (Worgall *et al.*, 1997). In addition, mainly because of clearance by Kupffer cells in the liver, the adenovirus half-life is approximately 2 min (Worgall *et al.*, 1997; Alemany *et al.*, 2000a).

The role of the immune system in adenovirus vector efficacy is not clear. There may be dual effects of the immune system. On the one hand, interference with viral function may occur via neutralizing antibodies or macrophage-mediated phagocytosis; on the other hand, activation of a virus-induced tumor-specific cytotoxic T lymphocyte (CTL) response may help to eradicate tumor cells. In addition, interactions between the growing tumor, the amount of replicating viruses, and antiviral immune responses are highly complex and nonlinear. An attempt has been made to describe these interactions in a mathematical model (Wodarz, 2001). In this model, the outcome of viral therapy is dependent on the balance between several host and viral parameters, including the growth and death rates of infected and noninfected tumor cells and the speed of viral replication. Such a model predicts optimal antitumor activity at the highest possible level of oncolytic activity and in the absence of an immune response. Also, a high growth rate of the tumor is expected to reduce efficacy and conventional treatments such as chemotherapy may be combined to decrease tumor growth, al-

lowing the virus to eliminate as many tumor cells possible (Wodarz, 2001).

Other characteristics of the tumor mass may also obstruct spreading of the CRAD, such as the architecture of the tumor. In general, it has been noted that established tumors appear to be more difficult to eliminate by CRADs than when tumor cells are premixed with virus before injection in mice for xenograft models or when treatment is started early after tumor growth is detected. "Older" tumors are more difficult to eradicate because of infiltrating macrophages and fibroblasts resulting in connective tissue formation, the ingrowth of blood capillaries, the tumor matrix, and the presence of necrotic areas, all of which impose blocks to the spreading of CRADs. It is currently unknown to what extent these obstacles interfere with CRAD efficacy but they will need to be addressed.

VALIDATION OF CRADs IN ANIMAL MODELS AND IN THE CLINIC

It is important to measure CRAD infection, replication, and selectivity to evaluate the efficacy of the agent. A major limitation in assessing the efficacy of CRADs in animal models is the inability of CRADs derived from human serotypes to replicate in nonhuman tissue. CRAD activity can thus be determined only in human xenografts models, predominantly in mice, in terms of antitumor effect and general toxicity of the virus. On this basis, determination of CRAD selectivity remains unaddressed. The molecular basis of the inability of human CRADs to replicate in nonhuman host cells is currently unknown. Until this is resolved and animal cells can be modified to become replication permissive, an alternative may be the generation and use of species-specific CRADs, such as a murine adenovirus in mice. In the clinic, viral infection has been determined in tumor biopsy samples by *in situ* hybridization with adenoviral DNA. Replication was demonstrated by testing blood from patients, using a quantitative polymerase chain reaction (PCR), for the presence of adenovirus sequences on day 3 after treatment, on the assumption that this reflects replicating virus because the initial inoculate at this time will be cleared from the blood. Determining infection and replication of adenovirus in tumor biopsies by histologic analysis has its drawbacks. Biopsies provide only a small amount of tissue, thus increasing the chance for false negatives, not to mention the ethical and practical matters associated with acquiring samples. For these reasons PCR is the method of choice, although both methods can be complementary.

The use of noninvasive methods to determine infection and replication is an important area of research that may help to evaluate CRAD efficacy. Methods that are currently being developed are adenovirus imaging systems for *in vivo* detection of infected cells, such as by incorporation of a transgene expressing the receptor for somatostatin subtype 2 (SSTR2) into the virus, allowing detection with intravenously administered radiolabeled tracer (Rogers *et al.*, 1999). The HSV-TK cassette has been used in combination with this approach, allowing the detection of TK on exposure of cells to ^{131}I-labeled 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (^{131}I-labeled FIAU) with γ-camera imaging (Zinn *et al.*, 2001). Other strategies employ photon-emitting reporter genes, including luciferase and green fluorescent protein in conjunction with a charged-coupled device (CCD) camera for *in vivo* imaging (Honigman *et al.*, 2001 and references therein).

FUTURE DIRECTIONS FOR IMPROVEMENTS

The current limitations to CRADs being effective single anticancer agents occur at multiple levels, including macrocellular, cellular, and molecular. Effective systemic delivery of these agents, being preferable in more advanced metastatic cancer, is hampered by clearance of the virus and binding/uptake by nontumor cells. The development of strategies to detarget the liver and other organs, in order to obtain the best possible ratios of tumor to nontumor targeting, is crucial for this application. This will require ongoing efforts to modify the viral coat to detarget the liver while at the same time increasing tumor specificity via tumor-targeting approaches such as the incorporation of targeting domains in the fiber knob or the use of targeting moieties, including bispecific single-chain antibodies. At the same time these methods will bypass the often-occurring CAR deficiency of cancer cells. These approaches may be used in conjunction with TSPs, which can drive both essential adenoviral genes and/or therapeutic genes to obtain liver-off/tumor-on characteristics. Enhancing replication and oncolytic properties of CRADs to overcome possible structural barriers in the tumor will be necessary to improve tumor-eradicating activity. The optimization between selectivity and oncolytic activity by means of small and subtle gene deletions and/or modifications, coupled with the use of TSPs, will probably provide the best and safest adenoviral platform to build in additional improvements. The combined use of enzyme–prodrug strategies or proapoptotic genes with such CRADs, thereby ensuring onset of expression at later stages of replication, may improve oncolysis and spreading of the virus in the tumor mass and may help to overcome structural barriers. Moreover, for additional rationalized approaches to improve CRADs more basic research on the factors involved in adenoviral oncolysis will be required, including the unraveling of the mechanism(s) responsible for synergy between chemotherapy and CRADs that may be instrumental for designing better agents. Studies to understand the way in which rodent cells block adenovirus replication may lead to the development of transgenic mouse models that are permissive for CRAD replication, thus providing better model systems. Alternatively, other species-specific adenoviruses may be used to examine CRAD characteristics. Finally, the incorporation of reporter genes in CRADs to allow noninvasive imaging techniques will be helpful in determining CRAD efficacy.

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Address reprint requests to:
Dr. D.T. Curiel
Division of Gene Therapy
Departments of Medicine, Pathology, and Surgery
Gene Therapy Center
University of Alabama at Birmingham
WTI 620, 1530 3rd Avenue S.
Birmingham, AL 35294-3300

E-mail: David.Curiel@ccc.uab.edu

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APPENDIX F

Title: **Infectivity Enhanced, Cyclooxygenase-2 Promoter-Based Conditionally Replicative Adenovirus for Pancreatic Cancer**

Subtitle: **Enhanced COX-2 CRAd for Pancreatic Cancer**

Authors: Masato Yamamoto*, Julia Davydova*, Minghui Wang*, Gene P. Siegal[‡§], Victor Krasnykh*, Selwyn M. Vickers[§] and David T. Curiel*.

***Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center, Department of [‡]Pathology, Cell Biology and [§]Surgery, University of Alabama at Birmingham, Birmingham, AL 35294-2172.**

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Abbreviations

CRAd, Conditionally replicative adenovirus; COX-2, cyclooxygenase-2; Ad, Adenoviral vector; CAR, coxsackie-adenovirus receptor; TSP, tumor specific promoter; GI, gastrointestinal; ATCC, American Type Culture Collection; PCR, viral particle; vp, polymerase chain reaction; RT-CPR, reverse transcription and polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Correspondence

Corresponding Author: Masato Yamamoto, M.D., Ph.D., Division of Human Gene Therapy, University of Alabama at Birmingham, BMR2-408, 901 19th Street South, Birmingham, AL 35294-2172. e-mail: Masato.Yamamoto@ccc.uab.edu; Fax: 205-975-8565.

Abstract

Background & Aim: Pancreatic cancer is one of the most aggressive human malignancies. Conditionally replicative adenoviruses (CRAds) have shown some promise in the treatment of the cancers, but to date their application for pancreatic cancer has met several obstacles: one is lack of a good control element to regulate replication and the other is relatively low adenoviral infectivity. Thus, we constructed infectivity enhanced cyclooxygenase-2 (COX-2) promoter-based CRAds to develop a safe and effective therapeutic modality. **Methods:** The CRAds were designed to achieve COX-2 promoter-controlled E1 expression for regulated replication (COX-2 CRAds). The infectivity enhanced CRAds also have an RGD-4C motif in the HI-loop of the adenoviral fiber knob region. The selectivity and efficacy of these constructs were analyzed with cell lines *in vitro*. The *in vivo* therapeutic effect was analyzed with a xenograft model. For *in vivo* toxicity analysis, pathology of the major organs and E1 RNA levels in the liver were studied after systemic administration. **Results:** The COX-2 CRAds demonstrated selective cytocidal effect *in vitro* in COX-2 positive cells and killed most of the pancreatic cancer cells. *In vivo*, intratumoral administration of the infectivity enhanced COX-2 CRAds (10^8 particles) showed a strong anti-tumor effect comparable to wild type virus, while the COX-2 CRAds without infectivity enhancement showed a limited effect. Systemic administration did not cause any detectable toxicity; the E1 RNA level in the liver after COX-2 CRAd administration was minimal. **Conclusions:** The infectivity enhanced COX-2 CRAd is a promising agent for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most aggressive and devastating human malignancies. Its aggressiveness is represented by a tightly matched number of estimated new cancer cases and actual cancer deaths [1], and 5-year survival rate is devastatingly low (<2%) [2]. In addition, pancreatic cancer ranks as the fourth leading cause of cancer death and the eighth most frequent type of solid tumor in North America [1]. So far, neither early detection nor treatment of advanced disease is possible. Ninety-one percent of lesions are unresectable at the time of diagnosis, showing an average survival time of 4-5 months [3]. Gemcitabine is currently most effective chemotherapeutic agent for pancreatic cancer, demonstrating a better one-year survival rate when compared to 5-fluorouracil [4]. However, even after the introduction of gemcitabine, the most advanced combination chemotherapy results in a median survival of less than one year [5]. This clearly indicates that development of new modalities is needed, and gene therapy represents one such strategy.

Adenoviral vectors (Ads) have been employed in a large number of gene therapy approaches due to their capacity to accomplish effective *in vivo* gene delivery [6]. However, the numbers of cancer gene therapy clinical trials carried out to date have fallen short with respect to initial expectations for demonstrable therapeutic outcomes. Of note, these trials have revealed limited tumor transduction frequencies and have also suggested that this limitation may represent a fundamental barrier to realizing the benefits of cancer gene therapy [7-10]. Notably in the field of gastrointestinal cancers (especially in pancreatic cancers), the transduction efficiency of adenoviral vectors is suboptimal due to the limited expression of the primary adenoviral receptor (coxsackie-adenovirus receptor, CAR) [11]. The insufficient infectivity is a critical issue for clinical application because it leads to a higher administrative dose to achieve the requisite level of gene transfer for the therapeutic outcome. At the high dose, there is increased risk of a vector-induced innate immune reaction and toxic side effects [12-17], which have resulted in a lethal systemic inflammatory response syndrome in the case of an ornithine transcarbamylase deficiency patient [18].

We have endeavored to overcome this transduction efficiency problem by rational engineering of Ad agents. As a natural consequence that the most widely used vectors have been rendered replication-

incompitent, effective tumor transduction has been limited. In comparison with conventional non-replicative vectors whose endpoint is the transgene expression, conditionally replicative adenoviruses (CRAds), which can replicate selectively in a tumor and infect neighboring tumor cells with the progeny virus, can spread in the tumor in a multiplicative fashion and eventually lead to the lysis of the entire tumor mediated by the oncolytic function of adenovirus [19, 20]. Initially, several CRAds with a mutation or deletion in the E1 region were developed (e.g. dl1520/ONYX-015, AdΔ24) [21, 22], and have been employed in clinical trials. Also, tumor specific promoters (TSP) have been applied to achieve a tumor selective replication of the adenoviral agents, and such an outcome has been accomplished by restricting the expression of key adenoviral genes with a TSP [6, 23-26]. For cancer of the pancreas, a number of candidate TSPs have been proposed [27-33], but none of the described promoters has exhibited the levels of activity and specificity required for the development of CRAds for pancreatic cancer eradication.

In the field of gastrointestinal (GI) cancers, cyclooxygenase-2 (COX-2) is frequently expressed in neoplastic tissues and plays an essential role in many aspects of cancer progression directly and indirectly [34-36]. Especially in the field of pancreatic cancer, the differential expression between cancerous and normal pancreatic tissues, along with the *in vitro* antitumor effect of COX-2 inhibitors, have led to the recognition of the importance of COX-2 as a potential target of anti-cancer therapy [37-42]. We have been studying promoter-based targeting in adenoviral gene therapy, and have established the COX-2 promoter as a promoter with an optimal activity profile for GI cancers [43, 44]. We have reported that the COX-2 promoter in an adenoviral construct shows a uniquely beneficial "tumor-ON / liver-OFF" profile for pancreatic cancers [45]. This data suggested that the COX-2 promoter is promising as a control element for the construction of pancreatic cancer CRAds. In this study, we constructed such CRAds by controlling the E1 gene expression with the COX-2 promoter.

In the context of infectivity enhancement *via* the augmentation of viral binding, we have reported that adenoviral vectors with the RGD-4C motif configured in the HI-loop of the fiber-knob region showed superior infectivity in the cells showing low CAR expression in comparison with the vectors with wild type Ad5 fiber.

This enhancement is mainly mediated by the binding of the RGD motif onto integrins which are frequently overexpressed on the surface of the target cancer cells [46]. Furthermore, our previous studies have indicated that low CAR expression and integrin overexpression is typically observed in pancreatic cancers [11]. When the RGD modification was applied to another CRAd (Δ24) [22], the viral replication and cytocidal effect was dramatically enhanced [47]. These studies clearly indicate that the RGD-4C motif-based infectivity enhancement should augment the therapeutic potential of the COX-2 CRAds in pancreatic cancer.

In this study, we constructed CRAds controlled by the COX-2 promoter, and also enhanced its infectivity by RGD-modification of the fiber protein. We evaluated these CRAds *in vitro* and *in vivo* to assess their potential therapeutic utility. These studies establish that the infectivity enhanced, COX-2 promoter-driven CRAd is a promising agent for the therapy of pancreatic cancer.

Materials and Methods

Cells and animals

The MIA PaCa-2, Capan-1, Hs 766T, and PANC-1 pancreatic cancer cell lines (CRL-1420, HTB-79, HTB-134 and CRL-1469, American Type Culture Collection, ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) with 10% fetal calf serum (HyCclone, Logan, UT). The A549 (COX-2 positive lung cancer cell line, CCL-185, ATCC), MKN45 (COX-2 positive gastric cancer cell line, JCRB0254, Japanese Collection of Research Bioresources, Tokyo, Japan), and BT474 (COX-2 negative breast cancer cell line, HTB-20, ATCC) cells were maintained in RPMI 1640 (Mediatech) containing 10% fetal calf serum. The medium of BT474 cells was supplemented with bovine insulin (0.01 mg/ml, Life Technologies, Rockville, MD). The 911 cells (a kind gift from Dr. Van Der Eb, Leiden University, the Netherlands) [48] were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Each medium was also supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml). Cells were incubated in a 37°C and 5% CO_2 environment under humidified conditions.

Female C57BL/6 mice (Charles River, Wilmington, MA) and female athymic NCr-nu nude mice (Frederick Cancer Research, Frederick, MD) (6–8 weeks of age) were used in the *in vivo* experiments. All animals received humane care based on the guidelines set by the American Veterinary Association. All the experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Vector construction and structure confirmation

The CRAd genomes were constructed *via* homologous recombination in *Escherichia coli* [49, 50] (Figure 1). The COX-2 CRAds had the sequence nt 1 - 358 of Ad5 as a left end structure (containing left inverted terminal repeat and viral packaging signal) and the COX-2 promoter controlled E1 expression cassette in place of the original E1 region of the Ad genome. Except for RGD-4C insertion into the Ad fiber-knob region, other parts (correspond to Ad5 nt 3511 and later) are identical to adenovirus type 5 [46, 51]. Specifically, we

constructed the shuttle vectors for COX-2 CRAd generation using pShuttleGL3Bcox-2L, a shuttle vector for COX-2 promoter-driven luciferase expression vector AdCox2L Luc, as a starting material. This plasmid contains the -1432/+59 region of COX-2 promoter derived from phPES2 (Drs. Inoue and Tanabe at the National Cardiovascular Center Research Institute, Suita, Japan [52, 53]), which shows high selectivity in an adenoviral vector configuration [43], and a simian virus 40 polyadenylation signal (see reference 43 for detail). Firstly, to restore the adenoviral protein IX promoter, the fragment nt 3511-3924 containing intact protein IX promoter was amplified by the polymerase chain reaction (PCR) using a high fidelity polymerase (*LA Taq*, PanVera/TaKaRa, Madison, WI) with primers 3511-Hc-S (5' gcgtcgacagatcttgtactgaaatgtgtgggcgtggctt 3') and 3511-Hc-AS (5' gtgccaaaagagccgtcaactt 3'), and treated with SalI and HincII. Then, the SalI-HincII region of the pShuttleGL3Bcox-2L was replaced with the new fragment. Next, the KpnI site at the 5' end of the COX-2 promoter was converted to a SalI site by using SalI linker for later construction of vectors with the E1 expression cassette in reverse orientation. The E1 region (Ad5 nt 556-3512) was amplified by PCR using a high fidelity polymerase (*LA Taq*) with primers E1-Hd-S (5' cccaagcttgaaaatgagacatattatctggcacgga 3') and E1-Xba-AS (5' gctctagaacctcaatctgtatcttcatcgctaga 3'). After confirmation of the sequence, the 3'side 1/3 of E1 gene (HindIII-XbaI fragment, from nt 2805 to the 3' end) was cloned into the shuttle vector in place of the luciferase gene (HindIII-XbaI), and then the rest of the E1 gene (HindIII-HindIII fragment, from the 5' end to nt 2805) was cloned into the HindIII site of the resultant vector, resulting in pShuttle cox-2L E1 F. The vector with the E1 expression cassette in the opposite direction was constructed by cleavage and re-ligation with two SalI sites at both ends of the E1 expression cassette (pShuttle cox-2L E1 R). After cleavage with PmeI, the shuttle vectors were recombined with Ad5 DNA and ClaI linearized pVK503 [46] to generate the CRAd genomes with a wild type fiber and for those with RGD-modified fibers, respectively. The resultant plasmids encoding COX-2 CRAds were linearized with PacI, and introduced into 911 cells using Superfect (Qiagen, Valencia, CA).

The structures of the CRAds were analyzed for several critical vector components by PCR. The virus (5.0×10^8 vp) was processed with a Blood DNA kit (Qiagen), and 1/50 of the DNA solution was analyzed by

PCR for various regions with Taq polymerase (Qiagen). Thermal cycling conditions were: initial denaturation, 5 minutes at 95 °C, and 25 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, and 1 minute at 72°C, and a final extension for 10 minutes at 72°C. The primers used for each region were as follows: Forward direction CRAd (392 bp) (CRAdITR(S) 5' GATAATGAGGGGGTGGAGTTTGTG 3' and CRAdcoxL(AS) 5' CAGTTATGTCCTAAGTCCTTAGCATTACA 3'), Wild type Ad5 (485 bp) (CRAdITR(S) and CRAdWt(AS) 5' GAAAACTCTACTCGCTGGCACTCA 3'), COX-2 promoter (405 bp) (COX2p check (S) 5' CCCATCCAAGGCGATCAGTC 3' and COX2p check (AS) 5' GACGTGCTCCTGACGCTCACTGCAA 3'), E1a (338 bp) (E1a (S) 5' GAGACATATTATCTGCCACGGAGG 3' and (AS) 5' TTGGCATAGAAACCGGACCCAAGG 3') and fiber-knob region (wild type: 247 bp, RGD: 274 bp) (Fiber up 5' CAAACGCTGTTGGATTTATG 3' and Fiber down 5' GTGTAAGAGGATGTGGCAAAT 3').

Wild type Ad5 (Ad5) and its RGD modified version (Ad5wtRGD, generated from pVK503[46]) were used as ubiquitous replication control vectors, while AdCMVLuc (E1-deleted non-replicative luciferase expression vector) [43] and its RGD modified version (RGDCMVLuc) were utilized as non-replicative controls in CRAd analysis.

For infectivity enhancement analysis, a CMV promoter driven luciferase expression vector with the RGD modification (Ad5lucRGD) [46] and its counterpart with wild type fiber (Ad5Luc1) were used. These two vectors are identical except for the HI-loop of the fiber-knob region.

The viruses were propagated in the adenovirus packaging cell line, 911, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol. The vectors were titrated by plaque assay and viral particle (vp) number was determined based on the optical density, and stored at -80°C until usage.

Analysis of COX-2 RNA status.

The COX-2 RNA status of cell lines was analyzed by reverse transcription and polymerase chain reaction (RT-PCR) as described previously [43]. Briefly, total RNA was extracted from semi-confluent cell

cultures using the RNeasy mini RNA extraction kit (Qiagen) and analyzed for COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA with the GeneAMP RNA PCR Kit (Perkin–Elmer, Branchburg, NJ). Total RNA (500 ng) was reverse-transcribed with oligo(dT) primer and murine leukemia virus reverse transcriptase and amplified by PCR with 50 nM primers using a cycling program as described previously [43]. This RT-PCR analysis has been confirmed to be semi-quantitative [43].

Analysis of infectivity

The infectivity of the vectors with the wild type fiber and the RGD modified fiber was analyzed and compared employing CMV promoter-driven luciferase expression vectors with both types of fibers. One day after plating 50,000 cells per well on a 24-well plate, cells were infected with Ad5lucRGD and Ad5Luc1 using two different amounts of virus per cell (50 and 500 vp/cell) in Dulbecco's modified Eagle's medium with 5% fetal calf serum (infection medium). Two hours later, the infection medium was replaced with the appropriate complete medium. After 48 h of cultivation, the cells were lysed with Cell Culture Lysis Buffer (Promega) and the resultant lysates were analyzed with the Luciferase Assay System (Promega). The protein concentration was determined with the DC protein assay (Bio-Rad, Hercules, CA). All experiments were performed in triplicate.

In vitro analysis of cytocidal effect

The *in vitro* cytocidal effect of the COX-2 CRAds was analyzed by determining the viability of the cells after infection with crystal violet staining. One day after 25,000 cells per well were plated on a 12-well plate, cells were infected at 0.001 vp per cell with CRAds in infection medium. Two hours later, the infection medium was replaced with the appropriate complete medium. After 10 days of cultivation, the cells were fixed with 10% buffered-formalin for 10 minutes and stained with 1% crystal violet in 70% ethanol, for 20 minutes, followed by washing three times with tap water and air drying.

Analysis of viral replication and regulation

To analyze the viral replication and regulation of CRAd selectivity, 2.5 x 10^4 A549 and BT474 cells were inoculated onto 12 well plates and infected with 1 vp/cell of each vector the next day. Three days later, supernatant and cells were separately recovered for analysis. Encapsidated viral DNA in the supernatant was isolated as follows: the medium was recovered from the culture dish and spun in a microcentrifuge at 3000 rpm for 5 min, and 360 µl of supernatant was recovered. Then, 40 µl of 10x DNAse buffer (60mM $MgCl_2$, 400mM Tris-Cl [pH7.5], 20mM $CaCl_2$) and 10 U of DNase I (Roche Applied, Indianapolis, IN) were added followed by incubation at 37°C for 1 hour. To the mix, 20 µl of 0.5M EDTA, 20 µl of 10% SDS, and 10 µl of Proteinase K were added, and the samples were then incubated at 52°C for 2 hours. After phenol-chloroform extraction, the DNA was ethanol-precipitated together with 10µg of glycogen. After rinsing with 70% ethanol and air drying, the DNA was dissolved with 100 µl of 10mM Tris-Cl, pH7.4. For viral DNA isolation from the cells, after washing with PBS twice, the cells were scraped from the plate and the suspension was split in two. One half was directly processed with a QIAamp Blood DNA Mini Kit (Qiagen) for total cellular DNA. The other half was processed by a spermine-HCl method for encapsidated viral DNA isolation [54]. Briefly, the cells were resuspended with 50 µl of 100mM Tris-Cl [pH 9.0], and then lysed with 50 µl of DOC lysis buffer (0.4% Sodium Deoxycholate, 0.1M Tris-Cl [pH9.0], 20% ethanol) followed by incubation with 1 µl of 500 mM spermine on ice for 10 minutes. After centrifugation at 14000 rpm at 4°C for 4 minutes, the supernatant was recovered and processed with a QIAamp Blood DNA Mini Kit.

The viral DNAs isolated by aforementioned methods were analyzed by real-time PCR analysis to determine the adenoviral DNA copy number at the UAB Gene Therapy Center Correlative Labs [55]. Briefly, 1µl of extracted DNA sample was added to 9 µl per reaction of a master mix containing 1X Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1nM probe and 0.025% BSA in each reaction capillary. For the standard curve to quantify the E4 copy numbers, E4 template DNA with known copy number (10^8, 10^6, 10^4 and 10^2/µl) was also

analyzed. All PCR p\reactions were carried out using the LightCycler™System (Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer. Thermal cycling conditions were: initial denaturation, 10 minutes at 95 °C, and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Data was analyzed with the LightCycler software. The primers were designed to detect a 68 bp region in E4: forward primer (5' GGAGTGCGCCGAGACAAC 3', nt 34007-34024), reverse primer (5' ACTACGTCCGGCGTTCCAT 3', nt 34074-34056) and 6-FAM labeled probe (6FAM-TGGCATGACACTACGACCAACACGATCT-TAMRA, nt 34054-34027).

In vivo anti-tumor effect

To analyze the anti-tumor effect of the CRAds in an *in vivo* model, 2 x 10^7 Hs766T cells were inoculated subcutaneously into the flank of female athymic NCr-nu nude mice, and a single dose (10^8 vp) of CRAds or control viruses were injected into the tumors after they reached a size of 6- to 8-mm in maximum diameter. The Hs766T cells were selected because this cell line produced the most constant subcutaneous tumors among the cells tested. The condition of the mice was monitored daily and the tumor diameter was measured twice a week with calibers. The tumor volume was calculated using a formula: tumor volume = width^2 x length/2. The mice were euthanized 32 days after the viral injection in accordance with the institutional approved animal experimental protocol due to the volume of the tumors in the control group.

In vivo toxicological study

To investigate the potential toxicity of the COX-2 CRAds, each group of CRAds and control viruses were injected into the tail veins of female C57BL/6 mice (Charles River, Wilmington, MA). The mice were carefully observed for 5 days and sacrificed under approved protocols. For histopathological analysis, the major organs were fixed with 10% buffered formalin, paraffin-embedded, and cut at 4 μm, followed by deparaffinization and staining with hematoxylin and eosin under standard conditions. To analyze the E1 RNA level, total RNA from the liver was analyzed. The liver tissue was frozen on dry ice

avoiding contamination and stored at –80°C until assayed. On the day of analysis, tissues were ground into fine powder with a pestle and mortar in an ethanol/dry ice bath and total RNA was extracted with the RNAeasy Mini Kit (Qiagen). After adjusting the concentration to 100ng/μl, the RNAs were analyzed with the TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA). Briefly, 1 μl of RNA sample was added to 9 μl of PCR mixture (1 x TaqMan EZ buffer, 3 mM of $Mn(CH_3COOH)_2$, 300 μM of dATP, dCTP, dGTP, 600 μM of dUTP, 100 nM of forward and reverse primers, and probe, 0.1 U/μl of r*Tth* DNA Polymerase, 0.01 U/μl and 0.025 %BSA in RNase-free water) into each reaction capillary. Known amounts of control E1a RNA (10^8, 10^6, 10^4 and 10^2/μl) were also analyzed to obtain a standard curve to quantify the E1A copy numbers in the unknown samples.

An RT-PCR reaction was carried out using the LightCycler™System. Thermal cycling conditions included reverse transcription (2 minutes at 50°C, 30 minutes at 60°C, and 5 minutes at 95°C) and 40 cycles of 20 seconds at 94°C and 1 minute at 62°C. Data was analyzed with the LightCycler software. The control E1a RNA was prepared by *in vitro* transcription (MAXIscript In Vitro Transcription Kit, Ambion, Austin, TX) with T7 polymerase from a cloned E1a template as described by the manufacturer. TaqMan primers and probe design for the E1a gene were as follows: the forward primer (5' AACCAGTTGCCGTGAGAGTTG 3'), reverse primer (5' CTCGTTAAGCAAGTCCTCGATACAT 3'), and 6-FAM labeled probe (5' CACAGCCTGGCGACGCCCA-TAMRA 3').

Results

Attributes of COX-2 CRAds

The vectors shown in figure 1 were constructed and amplified using 911 cells. The yields were between 0.5 and 1.5 x 10^{11} vp (per 20 15 cm dishes), comparable to the yields of ordinary E1 deleted vectors grown in 911 cells. The vp/pfu ratios were in the range of 3.3 to 11.9. The vector structure was confirmed by PCR of the viral DNA. All vectors lacked the wild-type left end sequence (Figure 2A). This indicated the absence of the wild type vector as a result of unfavorable homologous recombination during vector propagation. These vectors possessed the E1a gene and the COX-2 promoter sequence as part of the E1 expression cassette (Figure 2B and 2C). The 392 bp fragment was amplified from CRAdcox2F and RGDCRAdcox2F with the sense primer corresponding to the left inverted terminal-repeat sequence and the antisense primer corresponding to the COX-2 promoter sequence (Figure 2D). In the analysis of the fiber region, the vectors with wild type Ad5 fiber (CRAdcox2F and CRAdcox2R) gave a signal at 247bp, while the vectors with the RGD modified fiber (RGDCRAdcox2F and RGDCRAdcox2R) yielded a band at 274bp, representing the 9 amino acid insertion in the HI-loop. These data confirmed the structural accuracy of the CRAd constructs.

COX-2 RNA status of the pancreatic cancer cell lines

The COX-2 RNA status of the cell lines used for this experiment was analyzed by RT-PCR (Figure 3). Because the primers were designed to have three introns between them, the signal detected at 723 bp represented a complementary DNA sequence that was reverse transcribed from RNA. The BT474 breast cancer cell line (COX-2 negative control) was appropriately negative for COX-2 RNA, while the MKN45 gastric cancer cell line was strongly positive. The pancreatic cancer cell lines Hs766T and Capan-1 were positive for COX-2 RNA, while MiaPaca-2 and Panc-1 were negative for COX-2 mRNA. The GAPDH RNA levels analyzed by RT-PCR (Figure 3 lower panel) were the same among all the cells tested, serving as a control for RNA isolation and RT-PCR procedure.

Enhancement of the adenoviral infectivity in pancreatic cancer cells by incorporation of RGD-4C

The infectivity enhancement was analyzed using CMV promoter driven luciferase expression vectors. The vectors Ad5Luc1 and Ad5lucRGD have the same structure except that the fiber of the latter vector has a 9 amino acid insertion in the HI-loop corresponding to the RGD modification. The luciferase activities with Ad5lucRGD were higher than that of Ad5Luc1 in 4 out of 4 human pancreatic cancer cell lines (2.2-13.6 fold increase). Moreover the enhancements in each cell line were consistent regardless of the viral dose upon infection (50 and 500 vp/cell) (Figure 4). This indicated that the RGD modification confers infectivity enhancement in a wide range of pancreatic cancer cells.

In vitro oncolytic potency of COX-2 CRAds

To analyze the oncolytic potency in conjunction with viral replication, we infected each cell line with a low dose of the CRAds (0.001 vp/cell) and analyzed the cells by crystal violet staining at day 10 (Figure 5). In this experiment, the result demonstrates the presence of viral replication and spread since the toxicity based on the viral protein expression (e.g. E1a) does not cause detectable cytocidal effect at this low inoculation dose. In A549 cells (COX-2 positive), all four CRAds (CRAdcox2F, CRAdcox2R, RGDCRAdcox2F, and RGDCRAdcox2R) showed a strong cytocidal effect comparable to wild type virus (Ad5) and its RGD modified version (Ad5wtRGD), while the non-replicative luciferase expression vectors did not show any cytocidal effect. None of these COX-2 CRAds showed detectable cytocidal effect in COX-2 negative BT474 cells. In pancreatic cancer cells, CRAds with the left to right-direction E1 expression cassettes (CRAdcox2F and RGDCRAdcox2F) showed a cytocidal effect in 4 out of 4 cell lines, while the CRAds with the right to left-direction E1 expression cassette (CRAdcox2R and RGDCRAdcox2R) showed a cytocidal effect in 2 out of 4 cell lines. In the context of infectivity enhancement, the infectivity enhanced vectors showed more cytocidal effect in pancreatic cancer cells in general. However, under this *in vitro* condition, the magnitudes of the enhancement and the time points that showed the largest difference were variable depending on each cell

and CRAd. Microscopically, the cells undergoing cytocidal effect indicated the typical characteristic of adenoviral cytopathic effect (data not shown).

Regulation of the COX-2 CRAd replication

To determine which step(s) plays a major role in the replication control of the COX-2 CRAds, we quantified the viral DNA in three different stages of viral replication. The first was the total cellular DNA representing virion encapsidated and un-encapsidated DNA in the cells, the second was the encapsidated DNA in the cells, and the third was the encapsidated DNA within the culture medium (Figure 6). The total and encapsidated viral DNA levels after CRAd infection were lower than that of wild type controls in both COX-2 positive and negative cells. Also, those in COX-2 negative cells are lower than those in COX-2 positive cells. This tendency was more remarkable in CRAd cox-2R than CRAdcox-2F. The encapsidated viral DNA in the supernatant in BT474 cells was minimal and remarkably lower than that in A549 cells. The difference of the encapsidated DNA in the culture medium between COX-2 positive and negative cells were more evident than other franctions. This data suggested that promoter controlled E1 expression affects multiple steps of viral replication and that the release of the virus was one of the strongest control points in this type of CRAds. The same experiment with RGD-modified vectors showed the same tendency.

In vivo anti-tumor effect of COX-2 CRAds

The anti-tumor effect was analyzed *in vivo* using Hs766T pancreatic cancer subcutaneous xenografts. After establishment of the tumor (6-8 mm maximum diameter), the CRAds (10^8 vp) were injected into the tumors. This dose is more than 2 orders of magnitude lower than the dose we have been using for suicide gene therapy with non-replicative vectors. In comparison with the untreated group and the group that received a non-replicative luciferase expression vector, the groups with CRAdcox2F and CRAdcox2R showed tumor growth suppression but the effect was not statistically significant (Mann-Whitney: $p>0.05$). The RGDCRAdcox2F and RGDCRAdcox2R, however, showed stronger tumor growth suppression comparable to

that of wild type virus, with statistical significance when compared to the untreated group and the group with the non-replicative control (Mann-Whitney: $p<0.01$) (Figure 7). This experiment was repeated three times and showed the same results. This data indicates that COX-2 CRAds possess an *in vivo* therapeutic effect and moreover that the oncolytic potential of RGD modified COX-2 CRAds was significant. Some tumors that received COX-2 CRAds experienced tumor necrosis and consequent tumor regression (typical finding: Figure 8A), while the tumors of the untreated group and the group with the non-replicative control showed consistent growth without any tumor necrosis (typical finding: Figure 8B).

Minimal toxicity of COX-2 CRAds in rodents

In vivo toxicological studies were performed in mice to obtain preliminary safety data. We injected the CRAds (10^8 vp) (CRAdcox2F, CRAdcox2R, RGDCRAdcox2F, and RGDCRAdcox2R) or control vectors (AdCMVLuc, wild type Ad5, Ad5lucRGD, and Ad5wtRGD) into the systemic circulation *via* the tail vein, and the mice were sacrificed three days later for analyses. The organ specimens were processed for pathologic analysis, and the liver tissue was also processed for adenoviral E1 RNA analysis. By gross and histopathological analysis, systemic administration of COX-2 CRAds at the above dose did not result in any remarkable findings of toxicity in the major organs. Although slightly increased numbers of acute inflammatory cells in the liver parenchyma in the groups that receiving CRAdcox2F, RGDCRAdcox2F, wild type and RGD wild type were observed, these numbers were far below the level of inflammation associated with significant toxicity. Other than this point, the pathological findings were identical to the tissues from untreated mice. The findings for RGDCRAdcox2F are shown in Figure 9 a-d.

Next, we analyzed the E1 RNA level in the liver to obtain key information regarding replication specificity of the COX-2 CRAds. In comparison with the wild type controls (wild type and Ad5wtRGD), the E1 RNA levels in the liver for COX-2 CRAds, whose E1 gene is under the control of the COX-2 promoter, were much lower and near background level, regardless of the fiber modification (Figure 10). Since the

selectivity of the COX-2 CRAds is based on the selectivity of E1 expression, this provides encouraging critical information with respect to the *in vivo* replication selectivity of the COX-2 CRAds.

Discussion

The prognosis of pancreatic cancer is devastating, showing minimal long-term survival [2, 56]. This fact has motivated many efforts in the development of novel cancer therapeutic strategies. For almost 5 decades, various replicative viruses have been tried in the context of cancer therapy [57-64]. Recently, several conditionally replicative viral agents (adenovirus and herpes simplex virus), designed with the advanced molecular and biological understanding of the viruses, have been tested in pancreatic cancer [59, 65, 66]. In the field of adenovirus, *dl1520* / ONYX-015 virus showed good safety data in a phase I clinical trial. However, the therapeutic efficacy in pancreatic cancer was not as noteworthy as that seen in head-neck cancers [67, 68]. Thus, the further development of CRAds designed specifically for pancreatic cancer is needed.

In this study, we designed CRAds based on COX-2 promoter selectivity [43, 45] and enhanced the infectivity by incorporating an RGD-4C motif in the HI-loop of the fiber-knob region with the expectation of increased efficacy [11, 46, 47, 51]. During vector amplification, the COX-2 CRAds produced consistent replication regardless of the existence of the RGD modification, and the yield was comparable to regular ΔE1 vectors. Also, the resultant viruses maintained their correct genomic structures and were free from unconditionally replicative recombinants containing the wild type E1 control region (Figure 2). These data indicate that these vectors fulfill the required features for clinical grade vector production.

We have reported that the COX-2 promoter in an adenovirus vector delivers strong activity in all the established and near primary pancreatic cancer cell lines that we have tested [45]. Interestingly, the RT-PCR analysis of COX-2 RNA in the pancreatic cancer cell lines showed that COX-2 RNA is negative in two cell lines (Figure 3, MiaPaca-2 and Panc-1). Since the COX-2 promoter was confirmed to be active in these cells (data not shown), there is a discrepancy between the COX-2 RNA status and the activity of an extrinsically delivered COX-2 promoter. In pancreatic cancer cells, structural rearrangements in chromosome 1 are one of the most frequently observed phenomena [69], and the COX-2 gene and its promoter are in the 1q25.2-3 region of human chromosome 1 (see D28235 by Tanabe, DDBJ/EMBL/GenBank). The functional loss of the COX-2 gene or its promoter, a result of its deletion or the alteration of adjacent regions, could lead to the lack of

COX-2 expression in these cells but would not necessarily influence the positive control signal of the COX-2 promoter. Thus, this might explain the discrepancy between the COX-2 RNA status and the COX-2 promoter activity profile in some cells.

In the context of infectivity enhancement, we re-evaluated infectivity enhancement using a new set of vectors with and without the RGD-4C motif in the HI-loop region. In these CMV promoter-driven luciferase expression vectors, the genetic structure is identical except for the HI-loop. Additionally, infection with adenoviral vectors (with and without RGD modification) did not affect the CMV promoter activity (data not shown). Thus, luciferase activity obtained from this experiment truly represents the infectivity of each vector. As expected from the fact that the pancreatic cell lines used in this experiment express minimal CAR but much integrin(s) (either αvβ3 or αvβ5 integrin) [11, 46], the RGD-modified vectors showed more infectivity than the vectors with the wild type fiber. In fact, in HepG2 (hepatocellular carcinoma) cell line, which does not express αvβ3 or αvβ5 integrin, the infectivity enhancement with the RGD modified vector were minimal (data not shown). This suggests the infectivity enhancement with RGD modification correlates with αvβ3 and αvβ5 integrin expression.

The COX-2 CRAds indicated *in vitro* cytocidal effect selectively in COX-2 positive cells (Figure 4, BT474 and A549) and the same selectivity dependent on cellular COX-2 promoter status has been observed in many other cell lines (unpublished data). Since COX-2 CRAds lack cytocidal effect in COX-2 negative cells (BT474) which shows high adenoviral infectivity, the oncolytic effect clearly depends on the COX-2 promoter status. In pancreatic cancer cell lines, the COX-2 CRAds with E1 expression cassettes in the left to right direction (F) showed stronger cytocidal effect than those with the reverse direction cassettes (R). This difference is believed to be due to the difference of promoter strength because the COX-2 promoter placed in the left to right direction shows more activity than when it is placed in a right to left direction (unpublished data). In the context of infectivity enhancement, RGD-modified CRAds clearly showed a greater cytocidal effect than the unmodified versions although complete CPE of the entire cell culture in some wells makes the detection of the differential difficult.

To further characterize the mechanism of the selective cytocidal effect, we quantified the viral DNA in different stage of viral replication. Interestingly, the encapsidated viral DNA in the supernatant in COX-2 negative cells was minimal and remarkably lower than that in COX-2 positive cells, abd the total and encapsidated viral DNA levels after CRAd infection in COX-2 negative cells are lower than those in COX-2 positive cells to some extent. This data suggests that the replication of the CRAds based on the promoter controlled E1 expression is controlled at multiple points of viral replication, and the viral release is one of the most crucial control points. The RGD-modified vectors showed the same tendency. This result suggests that some degree of replication of the DNA and packaging does take place in the cell with no (or minimal) E1 expression, as reported by Steinwaeder et al. [70]. Conversely, the release of the encapsidated virus may require considerable amount of E1 expression in the cells. Since the spread of the cytocidal effect requires the effective release of the progeny viruses and their infection of the surrounding cells [20], it is reasonable to conclude that the *in vitro* cytocidal effect was observed only in COX-2 positive cells (Figure 5).

In the subcutaneous xenograft model of pancreatic cancer, the COX-2 CRAds showed a therapeutic effect upon intratumoral injection of 10^8 vp per tumor. In previous suicide gene therapy experiments with adenoviral vectors expressing HSV-TK, the minimum dose resulting in a clear therapeutic effect in the gastrointestinal cancer subcutaneous xenograft model with low CAR expression was approximately 10^9 plaque forming unit (approximately 10^{10} viral particle) per site [43, 71]. Thus, the COX-2 CRAds showed an antitumor effect with a 2-order lower dose. This is clearly beneficial since the innate immune response upon high dose administration can be avoided or minimized by reducing the administration dose [12-14]. In xenograft experiment, RGD-modified CRAds showed a much stronger anti-tumor effect than the fiber-unmodified CRAds. The benefit of the infectivity enhancement was bigger than that seen in the *in vitro* experiment. In clinical trials with replicative adenoviruses, local viral replication was minimally detected [68]. This suggests that *in vivo* circumstances are much more stringent for adenoviral replication than that *in vitro*. Thus, in this situation, infectivity enhancement of the CRAds may give a clear advantage for replication based cytocidal effect.

So far there is no practical rodent model to directly analyze adenoviral replication and replication-based toxicity since human adenovirus does not replicate in rodents except in defined contexts [72]. However, the E1 RNA level is a key determining factor of the replication of our CRAds since their replication selectivity depends on the selective expression of the E1 gene mediated by the COX-2 promoter. Thus, analysis of the E1 RNA after administration into mice provides us with key information about the *in vivo* replication potency and its toxicity as a consequence. In this experimental system, the vast majority of the intravenously administered adenovirus localizes to the liver, and the liver is the primary target of adenoviral toxicity. After injection of 10^8 vp of the CRAds, the E1 RNA level in the liver was close to background. No toxicity-related event was observed in those mice's major internal organs. This indicates that COX-2 CRAds maintain replication control functionality *in vivo*, a key finding which predicates the relative non-toxicity of our CRAd agents.

In the present study we have established a novel conditionally replicative adenovirus using the COX-2 promoter to control its replication and shown that the incorporation of an RGD-4C motif into these CRAds can enhance the antitumor efficacy, especially *in vivo*. The COX-2 CRAds presented a good selectivity profile *in vitro* and *in vivo*, showing no toxicity in major organs. Thus, these CRAds have the potential to be a clinically usable agent for pancreatic cancer. Also, the COX-2 CRAds are promising not only for local injection but also for transcatheter intra-arterial administration to target intrahepatic lesions, where transgene expression in the liver can cause safety problems but avoided with a COX-2 controlled vector [73]. To make gene therapy not only feasible but also clinically useful, strategies to ensure both key safety and efficacy endpoints are required. In this regard, the COX-2 CRAds, which confer selectivity of the COX-2 promoter, are useful in conferring selective replication to reduce nonspecific adverse effects and have widespread applicability for many kinds of COX-2-positive cancers [34, 38, 74-76].

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Figure Legends

Figure 1. Structure of conditionally replicative adenoviruses.

The vectors were constructed based on human adenovirus type 5 sequences. After restoration of the pIX promoter region, a COX-2 promoter driven E1 expression cassette was inserted in both orientations, respectively (CRAdcox2F and CRAdcox2R). The infectivity enhanced versions of COX-2 CRAds were also generated (RGD CRAdcox2F and RGD CRAdcox2R). The E3 region was maintained in all vectors.

Figure 2. Confirmation of the CRAd structure by PCR.

To confirm structural accuracy, the viral DNAs were analyzed using sets of primers corresponding to several important regions of the virus. (A) Detection of wild type Ad5. The primers that recognize wild Ad5 left-end sequence did not amplify any fragments from any of the four COX-2 CRAds. (B) Detection of the E1a sequence. All four COX-2 CRAds have the E1a sequence as they possess COX-2 promoter-driven E1 expression cassettes. (C) Detection of the COX-2 promoter sequence. All four CRAds contain the COX-2 promoter sequence to drive E1 expression. (D) Direction of the E1 Expression Cassette. A set of the primers recognizing the COX-2 promoter, placed in a left to right direction, amplified the sequence only in CRAdcox2F and RGDCRAdcox2F. (E) Fiber structure. The primers designed to distinguish the presence of an RGD motif in the HI-loop region amplified a 247 bp fragment from CRAdcox2F and CRAdcox2R, while they amplified a 274 bp fragment containing a 9 amino acid insertion from RGDCRAdcox2F and RGDCRAdcox2R. Lane 1: CRAdcox2F, lane 2: RGDCRAdcox2F, Lane 3: CRAdcox2R and lane 4: RGDCRAdcox2R.

Figure 3. COX-2 RNA profile of the pancreatic cancer cells.

The RNA of the cell lines used in these experiments was analyzed by reverse transcription followed by polymerase chain reaction. (Upper panel) The signal for cyclooxygenase-2 RNA was detected at the position

of 723 bp with COX-2 sense (5' ggtctggtgcctggtctgatgatg 3') and COX-2 antisense (5' gtcctttcaaggagaatggtgc 3') primers. (Lower panel) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was detected with GAPDH sense (5' caactacatggtttacatgttccaa 3') and GAPDH antisense (5' gccagtggactccacgacgt 3') primers. Lane 1: BT474, lane 2: MKN45, lane 3: Hs766T, lane 4: Panc-1, lane 5: Capan-2, lane 6: MiaPaca-2, and lane 7: H_2O

Figure 4. Infectivity enhancement by RGD modification in pancreatic cancer cells.

The infectivity enhancement was analyzed using CMV promoter driven luciferase expression vectors with and without RGD modification. Two days after infection at 50 and 500 vp/cell, the cells were analyzed for luciferase expression. The result is shown as a percentage infectivity of that of Ad5Luc1 used as the control. In all pancreatic cancer cell lines tested, RGD modified vectors indicated a 2.2-13.6 fold increase in transduction. This was seen consistently regardless of the moi used for the experiment.

Figure 5. *In vitro* analysis of cytocidal effect.

The COX-2 CRAds were analyzed for their cytocidal effect. The cells were infected at 0.001 virus particle/cell and cultivated for 10 days. Cytotoxicity was assessed by crystal violet staining. The virus used in each well is indicated in left lower panel. E1 deleted vectors (AdCMVLuc and RGDCMVLuc: non-replicative) did not kill any cells. The wild type viruses (Ad5 and Ad5wtRGD) killed all cells. Both COX-2 CRAds killed COX-2 positive control cells (A549), while neither of them killed COX-2 negative control cells (BT474). In pancreatic cancer cells, the CRAds with the left to right expression cassette (CRAdcox2F and RGDCRAdcox2F) showed cytocidal effect in 4 out of 4 cells, while those with the cassette in the opposite orientation (CRAdcox2R and RGDCRAdcox2R) killed only 2 out of 4 cells. In the context of infectivity enhancement, RGD CRAds showed a slightly better cytocidal effect that their non-RGD counterparts.

Figure 6. Analysis of the replication control.

To analyze which step of replication is controlled by these CRAds, the viral DNA at different replication stages was analyzed by quantitive PCR. The total viral DNA in the cell represents the total of un-encapsidated and encapsidated viral DNA in the cells. While the total and encapsidated viral DNA in COX-2 negative BT474 cells was lower than that present in COX-2 positive A549 cells, encapsidated viral DNA in the supernatant in BT474 cells was remarkably lower than that in A549 cells. This data suggested that the release of the virus is one of the control points of the COX-2 CRAds.

Figure 7. *In vivo* antitumor effects in a xenograft model

The *in vivo* antitumor effect of the COX-2 CRAds was analyzed in a Hs766T human pancreatic cancer cell subcutaneous xenograft model. When the tumor reached the size of 6-8 mm in maximal diameter, 10^8 vp of the COX-2 CRAds or control vectors was injected into the tumor. The COX-2 CRAds with unmodified fiber (CRAdcox2F and CRAdcox2R) demonstrated some therapeutic effect, but it was not statistically significant from the control ($*P>0.05$, Mann-Whitney test). The RGD-modified COX-2 CRAds (RGD CRAdcox2F and RGD CRAdcox2R) showed a stronger antitumor effect which was statistically significant compared to the untreated group and the group that received non-replicative control vectors ($*P<0.01$, Mann-Whitney test).

Figure 8. Tumors treated with the COX-2 CRAds.

Two weeks after CRAd administration, tumor regression was observed in mice treated with CRAds. (A) A tumor on day 25 after intratumoral administration of RGD CRAdcox2F (B) A tumor treated with a non-replicative control vector on the same day as (A).

Figure 9. Microscopic analysis of major organs after systemic CRAd administration

Major organs after systemic adminiatration of CRAds were analyzed histopathologically. Three days after injection of CRAds (10^8 vp) (CRAdcox2F, CRAdcox2R, RGDCRAdcox2F, or RGDCRAdcox2R) and control vectors (AdCMVLuc, wild type Ad5, RGDAdCMVLuc, and Ad5wtRGD), the major organs were analyzed

pathologically. Although a slight increase in inflammatory cells was noted in the parenchyma of the liver, in the groups which received CRAdcox2F, RGDCRAdcox2F, wild type or Ad5wtRGD, this was far below the level of significant hepatotoxicity. Other than this feature, the histological findings were identical among all groups examined. Importantly, there were no remarkable findings of toxicity in any of the major organs. The findings with RGDCRAdcox2F, which indicated most the promising result in the therapeutic experiments is shown in Figure 9 (a: liver, b: lung, c: spleen and d: kidney).

Figure 10. E1 RNA level in the liver after systemic CRAd administration.

Five days after systemic CRAd administration of 10^8 vp, RNA was isolated from the liver and analyzed by quantitive RT-PCR for the E1a gene. The result is indicated as E1a RNA copy number per 1 μg total RNA. The E1a RNA level in COX-2 CRAds was minimal in comparison with that of the wild type Ad5 and was close to the background level.

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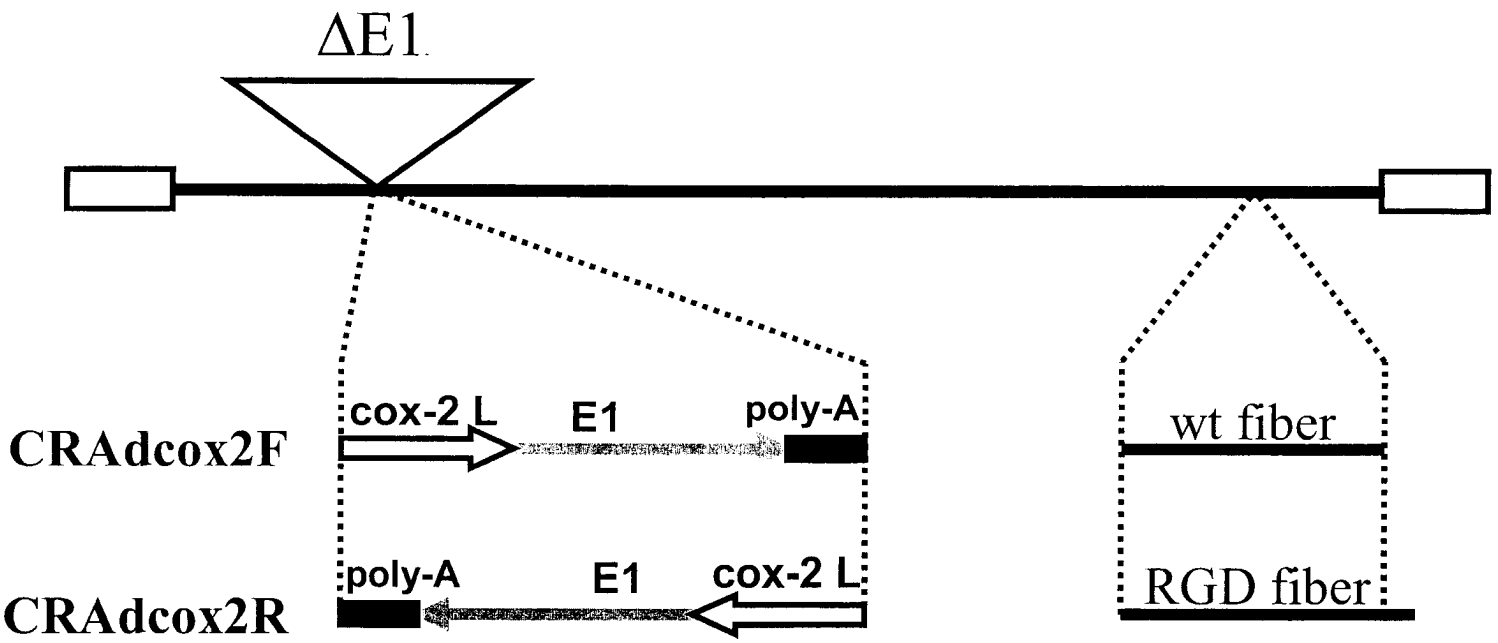
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poly-A
E1
cox-2 L
wt fiber
RGD fiber

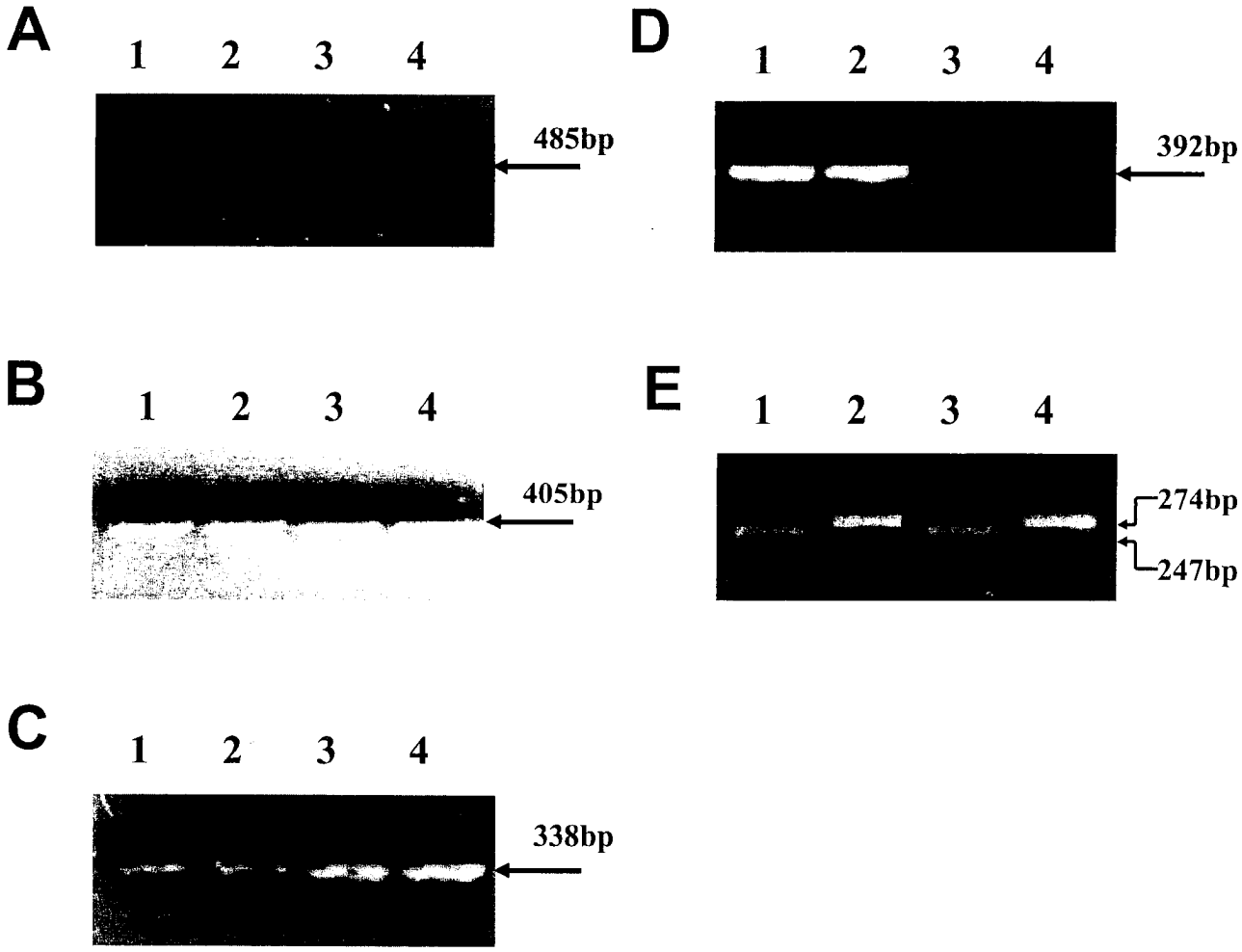
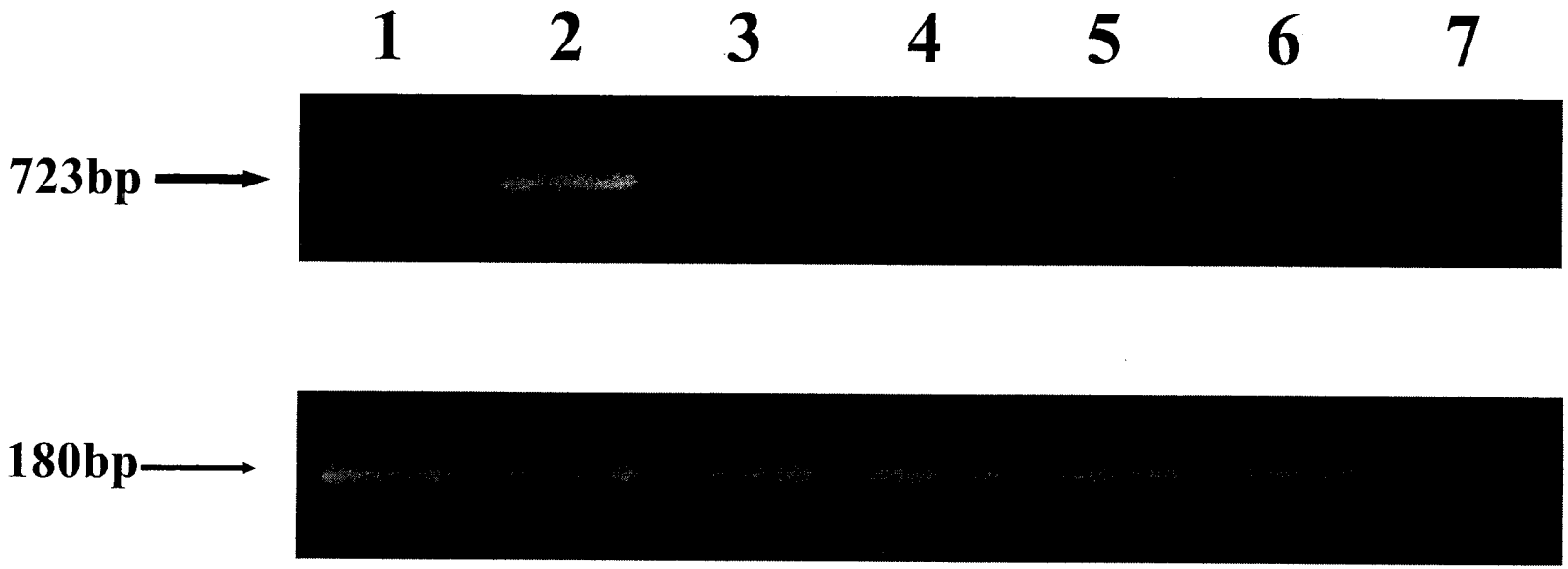
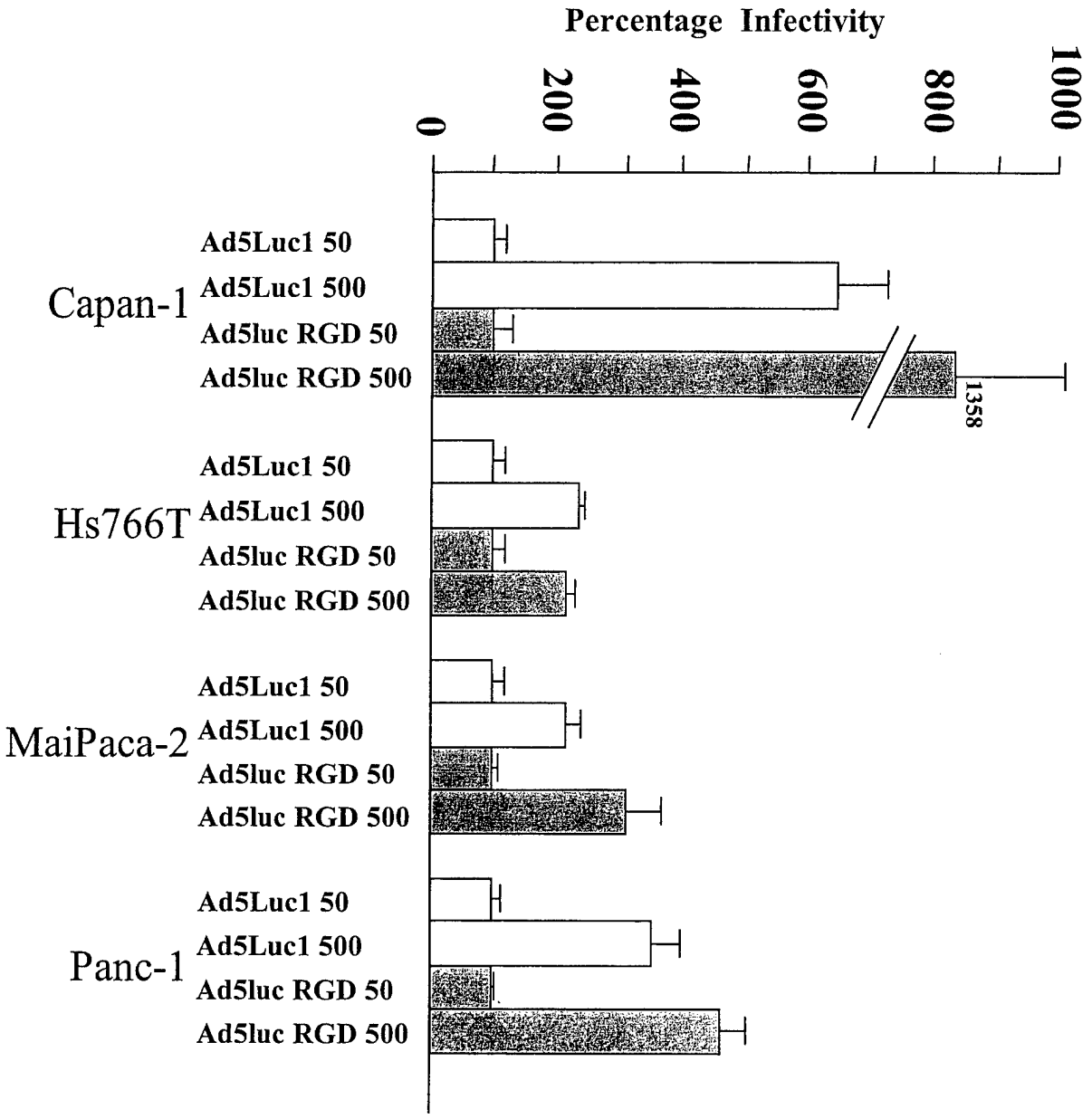

A
1 2 3 4
485bp
D
1 2 3 4
392bp
B
1 2 3 4
405bp
E
1 2 3 4
274bp
247bp
C
1 2 3 4
338bp

Figure 3




Percentage Infectivity
0
200
400
600
800
1000
Capan-1
Ad5Luc1 50
Ad5Luc1 500
Ad5luc RGD 50
Ad5luc RGD 500
1358
Hs766T
Ad5Luc1 50
Ad5Luc1 500
Ad5luc RGD 50
Ad5luc RGD 500
MaiPaca-2
Ad5Luc1 50
Ad5Luc1 500
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Ad5luc RGD 500
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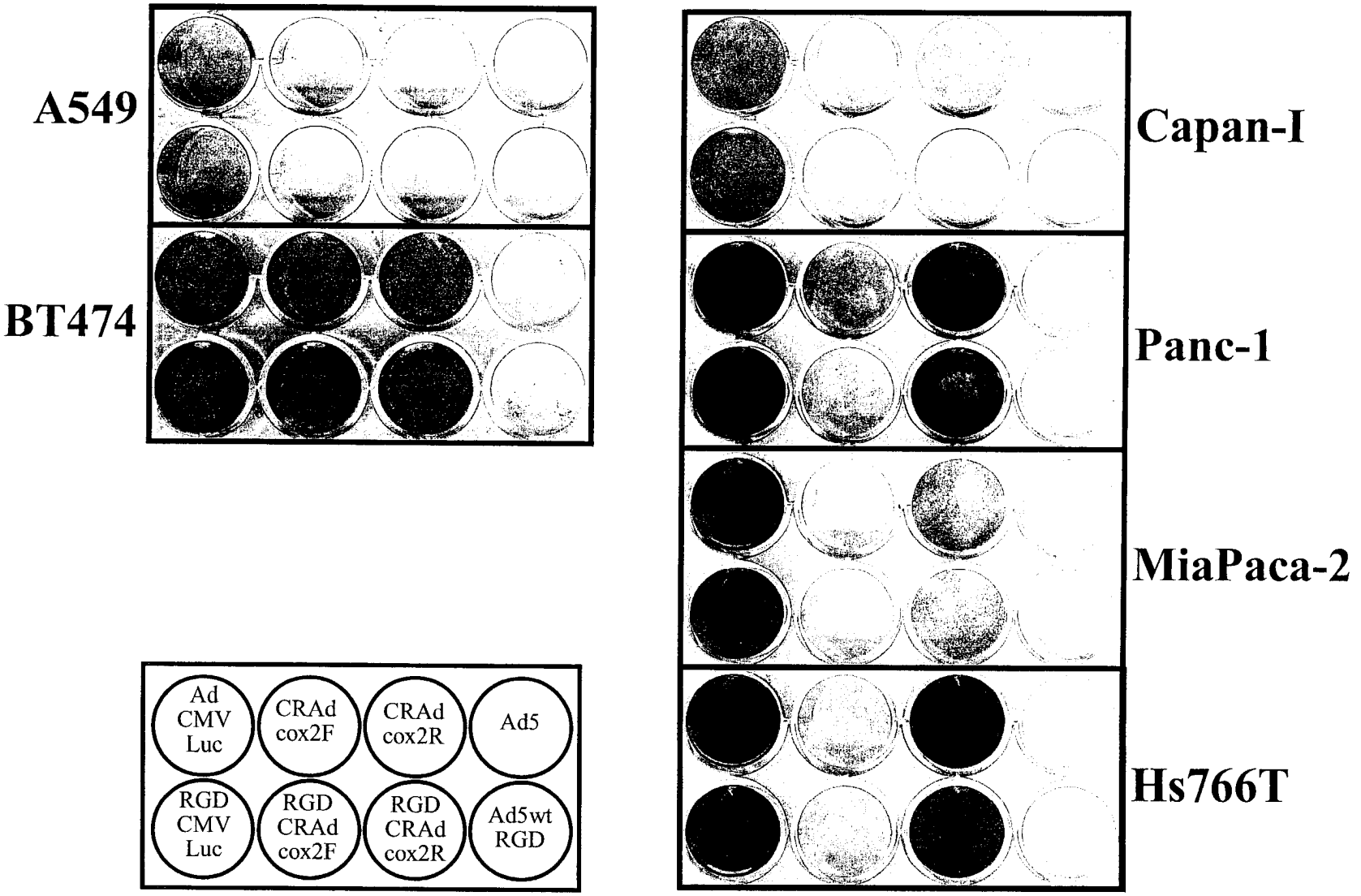
Ad5luc RGD 50
Ad5luc RGD 500


A549
BT474
Capan-I
Panc-1
MiaPaca-2
Hs766T
Ad CMV Luc
CRAd cox2F
CRAd cox2R
Ad5
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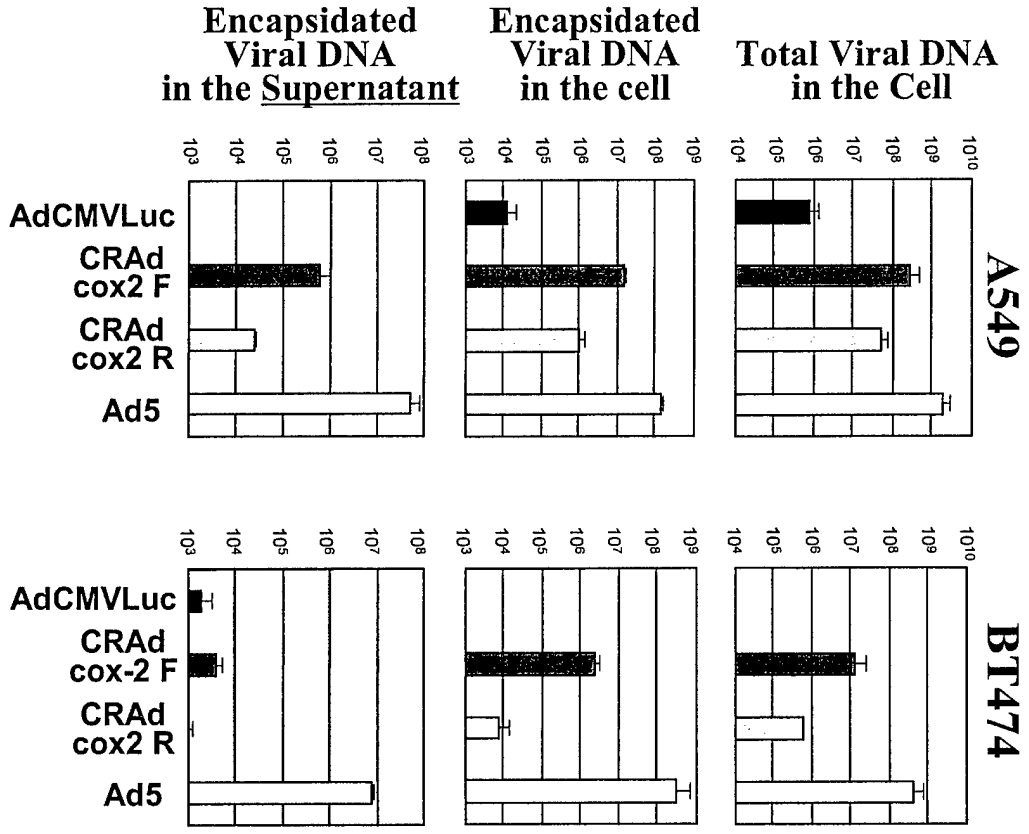
RGD CRAd cox2F
RGD CRAd cox2R
Ad5wt RGD


Encapsidated Viral DNA in the Supernatant
Encapsidated Viral DNA in the cell
Total Viral DNA in the Cell
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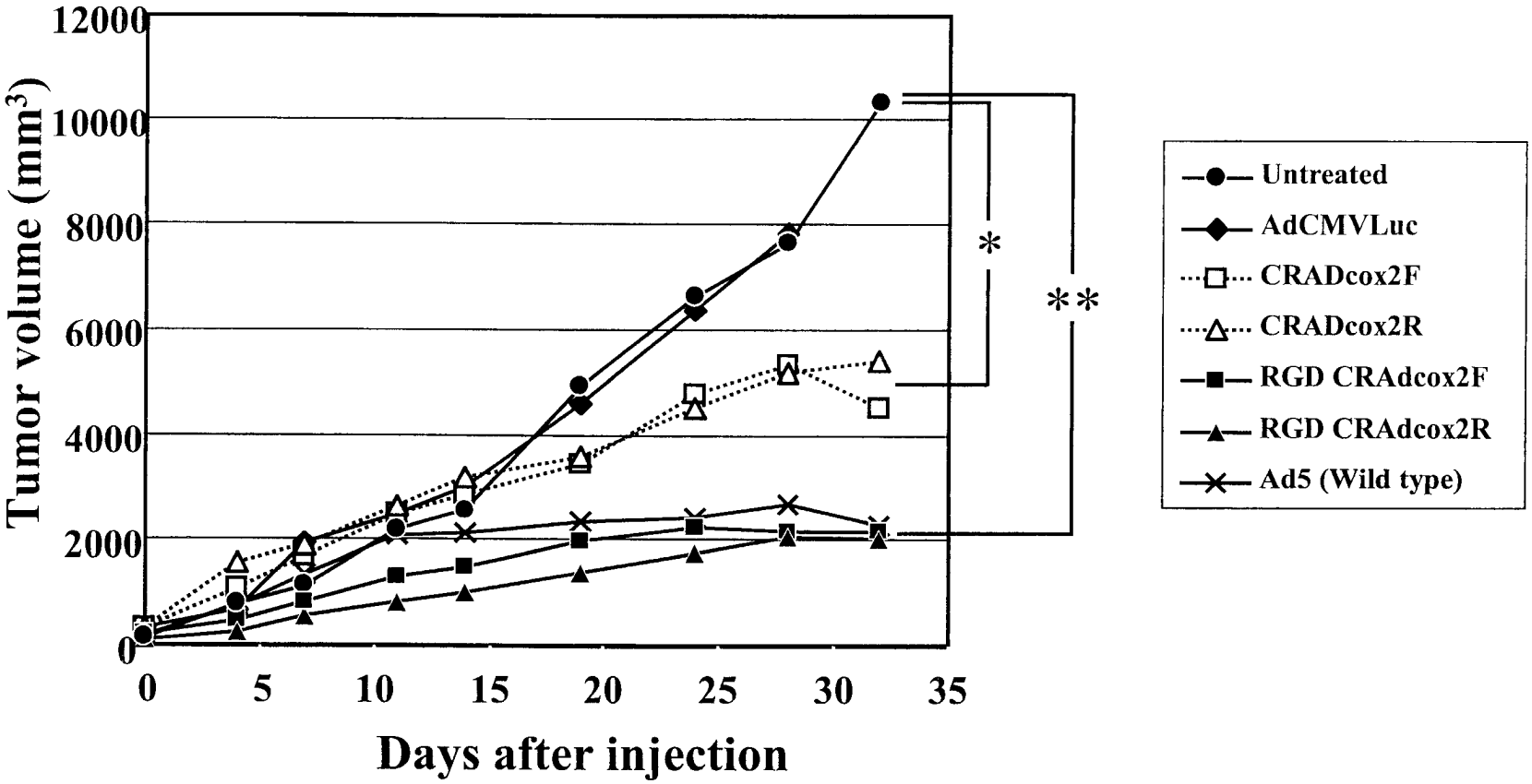
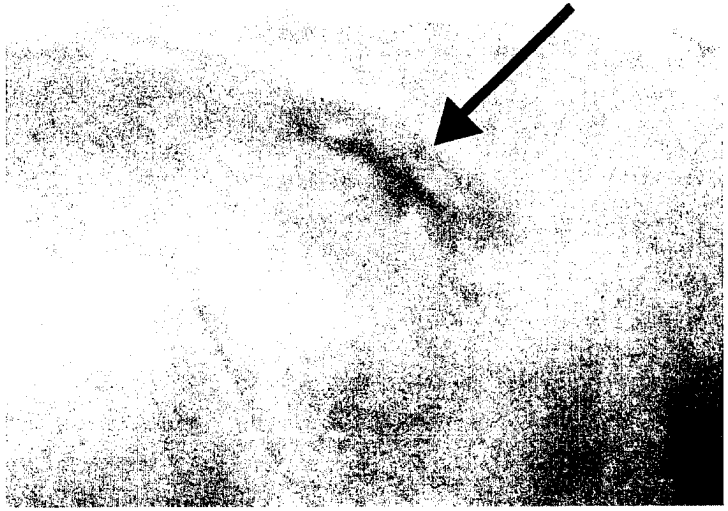
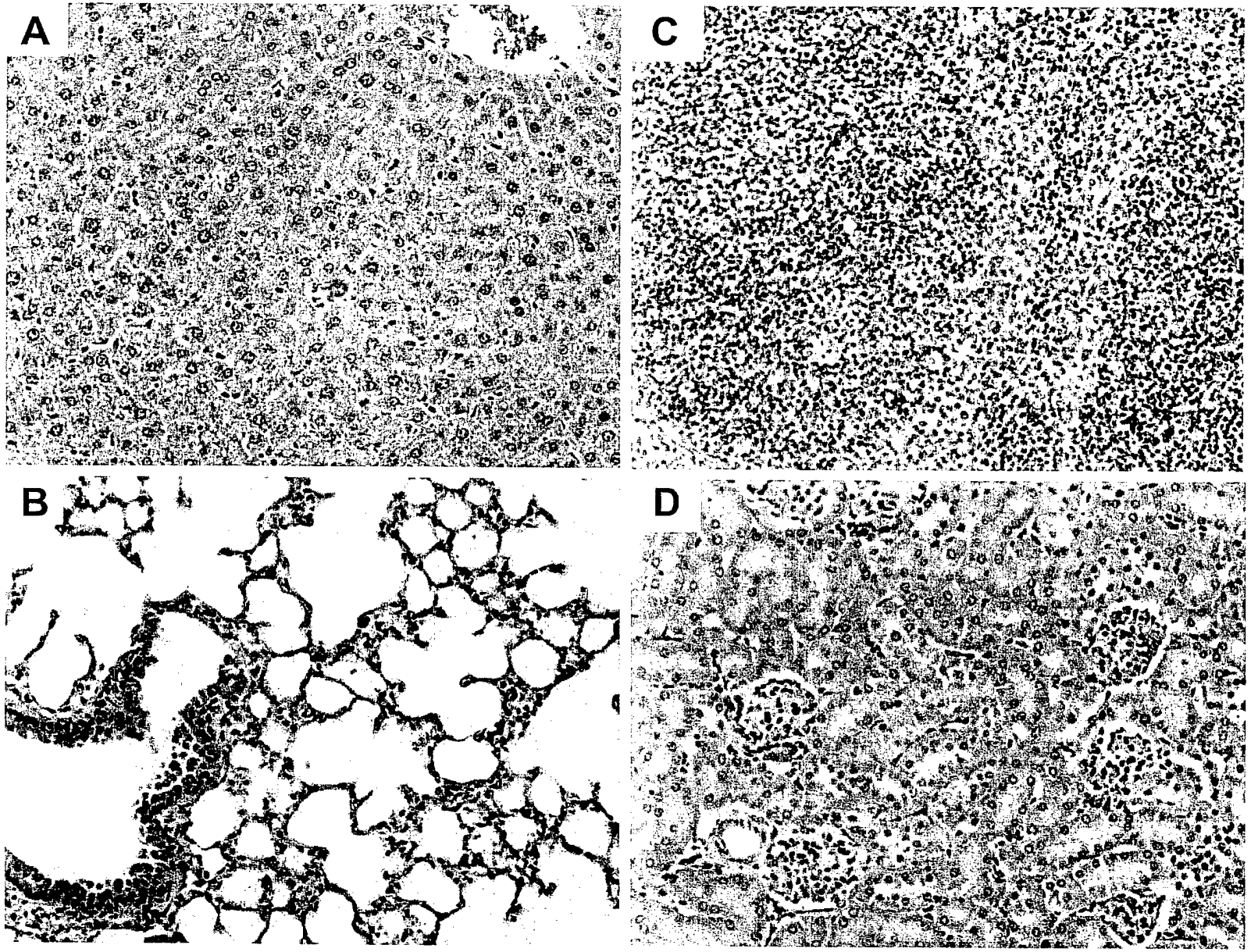
CRAd cox2 F
CRAd cox2 R
Ad5
A549
AdCMVLuc
CRAd cox-2 F
CRAd cox2 R
Ad5
BT474

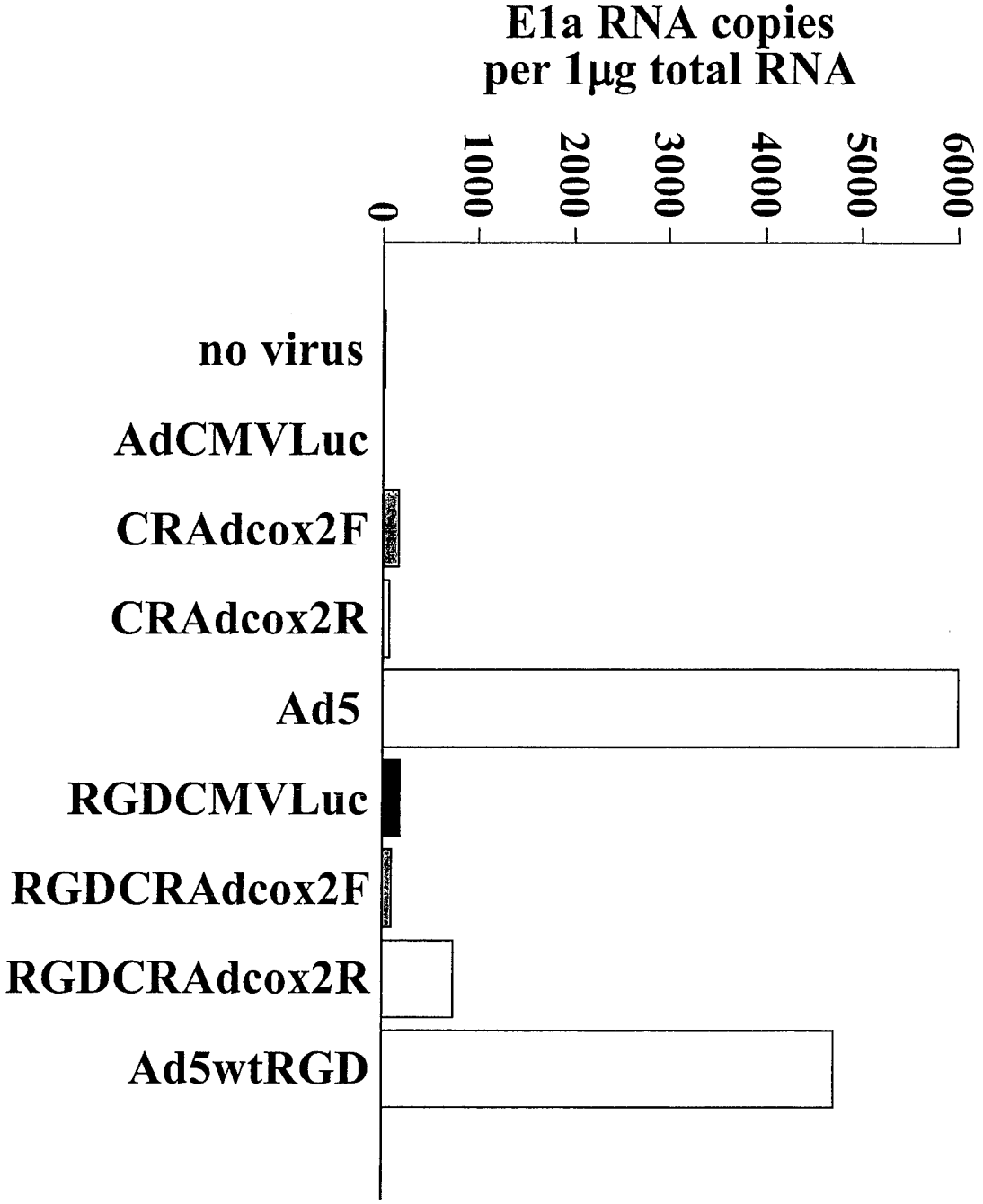

12000
10000
8000
6000
4000
2000
0
Tumor volume (mm3)
0
5
10
15
20
25
30
35
Days after injection
*
**
Untreated
AdCMVLuc
CRADcox2F
CRADcox2R
RGD CRAdcox2F
RGD CRAdcox2R
Ad5 (Wild type)

A



B


A
C
B
D


E1a RNA copies
per 1µg total RNA
0
1000
2000
3000
4000
5000
6000
no virus
AdCMVLuc
CRAdcox2F
CRAdcox2R
Ad5
RGDCMVLuc
RGDCRAdcox2F
RGDCRAdcox2R
Ad5wtRGD

APPENDIX G

VEGF Promoter-based Conditionally Replicative Adenoviruses for Pan-Carcinoma Application

Koichi Takayama, [1,$] Paul N. Reynolds, [1,#] Yasuo Adachi, [1] Lioudmila Kaliberova, [1] Victor Krasnykh, [1,2] Yoichi Nakanishi, [$] and David T. Curiel, [1,2,*]

Division of Human Gene Therapy [1], Departments of Medicine, Pathology and Surgery, and the Gene Therapy Center [2],
The University of Alabama at Birmingham, Birmingham, Alabama 35294-2172

Chest Clinic, Royal Adelaide Hospital [#], 275 North Terrace, Adelaide South Australia 5000, Australia.

Reseach Instituite for Diseases of the Chest[$], Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Fukuoka 812-8582, Japan.

Running title: VEGF promoter-based replicative adenovirus

Key words: VEGF, replicative adenovirus, angiogenesis, chimeric vector

*Corresponding author. Mailing address: Division of Human Gene Therapy
901 South 19th Street, BMR2-508, 2172
University of Alabama at Birmingham, Birmingham, AL 35294-2172.
Phone: (205) 934-8627.
Fax: (205) 975-7476. E-mail: david.curiel@ccc.uab.edu

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[2] To whom requests for reprints should be addressed, at Division of Human Gene Therapy, Department of Medicine, Surgery and Pathology, University of Alabama at Birmingham, 901 South 19th Street, BMR2-508, 2172, Birmingham, AL 35294-2172. Phone: (205) 934-8627; Fax: (205) 975-2961; E-mail: david.curiel@ccc.uab.edu

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; bFGF, basic fibroblast growth factor; IL-8, interleukin-8; PD-ECGF, platelet-derived endothelial cell growth factor; VEGF, vascular endothelial growth factor; RCV, replicative competent virus; CAR, coxsackie and adenovirus receptor; Ad5, serotype 5 adenovirus; Ad3, serotype 3 adenovirus; Ad5/3, Ad5 containing a chimeric fiber protein possessing the Ad3 knob; CRAd, conditionally replicative adenovirus; E1, early region 1; E4, early region 4; ITR, inverted terminal repeat; RT-PCR, reverse transcription-PCR; CMV, cytomegalovirus; CsCl, cesium chloride; VP, viral particle; pfu, plaque forming unit.

ABSTRACT

Treatment of advanced lung cancer is one of the major challenges in current medicine because of the high morbidity and mortality associated with this disease. In this regard, advanced stage lung cancer is refractory to conventional therapies and has an extremely poor prognosis. On this basis, it is clear that new therapeutic approaches are needed. Lung tumor formation depends on angiogenesis in which VEGF produced by cancer cells plays a pivotal role. Therapeutic interventions based upon neutralizing VEGF with soluble VEGF receptor can suppress tumor growth, however, the anti-cancer effect with this therapy may be attenuated after the intratumoral vascular network is completed. In this study, we exploited the expression of VEGF of tumors for therapeutic advantage using a conditionally replication-competent adenovirus (CRAd) in which the expression of the adenoviral E1 gene is controlled by the human VEGF promoter. This virus achieved high levels of viral replication in lung cancer cells and induced a substantial anti-tumor effect *in vitro* and *in vivo*. Further enhancement of the anti-cancer cell killing effect was achieved with tropism modification of the virus via serotype chimerism of the adenoviral fiber knob. These infectivity-enhanced VEGF promoter-based CRAds also showed a significant cell killing effect for various types of cancer types other than lung cancer. In this regard, a dysregulated VEGF axis is characteristic of many carcinomas. On this basis, this current CRAd agent may be useful as a "pan-carcinoma" therapeutic agent.

INTRODUCTION

Lung cancer is one of the most common malignant tumors in the world and is the leading cause of cancer deaths in many countries (1). Surgical resection remains the standard of care for patients with early-stage disease. Unfortunately, over 70% of all patients with non-small cell lung cancer (NSCLC) have inoperable disease at the time of diagnosis, and over 50% of patients who undergo resection eventually will experience local or distant failure after radical therapy. Despite the use of optimal chemotherapy and radiotherapy regimens, the outcome for patients with advanced disease is extremely poor, and little progress has occurred in the last decade (2). These considerations suggest a need for developing more effective treatments.

Anti-angiogenesis approaches represent a promising alternative with potentially broad relevance as several studies have shown that angiogenesis is one of the key control factors in the growth, progression, and metastasis of solid tumors (3, 4). Among the many known angiogenic factors, such as bFGF, angiogenin, IL-8, PD-ECGF and vascular endothelial growth factor (VEGF), VEGF is now believed to play a pivotal role in tumor-associated angiogenesis in lung cancer, as well as other solid tumors. In this regard, several studies have reported that resected lung cancer tissue expresses VEGF mRNA and protein abundantly, based on immunohistochemical and RT-PCR analysis (5-9). Moreover, the elevated expression levels of VEGF mRNA and protein can be correlated with poor prognosis in lung cancer patients (5-7), providing a definitive link between lung cancer pathobiology and dysregulated expression of VEGF. We have also reported the negative correlation between VEGF expression and dendritic cell infiltration in the tumor, suggesting an immunosuppressive function of VEGF which may also serve to

promote tumor growth (10). These clinical findings are consistent with the concept that VEGF represents a key target molecule for novel lung cancer treatments. Exploiting this axis, tumor suppression has been achieved in animal model studies by inhibiting VEGF signaling via neutralizing antibody to VEGF (11-15), antisense oligonucleotide to VEGF (16, 17), truncated FLK-1 as a dominant negative VEGF receptor (18), soluble VEGF receptor protein (19) and VEGF-R tyrosine kinase inhibitor (20). We have also reported that soluble VEGF receptor gene transfer is able to neutralize the angiogenic effect of VEGF and suppress tumorigenesis *in vivo* (21-23). Of note, the anti-tumor effect of anti-VEGF treatment is attenuated in well-established tumors that have already developed advanced blood vessel networks. Nevertheless, the fact that established tumors still express higher levels of VEGF than normal tissues suggests that approaches to exploit this differential for therapeutic advantage are relevant.

An emerging strategy for cancer therapy is the use of conditionally replicative adenoviruses (CRAds) that are designed to exploit key differences between tumor cells and normal cells to allow tumor-selective viral replication and oncolysis. Two basic strategies have been employed to generate CRAds (24-26). A type I approach, such as Ad-dl1520 (ONYX-015) or AdΔ24, involves directly mutating Ad genes, such as E1, to take advantage of the disordered cell cycle regulation in tumor cells with functionally deficient p53 or RB signaling, respectively (27, 28). The type II approach involves replacement of wild-type Ad promoters with tumor-specific promoters to drive the expression of genes essential for Ad replication. The promoters of alpha-fetoprotein (29), prostate specific antigen (30), osteocalcin (31) and MUC1 (32) have previously been employed to generate of this type CRAds for the treatment of hepatocellular carcinoma,

prostate cancer, and breast cancer, respectively. We also reported that midkine promoter and tyrosinase promoter-based CRAds are useful for the treatment of pediatric solid tumors and melanoma, respectively (33, 34). Of note, the CRAd agents ONYX-015 and CV706 (PSA promoter–based) have been shown to be safe in human clinical trials (35-37).

Based on these findings, we utilized the VEGF promoter, which is activated in advanced lung cancer, to generate a type II CRAd to achieve selective replication and oncolysis in VEGF positive tumors. To test our hypothesis, we replaced the native E1A promoter with the human 2.6kb VEGF promoter and evaluated the oncolytic effect in various cell lines and *in vivo*. Furthermore, in view of the increasing evidence that levels of the coxsackie and adenoviral receptor (CAR) are low in many tumor types, we also generated a tropism-modified variant VEGF promoter CRAd containing the receptor binding fiber knob domain of Ad serotype 3 in place of the Ad5 knob. We show here that these infectivity-enhanced VEGF promoter CRAds have broad utility for achieving anti-tumor effects in the context of lung cancer and other tumor types. The broad prevalence of dysregulation of the VEGF axis in diverse carcinomas suggests that the described CRAd agent may have broad utilities as a "pan carcinoma" agent.

MATERIALS AND METHODS

Cell culture.

The NCI-H82, NCI-H460, NCI-H157, NCI-H322, NCI-H522, NCI-H1299, NCI-H358, NCI-N417, A427, A549, lung cancer cell lines, BEAS-2B, normal human bronchial epithelial cell line, Panc-I, pancreas cancer cell line, HEK293 adenoviral transformed human embryonic kidney cell line were obtained from ATCC (American Type Culture Collection, Manassas, VA). QG56 and QG90 were provided by National Kyushu Cancer Center, Fukuoka, Japan). Human ovarian adenocarcinoma cell line SKOV3.ip1 was obtained from Dr. Janet Price (M.D. Anderson Cancer Center, Houston, TX). The MeWo cell line was a gift of Dr. Ian R. Hart (St. Thomas Hospital, London, UK). Cells were cultured in the media recommended by each provider and incubated at 37°C and 5% CO_2.

Adenovirus vectors.

The recombinant adenoviral vectors that express firefly luciferase were constructed through homologous recombination in *Esherichia coli* using the AdEasy system (38). The 2.6kb human VEGF promoter region derived from pVEGF-kpnI (provided from Dr. Semenza at the Johns Hopkins University, Baltimore, MD) (39) was placed in front of the firefly luciferase gene in an Ad E1 shuttle vector, recombined with the E1- and E3-deleted adenoviral backbone vector pAdEasy 1, then transfected into 293 cells by standard techniques to form Ad5VEGFLuc. The luciferase gene and simian virus 40-polyadenylation signal were derived from pGL3 Basic (Promega, Madison, WI). As a control, a vector that containing the ubiquitously active cytomegalovirus (CMV) immediate early promoter (derived from plasmid pCEP4; Invitrogen, Carlsbad, CA)

instead of the VEGF promoter was also constructed and named Ad5CMVLuc. The replication competent adenovirus, Ad5VEGFE1 was also generated from the same E1- and E3-deleted adenoviral backbone vector. Briefly, the fragment corresponding 489 bp to 3533 bp from the left end of the type 5 adenoviral genome was amplified by PCR and inserted in the E1 deleted region of the backbone vector. This fragment contains the transcriptional start site of the E1A gene but not the native E1A promoter. The 2.6kb VEGF promoter region was placed upstream of this fragment. A control vector was also constructed in which the CMV promoter was placed in the same position upstream of E1A. The strategy for these three constructs is summarized in Fi.g1. Fiber modified CRAd, Ad5/3VEGFE1 was generated in similar manner as Ad5VEGFE1 but using Ad5/3E1- E3-deleted backbone vector derived from Ad5/3luc1 containing Ad3 knob in place of Ad5 wild-type knob gene as described previously (40). To compare the difference in infectivity between the Ad5 and Ad5/3 chimeric vectors on our target cells, an Ad vector (Ad5/3luc1) that contains a CMV driven luciferase gene in E1 was compared to AdCMVLuc. Wild type p53 protein expressing adenovirus, Ad5p53 which contains CMV driven p53 cDNA was provided from Dr. Ueno (University of Occupational and Environmental Health, Kitakyusyu, Japan) (41)

The viruses were propagated in the adenovirus packaging cell line, 293HEK, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline with 10% glycerol. The viral particle (VP) concentration was determined spectrophotometrically, using a conversion factor of $1.1x10^{12}$ viral particles per absorbance unit at 260 nm (42), and standard plaque assays on 293 cells were performed to determine infectious particles (43).

Analysis of VEGF RNA expression.

The VEGF RNA status of cell lines was analyzed by reverse transcription and polymerase chain reaction (RT-PCR) as described previously (5). Total cellular RNA was extracted from $1x10^7$ cells using the RNeasy kit (Qiagen, Valencia, CA) and analyzed for VEGF and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) RNA with the GeneAmp RNA PCR core kit (Applied Biosystems) as described by manufacturer. Briefly, 500 ng of total RNA was reverse-transcribed with the random hexamer and murine leukemia virus reverse transcriptase (50°C 30min) and amplified by PCR with 50 nM of primer pairs described below using a cycling program (initial step of 95°C for 15min, 27 cycles of 95°C for 1 min and 60°C for 1 min and 72°C for 1 min, final step of 72°C for 10 min). The primers used for the analyses were as follows; VEGF sense (5'GAAGTGGTGAAGTTCATGGATGTC3'), VEGF antisense (5'CGATCGTTCTGTATCAGTCTTTCC3'), GAPDH sense (5'CCTTCATTGACCTCAACTA 3'), GAPDH antisense (5'GGAAGGCCATGCCAGTGAGC3').

Measurement of VEGF protein in Culture Media.

The VEGF protein expression was evaluated as described previously (22). Briefly, $1x10^5$ cancer cells were cultured for 24 h in the serum free media, and then the medium was collected. After centrifugation, the supernatant was stored at -80 °C until the assay. The VEGF protein in the culture medium was determined using an ELISA kit (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Each of the values given here is the mean of triplicate determination with respect to standardized cell numbers, $1x10^5$ cells.

***In vitro* analysis of VEGF promoter activation.**

The activity of the VEGF promoter in an adenovirus context was analyzed by infection of cells with luciferase expression vectors as reported previously (33). Briefly, cells were plated in 12-well plates in triplicate at the density of $1x10^5$ cells/well. The next day, the cells were infected with Ad5VEGFLuc or Ad5CMVLuc at the MOI of 10 pfu/cell in DMEM with 2% FCS for 3 h and then maintained in complete medium. The infected cells were harvested and treated with 100μl of lysis buffer (Promega, cat #E153A) after 2 days culture. A luciferase assay (Luciferase Assay System; Promega) and a FB12 luminometer (Zyluc corporation) were used for the evaluation of luciferase activities of Ad-infected cells. Luciferase activities were normalized by the protein concentration in cell lysate (Bio-Rad DC Protein Assay kit).

***In vivo* analysis of VEGF promoter activation.**

For determination of luciferase gene expression in mouse organs, nude mice (Charles Rivers) received $1x10^9$ pfu of Ad5CMVluc or Ad5VEGFLuc by tail vein injection as reported previously (33). Two days later, mice were sacrificed, and livers, kidneys, lungs, spleens were resected to measure the luciferase gene expression. The resected organs were placed in the polypropylene tubes, and immediately frozen in ethanol/dry ice. Frozen tissues ground to a fine powder was lysed using a tissue lysis buffer (Promega), and then luciferase activity was determined using a luciferase assay kit (Promega). The luciferase activity was normalized by protein concentration in the tissue lysate.

Analysis of viral genome amplification.

Viral DNA amplification was assessed as reported previously (33). Cells were plated in a 12-well culture plate in triplicate at the density of $1x10^5$ cells/well. After overnight culture, cells were infected with replication-competent Ads (Ad5VEGFE1, Ad5CMVE1 or Ad5wt) or non-replicative Ad (Ad5CMVLuc) at the MOI of 10 for 3 h and then cultured for 24 h. The harvest of infected cells was followed by viral DNA isolation using Blood DNA kit (Qiagen, Valencia, CA). Viral DNA was eluted with 100μl of elution buffer [10mM TrisCl (pH 8.5)]. Eluted samples (1μl) were analyzed by real-time PCR analysis to evaluate Adenoviral E4 copy number using a LightCycler (Roche). Oligonucleotides corresponding to the sense strand of Ad E4 region (5'-TGACACGCATACTCGGAGCTA-3': 34885-34905 nt), the antisense strand of E4 region (5'-TTTGAGCAGCACCTTGCATT-3': 34977-34958 nt), and a probe (5'-CGCCGCCCATGCAACAAGCTT-3': 34930-34951 nt) were synthesized, used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20s), annealing (55°C, 20s), and extension (72°C, 30s). Adenovirus backbone vector pTG3602 (Chartier; Transgene, Strasbourg, France) was available for making a standard curve for Ad E4 DNA copy number. E4 copy numbers were normalized by the β-actin DNA copy number.

***In vitro* cytotoxicity assay.**

For determination of virus-mediated cytotoxicity, $5x10^3$ cells were plated in 96-well plates in triplicate. After overnight culture, cells were infected with each Ads at various MOI for 3 h. The infection medium was then replaced with RPMI1640 containing 10%FCS. Viable cells using MTS assay (CellTiter 96 Aqueous Non-

Radioactive Cell Proliferation Assay; Promega) were evaluated every 3 days. The MTS color development was quantified as optical density at 490 nm by an EL 800 Universal Microplate Reader (Biotec Instruments Inc.). To visualize the cytotoxic effect, crystal violet staining was also performed. $2x10^5$ cells were plated in 12-well plates and infected with each Ad at various MOI for 3 h. The infection medium was replaced with growth medium the next day. When cell lysis was observed, cells were fixed and stained with 1% crystal violet in 70% ethanol for 45 min, followed by washing with tap water to remove excess color. The plates were dried, and images were captured with a Kodak DC260 digital camera (Eastman Kodak, Rochester, NY). All experiments were performed in duplicate wells.

***In vivo* studies - tumor formation in nude mice.**

All animals were used in accord with protocols approved by the animal care committees of Kyushu University. The experiment was carried out under both the Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese government. Tumor suppressive effect *in vivo* was analyzed as described previously (22). Briefly, H157 cells ($5x10^6$) were injected s.c. into the dorsal skin of nude mice, and tumor growth was monitored for 25 days. Tumor volume was calculated according to the formula $a^2 \times b$, where a and b are the smallest and largest diameters, respectively as described previously. When tumor formation was seen 10 days after inoculation, 1×10^8 pfu of each virus was injected into the tumor directly. Student's t test was used to compare tumor volumes, with $p<0.05$ being considered significant.

RESULTS

VEGF mRNA and protein expression in various cell lines.

We wished to develop a strategy for the therapy of NSCLC based on the use of a CRAd in which the VEGF promoter controls the expression of E1(Fig.1). We first investigated a panel of twelve non-small cell lung cancer cell lines, one bronchial epithelial cell line (BEAS-2B) as a normal cell control, one ovarian cancer cell line (SKOV3.ipl), one gastric cancer cell line (MKN28), and a pancreatic cancer cell line (Panc-I) for VEGF mRNA expression using a RT-PCR method, as reported previously (5). In this regard, there are four structural variants of VEGF (*VEGF*121, *VEGF*165, *VEGF*189, and *VEGF*206) resulting from alternative mRNA splicing in the regions encoding the cytoplasmic domains. Fig.2A shows amplification of a 408bp fragment (representing *VEGF*121 cDNA) and a 541bp fragment (representing *VEGF*165 cDNA) in all cell lines tested. The intensity of each band (*VEGF*121 and *VEGF*165) was similar in all cancer cells tested. The PCR bands corresponding to *VEGF*189 (615bp) and *VEGF*206 (666bp) were minimal or not detected, indicating *VEGF*121 and *VEGF*165 were the dominant isoforms in these cell lines. These results are consistent with those of previous similar studies of primary lung cancer tissues (5). Of the cells tested, H157, A427, N417, H358 and SKOV3.ipl showed relatively high expression of VEGF mRNA, while the contral normal cell line BEAS-2B showed a less intense band than the cancer cell lines, although the band corresponding to VEGF121 was detected at very low levels. We also investigated the correlation between mRNA expression and protein expression for VEGF. As shown in Fig.2B, the VEGF protein expression levels also varied between cell lines. H157 secreted the highest amount of VEGF protein into the culture media, and

the concentration was over 100 times higher than that of BEAS-2B. Comparison between Fig.2A and 2B revealed that the VEGF mRNA expression level positively correlated with VEGF protein expression level. These results thus suggested the VEGF promoter activity can be predicated from the VEGF protein concentration of tumor cellular substrates.

Transgene expression by VEGF promoter in the Ad context *in vitro*.

Candidate tumor-specific promoters may lose their specificity when placed in the context of the Ad genome. Thus, for our next analysis we assessed the VEGF promoter activity in an Ad vector (Ad5VEGFluc) containing the luciferase gene as a reporter. This was endeavored in several cell lines which represented the range of VEGF levels we detected in Fig 2. In all of the cells lines tested, luciferase expression was achieved using the positive control Ad5CMVLuc, which contains the luciferase gene driven by the non-selective viral CMV promoter. These results demonstrate that the A247 and H157 cells were most susceptible to Ad5 infection, exhibiting luciferase levels over 100 times higher than these of H460, as shown in upper panel of Fig3, consistent with the findings of our previous report (41). To standardize the differentiative susceptibility to Ad5 infection between cell lines, VEGF promoter activity is thus shown as the percentage of luciferase activity of Ad5VEGFLuc relative to Ad5CMVLuc. As shown in the lower panel in Fig.3, H157 cells showed the strongest VEGF promoter activity which was 28% of CMV promoter activity. In contrast, BEAS-2B cells, which presented the lowest VEGF promoter activity, was less than 0.1% of CMV. This low transgene expression seen with the VEGF promoter in the adenoviral context with BEAS-2B was consistent with the other recent reports. Other cell lines demonstrated various VEGF promoter activities

which correlated with the mRNA expression level for each cell lines tested (Fig.2A). Based on these data, we concluded that the VEGF promoter was able to induce transgene expression in VEGF producing cells and, importantly, that the promoter retained its specificity when configured in the Ad genome context.

Transgene expression by VEGF promoter in the Ad context *in vivo*.

A key limitation of adenovirus-mediated cancer gene therapy is the potential for toxicity to non-target organs. Because Ad exhibits a marked tropism for the liver, we were especially interested to determine whether the VEGF promoter would have low activity in the liver *in vivo*. Such a "liver off" phenotype would be critical to avoid any toxic effects of VEGF promoter CRAd therapy. In this regard, normal liver has been reported to exhibit minimal VEGF expression. On this basis, Ad5VEGFLuc or Ad5CMVLuc (as a positive control) were injected i.v. via the tail vein into mice and the level of transgene expression at day 2 was determined (Fig.4.) In this assay, transgene expression in the liver induced by the VEGF promoter was a mean 270-fold less than that seen with the CMV promoter. These results thus confirm the key property of VEGF promoter fidelity *in vivo* in the context of the Ad vector used. Further, the "liver off" phenotype of the VEGF promoter feasibilizes the potential use of a VEGF promoter CRAd is a systemic delivery context.

VEGF promoter driven CRAd shows replication specificity.

To exploit the cell specificity of the VEGF promoter in a CRAd context, we then constructed a recombinant Ad (Ad5VEGFE1) in which the native E1promoter was

replaced with the 2.6kb human VEGF promoter. The genomic structures of replication-competent Ads used in this study are depicted in Fig.1. In addition to using the VEGF promoter to regulate E1 expression, we employed an Ad in which E1 expression is controlled by the non-selective viral CMV (Ad5CMVE1) promoter as control (33). These viruses are deleted in the E3 region required to accommodate the large VEGF promoter and the E1A gene region. The deleted E1A promoter region, containing the native E1A TATA box, was replaced with either the VEGF promoter or CMV enhancer/promoter, to produce the viruses Ad5VEGFE1 or Ad5CMVE1, respectively.

To determine the specificity of replication of the AdVEGFE1, we infected the high VEGF expressing cell line (H157) and low expressing cell line (BEAS-2B), and then employed quantitative real-time PCR to determine the level of amplification of viral DNA. The non-replicative Ad5CMVLuc and wild-type Ad5 virus (Ad5wt) were used as negative and positive controls, respectively. Since all viruses tested contained the Ad E4 region, viral DNA was quantified by E4 copy number via real-time PCR. As shown in the upper panel of Fig.5, the Ad5VEGFE1 viral genome replicated in the high VEGF producing cancer cells H157 to a similar extent as did the Ad5CMVE1 genome. The non-replicative Ad5CMVLuc showed a background level of E4 signal, indicating no replication in this cell line. Importantly the replicative capacity of Ad5VEGFE1 decreased in the low VEGF expressing BEAS-2B cells, with values 3-logs lower than for Ad5CMVE1 (lower panel in Fig.5). These results indicate that the VEGF promoter retains fidelity in the replication-competent adenoviral context and mediates tumor-specific adenoviral replication.

Specific cell killing efficacy of VEGF promoter driven CRAd.

We next investigated the ability of Ad5VEGFE1 to achieve cell killing in the VEGF-positive cell lines using a MTS assay. The viability of the high VEGF expressing H157 cells and the low VEGF expressing BEAS-2B cells was quantified every three days after virus infection as shown in Fig.6A. For the H157 cells, Ad5VEGFE1 showed as strong cytotoxic effect as did the Ad5CMVE1 positive control virus. All cancer cells were killed by day 9 with infection at a low MOI. The relatively steep fall in the survival curve after day 5 suggested a minimal temporal requirement before sufficient replication occurred to induce toxicity. To reconcile these results with an alternative gene-based approach to cancer treatment which has been proposed, we also compared the cytotoxic effect of Ad5VEGFE1 with Ad5p53, which encodes the wild-type p53 gene and has been employed in human clinical trials. In this regard, we have previously shown that H157 cells, which have a mutated p53 gene, undergo apoptosis when infected with Ad5p53 (41). Ad5p53 infection of H157 cells at MOI 0.1 showed a weak cytotoxic effect compared with Ad5VEGFE1. Similar results were obtained with A427 cells (data not shown). In contrast to the effect in the cancer cells, BEAS-2B cells were resistant to Ad5VEGFE1 toxicity even with infection at a high MOI of 10. These data were consistent with the crystal violet staining appearance as shown in Fig.5B.

Tumor growth suppression by Ad5VEGFE1 *in vivo*.

We next investigated whether Ad5VEGFE1 could suppress tumor growth *in vivo*. To this end, we established subcutaneous tumors in nude mice then directly injected either Ad5CMVLuc, Ad5VEGFE1 or Ad5p53 into the tumor. Tumors become visible

and injectable 10 days after subcutaneous inoculation. Our previous work revealed that the inoculated H157 cells have completed angiogenesis at this time, and in this regard resemble advanced human tumors (22). For these studies, 1 x 10^8 pfu of each virus was injected into the tumor directly and each tumor was observed for 2 weeks. As shown in Fig.7, the tumor injected with Ad5CMVLuc increased in size. Ad5p53 suppressed the tumor growth partially, however, the suppressive effect was minimal. In contrast, Ad5VEGFE1 suppressed the tumor growth to a significantly greater degree than Ad5p53. These findings suggested the CRAd may be a more efficacious agent than a non-replicative virus-based gene therapy approaches such as Ad5p53.

Improvement of CRAd potency via fiber modification.

The oncolytic effect of any CRAd is dependant on the infectivity of the cancer cells as well as promoter activation specificity. Based on these concepts, we endeavored to achieve improvement of the adenovirus infectivity as a means to enhance the anticancer effect achieved via the CRAd agent(46). In this study, we noted that adenovirus infectivity for H460 lung cancer cells and SKOV3.ipl ovarian cancer cells was almost 2 orders of magnitude lower than H157 and A427 lung cancer cells (Fig 3). This infectivity differentiated is the likely basis of the differential CRAd efficacy noted in these contexts. In this regard, we have previously reported that infectivity of serotype 5 adenovirus can be improved by fiber modifications. For example, a modified adenovirus with a chimeric fiber which expresses Ad3 knob instead of Ad5 knob, (Ad5/3) showed enhanced infectivity for various tumor cells (41, 47, 48) that was otherwise Ad refractory. We therefore analyzed the effect of infectivity enhancement via knob serotype chimerism

for the cell lines tested in this study. As shown in Fig.8, the luciferase activities with the Ad5/3 vector increased in all 6 cell lines tested. The increases observed were between 5.1 times in Panc I cells and 39.4 times in A549 cells. These findings led us to construct a Ad5/3VEGFE1 in which the Ad5 knob is replaced with Ad3 knob. Ad5/3VEGFE1 was generated and propagated as described in Materials and Methods. The oncolytic effect of Ad5/3VEGFE1 relative to Ad5VEGFE1 for the various cancer cells was evaluated using infection at 1 MOI (Figure 9). Cytopathic effect with Ad5/3VEGFE1 infection was seen rapidly, almost 2 days earlier than with Ad5VEGFE1, in all cell lines. In this experiment, at day 9 after infection complete cell death was seen for all lines infected with Ad5/3VEGFE1 whereas a significant number of cells survived with Ad5VEGFE1 infection. Moreover, Ad5/3VEGFE1 showed a stronger cell killing effect for H322 cells and SKOV3.ipl cells compared with Ad5CMVE1. These results suggested that the infectivity enhancement with the modified adenovirus fiber could improve the cell killing effect of the VEGF promoter CRAd.

DISCUSSION

Conditionally replicative adenoviruses represent a promising new therapeutic approach for malignancies resistant to conventional treatments. In the current report we demonstrate a strategy based on the use of a replication-competent Ad controlled by a VEGF promoter. Furthermore, we demonstrated that AdVFEGFE1 is applicable for the treatment of a wide spectrum of tumors. With regard to gene therapy of lung cancer, replication-incompetent Ad expressing wild-type p53 is currently being employed in human clinical trials. While replication-incompetent viral vectors have demonstrated great promise as anticancer agents in preclinical studies, this has not been translated into patient benefit in the clinical setting (49). The poor anticancer effect with replication-incompetent Ad is partly dependent to the limited penetration of the vector into the tumor mass. In this regard, CRAd agents are designed to achieve intratumoral spread and penetration by virtue of their replicative capacity (50).

For clinical application, prevention of hepatic toxicity by adenoviral agents is also an important consideration. Tumor cells infected with replication-competent Ad may release new viruses *in vivo*. Such dissemination could predicate treatment related-toxicity, especially in the context of the liver as this is the predominant site of Ad vector localization after systemic injection (51, 52). In this regard, we show that the VEGF promoter exhibits extremely limited promoter activity in the liver and thus may avoid untoward hepatic injury. Since AdVEGFE1 exhibited a high degree of specificity in both replication and cytotoxicity, which correlated with target cell VEGF expression, it would be predicated to be less toxic to the liver compared with AdCMVE1 or wild-type Ad. Unfortunately, at this time no suitable animal models exist for the assessment of CRAd

toxicity *in vivo*. On the other hand, the results of a phase I clinical trial with VEGF inhibitors showed that these agents were well tolerated (53), indicating a marginal role for VEGF signaling in normal organs under physiological conditions except the ovary during the menstrual cycle (54).

Another consideration for the clinical employment of a type II CRAd is that the relevant promoter activity in each tumor should be confirmed before treatment. In this regard, it is clear that tumors with low promoter activity are resistant to type II CRAds containing that promoter (31-34). Therefore it is important to evaluate the promoter activity a priori to avoid potentially non-indicated therapy. Analysis for RNA status requires tissue obtained from the patient to prepare RNA samples for RT-PCR or northern blotting. Precise evaluation of promoter activity with a reporter gene such as luciferase is more difficult in the clinical setting generally. Considered in this context, it is clear that the VEGF promoter has an advantage for its activity evaluation. As shown in Fig.2 and 3, we demonstrated that there is a positive correlation between VEGF mRNA expression level, VEGF protein expression level, and transgene activation for the VEGF promoter, which is consistent with a previous report using clinical samples (55). Taken together these data suggest that the VEGF promoter activity within a tumor can be predicted from VEGF protein expression levels. Of note, VEGF protein is easily detectable in clinical samples by ELISA evaluation of fluid samples (56) and immunohistochemical staining of tissue samples (10). Thus these tests can potentially be employed to prospectively select the most appropriate patients for consideration of VEGF promoter CRAd therapy in the clinical setting.

VEGF production is an important mechanism for the development of tumor-associated angiogenesis in many types of tumors. In fact, many types of cancer are already known to express the VEGF protein at significant levels and this VEGF expression is associated with poor prognosis in several disease contexts including leukemia, breast cancer, colorectal cancer, hepatocellular carcinoma, ovarian cancer and non-small cell lung cancer (53). It appears that more advanced stage tumors actually express higher levels of VEGF protein. Of note, VEGF gene expression is known to be regulated transcriptionally. Although several transcription factors bind to the *cis*-elements on the promoter, hypoxia inducible factor (HIF) is the key factor for activation of the promoter (39). In this regard, the central regions of tumors are often hypoxic and necrotic due to decreased blood flow; immunohistochemical analysis of primary tumor samples shows that VEGF protein expression is enhanced in the tumor tissue adjacent to necrotic regions (57). On the other hand, some types of cancer are known to express the HIF protein constitutively despite the oxygen tension, leading an increase VEGF promoter activation (58, 59). Taken together these findings suggest that the antitumor effect of AdVEGFE1 may be even more efficacious in large *in vivo* tumors than under the normoxic conditions under which our *in vitro* experiments were performed.

The cell killing effect of a type II CRAd may be improved by several mechanisms such as promoter induction, infectivity enhancement, or an armed CRAd strategy (60). A major obstacle to be overcome in Ad5-based cancer gene therapy has been the paucity of the primary receptor, CAR, which frequently characterizes human primary tumor cells. In this regard, decreased expression of CAR has been documented in many cancer types (47, 61-65). Furthermore, down regulation of CAR may be associated with a more

malignant phenotype (66). Due to variable expression of CAR on human primary cancer cells, the utility of Ad5 as a cancer gene therapy vector may be compromised, limiting the overall efficacy of any Ad-based cancer gene therapy, including the use of CRAds agents. On this basis, approaches to circumvent tumor-associated CAR deficiency are required. In this regard, the native Ad5 tropism can be modified to enhance Ad infectivity. One approach is pseudotyping, i.e., retargeting Ad by creating chimeric fibers possessing knob domains derived from alternate serotypes which bind to receptors other than CAR. To this end, we have constructed nonreplicating Ads containing chimeric fibers with the tail and shaft domains of Ad serotype 5 and the knob domain of serotype 3 (40). Our previous work has revealed that a distinct Ad3 receptor exists in ovarian cancer cells based on a novel knob binding assay, and that the Ad5/3 chimeric vector is retargeted to the Ad3 receptor (47). Based on these findings we constructed a CRAd exploiting the Ad5/3 chimeric approach in this study. As we hypothesized, Ad5/3VEGFE1 showed a stronger cell killing effect than that of the Ad5-based CRAd, likely on this basis of the conferred infectivity enhancement.

In conclusion, we believe that the data presented here provides the basis for the advancement of replication-competent adenovirus strategies based on the VEGF promoter for the therapy of various cancers. Furthermore, a CRAd based on the Ad5/3 chimeric vector is promising way to enhance the anti-tumor potency via infectivity enhancement for cancer cells. Given the relevance of a dysregulated VEGF axis in a broad spectrum of tumor types, as well as the frequency of deficient adenoviral receptor CAR in the context of epithelial neoplasms, the current infectivity enhanced VEGF promoter CRAd may represent a "pan-carcinoma" CRAd with broad potential utilities.

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Figure Legends

Fig.1

Schematic diagram of vector construction. These vectors are constructed from an E3 region-deleted Ad5 backbone and do not contain the Ad E1A promoter region (from nucleotides 324 to 488 of the Ad genome). Deletion of the E3 region was necessary due to the length of the 2.6kb VEGF. AdCMVE1 and AdVEGFE1 differ in the promoter driving E1A expression. We also used wild type Ad5 (Ad5wt) as a control.

Fig.2

A. Evaluation of VEGF mRNA expression in cell lines. H82, H460, H157, H322, H522, H1299, QG56, QG90, A427, H358, A549 and N417 are lung cancer cell lines. BEAS-2B is a normal bronchial epithelial cell line. SKOV3.ipl, MeWo and Panc-I are cell lines derived from ovarian cancer, melanoma, and pancreatic cancer respectively. The RT-PCR product for *VEGF*121(408bp), *VEGF*165 (541bp) or GAPDH (574bp) is shown in upper or lower panel respectively. **B.** Evaluation of VEGF protein expression of the each cell lines. $1x10^5$ cancer cells were cultured for 24 h in the serum free media. The VEGF protein concentration in the media was measured by ELISA. Mean + SE of triplicate determination is shown. Cell line in each lane is as for A.

Fig.3

Upper panel, Luciferase activities in various cell lines infected by Ad5CMVLuc or Ad5VEGFLuc. $1x10^5$ cells of each cell line were infected with Ad5CMVLuc or Ad5VEGFLuc for 3 h at MOI 10. 48 h after infection cells were harvested and lysed in

100μl of lysis buffer. 10μl of each lysate was used for luciferase assay. Mean + SE of triplicate determination is shown. Lower panel, The ratio of VEGF promoter activity to CMV promoter activity. To standardize the VEGF promoter activity in each cell line, the luciferase activity with Ad5VEGFLuc was expressed as the percentage of luciferase activity with Ad5CMVLuc.

Fig. 4

Tissue specificity of the VEGF promoter in the adenoviral context. Mice received 1×10^9 pfu of Ad5VEGFLuc or Ad5CMVLuc via tail vein injection (three per group). Two days after virus injection, mice were sacrificed to obtain the organ samples. Each organ lysate was assayed for lucifrase activity and normalized for protein concentration. Mean + SE of triplicate determination is shown.

Fig.5

Assessment of viral DNA replication 24 h after infection. $1x10^5$ cells were infected with replication-competent Ads (Ad5VEGFE1, Ad5CMVE1 or Ad5wt) or non-replicative Ad (Ad5VEGFLuc) at an MOI of 10 for 3 h and then cultured for 24 h. Viral DNA was isolated from the cells and analyzed by real-time PCR analysis to evaluate Adenoviral E4 copy number as described in Materials and Methods. E4 copy numbers were normalized by the β-actin DNA copy number. Mean + SE of triplicate determination is shown.

Fig.6

Cell killing effect of AdVEGFE1 and control. A. Cell killing effect was evaluated by MTS assay. $5x10^3$ H157 cells were infected with Ad5CMVLuc (negative control), Ad5CMVE1 (positive control), or Ad5VEGFE1 at MOI of 0.1. After infection cell viability in each well was quantified by MTS assay every three days. The cell viability of cells infected with Ad5VEGFE1 or Ad5CMVE1 is expressed as the percentage of the OD490 value to control cells infected with Ad5CMVLuc (100%). BEAS-2B cells were infected with each Ad at MOI 10 and evaluated by MTS assay in the same manner. B. $2x10^5$ H157 cells and BEAS-2B cells were infected with each Ad at MOI 0.1, 1.0 or 10. 9 days after infection all wells were stained by crystal violet to visualize the viable cells.

Fig.7

Ad5VEGFE1 suppressed tumor growth *in vivo*. Intact H157 cells ($5x10^6$) were injected s.c. into nude mice. When tumor formation was seen 10 days after inoculation, 1 x 10^8 pfu of each virus (diamond, Ad5CMVLuc; circle, Ad5VEGFE1; square, Ad5p53) was injected into the tumor directly. Three similar sized tumors were injected with each virus, and the mean volume + SE is shown.

Fig.8

Enhancement of infectivity to cancer cells with Ad5/3 chimeric vector. Luciferase activities in various cell lines infected by Ad5CMVLuc or Ad5/3luc1 containing CMV driven luciferase gene. $1x10^5$ cells of each cell line were infected by Ad5CMVLuc or Ad5/3luc1 at MOI 10. 48 h after infection infected cells were harvested

and lysed in 100μl of lysis buffer. 10μl of each lysate was used for luciferase assay. Mean + SE of triplicate determination is shown.

Fig.9

Enhancement of cell killing effect with Ad5/3 chimeric CRAd. Cell killing effect was evaluated by MTS assay. $5x10^3$ cells of each cell line were infected with Ad5CMVLuc (negative control), Ad5CMVE1 (positive control), Ad5VEGFE1 or Ad5/3VEGFE1 at MOI of 1.0. After infection cell viability in each well was quantified using OD490 by MTS assay every three days. The viability of cells infected with Ad5CMVE1, Ad5VEGFE1 or Ad5/3VEGFE1 was expressed as a percentage of cells infected with Ad5CMVLuc (100%).

Fig.1

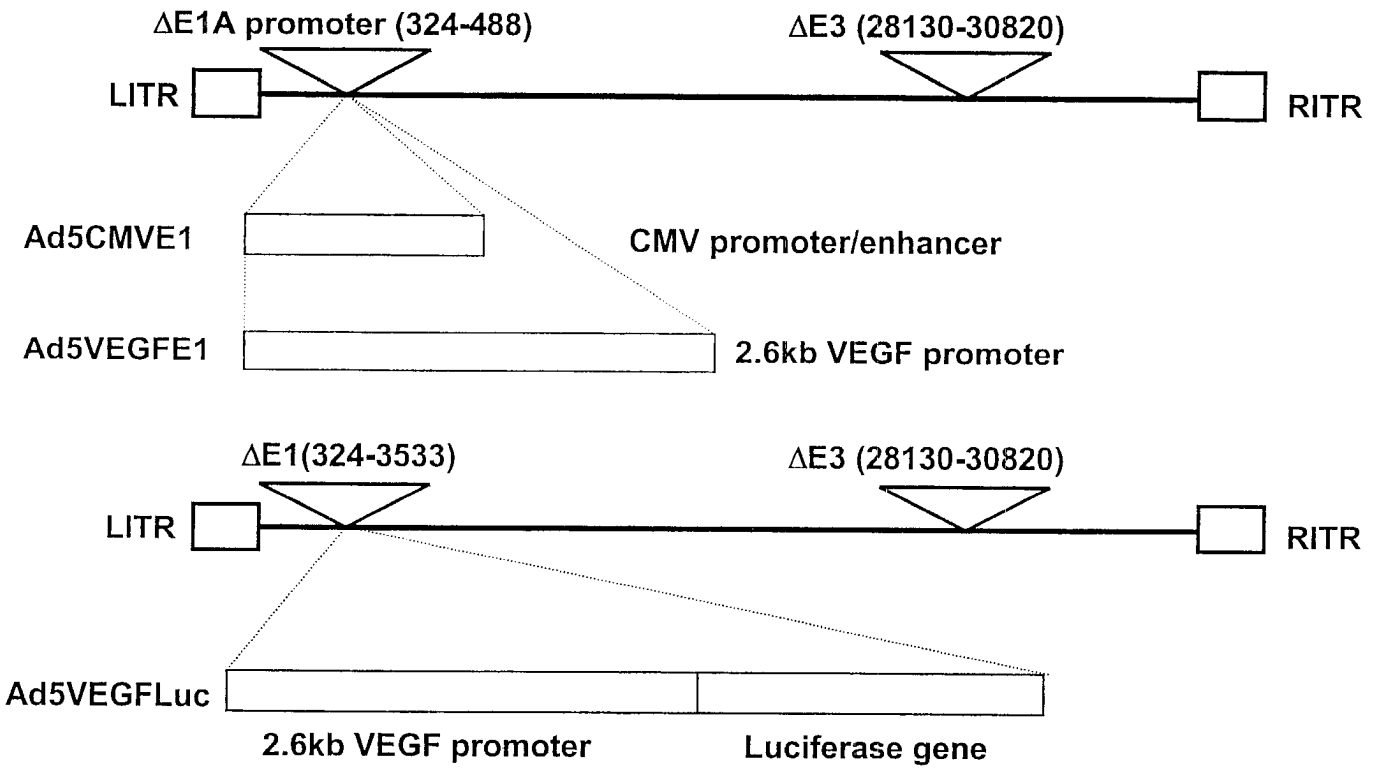
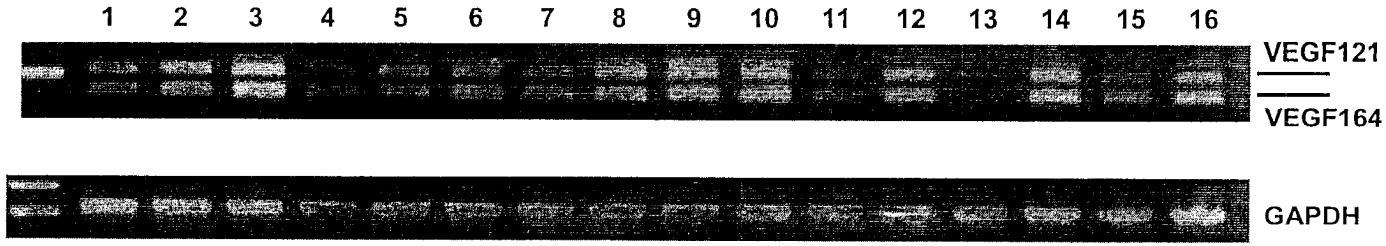

ΔE1A promoter (324-488)
ΔE3 (28130-30820)
LITR
RITR
Ad5CMVE1
CMV promoter/enhancer
Ad5VEGFE1
2.6kb VEGF promoter
ΔE1(324-3533)
ΔE3 (28130-30820)
LITR
RITR
Ad5VEGFLuc
2.6kb VEGF promoter
Luciferase gene

Fig.2

A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
VEGF121
VEGF164
GAPDH

B

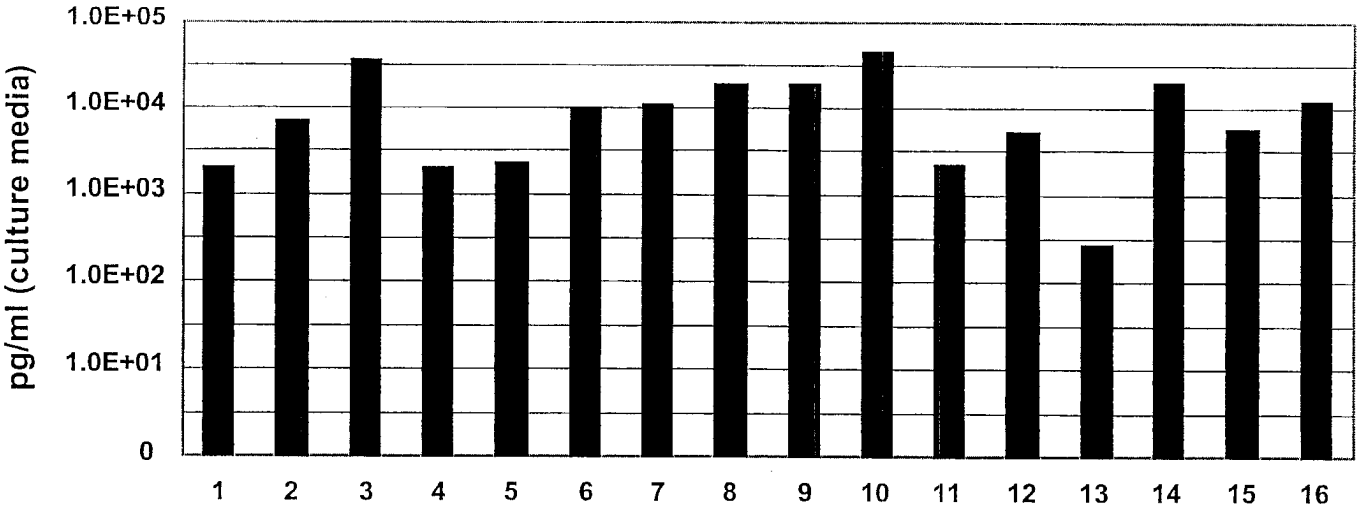

pg/ml (culture media)
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig.3

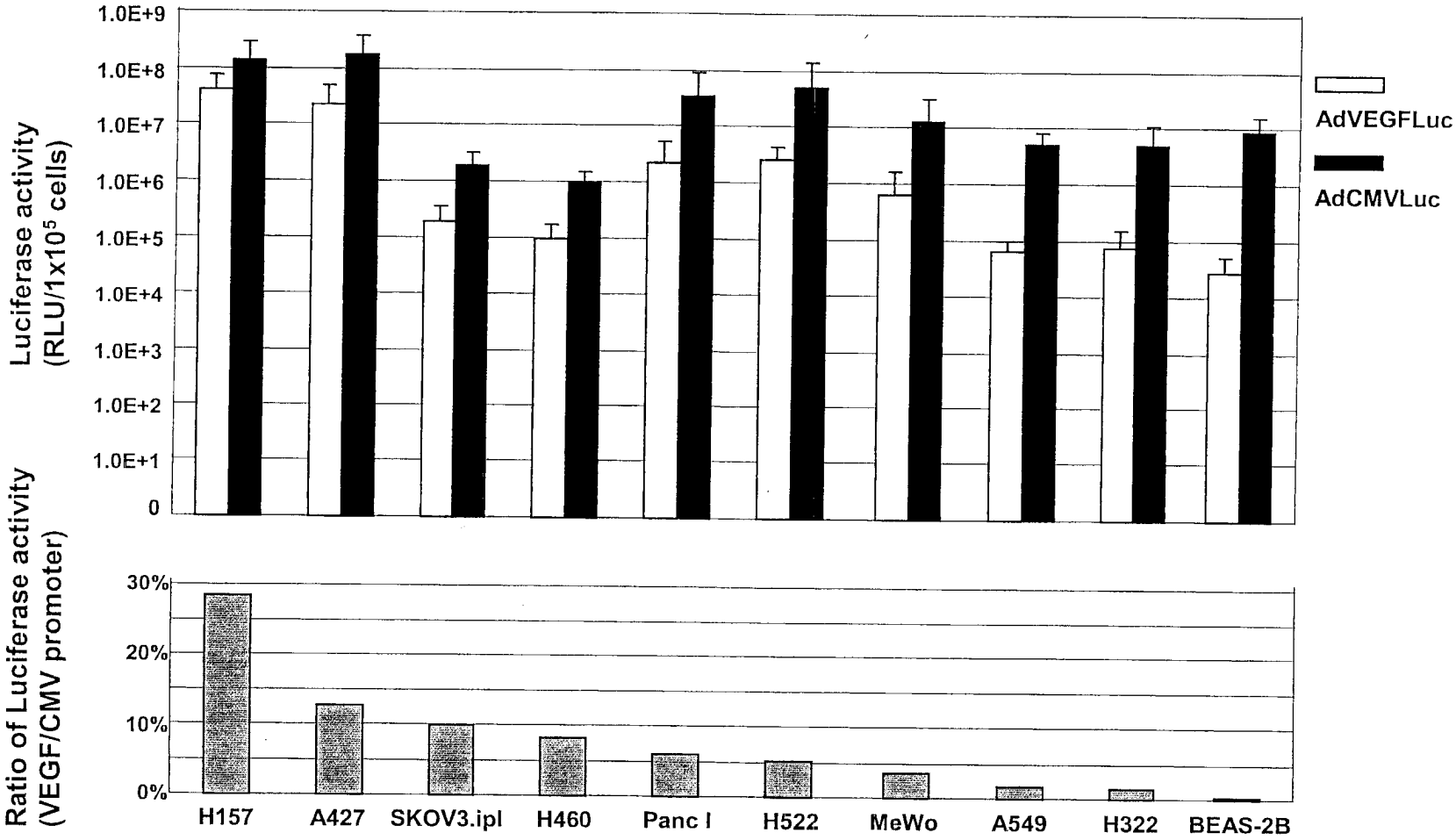

Luciferase activity
(RLU/1x10^5 cells)
1.0E+9
1.0E+8
1.0E+7
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
0
AdVEGFLuc
AdCMVLuc
Ratio of Luciferase activity
(VEGF/CMV promoter)
30%
20%
10%
0%
H157
A427
SKOV3.ipl
H460
Panc I
H522
MeWo
A549
H322
BEAS-2B

Fig.4

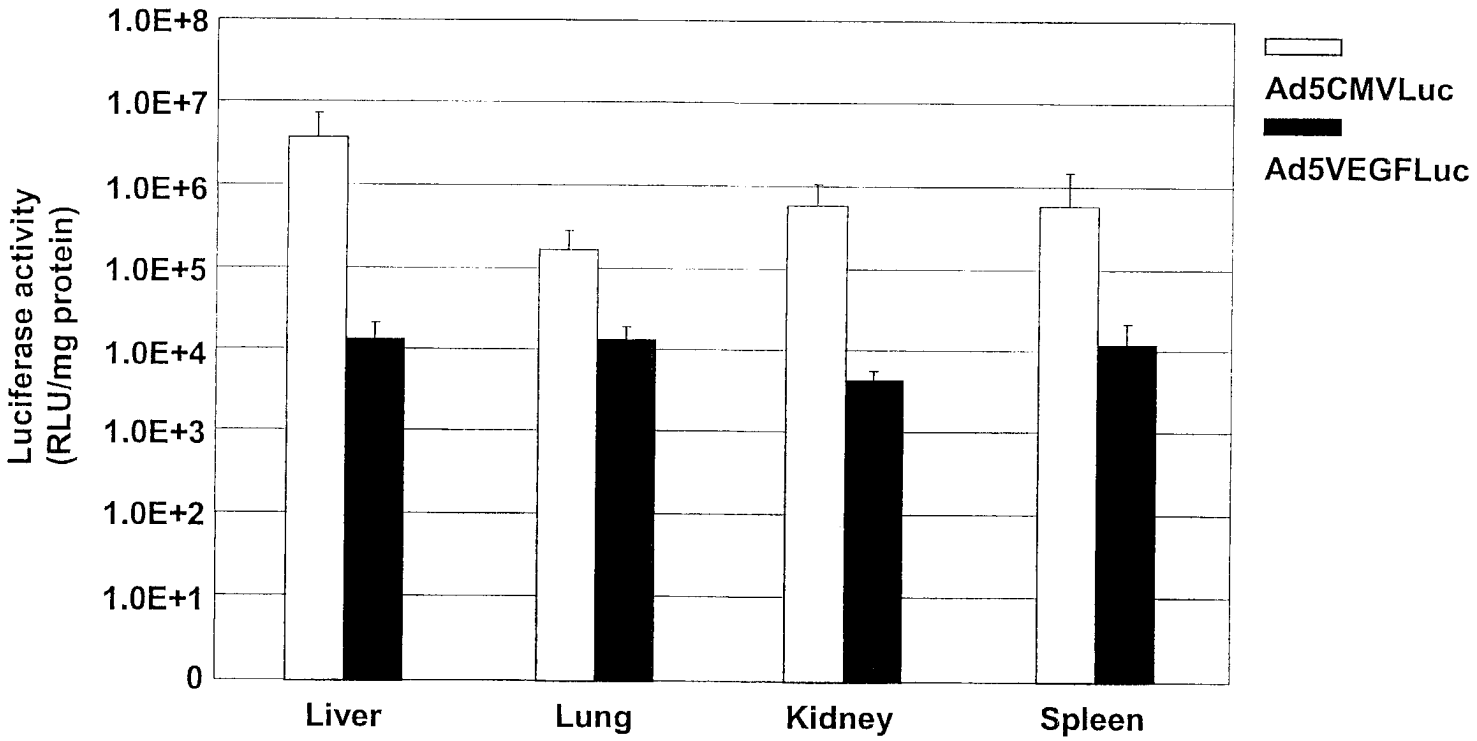

1.0E+8
1.0E+7
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
0
Luciferase activity
(RLU/mg protein)
Liver
Lung
Kidney
Spleen
Ad5CMVLuc
Ad5VEGFLuc

Fig.5

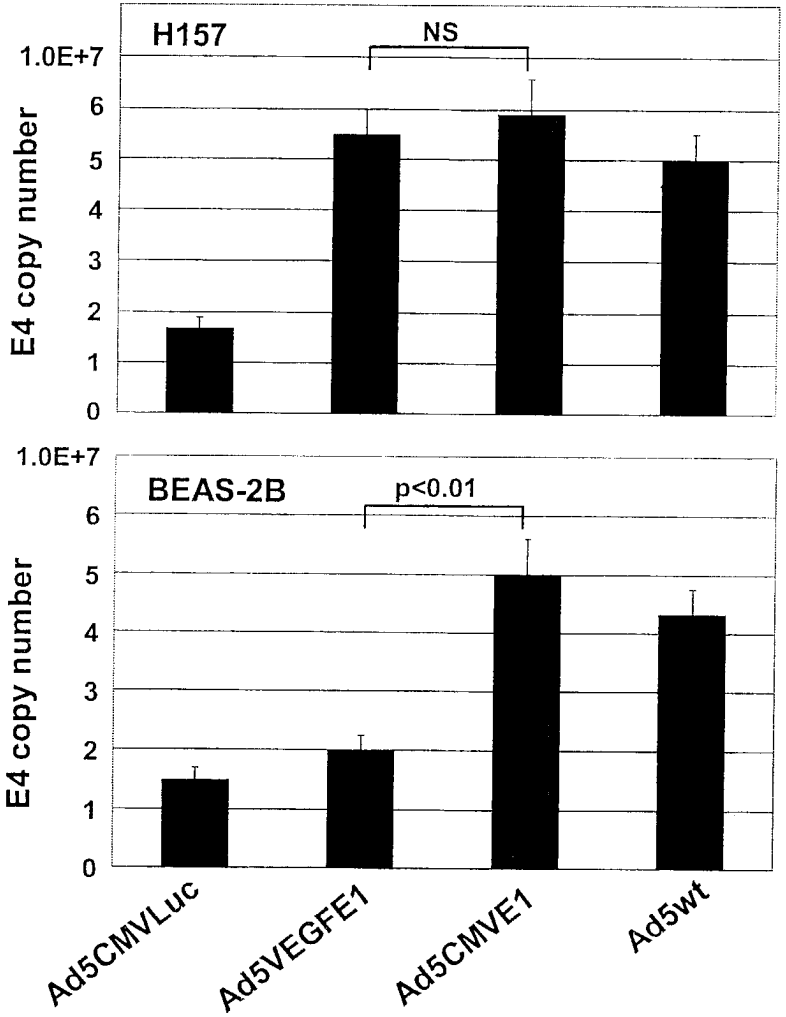
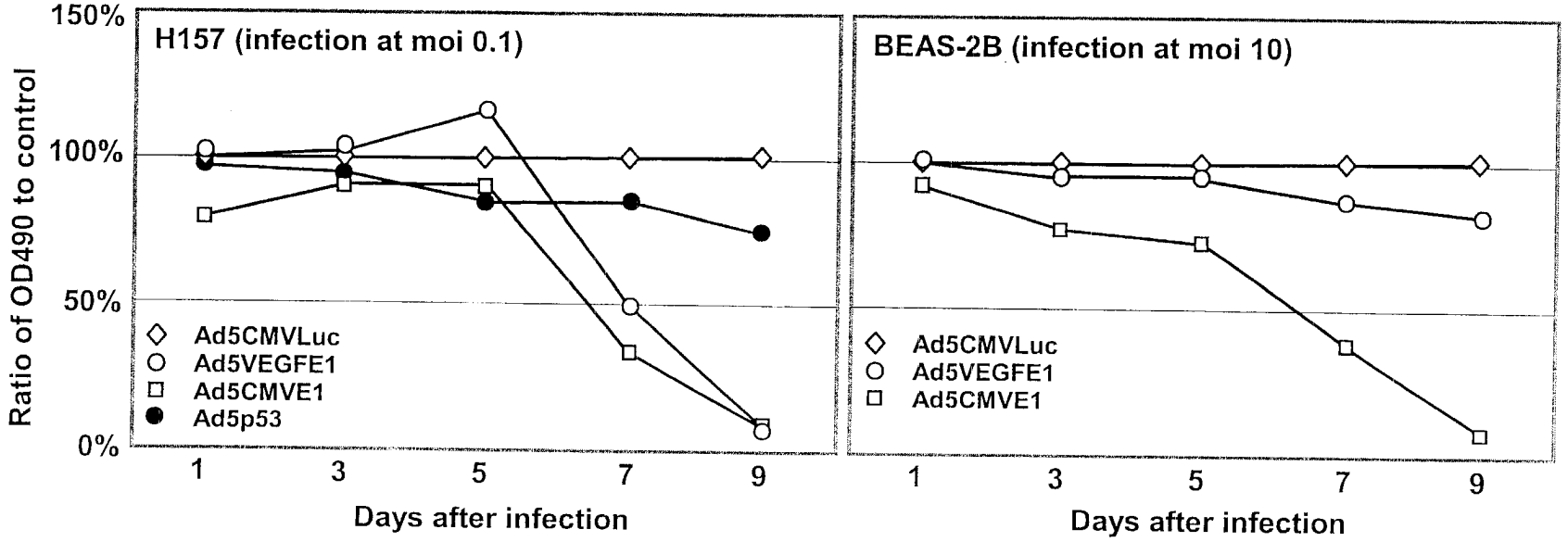

H157
NS
1.0E+7
6
5
4
3
2
1
0
E4 copy number
BEAS-2B
p<0.01
1.0E+7
6
5
4
3
2
1
0
E4 copy number
Ad5CMVLuc
Ad5VEGFE1
Ad5CMVE1
Ad5wt

Fig.6

A


150%
100%
50%
0%
Ratio of OD490 to control
H157 (infection at moi 0.1)
Ad5CMVLuc
Ad5VEGFE1
Ad5CMVE1
Ad5p53
1
3
5
7
9
Days after infection
BEAS-2B (infection at moi 10)
Ad5CMVLuc
Ad5VEGFE1
Ad5CMVE1
1
3
5
7
9
Days after infection

B

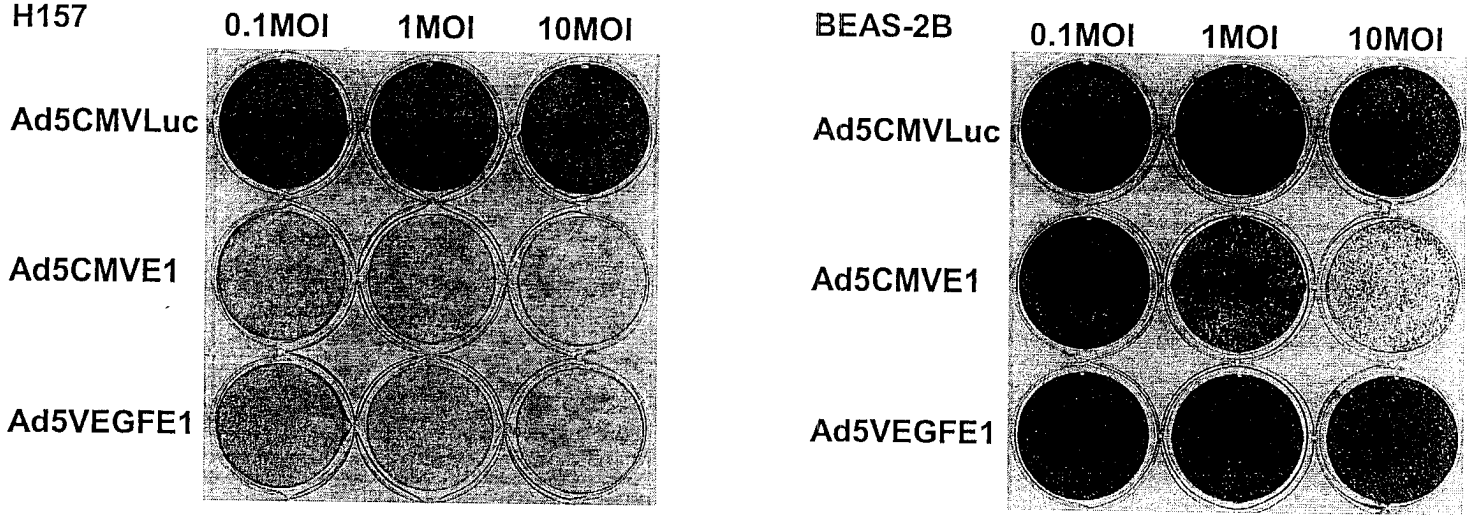

H157
0.1MOI
1MOI
10MOI
Ad5CMVLuc
Ad5CMVE1
Ad5VEGFE1
BEAS-2B
0.1MOI
1MOI
10MOI
Ad5CMVLuc
Ad5CMVE1
Ad5VEGFE1

Fig.7

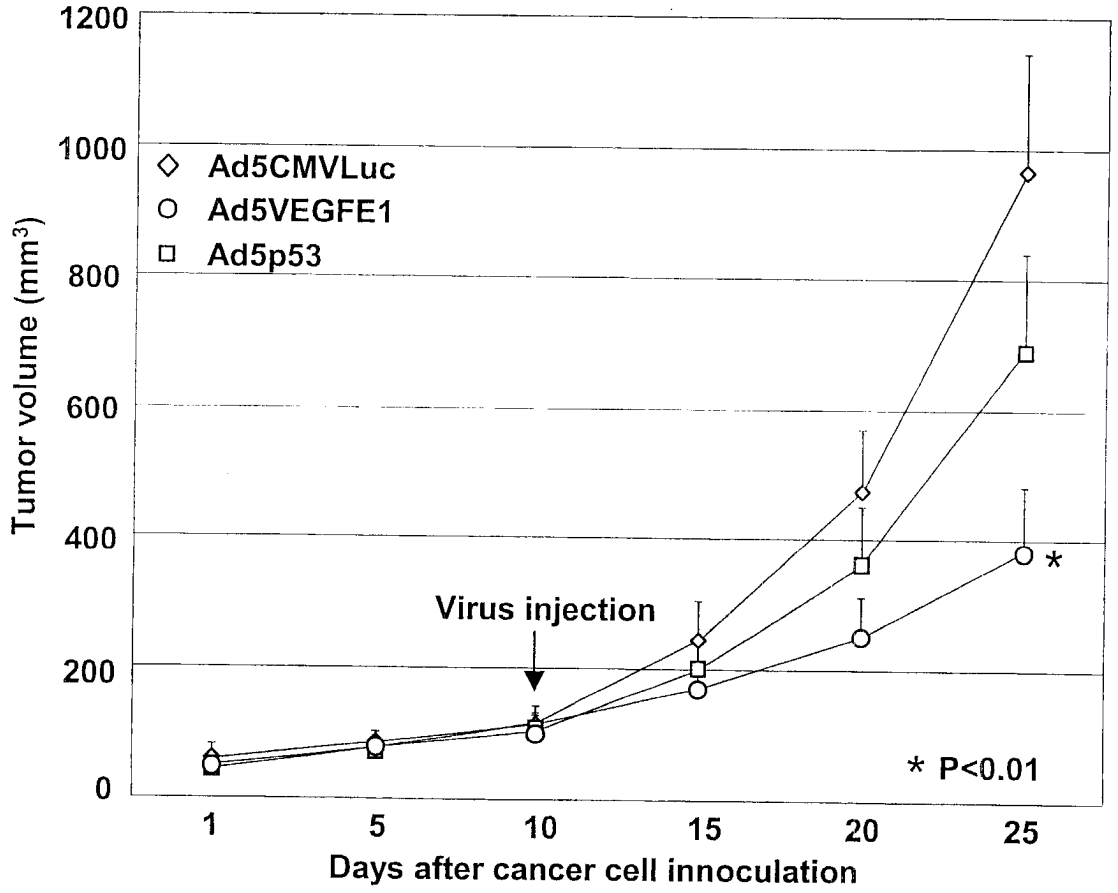

1200
1000
800
600
400
200
0
Tumor volume (mm3)
Ad5CMVLuc
Ad5VEGFE1
Ad5p53
Virus injection
*
* P<0.01
1
5
10
15
20
25
Days after cancer cell innoculation

Fig.8

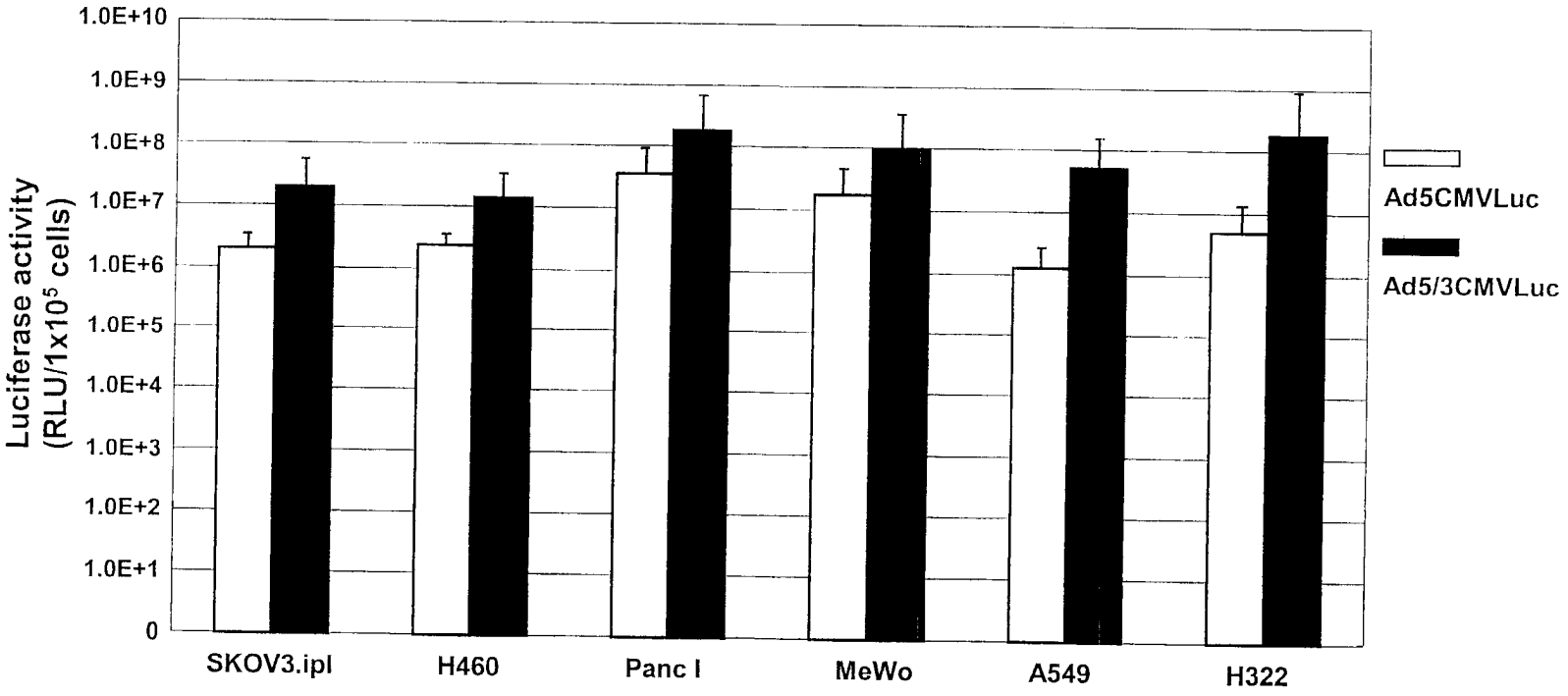
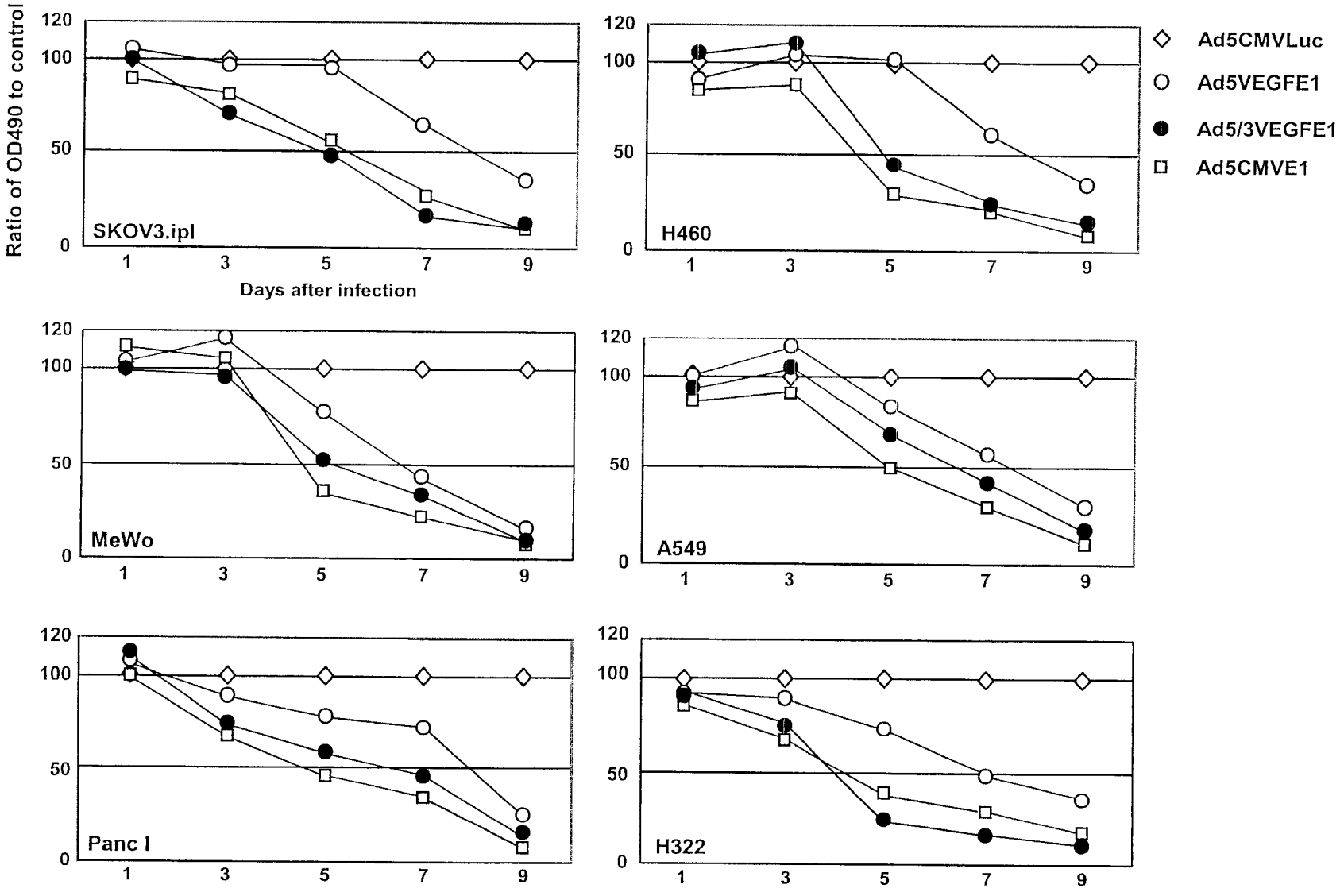

1.0E+10
1.0E+9
1.0E+8
1.0E+7
1.0E+6
1.0E+5
1.0E+4
1.0E+3
1.0E+2
1.0E+1
0
Luciferase activity (RLU/1x10^5 cells)
SKOV3.ipl
H460
Panc I
MeWo
A549
H322
Ad5CMVLuc
Ad5/3CMVLuc

Fig.9


Ratio of OD490 to control
120
100
50
0
1
3
5
7
9
Days after infection
SKOV3.ipl
H460
MeWo
A549
Panc I
H322
Ad5CMVLuc
Ad5VEGFE1
Ad5/3VEGFE1
Ad5CMVE1

APPENDIX H

Augmentation of the Efficacy of Anti-Tumor Replicating Adenovirus via Inhibition of TGF-β Signaling [1]

Yasuo Adachi, Koichi Takayama, Paul N. Reynolds, William E. Grizzle, Larisa Pereboeva, and David T. Curiel[2]
The Division of Human Gene Therapy, Department of Medicine, Surgery, and Pathology, and Gene Therapy Center [Y. A., K. T., L. P., D. T. C.], Department of Pathology [W. E. G.], University of Alabama at Birmingham, Birmingham, Alabama 35294; Royal Adelaide Hospital Chest Clinic, University of Adelaide, 275 North Terrace, Adelaide, South Australia, Australia 5000 [P. N. R.]

INDEX WORDS: Replicating adenovirus, oncolysis, transforming growth factor-β (TGF-β), soluble TGF-β receptor, and intratumor matrix

Running title: Replicating adenovirus expressing soluble TGF-β receptor

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[2]To whom requests for reprints should be addressed: Division of Human Gene Therapy, Departments of Medicine, Surgery and Pathology, University of Alabama at Birmingham, 508 BMR II, 901 19th Street South, Birmingham, Al 35294. Phone: (205) 934-8627; Fax: (205) 975-7476

[3]The abbreviations used are; CRAd, conditionally replicative adenovirus; Ad, adenovirus; sTβRII, soluble TGF-β type II receptor; ECM, extracellular matrix; TGF-β, transforming growth factor-β; DMEM, Dulbecco's Modification of Eagle's Medium; FCS, fetal calf serum; CMV, cytomegalovirus; pfu, plaque forming units; MOI, multiplicity of infection; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidone difluoride; HRP, horseradish peroxidase; PAI, plasminogen activator inhibitor; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; IP, intraperitoneal; IT, intratumoral

Abstract

Conditionally replicative adenoviruses (CRAds) are a promising therapeutic strategy for cancers refractory to conventional treatment. However, the clinical efficacy of this approach alone is in many cases disappointing, possibly due to physical factors limiting intratumoral spread of virus. We present herein the achievement of augmented efficacy of *in vivo* tumor regression by combining an oncolytic adenovirus (Ad) with expression of a soluble, dominant negative TGF-β type II receptor (sTβRII). We constructed a replication competent Ad expressing sTβRII (AdCMVsTβR-E1) and compared its anti-tumor effects to an Ad (AdCMVE1) which does not express sTβRII. The oncolysis induced by AdCMVsTβR-E1 infection *in vitro* in G401 (Wilms' tumor) or SK-ES-1 (Ewing's sarcoma) cells was similar to that seen with AdCMVE1. However, in vivo efficacy in subcutaneous tumors in a mouse model was much greater with AdCMVsTβR-E1. These results indicate that the combination of the oncolytic properties of Ad with inhibition of extracellular matrix (ECM) formation may ultimately lead to a more effective clinical agent.

Introduction

Conditionally replicative adenoviruses (CRAds) represent a promising new class of anti-tumor agent (1,2). In this regard, CRAd approaches have been rapidly translated to the context of human clinical trials for a variety of disease contexts (3). While demonstrating the overall safety of this approach, these trials have revealed the limits of current systems whereby only limited clinical responses have been made where CRAds have been employed as single modality agents. On this basis, a number of distinct approaches have been endeavored to identify the precise biologic factors limiting CRAd efficacy and to design advanced generation CRAd agents which address these limiting features and thereby embody enhanced anti-tumor potency (3-6).

The critical determinants of CRAd efficacy are the ability of the adenovirus to efficiently infect tumor cells, replicate selectively within tumor cells, and to lateralize within the tumor mass for the achievement of multiplicative effects (2). In the former regard, the recognition of a deficiency of the primary adenovirus receptor CAR on tumor cells as a limiting factor to infection efficiency has led to the design of CRAD agents capable of CAR-independent infection (7). The dramatic gains in overall CRAd efficacy accrued in this context serve to highlight the magnitude of lateralized potential embodied in CRAd agents. By extension of this logic, it is clear that additional yield in CRAd efficacy may be realized via methods to address other key biologic predicates of CRAd efficacy.

With respect to further improvements of CRAd design, it is clear that effective lateralization of progeny viruses is critical for the realization of the desired multiplicative effects embodied in the concept of replicating viral agents. Potential limits to this process are connective tissue components of the tumor stroma (5). We endeavored a strategy to imbue CRAd agents with the capacity to inhibit tumor-essential matrix development via specific inhibition of TGF-β. TGF-β is one of the key factors responsible for the production and accumulation of extracellular matrix (ECM) (8,9). We hypothesized that inhibition of TGF-β signaling would reduce ECM formation, thereby improving intratumoral spread of virus and therapeutic oncolysis. In this regard, this strategy could be interpreted as an interruption of the cross-talk between tumor cells and non-tumor cells. This study thus serves to highlight the therapeutic gains which may be accrued via CRAd design advancement which address biologic limits to their anti-tumor effects.

Materials and Methods

Cells and cell culture

The mouse fibroblast NIH3T3, Wilms' tumor G-401 and Ewing's sarcoma SK-ES-1 cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in the medium recommended by the manufacturer. The E1 transcomplementing 911 cell line (10) was obtained from Dr. Alex J. van der Eb, University of Leiden, The Netherlands, and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS).

Viruses and Viral Techniques

The recombinant Ad vectors AdCMVsTβR and AdCMVsTβR-E1, encoding the sequence of the type II TGF-β receptor extracellular domain fused to the human immunoglobulin Fc fragment under the control of cytomegalovirus (CMV) enhancer/promoter, were constructed using the "AdEasy" method reported previously (11). The vector including the sequence of this fusion protein was kindly gifted by Prof. Hikaru Ueno (University of Occupational and Enviromental Health School of Medicine, Japan). We already reported an AdCMVE1, including adenoviral E1A region under the control of CMV enhancer/promoter with a deletion of Ad E3 region (Ad bases 28130-30820), as a replicating competent adenovirus. Based on this vector, we inserted the sequence of the fusion protein gene under the control of CMV enhancer/promoter just before the CMV promoter in AdCMVE1 to generate AdCMVsTβR-E1. The recombinant Ad vector AdCMVLuc, encoding firefly luciferase gene under the control of CMV promoter/enhancer, was used as a replication deficient Ad control (12).

Western Blot

911 cells were infected with Ad vectors used in this study at a multiplicity of infection (MOI) of 10 for 1 hour. After infection, the medium was replaced with DMEM supplemented with 1% of FCS after washing with phosphate buffer saline (PBS) and cells were incubated for a further 24 hours. Protein A sepharose CL-4B (Sigma) was added to the supernatant and then incubated for 1 hour at 4°C. The precipitate was washed, boiled for 5 min, and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins in the gels were transferred to polyvinylidone difluoride (PVDF) transfer membrane (Scheicher & Schuell). After the membrane was blocked for 30 min with 0.5% Casein in PBS, the membrane was incubated with either affinity-purified polyclonal rabbit antibody to human type II TGF-β receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or polyclonal antibody to human IgG (A0424, DAKO, A/S, Denmark) as primary reaction. Affinity purified goat anti-rabbit polyclonal antibody conjugated with horseradish peroxidase (HRP) (DAKO, A/S, Denmark) was available as secondary antibody.

TGF-β signaling

Human TGF-β1 was purchased from R & D Systems. A TGF-β responsive region, which extends from –799 to +71 in the human plasminogen activator inhibitor 1 (PAI-1) gene (13), was isolated from the genomic DNA extracted from 911 cells by virtue of the polymerase chain reaction (PCR) procedure. This PAI-1 promoter sequence was subcloned to the multicloning site of the promoterless luciferase plasmid (pGL3basic vector, Promega) to form pGL3-PAI-1. To prepare the test sample for the blocking of

TGF-β signaling by sTβRII, serum samples were harvested from the athymic nude mice (Charles Rivers) injected with 2 x 10^9 pfu of AdCMVsTβR or 200 μl of phosphate buffer saline (PBS) intraperitoneally 3 days prior to the blood sampling. The concentration of sTβRII in serum from the mouse injected with AdCMVsTβR was measured by an enzyme-linked immunosorbent assay (ELISA) method reported previously (14).

NIH3T3 cells were plated in 12-well plates in triplicate at the density of 100,000/well. After overnight culture, the cells were transfected with pGL3-PAI-1 at the DNA concentration of 500ng/well using Superfect (Qiagen) for 2 hours, and transfection mixture was replaced with complete medium. The following day, the transfected cells were exposed to the DMEM medium containing 1% FCS with mouse serum described above for 20 hours. The cells were harvested and treated with 200 μl of lysis buffer (Cell Culture Lysis Reagent, Promega, part# E153A). A luciferase assay (Luciferase Assay System; Promega) and a FB12 luminometer (Zylux Corporation) were used for the evaluation of luciferase activities.

Oncolysis assay

G401 or SK-ES-1 cells were plated in 12-well plates at the density of 1 x 10^5 /well. After overnight culture, cells were infected with 4 types of Ad vectors at several MOIs (multiplicity of infection) for 3 hours. The infection medium was replaced with DMEM supplemented with 5% FCS. Then, G401 or SK-ES-1 cells were cultured without medium change for 5 or 8 days, respectively. The adherent cells were fixed with 10% buffered formaldehyde, and then stained with 1% crystal violet solution.

MTS assay was used to evaluate the oncolytic efficacy of the Ad vectors used in this study. Tumor cells were plated in 96-well plates in triplicate at a density of 3000/well. After overnight culture, cells were infected with 4 types of Ads at various MOIs for 3 hours. The infection medium was then replaced with complete media. Viable G401 or SK-ES-1 cells were quantitated using MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) at 5 or 8 days after infection, respectively. The MTS color development was analyzed by an EL 800 Universal Microplate Reader (Biotec Instruments Inc.).

In vivo treatments

For tumor implantation, 2.0 $\times 10^7$ cells of G401 or SK-ES-1 were injected into the flank of athymic nude mice (Charles Rivers). Tumors were allowed to grow for at least 1 week before randomizing mice by tumor size. Tumor volumes were calculated by the formula $0.5 \times L \times W^2$ (L: length of the tumor, W: width of the tumor). All tumors were at least 150 mm^3 at the day of the first virus injection. Tumor bearing mouse were classified into 4 groups according to the treatment regimen, AdCMVLuc, AdCMVTβR, AdCMVE1, and AdCMVTβR-E1. Each group consists of 6 mice. Mice received intratumoral virus injection at the dose of 2.0×10^8 pfu per each tumor, and tumor volume was monitored. A second intratumoral virus injection was performed 6 days after first injection. Since tumor sizes were randomized, the initial tumor volumes calculated at the day of first virus injection (day 0) were given a value of 1.0. All subsequent measurements are expressed as a ratio to this value.

Results

Detection of soluble TβRII fusion protein

We sought to develop CRAd agents which embodied anti-matrix assembly effects via the expression of an inhibitor of TGF-β signaling. To this end CRAd agents were designed which encoded a fusion between the extracellular domain of the TGF-β type II receptor (amino acids; 1-159) and the Fc portion of the human IgG Fc fragment. The adenoviruses constructed and employed for this study are shown in Figure 1-A. We initially accessed the expression profiles of the various adenoviruses in the supernatant of infected 911 cells via Western blot. The soluble fusion could be precipitated with the protein A sepharose CL-4B based on the affinity of the fusion's IgG Fc domain for the protein A. Precipitated material has thus analysed by Western blot via rabbit anti-human TβR-II antibody. Extraction of supernatant of cells infected with AdCMVsTβR and AdCMVsTβR-E1 clearly demonstrated the secretion of the encoded soluble fusion (Figure 1-B). A corresponding band was not detected in the context of the control adenoviruses. These studies confirm our derivation of a CRAd agents expressing the anti-matrix protein sTβRII.

Inhibition of TGF-β 1 signaling

TGF-β is known to induce PAI-1 expression in various cells. On this basis, the promoter of the PAI-1 has been exploited as a means to evaluate the concentration of TGF-β or function of TGF-β type II receptor in target cells. The promoter region extending from –799 to +71 in the human PAI-1 gene was reported as a TGF-β specific responsive region that does not respond to other growth modulators, such as platelet-

derived growth factor. We isolated this region and subcloned it into a promoterless luciferase plasmid to form pGL3-PAI-1 as a means to assess the effects of sTβR on TGF-β signaling. The concentration of sTβRII in the serum from mice injected with the sTβR-expressing Ad vector AdCMVsTβR was about 60 nM. As shown in Figure 2, a dose-dependent induction of the pGL-PAI-1 was observed by the addition of TGF-β 1, confirming the utility of our indicator system. We next assessed the inhibition of TGF-β signaling by the addition of 2 nM sTβRII. The addition of mouse serum containing sTβRII suppressed the luciferase induction by the pGL-PAI-1. To exclude the possibility that alternative modulators were functioning to suppress the pGL-PAI-1 in mouse serum, the same amount of the serum harvested from the mouse injected with PBS intraperitoneally 3 days prior to the blood sampling was added to this system. As shown in Figure 2, the profile of dose-dependent luciferase induction by TGF-β1 was almost same as a control. These studies thus confirm that the sTβRII encoded in the constructed adenovirus was capable of inhibition of TGF-β signaling.

Oncolytic Efficacy of sTβR-II-Encoding Adenovirus In Vitro

To investigate the ability AdCMVsTβR-E1 to achieve *in vitro* tumor cell killing, crystal violet staining was used to visualize surviving cells in direct assays of adenovirus-induced oncolysis (Figure 3). Surviving G401 or SK-ES-1 cells were assessed 5 or 8 days after Ad infection, respectively. These results indicated that the oncolysis induced by AdCMVsTβR-E1 infection was comparable to that induced by AdCMVE1 *in vitro*. Although AdCMVE1 and AdCMVsTβR-E1 are both replicative Ads, AdCMVE1 does not contain the sTβRII sequence under the control of CMV promoter as is the case for

AdCMVsTβR-E1. These results were then confirmed using the more quantitative MTS assay. MTS assay of G401 or SK-ES-1 were performed 5 or 8 days after Ad infection, respectively. The MTS results were comparable to those of crystal violet assay.

Oncolysis of the targets induced by AdCMVsTβR or AdCMVE1 in this study was of the same efficiency, suggesting that the expression of sTβRII does not exert any adjunctive anti-tumor effects in contexts whereby matrix components are not involved.

Oncolytic Effect of sTβR-II-Encoding Adenovirus In Vivo

To examine the therapeutic effects of the combination system (replicative Ad combined with sTβRII) *in vivo*, subcutaneous nodules were established in nude mice using the stroma rich G-401 Wilms' tumor or SK-ES-1 Ewing's sarcoma cell line. These tumors were then treated with intratumoral injection of Ad vectors (2.0×10^8 pfu per each tumor), and tumor volume was monitored. A second intratumoral virus injection was performed 6 days after first injection. During this treatment study, none of the study animals manifested any clinical evidence of toxicity. The results of treatment are depicted in Figure 4. The size of the subcutaneous G401 tumors in the non-replicative Ad treated groups increased over time. Surprisingly, IT injection of AdCMVE1, which showed a strong oncolysis against G401 cells in the *in vitro* assay, did not reduce the G401 tumor growth compared with non-replicative Ads. On the other hand, significant inhibition of G401 tumor growth was observed in the groups that received AdCMVsTβR-E1 (Figure 4A). AdCMVE1 did not reduce the growth of subcutaneous SK-ES-1 tumors. However, IT injection of AdCMVsTβR-E1 reduced the SK-ES-1 tumor growth compared with other Ad vectors (Figure 4B). These results are consistent with the concept that the anti-

tumor effects of sTβRII expression were adjunctive to CRAd efficacy exclusively in the context of matrix-forming tumor targets.

Discussion

This report has clearly established that CRAd with expression of sTβRII can augment the anti-tumor potency of the replicative agent. In this regard, a variety of approaches have been exploited to improve CRAd efficacy (3-6). These strategies have included the incorporation of heterologous transgenes which embody distinct anti-tumor mechanisms for the purpose of additive or synergistic effects with the parental CRAd agents. Specific methods have sought to exploit anti-tumor toxic gene systems such as herpes simplex virus thymidine kinase or cytosine diaminase in conjugation with CRAd agents (4,6). The utility of these methods have not been unambiguous as suggested by the various reports exploring this strategy to date. Of note, toxic gene expression may directly limit new virus production via the delivered CRAd due to suboptimal survival of the primary target cell infectants which are required to function as Ad producer cells in the CRAd context. Such limits may also accrue in instances where incorporated transgenes effect apoptosis/anti-apoptosis pathways. In the aggregate, these studies suggest that CRAd expressed transgenes must not operate at biologic cross-purposes to CRAd efficacy determinants for therapeutic gene to be realized.

Of note, tumor cell stromal physiology represents a direct barrier against realizing the full benefit of CRAd agents. In this regard, studies by Harrison *et al.* have demonstrated a clear delimitation effect on CRAd efficacy as the stroma cell function of tumor nodules is increased (5). In addition, capsid components of the adenovirus can potentially interact with stromal cellular components including integrins and matrix aspects such as heparan sulfate-glycosaminoglycan (15). These various factors may serve to conspire against the CRAd requirement of effective lateralization within the tumor's three dimentional

structure. For these reasons, we explored a strategy of CRAd-based expression of a transgene designed to limit the impact of stroma/matrix factors in CRAd-based oncolytic intervention. Such a strategy would be consistent with the concept of CRAd-expressed transgene operating in a manner consistent with CRAd efficacy determinants, in this instance augmenting the capacity of new viruses to lateralize.

In this study, we chose to block TGF-β1 signaling to inhibit ECM development in tumor tissue. TGF-β is one of the key regulators of the production and accumulation of ECM proteins, especially during tissue repair (8,9). TGF-β1 signaling is initiated by binding to a TGF-β type II receptor dimer that heterotetramerizes with a TGF-β type I receptor homodimer thereby activating a cascade involving Smad proteins (Smad 2, Smad 3, and Smad 4) (9). Ueno *et al.* constructed an Ad vector expressing a sTβRII and confirmed that this receptor served as a dominant negative, inhibiting TGF-β signaling by generating a dysfunctional receptor-ligand complex (14). The administration of this vector prevented liver fibrogenesis in the dimethylnitrosamine-treated rat model (14,16). Rowland-Goldsmith *et al.* reported that the sTβRII strategy successfully attenuated the invasion and tumorigenic capacity of a pancreatic cancer COLO-357 (17). Thus, we constructed a replicating, oncolytic Ad expressing sTβRII (AdCMVsTβR-E1) with the twin aims of improving intratumoral viral spread and directly inhibiting tumor growth via ECM disruption.

It is noteworthy that recent approaches to increase the stringency of the replicative specificity of CRAd agents have allowed clear improvement in the therapeutic index achieved the model systems. Further, strategies to enhance the infectivity of CRAd agents via tropism modification of the parent adenovirus have likewise allowed the realization of

dramatic enhancement of therapeutic efficacy (18). The requirement for effective lateralization has more recently been recognized as a key predictor of CRAd efficacy. In this regard, specific strategies to alter the physiology of Ad-induced cell killing and release have been explored as a means to address this issue. The approach we report here presents an improvement adjunctive to these earlier methods. It is likely that strategies that facilitate the steps of virus release and interruption of stroma/matrix barriers will operate synergistically to augment CRAd efficacy. In an even larger context, it is further likely that a combination of the aforementioned strategies to improve target cell infection, replication, specificity, and lateralization will allow the realization of advanced generation CRAd that embody the maximized anti-tumor potentially embodied the strategy of replicative viral agents.

Acknowledgements

We wish to thank Prof. Hikaru Ueno (University of Occupational and Enviromental Health School of Medicine, Japan) for providing us with the Ad vector expressing soluble TGF-β type II receptor. We are grateful to Dr. Alex J. van der Eb (University of Leiden, the Netherlands) for the 911 cell line. We also thank Joel N. Glasgow, Yosuke Kawakami, Lioudmila Kaliberova, Svetlana Komarova, Minghui Wang, and Masato Yamamoto for their excellent technical support and expert advice.

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Figure legends

Fig. 1A Schema of the Ad vectors used in this study. These vectors are constructed from E3 region deleted Ad5 backbone and do not contain the Ad E1A promoter region expanding from nucleotide 324 to 488 of the Ad genome. AdCMVE1 and AdCMVsTβR-E1 contain Ad E1 gene under the control of CMV promoter/enhancer. AdCMVLuc and AdCMVsTβR are the non-replicating Ads. LITR or RITR stands for left or right inverted terminal repeat, respectively.

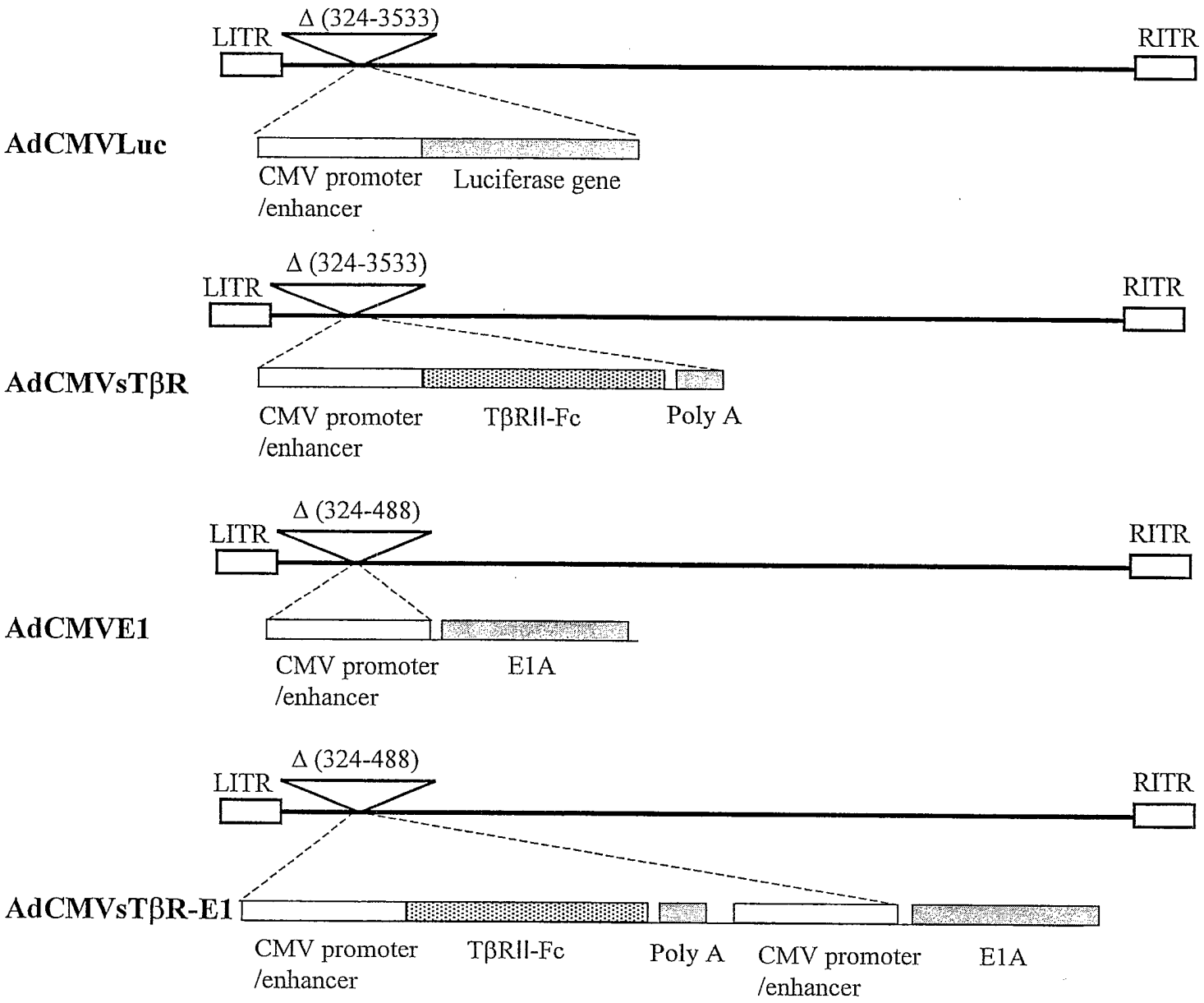
Fig. 1B Expression of sTβRII-Fc fusion protein in the supernatant of 911 cells infected with Ads. Western blot analysis was available to detect the band corresponding to TβRII-Fc. Sigma Fast 3,3'-diaminobenzidine tablet sets (Sigma) were used for the staining. The arrows indicate sTβRII portion. A top panel (B) shows the results using antibody for the detection of TGF-β type II receptor as a primary reaction. A bottom panel (C) shows the results for detecting Fc portion. No corresponding bands were detected in the supernatant of 911 cells infected with mock (lane 1), AdCMVLuc (lane 2), or AdCMVE1 (lane 3). Infection of AdCMVsTβR or AdCMVsTβR-E1 (lane 4 or 5, respectively) induced the expression of sTβRII-Fc. M stands for marker. Molecular sizes are in kilodaltons.

Fig. 2 Luciferase induction in NIH3T3 cells transfected with pGL3-PAI-1 by rTGF-β1. A) without mouse serum. B) addition of mouse serum containing sTβRII. C) addition of control mouse serum. Each column shows the mean induction (±SD).

Fig. 3A *In vitro* oncolytic efficacy of Ads evaluated by the crystal violet staining. Survived cells of G401 or SK-ES-1 were stained in violet.

Fig. 3B Oncolytic efficacy induced by the Ad infection. Cell killing efficiency is evaluated by MTS assay. The conversion of MTS into formazan was measured by absorbance (OD) at 490 nm. We made blank controls of culture medium without cells and measured the absorbance (blank OD) following MTS procedure. ΔOD (OD - blank OD) reflects to the surviving cell number. 1) Surviving cells of G401 5 days after infection. 2) Surviving cells of SK-ES-1 8 days after infection. In both cell lines, anti-tumor replicating Ad, AdCMVE1 (open triangles) and AdCMVsTβR-E1 (closed circles), induced remarkable cell killing compared with replicating deficient Ad, AdCMVLuc (open circle) and AdCMVsTβR (closed squares).

Fig. 4. A) Subcutaneous G-401 nodule regression by AdCMVsTβR-E1 (closed circles) treatment. B) Growth suppression of SK-ES-1 nodule induced by AdCMVsTβR-E1 (closed circles) IT injection. Results are shown as fractional tumor volumes (V/V0, where V = volume at each time point, and V0 = volume at adenovirus injection). IT injection of AdCMVsTβR (closed squares) or AdCMVE1 (open triangles) did not reduce the tumor size of G401 or SK-ES-1 compared with AdCMVLuc (open circles). Each line shows the mean (±SD) of six nodules. In the case of G401 nodules, there was a statistically significant difference between AdCMVsTβR-E1 and other virus injection (Wilcoxon-Mann-Whitney test). (*) $p < .05$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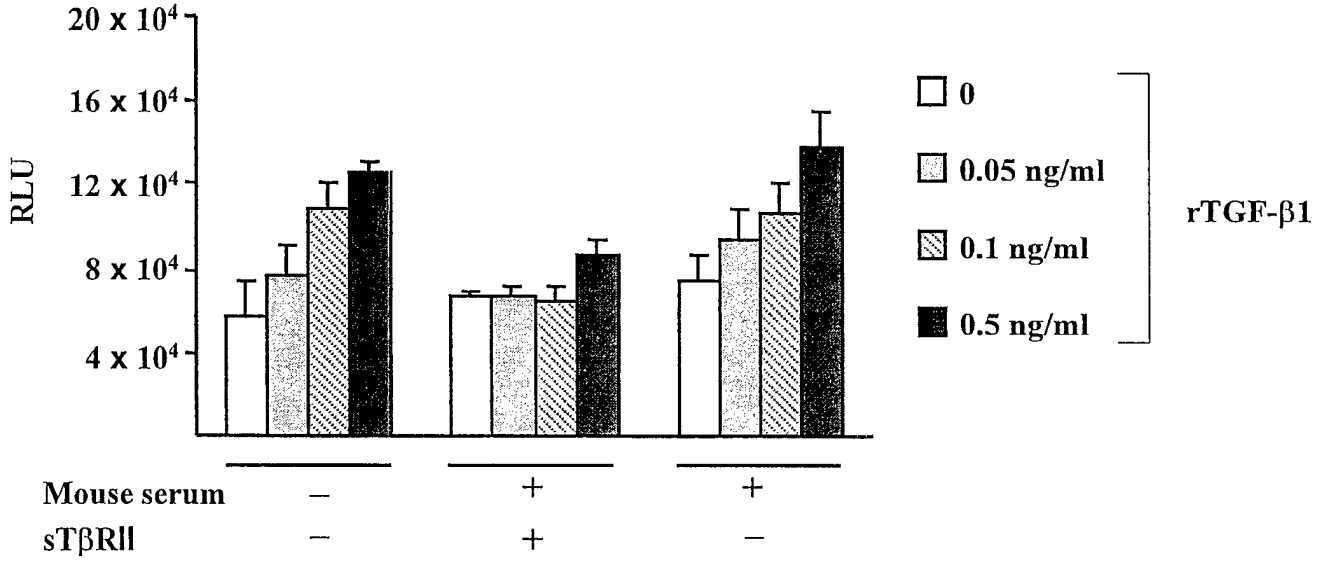
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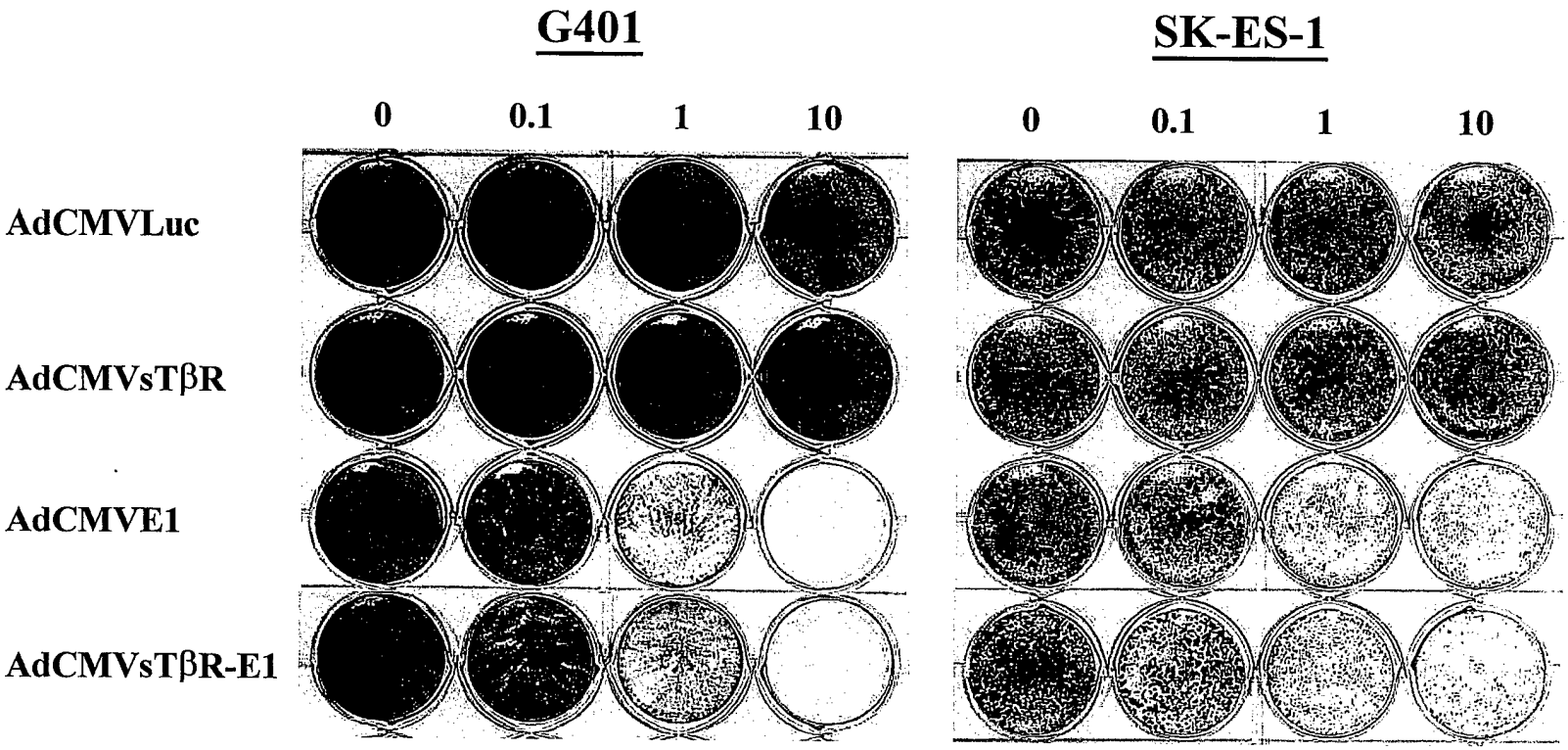
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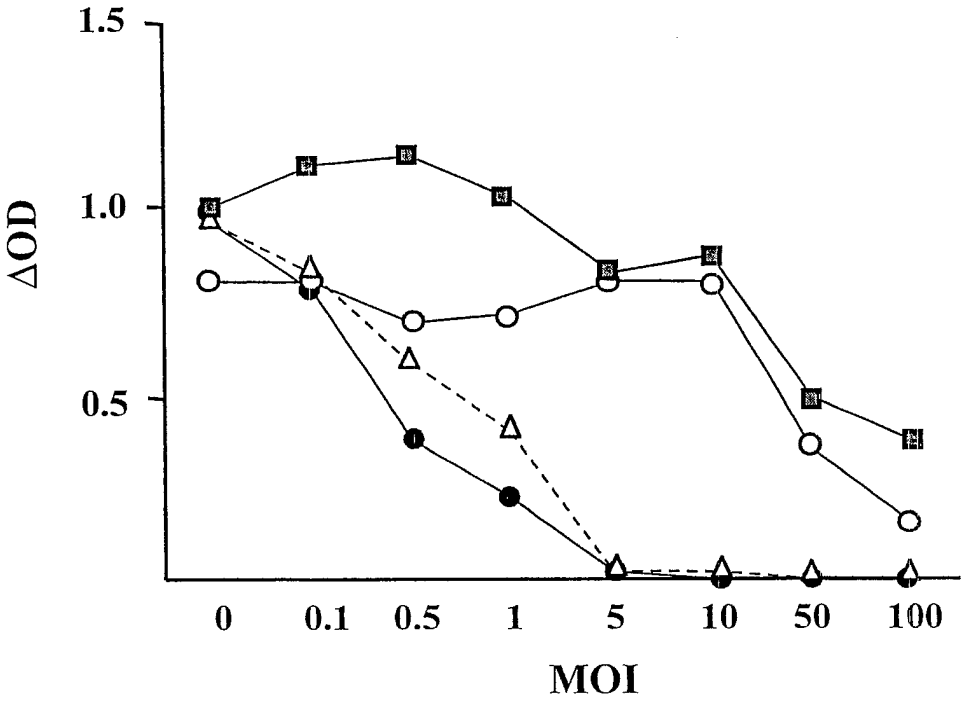
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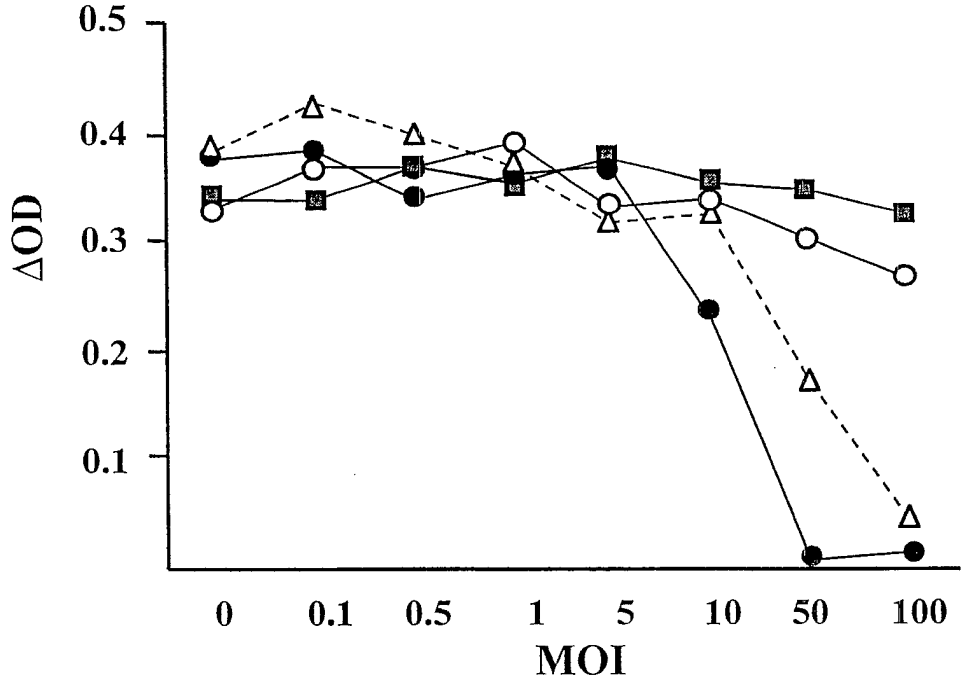
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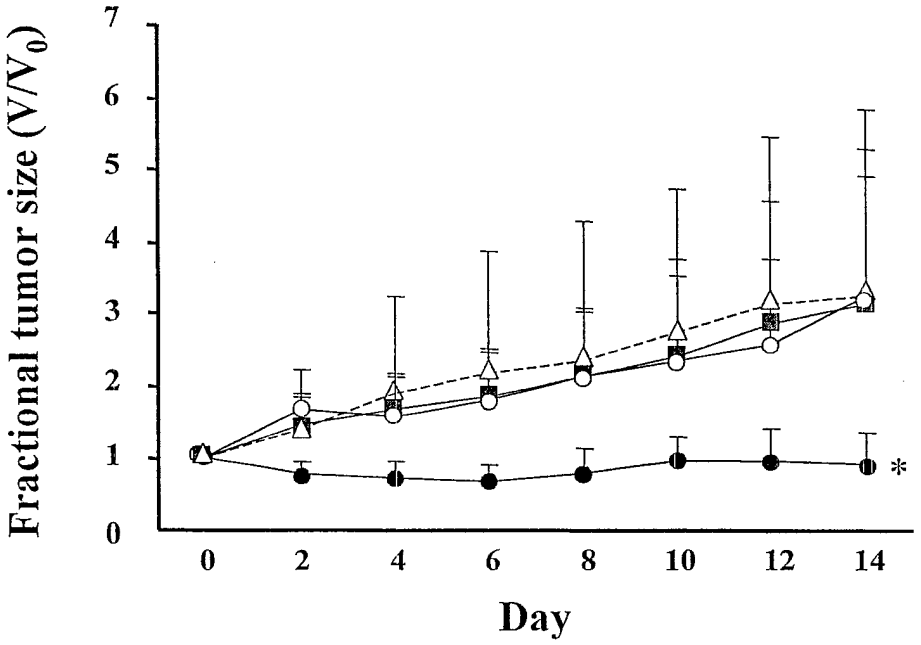
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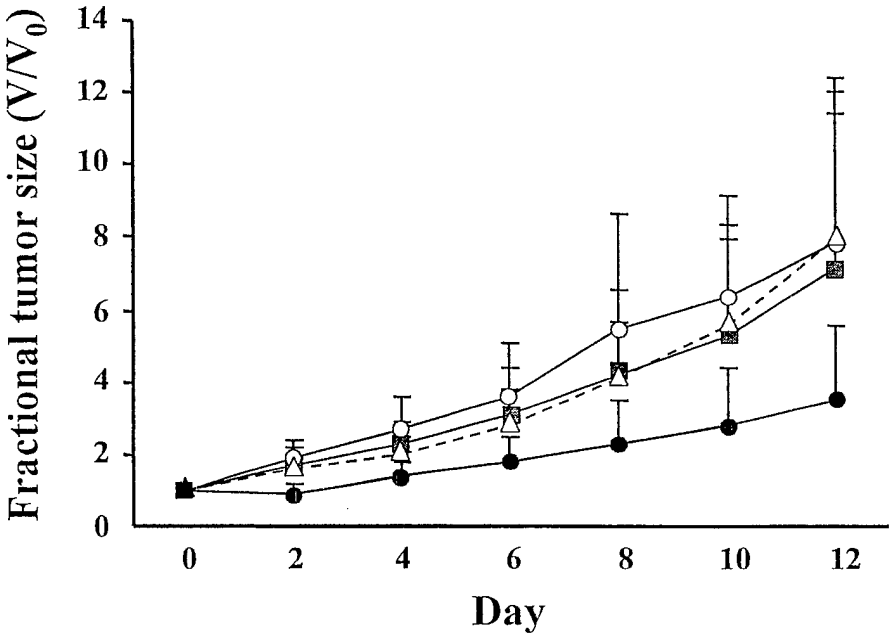
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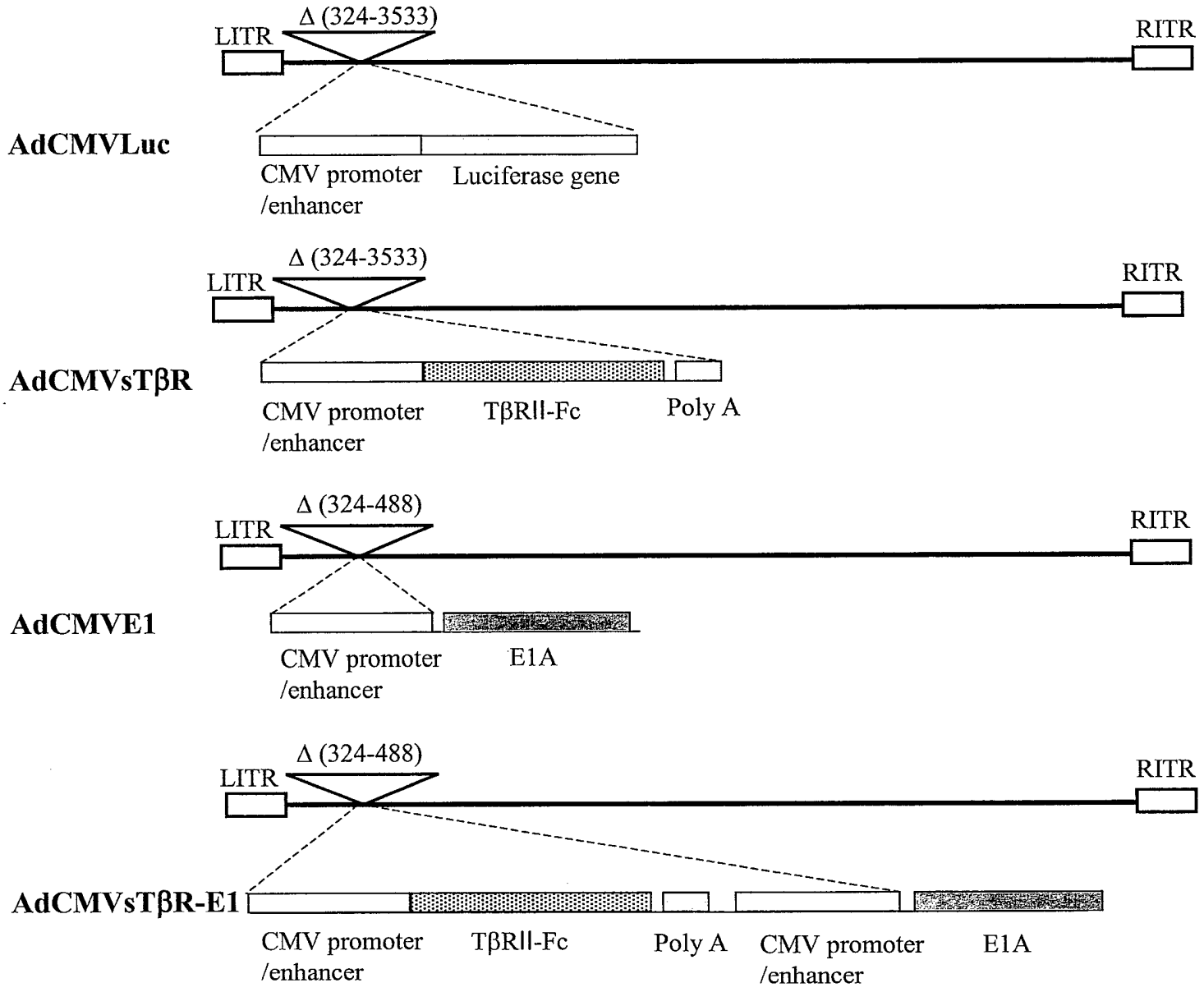
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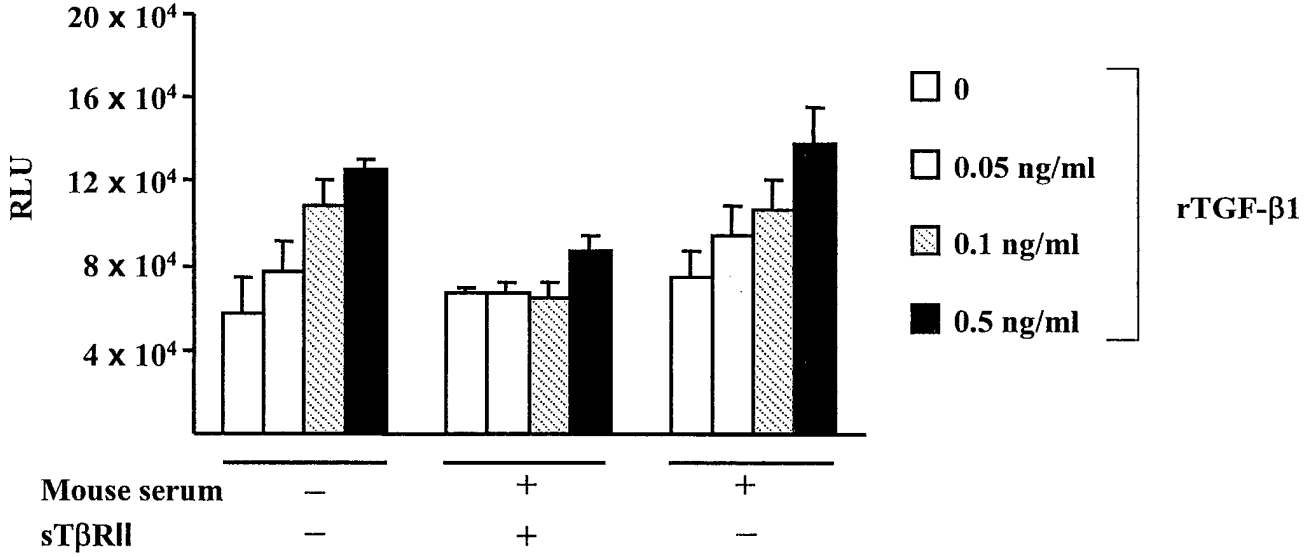
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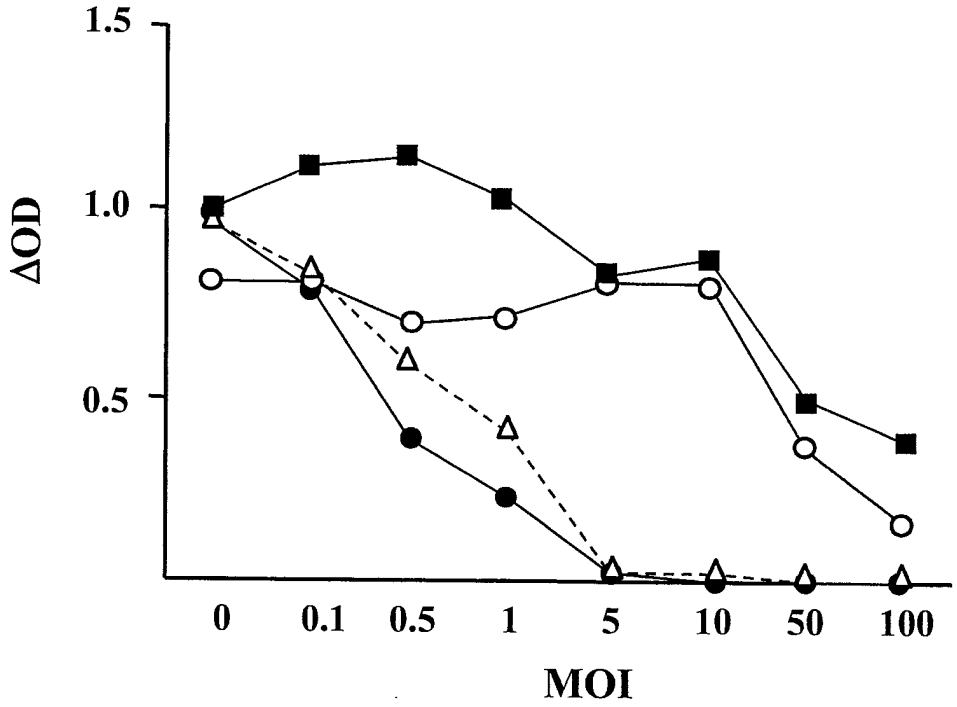
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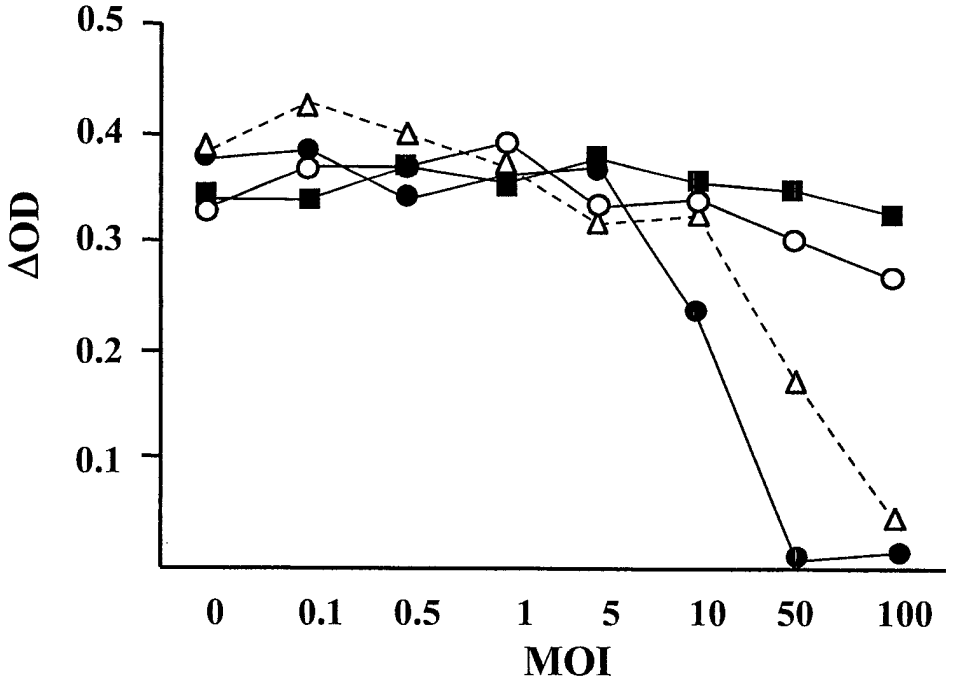
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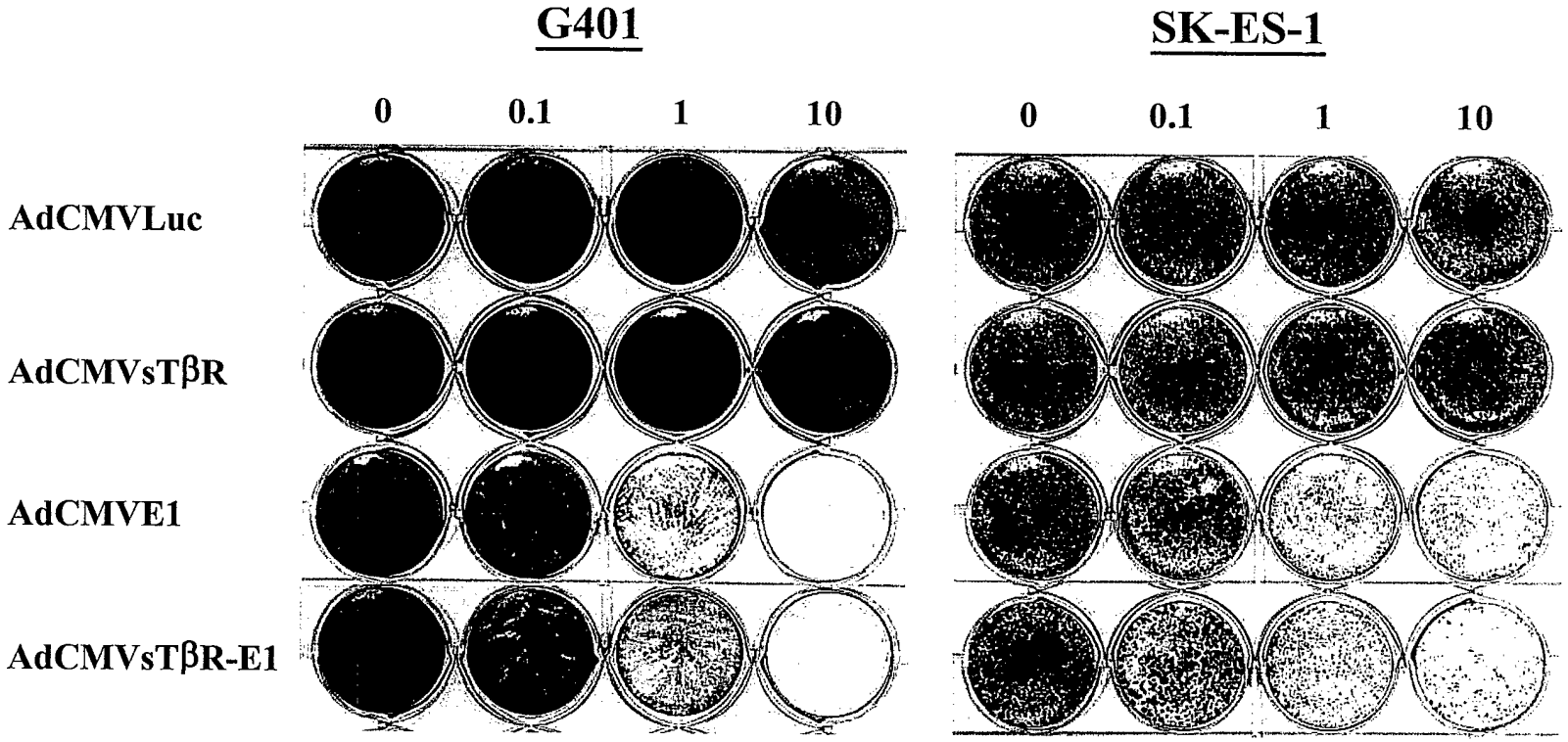
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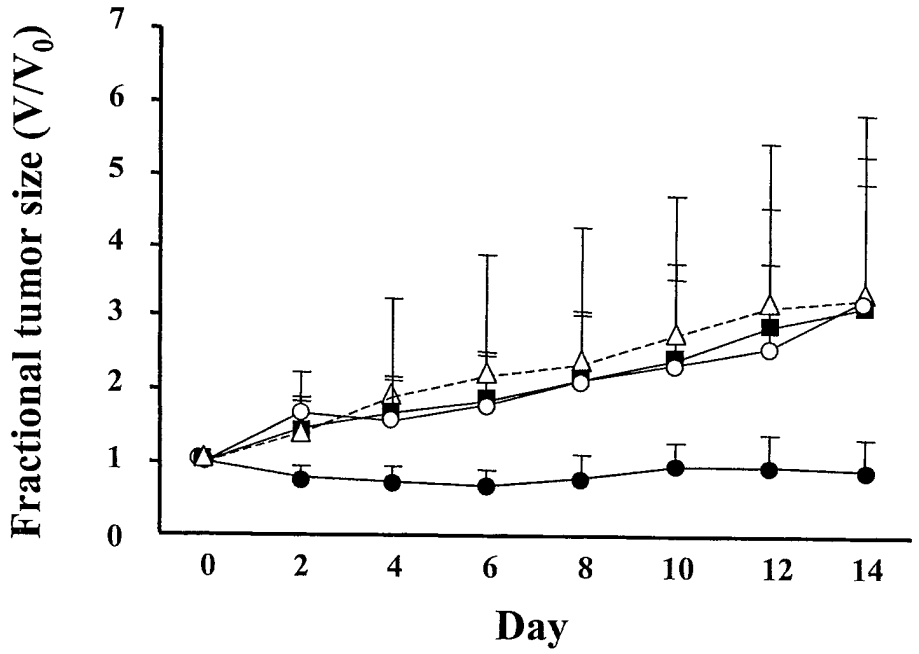
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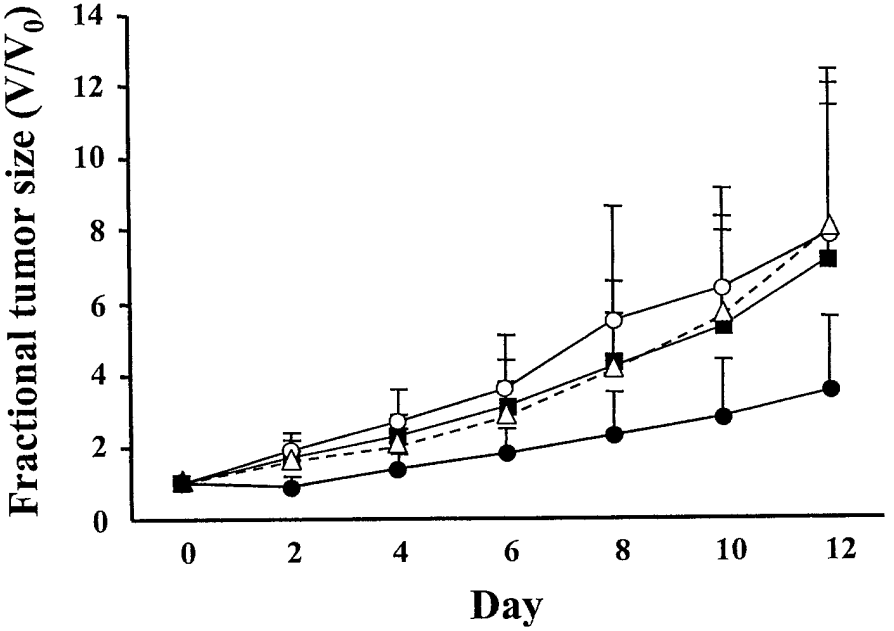
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APPENDIX I

Deletion of E1B 19k improves the efficacy of a midkine-promoter based conditionally replicative adenovirus for neuroblastoma cells[1]

Yasuo Adachi, Minghui Wang, Koichi Takayama, David T. Curiel[2], and Paul N. Reynolds
The Division of Human Gene Therapy, Department of Medicine, Surgery, and Pathology, and Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama 35294 [Y. A., W. M., K. T., D. T. C., P. N. R.]

INDEX WORDS: Replicating adenoviral vector, midkine promoter, and neuroblastomas

Running title: Replicating adenoviral vector controlled by midkine promoter

[1]This work was supported by grants from the US Army Department of Defense DAMD17-00-1-0002, DAMD17-98-1-8571, the National Institute of Health R01 CA83821, P50 CA83591, the Grant CA83821, the Lustgarten Foundation LF043, and the CapCure Foundation to David T. Curiel.

[2]To whom requests for reprints should be addressed: Division of Human Gene Therapy, Departments of Medicine, Surgery and Pathology, University of Alabama at Birmingham, 620 Lurleen B. WTI, 1824 Sixth Ave South, Birmingham, Al 35294. Phone: (205) 934-8527; Fax: (205) 975-7476

[3]The abbreviations used are; NB, neuroblastoma; Ad, adenovirus; MK, midkine; CRAd, conditionally replicative adenovirus; RT, reverse transcriptase; PCR, polymerase chain reaction; GAPDH, glyceraldehydes 3-phosphate dehydrogenase CMV, cytomegalovirus; MOI, multiplicity of infection; DMEM, Dulbecco's Modification of Eagle's Medium; FCS, fetal calf serum; PBS, phosphate buffer saline; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium: tsp, tumor specific promoter.

Abstract

Conditionally replicative adenoviruses (CRAds) are being considered as novel therapies for a variety of malignancies, and several clinical trials have been undertaken. Since neuroblastomas (NBs) are sensitive to adenoviral infection, and advanced NB is refractory to conventional therapy, we have evaluated the use of CRAds for this disease. We previously constructed a replication competent adenovirus (AdMKE1) that achieves tumor-specific replication by virtue of the replacement of the native viral E1 promoter with the tumor specific midkine (MK) promoter. This vector was shown to have utility in an in vitro bone marrow tumor cell purging model. However, clinical trials are revealing that the efficacy of first generation CRAds is suboptimal, and that further modifications or complementary approaches may be necessary. In this regard, it has been reported that replication competent Ad with a deletion of the E1B 19kD gene achieves more effective oncolysis than vectors with intact E1. Thus, we constructed a new virus, AdMKE1(19kdel), in which the modified E1 region is under the control of the MK promoter, and evaluated the oncolytic effect and replication properties of this virus in neuroblastoma cells in vitro. AdMKE1(19kdel) showed a significant improvement in cell killing effect in the SK-N-SH NB cell line as compared to the original AdMKE1 virus, whilst retaining its cell killing specificity. This improvement was not related to the replication efficiency of this virus, but possibly due to the early cell burst and early virus release from the NB cells. Thus, the combination of the MK promoter and the E1B modification may ultimately lead to a more effective clinical agent.

Introduction

Advanced NB is refractory to conventional therapies and the treatment of this condition is one of the most challenging problems in pediatric oncology (1-3). We have been investigating a new strategy to treat advanced NB using conditionally replicative adenoviruses (CRAds) (4). CRAds are modified viruses designed to replicate exclusively in the tumor cells, thus achieving therapeutic cytolysis whilst being non-toxic to normal tissues (5-7). Several clinical trials using CRAds have been undertaken, and the early evidence suggests that while this approach is safe, the tumor cell killing efficacy is often suboptimal. Thus, further improvements in the basic strategy are required.

We previously established a novel virus (MK-CRAd) whose E1A transcription is controlled by tumor specific midkine (MK) promoter (4). MK is a heparin-binding growth factor (8,9) with properties including a neurotrophic function (10), anti-apoptotic activity (11), and angiogenic activity (12). The use of the MK promoter as the basis for oncolytic therapy for the NB is logical because MK is over-expressed in NBs and strong expression of MK is correlated with poor prognosis (13). Moreover, this promoter retains a desirable feature of minimal activity in the liver even in the Ad context (14). In the case of intravenous Ad vector injection, mitigation of liver toxicity is a key property, because the liver is the predominant site of Ad vector localization after systemic administration (14-16). Although the use of non-replicating Ad vectors carrying a suicide gene may be an effective approach (17,18), it is questionable as to whether these anticancer strategies can transduce therapeutic genes to NB with sufficient efficacy for an optimal therapeutic effect. This limitation is of particular importance in these tumor types, which have less

cell-to-cell contact than epithelial neoplasms, and therefore less bystander effect, which is an important factor in the efficacy of suicide gene therapy using the HSV-TK system (19). On the other hand, a CRAd strategy may circumvent the limitations of the loose cell contact observed in NB tumors. Furthermore, NB has less abnormality of p53 compared with adult cancers (20), which implies that NB may not be susceptible to Ad p53 therapy. Based on these rationales, we evaluated a MK-CRAd as a therapeutic agent for NB (4). However, the NB cell killing effect induced by the MK-CRAd infection was lower than that observed in the other MK-postive tumors tested (Ewing's sarcomas or Wilms' tumors). Some modifications of the MK-CRAd seemed to be required to achieve sufficient oncolysis of NBs. One such approach involves more extensive manipulation of the Ad genome, particularly with respect to the early genes. The Ad genome carries five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units, and one late unit. E1A expression activates every viral early gene promoter and plays an important role in the regulation of viral replication and propagation. Recently, it has been reported that a replicating Ad deleted for the E1B19k gene showed enhanced cell killing efficacy compared with a replicating Ad having an intact E1 region (21,22). This property may be related to earlier viral release from infected cells and improved cell to cell spread. Thus, we have modified the MK-CRAd by deleting E1B19k region from original MK-CRAd and evaluated the oncolytic efficiency of these vectors against NB SK-N-SH cells

Materials and Methods

Cells and cell culture

The NB SK-N-SH and Wilms' tumor G-401 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in the medium recommended by the manufacturer. The melanoma MeWo cell line was a kind gift of Prof. Ian R. Hart (St. Thomas Hospital, London, United Kingdom). The 911 cell line (23), a human embryonic retinoblastoma line transformed with a plasmid containing E1 region of the Ad5 genome, was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and used for initial virus generation and propagation. All media and FCS using in this study were purchased from Mediatech/Cellgro (VA).

Viruses and Viral Techniques

The construction of the replication competent Ad AdMKE1, including adenoviral E1A region under the control of human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5' flanking region of the human MK gene (24), was described in the previous report (4). A deletion of the adenoviral E3 region was required to insert the 2.3kb of the MK promoter into the Ad genome. To generate an E1B modified Ad, we made shuttle vectors containing E1 region with a deletion of E1B 19k coding region (1712-2000) using PCR methods. These shuttle vectors were then used for homologous recombination with an Ad backbone plasmid (pAdEasy1) (25). The recombinants were transfected into the E1 transcomplementing 911 cell line to generate AdMKE1(19kdel). These MK-CRAds were propagated in 911 cells and purified by double CsCl density centrifugation. We also constructed AdCMVE1, replacing MK promoter in AdMKE1 by CMV enhancer/promoter, as a control vector. The recombinant Ad vector AdMKLuc (14),

encoding firefly luciferase gene under the control of human MK promoter, was used as a replication deficient control. Wild-type Ad (Adwild) was used as a replication competent control. Virus titers (plaque forming units, pfu) were determined by counting the plaque formation in 911 cells 14 days after infection.

RNA preparation and Reverse Transcription of RNA and PCR amplification of cDNA (RT-PCR)

Total RNA of Ad infected tumor cells was extracted using an RNeasy kit (Qiagen). GeneAmp RNA PCR core kit (Applied Biosystems) was available for cDNA synthesis and PCR amplification of cDNA products. DNase 1 digestion followed by heat inactivation procedure was performed to avoid viral genome DNA contamination in the RNA samples. Oligonucleotides for primer to detect the transcripts of Ad E1A, E1B(19k), E1B(55k), and human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (26) are as follows, Ad E1A; sense strand (5'-ACGGTTGCAGGTCTTGTCATTATC-3': 1014-1038), antisense strand (5'-AAGCAAGTCCTCGATACATTCCA-3': 1494-1472): Ad E1B(19k); sense strand (5'-GCTTGGGAGTGTTTGGAAGATTT-3': 1720-1742), antisense strand (5'-CTGCTCCTCCGTCGGTATTAT-3': 2160-2140): Ad E1B(55k); sense strand (5'-GAATGAATGTTGTACAGGTGGCT-3': 2239-2261), antisense strand (5'-AGGAAAACCGTACCGCTAAAATTG-3': 2773-2750) (27): human GAPDH; sense strand (5'-TCCCATCACCATCTTCCA-3': 276-293), antisense strand (5'-CATCACGCCACAGTTTCC –3': 655-638). Although there are two kinds of transcripts (12S and 13S) originated from E1A region, the PCR primers for E1A can only detected the 13S transcript and cannot detect the 12S transcript. Five hundred ng of total RNA of

tumor cells were applied to a standard RT-PCR protocol. The PCR condition was 30 or 35 cycles of denaturation (95°C, 1 min); annealing (52°C, 1 min) and extension (72°C, 1 min). PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide staining.

Real-time PCR (LightCycler, Roche) analysis combined with reverse transcriptase preparation was also used for the measurement of E1A transcripts. Oligonucleotides corresponding to the sense strand of Ad E1 region (5'-AACCAGTTGCCGTGAGAGTTG-3': 1433-1453), the antisense strand of E1 region (5'-CTCGTTAAGCAAGTCCTCGATACA-3': 1500-1476), TaqMan probe (5'-CACAGCCTGGCGACGCCCA-3': 1473-1455) (27) were synthesized and used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20 sec), annealing (55°C, 20 sec) and extension (72°C, 30 sec). Ad backbone vector pTG3602 (Transgene, Strasbourg, France) was available for making a standard curve for Ad E1A DNA copy number. E1A copy numbers were normalized by the total RNA (ng).

Oncolysis assay

SK-N-SH or MeWo cells were plated in 12-well plates at the density of 2×10^5 /well or 1×10^5 /well respectively. After overnight culture, cells were infected with non-replication or replication-competent Ads at several MOIs (multiplicity of infection) for 3 hours. The infecting medium was then replaced with complete medium. Eight days after infection, the adhesion cells were washed gently with PBS (phosphate buffer saline) and

fixed with 10 % buffered formaldehyde, and then processed into the staining with 1 % crystal violet solution.

MTS assay was also available for the evaluation of oncolytic efficacy induced by CRAd infection. Tumor cells were plated in 96-well plates in triplicate at the density of 3000/well. After overnight culture, cells were infected with non-replication or replication competent Ads at various MOIs for 3 hours. The infecting medium was then replaced with complete media. Viable cells using MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) were evaluated at 8 days after virus infection. The MTS color development was analyzed by an EL 800 Universal Microplate Reader (Biotec Instruments Inc.).

Assessment of viral DNA replication in NB cells

SK-N-SH cells were plated in 6-well plates in triplicate at the density of 300,000/well. After overnight culture, cells were infected with non-replication Ad or replication competent Ads at the MOI of 1 for 3 hours and then cultured in complete medium. The infected cells were harvested with their supernatant 1, 3, and 5 days after infection. The viruses were extracted from cell suspension by three times of freeze-thawing method. DNase 1 preparation was performed to remove the naked DNA floating in the virus solution. Then, EDTA, SDS, and proteinase K were added to the solutions (final concentration: EDTA, 20 mM; SDS, 0.5 %; proteinase K, 0.2 mg/ml) for inactivation of DNase 1 and isolation of encapsidated viral DNA. The mixtures were incubated at 52^0 C for 1 hour, and followed by phenol/chloroform extraction. The purified viral DNA was dissolved in distilled water. Real-time PCR analysis was available for evaluating Ad copy number. An Ad E4 copy number was evaluated as a viral replication, because all vectors

using in this study contained Ad E4 region. Oligonucleotides corresponding to the sense strand of Ad E4 region (5'-TGACACGCATACTCGGAGCTA-3': 34885-34905), the antisense strand of E4 region (5'-TTTGAGCAGCACCTTGCATT-3': 34977-34958), TaqMan probe (5'-CGCCGCCCATGCAACAAGCTT-3': 34930-34951) (4) were synthesized and used as primers and probe for real-time PCR analysis. The PCR conditions were as follows: 35 cycles of denaturation (94°C, 20 sec); annealing (55°C, 20 sec) and extension (72°C, 30 sec). The plasmid pTG3602 (containing the entire Ad genome) was available for making a standard curve for Ad E4 DNA copy number. We also evaluated encapsidated viral DNA copy number in the supernatant using the similar procedure.

Results

The Ads used in this study are depicted in figure 1. G-401 Wilms' tumor cells were chosen for the initial evaluation of these vectors, because this cell line is well characterized as a MK positive tumor and is highly sensitive to the Ad infection. We first assessed the profiles of Ad E1A, E1B19k, or E1B55k mRNA expression in G-401 cells after Ad vector infection using RT-PCR. PCR primers for E1A transcripts were designed to detect only 13S transcript in the E1A transcripts. The RT-PCR analysis showed that the cells infected with AdMKE1, AdCMVE1, or Adwild expressed all of E1 components. Although the cells infected with AdMKE1(19kdel) expressed the mRNA corresponding to E1B 55k, they did not express E1B 19k mRNA (Fig. 2).

Next, we investigated the E1A expression level in SK-N-SH cells infected with Ads (Fig. 3A). This experiment was repeated three times, and reproducible results were obtained. Real time RT-PCR analysis was also used for the measurement of E1A transcript levels (Fig. 3B). Previously, we reported that MK promoter activity was about 15 % of CMV activity in SK-N-SH cells in the context of Ad vectors carrying reporter genes (14). The difference of E1A expression between AdMKE1 and AdCMVE1 in this study was consistent with the previous data. E1A expression levels seen with the MK vectors were comparable to that seen with wild-type Ad. Thus, we confirmed the ability of the MK promoter to drive E1A expression in the neuroblastoma line.

To investigate the ability AdMKE1(19kdel) to achieve NB cell killing, crystal violet staining was used to visualize the surviving cells 8 day after infection. As compared with

AdMKE1, AdMKE1(19kdel) achieved a substantial improvement in oncolysis of SK-N-SH cells. It was notable that AdMKE1(19kdel) induced oncolysis more effectively than AdCMVE1 and its efficacy was comparable to that of Adwild (Fig. 4a). To assess the specificity of oncolytic efficacy induced by AdMKE1(19kdel), MeWo cells (MK negative melanoma cell line) were infected with the viruses and the cell killing efficiency was evaluated. The cell-killing effect in the MeWo cells induced by AdMKE1(19kdel) infection was insignificant and comparable with that induced by AdMKE1 which has been reported previously (4). In contrast, infection with AdCMVE1 or Adwild resulted in significant cell killing of MeWo cells (Fig. 4b). Thus, the 19k deletion had achieved an improvement in the cell killing effect for MK positive cells without any loss of specificity. These results were then confirmed using the more quantitative MTS assay. Eight days after virus infection, the advantage of AdMKE1(19kdel) compared to AdMKE1 for cell killing of SK-N-SH is clear (Fig 5A), as is the specificity shown by the relative lack of toxicity for the MK negative MeWo line (Fig 5B).

To examine the correlation between viral replication and oncolytic efficiency, viral DNA production was measured with the passage of time after virus infection (Fig. 6). Viral copy number in the cells infected with AdCMVE1 was about 100 times higher than that with AdMKE1 on day 1 after infection and about 10 times higher on day 3 and 5. Thus, the improved cell killing of AdCMVE1 vs AdMKE1 could be explained on the basis of faster viral replication. On the other hand, AdMKE1(19kdel) did not replicate more effectively than AdMKE1 in SK-N-SH cells, despite the fact that the cell killing effect of AdMKE1(19kdel) was significantly greater than AdMKE1 and was in fact comparable to

AdCMVE1. This would suggest that the efficacy gains seen with AdMKE1(19kdel) are not simply due to increased viral replication.

Previously, it was reported that an E1B-19kD mutant virus is released more rapidly from the tumor cells and that may lead to the improvement of the cell killing efficiency (21,22). Thus, we investigated whether AdMKE1(19kdel) might be released from NB cells earlier than other viruses after infection. SK-N-SH cells were plated in 6-well plates in triplicate at the density of 100,000/well. After overnight culture, cells were infected with Ads at the MOI of 10 for 3 hours and then cultured in complete medium. Supernatants were harvested 3 and 5 days after infection and the DNA copy number of encapsidated virus in the supernatants were measured (Fig. 7). Notably, virus DNA copy number in the supernatant of AdMKE1(19kdel) infection group was higher than that of AdMKE1 or AdCMVE1 at day 3. These data suggest that AdMKE1(19kdel) is released from NB cells earlier compared with other CRAds lacking the Ad E3 region. However, the amount of released virus from the NB cells infected with Adwild was comparable to that of AdMKE1(19kdel) (Fig. 7a). The prominent number of released virus observed in the Adwild infection possibly due to the efficacious virus production peculiar to native viruses (Fig.7b), especially the fact that the WT virus contains E3, which is known to enhance cytolytic effect.

Discussion

The potential utility of Ad as a therapeutic oncolytic agent has been considered at least since the middle of 20th century. The accumulation of knowledge about Ad in recent years has seen the re-emergence of this concept with the development of CRAds for cancer therapy. The key concept of the CRAd strategy is achievement of tumor cell killing via tumor-specific virus replication (5-7, 28), and control of E1A expression by using a tumor specific promoter (tsp) is a promising method to confer upon Ads tumor specific replication properties (29,30). Many tsps, including MK promoter, have been evaluated for this approach, and a virus containing the prostate specific antigen (PSA) promoter has already been employed in human clinical trial (31).

We have been evaluating the development of therapeutic viral agents for advanced NBs or other pediatric tumors. The treatment of advanced NBs is one of the most difficult problems in the pediatric surgical field. Fortunately, NBs, even the case of primary tumors, are susceptible to the Ad infection (32) and several authors have proposed Ad vector-based gene therapy for these conditions (14,17,33). We have extended the potential use of Ads in this disease now to include CRAds based on the MK promoter. Our previous study illustrated the potential utility of the CRAd approach for bone marrow purging in a model system which has relevance for the high dose chemotherapy/bone marrow transplant approach for advanced NB or Ewing's sarcoma patients (4). However, NB SK-N-SH cell killing effect induced by AdMKE1 infection was lower than that observed in Ewing's sarcomas or Wilms' tumors.

The Ad 19k protein encoded in E1B region is an Ad homologue of Bcl-2 protein, an anti-apoptotic agent (34,35). Hay *et al.* reported that the replicating Ad without E1B 19k units showed enhanced cell-killing efficacy compared with the CRAds having an intact E1 region (21,22). Thus, we have modified the MK-CRAd by deleting the E1B 19kD protein coding region to improve the oncolytic efficiency against SK-N-SH cells. We presented in this report that AdMKE1(19kdel) actually killed SK-N-SH cells more effectively than AdMKE1. Especially, cell-killing effect of AdMKE1(19kdel) was comparable to that of AdWT. On the other hand, the MeWo cells which we chose as an Ad-infectable MK negative control showed insignificant damage by the AdMKE1(19kdel) infection. These data indicate that E1B modification in the context of a tsp-controlled CRAd is a useful candidate approach to improve oncolytic efficacy whilst retaining specificity.

A multitude of factors contribute to the oncolytic efficacy of CRAds, including the initial infectivity of the vector for the target cells (36,37), efficiency of viral replication within the cell and efficiency of release of the viral progeny and dissemination throughout the tumor mass. We speculated that a higher level of the viral replication might be observed in the cells infected with E1B modified CRAds compared with AdMKE1, because we believed that the viral replication induced the oncolytic effect on SK-N-SH and the viral replication speed was correlated with oncolytic potency. In the case of AdCMVE1, this did indeed seem to be the case – much higher levels of viral DNA were seen at early time points compared to the other viruses. However, the viral replication speeds of E1B modified CRAds were same or less than AdMKE1 in SK-N-SH host cells. Since the E1B 19k protein serves as an anti-apoptotic agent (34), it may be that the Ad vectors defective

in E1B 19k expression show improved cell killing effect without augmentation of the viral replication. As the previous report, the improvement of cell killing effect was mainly due to the early cell burst and early virus release from the NB cells.

It was also noted that Adwild could replicate and induce cell death most efficiently in spite of the lower level of E1A expression in the cells infected with Adwild vs AdCMVE1. We believe this is most likely due to the fact that the wild-type Ad contains E3 whereas the other viruses do not. In this regard, the death protein encoded in E3 region has been reported as the important trigger for the late phase cell burst (38). In the absence of E3, we speculate that E1A expression could play a central role in the cell killing mechanism. The E1A proteins are known as not only key triggers of Ad replication but also strong apoptosis agents *per se* (39,40). Thus, because the E1B 19k protein ordinarily acts to inhibit apoptosis induced by E1A proteins (41), the enhanced cell killing effect seen with AdMKE1(19kdel) may result from the unopposed apoptotic activity of E1A proteins. Re-introduction of E3 protein function into the AdMKE1(19kdel) CRAd (within the size constraints of the genome) may still further improve the utility of this agent.

The clinical application of CRAds is well underway in human trials. The basic safety appears to have been established, but in many cases efficacy has been disappointing. Efforts to improve efficacy by combining CRAds with conventional therapies are now being evaluated. We show here that further improvements in CRAd efficacy can be achieved through 19k deletion – importantly without any loss of specificity of cell-killing

effect. Thus, this approach may be one of several improvements that together achieve greater therapeutic utility for cancer treatment, and in the current context for neuroblastoma (and other MK positive tumors) in particular.

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Figure Legends

Figure 1. Schema of the Ad vectors used in this study. All vectors shown here are constructed from E3 region (nucleotide 28130 to 30820 of Ad genome) deleted Ad type 5 backbone. Ad E1A region is essential to the virus replication, non-replicating virus (AdMKLuc) due to the replacement of Ad E1 region (nucleotide 324-3533 of Ad genome) to MK promoter-luciferase gene sequence. AdMKE1, AdCMVE1, and AdMKE1(19kdel) are the CRAd vectors used in this study and do not contain the Ad E1A promoter region expanding from nucleotide 324 to 488 of the Ad genome. AdCMVE1 and AdMKE1 differ in the promoter driving E1A expression. AdMKE1(19kdel) is defective in the E1B 19kD protein coding region. We also used an adenovirus type 5 as a wild type Ad control (Adwild).

Figure 2. Evaluation of Ad viral E1 expression in G-401 cells after Ad vector infection. M stands for a marker DNA. Lane1, AdMKLuc; Lane 2, AdMKE1; Lane 3, AdMKE1(19Kdel); Lane 4, AdCMVE1; Lane 5, Adwild. These data meant that each Ad vector expressed the anticipated E1 mRNAs.

Figure 3a. Evaluation of E1A expression in the SK-N-SH cells after Ad vector infection by virtue of RT-PCR method. M stands for a marker DNA. Lane1, AdMKLuc; Lane 2, adMKE1; Lane 3, AdMKE1(19kdel); Lane 4, AdCMVE1; Lane 5, Adwild; Lane 6, mock infection.

Figure 3b. Quantitative evaluation of E1A transcripts after Ad vector infection. the quantitative real-time PCR method was available for the measurement of the E1A mRNA.

Figure 4. Oncolytic efficacy induced by the infection with CRAds and controls. Cell killing efficiency was evaluated by the crystal violet staining. Survived cells on 8 days after Ad vector infection were stained in violet.

Figure 5. Oncolytic efficacy evaluated by MTA assay. The formazan product was measured by absorbance (OD) at 490 nm. We made blank controls of culture medium without cells and measured the absorbance (blank OD) after MTS procedure. ΔOD =OD – blank OD.

Figure 6. Assessment of viral DNA replication in course of time after infection. Since all vectors tested here contained complete E4 region, the viral DNA replication rate was evaluated by measuring the E4 copy number using the quantitative real-time PCR method. Data are means of triplicate determinations.

Figure 7. Encapsidated virus DNA copy number in SK-N-SH supernatants. Data are means of triplicate determinations. E4 copy number was measured as Ad virus DNA copy number. A), the results 3 days after infection. B), the results 5 day after infection.