A Conditionally Replicative Adenovirus with Enhanced Infectivity Shows Improved Oncolytic Potency[1]

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ABSTRACT

The absence or the presence of low levels of the Coxsackievirus and adenovirus receptor (CAR) on several tumor types might limit the efficacy of recently proposed tumor-specific or conditionally replicative adenoviruses (CRAds). To address this issue, we used a genetic modification of the fiber knob in the context of an E1A-defective CRAd to allow CAR-independent target cell infection as a means to enhance oncolytic potency. Such infectivity-enhanced CRAd showed higher replication, more efficient infection, and lysis of tumor cells *in vitro*. Of note, the improved antitumor effect of the fiber-modified CRAd could be demonstrated *in vivo*. We conclude that the combination of genomic modification to achieve tumor-selective replication and capsid modification to enhance infectivity yields more potent oncolytic adenoviruses for use in cancer treatment.

INTRODUCTION

CRAds[3] represent a novel and promising approach for treating neoplastic diseases (1, 2). The use of CRAds offers two advantages over conventional gene therapy. First, CRAds have an intrinsic amplification capacity that allows extensive tumor infection, leading to expansive oncolysis by reason of the actual cytopathic effect of the virus. Second, the restriction of viral replication to tumors avoids damage to normal host tissues and improves the therapeutic index. Two strategies have been implemented to achieve specificity: the control of the expression of an essential early viral gene by using tumor-specific promoters (3); and deletions in viral genes encoding proteins that interact with cellular proteins necessary to complete the viral lytic life cycle in normal cells, but not in tumor cells (4). Both CRAd-based strategies have been rapidly translated into clinical trials (5, 6).

However, realization of the full utility of CRAds in cancer therapeutics depends on their ability to infect human tumors. Previous studies on adenovirus-mediated gene delivery to human tumor cells have pointed out the highly variable expression of primary adenoviral receptor, CAR, in neoplastic cells (7, 8), and this variation may curtail the initial infection and lateral propagation of CRAds (9). On the basis of these data, it has been proposed that gene delivery via CAR-independent pathways is required to overcome this aspect of tumor biology (10, 11). We have focused on α_V integrins as enhancers of adenoviral infection according to a previous report on the correlation of the levels of α_V integrins expressed by tumor cells with the efficiency of adenovirus-mediated gene transfer (12). Furthermore, previous studies demonstrate that α_V integrins are aberrantly expressed in several types of cancer (13, 14) and are present in tumor blood vessels of breast cancer and malignant melanoma (15).

Modifications of capsid proteins responsible for adenovirus binding to target cells can alter its tissue tropism. These data favor the incorporation of an Arg-Gly-Asp (RGD) sequence, known to interact with α_V integrins, into the adenovirus fiber to enhance tumor infection. Recently, we developed an approach based on the genetic incorporation of a sequence encoding an RGD peptide into the HI loop of the fiber knob. The addition of RGD-integrin interactions on primary CAR binding confers an expanded tropism to the fiber-modified adenovirus, and this effect has been demonstrated in previous studies (16, 17).

In this study, we combined the fiber knob modification strategy with a CRAd based on a partial deletion of the *E1A* gene, which synthesizes a defective protein unable to bind host cell Rb protein. The selectivity of this mutant adenovirus has been previously demonstrated by Fueyo *et al.* (18) and recently by another group that uses a virus with the same deletion (19). Our results demonstrated that the incorporation of the RGD motif into the fiber of a CRAd enhances its oncolytic potency *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines

A549 human lung adenocarcinoma and LNCaP human prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines are defective

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[3] The abbreviations used are: CRAd, conditionally replicative adenovirus; BrdUrd, bromodeoxyuridine; CAR, Coxsackievirus and adenovirus receptor; i.t., intratumoral/intratumorally; Rb, retinoblastoma; FBS, fetal bovine serum; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide.

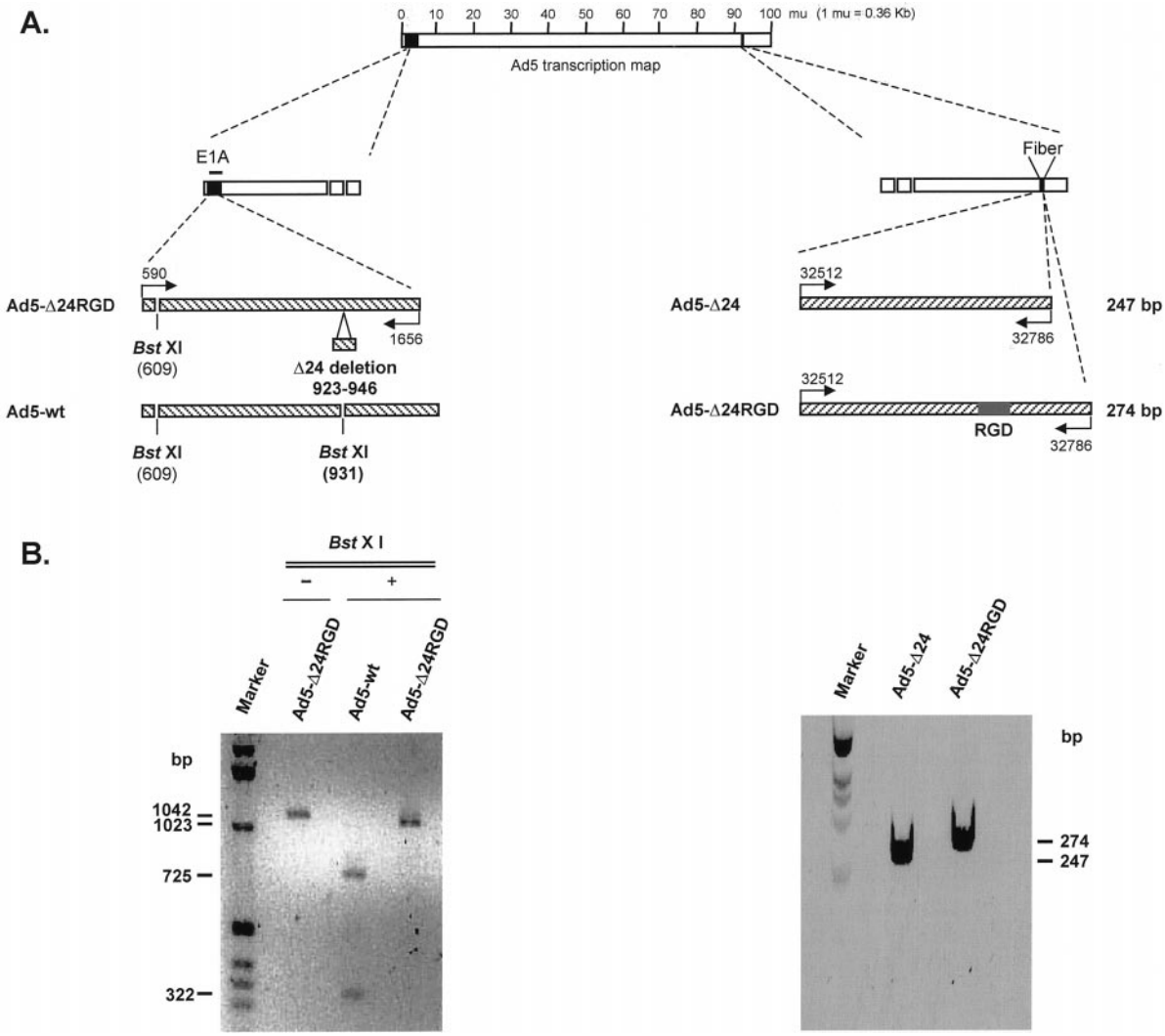

Fig. 1 Analyses of adenoviral DNA. *A*, map of E1A- and fiber-encoding regions of Ad5-Δ24RGD amplified by PCR, showing the 24-bp deletion and the introduced RGD-encoding sequence. *B*, restriction analysis of Ad5-Δ24RGD. The presence of the 24-bp deletion was confirmed by *Bst*XI digestion of the PCR product of the E1A region. The fragments were resolved on a 2% agarose gel, and visualized by UV fluorescence. *Left*, *Marker* (Life Technologies, Inc.), 1-kb DNA ladder. The presence of uncleaved PCR product verified the presence of the deletion. PCR amplification products of the region encoding the fiber from Ad5-Δ24 and Ad5-Δ24RGD were resolved on a 6% acrylamide gel. *Right*, *Marker* (Life Technologies, Inc.), 100-bp DNA ladder. The bigger size (27 bp) of Ad5-Δ24RGD band indicates the presence of the sequence encoding RGD.

in the Rb pathway because of a deficiency in $p16^{INK4}$ (20–22). The cells were cultured in DMEM supplemented with 5% heat-inactivated FBS, 100 I.U./ml penicillin, and 100 μg/ml streptomycin.

Virus Construction

Ad5-Δ24 Mutant. The replication-competent Ad5-Δ24 adenovirus was provided by J. F. (The University of Texas M. D. Anderson Cancer Center, Houston, TX). This virus contains a 24-nucleotide deletion, from Ad5 bp 923 to 946 (both included), corresponding to the amino acid sequence L_{122}TCHEAGF$_{129}$ of the E1A protein known to be necessary for Rb protein binding (23). Details of the tumor-specific replication of this virus are presented elsewhere (18, 19).

RGD Modification of Ad5luc and Ad5-Δ24. Ad5lucRGD is an E1-deleted virus containing the recombinant RGD fiber and expressing the firefly luciferase. This vector was constructed by homologous recombination of the E1 region containing the *luciferase* gene into the plasmid pVK503 that contains the modified fiber (15). A similar procedure was followed to construct the RGD-modified version of Ad5-Δ24. Briefly, an E1 fragment containing the 24-bp deletion was isolated from the plasmid pXC1-Δ24, originally used to construct Ad5-Δ24 (18), and cloned by homologous recombination into the *Cla*I–digested plasmid pVK503 containing the RGD fiber (15). The genome of the new virus was released from the plasmid backbone by digestion with *Pac*I, and the resulting fragment was used to transfect 293 cells to rescue the Ad5-

Δ24RGD. The presence of the RGD motif in Ad5-Δ24RGD and Ad5lucRGD was confirmed by PCR with the fiber primers FiberUp (5′-CAAACGCTGTTGGATTTATG-3′) and Fiber-Down (5′-GTGTAAGAGGATGTGGCAAAT-3′). The Δ24 deletion was analyzed by PCR with primers E1a-1 (5′-ATTAC-CGAAGAAATGGCCGC-3′) and E1a-2 (5′-CCATTTAA-CACGCCATGCA–3′) followed by *Bst*XI digestion.

Virus DNA Replication. A549 cells, cultured in 6-well plates, were infected with Ad5-Δ24 or Ad5-Δ24RGD at a dose of 0.01 viral particles/cell. The cells were maintained in DMEM-5% FBS with 1 μCi/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested at 2, 4, 6, and 8 days after infection, and encapsidated viral DNA was purified by the spermine-HCl method (24). The DNA was digested with *Hin*dIII and resolved in 1% agarose gel. The BrdUrd incorporated into the DNA resulting from viral replication was detected by Southwestern blot using mouse anti-BrdUrd IgG (DAKO, Carpinteria, CA) and peroxidase-labeled antimouse antibody (Amersham). The membrane was exposed to Kodak Biomax ML film and developed in an auto-mated processor.

Adenovirus Yield Assay. A549 cells cultured in 6-well plates were infected with 0.01 particle/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, and maintained in DMEM-5% FBS. After 8 days, cells and media were harvested, and the titer was determined by plaque assay.

Oncolysis Assay. A549 and LNCaP cells cultured by triplicate in 6-well plates were infected with one of the three types of adenovirus at doses of 0.001 or 0.01 viral particles/cell. Eight (A549) and 10 (LNCaP) days after infection, the cells were fixed and stained with crystal violet solution.

***In Vitro* Cytotoxicity Assay (XTT).** A549 and LNCaP cells were seeded and infected in parallel with the ones used for the oncolysis assay described above. Eight and 10 days after infection, cell survival was determined using XTT (Sigma, St. Louis, MO). The number of living cells was calculated from noninfected cells cultured and treated with XTT in the same way as were the experimental groups.

s.c. Tumor Xenograft Model in Nude Mice. Female athymic nu/nu mice (Frederick Cancer Research, Frederick, MD), 8–10 weeks old, were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Eight million A549 cells were xenografted under the skin of each flank in anesthetized mice. When the nodules reached 60–100 mm^3, a single dose of 10^9 viral particles (high-dose experiment; $n = 5$) or 10^7 viral particles (low-dose experiment; $n = 4$) of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS was administered i.t. Tumor size was monitored twice a week, and fractional volume was calculated from the formula: (length × width × depth) × 1/2. The mice were euthanized 35 days after the treatment because of the size of the tumors 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. Statistical differences among groups were assessed with Student's *t* tests.

Adenovirus Hexon Immunodetection. The presence of adenovirus hexon in the treated tumor xenografts was assessed by immunofluorescence. A549 tumor sections were treated with goat antihexon (Chemicon Inc., Temecula, CA) and Alexa Fluor 488-labeled donkey antigoat (Molecular Probes, Eugene, OR) antibodies, and were counterstained with Hoechst 33342 (Molecular Probes). The slides were analyzed under a fluorescent microscope (Leitz Orthoplan).

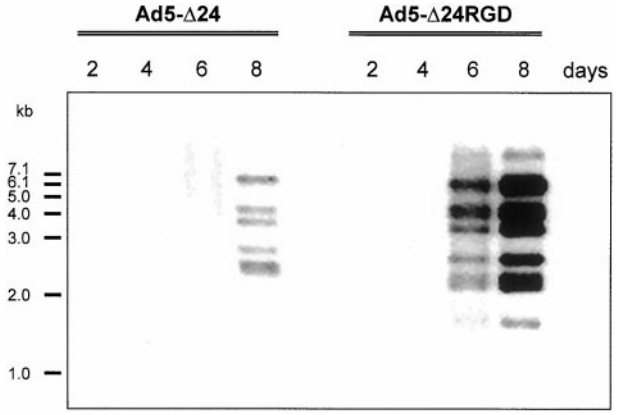

Fig. 2 Propagation efficiency of Ad5-Δ24 *versus* Ad5-Δ24RGD. A549 cells were infected with 0.01 particles/cell Ad5-Δ24 or Ad5-Δ24RGD and incubated in medium containing 1 μCi/ml BrdUrd. At the indicated times after infection, the cells were harvested, and the encapsidated DNA was purified by the spermine-HCl method. Viral DNA from 6×10^5 infected cells was digested with *Hin*dIII and electrophoresed, and the resulting fragments were blotted into a membrane that was processed with a mouse anti-BrdUrd antibody. The amount of BrdUrd incorporated into viral DNA indicated that Ad5-Δ24RGD propagation is more efficient than that of Ad5-Δ24.

RESULTS

Propagation Advantage of an RGD-modified CRAd. The Δ24 deletion of E1A and the RGD insertion in the fiber knob were combined into a unique viral genome by homologous recombination, and the resulting Ad5-Δ24RGD was propagated efficiently in A549 cells. The 24-bp deletion in the *E1A* gene and the RGD-encoding sequence in the fiber were verified by PCR (Fig. 1). Of note, no adenoviruses having wild-type E1 or wild-type fiber appeared throughout the propagation of Ad5-Δ24RGD, a finding that confirms the lack of endogenous adenoviral sequences in A549 cells.

After structural confirmation, the replication capacity of Ad5-Δ24RGD and Ad5Δ24 was compared. A549 cells were infected with 0.01 viral particle per cell of each virus and were maintained in medium with BrdUrd throughout the 8-day incubation period. The encapsidated viral DNA was purified on days 2, 4, 6, and 8 postinfection, and the samples were analyzed by Southwestern blot as described in "Materials and Methods." As indicated by the BrdUrd incorporated into replicating viral DNA, Ad5-Δ24RGD propagation was more efficient than that of Ad5-Δ24 (Fig. 2). The Ad5-Δ24RGD DNA can be detected not only sooner (day 6) compared with Ad5-Δ24 DNA (day 8) but in greater amounts. Thus, the infectivity advantage conferred by RGD incorporation into the fiber knob increased adenovirus propagation in target cells.

Increased Viral Yield of Infectivity-enhanced CRAd *in Vitro*. On the basis of the previous experiment, we decided to compare the amount of infectious virus produced by

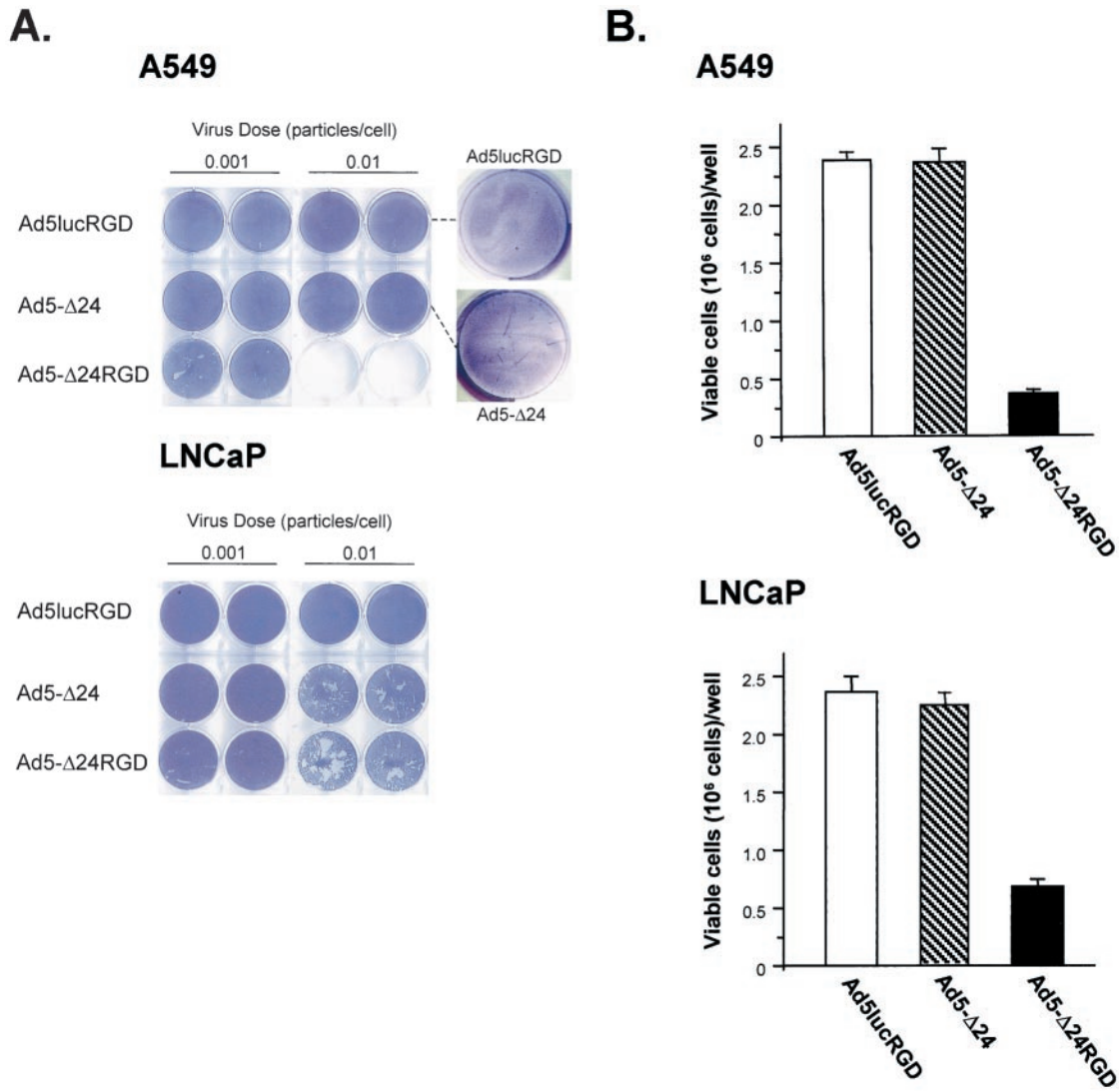

Fig. 3 Oncolytic potency of the RGD-modified virus. In *A*, A549 and LNCaP cells were infected with 0.001 or 0.01 particles/cell Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD. Eight (*A549*) and 10 days (*LNCaP*) later, the cells were fixed and stained with crystal violet. A higher magnification of two wells is presented to show the incipient cytopathic effect of Ad5-Δ24. In *B*, in parallel, cell viability was analyzed with an XTT colorimetric assay. In both cell lines, Ad5-Δ24RGD had higher lytic potency than did its unmodified counterpart, as shown by the percentage of viable cells remaining in the corresponding treatment conditions.

Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD in A549 cells at 8 days after infection by plaque assay. Ad5-Δ24RGD produced a viral yield of 3.75×10^9 plaque-forming units/ml which was 43 times higher than that of its unmodified Ad5-Δ24 counterpart (8.75×10^7 plaque-forming units/ml). No virus was obtained from the nonreplicative control Ad5lucRGD-infected cells. These results are consistent with the fact that modifying the fiber knob with an RGD motif led to enhancement of viral infectivity and an increase in the production of infectious adenovirus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vitro*. To demonstrate the increased lytic potency of Ad5-Δ24RGD, we infected A549 and LNCaP cells with small amounts of each virus to allow multiple cycles of viral replication over the ensuing 8 days, then stained the attached cells with crystal violet and counted viable cells by XTT assay. In both cell lines, the fewest viable cells were detected in the Ad5-Δ24RGD-infected group (Fig. 3, *A* and *B*). The cell lysis capacity of Ad5-Δ24RGD is 7 times higher in A549, and 3.5 times higher in LNCaP compared with Ad5-Δ24. These results demonstrate that the fiber knob modification enhanced adenoviral lytic potency over that of the Ad5-Δ24 virus.

Increased Oncolytic Potency of Infectivity-enhanced CRAd *in Vivo*. The ultimate goal of this study was to demonstrate the oncolytic superiority of infectivity-enhanced CRAds over that of unmodified adenoviruses *in vivo*. Because low doses of virus allow several cycles of replication along with destruction of tumor cells, even a single dose

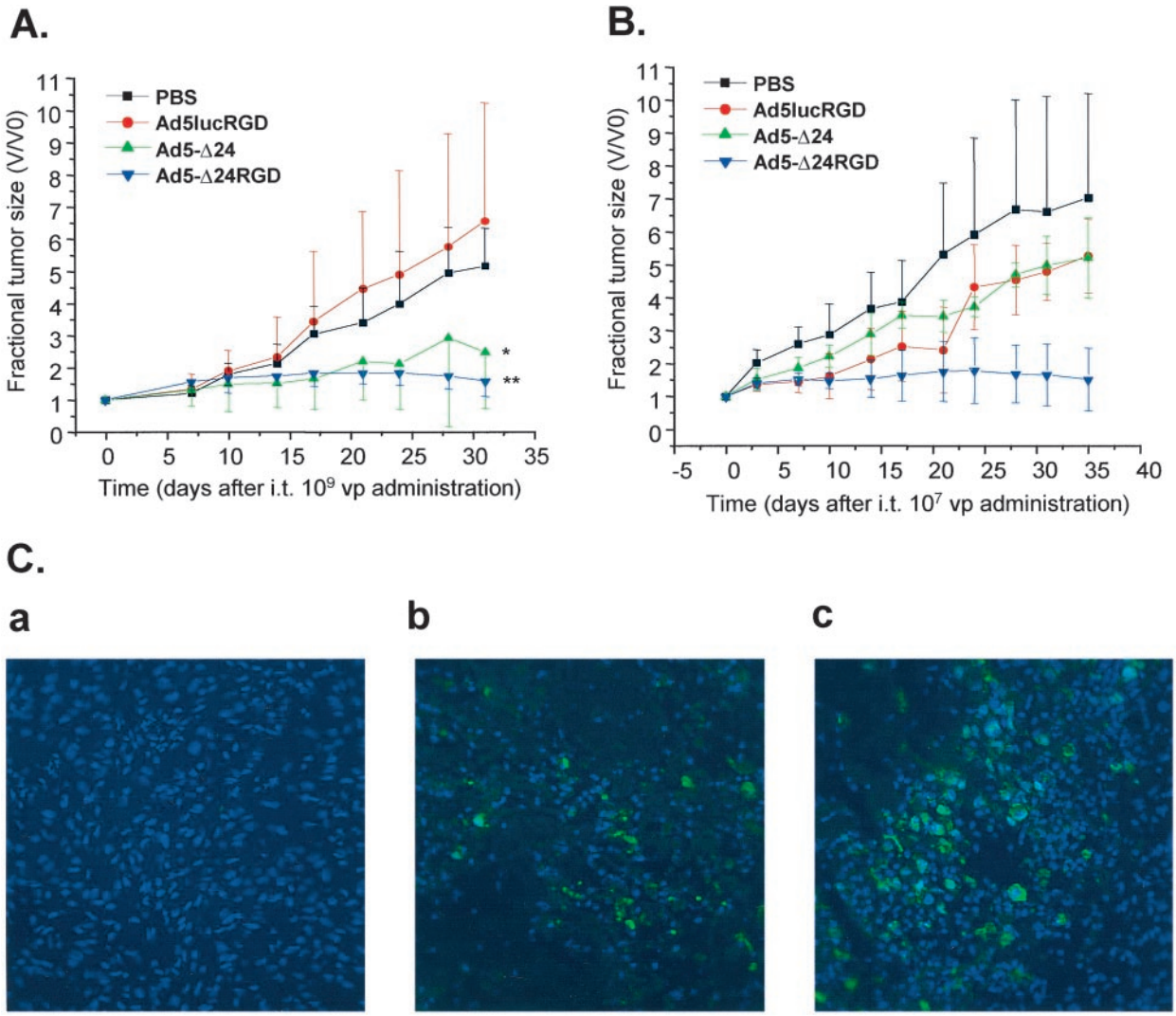

Fig. 4 *In vivo* oncolysis by high and low doses of infectivity-enhanced CRAds. s.c. A549 xenografts in nude mice were treated with a single i.t. injection of (*A*) 10^9 viral particles or (*B*) 10^7 viral particles of Ad5lucRGD, Ad5-Δ24, or Ad5-Δ24RGD, or with PBS alone. Tumor size was measured twice a week. Results are shown as fractional tumor volumes (*V*/*V0*, where *V* = volume at each time point, and *V0* = volume at adenovirus injection); *each line*, the mean of five tumors (±SD) in the high-dose group, and 4 tumors (±SD) in the low-dose group. In the high-dose experiment, both CRAds show a similar oncolytic effect that results in smaller tumors compared with PBS-treated groups (*, Ad5-Δ24, $P < 0.05$; **, Ad5-Δ24RGD, $P < 0.01$). However, in the low-dose experiment, tumors treated with Ad5-Δ24 followed a growth curve similar to that of tumors treated with nonreplicative Ad5lucRGD, whereas tumors treated with Ad5-Δ24RGD did not grow ($P < 0.01$ compared with PBS). *C*, detection of adenovirus hexon in tumor xenografts by immunofluorescence. Frozen sections of tumor specimens injected with (*a*) Ad5lucRGD, (*b*) Ad5-Δ24, and (*c*) Ad5-Δ24RGD were treated with goat antihexon antibody and Alexa Fluor 488-labeled donkey antigoat antibody, and nuclei were counterstained with Hoechst 33342. Images were captured from Leitz fluorescence microscope (×100) with a double filter. Sections taken from tumors treated with CRAds were positive for adenovirus presence (*green dots in b* and *c*); Ad5-Δ24RGD signal was stronger than that of Ad5-Δ24. Samples taken from tumors treated with PBS (not shown) or Ad5lucRGD exhibited no hexon signal (*a*). *vp*, viral particles; *Ad*, adenovirus.

would produce an exponential rise in the number of killed cells, which would extend to the entire tumor. To demonstrate this hypothesis, we treated A549 xenografts in nude mice with a single i.t. injection (10^9 viral particles) of one of the three viruses or with PBS. At 32 days after injection, both CRAds had an oncolytic effect in the tumors opposite to that of those treated with nonreplicative virus or with PBS (Ad5-Δ24, $P < 0.05$; Ad5-Δ24RGD, $P < 0.01$ compared with PBS group; Fig. 4*A*). Given these results, we conducted another experiment in which we administered a 100-fold lower dose (10^7 viral particles) of the viruses. At this dose, Ad5-Δ24 treatment did not show a statistically significant difference compared with either PBS or AdlucRGD. However, it demonstrated that the oncolytic effect of Ad5-Δ24RGD is maintained (Ad5-Δ24RGD *versus* PBS, $P < 0.01$; Ad5-Δ24RGD *versus* Ad5-Δ24, $P < 0.05$). These variations observed between high-dose and low-dose experiments suggest that a threshold dose over 10^7 viral particles of Ad5-Δ24 is required

to obtain an oncolytic effect in tumor nodules (Fig. 4*B*). To confirm that the CRAds were present in the tumor tissue, we used immunofluorescence to detect the virus hexon in tumor samples collected after the low-dose experiment (35 days postinjection). Ad5-Δ24RGD was present in the tumor nodules, as was Ad5-Δ24 to a lesser extent. PBS- and Ad5lucRGD-treated nodules showed no hexon signal (Fig. 4*C*). These results corroborated the observation that the partial reduction of tumor mass was attributable to virus replication and that the RGD modification of the fiber knob conferred infectivity and oncolysis advantage to a CRAd *in vivo*.

DISCUSSION

CRAds are novel and promising agents for cancer therapy. However, their efficacy is predicated on efficient tumor infection, specific replication, and lateral spread. The deficiency of CAR in a variety of tumor targets is a limitation to adenovirus infection. In a previous report, we demonstrated that the insertion of an RGD motif into the HI loop of the fiber knob of nonreplicative adenoviruses enhances tumor infection (16, 17). This proves that CAR-independent entry represents a viable way to circumvent CAR deficiency in some tumor types.

In this report, we have demonstrated that the genetic introduction of an RGD sequence in the fiber of a CRAd, such as previously characterized Ad5-Δ24 (18), allows CAR-independent infection that leads to the enhancement of viral propagation and oncolytic effect *in vitro* and *in vivo*. The increased initial virus entry into the cells rendered by the RGD modification results in earlier detection and augmented yields of encapsidated DNA of Ad5-Δ24RGD compared with the unmodified Ad5-Δ24 (Fig. 2). Because this tropism modification is not anticipated to alter fundamental aspects of the viral replication cycle, this effect was likely attributable to the infectivity enhancement allowed by delivering the virus through CAR-independent pathways. Subsequently, we studied the oncolytic potency of CRAds in two cell lines and concluded that Ad5-Δ24RGD potency is higher than that of the unmodified virus. Although the XTT assay was not sensitive enough to demonstrate the lytic effect of Ad5-Δ24 compared with the nonreplicative Ad5lucRGD, the crystal violet showed early comet-like cytopathic areas in Ad5-Δ24-treated A549 and LNCaP cells, which indicated the presence of an incipient lytic effect, whereas Ad5lucRGD-treated cells were intact (Fig. 3*A*). The less notable difference between Ad5-Δ24RGD and Ad5-Δ24 seen in LNCaP cells is explained by the absence of the $\alpha_V\beta_3$ integrins (25), compensated by the presence of other types of RGD-binding integrins ($\alpha_3\beta_1$ and $\alpha_5\beta_1$; Ref. 26) that were rapidly saturated (Fig. 3).

Our ultimate goal was to demonstrate the superior oncolytic effect of Ad5-Δ24RGD in an *in vivo* model. To this end, A549 cells xenografted in nude mice were treated with single, high-dose (10^9 viral particles), i.t. injections of Ad5lucRGD, Ad5-Δ24, Ad5-Δ24RGD, or PBS, and the results showed that both CRAds (modified and unmodified) yielded similar oncolysis (Fig. 4*A*). However, when a 100-fold lower dose (10^7 viral particles) was administered, it became clear that the oncolytic effect of Ad5-Δ24RGD was higher than that of Ad5-Δ24 ($P < 0.05$; Fig. 4*B*). Furthermore, we were able to correlate the observed oncolytic effect with the presence of virus progeny in the tumor samples by immunofluorescent detection of adenoviral hexon. Hexon was not detected in PBS- (not shown) and Ad5lucRGD-treated nodules (Fig. 4*C*, *a*), whereas it was detected throughout the tumors treated with CRAds. The comparison between the two CRAds showed that fluorescence in Ad5-Δ24RGD-treated tumors was stronger than the one observed in Ad5-Δ24-treated tumors (Fig. 4*C*, *b* and *c*, respectively). The lack of fluorescent staining in tumors treated with the nonreplicative control Ad5lucRGD indicates that the detected hexon belongs to the viral progeny of Ad5-Δ24 and Ad5-Δ24RGD, and not to the initial inoculum. As regards the high divergence of the volumes of PBS- and Ad5lucRGD-treated tumors, factors such as highly heterogeneous cell replication rates and hypoxic and necrotic areas are known to affect individual tumor volume after a critical size is reached. These differences have been noted previously when using oncolytic viruses (27, 28). Nevertheless, total resolution of the tumors in the s.c. xenograft model was seen only in some nodules treated with Ad5-Δ24RGD, which indicated that administration volume and schema adjustments, such as the ones suggested recently by Heise *et al.* (29), might be necessary to achieve complete oncolysis.

As presented here and elsewhere (30), the efficacy of replication-competent viruses used as oncolytic agents can improved at the level of infectivity. As other tumor-binding peptides are isolated (30, 31), modifications in addition to the RGD insertion can be considered as well. Of note, the RGD modification described here does not preclude the binding of the fiber to CAR, and the modified virus can enter the cells through α_V integrins and CAR. One approach to improve specific tumor infection/transduction would be the combination of CAR ablation and tumor-specific ligands to redirect the virus tropism. Recently, the adenovirus fiber amino acids crucial for CAR-binding abrogation and new tumor-selective peptides have been defined (15, 31, 32). This combination will generate truly targeted viruses, but the efficiency of their propagation will depend on the amount of the targeted receptor in the same way as the propagation of the unmodified virus depends on CAR.

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