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Advances in Brief

Targeting Oncolytic Adenoviral Agents to the Epidermal Growth Factor Pathway with a Secretory Fusion Molecule[1]

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Abstract

Cancer gene therapy with conditionally replicating adenoviruses is a powerful way of overcoming low tumor transduction. However, one of the main remaining obstacles is the highly variable level of the coxsackie-adenovirus receptor expression on human primary cancers. In contrast, the epidermal growth factor receptor (EGFR) is overexpressed in various tumor types, and its expression correlates with metastatic behavior and poor prognosis. We constructed an adenovirus expressing a secretory adaptor capable of retargeting adenovirus to EGFR, resulting in a more than 150-fold increase in gene transfer. A replication-competent dual-virus system secreting the adaptor displayed increased oncolytic potency *in vitro* and therapeutic gain *in vivo*. This approach could translate into increased efficacy and specificity in the treatment of EGFR overexpressing human cancers.

Introduction

CRADs[3] are a promising and novel way of overcoming low tumor transduction, which is the main obstacle preventing effective gene transfer and therapeutic effect in clinical applications of cancer gene therapy (1). However, one of the main reasons why the unparalleled transduction efficacy of Ads has not translated into similar results in humans is the variable level of the CAR on primary cancers (2–9) *in vivo*. CAR is ubiquitously expressed on normal epithelial tissues and is the main receptor mediating binding of the most commonly used Ad serotypes 2 and 5. Expression of CAR may be the major factor determining the rate of transduction (4, 6, 9–11). Importantly, recent evidence (11) suggests that CAR expression may inversely correlate with the malignant potential of tumors, resulting in low infectivity of highly aggressive tumors. In contrast to the expression profile of CAR, EGFR, the prototype of cancer-associated receptors, is commonly overexpressed in many if not most carcinomas with correlation to metastatic behavior and poor prognosis (12). A powerful approach for increasing tumor transduction could be combining the tissue-penetrating capability of CRADs with the transductional control provided by retargeting moieties. In support of this hypothesis, an artificial receptor system has been used to demonstrate that the effect of Ad dispersion and subsequent oncolysis critically depends on receptor expression (13). We have constructed a novel virus that mediates secretion of a fusion molecule consisting of the extracellular domain of CAR and EGF. We then explored the capability of the sCAR-EGF to retarget Ad to EGFR. Finally, we demonstrated that infection of cancer cells with a sCAR-EGF-retargeted replication-competent dual-virus system resulted in increased oncolysis *in vitro* and a therapeutic benefit *in vivo*.

Materials and Methods

Viruses. For construction of AdsCAR-EGF, a replication-deficient Ad with sCAR-EGF in E1, the gene coding for sCAR-EGF was cloned from pFBshCAR-EGF (14) into pShuttle-CMV (Quantum, Montreal, Quebec, Canada). Homologous recombination with pAdEasy-1 (Quantum) was performed in *Escherichia coli*, followed by confirmation of structure with *Eco*RV and *Pac*I digestions, PCR, and sequencing of the transgene (data not shown). The viral genome was transfected into 293 cells for plaque purification, followed by cesium chloride purification and standard titering with OD260 and plaque assay. Resulting titers were 3.8×10^{11} VPs/ml and 1.0×10^{10} plaque-forming units/ml, ratio = 38.4 VPs/plaque-forming unit. Large-scale preparations of AdCMVLuc (a nonreplicating Ad-expressing luciferase; courtesy of Dr. Robert Gerard, Texas Southwestern Medical Center, Dallas, TX), AdsCAR6H (a nonreplicating Ad-mediating secretion of sCAR6H),[4] and Δ24 (an Ad with a 24-bp deletion in *E1A*, allowing selective replication in cells mutant in the *Rb-p16* pathway; Ref. 15) were performed with standard methods on 293 cells (or A549 cells for Δ24).

CRAdsCAR-EGF denotes a replication-competent, sCAR-EGF-secreting dual-virus system consisting of equal VPs of AdsCAR-EGF and Δ24 mixed immediately before administration to cells. CRAdCMVLuc is the respective combination of Δ24 and AdCMVLuc. Δ24 has been characterized previously (15). Validating the dual-virus strategy, it has been demonstrated that transcomplementation of E1 proteins from a plasmid or replication-competent virus results in replication of E1-deleted viruses present in the same cell (9, 16–18).

Cell Lines. 293 cells were purchased from Microbix (Toronto, Ontario, Canada). A549 (lung cancer), HeLa (cervical cancer), U118 (glioma), A431 (squamous cell skin cancer), BT474, and MB-453 (breast cancer) were obtained from the American Type Culture Collection (Rockville, MD), and SKOV3.ip1 cells (ovarian cancer) are from Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX). Cell lines were propagated in the recommended conditions.

Protein Detection. HeLa cells were infected overnight with 50 VP/cell, and BT474 and MB453 cells were infected with 500 VP/cell of AdsCAR-EGF, AdsCAR6H, and AdCMVLuc. Supernatants were collected at 48 h, and cellular debris was removed by centrifugation. Dilutions in a volume of 300 μl were transferred onto a nitrocellulose membrane using the Bio-Dot apparatus (Bio-Rad). BSA (3%) was used for blocking, followed by detection with a 1:5000 polyclonal mouse anti-CAR antibody (14) and 1:2000 goat antimouse alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in 3% BSA. Western blot analysis on the supernatants was performed with standard methodology in a 12% two-phase gel, and proteins were detected as above. Baculovirus-expressed and -purified sCAR-EGF and sCAR6H (14) were used as controls.

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[3] The abbreviations used are: CRAD, conditionally replicative adenovirus; Ad, adenovirus; CAR, coxsackie-Ad receptor; EGF, epidermal growth factor; EGFR, EGF receptor; sCAR-EGF, secretory CAR-EGF fusion molecule; CMV, cytomegalovirus immediate early promoter; VP, viral particle; sCAR6H, secretory CAR with 6-histidine tag.

[4] I. Dmitriev, unpublished observations.

Retargeting Assays. SKOV3.ip1, U118, and A431 cells were infected in duplicate with AdCMVLuc preincubated for 30 min with aliquots of supernatants (see above). Twenty VP/cell were used for SKOV3.ip1, whereas 200 VP/cell were used for U118 and A431. Luciferase assay was performed 48 h later (Luciferase Assay System; Promega, Madison, WI).

Cell-killing Assays. SKOV3.ip1 cells were plated in triplicate and infected with Δ24 or AdCMVLuc for 1 h. Aliquots (25 μl) of AdsCAR-EGF or AdCMVLuc supernatant (from protein detection assays) were added each day. At 20 days, cells were fixed with formalin and stained with crystal violet.

Next, A431 and SKOV3.ip1 cells were infected with the CRAdCMVLuc or CRAdsCAR-EGF for 1.5 h. After incubating for 20 days with medium changes, the remaining cells were fixed and stained as above.

Preliminary toxicity analysis was performed by infecting A431 and SKOV3.ip1 cells with 50, 100, 200, 500, and 1000 VP/cell of AdsCAR-EGF and AdsCAR6H, followed by medium changes every 2 days for 20 days.

Animal Experiments. Initially, A431 cells were infected *ex vivo* with 50 VP/cell of CRAdsCAR-EGF for 1 h followed by a 5-h incubation. Cells were then collected and mixed with uninfected cells, and a total of 10^7 cells were injected into flanks of athymic CD-1/nu mice (Charles River Laboratories, Wilmington, MA; n = five/group). Tumor size was determined as the mean of the shortest and longest diameter (to avoid variability attributable to difficulty with estimation of height).

To compare CRAdsCAR-EGF to CRAdCMVLuc *in vivo*, s.c. tumors were established by injecting 5×10^6 A431 cell into both flanks of athymic mice (n = five/group). When tumors were ~5 mm ("day 0"), viruses were injected intratumorally in a 15-μl volume and tumors were measured as above. Each mouse was checked daily for the absence of pain or distress (19).

Statistics. Upon termination of the experiment, mean tumor size and SDs were calculated for each group of animals for each time point. The nonparametric change-point test (20) was used to show a systematic change in the pattern of observations as opposed to fluctuation attributable to chance. The mixed model (21) was used to longitudinally model the data on each tumor. The variance covariance structure was determined by using Akaike's Information Criteria (22). The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth.

Results

Infection of Cells with AdsCAR-EGF Results in Secretion of sCAR-EGF. Initially, infection of high EGFR HeLa cells (23) with AdsCAR-EGF produced no evidence of secretion, whereas sCAR6H was secreted (Fig. 1). With low EGFR-expressing cells MB453 and BT474 (14, 24), secretion of sCAR-EGF was detected. The amount of protein was estimated at 110 ng/ml (75-cm^2 flask; 12 ml of medium). Western blot confirmed secretion (Fig. 1*B*). The altered migration rate of the protein in comparison with baculovirus-expressed sCAR-EGF perhaps resulted from altered charge caused by different glycosylation by insect cells. A preliminary investigation on sCAR-EGF toxicity was performed by infecting SKOV3.ip1 and A431 cells with various amounts of AdsCAR-EGF and AdsCAR6H without significant differences in cell viability (data not shown).

Secreted sCAR-EGF Mediates Retargeting of Ad to EGFR. Aliquots of supernatant from AdsCAR-EGF-infected BT474 cells were incubated with AdCMVLuc. The virus-supernatant mix was used for infection of SKOV3.ip1, U118, and A431 cells, which display moderate (SKOV3.ip1 and U118) to high (A431) EGFR expression and moderate (A431) to low (SKOV3.ip1 and U118) CAR expression (4, 14). A supernatant dose-dependent increase in luciferase expression was seen, with the highest readings 17.1-, 20.2-, and 158-fold higher than without retargeting for SKOV3.ip1, U118, and A431 cells, respectively (curves with triangles in Fig. 2).

With the highest amounts of supernatant from AdsCAR6H-infected cells, luciferase expression was reduced to 73%, 48%, and 65% (on SKOV3.ip1, U118, and A431, respectively) of the highest values for the series (curves with squares in Fig. 2). sCAR6H binds to Ad fiber but does not mediate binding to EGFR, thus modeling blockage of CAR-binding with sCAR-EGF.

Retargeting Replication-competent Ad to EGFR Results in Increased Cell Killing *in Vitro*. To validate Δ24 replication in SKOV3.ip1 cells, infections were performed at 0, 0.01, 0.1, or 1 VP/cell, and aliquots of supernatant (from BT474 cells infected with

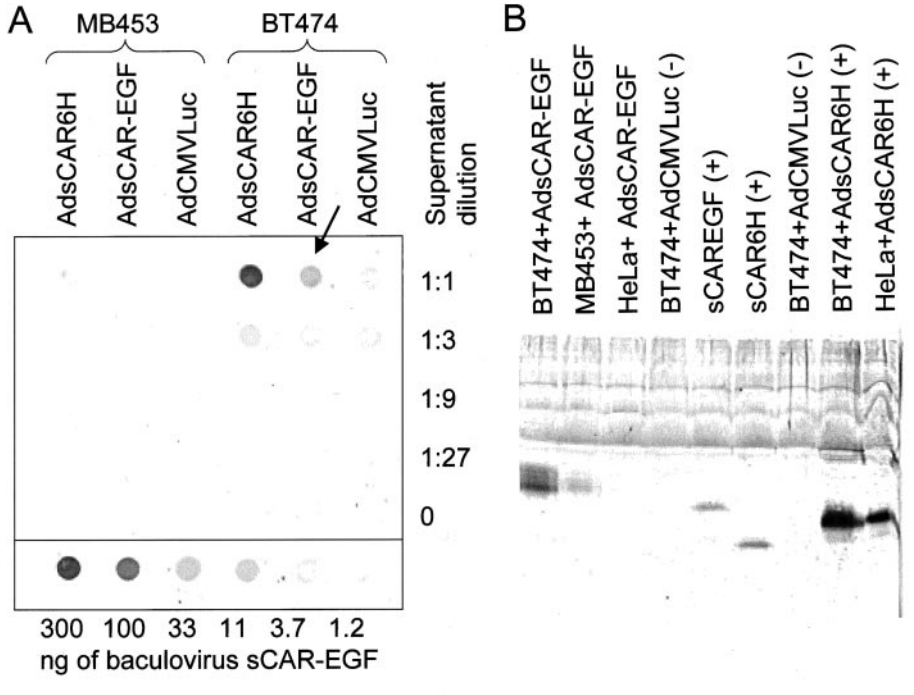

Fig. 1. Secretion of sCAR-EGF from human cancer cells infected with AdsCAR-EGF. *A*, supernatant from MB453 and BT474 (both low EGFR breast cancer lines) cells infected with AdsCAR6H (codes for human CAR ectodomain, positive control), AdsCAR-EGF, or AdCMVLuc (negative control) was centrifuged and then transferred onto a membrane. *Arrow*, the signal for sCAR-EGF. When compared with known amounts of sCAR-EGF (*lowest row*), the amount of secretion could be estimated at 110 ng/ml. *B*, Western blot suggested that the sCAR-EGF secreted from BT474 and MB453 cells (*Lanes 1–2*) was close in size to baculovirus-expressed sCAR-EGF (*Lane 5*). High EGFR HeLa cells (*Lane 3*) did not show evidence of sCAR-EGF secretion, but the positive control sCAR6H was secreted (*Lane 9*). −, supernatants collected from cells infected with AdCMVLuc (*Lanes 4 and 7*). These serve as negative controls. +, the positive controls, including *Lane 8*, which has supernatant from BT474 cells infected with AdsCAR6H. sCAR-EGF and sCAR6H (*Lanes 5–6*) are purified baculovirus-expressed proteins.

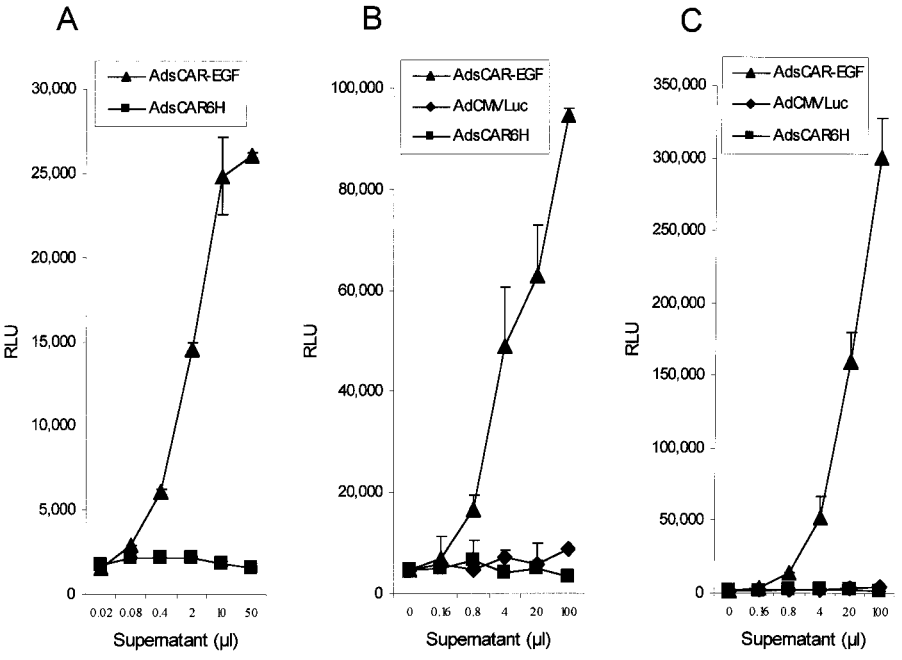

Fig. 2. Secreted sCAR-EGF mediates retargeting of Ad to EGFR. Increasing amounts of supernatant collected from cells infected with AdsCAR-EGF (curves with ▲) were incubated with AdCMVLuc, and then the mix was added to (*A*) SKOV3.ip1 (ovarian cancer), (*B*) U118 (glioma), or (*C*) A431 (squamous cell skin cancer) cells. These cells express moderate to high EGFR and thus resemble many aggressive human cancers. Supernatant from cells infected with AdCMVLuc (◆) or AdsCAR6H (■) were used as controls. Relative light units (*RLU*) are means of duplicate experiments (±1 SD). With the highest amounts of supernatant, luciferase readings were 17.1-fold (*A*), 20.2-fold (*B*), and 158-fold (*C*) higher with retargeting. The slope of the AdsCAR-EGF curves suggests that maximum retargeting potential was not reached.

AdsCAR-EGF or AdCMVLuc) were added daily. At 20 days, cell killing and partial loss of monolayer was seen only with cells that had been infected with 1 VP/cell and subjected to AdsCAR-EGF/BT474 supernatant (data not shown).

To study the effect of continuous sCAR-EGF secretion on the oncolytic potential of CRADs, we infected SKOV3.ip1 and A431 cells with the CRAdsCAR-EGF and CRAdCMVLuc dual-virus systems. On both cell lines, infection with CRAdsCAR-EGF resulted in cell killing with one to two orders of magnitude less virus than with CRAdCMVLuc (Fig. 3).

Targeting Replicative Ad to EGFR Results in a Therapeutic Advantage *in Vivo*. Various proportions of infected and uninfected A431 cells were mixed and injected s.c. (Fig. 4*A*). One percent of infected cells was sufficient to inhibit tumor growth, and 5% or more resulted in healing of tumors. None of the mice showed signs of illness or distress, suggesting that the secretion of sCAR-EGF did not cause overt toxicity.

To evaluate sCAR-EGF retargeting *in vivo*, CRAdsCAR-EGF or CRAdCMVLuc were administered with a single intratumoral injection into established A431 tumors (Fig. 4, *B–C*). The change-point test (20) revealed that the tumor growth pattern changed at 13 days for 10^9 VP CRAdsCAR-EGF ($P = 0.0045$), 21 days for 10^9 VP CRAdCMVLuc ($P = 0.0012$), 17 days for 10^8 VP CRAdsCAR-EGF ($P = 0.0026$), and 25 days for 10^8 VP CRAdCMVLuc ($P = 0.0011$), *i.e.*, 8 days earlier for CRAdsCAR-EGF with both doses.

The change-point test and the test of fixed effects (22) showed that there was a significant correlation between observations of tumor size and time ($P < 0.0001$ for all of the groups). A polynomial equation was fit for each group, thereby creating a mathematical model for each growth pattern ("modeled" in Fig. 4). The mixed model (22) was used

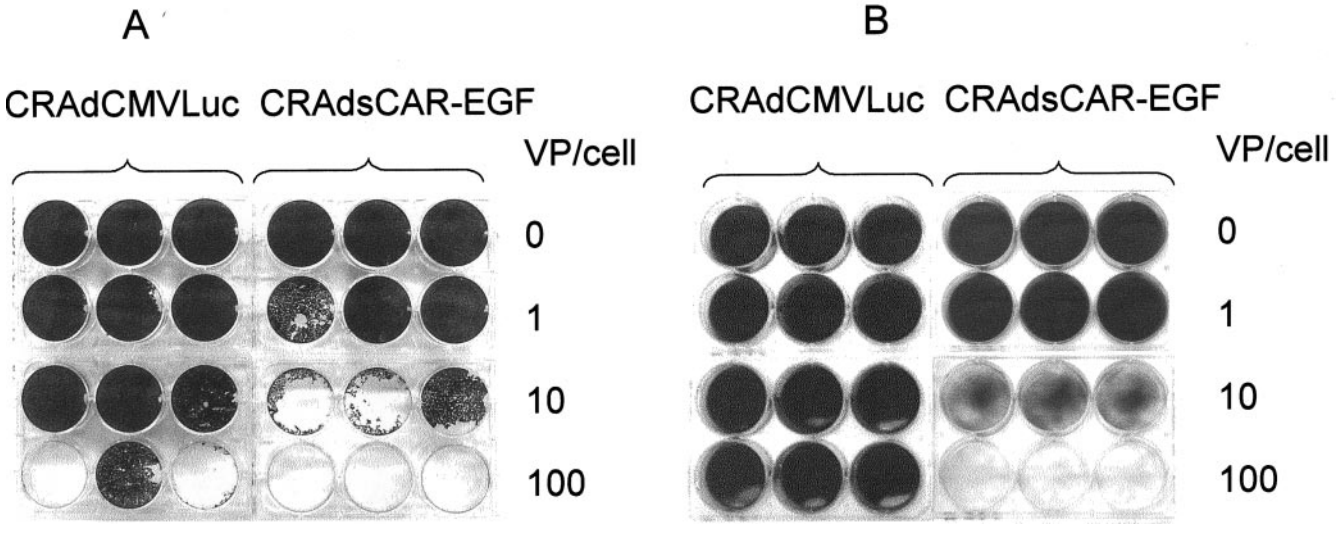

Fig. 3. Targeting oncolytic Ad to EGFR with sCAR-EGF results in an increased oncolytic effect. (*A*) SKOV3.ip1 or (*B*) A431 cells were infected with CRAdsCAR-EGF, a replication-competent sCAR-EGF-secreting dual-virus system. Oncolytic potency was compared with CRAdCMVLuc, which is isogenic in regard to replicativity but does not secrete a retargeting molecule. A similar effect on cells was observed with 1 VP/cell of CRAdsCAR-EGF as with 100 VP/cell of CRAdCMVLuc, suggesting increased oncolysis because of sCAR-EGF secretion-mediated EGFR targeting.

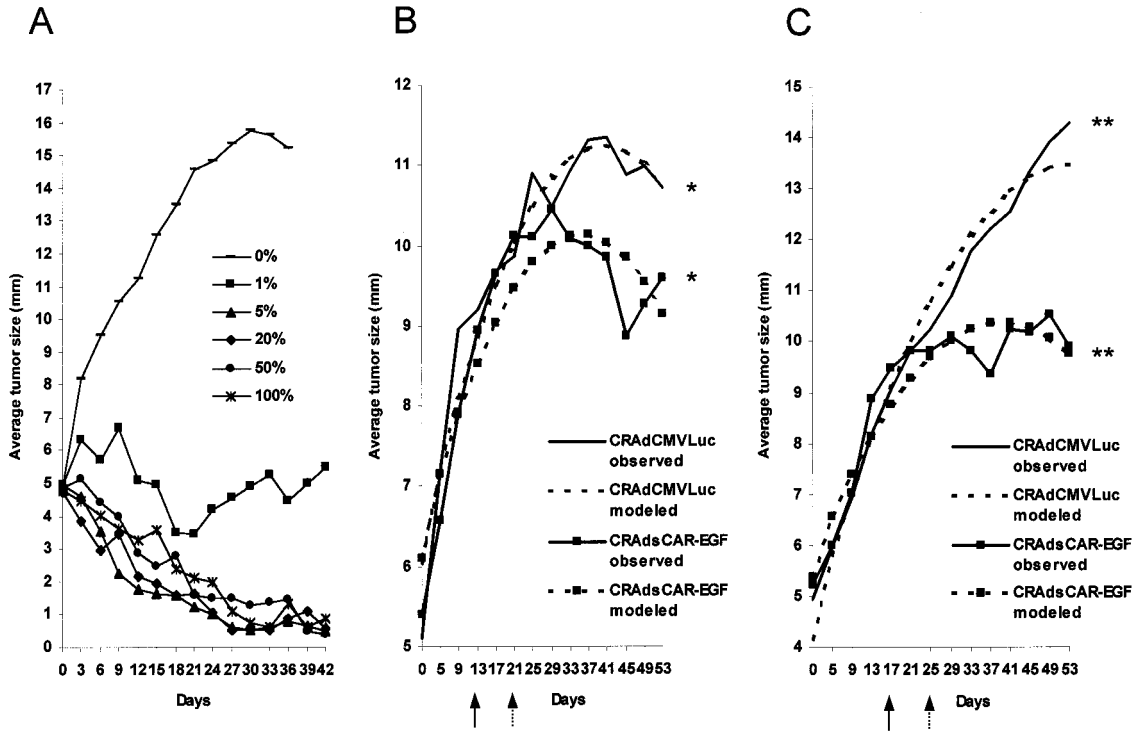

Fig. 4. sCAR-EGF secretion results in therapeutic efficacy *in vivo*. *A*, various percentages of A431 cells were infected *ex vivo* with CRAdsCAR-EGF and mixed with uninfected cells. One percent of infected cells was sufficient to inhibit tumor growth, whereas 5% or more resulted in healing of mice. Single intratumoral injections of (*B*) 10^9 or (*C*) 10^8 VP of CRAdsCAR-EGF or CRAdCMVLuc dual-virus systems were performed into established A431 xenografts. *Arrows* (*solid* for CRAdsCAR-EGF, *dotted* for CRAdCMVLuc) indicate the change-point in tumor growth characteristics, which represents the time point when oncolysis changes the initial pattern of tumor growth. On the basis of tumor size measurements (*observed*), the growth patterns were mathematically modeled for statistical comparison (*modeled*), and CRAdsCAR-EGF was found to be more oncolytic. *, $P < 0.05$; **, $P < 0.0001$.

to allow for the curvature of the plots, which was caused by the opposite effects of oncolysis and cell division. For the mice that received injections with 10^9 VP, groups were significantly different from day 29 onwards ($P < 0.05$; Fig. 4*B*). When 10^8 VP was used (Fig. 4*C*), the differences were significant from day 25 onwards ($P = 0.0066$ and 0.0003 on days 25 and 29, respectively, and < 0.0001 on days 33–53).

Discussion

In this study, we report construction of the first human Ad secreting a paracrine adaptor molecule. Secretion of sCAR-EGF was demonstrated with low EGFR cells (*Lanes 1 and 2*, Fig. 1), but not with high EGFR HeLa cells (23). In contrast, secretion was detected when HeLa cells were infected with AdsCAR6H, which codes for ectodomain of CAR but not EGF (*Lane 9*, Fig. 1*B*). EGF exhibits high affinity binding to EGFR, which leads to rapid internalization but no recycling of the receptor-ligand complex (12). Thus, perhaps secreted sCAR-EGF also internalizes. Alternatively, binding without internalization would also limit the amount of sCAR-EGF in the supernatant.

Supporting the capacity of sCAR-EGF to mediate binding and subsequent internalization of Ad, supernatant containing the fusion molecule resulted in dose-dependent increases in marker gene expression (Fig. 2). The shape of the curves suggests that the upper limit of retargeting potential was not reached. In an *in vitro* system, it is difficult to assess the capability of a secreted fusion molecule to block fiber-CAR interaction, because in the absence of CAR binding, uptake of Ad into cells can also occur via alternative mechanisms (10). However, we observed up to 52% reduction in luciferase expression with sCAR6H, which could translate into partial blocking of CAR-mediated internalization (into normal cells) by sCAR-EGF *in vivo*, but additional studies are needed.

We used a dual-virus system (CRAdsCAR-EGF) to evaluate the combination of oncolysis and EGFR targeting and saw dramatically increased killing of cells relatively low in CAR but high in EGFR expression, a combination commonly seen with primary cancer cells (Fig. 3). *In vitro*, the isogenic control virus (CRAdCMVLuc) is expected to enter cells even if they are low in CAR (10). The observed difference in oncolysis may result from more rapid internalization of the retargeted virus because of a higher number of receptors. In a living organism, extracellular viruses are at risk for neutralization by immune defenses or being swept away into organs responsible for Ad clearance. Thus, the advantage of rapid binding and internalization could be more pronounced.

s.c. xenografts are a stringent model for testing an oncolytic effect, because viral replication is balanced against rapid tumor growth. Here, we demonstrated significantly improved therapeutic efficacy of CRAdsCAR-EGF in comparison with the isogenic control not secreting sCAR-EGF (Fig. 4).

No signs of sCAR-EGF-causing toxicity were evident when cells were infected with AdsCAR-EGF in comparison with AdsCAR6H. When sCAR-EGF was added daily to SKOV3.ip1 cells infected with a CRAD, no evidence of toxicity to cells was seen. Moreover, obvious signs of toxicity were absent in mice whose xenografts were infected with CRAdsCAR-EGF. Additional studies will show whether the adaptor molecule has an effect of cell growth or whether there is toxicity *in vivo*. Also, it remains to be seen whether sCAR-EGF mediates Ad internalization via the EGFR pathway or merely substitutes for CAR in binding Ad for the native entry mechanism via penton base arginine-glycine-aspartic acid and cellular integrins.

This is the first report of a retargeting molecule secretory from human cells, but this strategy could be feasible with various high-affinity, cancer-specific ligands. Because rapid screening methods allow recognition of large numbers of cancer-specific features, unlimited possibilities for retargeting with secretory-targeting moieties

may soon be available. The dual-virus system used here provides a useful model for rendering AdsCAR-EGF replicative and investigating the combination of oncolysis and retargeting, but efficacy could be improved when *sCAR-EGF* is genetically incorporated into a CRAD.

In conclusion, we show that retargeting of replicating Ad to a receptor overexpressed in cancers is a powerful way of increasing tumor transduction and allows overcoming the lack of the primary Ad receptor. Clinical translation of this approach may be effective in treatment of a variety of human cancers that overexpress EGFR.

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