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A conditionally replicating adenovirus with strict selectivity in killing cells expressing epidermal growth factor receptor

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Abstract

Virotherapy of cancer using oncolytic adenoviruses has shown promise in both preclinical and clinical settings. One important challenge to reach the full therapeutic potential of oncolytic adenoviruses is accomplishing efficient infection of cancer cells and avoiding uptake by normal tissue through tropism modification. Towards this goal, we constructed and characterized an oncolytic adenovirus, carrying mutated capsid proteins to abolish the promiscuous adenovirus native tropism and encoding a bispecific adapter molecule to target the virus to the epidermal growth factor receptor (EGFR). The new virus displayed a highly selective targeting profile, with reduced infection of EGFR-negative cells and efficient killing of EGFR-positive cancer cells including primary EGFR-positive osteosarcoma cells that are refractory to infection by conventional adenoviruses. Our method to modify adenovirus tropism might thus be useful to design new oncolytic adenoviruses for more effective treatment of cancer.

Keywords: Gene therapy; Oncolytic adenovirus; Targeting; Coxsackie and adenovirus receptor; Epidermal growth factor receptor; Osteosarcoma

Introduction

Conditionally replicating adenoviruses (CRAds) are being explored for treatment of cancer. They kill cancer cells by specifically replicating in and lysing these cells, allowing lateral spread of progeny virus. The amplification of input dose and the mechanism of cell kill are clearly different from conventional treatment, which make CRAds a promising, complementary approach in anti-tumor therapy. So far, clinical trials using local, loco-regional, or systemic application of CRAds have demonstrated biological activity but only modest anti-cancer efficacy (Kirn, 2001; Reid et al., 2002). The utility of CRAds is in part hampered by the promiscuous tropism of the commonly used adenovirus type 5 based vectors, which results in uptake by normal tissues. This limits the amount of virus available for infection of tumor cells and may cause toxic side effects, a concern especially relevant for systemic treatment. Moreover, several tumor cell types including glioblastoma, melanoma and osteosarcoma were found to be refractory to adenovirus transduction because of low expression of the high-affinity

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receptor, coxsackie and adenovirus receptor (CAR) (Grill et al., 2001; Hemmi et al., 1998; Miller et al., 1998; Witlox et al., 2002). Furthermore, primary ovarian and brain tumors showed a large degree of heterogeneity in CAR expression between different specimens but also within one tumor (Grill et al., 2001; Zeimet et al., 2002). The relatively low and heterogeneous expression of the native adenovirus receptor might compromise cancer gene therapy with oncolytic adenoviruses. Indeed, it has been found that the efficiency by which a CRAd is able to destroy tumor cells in vitro and in vivo is critically dependent on CAR expression status (Douglas et al., 2001). CRAd efficacy can be improved by targeting the vector to tumor tissue independently from CAR (Bernt et al., 2003; Suzuki et al., 2001; Van Beusechem et al., 2003). Proper targeting of CRAds to tumor cells should fulfill two requirements; i.e., the virus should be engineered to transduce the cells of interest with high efficiency and its natural tropism towards nontargeted tissues should be abolished.

Several strategies have been employed to broaden the tropism of Ad5-based gene transfer vectors (Mizuguchi and Hayakawa, 2004) and some of these strategies have been incorporated in CRAds to enhance oncolytic efficacy. The approaches can be roughly divided in those that involve direct genetic modification of the capsid proteins and those that rely on conjugating adenovirus with adapter molecules. CRAds carrying foreign peptides in the fiber exhibited an expanded tropism leading to augmented oncolysis in vitro and in vivo (Shinoura et al., 1999; Suzuki et al., 2001). Also CRAds carrying chimeric fibers that were created by exchanging parts of the Ad5 fiber protein with that of different adenovirus serotypes displayed augmented infectivity on CAR-deficient cancer cells (Bernt et al., 2003; Haviv et al., 2002). Although these studies clearly demonstrate the benefits of targeting via CAR independent pathways, the applicability of these targeting approaches is not without limitations. Insertions in the fiber are restricted by so far not fully understood structural demands for retaining proper trimerisation of the fiber and pseudotyped viruses are limited by the targeting repertoire of naturally occurring adenovirus serotypes (Krasnykh et al., 2000). In contrast, approaches that use a bispecific adapter molecule to bind the virus to a cell-type specific protein are more flexible (Dmitriev et al., 2000; Haisma et al., 2000; Nettelbeck et al., 2001). The cell-binding component can be for example a single chain monoclonal antibody (scFv). This antibody-based approach combines excellent selectivity and affinity with versatility. A disadvantage of this two-component approach is that the targeting moiety is not an integral part of the adenovirus capsid. Utility in the CRAd context requires that the gene encoding the adapter protein is incorporated in the CRAd genome to ensure that the targeting property is retained upon in situ replication and spread through the tumor mass. Previously, we confirmed utility of this concept. To that end, we developed the CRAd AdΔ24-425S11 that encodes a bispecific single-chain antibody (scFv) consisting of anti-epidermal growth factor receptor (EGFR) scFv 425 and anti-adenovirus fiber knob scFv S11 (Van Beusechem et al., 2003). This CRAd produced 425-S11 scFv during replication in cancer cells yielding progeny with enhanced infectivity and oncolysis properties on EGFR-positive, CAR-deficient tumor cells. In addition to infection via EGFR, AdΔ24-425S11 retained native infection capacity via binding to CAR and integrins. To achieve strict targeting, the native tropism of the CRAd should be abolished. This is especially important for systemic delivery of CRAds because the vast majority of intravenously injected adenovirus is sequestered in the liver (Bernt et al., 2003; Einfeld et al., 2001). Detargeting adenovirus from normal tissue upon intravenous injection in mice and rats required ablation of both CAR and α_v integrin-binding sites (Einfeld et al., 2001; Nicol et al., 2004). Therefore, we introduced mutations that abolish CAR- and integrin-binding in the genome of AdΔ24-425S11. This should produce a CRAd lacking CAR and α_v integrin-binding sites capable of producing EGFR-targeted progeny upon replication in cancer cells. We show here that the new CRAd AdΔ24P*F*-425S11 exhibited a strictly EGFR-dependent targeting profile. In contrast to AdΔ24-425S11 with intact native binding sites, the new virus showed reduced replication and thus improved safety on EGFR-negative cells. Similar to AdΔ24-425S11, it exhibited enhanced oncolytic potency on CAR-negative, EGFR-positive cancer cell lines and on primary osteosarcoma specimens. Additionally, we evaluated the targeting properties of AdΔ24P*F*-425S11 on human liver tissue using a recently developed ex vivo liver slice model.

Results

*Construction and characterization of the CRAd AdΔ24P*F*-425S11*

Fig. 1 shows a schematic representation of the viruses used in this study. All three CRAds carry the Δ24-deletion in the E1A CR2 domain responsible for binding Rb protein (Fueyo et al., 2000). AdΔ24-425S11 contains an expression cassette for the EGFR-targeting bispecific antibody 425-S11 (Van Beusechem et al., 2003). AdΔ24-425S11-infected cells secrete 425-S11 adapter protein. Following oncolysis, released virus progeny bind adapter protein, which confers targeted tropism.

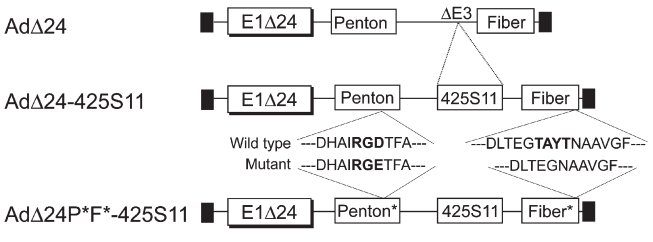
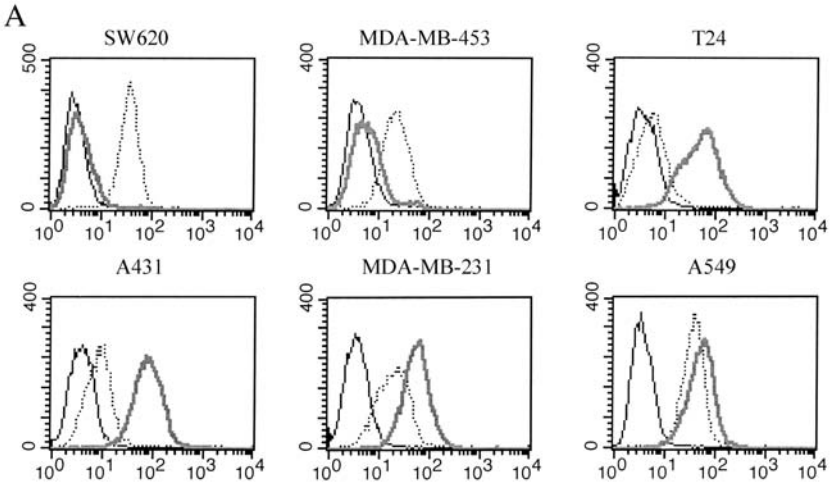


Fig. 1. Schematic representation of the viruses used in this study. The parental CRAd AdΔ24 carries a deletion of 24 base pairs in the E1 region to restrict replication to cells with a defective Rb pathway and a deletion of the E3 region. The CRAd AdΔ24-425S11 is identical to AdΔ24 except for an expression cassette inserted in place of the E3 region encoding the bispecific single chain antibody 425-S11 to allow infection via EGFR. The CRAd AdΔ24*F*-425S11 is identical to AdΔ24-425S11 except that it encodes a mutant penton base protein with an RGD to RGE substitution that ablates the integrin binding site and a fiber protein with a TAYT deletion that abrogates CAR binding.

The new CRAd AdΔ24P*F*-425S11 was derived from AdΔ24-425S11 by introducing two mutations that abolish CAR- and integrin-binding sites in its fiber and penton genes, respectively (Einfeld et al., 2001; Roelvink et al., 1999). To allow propagation of this CRAd in the absence of native virus–receptor interactions, we generated the 911-S11 cell line. This E1-complementing cell line expresses an artificial receptor that binds adenovirus fiber knob distinct from its CAR-binding site. To compare the targeting properties of the three CRAds, we selected a panel of 6 cancer cell lines differing in CAR- and EGFR-expression. Fig. 2A shows flow cytometry analysis for CAR and EGFR expression by these cells. Relative median fluorescence (RMF) values were used to categorize cells as negative or positive for expression (Fig. 2B). T24 cells did not express detectable CAR, but did express EGFR. In contrast, SW620 and MDA-MB-453 cells were positive for CAR and negative for EGFR. The other three cell lines all expressed EGFR and also expressed low (A431), intermediate (MDA-MB-231) or high (A549) levels of CAR.

AdΔ24, AdΔ24-425S11 and AdΔ24P*F*-425S11 virus stocks essentially lacking 425-S11 protein were prepared by releasing virus from production cells in fresh culture medium, followed by CsCl gradient banding. A549 (CAR+/EGFR+), T24 (CAR−/EGFR+) and SW620 (CAR+/EGFR−) cells were infected with these viruses and stained for expression of adenoviral hexon 2 days after infection (Fig. 3). On the CAR-negative cell line T24, all three CRAds showed low infection efficiencies, whereas AdΔ24 and AdΔ24-425S11 infected CAR-positive A549 and SW620 cells with high efficiency. In sharp contrast, the native tropism-ablated AdΔ24P*F*-425S11 exhibited low infection efficiencies on all three cell lines. These findings thus demonstrated that the mutations introduced in the penton and fiber genes of AdΔ24P*F*-425S11 effectively reduced native infectivity. They also confirmed that the virus particles were largely devoid of bispecific 425-S11 scFv. To mimic in situ produced 425-S11-carrying virus progeny, we pre-incubated virus with recombinant 425-S11 prior to inoculation of the cells (Fig. 3). Precomplexation of the control viruses AdΔ24 and AdΔ24-425S11 led to a minor increase in transduction efficiency on CAR-positive A549 cells and a clear increase on CAR-negative EGFR-positive T24 cells. Precomplexation of AdΔ24P*F*-425S11 with 425-S11 protein also increased infection of EGFR-positive A549 and T24 cells, but most importantly, infection of SW620 cells that are negative for EGFR remained low. The specificity of this retargeting was further confirmed using soluble EGFR to block EGFR-mediated entry. Incubating AdΔ24P*F-425S11 complexed with 425-S11 with soluble EGFR negated infection of A549 and T24 cells. Together, these results strongly suggest that the new EGFR-targeted CRAd AdΔ24P*F*-425S11 with ablated native tropism, has a targeting phenotype strictly dependent on the EGFR-targeting antibody and cellular EGFR-expression.



	CAR	EGFR	
SW620	12.4	1.2	CAR + ; EGFR -
MDA-MB-453	5.6	1.4	
T24	1.5	12.9	CAR - ; EGFR +
A431	2.2	19.1	CAR +; EGFR +
MDA-MB-231	5.6	14.9	
A549	11.1	14.3	

Fig. 2. CAR and EGFR expression of the cell lines used in this study. (A) Flow cytometry histograms of A431, A549, MDA-MB-231, MDA-MB-453, SW620 and T24 cells that were analyzed for CAR (dotted lines) and EGFR (bold grey lines) expression. Second-antibody only controls are indicated by the black lines. (B) CAR and EGFR expression on the cell surface are given as the median fluorescence intensities of anti-CAR or anti-EGFR antibody-stained cells divided by the median fluorescence of second antibody-stained control cells (RMF).

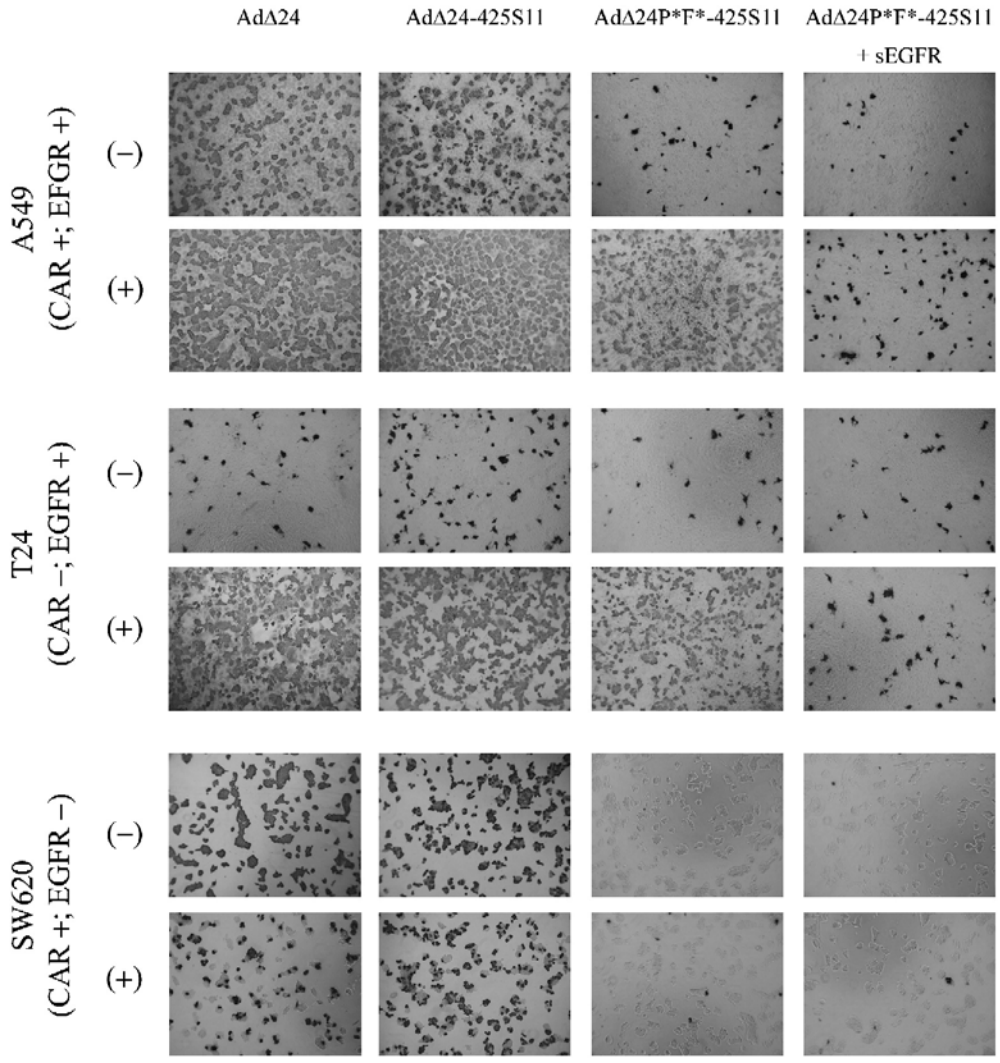


Fig. 3. Native tropism-ablated AdΔ24P*F*-425S11 complexed with recombinant 425-S11 scFv selectively infects EGFR-positive cells independent of CAR-status. A549, T24 and SW620 cells with different expression of CAR and EGFR as indicated, were infected at 1000 VP/cell with AdΔ24, AdΔ24-425S11 or AdΔ24P*F*-425S11. Prior to infection, purified virus was either untreated (−) or allowed to conjugate to recombinant scFv 425-S11 (+). Where indicated, soluble EGFR (sEGFR) was added to the virus mixture. Two days after infection, cells were immunohistochemically stained for expression of hexon protein.

*Selective oncolytic potency of AdΔ24P*F*-425S11 on EGFR-positive cancer cells*

Next, we examined whether reduced native infectivity of AdΔ24P*F*-425S11 virus translated into reduced cytotoxic replication on CAR-positive, EGFR-negative cells. Monolayers of cells varying in CAR and EGFR status were infected with 425-S11-precomplexed CRAds at various multiplicities of infection (MOIs). Subsequently, CRAds were allowed to replicate for 2 to 3 weeks, after which monolayer cytolysis was examined by crystal violet staining (Fig. 4A). Cells expressing CAR and EGFR could be killed by all three CRAds, with AdΔ24-425S11 capable of infecting via both molecules being generally most effective. Monolayers of CAR-positive, EGFR-negative SW620 and MDA-MB-453 cell lines were eradicated effectively following infection with AdΔ24 or AdΔ24-425S11. In striking contrast, the monolayers of these cells infected with AdΔ24P*F*-425S11 remained intact even at very high MOI. This suggested that the mutations present in the fiber and penton base of this virus effectively blocked its ability to infect and kill CAR-positive EGFR-negative cells. Importantly, AdΔ24P*F*-425S11 exhibited clearly improved oncolytic potency compared to AdΔ24 on CAR-negative EGFR-positive T24 cells. This showed that in situ production and secretion of 425-S11 from the expression cassette present in AdΔ24P*F*-425S11 restored its oncolytic potency on EGFR-positive cells. To confirm these findings in an independent manner, the infections were repeated three times, following which cell viability was quantified by measuring WST-1 conversion. Fig. 4B shows a representative example of these experiments, which confirmed the crystal violet CPE assay data. Relative oncolytic potencies of the CRAds were determined on the basis of $TCID_{50}$ values. On the two EGFR-deficient cell lines, AdΔ24P*F*-425S11 was 192- and 294-fold attenuated compared to AdΔ24. In contrast, AdΔ24P*F*-425S11 was 160-fold more potent than AdΔ24 against CAR-deficient

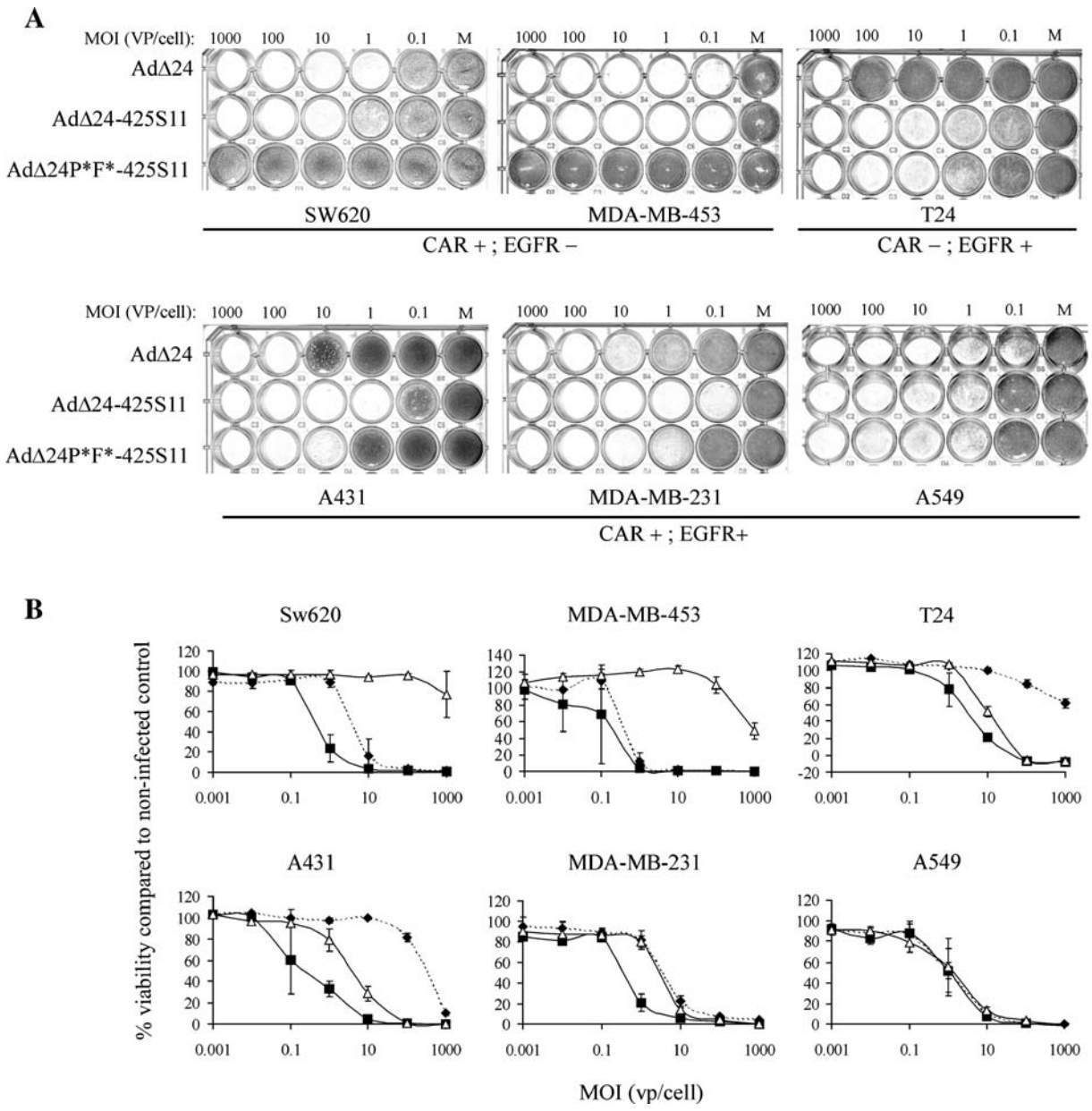
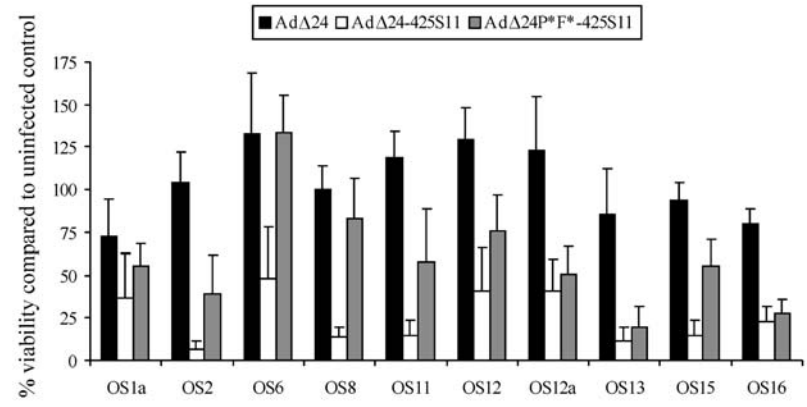


Fig. 4. AdΔ24P*F*-425S11 exhibits selective oncolytic potency on EGFR-positive cells and improved safety on CAR-positive, EGFR-negative cells. (A) Human cancer cell lines with different expression of CAR and EGFR as indicated, were infected with AdΔ24, AdΔ24-425S11 or AdΔ24P*F*-425S11 at the indicated multiplicity of infection (MOI) or mock infected (M). Two to three weeks after infection, surviving cells were stained using crystal violet. Each cell line was tested at least twice. Data shown are from representative experiments. (B) Cells were infected at indicated MOI with AdΔ24 (closed diamonds), AdΔ24-425S11 (closed squares) or AdΔ24P*F*-425S11 (open triangles). Two to three weeks after infection, cell viability was measured using a WST-1 conversion assay. Values given are percentages of uninfected control cultures. Data are the mean values±S.D. from a typical experiment performed in triplicate.

EGFR-positive T24 cells. We could thus conclude that AdΔ24P*F*-425S11 exhibited a stringent EGFR-dependent oncolytic potency.

*AdΔ24P*F*-425S11 kills primary CAR-deficient human osteosarcoma cells*

Similar to what has been reported for primary cells isolated from tumors derived from other tissues, primary high-grade osteosarcomas are often CAR-negative and EGFR-positive (Graat et al., 2005; Witlox et al., 2002). We evaluated the utility of AdΔ24P*F*-425S11 on short-term cultures of primary high-grade osteosarcomas isolated from patient biopsies. FACS analysis for CAR revealed that all samples were deficient for CAR with relative median fluorescence values ranging from 1.0 to 1.2. FACS analysis for EGFR revealed low to moderate expression ranging from 1.8 to 3.8 (Fig. 5). As a stringent test for anti-cancer efficacy of administered oncolytic adenovirus, primary osteosarcoma samples were infected with AdΔ24, AdΔ24-425S11 or AdΔ24P*F*-425S11 without adding bispecific scFv targeting molecules. Following culture for up to 27 days, during which the CRAds may replicate and produce



	OS1a	OS2	OS6	OS8	OS11	OS12	OS12a	OS13	OS15	OS16
CAR	1.1	1.1	1.1	1.1	1.0	1.1	1.2	1.1	1.1	1.1
EGFR	1.8	1.9	2.1	3.0	2.9	3.3	2.9	2.6	3.3	3.8

Fig. 5. AdΔ24P*F*-425S11 exhibits enhanced oncolytic potency on primary CAR-deficient human osteosarcoma specimens. Expression of CAR and EGFR on 10 primary osteosarcoma short-term cultures was measured by FACS analysis. The expression of CAR and EGFR is indicated by their relative median fluorescence value. All samples were negative for CAR while EGFR expression was low to moderate. Cells were subjected to infection with AdΔ24, AdΔ24-425S11 or Ad24ΔP∗F∗-425S11 at 100 VP/cell and cultured for 16 to 27 days. Cell viability was measured by WST-1 conversion assay and is given as percent viability compared to uninfected control cultures. Data are means + S.D. of sextuplicate measurements. Compared to AdΔ24, the EGFR-targeted AdΔ24-425S11 exhibited significant enhancement of oncolytic potency in all tested specimens ($P<0.05$). The native tropism-ablated AdΔ24P*F*-425S11 led to significantly enhanced oncolytic potency compared to AdΔ24 in 7 out of 10 specimens tested ($P<0.05$), the exceptions being OS1a, OS6 and OS8.

425-S11 adapter protein, cell viability was measured using WST-1 and compared to the viability of uninfected controls (Fig. 5). No or only a small decrease in viability was observed in primary osteosarcoma cells that were infected with the parental virus AdΔ24. In contrast, AdΔ24-425S11 efficiently killed these cells leading to a significant decrease in viability

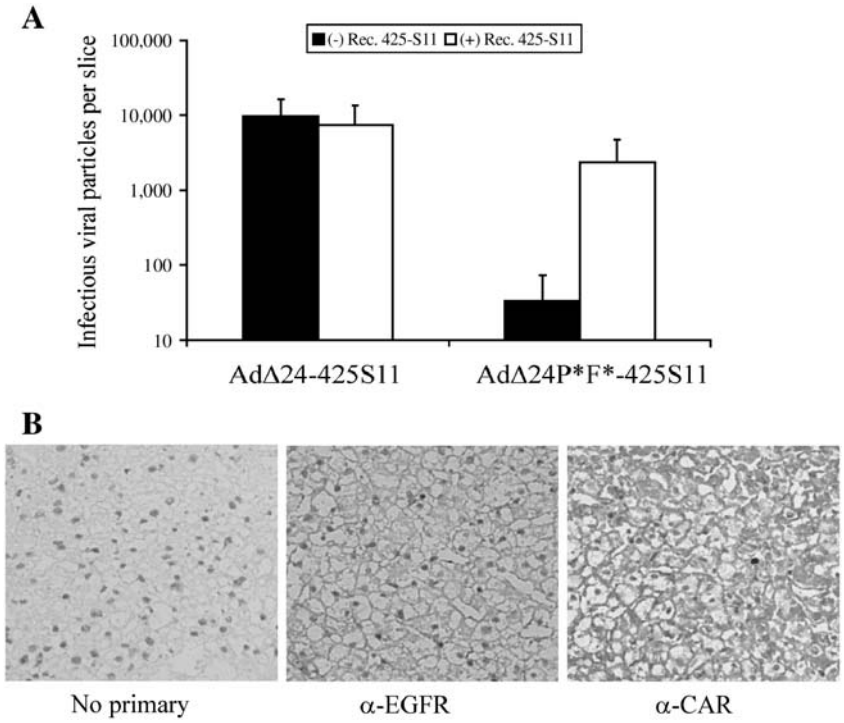


Fig. 6. Ablated native tropism of AdΔ24P*F*-425S11 reduces infection of human liver slices. (A) Fresh human liver samples were obtained and thin-slices were made with Krumdieck thin-slice tissue slicer. Slices were infected at 1×10^8 VP/slice with AdΔ24-425S11 or AdΔ24P*F*-425S11. Prior to infection, purified virus was either untreated (black bars) or allowed to complex with recombinant scFv 425-S11 (white bars). Two days after infection, lysates were prepared and the number of infectious particles present in the slices was determined by limiting-dilution infection of 911-S11 cells. Data shown are the mean values+ S.D. of six to nine infections of slices derived from three human livers. (B) Immunohistochemical staining of 4 μm sections of human liver using antibodies directed against EGFR or CAR. The negative control underwent the same procedure without primary antibody incubation. Sections were counterstained with hematoxylin.

compared to AdΔ24 in all tested specimens. Moreover, its native tropism-ablated derivative AdΔ24P*F*-425S11 also infected and killed these osteosarcoma samples. Although AdΔ24P*F*-425S11 was somewhat less effective than AdΔ24-425S11, it caused a significant decrease in viability compared to parental AdΔ24 in 7 out of 10 specimens. Hence, EGFR-targeting via 425-S11 scFv enhances oncolytic potency on CAR-negative EGFR-positive primary cancer cells also in the context of a native tropism ablated CRAd.

*Infectivity of AdΔ24P*F*-425S11 on human liver slices*

As adenovirus localizes largely to the liver upon systemic administration, it is worthwhile to examine transduction of liver tissue. Because the 425-S11 scFv molecule binds human EGFR only, such a study should be done on human liver material. Therefore, we employed a recently developed model using precision-cut human liver slices, which can be maintained ex vivo for a short period of time after preparation (Rots et al., 2005). Freshly prepared human liver slices were infected with AdΔ24-425S11 or AdΔ24P*F*-425S11. Two days after infection, lysates were prepared from the slices and the amount of infectious virus particles in the slices was determined. As shown in Fig. 6A, infection with native tropism-ablated AdΔ24P*F*-425S11 CRAd resulted in 289-fold reduced recovery of virus from the liver slices compared to AdΔ24-425S11. This suggested that injected AdΔ24P*F*-425S11 would be considerably less sequestered by the human liver than a CRAd retaining native binding sites. Interestingly, however, when AdΔ24P*F*-425S11 was complexed with bispecific scFv 425-S11 prior to inoculation, virus output was elevated 71-fold to levels only slightly lower than observed with AdΔ24-425S11. To investigate if this could be explained by liver cells expressing EGFR, we performed immunohistochemical staining on human liver slices using antibodies recognizing CAR and EGFR (Fig. 6B). Positive staining with both antibodies was observed, which is in line with previous studies that reported CAR and EGFR expression in human liver tissue (Reeves et al., 1994; Tomko et al., 1997). Hence, while systemically injected AdΔ24P*F*-425S11 is expected to exhibit strongly reduced liver tropism, its in vivo produced 425-S11-carrying progeny is likely to be sequestered by the liver via binding to EGFR.

Discussion

Proper targeting of oncolytic viruses to tumor cells requires redirecting the virus to a tumor-specific antigen while abolishing its native tropism. We have shown previously that a CRAd expressing an EGFR-targeting bispecific adapter molecule (425-S11) exhibited enhanced oncolytic potency on CAR-deficient tumor cells in vitro and in vivo (Geoerger et al., 2005; Van Beusechem et al., 2003). This infectivity-enhanced virus retained ability to enter cells via CAR, thus potentially limiting its utility due to its sequestration in normal tissue. Other previously reported CRAds with enhanced oncolytic potency on CAR-negative tumor cells include CRAds containing a cyclic RGD peptide insertion in the fiber HI loop, a stretch of lysine residues added to the carboxy-terminus of the fiber protein and Ad5/Ad3 or Ad5/Ad35 fiber chimeras (Bernt et al., 2003; Rivera et al., 2004; Shinoura et al., 1999; Suzuki et al., 2001). It is important to note that in most of these infectivity-enhanced CRAds the tropism of the virus is expanded rather than restricted to tumor cells. In fact, transduction of liver cells and hepatotoxicity was significantly higher with replicating adenoviruses containing the Ad5/Ad3 chimeric fiber compared to the parental virus with wild-type Ad5 fibers (Rein et al., 2005).

In this study, we describe a next generation targeted CRAd that carries mutations in its fiber and penton genes to ablate CAR- and integrin-dependent entry and expresses a bispecific targeting-antibody. We predicted that this type of virus should exhibit reduced native tropism, while its bispecific antibody-carrying progeny would have targeted tropism. As prototype, we made an EGFR-targeted variant expressing the 425-S11 adapter molecule. To the best of our knowledge, this CRAd is the first oncolytic virus with abolished native tropism. We evaluated infection and replication properties of this virus on cancer cell lines, primary osteosarcoma cells and normal liver cells. The new CRAd exhibited a clearly reduced transduction efficiency on CAR-positive cells, which is in line with previous findings with replication-deficient vectors carrying similar native tropism ablating mutations (Einfeld et al., 2001; Nettelbeck et al., 2004; Van Beusechem et al., 2002a). Compared to non-ablated viruses, safety of the novel CRAd on CAR-positive, EGFR-negative cells was considerably improved rendering the virus unable to infect and kill these cells even at high MOI. Importantly, retargeting the native tropism-ablated virus towards EGFR-positive cells using in situ produced bispecific 425-S11 antibody resulted in much higher (on CAR-negative cells) or comparable (on CAR-positive cells) oncolytic potencies compared to non-targeted parental AdΔ24 virus. Experiments using cells with different CAR and EGFR expression profiles and competition with soluble EGFR showed that infection by AdΔ24P*F*-425S11 was strictly EGFR-mediated. Hence, we showed that a CRAd with native tropism ablation that expresses its own targeting adapter displays a highly selective targeting profile.

The targeted replication of AdΔ24P*F*-425S11 was verified on freshly isolated, primary osteosarcoma cells which may represent clinically more relevant substrates compared to established cell lines. It has been reported previously that the CAR expression on cells derived from primary high-grade tumors is generally low. Indeed, all 10 osteosarcoma specimens tested showed undetectable CAR expression. To mimic clinical application of the new type of targeted CRAd, we subjected these cells to virus without adding recombinant adapter antibody. In this setting, the benefit of EGFR-targeting relies entirely on adapter protein production during oncolytic replication. In 7 out of 10 samples, the native tropism-ablated, EGFR targeted virus was significantly more effective in killing primary osteosarcoma cells through multiple rounds of infection and replication compared to the parental CRAd. Interestingly, on several specimens AdΔ24-425S11 was more oncolytic than AdΔ24P*F*425S11. Although we cannot formally exclude that

this difference might be caused by differences in functional virus titers, we favor the explanation that AdΔ24-425S11 was more effective because the primary OS cells expressed α_v integrins. Also in the absence of CAR, these molecules may serve as entry receptors for AdΔ24-425S11 with intact penton base, but not for AdΔ24P*F*-425S11 with mutated penton base. In fact, we have previously shown that the infectivity-enhanced CRAd Ad5-Δ24RGD, which targets α_v integrins, efficiently infects primary OS cells (Witlox et al., 2004). For this reason, a somewhat higher oncolytic potency of AdΔ24-425S11 than AdΔ24P*F*-425S11 was not unexpected.

It has been reported previously that bispecific adapter molecules, including S11-based proteins, that bind to the adenovirus fiber knob can block CAR-dependent entry (Haisma et al., 1999; Kashentseva et al., 2002; Watkins et al., 1997). If this block were always efficient, there would be no need to further ablate CAR-binding by introduction of mutations in the fiber. In our experiments, addition or stable expression of 425-S11 did not abolish oncolytic replication of CRAds on CAR-positive, EGFR-negative cell lines. This suggests that bispecific antibody concentrations required for neutralization were not reached. Presumably, binding of 425-S11 to only few fiber proteins per virus particle is already sufficient to mediate adenovirus entry via EGFR, whereas neutralization of CAR-mediated adenoviral transduction requires excess antibody to cover all fiber proteins. These considerations are especially relevant for in vivo applications where virus-adapter binding cannot be controlled and antibody adapters might dissociate over time. As shown herein, genetic ablation of CAR- and integrin-binding causes a strong reduction in native infection and oncolysis, obviating the need to control neutralization by the bispecific antibody.

The use of bispecific adapter molecules based on monoclonal antibodies allows flexibility in the choice of tumor antigen. By way of example, we chose the 425 monoclonal antibody that is directed against EGFR. The validity of EGFR as specific target is supported by the clinical development of EGFR inhibitors of which the human-mouse chimeric version of anti-EGFR monoclonal antibody C225 has been evaluated through phase III trials (Herbst and Shin, 2002) and monoclonal antibody 425 is being evaluated in phase I and II trials. Although EGFR is frequently overexpressed in tumor cells, also normal cells express EGFR including liver and skin (Herbst and Shin, 2002; Reeves et al., 1994). Indeed, while the native tropism-ablated virus AdΔ24P*F*-425S11 showed substantial detargeting in the human liver slice model, complexation with 425-S11 largely restored infection of liver cells. It is likely that this was caused by EGFR expression in the liver slices, which we detected using immunohistochemistry. This finding discourages systemic delivery of oncolytic viruses carrying EGFR-binding ligands. Our targeting approach, where the EGFR-targeting moiety is provided only upon oncolytic virus replication, might perhaps circumvent this obstacle. Alternatively, EGFR-targeting should primarily be considered for local and loco-regional virotherapy approaches. Various such strategies are clinically relevant and currently being evaluated. In particular, malignant brain tumors are often CAR−/EGFR+, while normal brain tissue is CAR+/EGFR− (Grill et al., 2001; Miller et al., 1998). A truly EGFR-targeted CRAd could be very useful in this context. Moreover, the flexibility of the antibody-based approach readily allows targeting to different antigens specific for various types of cancer. Since we found that ablation of native tropism decreased liver cell infection considerably, our targeting concept might prove most useful if targeting molecules are employed that are not expressed on liver cells. Such specific targets have been described. A good example is carbonic anhydrase IX or G250, which is specifically expressed on renal cell carcinomas (RCC) and in hypoxic tumor areas. Successful retargeting of a non-replicating adenovirus vector towards RCC using a bispecific adapter molecule directed to G250 was already reported (Jongmans et al., 2003).

Oncolytic adenoviruses owe their tumor-selectivity to specific mutations introduced in their genome. The AdΔ24 CRAd that we used as backbone to construct the targeted CRAd contains a deletion in the pRb-binding CR2 region of the adenoviral E1A protein that confers selective replication in cells with abnormalities in cell-cycle checkpoints regulated by pRb (Fueyo et al., 2000). Because pRb malfunction is shared by many tumors, the replication efficacy and oncolytic potency of AdΔ24 type CRAds could be demonstrated on a broad range of cancer cells (Balague et al., 2001; Fueyo et al., 2000; Geoerger et al., 2004, 2005; Johnson et al., 2002; Suzuki et al., 2001; Van Beusechem et al., 2002b). In the present study, we aimed to enhance tumor-selective replication of the potent CRAd AdΔ24 by tumor-selective infection via EGFR and we demonstrate that this indeed abolished replication and cell kill on CAR-positive EGFR-negative cells. Because neither transductional targeting nor selective replication is perfect by itself, combining these approaches may achieve a higher degree of tumor-specificity. The targeting approach presented herein might therefore contribute to the development of safer and more potent CRAds for cancer virotherapy.

Materials and methods

Cell lines and primary osteosarcoma specimens

A431 squamous skin carcinoma cells, A549 lung carcinoma cells and MDA-MB-231 and MDA-MB-453 breast carcinoma cells were all obtained from the ATCC (Manassas, VA, USA). T24 bladder cancer cells and SW620 colorectal carcinoma cells were kindly provided by Dr H.G. van der Poel (Department of Urology, NKI, Amsterdam, Netherlands) and M.L. Janmaat (Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands), respectively. Adenovirus E1-complementing 911 cells were obtained from IntroGene, Leiden, The Netherlands.

911-S11 cells were derived from 911 cells by transfecting 911 cells with the plasmid pDisplay-S11. pDisplay-S11 was made by releasing the single chain anti-fiber antibody S11 encoding fragment from pSTCF-S11 (Haisma et al., 2000) using SfiI and SalI and inserting it into pDisplay (Invitrogen, Carlsbad, CA) digested with the same restriction enzymes.

Stable transfectants were selected using geneticin (Invitrogen) at a concentration of 500 μg/ml and a clonal cell population was obtained by limiting dilution. Expression of the S11 scFv at the surface of the cells was verified using immunocytochemistry. Infection of 911-S11 cells via interaction of adenovirus fiber with S11 allows functional titer assessment of CAR-ablated viruses.

Fresh tumor material from patients suspected of having a high-grade OS was processed directly after open biopsy surgery as described earlier (Witlox et al., 2002). Cells were used for experiments at passage 0–10. Confirmation of OS tumor cell morphology of all short-term cultures was performed by histopathology.

MDA-MB-453 cells were maintained in Leibovitz's L-15 medium (ATCC) supplemented with 10% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Paisley, United Kingdom). All other cells were maintained in DMEM:Ham's F-12 supplemented with 10% FCS, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies).

Adenovirus plasmid construction

To ablate CAR-binding, the plasmid pBHG11 (Bett et al., 1994; Microbix Biosystems, Toronto, Canada) was modified to contain the four amino acid deletion $T_{489}AYT_{492}$ in the FG loop of the fiber. This deletion has been shown to ablate binding to CAR (Roelvink et al., 1999) and will be referred to as F*. To facilitate cloning, subclones of pBHG11 were made by digestion of pBHG11 with *Xba*I and *Spe*I and religation to form pBHG11ΔXbaΔSpe and digestion with *Asc*I and religation to form pBHG11ΔAsc. Site-directed mutagenesis was performed using overlap extension PCR (Ho et al., 1989). For this, two separate PCR reactions were performed with plasmid pBHG11 as template using either primers 5′-GGCGGACGGCTACGACTG-3′ and 5′-CATAAATCCAACAGCGTTGCCTTCAGTAAGGTCTCC-3′ or 5′-GGAGACCTTACTGAAGGCAACGCTGTTGGATTTATG-3′ and 5′-CGGGAGGGTGCTATTTTGCC-3′. Subsequently, the PCR products were combined and used as template for a PCR reaction using primers 5′-CGGGAGGGTGCTATTTTGCC-3′ and 5′-GGCGGACGGCTACGACTG-3′. The resulting PCR product that contains the mutated fiber was digested with *Pac*I and *Avr*II and ligated in pBHG11ΔXbaΔSpe digested with the same enzymes. Subsequently, the *Cla*I/*Pac*I fragment was released and introduced in full-length pBHG11 to generate pBHG11F*. To abrogate integrin-binding, the penton motif $R_{340}GD_{342}$ was changed into the non-binding sequence $R_{340}GE_{342}$ (Obara et al., 1988). This mutation will be referred to as P*. Site-directed mutagenesis was performed using the primers 5′-GCCATCCGCGGCGAGACCTTTGCCACAC-3′, 5′-TCACTGACGGTGGTGATGG-3′, 5′-GGCAGAAGATCCCCTCGTTG-3′ and 5′-GTGTGGCAAAGGTCTCGCCGCGGATGGC-3′ with the pBHG11 plasmid as template. The resulting PCR product containing the desired mutation in the RGD motif was digested with *Pme*I and *Asc*I and introduced in pBHG11ΔAsc. Subsequently, the original AscI fragment was reintroduced by ligation to form full-length pBHG11P*. To construct pBHG11 containing both mutations, the *Cla*I/*Pac*I fragment of pBHG11F* was introduced in pBHG11P* digested with the same enzymes. This construct was named pBHG11P*F*.

To construct pBHG11P*F*-425S11-R, the 4.1 kb *Pac*I fragment from pABS.4-425S11 (Van Beusechem et al., 2003) carrying the CMV-425S11 cassette and kanamycin resistance gene was inserted into pBHG11P*F*. A clone with an insert in the orientation that places the CMV-425S11 cassette on the adenovirus R-strand was isolated and the kanamycin resistance gene was removed by digestion with SwaI followed by self-ligation, yielding pBHG11P*F*-425S11-R.

Recombinant adenoviruses

The CRAds AdΔ24 and AdΔ24-425S11 were described before (Van Beusechem et al., 2002b, 2003). The native tropism-ablated EGFR-targeted CRAd AdΔ24P*F*-425S11 was made by homologous recombination in 911 cells between pXC1-Δ24 (a generous gift of Dr Ramon Alemany, Gene Therapy Center, UAB, AL, USA) and pBHG11P*F*-425S11-R. AdΔ24, Ad24-425S11 and AdΔ24PF-425S11 carry the same 24 bp E1A deletion in the pRb-binding CR2 domain in E1A (Fueyo et al., 2000) and have identical deleted E3 regions (Ad5 nt 27865–30995). They only differ in the CMV-425S11 cassette insertion present in Ad24-425S11 and AdΔ24PF-425S11. Additionally, AdΔ24PF-425S11 contains the four amino acid deletion $T_{489}AYT_{492}$ in the FG loop of the fiber and the $R_{340}GD_{342}$ to $R_{340}GE_{342}$ substitution in the penton base that abolish interaction of the virus with CAR and integrins, respectively (Obara et al., 1988; Roelvink et al., 1999). Viruses were propagated on A549 cells and released from cells experiencing cytopathic effect by multiple freeze–thaw cycles in fresh culture medium. This yields particles that are essentially free from bispecific adapter protein. Purified virus stocks were prepared by two successive bandings on CsCl gradients. Viruses were stored at −80 °C in storage buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol) until use. The E1Δ24, P* and F* mutations and CMV-425S11 insertion were confirmed by PCR on the final products. Physical viral particle (VP) titers were determined by measurement of optical density at 260 nm. Functional titers in plaque forming units (PFU) were determined by limiting-dilution titration according to standard techniques on 911 cells and on 911-S11 cells. The VP/PFU ratios determined on 911 and 911-S11 were respectively 48 and 49 for AdΔ24, 9 and 13 for AdΔ24-425S11, and 2450 and 121 for AdΔ24PF-425S11. Since functional titers of viruses with different tropism cannot be directly compared, all infections were normalized by VP titers.

FACS analysis

CAR and EGFR expression levels on cell lines were analyzed after incubating cells with RmcB anti-CAR MoAb (Hsu et al., 1988) or 425 anti-EGFR MoAb (culture supernatant from the 425 hybridoma cell line; ATCC), respectively, followed by RbaMIgG-FITC (DAKO, Glostrup, Denmark). Negative controls were incubated with second antibody only.

Cells were analyzed on a FACScan (Beckton-Dickinson, Erembodegem-Aalst, Belgium), using CellQuest software (Beckton-Dickinson). Relative median fluorescence (RMF) signal intensity is defined as the ratio of the median fluorescence signal intensity of anti-CAR MoAb or anti-EGFR MoAb stained cells divided by that of the negative control stained cells.

Assessment of CRAd transduction efficiency by immunohistochemistry for adenoviral hexon protein expression

Cancer cells were seeded 1×10^4 per well in 96-well plates. Where indicated, virus was pre-complexed 30 min at room temperature with undiluted recombinant bispecific scFv 425-S11 and/or soluble EGFR before inoculating the cells. Soluble EGFR secreted by A431 cells (Weber et al., 1984), was isolated from culture medium by centrifugation over an Amicon ultra-15 centrifugal filter device (Millipore, Carrigtwohill, Ireland). The filtrate (without soluble EGFR) was used as negative control. Cells were inoculated at 1000 VP/cell with AdΔ24, Ad24-425S11 or AdΔ24P*F*-425S11 for 1 h at 37 °C, after which virus-containing medium was replaced with fresh medium. Two days after infection, cells were fixed with methanol and immunohistochemically stained using the anti-hexon antibody and other reagents provided in the Adeno-X rapid titer kit (BD Bioscience, Clontech, Palo Alto, CA) according to the manufacturer's protocol. Photos were taken using a 10× objective.

Assessment of CRAd oncolytic potency by crystal violet CPE assay and WST-1 cell viability assay

Cancer cells were seeded 5×10^4 per well in 24-well plates for crystal violet assay or 5×10^3 per well in 96-well plates for WST-1 assay. Before infection, virus was pre-complexed 30 min at room temperature with recombinant bispecific scFv 425-S11. Subsequently, cells were subjected to infection with AdΔ24, Ad24-425S11 or AdΔ24P*F*-425S11 at a range of MOIs for 1 h at 37 °C, after which virus-containing medium was replaced with fresh medium. Cells were cultured for 2–3 weeks with 50% medium refreshment every 4–5 days. For crystal violet staining, the culture medium was then removed and adherent cells were fixed for 15 min at room temperature with 4% formaldehyde in PBS, and subsequently stained using 1% crystal violet dye in 70% ethanol for 30 min at room temperature. After several washes with water, the culture plates were air-dried and scanned on a Bio-Rad GS-690 imaging densitometer. For the colorimetric WST-1 cell viability assay (Roche Diagnostics, Mannheim, Germany), the culture medium was replaced by 100 μl of 10% WST-1 in culture medium. Depending on the cell type, the formation of the formazan dye was allowed to proceed for 30–60 min at 37 °C after which the A450 was measured on a Bio-Rad (Hercules, CA, USA) model 550 microplate reader. WST-1 conversion was expressed relative to the conversion by uninfected control cells, after subtraction of background values of WST-1 incubated in the absence of cells. The 50% tissue culture inhibiting dose ($TCID_{50}$; i.e., the MOI causing 50% reduction in WST-1 conversion) was determined using GraphPad Prism4 software. Relative oncolytic potencies of AdΔ24P*F*-425S11 compared to AdΔ24 on different cell lines were estimated by calculating the average ratios of the $TCID_{50}$ of AdΔ24 over that of AdΔ24P*F*-425S11 from three independent experiments.

Replication and cytotoxicity of CRAds on primary osteosarcoma specimens

Primary, short-term osteosarcoma cell cultures were seeded 5×10^3 cells per well to prepare subconfluent monolayers in 96-well plates. The next day, the cells were infected at 100 VP/cell with AdΔ24, Ad24-425S11 or AdΔ24P*F*-425S11 for 1 h at 37 °C,without adding bispecific scFv targeting molecules. Subsequently, cells were cultured at 37 °C for 16–27 days before analysis of cell survival by WST-1 conversion assay. Cell survival was assessed using WST-1 as described by the manufacturer (Sigma, St. Louis, MO). Survival is expressed as a percentage of control uninfected cells. Statistical significance of enhanced oncolysis by Ad24-425S11 and AdΔ24P*F*-425S11 compared to AdΔ24 was tested by two-tailed Students *t*-test.

Recovery of infectious viral particles from human liver slices

Human liver samples were obtained from the Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation of the University Hospital Groningen after obtaining approval of the local medical ethical committee and informed consent. Samples were stored in University of Wisconsin organ preservation solution (UW, Du Pont Critical Care, Waukegan, IL) at 4 °C until slicing. Slices were prepared as described previously (Olinga et al., 1998). In short, cores of 8 mm were drilled from the tissues, which subsequently were sliced using a Krumdieck slicer (Alabama R&D, Munfort, AL) for optimal and reproducible preparation of liver slices. The thickness of the slices was adjusted to 200–250 μm (10–14 mg). The slicing procedure itself was performed in ice-cold physiological Krebs buffer. Slices were washed and pre-incubated at 37 °C for 1 h in Williams' Medium E (WME) supplemented with D-glucose (25 mM) and gentamycin (50 μg/ml), saturated with 95% O_2/5% CO_2 before experiments were started. Slices were infected by adding virus to 3.2 ml of fresh oxygenated WME in 6-well plates. Where indicated, virus was pre-complexed 30 min at room temperature with recombinant bispecific scFv 425-S11 before inoculation. Subsequently, cells were inoculated with AdΔ24, Ad24-425S11 or AdΔ24P*F*-425S11 at 1×10^8 VP/slice for 2 h at 37 °C after which infection medium was replaced with fresh medium. Slices were further incubated in 95% O_2/5% CO_2 for 48 h at 37 °C under continuous shaking. Slices were harvested 2 days after infection and lysates were prepared in reporter lysis buffer (Promega, Madison, WI, USA). The number of infectious particles present in the lysates was determined using a limiting-dilution titration assay on 911-S11 cells using the Adeno-X rapid titer kit (Clontech) according to the manufacturer's protocol.

Immunohistochemistry on human liver slices

Human liver material that remained after preparation of the slices used for infection was frozen and sectioned at 4-μm-thick for immunohistochemical staining for EGFR and CAR expression. Frozen sections were fixed in acetone and incubated with the primary antibody against CAR (RmcB 1:100) or EGFR (clone EGFR.113, Novacastra, Newcastle upon Tyne, UK) at room temperature for 1 h. Subsequently, the sections were stained using the Envision, Peroxidase/DAB detection kit (DAKO) according to manufacturer's protocol. The sections were counterstained with hematoxylin, dehydrated and cover slipped. Tissue from normal skin was used as a positive control for EGFR staining. As a negative control, the primary antibody was replaced by PBS. Photos were taken using a 20× objective.

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