**Featured Article**

**Conditionally Replicative Adenovirus with Tropism Expanded towards Integrins Inhibits Osteosarcoma Tumor Growth in Vitro and in Vivo**

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Abstract

**Purpose:** The clinical course of osteosarcoma (OS) demands the development of new therapeutic options. Conditionally replicative adenoviruses (CRAds) represent promising agents for the treatment of solid tumors, because CRAds have an intrinsic replication capacity that allows in situ amplification and extensive tumor infection through lateral spread. The CRAd AdΔ24 has been developed to replicate selectively in cells with a defective retinoblastoma (Rb) pathway. Because genetic alterations in the Rb pathway are frequently observed in OS, AdΔ24 might be useful in the treatment of this cancer.

**Experimental Design:** Because the lack of Coxsackie adenovirus receptor on OS cells limits the efficacy of CRAd treatment, we explored alternative target molecules on OS. Insertion of an Arg-Gly-Asp (RGD-4C) integrin-targeting motif into the adenovirus fiber knob expanded tropism toward the α5β1 and αvβ3 integrins. The oncolytic capacity of the CRAd Ad5-Δ24RGD was tested on primary OS cells in vitro and in vivo.

**Results:** The α5β1 and αvβ3 integrins are abundantly expressed on OS cells. RGD-mediated infection augmented adenovirus infection of primary OS cells by two orders of magnitude. Ad5-Δ24RGD was shown to be highly active in killing human OS cell lines, as well as primary cell cultures. Furthermore, intratumoral injections with Ad5-Δ24RGD into established human OS xenografts that were derived directly from a patient with OS refractory for chemotherapeutic treatment caused a significant tumor-growth delay. Furthermore, adenoviral particles could be detected in tumor cells 25 days posttumor injection.

**Conclusions:** Targeting adenovirus toward integrins rendered OS cells more sensitive to killing by Ad5-Δ24RGD. These findings suggest that Ad5-Δ24RGD has potential for use in OS treatment.

Introduction

Osteosarcoma (OS) is one of the most common primary tumors of bone. It is highly malignant and typically affects children and young adults (1). Despite improvements in the treatment of OS, there are still too many patients who cannot benefit from current treatment modalities (2, 3). The overall survival with an aggressive chemotherapy regimen before and after surgery now varies between 50% and 65% (4–6). In addition, current chemotherapeutic agents have a broad spectrum of side effects (7). Therefore, new therapeutic options, such as gene therapy, are warranted. In the field of adenoviral vector (Adv)-based gene therapies for OS, several approaches have been explored, such as suicide gene therapy (8), tumor suppressor gene therapy (9), and cytokine-based gene therapy (10). Results of these studies have not yet been translated into clinical trials. Important limitations in this regard are the failure of nonreplicating Adv to achieve sufficient tumor-cell transduction and effective solid-tumor penetration.

The development of conditionally replicative adenoviruses (CRAds) has addressed these limitations. CRAds represent promising agents for the treatment of solid tumors, because CRAds have intrinsic replication capacity that allows in situ amplification and extensive tumor infection through lateral spread (11). CRAds have been designed following two different strategies, i.e., by controlling expression of essential early adenovirus genes with tumor-specific promoters (e.g., see Refs. 12, 13) and by deleting viral genes encoding proteins that are necessary to complete the viral lytic cycle in normal cells, but not in cancer cells (14–16).

A critical step determining CRAd efficacy is the infection efficiency dependent on expression of the primary adenovirus receptor, the Coxsackie adenovirus receptor (CAR; Ref. 17). Low CAR expression on primary-tumor cells has been described to limit the efficacy of adenovirus-based therapy for several types of cancers, including brain, bladder, and pancreatic cancer (18–21). Recently, we demonstrated that Adv-infection efficiency is also compromised in OS, because of low or absent
CAR expression (22). Therefore, other, more effective routes of infection for OS should be explored. Previously, it has been shown that incorporation of a cyclic Arg-Gly-Asp (RGD-4C) sequence that interacts with \( \alpha_v \) integrins into the adenovirus fiber-knob circumvents low CAR expression by providing an alternative viral entry pathway (23). In addition, recent studies have shown that a CRAd encoding retinoblastoma protein binding-deficient E1A proteins and the RGD-4C-fiber, Ad5-\( \Delta \)24RGD (24), exhibited oncolytic potency on CAR deficient cancer cells (25, 21). Therefore, we hypothesized that Ad5-\( \Delta \)24RGD could also be effective in OS tumors.

In this study, we first explored the expression of integrins on OS cell lines and primary OS cells. Next, we tested the effect of the RGD-4C insertion in the fiber knob of a nonreplicative Adv on OS transduction. Finally, we assessed the efficacy of Ad5-\( \Delta \)24RGD on a broad panel of primary OS cell cultures in vitro and on primary OS s.c. xenografts in vivo.

Materials and Methods

Cells and Culture Conditions. MG-63 (Ref. 26; courtesy of Dr. C. Löwik, Leiden University Medical Center, The Netherlands), MNNG-HOS (American Type Culture Collection, Manassas, VA; Ref. 27), SaOs-2 (Ref. 28; courtesy of Dr. F. van Valen, Westfalische Wilhelms-Universität Münster, Germany), and CAL-72 (Ref. 29; courtesy of Dr. J. Gioanni, laboratoire de Cancerologie, Faculté de Medicine, Nice, France) OS cell lines were maintained in DMEM supplemented with 10% FCS, 50 \( \mu \)g/ml streptomycin, and 2 \( \mu \)g/ml penicillin, 50 \( \mu \)g/ml amphotericin B, and on primary OS s.c. xenografts in vivo.

Patient Material. Fresh tumor material from patients suspected of having a classic high-grade OS was processed directly after open biopsy surgery and kept under sterile conditions and fed a standard laboratory diet (Hope Farms, Madison, WI). Medium was removed from the cells, and luciferase reporter lysis buffer was added to the wells followed by a freeze thaw step. Luminescence was measured during 10 s immediately after initiation of the light reaction in a Luminat LB 9507 luminometer (EG\&G Berthold, Bad Wildbad, Germany). Values were protein normalized (Bio-Rad Protein Assay, Bio-Rad laboratories, Veenendaal, The Netherlands). Results were expressed as relative light unit(s) (RLU) per microgram of protein.

Colorimetric WST-1 Cell Viability Assay. Cell viability was quantified using the colorimetric WST-1 conversion assay, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Diagnostics, Manheim, Germany), 7 or 11 days after infection. The culture medium was removed and replaced by 100 \( \mu \)l of 10% WST-1 in culture medium. Culturing was continued until the formation of formazan dye was optimal. The \( A_{450} \) was measured on a Bio-Rad Model 550 (Hercules, CA) microplate reader. WST-1 conversion was expressed as a percentage of the conversion by uninfected control cells.

In Vivo Experiment. Female athymic nu/nu mice, weighing 25–35 g, obtained from Harlan-Cpb (Austerlitz, The Netherlands) were used. Animals were kept under pathogen-free conditions and fed a standard laboratory diet (Hope Farms, Woerden, The Netherlands) ad libitum. The experimental protocols adhered to the rules outlined in the Dutch Animal Exper-
Table 1  Fluorescence-activated cell sorter analysis of integrin expression on osteosarcoma (OS) cell lines and primary OS cells

<table>
<thead>
<tr>
<th>OS cells</th>
<th>Relative median expression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>αβ3</th>
<th>αβ5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63</td>
<td></td>
<td>3.1</td>
<td>11.6</td>
</tr>
<tr>
<td>MNNG-HOS</td>
<td></td>
<td>2.4</td>
<td>12.6</td>
</tr>
<tr>
<td>CAL-72</td>
<td></td>
<td>2.3</td>
<td>10.5</td>
</tr>
<tr>
<td>SaOs-2</td>
<td></td>
<td>2.9</td>
<td>7.6</td>
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<tr>
<td>OS-1</td>
<td></td>
<td>5.2</td>
<td>2</td>
</tr>
<tr>
<td>OS-1a</td>
<td></td>
<td>5.2</td>
<td>2</td>
</tr>
<tr>
<td>OS-2</td>
<td></td>
<td>5.8</td>
<td>9.2</td>
</tr>
<tr>
<td>LOS-5</td>
<td></td>
<td>2.6</td>
<td>2.3</td>
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<tr>
<td>OS-6</td>
<td></td>
<td>6.2</td>
<td>5.4</td>
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<tr>
<td>OS-6a</td>
<td></td>
<td>2</td>
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<sup>a</sup> Relative median fluorescence intensity of integrin stained cells over control (non-stained) cells. Values ≥2 were considered positive.

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Results

Targeting Toward α Integrins Enhanced Infection of OS Cells. Previously, we examined CAR expression on OS cell lines and primary OS cell cultures (22). All OS cell lines, except SaOs-2, and all primary cells showed low or even absent CAR expression. In search of alternative target molecules on OS that would allow more efficient CRAd infection, we examined αβ3 and αβ5 integrin expression by fluorescence-activated cell sorter analysis (Table 1). All OS cell lines and primary OS cell cultures expressed at least one of these integrins. To study the utility of integrins as targets for adenovirus infection of OS, we compared the gene-transfer efficiency of an adenovirus vector with native tropism (AdCMVLuc) to that of a derivative vector with RGD-4C fibers (AdLucRGD). As shown in Fig. 1, targeting toward integrins strongly enhanced gene-transfer efficiency on OS cell lines and primary OS cell cultures. In particular, the on-average two orders of magnitude augmented transduction of primary cells by AdLucRGD held promise for the utility of integrin-targeted CRAds for the treatment of OS.

Oncolytic Effect of AdΔ24 and Ad5-Δ24RGD in Vitro. To evaluate the efficacy of the CRAd AdΔ24 on OS cells, we infected four OS cell lines at high MOI (Fig. 2A). AdΔ24 effectively killed all OS cell lines, suggesting that a CRAd with the Δ24-mutation could be effective for the treatment of OS. However, when we infected the same panel of OS cell lines or OS primary cell cultures with AdΔ24 at low-MOI, only CAR-positive SaOs-2 cells were killed (Fig. 2B). In contrast, integrin-targeted Ad5-Δ24RGD was highly active in killing all OS cell lines and primary cell cultures at low MOI (Fig. 2B). Thus, only Ad5-Δ24RGD was capable of effectively killing CAR-deficient primary OS cells.

Inhibition of OS Tumor Growth by Ad5-Δ24RGD Treatment in Vivo. Finally, we investigated if Ad5-Δ24RGD was also effective against CAR-deficient primary human OS tumors in vivo. Nude mice carrying established OS-1a xenografts derived from a chemotherapy-resistant primary tumor were treated by intratumor injections with a total virus dose of 5 × 10⁷ pfu Ad5-Δ24RGD. The growth curves of tumors injected with virus or control medium are shown in Fig. 3. The median tumor growth rate was calculated for both treatment groups. Control tumors had a median tumor growth rate of 14 ± 4 days, whereas tumors injected with Ad5-Δ24RGD exhibited a significant growth delay (median tumor growth rate of 25 ± 4 days; P < 0.05).

![Fig 1](link) Targeting an adenovirus vector toward integrins on osteosarcoma (OS) cell lines and primary OS cells. OS cell lines MG-63, MNNG-HOS, CAL 72, and SaOs-2 and five different primary OS cell cultures were transduced with AdCMVLuc (white bars) or AdCMVLucRGD (black bars) at a multiplicity of infection of 100 plaque-forming units/cell. Luciferase expression was measured after 24 h. Data shown are mean ± SD from a representative experiment done in triplicate. BLU, relative light unit(s).
Histological analysis of tumors exceeding 1000 mm$^3$ showed classical OS features with osteoid calcified areas. In tumors retrieved from Ad5-H9004$_{24}$RGD-treated animals, classic OS features could still be observed, together with posttherapeutic changes of necrosis within viable tumor fields surrounded by fibrosis (Fig. 4A). Anti-adenovirus staining revealed viral replication throughout treated tumors, mainly in tumor cells at the border of areas with necrosis (Fig. 4B). Hexon staining was absent in nontreated tumors (data not shown). Areas with positive anti-adenovirus staining were sampled for electron microscopy, which showed 65-nm dense, rounded adenovirus particle inclusions in tumor cells (Fig. 4, C and D). Electron microscopy of nontreated tumors did not show such inclusions (data not shown). Altogether, these findings showed a presence and replication of Ad5-$\Delta 24$RGD in treated tumors for up to 25 days postinjection.

**Discussion**

The clinical course of OS demands the development of new therapeutic options. CRAds represent promising agents for the treatment of solid tumors. However, their efficacy in OS tumors may be hampered by inefficient cell entry because of a lack of CAR expression (22). We, thus, hypothesized that the efficacy of CRAds against OS could be improved by expanding their tropism toward $\alpha v$ integrins. This was supported by our findings described herein that $\alpha v$ integrins are expressed on primary OS cells and that a replication-deficient Adv, which was genetically modified to expose the RGD-4C peptide in the fiber knob, exhibited dramatically enhanced gene transfer into OS cell lines and, more importantly, into primary OS cells.

We chose to investigate the effect of CRAd integrin targeting using Ad$\Delta 24$ as a backbone. Ad$\Delta 24$ encodes E1A proteins with a deletion of eight amino acids in the pRb-binding CR2 domain that disables their capacity to release E2F from preexisting pRb/E2F complexes, thereby conferring selective replication in cells with already dysfunctional pRb control (13). It has already been shown that infection with Ad$\Delta 24$-type CRAds effectively kills cancer cells in vitro and inhibits tumor growth in vivo, and replication is reduced in nonproliferating normal cells and in cancer cells with restored pRb function (13, 14). Because genetic alterations in the Rb pathway are frequent in OS (24, 33), Ad$\Delta 24$-type CRAds appear to be useful agents for treatment of this disease. Notably, however, the concept of integrin targeting is relevant for any kind of CRAd, irrespective of the genome modification conferring selective replication.

Enhancing the infection efficiency of Ad$\Delta 24$ by integrin targeting translated into increased oncolytic effects. On a broad panel of primary OS cells infected at low MOI, Ad5-$\Delta 24$RGD
showed complete oncolysis, whereas AdΔ24 induced no detectable cell kill at all. Because AdΔ24, in contrast to Ad5-Δ24RGD, lacks the adenovirus E3 region, differences in oncolytic effect may not only be explained by the expanded tropism of Ad5-Δ24RGD. However, AdΔ24 did effectively kill the CAR-positive OS cell line SaOS-2 at low MOI, suggesting that the infection enhancement of Ad5-Δ24RGD contributed to its efficacy on CAR-negative OS cells.

Primary cells derived directly from patient tumor samples represent an in vitro model closer to the patient compared with cell lines. Therefore, the observation that Ad5-Δ24RGD showed complete oncolysis of primary OS cells at an MOI of only 0.1 pfu/cell, whereas AdΔ24 required 100 pfu/cell for this effect, is of significant importance. The in vitro efficacy of Ad5-Δ24RGD was confirmed in a relevant in vivo OS tumor model. Ad5-Δ24RGD caused a significant tumor-growth delay of human primary, chemotherapy-resistant, CAR-deficient OS xenografts in nude mice. Histological analysis showed that Ad5-Δ24RGD replicated in OS tumor cells for at least 25 days. However, the CRAd did not completely eradicate the s.c. xenografts. This can best be explained by inefficient dispersion of the CRAd through the tumor mass after intratumoral injection. Barriers within the established tumor, such as connective tissue and tumor matrix, may limit the spread of virus (34). In OS, osteoid calcification could be such a hurdle for viral therapy that needs to be overcome.

Altogether, our data demonstrate that targeting a CRAd toward integrins enhances its cytotoxicity on OS dramatically and warrants further exploration of Ad5-Δ24RGD for its utility in OS treatment. The main challenge in the treatment of OS, however, is the eradication of metastases. Patients with OS do not die from the primary tumor; they die from metastatic disease, typically in the lungs. Notably, we have found that α3,β3 and αv,β5 integrins are expressed on OS lung metastases (data not shown). Clearly, intratumoral injection as evaluated in this study is not a feasible option for treatment of lung metastases. However, systemic delivery is now being explored with several viro-therapy agents in clinical trials (35), and, thus, the use of Ad5-Δ24RGD could perhaps be considered for the treatment of OS.

Fig. 4 Detection of adenovirus replication in OS-1a tumor xenografts. At a tumor size of >1000 mm³, mice were sacrificed and tumors were dissected. Tumor sections of an Ad5-Δ24RGD treated tumor were stained with hematoxylin-eosin (A) and with an anti-adenovirus hexon antibody (B). Viable tumor fields are surrounded by fibrosis (fb). Large areas of necrosis (nc) are surrounded by hexon stained cells (arrows), indicative of adenovirus replication. Areas stained positive for hexon were sampled for electron microscopy (C and D). Adenovirus particle inclusions are indicated by arrows. Original magnifications: ×20 (A and B); ×24,000 (C); and ×108,000 (D). ECM, extracellular matrix.
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References


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