Chapter 4

Highly efficient and carcinoma-specific adenoviral replication restricted by the EGP-2 promoter

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Abstract

Although some successes have been reported using adenoviral vectors for the treatment of cancer, adenoviral cancer gene therapy is still hampered by the lack of sufficient tumor cell killing. To increase the efficiency, adenoviruses have been modified to replicate specifically in tumor tissues by using tumor specific promoters controlling genes essential for adenoviral replication. However, many conditionally replicating adenoviral vectors replicate in one tumor type only, which limits their application. The epithelial glycoprotein-2 (EGP-2) promoter is active in a broad variety of carcinomas, the most common type of cancer. We utilized this promoter to restrict adenoviral replication. In this report we demonstrate that the potency of the replication-competent adenovirus AdEGP-2-E1 to specifically lyse EGP-2 positive cells is comparable to wild-type adenovirus (AdWT). In addition, we show that \textit{in vivo} AdEGP-2-E1 replicates as efficient as AdWT in EGP-2 positive tumor cells. On the contrary, in EGP-2 negative cell lines as well as in primary human liver samples, the replication was attenuated up to 4-log in comparison to wild-type virus. This report clearly shows the potency of the EGP-2 promoter to mediate highly efficient and specific adenoviral replication for carcinoma gene therapy.
Introduction

Cancer still is a major cause of death in the western world. This signifies that current therapies are not sufficiently effective and novel treatments need to be developed. In this regard, adenoviral vectors have been proposed as promising cancer gene therapeutic agents. The incorporation of suicide genes into the adenoviral genome, for example, allows relative efficient delivery of such genes into the malignant tissue, which ultimately results in cell killing. After systemic administration however, most of the adenovirus homes to the liver. The expressed transgene can subsequently cause severe hepatotoxicity. To avoid the expression of therapeutic genes in normal healthy cells, tumor specific promoters (tsp) have been used. These promoters are active in tumor cells, but show limited activity in normal tissues. This results in the selective expression of suicide genes within the malignant cells.

Although the adenovirus is among the most efficient vectors currently available, it can not efficiently penetrate into the solid tumor mass after intratumoral administration. This results in a limited number of malignant cells expressing the therapeutic gene. To this end, replication-competent adenoviruses have been developed to improve the therapeutic efficacy of adenoviral agents. Replication of the adenovirus will result in cell lysis and subsequent infection of neighboring cells. This augmentation of the initial infection results in a substantially increased number of infected cells and subsequent cell killing.

To increase specificity, conditionally replicating adenoviruses (CRAds) have been designed, which replicate preferentially in tumor cells. One approach to restrict adenoviral replication to tumor cells is to control the expression of genes essential for adenoviral replication by tsp. Activity of the tsp only in malignant cells will result in the selective expression of replication-essential adenoviral genes. Subsequently, adenoviral replication will only occur in these malignant cells, and not in healthy tissues. Several different tsp have already been utilized to control adenoviral replication such as the tyrosinase, α-fetoprotein, osteocalcin, uroplakinII, and prostate-specific antigen (PSA) promoter. Although many tsp show great potential to selectively control adenoviral replication, their applications are limited to specific cancer types.

To broaden the spectrum of cancer types to be treated by conditionally replicating adenoviruses, we used the epithelial glycoprotein-2 (EGP-2) promoter, also known as Ep-CAM or GA733-2, is a trans-membrane protein and is over-expressed in the majority of epithelial derived cancers, such as pancreatic, colorectal, ovarian, prostate, and lung cancer. Most importantly for retargeting purposes, mature hepatocytes do not express EGP-2. Our previous work demonstrated that the EGP-2 promoter controlled highly selective and efficient transtene expression. Moreover, when the EGP-2 promoter was utilized in an adenoviral context to control the expression of a suicide gene, we observed selective and efficient cell killing.

This specificity and efficiency of the EGP-2 promoter, as well as the feasibility of targeting a broad range of tumor types makes this promoter an attractive device to control adenoviral replication. In this study, we show the potential of an EGP-2-based CRAd to specifically replicate and eradicate EGP-2 positive tumor cells, while sparing healthy liver tissue.
Materials and methods

Cell and tissue culture

The human colon carcinoma cell lines WiDR and LS174T, and the human colorectal adenocarcinoma cell line SW948 were all cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM glutamine ( Gibco Invitrogen Corporation, Breda, The Netherlands), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Bio Whittaker Europe, Verviers, Belgium), penicillin (100 U/ml) and streptomycin (100 U/ml) (Gibco). The human ovarian cancer cell line Ovcar-3, the human hepatocellular carcinoma cell line HepG2, and the human cervical cancer cell line HeLa were cultured in the same medium. The human astrocytoma cell line U373 and the transformed human embryonic kidney cell line 293 were cultured in DMEM/nut mix-F12 (Gibco) supplemented with 10% FBS (Bio Whittaker Europe), 2 mM glutamine (Gibco), penicillin (100U/ml), and streptomycin (100U/ml) (Gibco). Cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

Human liver samples were obtained from the Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation of the University Medical Center Groningen, after approval of the local medical and ethical committee and informed consent. Samples were stored at 4°C in University of Wisconsin organ preservation solution (UW, Du Pont Critical Care, Waukegan, IL, USA) until slicing. Slices were prepared as described previously.  

Construction of AdEGP-2-E1

All recombinant vectors were constructed through homologous recombination in Escherichia Coli using the AdEasy system. The transgene cassettes were cloned in the E1 region of the E1- (480-3533) and E3- (28130-30820) deleted backbone. Figure 1 gives an overview of the adenoviral vectors used in this study. The construction of pShGL3BEGP-2 and pShGL3BCMV is described elsewhere. Briefly, these constructs are based on pShuttle but contain the multiple cloning site, luciferase gene, and the simian virus 40 polyadenylation signal from pGL3Basic (Promega, Madison, WI). In the multiple cloning site, the expression of the luciferase gene is controlled by respectively the EGP-2 promoter or the CMV promoter. To determine the EGP-2 promoter activity in different cell lines, a 1.2 kb EGP-2 fragment was used to control the expression of the luciferase gene. An adenovirus containing a larger 3.4 kb EGP-2 fragment driving the expression of luciferase was used as a non-replicating adenovirus in the experiments with the human liver slices. There was no difference in activity and specificity between the 1.2 kb and 3.4 kb EGP-2 promoter fragment. AdTL contains two CMV-driven expression cassettes, for the green fluorescent protein and for luciferase. The wild-type virus (AdWT) used in this study contained the complete adenoviral genome and the adenoviral vector dl7001 contained all adenoviral sequences except for the E3 region.

In order to obtain the replication-competent adenovirus AdEGP-2-E1, pShGL3BEGP-2 was restricted with KpnI and XhoI, and the EGP-2 fragment was cloned into the KpnI-XhoI sites of pShuttle generating pShEGP-2. To clone the E1 gene downstream of the EGP-2 promoter, first the E1 region of the Ad5 genome (459-3533) was subcloned in the EcoRV sites of pcDNA 3 (Invitrogen) (kind gift from Dr. R. Alemany,
Translational Research Laboratory, Institut Catala d’Oncologia, Barcelona, Spain), subsequently subcloned in the NotI and BglII sites of pShTyr, followed by restriction with SpeI and Pmel to clone the E1 fragment into the SpeI and Pmel sites of pShEGP-2 generating pShEGP-2-E1. The resulting virus was named AdEGP-2-E1.

All viruses used in this study were purified by double density gradient centrifugation, followed by dialysis against 5% sucrose in 10 mM Tris pH 8.0 and 2 mM MgCl₂. The viruses were aliquoted and stored at −80°C until usage. The virus titer was determined by conventional limiting dilution on 293 cells. The vp/pfu ratios were as follows, AdGL3BCMV: 23, AdGL3BEGP-2: 164, AdTL: 90, AdEGP-2-E1: 72, and AdWT: 18.

Assay to determine the EGP-2 promoter activity in a panel of cell lines

To fully establish the activity of the EGP-2 promoter, a previously reported panel of cell lines was extended. Cells were plated at a density of 50.000 cells/well in a 24-wells plate (Gibco). The following day, the cells were infected at an m.o.i. of 100 pfu/cell with the luciferase-encoding viruses AdGl3BEGP-2 or AdGL3BCMV, in medium containing 2% FBS. After 48 h the cells were lysed with cell culture lysis buffer (Promega) and the lysates were analyzed for luciferase activity on a Lumicount luminometer (Packard, Groningen, The Netherlands). Data are also depicted as percentage of CMV expression to correct for the difference in infection efficiency between different cell lines.

Assay to validate the oncolytic potential of AdEGP-2-E1

To examine the specificity and efficiency of AdEGP-2-E1-induced oncolysis, EGP-2 positive and EGP-2 negative cell lines were plated 10.000 cells/well on a 96-well plate (Gibco). The following day, cells were infected with AdWT, AdEGP-2-E1, or AdTL with an m.o.i. of 10, 1, 0.1, 0.01 or 0.001 PFU per cell in culture medium containing 5% FBS. After 10 days, the remaining cells were fixated with formalin solution (Sigma, Zwijndrecht, The Netherlands), stained with gentian violet (Sigma) (1% in 70% EtOH) and photographed.

Virus yield assay

To determine the amount of functional viral particles formed after infection with AdEGP-2-E1 or AdWT, 300.000 cells of the EGP-2 positive cell line WiDR or of the EGP-2 negative cell line U373 were plated in a 6-well plate (Gibco). The following day, cells were infected with 5 pfu/cell AdEGP-2-E1 or AdWT in culture medium containing 5% FBS. Two hours post-infection, cells were washed twice with pre-warmed PBS (Invitrogen) and new medium containing 5% FBS was added. Each day during a period of 10 days cells and medium were harvested. Cells were cracked by three cycles of freeze-thawing and the remaining cell debris was spun down. The virus titer was determined separately for the collected cells and medium by using conventional limiting dilution techniques. Total virus production was calculated as 1/7*(pfu/ml cells) + 6/7*(pfu/ml medium) as 500 µl medium contained virus production in cells and 3000 µl medium contained virus production in medium for each well.
Virus production in human liver samples

To determine the ‘liver-off’ profile of AdEGP-2-E1, human liver slices were prepared from two different human liver samples. The vitality of these samples was checked by measuring the ATP concentration and the metabolic capacity. After pre-incubation for 1 h, human liver slices were either left untreated or were infected with the E1-deleted virus AdGL3BEGP2, AdWT, or AdEGP-2-E1 by adding $1 \times 10^6$ vp/slice of the indicated viruses (corresponds to circa 1 vp/hepatocyte) to 3.2 ml fresh oxygenated Williams’ medium E (WME) supplemented with D-glucose (25 mM) (Merck, Amsterdam, The Netherlands), gentamycin (10 µg/ml) (Gibco), penicillin (100 U/ml) (Gibco), streptomycin (100 U/ml) (Gibco), and fungizone (25 µg/ml) (Gibco) in 6-well plates (Gibco). Slices were incubated in an atmosphere of 95% O$_2$ / 5% CO$_2$ at 37 °C under continuous shaking for 96 h or 168 h. Extra antibiotics were added every 48 h. After incubation, the medium was snap-frozen and stored at −80 °C. At a later time point, the medium was titrated on 293 cells by conventional limiting dilution to determine the virus concentration. The virus concentration found in the medium after infection with AdGL3BEGP-2 was set as background and subtracted from the virus concentration of AdEGP-2-E1 or AdWT in the medium. The number of slices used per virus is indicated in Figure 4. Values are expressed as mean of the different human liver slices.

To determine possible differences in virus production between AdWT and dl7001, slices were infected with 1 pfu/hepatocyte and treated as described above.

Animal experiments

For in vivo experiments, female BALB/c nu/nu mice were used. All in vivo experiments were reviewed and approved by the Animal Experiments Committee (DEC) of the University of Groningen, The Netherlands.

$5 \times 10^6$ Cultured LS174T cells were inoculated into the flank of nude mice to determine the replication efficiency of AdEGP-2-E1 in comparison the AdWT replication in vivo. After 10 days all tumors were palpable, and either sucrose buffer (n=6), $1 \times 10^8$ pfu AdTL (n=5), AdEGP-2-E1 (n=6), or AdWT (n=6) was injected. The injections were repeated after 3 and 6 days. Tumor growth was monitored three times a week and the mice were sacrificed when the tumor volume became excessive. Tumors and livers were isolated, divided in two parts, and frozen in liquid nitrogen for further analysis.

Real-time PCR to determine the hexon copy number in liver and tumor tissue

To examine the potency of AdEGP-2-E1 to replicate in vivo, hexon copy numbers were determined on one part of the isolated tumor and liver tissue after i.t. injection with AdEGP-2-E1, AdWT, or AdTL. Total DNA was isolated out of 10-25 mg of mouse liver or tumor tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the instructions of the supplier. Quantitative real-time PCR specific for the hexon-gene was performed on the DNA isolate with the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Molecular Biochemicals). PCR was performed as described by the manufacturer. The primers hexon Fw (5’-CTTCgATgATgCCgCAgTg-3’) and hexon Rv (5’-ggCTCAggTACTCCAgg-3’) were used as forward and reverse primer. Primers were obtained from TibMolbiol (Berlin, Germany). Wildtype adenovirus serotype 5 DNA (1×10$^1$
to $1\times10^6$ copies/$\mu$l as a 10-fold dilution series) was used as an external standard to generate a calibration curve for quantitation of the test samples.

Hexon copy numbers detected in the liver of each mouse were set as background as mouse liver cells do not support adenoviral replication and these background values were subtracted from the values found in the tumor tissue of the same mouse.

**Immunohistochemistry to determine the presence viral progeny in tumor tissue in vivo**

To determine viral progeny production after i.t. administration of AdTL, AdEGP-2-E1 or AdWT, immunohistochemical staining of adenoviral hexon protein was performed in one cross-section of the remaining part of the frozen tumor sections. The acetone fixed cryosections were obtained from the excised tumors of mice sacrificed 24 or 30 days after the first injection. Briefly, immunohistochemical staining was performed using the goat anti-adenoviral hexon protein antibody AB1056 (Chemicon International, Hampshire, United Kingdom). Bound primary antibodies were detected with horseradish peroxidase-conjugated rabbit anti-goat antibodies (Dako Cytomation B.V., Heverlee, The Netherlands) and exposure to chromogens 3-amino-9-ethylcarbazole followed by counterstaining with Mayer’s hematoxilin. Negative controls were not incubated with the primary antibody.

**Results**

**Adenoviral vectors**

The adenoviral vectors used in this study are depicted in Figure 1. AdGL3BEGP-2 1.2 kb and AdGL3BEGP-2 3.4 kb contain respectively the 1.2 kb EGP-2 promoter fragment or the 3.4 kb EGP-2 promoter fragment controlling the expression of luciferase. There is no difference in the activity or specificity between the two promoter fragments. AdGL3BCMV contained the constitutively active CMV promoter driving the expression of luciferase. The 1.2 kb EGP-2 promoter fragment was utilized to control the expression of the replication-essential gene E1, generating the conditionally replicating adenovirus AdTL.
AdEGP-2-E1. As a negative control for replication AdTL was used, which contained in the E1 region the CMV promoter controlling the expression of both luciferase gene and the green fluorescent protein. As a positive control for replication, we utilized wild-type adenovirus (AdWT).

EGP-2 promoter activity in a panel of human cell lines

To determine the activity of the EGP-2 promoter in vitro in an adenoviral context, several human cell lines were infected with adenoviruses containing the luciferase gene under the control of either the CMV or the EGP-2 promoter. The EGP-2 expression status of all cell lines has been confirmed previously, on the mRNA level and/or on the protein level.16,27,28,29 The human ovarian cancer cell line Ovcar-3, as well as the human colon cancer cell lines LS174T and WiDR demonstrated very high EGP-2 promoter activity when expressed as relative light units (Table 1). Compared to the strong and constitutive active cytomegalovirus (CMV) promoter, the EGP-2 promoter activity in the cell line Ovcar-3 was 4% of CMV activity. The relative promoter activity was up to 10-fold higher for the human cell lines LS714T, WiDR, and SW948 (31-40% of CMV activity). In contrast, the hepatoma cell line HepG2, the astrocytoma cell line U373, as well as the cervical cancer cell line HeLa showed very limited activity of the EGP-2 promoter (0.06-0.62% of CMV activity).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGP-2 promoter activity (relative light units ± s.e.m.)</th>
<th>EGP-2 promoter activity (relative to CMV activity ± s.e.m.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovcar-3</td>
<td>5.1<em>10^5 ± 7.6</em>10^4</td>
<td>4.0% ± 0.1</td>
</tr>
<tr>
<td>LS174T</td>
<td>4.4<em>10^5 ± 1.0</em>10^5</td>
<td>39.7% ± 3.3</td>
</tr>
<tr>
<td>WiDR</td>
<td>3.6<em>10^5 ± 7.5</em>10^4</td>
<td>30.8% ± 4.3</td>
</tr>
<tr>
<td>SW948</td>
<td>7.4<em>10^4 ± 5.6</em>10^3</td>
<td>37% ± 1.3</td>
</tr>
<tr>
<td>HepG2</td>
<td>5.7<em>10^4 ± 8.1</em>10^3</td>
<td>0.62% ± 0.17</td>
</tr>
<tr>
<td>U373</td>
<td>1.8<em>10^5 ± 3.1</em>10^3</td>
<td>0.06% ± 0.04</td>
</tr>
<tr>
<td>HeLa</td>
<td>4.2<em>10^5 ± 1.0</em>10^5</td>
<td>0.18% ± 0.05</td>
</tr>
</tbody>
</table>

Table 1. Activity EGP-2 promoter in a panel of cell lines

Cells were infected with AdGL3BEGP-2 or AdGL3BCMV at an m.o.i. of 100 pfu/cell and luciferase activity was determined after 48 hr. Values are expressed as relative light units and as percentage of the activity of the CMV promoter in each cell line to correct for the different transfection efficiencies seen between cell lines. Values are the mean of at least three independent experiments performed in duplicate.

AdEGP-2-E1 causes selective oncolysis of EGP-2 positive cells

Adenoviral infection of tumor tissue will result in only a limited number of transduced cells. To increase the efficiency of cancer gene therapy, the EGP-2 promoter can be utilized to control viral replication. Therefore, the replication-competent adenovirus AdEGP-2-E1 was tested for its ability to specifically and efficiently lyse cancer cells. To
this end, different human cell lines were infected with various m.o.i. of AdEGP-2-E1 or AdWT. Infection with the various m.o.i. would initially result in a limited number of infected cells; consequently augmentation of the initial infection could be examined.

After 10 days, a clear difference in cell lysis was observed between AdEGP-2-E1 and AdWT in the EGP-2 promoter negative cell lines HeLa, HepG2, and U373 (Figure 2). In the EGP-2 negative cell line HeLa, the m.o.i. required to lyse the cells was 2-log higher for AdEGP-2-E1 compared to AdWT. Similar results were observed for the EGP-2 negative cell line U373. The difference in cell lysis caused by either AdWT or AdEGP-2-E1 in this cell line was even more clearly seen after 17 days, when AdWT caused complete cell lysis at an m.o.i. of 0.01 while cell lysis caused by AdEGP-2-E1 could only be detected at the highest m.o.i. tested (data not shown). For the EGP-2 negative cell line HepG2, the difference between AdEGP-2-E1 and AdWT was less distinct, although still more than a log difference was observed in the m.o.i. required to lyse all cells.

Importantly, in all cell lines that demonstrated high EGP-2 promoter activity based upon the luciferase assay data (Table 1), AdEGP-2-E1 eradicated cells as efficient as wild-type adenovirus after an incubation period of 10 days (Figure 2).
Under these conditions, complete cell death was only observed in the highest m.o.i. for Ovcar-3 and SW948 after infection with AdWT or AdEGP-2-E1. To visualize possible differences between AdWT and AdEGP-2-E1 mediated cell death in these cell lines, we increased the incubation period. After an extended incubation, both cell lines showed slightly more cell death after infection with AdWT in comparison to infection with AdEGP-2-E1 (data not shown).

Overall, we can conclude that these results demonstrate the exceptional potency of the constructed adenovirus AdEGP-2-E1 to specifically and efficiently lyse EGP-2 positive tumor cells.

*Selective replication of AdEGP-2-E1 results in virus progeny production in EGP-2 positive cells*

To study the replication efficiency and specificity of AdEGP-2-E1 in more detail, the kinetics of AdEGP-2-E1 virus production was investigated in an EGP-2 positive and EGP-2 negative cell line and compared to AdWT virus production. Wild-type adenovirus demonstrated efficient viral progeny production in the EGP-2 positive cell line WiDR as well as in the EGP-2 negative cell line U373 (Figure 3A). In WiDR cells, 10 days after infection with AdWT the cells and medium contained respectively $3.2 \times 10^6$ pfu/ml and $2.0 \times 10^8$ pfu/ml and after 10 days U373 cells infected with wild-type virus contained $7.9 \times 10^6$ pfu/ml and the medium contained $4.0 \times 10^7$ pfu/ml (Figure 3A and data not shown). Microscopically, in both cell lines 100% cell lysis was visible 10 days after infection (data not shown).
After infection of the EGP-2 positive cell line WiDR with AdEGP-2-E1, the virus titer increased rapidly (Figure 3B). After 10 days the cells contained $1.3 \times 10^5$ pfu/ml and the medium contained $4.0 \times 10^5$ pfu/ml (Figure 3B and data not shown). In addition, microscopically over 50% cell lysis was observed (data not shown). On the contrary, in the EGP-2 negative cell line U373 AdEGP-2-E1 virus production was up to 2-log attenuated in comparison to the virus production in the EGP-2 positive cell line WiDR. U373 cells infected with AdEGP-2-E1 contained only $1.3 \times 10^4$ pfu/ml 10 days after infection and at the same time point, the medium only contained $1.6 \times 10^3$ pfu/ml (Figure 3B and data not shown). Microscopically, even after 10 days no cytopathologic effects were observed (data not shown). This indicates that the EGP-2 promoter can be used to restrict replication to EGP-2 positive cells.

**Replication of AdEGP-2-E1 is highly attenuated in human liver slices**

To examine the ‘liver-off’ profile of the constructed adenovirus AdEGP-2-E1, human liver slices were incubated with AdWT, AdEGP-2-E1, the replication-incompetent virus AdGL3BEGP-2 or left untreated and after 96 h or 168 h the virus titer in the medium was determined by conventional limiting dilution assay. AdGL3BEGP-2 could only once be detected in the medium (2 pfu/ml in human liver sample 1 after 96 h incubation). In human liver sample 1, AdEGP-2-E1 replicated 3 log less efficient compared to AdWT as measured after 96 h of incubation (Figure 4). This considerable difference was maintained even after 168 h of incubation. In human liver sample 2, the same 3 log difference between AdWT and AdEGP-2-E1 was observed. The difference increased after 168 h of incubation, resulting in a 4 log difference between AdWT and AdEGP-2-E1 (Figure 4).

![Graph showing replication in liver samples](image-url)
To exclude that the observed difference between AdWT and AdEGP-2-E1 viral progeny production was solely due to a different E3 structure, we compared the virus production in human liver slices after infection with AdWT or dl7001,\textsuperscript{25} which lacks all E3 genes. Overall, the virus production differed less than 2 log between AdWT and dl7001 (t=96h, $5.4*10^6 \pm 2.0*10^5$, $7.0*10^3 \pm 0.9*10^4$ pfu/ml; t=168h, $2.6*10^5 \pm 4.2*10^4$, $1.2*10^5 \pm 1.6*10^3$ pfu/ml (n=3) for AdWT versus dl7001 respectively). The absence of E3 did therefore not solely explain the up to 4 log difference seen between AdWT and AdEGP-2-E1 virus production. The observed difference between AdWT and AdEGP-2-E1 is therefore also due to the controlled expression of the E1 gene. These data clearly demonstrate the outstanding specificity of AdEGP-2-E1 in clinically relevant primary human liver material.

\textit{AdEGP-2-E1 demonstrates efficient replication in a tumor xenograft model}

To investigate the potential of AdEGP-2-E1 to efficiently replicate \textit{in vivo}, subcutaneous tumors were injected with sucrose buffer, AdWT, AdEGP-2-E1, or the replication-incompetent virus AdTL. Mice were sacrificed when the tumor burden became excessive and hexon copy numbers were determined for tumor and liver tissues. As mouse liver cells do not support adenoviral replication, for each mouse the value found in the liver was set as background and subtracted from the value found in the tumor. The values found in the liver where similar for each group (mean AdTL: $2.2*10^5$, mean AdEGP-2-E1: $1.0*10^5$, mean AdWT: $1.3*10^5$ copy number / mg tissue) and did not vary between the different time points.

To determine the efficiency of AdEGP-2-E1 replication \textit{in vivo}, direct comparisons could be made for mice sacrificed 24 days (AdEGP-2-E1 vs. AdTL) or 30 days (AdEGP-2-E1 vs. AdWT) after the first injection (Figure 5). After 24 days, the hexon copy number in the tumor tissue was 4-log higher for AdEGP-2-E1 (mean: $1.3*10^8$ copy numbers / mg tissue) in comparison with the replication-incompetent virus AdTL, for which the hexon copy numbers were comparable to background levels. This difference

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{AdEGP-2-E1 replicates as efficient as wild-type virus \textit{in vivo}.\newline Adenoviral hexon copy numbers were detected in tumor tissues of mice injected with AdTL (n=4), AdEGP-2-E1 (n=6) or AdWT (n=6). Of each mouse, the hexon copy number / mg tissue was determined in the liver as well. This value was set as background and subtracted from the hexon copy number determined in the tumor tissue. Symbols represent individual mice.}
\end{figure}
indicates a high level of replication of AdEGP-2-E1 in the tumor cells. To determine if this potent replication was comparable to wild-type virus replication in the tumor, the hexon copy number could be compared for mice sacrificed 30 days after the first injection. The copy numbers seen in tumor tissue of mice treated with AdEGP-2-E1 reached similar levels compared to AdWT (mean: $2.0 \times 10^8$ copy numbers / mg tissue and $5.7 \times 10^7$ copy numbers / mg tissue respectively). These results indicate the high potency of AdEGP-2-E1 to replicate in tumor tissue in vivo.

In addition to the quantitative determination of the hexon copy numbers, immunostainings of the adenoviral hexon on frozen tumor sections were performed in one cross-section to determine the presence of viral progeny (Figure 6). 24 Days after the first administration, clusters with bright staining against the adenoviral hexon protein were detected in two out of three mice injected with AdEGP-2-E1 (Figure 6A). In contrast, the cryosections of the three tumors injected with the replication-incompetent virus AdTL and excised after 24 days did not show any stained clusters and in only one tumor sample hexon protein could be detected in a few single cells.

Moreover, even 30 days after the first administration with AdEGP-2-E1, tumor samples showed bright clusters of adenoviral hexon staining ($n=2$) (Figure 6B). Of the two mice injected with AdWT and sacrificed after 30 days, the tumor sample of only one mouse showed clear clusters of adenoviral hexon protein staining in the examined tumor cross-section. These results convincingly demonstrate that AdEGP-2-E1 efficiently replicates in vivo resulting in the spread of viral progeny to neighboring cells, and continuing the cycle of replication and cell lysis.

\[
\begin{array}{ccc}
\text{A} & \text{AdTL} & \text{AdEGP-2-E1} \\
\text{Day 24} & & \\
\text{B} & \text{AdWT} & \text{AdEGP-2-E1} \\
\text{Day 30} & & \\
\end{array}
\]

*Figure 6. Efficient AdEGP-2-E1 progeny production in vivo*

Immunohistochemical staining of adenoviral hexon protein was performed in one area of the tumor tissue of mice sacrificed on day 24 (A) or day 30 (B) after the first injection with AdTL, AdEGP-2-E1 or AdWT. Representative pictures are shown. A full color version of this figure can be found in the appendix.
Discussion

Cancer gene therapy is still hampered by a lack of efficient killing of the entire tumor tissue. To target and eradicate more tumor cells, replicating adenoviral agents are utilized. Replication of the adenovirus results in cell lysis and subsequent spread of progeny virus to the neighboring cells, repeating the cycle of infection and replication. This ongoing replication greatly enhances the efficiency of adenoviral vectors. This study shows the specific and efficient eradication of tumor cells by the novel conditionally replicating adenovirus (CRAd) AdEGP-2-E1.

Several conditionally replicating adenoviruses have previously been tested in a clinical setting. One example is Onyx-015, which is an E1B-55K-deleted adenovirus with oncolytic activity in cells with malfunctioning p53. Onyx-015 showed promising results in combination with chemotherapy after intra-arterial infusion. The same viral agent could elicit an anti-tumor response in distant tumor tissues after systemic administration in mice, demonstrating the power of these viruses for the treatment of metastasized cancer. Alternatively, tumor-specific promoters can be utilized to control adenoviral replication. Although there are some exceptions such as telomerase and E2F promoter based CRAds, many promising oncolytic viruses can only be used for one type of cancer. In this study we aimed to construct an adenovirus capable of targeting a broad spectrum of carcinomas. These epithelial derived tumors comprise the majority of cancers and have a poor prognostic overall survival rate at standard treatment therapy. To target a wide variety of carcinomas, we utilized the EGP-2 promoter to control the expression of E1. We and others have demonstrated EGP-2 promoter activity in a variety of tumor types, originating from breast, colon and ovarian cancer tissues. Moreover, EGP-2 promoter activity has been observed in primary tumor material from breast cancer tissue and from an adenocarcinoma of the stomach (unpublished results). As high over-expression of EGP-2 has been observed in the majority of carcinomas, we anticipate that our promoter fragment is active in a broad panel of tumor types. This would make the EGP-2 promoter an attractive promoter for controlling adenoviral replication in the most relevant cancer types.

Previously, we have demonstrated the outstanding specificity and efficiency of the tsp EGP-2 in a plasmid-based system and in an adenoviral context for suicide gene therapy. To increase the tumor killing efficiency necessary for the treatment of cancer, we constructed an EGP-2-based CRAd. In every EGP-2 positive cell line tested, cell death caused by AdEGP-2-E1 was at least as effective as caused by wild-type adenovirus as detected by crystal violet staining (Figure 2). Detailed investigation of the kinetics of virus replication using a different experimental set-up showed that in the EGP-2 positive cell line WiDR less functional viral particles were produced after infection with AdEGP-2-E1 in comparison to AdWT (Figure 3). This difference in kinetics between AdEGP-2-E1 and AdWT might be explained by the absence of the adenoviral death protein (ADP) in AdEGP-2-E1 due to the deletion of the E3 region. ADP was previously shown to increase the efficiency of CRAds after infection and reintroducing the ADP gene might therefore even further increase the efficiency of this EGP-2 based CRAd while retaining the specificity.

To investigate the efficiency of AdEGP-2-E1 replication in vivo, we employed a human xenograft mouse model. Although the experimental set-up of this subcutaneous tumor model did not allow demonstration of an increased survival of mice injected with
either AdWT or AdEGP-2-E1, we could show similar efficient viral progeny production of AdWT and AdEGP-2-E1, as measured by DNA copy numbers. In addition, immunohistochemical analysis of tumor sections demonstrated similar high level of adenoviral hexon protein expression after infection with AdWT or AdEGP-2-E1 even 30 days after administration. Moreover, quantitative PCR however did demonstrate a comparable presence of adenoviral AdWT and AdEGP-2-E1 DNA in every tumor, indicating similar efficient adenoviral replication.

Our results clearly demonstrate the high potency of AdEGP-2-E1 to replicate in vivo. Administration of AdWT does increase the survival rate in other subcutaneous tumor models, and because the efficacy of AdWT and AdEGP-2-E1 was similar in vivo, AdEGP-2-E1 is likely to increase the survival rate in other experimental tumor models as well.

In addition to this potent ‘tumor-on’ profile, the EGP-2 based CRAd should exhibit a strong ‘liver-off’ profile for a good therapeutic index, as after systemic administration most of the adenovirus homes to the liver. It is therefore of extreme importance that healthy human liver cells do not support replication of the constructed CRAd AdEGP-2-E1. To this end, we have developed a clinically relevant method to test the liver-off profile of adenoviral agents in a system using primary human liver material. Using this precision-cut tissue slice technology, viable human liver slices can be prepared that still contain the architecture and all cell types of the liver. In the present study, we demonstrate an up to 4 log difference between the replication of wildtype virus and AdEGP-2-E1 indicating an outstanding ‘liver-off’ profile of AdEGP-2-E1. To investigate the contribution of the E3 region, we compared AdWT with a virus containing a wildtype E1 region but deleted for the E3 region. Interestingly, removal of the E3 region diminished wild-type virus production in primary liver material nearly 2-log. Although a direct comparison between experiments is difficult, these results are in agreement with a study from Suzuki et al, in which also a 2-log difference in virus production was observed between the adenoviruses with and without E3 region, in vitro as well as in vivo. As the EGP-2 based CRAd produced up to 4-log less viral particles in comparison to AdWT virus production, the replication impairment of AdEGP-2-E1 in primary human liver material was not solely due to the removal of the E3 region but also a consequence of the specificity of the EGP-2 promoter.

The wild-type E1A promoter is a relatively weak promoter. However, even this promoter has demonstrated to excessively produce E1A, as only a very small amount of E1A is sufficient to induce adenoviral replication. AdEGP-2-E1 virus production was severely impaired in primary liver material in comparison to AdWT and dl7001 virus production, suggesting that the EGP-2 promoter activity is inferior to the wild-type E1A promoter activity in clinically relevant primary human liver material and therefore suitable to improve the specificity profile of replication-competent adenoviruses. The observed limited replication in EGP-2 negative cells might be due to upstream adenoviral sequences which can interfere with the selective activity profile of the introduced tumor-specific promoter. Deletion analysis of the EGP-2 promoter in a plasmid-based system has previously demonstrated that the epithelial specificity is retained within the first 700 bp upstream of the transcription start site. The length of the EGP-2 promoter fragment used in this study is 1.2 kb, and this extra length may serve as insulator DNA, reducing the expression of E1 by upstream adenoviral sequences.
In addition to the re-introduction of ADP, the potency of CRAds can be further enhanced by transductionally retargeting to increase the infection efficiency. For example, the viral capsid can be modified to display one or two retargeted fiber types, and also bi-specific antibodies can be used to increase the infection efficiency towards the tumor tissue. The combination of a CRAd which is expected to replicate in many different tumor types, such as AdEGP-2-E1, together with an increased infection profile will result in a powerful tool for the treatment of a high variety of tumor types.

This study shows the strong ‘liver-off’ and ‘tumor-on’ profile of the virus AdEGP-2-E1, resulting in a very potent therapeutic index. Overall, we can conclude that the EGP-2 promoter can be utilized to selectively and efficiently control adenoviral replication for carcinoma gene therapy.

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