The development of new treatment strategies for oesophageal cancer

Buskens, C.J.

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adenoviral cancer gene therapy: the lack of A genetically retargeted adenoviral vector enhances viral transduction in oesophageal carcinoma cell lines and the lack of adenoviral vector in the development of important problems.

In this study a cell culture of fresh human resection material was established to analyse primary cultured oesophageal resection specimens in (primary) oesophageal adenocarcinoma cells. An RGD retargeted adenoviral vector was used to transduce cells in an appropriate cell culture.
Oesophageal cancer is a highly aggressive disease with poor long-term outcome. So far, surgical resection is still the treatment of choice when curative is aimed at. However, this treatment is associated with high morbidity and mortality, and even after potentially curative surgery, five-year survival rates rarely exceed 25%. Therefore, new approaches in the treatment of this malignancy are searched for. Gene therapy with adenoviral vectors seems a promising novel treatment modality for various malignancies in preclinical studies, but so far clinical results are often disappointing. This is at least partly due to the following two important problems.

First, there is a significant discrepancy between the adenoviral vector efficacies observed in vitro using established cell lines and the tumour transduction rates achievable in vivo delivery. Although carcinoma cell lines are valuable tools for investigating various aspects of transduction efficiencies, only specific cell types can be studied. In addition, these cells consist of a monolayer of monoclonal tumour cells which are transformed due to multiple passages with limited similarity to the heterogeneous carcinoma cell population in vivo. Therefore, it would be desirable to have a culture system of primary cells to study adenoviral transduction in cells more comparable to the in vivo situation.

A second important limitation of the in vivo application of the current adenoviral cancer gene therapy, is the resistance of carcinoma cells to adenoviral infection. Adenoviral entry into target cells is the rate-limiting step of gene delivery. The initial binding of an adenovirus to the cell surface is a receptor-mediated process, and therefore efforts have been made to characterize the cellular receptors of the adenovirus. Recently, one of the adenoviral receptors on the surface of a host cell was identified as the Coxsackie and adenoviral receptor (CAR), which is a protein that is also involved in maintaining tight junction integrity. Upon binding of the knob domain of the viral fiber protein to CAR, the virion enters the cell through the interaction of its penton base with the $\alpha_\text{v}\beta_3$ and the $\alpha_\text{v}\beta_5$ integrins on the host cell surface. This is followed by the internalization of the virus within a clathrin-coated endosome. Finally, the virus escapes the endosome, translocates to the nuclear pore complex, and releases its genome into the nucleoplasm where subsequent steps of viral replication take place (Figure 1).

Several investigations have revealed a decrease in expression of the CAR protein in neoplastic cells, which may explain the low transduction efficiency of carcinoma cells seen in vivo. In order to overcome the limitations imposed
by the CAR dependence of adenoviral infection, targeting can be used by genetically modifying the fiber knob, enabling the virus to attach and infect via another cell surface receptor. Since tumour cells express integrins abundantly, a retargeted adenoviral vector with an Arginine-Glycine-Aspartate (RGD) peptide inserted into the HI-loop of the fiber knob might solve the problem of poor transduction of tumour cells by allowing a CAR independent gene transfer directly through integrins.
The first aim of this study was to establish a culture system from fresh surgical resection specimens to study adenoviral transduction in cells more comparable to the in vivo situation. An additional advantage of such a system is that apart from analysing infection percentages in (heterogeneous) carcinoma cells of various patients, the transduction efficiencies of adenoviral vectors in carcinoma cells can directly be compared to normal squamous mucosal cells of the same patient.

The secondary aim of this study was to analyse the efficiency of the genetically retargeted vector for oesophageal carcinoma in comparison to the native adenovirus. The RGD-retargeted adenovirus was tested on four established human oesophageal carcinoma cell lines, and on ten primary cell cultures from fresh surgical oesophageal resection material. Because primary cultures of human material will contain various cell types (especially fibroblasts, lymphocytes and hematopoietic cells) in addition to cancer cells, we subsequently characterized the origin of the cells that were infected by both vectors.

MATERIALS AND METHODS

Established oesophageal carcinoma cell lines
The human oesophageal adenocarcinoma cell lines OE19 and OE33 were purchased from the European Collection of Cell Cultures (Salisbury, UK). The squamous cell carcinoma cell lines TE1 and TE2 have been isolated and characterized at Tohuku University (Sendai, Japan). These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 300 μg/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM/10% FCS/L-glu/ Pen/Strep), at 37°C in a humidified, 10% CO₂ atmosphere.

Primary cell cultures of oesophageal resection specimens
This study was performed in accordance with the guidelines of the local ethics committee. In the Netherlands, a written informed consent is not required to perform additional experiments on primary resected material.

In patients who underwent subtotal oesophageal resection and proximal gastrectomy with curative intent for adenocarcinoma of the oesophagus or gastro-oesophageal junction or squamous cell carcinoma of the oesophagus, the left gastric artery was preserved until the end of the resectional phase of the operation to maintain maximal viability of the removed specimen. Samples of normal squamous oesophageal mucosa without the underlying submucosal connective tissue and proper muscle, and vital tumour (assigned by experienced
Gl pathologists GJAO or FTK) were collected, washed in phosphate-buffered saline (PBS), and minced into small pieces. The fragments of mucosa or tumour were resuspended in 10 ml Liver Digest Medium (LDM) (Life Technologies, Breda, The Netherlands) and floated in a shaking waterbath at 37°C. After two hours, the supernatant containing dissociated cells was decanted, supplemented with 50% FCS and centrifuged (1500 rpm/min. for 5 min. at 4°C). The single cells were resuspended in cold culture medium (DMEM/ 10% FCS/ L-glu/ Pen/ Strep) and stored on ice until use. The dissociation step was repeated with fresh LDM three-times. All dissociated cells obtained per tissue-sample were pooled and applied to tissue culture plates coated with collagen type IV.

Two x 10^6 cells in a well of a 24-well plate or in iso-concentration in 6 well plates were cultured for 24 hours in 0.5 ml DMEM/ 10% FCS/ L-glu/ Pen/ Strep supplemented with 0.1mg/ml fungizone, in a humidified atmosphere containing 10% CO₂ at 37°C.

**Adenoviral constructs**
The native adenoviral vectors (Ad), derived from human adenovirus type 5, were obtained from R.D. Gerard (University of Leuven, Leuven, Belgium). For adenoviral transduction analysis, the adenovirus was made replication deficient by deleting the E1 region. Into this deleted E1 region, the reporter genes luciferase (Adluc) and Green Fluorescent Protein (AdGFP) were cloned, and constructed to be driven by the human cytomegalovirus (CMV) promoter. These reporter genes were used to analyse viral transduction levels by measuring the amount of luciferase and GFP transcription.

The genetically retargeted adenoviral vectors containing the recombinant RGD (Arg-Gly-Asp) peptide in the Hl-loop of the fiber knob were generated by transfection of 293 cells with Pacl-digested pVK70314, and constructed to encode for either the luciferase or GFP reporter gene (AdlucRGD and AdGFPRGD respectively).* The viral vectors were propagated on the permissive cell line 293, and purified by double cesiumchloride gradients centrifugation. All virus preparations were dialyzed against PBS, aliquoted, and stored at -80°C until use. Adenovirus titers in plaque forming units (pfu) were determined in parallel by a plaque forming assay using 293 cells and the number of viral particles (vp) was measured by optical density-based physical titration. The vp/pfu ratios of the native vectors were 10-20 and those of the RGD modified vectors were 40-50.

**Adenoviral gene transfer**
The adenoviral transduction experiments on the four cell lines were performed in triplicate. Cell lines were grown overnight at a count of 1 x 10^6
cells/well in 6 well plates to allow adherence. The monolayers of cells were then washed with PBS, and incubated for one hour with the different adenoviral constructs at various multiplicity of infection (moi) of 1, 10 and 100 pfu/cell. Then, complete fresh medium was added, and after 24 hours of incubation, the luciferase and GFP expression was analysed for the different viral constructs. For all experiments a sample of cells in which no virus was added, was used as a negative control.

After cell lysis, quantitative levels of viral luciferase production in the cells were measured in a luminometer (Berthold Detection System, Pforzheim, Germany) using a luciferase assay system (Promega, Madison, WI, USA). The protein concentration of the lysates was determined with a protein assay (Pierce Biotechnology, Rockford, IL, USA).

Infection percentages were analysed by determining the percentage of GFP expressing cells by fluorescence activated cell scanning (FACS; BD Biosciences, San Jose, CA, USA). Cultured cells were trypsinized, washed in PBS, and fixated in 1 ml 2% paraformaldehyde (PAF). After centrifuging the cells at 1000 rpm for 5 min, the cells were resuspended in PBS with 1% bovine serum albumin. The percentage of transduced cells was determined by gating the right-hand tail of the distribution of the negative control sample for each individual cell line at a 1% positivity (based on the negative control results), which was considered as autofluorescence of the cell line.

To analyse adenoviral transduction levels in primary cells, the single cells were cultured for 24 hours after isolation. Then, cultures were rinsed with PBS and vital cells (i.e. cells attached to the collagen coating) were exposed to the different adenoviral vectors as described above. Dependent on the amount of primary cells available, the experiments were performed at least in duplicate at various moi’s. For quantitative viral expression levels, cells were infected with Adluc or AdlucRGD and lysed after 24 hours for luciferase measurement. The transduction ratio (i.e. luciferase activity per sample infected with AdlucRGD divided by the luciferase activity per sample infected with Adluc) was used to analyse the differences between gene transfer increase in carcinoma cells and normal epithelial cells from the same patient.

To analyse if the quantitative increase in viral expression levels was due to an increase in the number of transduced cells or an increase in the viral expression per cell, the primary cultures were also infected with AdGFP and AdGFPRGD and analysed by fluorescence microscopy.

Characterization of primary cultures
Three of the ten primary cultures infected with AdGFP and AdGFPRGD were used to characterize the origin of the different primary cells and to determine
the infection percentages of the various cell types by two-color flowcytometric analysis. Cells were trypsinized, washed, and resuspended in PBS-Tween (0.2%) to make the cells permeable for the detection of intracellular cytokeratin proteins by FACS analysis. Cytokeratins are part of the cytoskeleton of epithelial cells and are not present in fibroblasts and other stromal cells. All cells were incubated for 60 min with saturating concentration of the MNF116 mouse-anti-human cytokeratin monoclonal antibody (DAKO, Glostrup, Denmark), which reacts with human epithelial tissue and malignant epithelial lesions. Cells were then washed three times in PBS, and incubated for another 60 minutes with the secondary Fluorescein-isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin G antibody (Jackson, Westgrove, PA, USA). After three more washing steps, the cells were resuspended in 1 ml 2% paraformaldehyde. As a negative control, an aliquot of cells from each patient was used in which control IgG was added to the cells without previous labeling with MNF116. The percentage of transduced epithelial cells was analysed by determining the percentage GFP positivity and cytokeratine positivity per primary cultured tissue by quadrant statistics after two-color flowcytometry.

Statistical analysis
To analyse if the increase in luciferase activity with the retargeted adenoviral vector was significantly higher in carcinoma cells than in normal squamous epithelial cells, a parametric Student’s t-test was used to compare the transduction ratios of the primary cells. A p-value of 0.05 or below was considered statistically significant. The statistical analysis was performed using the Statistical Software Package version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS
Adenoviral gene transfer in established esophageal carcinoma cell lines
To compare the efficiency in gene transfer of the parental virus to the genetically modified virus, the four oesophageal carcinoma cell lines were infected with Adluc and its retargeted variant AdlucRGD as described in Material en Methods. A dramatic augmentation of luciferase activity was seen with the vector containing the Arg-Gly-Asp (RGD) peptide inserted into the HI-loop of the fiber knob (Figure 2). With a multiplicity of infection (moi) of 1 and 10 pfu/cell, AdlucRGD demonstrated in the oesophageal carcinoma cell lines OE19, OE33, TE1, and TE2 an approximately 250-, 30-, 300-, and 700-fold increase
respectively in luciferase activity, compared to the cells infected with the parental virus. With a moi of 100, this increase was less pronounced, but there was still a 80-, 10-, 150- and 400-fold enhancement in viral expression for the carcinoma cell lines with AdlucRGD.

To analyse whether this enhancement was due to an increase in luciferase expression per cell or an increase in number of cells infected, the experiments were repeated with the viruses encoding GFP. With all experiments, an increase in the percentage of infected cells with the AdGFPRGD vector was seen for all four cell lines (Figure 3).

**FIGURE 2**

Adenovirus mediated gene transfer of the parental adenoviral vector and the integrin retargeted adenoviral vector, both encoding luciferase (Adluc and AdlucRGD respectively) to human oesophageal carcinoma cell lines (OE19, OE33, TE1, and TE2). Cells were infected with either virus at a moi of 1, 10 or 100. Luciferase levels were corrected for the protein concentration and are shown in light units (LU)/mg protein. Background luciferase activity in the samples in which no virus was added (negative control) is also shown.
FIGURE 3
An example of flow cytometric analysis showing the percentage of infected cells from four oesophageal carcinoma cell lines (OE19, OE33, TE1, and TE2) comparing the parental to the retargeted virus encoding GFP (AdGFP and AdGFPRGD respectively). On the Y-axis, the number of counted cells is shown (counts), and on the X-axis, the intensity of the Green Fluorescent Protein expression (FL1-height) is depicted.
In addition, there was also an increase in the intensity of fluorescence per cell, as was seen by the shifting of the bulk of the cells to the right. This indicates that the increase in luciferase expression seen with AdlucRGD is caused by both an increased expression per cell, as well as a higher percentage of infected cells.

Adenoviral gene transfer in primary cell cultures of oesophageal resection specimens
Promising results in established cell lines do not always reliably predict efficient adenoviral transduction of carcinoma cell in vivo. Therefore, ten cultures of both normal oesophageal epithelium and carcinoma cells were established as described in Materials and Methods. This primary material was derived from 6 men and 4 women with a median age of 62 years (range 46 – 82). Three patients had a distal oesophageal adenocarcinoma developed in a Barrett segment, four patients had an adenocarcinoma of the cardia and three patients had a squamous cell carcinoma. The tumour characteristics are described in Table 1. All ten primary cultures were infected with the four viral constructs expressing either luciferase (Adluc and AdlucRGD) or GFP (AdGFP and AdGFPRGD). The increase in viral luciferase expression of the retargeted viral vector versus the parental vector is shown for both the carcinoma cells and the normal

Table 1
Characteristics and histopathologic findings of 10 tumours used to establish primary cultures.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Operation</th>
<th>Tumour type</th>
<th>Tumour length</th>
<th>Infiltration depth</th>
<th>Lymph node</th>
<th>Distant metastasis</th>
<th>Differentiation grade</th>
<th>Radicability status</th>
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<tr>
<td>1</td>
<td>THEa</td>
<td>Barrett</td>
<td>6.4 cm</td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
<td>poor</td>
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<td>N1</td>
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<td>poor</td>
<td>R0</td>
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<tr>
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<td>THE</td>
<td>Barrett</td>
<td>3.0 cm</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>good</td>
<td>R0</td>
</tr>
<tr>
<td>4</td>
<td>TTEb</td>
<td>AC cardia</td>
<td>4.5 cm</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
<td>moderate</td>
<td>R0</td>
</tr>
<tr>
<td>5</td>
<td>THE</td>
<td>AC cardia</td>
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<td>N0</td>
<td>M0</td>
<td>poor</td>
<td>R0</td>
</tr>
<tr>
<td>6</td>
<td>THE</td>
<td>AC cardia</td>
<td>7.0 cm</td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
<td>moderate</td>
<td>R0</td>
</tr>
<tr>
<td>7</td>
<td>THE</td>
<td>AC cardia</td>
<td>4.5 cm</td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
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<td>R0</td>
</tr>
<tr>
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<td>SSCc</td>
<td>8.0 cm</td>
<td>T3</td>
<td>N1</td>
<td>M1</td>
<td>poor</td>
<td>R0</td>
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<tr>
<td>9</td>
<td>THE</td>
<td>SSC</td>
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<td>M0</td>
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<td>R29</td>
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<td>T3</td>
<td>N0</td>
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<td>moderate</td>
<td>R0</td>
</tr>
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*aTranshiatal oesophageal resection; *bTransthoracic oesophageal resection; *cAdenocarcinoma of the distal oesophagus developed in a Barrett's segment; *dAdenocarcinoma of the gastric cardia; *eSquamous cell carcinoma of the oesophagus; *fMicroscopically radical resection; *gMacroscopically irradical resection
squamous epithelial cells in Figure 4A and B. In all patients an increase in viral expression was seen with AdLucRGD versus the native adenoviral vector, varying from 5- to almost a 100-fold increase. This transduction ratio for both normal squamous epithelial cells and carcinoma cells is shown in Figure 4C. The increase in gene transfer was significantly less pronounced in normal cells (p=0.03). In combination with the fact that the absolute luciferase expression of AdRGD was higher in carcinoma cells than in normal cells, this suggests a more specific transduction of oesophageal carcinoma cells by the RGD retargeted adenoviral vector.

To assess whether this increase in viral expression represented more transduced cells or an increase in viral expression per cell, all primary cultures were also infected with both the parental AdGFP and the genetically retargeted variant AdGFPRGD, and subsequently analysed by fluorescence microscopy. It was demonstrated that with the retargeted virus both the number of infected cells, and the fluorescence intensity per cell increased in all cultures (Figure 5, upper panels). However, the cultures of primary tumour material seemed to contain two cell populations with different morphology. About 50% of the cells revealed a fibroblast like morphology and most likely were stromal cells. The remaining cells showed a more epithelial cell like shape and probably were cancer cells (Figure 5, lower panels). These data therefore suggest that the increased transduction of the primary cultures might result from a better transduction of stromal cells, cancer cells, or both.

**Characterization of infected cell types**

To discriminate between tumour cells and stromal cells, the primary cultures of patient 1, 3, and 6, were analysed by flowcytometry for the epithelial cytokeratin marker MNF116. As expected, the primary cultures were indeed a mixture of cells from different origin. To analyse differences in the transduction efficiency of AdGFP and AdGFPRGD in both tumour cells and stromal cells, a two-color FACS analysis for GFP positivity and cytokeratine positivity was performed for the three primary cultures (Figure 6). The percentages of uninfected epithelial cells, infected epithelial cells, uninfected stromal cells, and infected stromal cells per culture were analysed by quadrant statistics. The increase in percentage of transduction with AdGFPRGD was determined for the different cell populations. In these three cultures, an increase was seen of 13.8%, 28.7% and 4.5% respectively in the percentage of transduced epithelial cells, and an increase of 30.0%, 23.3% and 8.1% respectively in the percentage of transduced stromal cells when compared to infection with AdGFP. These results indicate that the RGD retargeted adenovirus infected both primary epithelial cells and stromal cells more efficiently.
FIGURE 4
A: Luciferase levels in LU/mg protein representing the adenoviral gene transfer of Adluc and AdlucRGD in primary cultured normal squamous epithelial cells from resection material of 10 patients. B: Luciferase levels (LU/mg protein) in carcinoma cells of the same 10 patients. C: Transduction ratio (i.e. increase in luciferase expression) in normal squamous epithelial cells compared to carcinoma cells. The increase in gene transfer was significantly lower in normal cells (p=0.03).
DISCUSSION

In this study, the utility of a retargeted adenoviral vector containing an RGD peptide in the HI-loop of the fiber knob was examined for gene therapy for oesophageal carcinoma. It was shown that the retargeted virus had a 5-800 times more efficient gene transfer than the native virus in established oesophageal cancer cell lines and in primary cultured oesophageal carcinoma cells. Since the transduction increase was significantly less pronounced in normal cells with AdRGD and the adenoviral transgene levels were higher in carcinoma cells, the retargeted adenoviral vector was also demonstrated to be more efficient for carcinoma cells than the parental virus. The observation that a higher percentage of carcinoma cells is transduced with a more specific gene transfer is of clinical importance, because the therapeutic effect of gene therapy will depend on...
FIGURE 6
Example of two-color flowcytometric analysis to determine the percentage of transduced epithelial and stromal cells. The cultured carcinoma cells were infected with AdGFP and AdGFPRGD, and incubated with the cytokeratin antibody MNF116. The percentage of GFP positive cells (FL1-height) and cytokeratin positive cells (FL-2 height) was analysed by quadrant statistics for the upper left (uninfected epithelial cells), upper right (infected epithelial cells), lower left (uninfected stromal cells), and lower right quadrant (infected stromal cells) (UL, UR, LL, LR respectively).

efficient gene transfer to carcinoma cells and with fewer viral particles needed for the same therapeutic effect, the vector-related toxicity is likely to be decreased.

Gene therapy, although originally developed for correction of genetic deficiencies of inherited disorders, has become a promising therapeutic entity for various carcinomas. In cancer gene therapy, therapeutic genes are introduced into tumour cells aiming at arrest of tumour growth. To compete with conventional therapeutic modalities, cancer gene therapy should be both safe and effective. Targeting of vectors can be used to increase both the selective transduction and the transduction efficiency. Genetic targeting by inserting the tri-peptide RGD into the H1-loop of an adenoviral vector was previously demonstrated to enhance infection of ovarian, pancreatic, and head-and-neck carcinomas with promising in vitro results. Despite promising adenoviral cancer gene therapy results in pre-existing cell lines, so far only two of the more than 500 approved clinical trials, are being evaluated in a phase III clinical trial due to disappointing results in vivo. To be able to analyse gene therapy vectors in cells more resembling the in vivo situation, a culture system was successfully established from fresh surgical resection specimens. Culturing gastrointestinal mucosa has been proven to be extremely difficult. We also encountered several difficulties of this primary
culture system. It was noted that preserving the left gastric artery intact until the end of the resectional phase of the operation was important to maintain maximal viability of the oesophageal cells. Sometimes a fungal overgrowth developed (especially in specimens from patients with an obstructing tumour or candida oesophagitis), or the viability of the cultures was limited (especially in specimens from patients with an ulcerating tumour). However, ultimately our established culture system was reproducible and unique in allowing a comparison of gene transfer in primary carcinoma cells and normal squamous epithelial cells.

Apart from epithelial cells, primary cultures established from fresh surgical resection specimens were demonstrated to contain stromal cells which were also more efficiently transduced with the retargeted virus. Due to the artificial culturing conditions, these primary cultures will probably contain a relatively high percentage of fibroblasts, which are susceptible for viral infection. However, fibroblasts will also be present in the stroma of a primary tumour. Thus, transduction of stromal cells may also play a role in vivo. On the one hand, transduction of stromal cells might have a positive therapeutic effect because the vitality of a tumour is at least partly dependent on its stroma, and attacking these stromal cells may result in a more efficient gene therapy, but on the other hand, tumoural stroma is considered a barrier limiting tumour expansion, and tumours lacking stromal cells have been reported to be more aggressive and to induce more metastases. This phenomenon therefore implies that transduction of stromal cells may counteract the therapeutic possibilities of non-replicating cytotoxic adenoviral vectors in cancer gene therapy.

For treating neoplastic diseases, carcinoma cells should be efficiently transduced and killed. In previous studies of our group, it was demonstrated that the absolute luciferase expression after viral transduction is representative for the amount of cell kill with a suicide gene. However, non-replicating adenoviral vectors encoding suicide genes were demonstrated to be not the most efficient vectors to be used in a clinical setting. Nowadays, conditionally replicating adenoviruses (CRAds) represent a promising and novel way for cancer gene therapy. These agents are designed to replicate specifically in tumour cells, followed by the spread of the viral progeny to neighbouring cancer cells. This specific replication will at the same time prevent the adverse effects that could be caused by infection of stromal cells. An example is the ONYX-015 virus which has been modified in the early regulatory protein E1b that normally allows the virus to bind and inactivate the host p53 gene to promote its own replication. The mutated adenovirus however, only replicates in and lysis human cells with a defective p53 pathway. This virus is expected to be especially useful in oesophageal carcinomas, since for this malignancy a
p53 mutation is the most frequent alteration identified (75-100%). It has also been demonstrated that p53 mutations are an early event during the malignant degeneration of oesophageal cells. Therefore, this virus could even be used for the treatment of premalignant stages. Targeting such CRAds with the RGD tri-peptide, could improve the utility of this adenoviral vector by creating a CAR independent infection capability for tumour cells.

To our knowledge, this is one of the first reported studies which has investigated the possibilities for adenoviral gene therapy to treat a gastrointestinal malignancy in primary tumour cells. However, we acknowledge that although this culture system is more resembling to the in vivo situation than the established monoclonal cell lines, it is still an artificial monolayer test-system. Because the complex three-dimensional structure of a tumour might influence the transduction efficiency of an adenoviral vector, the development of a system using cultured biopsies to test viral infection is currently under investigation.

It should also be noted that using the RGD-virus will only partly solve the current difficulties with cancer gene therapy. Also with this retargeted adenoviral vector it seems impossible to transduce 100% of the tumour cells, which is necessary to create efficient cancer treatment and although this vector did establish a more efficient gene transfer in carcinoma cells in comparison to normal squamous epithelial cells, it did not create a selective viral transduction. Therefore, further research has to be focussed on more selective targeting motifs, before gene therapy could be incorporated in daily clinical practice.

In conclusion, this study demonstrates that adenoviral entry via the primary adenoviral receptor CAR is limited in oesophageal carcinoma cells. Adenoviral entry was increased when an integrin retargeted adenoviral vector was used. The increase in viral expression was not only due to an increase in the percentage of infected cells, but also to an increased gene expression per cell. Therefore, genetic targeting of the adenoviral vectors with an RGD tri-peptide seems a promising treatment strategy to optimize cancer gene therapy.

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