The development of new treatment strategies for oesophageal cancer
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Primary cultured oesophageal carcinoma cells are more resembling to the heterogeneous tumour cells in vivo but they still lack the complex 3-dimensional architecture.

Gene therapy for oesophageal carcinoma: Therefore, an oesophageal explant model using biopsies from patients with oesophageal cancer was established.

In the present study, the transduction efficiency of the genetically retargeted RGD adenoviral vector was analysed in this explant model which is more comparable to the in vivo situation.
INTRODUCTION

Over the last decades there has been a significant increase in the incidence of oesophageal adenocarcinoma.1 Curative treatment consists mostly of surgery, which is only feasible in a subset of patients. Due to distant metastases, local irresectability, or poor condition of the patient, only 20-30 percent of the patients are candidates for a potentially curative resection.1,3 The other patients receive palliative treatment with intraluminal radiotherapy or stenting. Recently (neo-)adjuvant modalities, such as chemotherapy and radiotherapy, have been explored for downstaging of the tumour and improving the prognosis after surgery.4

Patients with a so-called Barrett’s oesophagus, i.e. intestinal metaplasia of the oesophagus, are at increased risk for development of oesophageal adenocarcinoma.5 Malignant degeneration is characterized by a stepwise process from intestinal metaplasia through low grade dysplasia and high grade dysplasia into oesophageal adenocarcinoma. When high grade dysplasia is diagnosed, many guidelines still advise a surgical resection.6 A surgical resection, however, is accompanied by a significant morbidity and even mortality. Therefore other endoscopic modalities are being explored for, mainly, early lesions (e.g. photodynamic therapy, endoscopic mucosal resection).7

Gene therapy might play a future role in the management of patients with oesophageal carcinoma. Therapeutic genes can be selectively transduced into cancer cells and eradicate them by expression of a suicide gene.8 Adenovirus is most commonly used as a vector for cancer gene therapy because it can transduce both dividing and non-dividing cells and it is easy to produce in a high titer. Moreover, a large therapeutic gene can be inserted into its genome. Currently, several clinical trials using adenovirus as a cancer gene therapy vector are forthcoming.9 In the setting of oesophageal carcinoma, both the patient with metastatic disease and the patient with early malignant lesions in a Barrett’s oesophagus might potentially benefit from the use of local and/or systemic gene therapy.

In the clinical setting the use of adenovirus is hampered by a strong immunological response of the host. Moreover, the in vivo adenoviral transduction efficacy into cancer cells is still rather limited.10 Therefore, research is focused on adenoviral targeting in order to improve the transduction of cancer cells. For cell entry, the adenovirus is dependent on the presence of integrins and the Coxsackie and adenovirus receptor (CAR) on the surface of the host cell.11 In order to improve cell entry, genetic targeting is an attractive option. An adenovirus in which the peptide Arg-Gly-Asp (RGD) is
incorporated into the HI loop of the viral fiber knob revealed an improved transduction in different cancer cell lines.\textsuperscript{12,13} This modification results in an improved adenoviral transduction via integrins, and obviates the need for cell entry via CAR.

Different experimental models are available to study the targeting capacity of a gene delivering vector. Established cell lines are most commonly used for both \textit{in vitro} and \textit{in vivo} experiments. These cells, however, are transformed due to multiple passages and only one specific cell type can be studied, which does not reflect the \textit{in vivo} tissue heterogeneity of a tumour. Therefore it is desirable to have a more relevant model, which consists of primary patient material in which the original tissue architecture is preserved. For this reason, we set up an oesophageal explant model with oesophageal biopsies obtained from different patients. This model was used to study both non-targeted and RGD-targeted adenoviral transduction in different oesophageal tissues.

**MATERIALS AND METHODS**

**Adenoviral constructs**

An E1-deleted adenovirus type 5 expressing green fluorescent protein (AdGFP) under control of a CMV promoter, and an RGD-targeted adenovirus (AdGFP-RGD), with an RGD peptide cloned into the HI loop of the fiber knob, were kindly provided by Dr. V. Krasnykh (University of Alabama, Birmingham, AL, USA).\textsuperscript{12} The viruses were propagated and plaque titered on the permissive cell line 293 using standard techniques.\textsuperscript{14} The virus titer was expressed as plaque forming units (pfu). Purification was performed by centrifugation on a CsCl gradient and all virus aliquots were stored at -80 °C until use.

**FIGURE 1**

Histological viability of a biopsy with metaplastic intestinal metaplasia which was cultured for 24 hours. The viability was categorized in a good (A), moderate (B) and poor (C) viability. (H&E, 25x)
FIGURE 2
Flowcytometric transduction efficacy of adenoviral vectors in an oesophageal adenocarcinoma cell line OE33 (A-D) and an oesophageal squamous cell carcinoma cell line TE1 (E-H). The cell lines were incubated with a non-targeted adenoviral vector at an MOI of 1 and 10 (A, C and E, G) and an RGD-targeted adenoviral vector with an MOI of 1 and 10 (B, D and F, H).
Adenoviral transduction in established cell lines
Two established oesophageal cancer cell lines were used in an in vitro model to test the transduction of a non-targeted and RGD-targeted adenoviral vector. The OE33 cell line was originally derived from an oesophageal adenocarcinoma and obtained from the European Collection of Cell Cultures. The TE1 cell line was originally derived from an oesophageal squamous cell carcinoma and provided by Dr. M. Yamamoto (University of Alabama, Birmingham, AL, USA). The cell lines were propagated every 3 days and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (300μg/ml), 10% heat-inactivated foetal calf serum, penicillin (100 U/ml) and streptomycin (100μg/ml). To assess adenoviral transduction in cell lines, 10^5 cells/well were plated in a 24-well plate and incubated overnight to allow adherence. Then the cells were transduced with the adenoviral constructs. Before transduction the cells were washed with phosphate buffered saline (PBS) and incubated with the adenoviral vector for one hour with a multiplicity of infection (MOI) of 1 and 10 respectively. Fresh culture medium was added and 48 hours thereafter transduction efficacy was assessed by measuring GFP expression using flowcytometry. The cells were prepared for flowcytometry as follows. The cells were trypsinised and fixated with 4% buffered formalin. Then they were pelleted at 1200 rpm for 5 minutes and resuspended in PBS with 1% bovine serum albumin.
FIGURE 4
Adenoviral transduction in cultured oesophageal biopsies. Transduction was detected by immunohistochemical analysis of GFP. Transduction is located in the basal layer in normal squamous epithelium (A,25x; B,100x), is mainly stromal in metaplastic columnar mucosa (C,25x; D,100x), very limited in adenocarcinoma (E,25x; F,100x) and localized in both stromal and cancer cells in squamous cell carcinoma (G,25x; H,100x).
Resuspended cells were used for flowcytometry (Calibrite; Becton Dickinson Immunocytometry Systems; Franklin Lakes, NJ, USA). At least 10,000 cells were counted and data on the number of transduced cells were obtained by setting a 1% quadrant marker for nonspecific staining in the non-transduced cells. Transduction efficacy of AdGFPRGD was compared to AdGFP in both cell lines.

Patients
At our endoscopy unit patients with oesophageal carcinoma or Barrett’s oesophagus with high grade dysplasia, undergo endosonography for staging purposes. During this staging procedure additional biopsies were taken from normal looking oesophageal mucosa and, if present, from squamous cell carcinoma, adenocarcinoma or a columnar lined metaplastic segment. These biopsies were transported in culture medium and processed within 15 minutes. The medical ethical committee of the Academic Medical Center approved this protocol and all patients gave a written informed consent.

Explant model
Endoscopically obtained biopsies were cultured as has been previously described. After collection the biopsies were washed in PBS. Then they were placed on a stainless steel grid, which was mounted in a 10-mm transwell plate (Corning, New York, NY, USA). Culture medium (1 ml) was added until it just covered the surface of the biopsy. Culture medium consisted of DMEM supplemented with L-glutamine (300μg/ml), 10% heat-inactivated foetal calf serum, penicillin (100 U/ml) and streptomycin (100μg/ml).
The transwell plate was placed in an airtight jar (Oxoid, Basingstoke, England) which was continuously gassed with 95% O2 and 5% CO2. The jar was placed in a water bath with a temperature of 37°C. After 24 hours of culture the biopsies were fixated in 4% buffered formalin and embedded in paraffin. Staining was done with haematoxylin and eosin. Viability was assessed by an experienced GI pathologist (GJO). Histological signs of cell death included complete lyses of cells, swelling of cells and syncytial formation. Viability was scored and categorized in three groups: good viability, moderate viability and poor viability (Figure 1A, 1B and C).

Adenoviral transduction in the explant model
Biopsies were infected with an adenoviral construct one hour after initiation of the biopsy culture. One million pfu of the adenovirus was dissolved in 3 μl PBS and pipetted on the biopsy. Then the culturing conditions were re-established and the biopsies were evaluated histologically after 24 hours of culture. Only biopsies with a good or moderate viability were used to assess adenoviral transduction.

To assess the transduction of the adenovirus in the biopsies, immunohistochemical analysis for GFP expression was done using a three-step detection method. The paraffin-embedded biopsies were dewaxed, rehydrated in graded alcohols and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Non-specific staining was blocked with 10% normal goat serum. Then the slides were incubated overnight with a mouse monoclonal anti-GFP JL-8 (Clontech, Palo Alto, CA, USA) in a 1:1000 dilution. This step was followed by an incubation with rabbit anti-mouse HRP in a 1:100 dilution (Dako, Glostrup, Denmark) and a subsequent incubation with streptABComplex/HRP (Dako) was done according to the manufacturer's instructions. Peroxidase activity was measured with a diaminobenzidine reaction. The TE1 oesophageal squamous cell carcinoma cell line was transduced with AdGFP and used as a positive control. A non-transduced cell line and cultured biopsy were used as negative controls. These controls were fixed in 4% buffered formalin and embedded in paraffin. Transduction efficacy was scored and categorized in three groups: no transduction for biopsies without detectable GFP; minimal transduction for biopsies with less than 10 GFP positive cells per biopsy; moderate to good transduction for biopsies with more than 10 GFP positive cells per biopsy. All biopsies were endoscopically obtained using a standard jumbo forceps.
Differences in transduction efficacy between AdGFP and AdGFPRGD in the biopsies were tested for significance using the chi-square test. For cell entry, the adenovirus is dependent on the presence of CAR and integrins. We studied the presence of CAR immunohistochemically in normal oesophagus and Barrett’s oesophagus. This was done on a resection specimen of a patient operated on an oesophageal adenocarcinoma in a Barrett’s oesophagus. CAR 72 (ONYX Pharmaceuticals, Richmond, CA, USA) was used as a primary antibody and immunohistochemistry was performed as described previously. Briefly, the sections were incubated with 0.01% trypsin and microwaved in a citrate buffer for antigen retrieval. After blocking with goat serum, the sections were incubated for 12 hours with CAR 72, in a dilution of 1:7000. Subsequent steps were performed as described above. Incubation without the primary antibody was used as a negative control. The primary antibody CAR 72 was kindly provided by Dr. Qing Wang (ONYX Pharmaceuticals).

RESULTS

Adenoviral transduction in established cell lines

Established oesophageal cancer cell lines were used to investigate the adenoviral transduction and the effect of RGD-targeting in an in vitro model. Transduction efficacy was assessed by the detection of GFP positive cells using flowcytometry. The established oesophageal adenocarcinoma cell line, OE33, was readily transducable with the non-targeted adenovirus with a transduction rate of 29.2% and 79.1% at an MOI of 1 and 10 respectively (Figure 2A and C). In this cell line the RGD-targeted adenovirus showed a 3 fold increased transduction, with an efficacy of 84.0% and 98.5% at an MOI of 1 and 10 respectively (Figure 2B and D). Transduction of the established squamous cell carcinoma cell line, TE1, was poor using the non-targeted adenovirus, with a transduction rate of 14.5% and 66.9% with a MOI of 1 and 10 respectively (Figure 2E and G). This transduction improved markedly up to 5 times using the RGD-targeted adenovirus with an efficacy of 71.8% and 99.5% with an MOI of 1 and 10 respectively (Figure 2F and H). These results show an improved transduction with the RGD-targeted adenovirus for both the oesophageal adenocarcinoma cell line OE33 and the oesophageal squamous cell carcinoma cell line TE1.
Viability of the oesophageal explants
To validate the quality of the oesophageal explant model as it was described in the literature, we first performed biopsy cultures without adenoviral gene transfer and assessed the viability microscopically, categorized in good, moderate and poor viability (Figure 3). Forty-five biopsies were taken from 8 patients who underwent endoscopic staging for oesophageal carcinoma. Sixteen biopsies contained normal squamous epithelium of which 13 (81%) had a moderate to good viability. Nine biopsies contained metaplastic intestinal metaplasia, i.e. Barrett's mucosa, of which 8 (89%) had a moderate to good viability. Twenty biopsies contained oesophageal adenocarcinoma, of which only 4 (20%) had a moderate to good viability. After this initial period all subsequently procured biopsies were incubated with an adenoviral vector. Transduction with an adenovirus did not influence the viability of the explants. Fourteen biopsies contained oesophageal squamous cell carcinoma, and all these biopsies showed a moderate to good viability.

Adenoviral transduction in the oesophageal explants
The oesophageal explant system was used to study the transduction of adenoviral vectors on different types of primary human oesophageal tissues. Ninety-four oesophageal biopsies were obtained from 23 different patients (Table 1).

NORMAL SQUAMOUS EPITHELIUM Twenty-seven biopsies were taken from normal squamous epithelium in 7 patients. Four of these biopsies had a poor viability, which left 23 biopsies, with a moderate or good viability, suitable for assessment of adenoviral transduction. Twelve biopsies were transduced with AdGFP and 11 biopsies were transduced with AdGFPRGD. Transduction of either AdGFP or AdGFPRGD occurred only in the basal layer of the biopsies and no transduction was observed at the luminal side (Figure 4A and B). Most biopsies had a minimal to moderate transduction and there was no significant difference between the transduction efficacy of AdGFP and AdGFPRGD.

INTESTINAL METAPLASIA Twenty-seven biopsies were taken from an oesophageal Barrett's segment in 6 patients and contained intestinal metaplasia on histological examination. Three biopsies had a poor viability, which left 24 biopsies, with a moderate or good viability, suitable for assessment of adenoviral transduction. Eighteen biopsies were transduced with AdGFP and 6 biopsies were transduced with AdGFPRGD. Transduction was limited to stromal tissue and there was hardly any transduction of the epithelial layer (Figure 4C and D). Most biopsies had a minimal to moderate transduction. The transduction efficacy and localization was similar for both adenoviral constructs.
TABLE 1
Transduction of a non-targeted and RGD-targeted adenovirus in different oesophageal tissues in the oesophageal explant system. Only biopsies with a moderate to good viability were included for transduction analysis. Transduction was categorised in no transduction, minimal transduction and moderate to good transduction with no GFP positive cells, less than 10 GFP positive cells and more than 10 GFP positive cells in a biopsy respectively. Differences of transduction efficacy were tested for significance using the chi-square test.

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<tr>
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<th>AdGFP (%)</th>
<th>AdGFPRGD (%)</th>
<th>p-value</th>
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<td>Normal squamous epithelium</td>
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<td>12 (100)</td>
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<td>minimal transduction</td>
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<td>moderate transduction</td>
<td>8 (66)</td>
<td>3 (27)</td>
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</tr>
<tr>
<td></td>
<td>2 (17)</td>
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<tr>
<td>Intestinal metaplasia</td>
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<tr>
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<td>6 (100)</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td></td>
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<td>4 (57)</td>
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**OESOPHAGEAL ADENOCARCINOMA** Fourty-two biopsies were taken from oesophageal adenocarcinomas in 10 patients. Thirty-one biopsies had a poor viability and only 11 biopsies, with a moderate or good viability, were suitable to assess adenoviral transduction. Five biopsies were transduced with AdGFP and 6 biopsies were transduced with AdGFPRGD. Transduction was very limited, and in 6 biopsies no transduction was observed at all (Figure 4E and F). AdGFPRGD did not show any improved transduction.

**OESOPHAGEAL SQUAMOUS CELL CARCINOMA** Fourteen biopsies were taken from an oesophageal squamous cell carcinoma in 4 patients. None of these biopsies showed a poor viability, and therefore all biopsies were suitable to assess adenoviral transduction. Seven biopsies were transduced with AdGFP and 7
biopsies were transduced with AdGFPRGD. Overall the transduction was minimal to moderate and localized both in stromal cells and cancer cells (Figure 4G and H). Transduction efficacy of AdGFPRGD and AdGFP was similar.

**CAR IMMUNOHISTOCHEMISTRY** The presence of CAR was studied in normal oesophageal tissue and in tissue with intestinal metaplasia from a resection specimen of a patient with an oesophageal adenocarcinoma in a Barrett oesophagus. In the normal oesophagus CAR was present in the basal layer, and not at the luminal side (Figure 5A). This corresponds with the location of adenoviral transduction in normal oesophageal tissue, which occurred at the basal layer and not at the luminal side. In the Barrett's oesophagus CAR was mainly present in the crypts and not in the villi (Figure 5B). This did not correspond with the limited stromal transduction as we observed with adenoviral transduction in biopsies taken from a Barrett's oesophagus.

**DISCUSSION**

For the development of cancer gene therapy, experimental models play an essential role. Established cell lines are commonly used for these models. However, the use of established cell lines has its limitations. Cell lines consist of selected cell types, which have been transformed due to multiple cell passages. In addition, cell lines represent only one cell type and do not include stromal cells or normal epithelial cells. *In vitro* as a monolayer, cells are readily accessible in contrast to a dense mass of tumour cells. Moreover *in vitro*, cell lines are highly proliferative compared to the *in vivo* situation. This is important because the grade of proliferation plays an important role in transduction of a gene delivering vector. In addition, several *in vivo* models have been developed to study adenoviral gene transfer. The most popular model consists of subcutaneously injected tumour cells in nude mice. Again established, and thus transformed, cell lines are used in these models, which give a different histology and anatomy compared to the original tumour. In addition, this model does not represent normal non-cancerous tissue. Despite encouraging results using adenoviral vectors in these models, still limited progress has been made in the clinical setting. The large difference between these experimental models and the *in vivo* situation may explain this discrepancy.

Taking the limitations of the current models into account, it is desirable to apply models in which primary patient material is used to study the gene
transfer capacity of different adenoviral vectors. In this study we established such a model by using an oesophageal explant system. Oesophageal biopsies were cultured and transduced with adenoviral vectors. Culturing gastrointestinal mucosa has proven to be difficult. Under standard conditions only short-term experiments are feasible with marked epithelial necrosis and disintegration beyond 2-3 hours of culturing. Several techniques have been tested to extend the culture time of mucosal explants. An environment of 95% O₂ and 5% CO₂ appeared to be crucial to have a good viability of cultured intestinal explants up to 48 hours. In this explant system mainly normal mucosa of the colon and small intestine has been used. More recently this technique was used to study explants of intestinal metaplasia, obtained from a Barrett's oesophagus. Culturing oesophageal adenocarcinoma or squamous cell carcinoma has not been described previously. In our hands we obtained a good viability of the cultured oesophageal biopsies which contained normal squamous epithelium, intestinal metaplasia and squamous cell carcinoma. This in contrast to the viability of cultured oesophageal adenocarcinoma, which was very poor. Possibly the adenocarcinoma cells have a higher metabolic activity which might lead to a relatively high oxygen deficiency. In addition ischemic damage might have been present in the primary tumour at the beginning of the culture.

For attachment and internalization the adenovirus is dependent on the presence of integrins and CAR. Many cells, however, lack the presence of CAR and are therefore resistant to transduction of the adenovirus. To overcome this problem the adenovirus can be genetically retargeted. When an RGD peptide is cloned into the viral fiber knob of the HI loop, this will improve transduction via integrins, independently of the presence of CAR. This RGD-targeted adenovirus has shown an improved transduction in many different tumour cell lines. We tested the RGD-targeted adenovirus on two oesophageal cell lines: OE33 obtained from an oesophageal adenocarcinoma and TE1 obtained from an oesophageal squamous cell carcinoma. In both cell lines the RGD-targeted adenovirus showed an improved transduction.

To overcome the limitations of the established cell lines, we also tested the different adenoviruses in the oesophageal explant system. The main objectives were to study which cell types are transduced in the tissue culture and what the impact of RGD-targeting is. Overall the adenoviral transduction efficacy was limited and localized. In normal squamous epithelium, only the basal layer was transduced and no transduction was observed at the luminal side. Transduction in intestinal metaplasia was limited to stromal tissue, only one biopsy showed limited transduction in the epithelial layer.
Because of a poor viability only a few biopsies from oesophageal adenocarcinoma were suitable to assess adenoviral transduction, which was very limited. The transduction in the cultured squamous cell carcinoma was minimal to moderate and occurred both in stromal cells and cancer cells. This limited and localized transduction, underlines how important it is to study transduction histologically in primary material. Obviously, the important observation that adenoviral transduction in normal squamous epithelium is limited to the basal layer can only be made in primary material and not in established cell lines.

CAR is the main receptor for the transduction of adenoviral vectors. We studied the presence of CAR in normal oesophageal tissue and it appeared to be present in the basal layer which corresponds with the localization of adenoviral transduction. In addition, cells in the basal layer are relatively undifferentiated and have a high integrin expression. The combination of the presence of CAR and integrins might explain the selective adenoviral transduction in the basal layer. Stromal cells are rather undifferentiated compared to epithelial cells, which might explain the preferential stromal adenoviral transduction in the biopsies obtained from a Barrett's oesophagus. This did not correspond with the presence of CAR, which is mainly present in the crypts, and not in the stroma of a Barrett's oesophagus. Transduction in Barrett's oesophagus, however, was too limited in order to localize staining and correlate it to the presence of CAR.

We compared the transduction efficacy of the non-targeted and RGD-targeted adenoviruses in the oesophageal explant model. In the different oesophageal tissues we observed a similar transduction efficacy and localization of the two adenoviral vectors. While the RGD-targeting showed a clear advantage in the two established cell lines, this advantage did not hold up for the ex vivo situation. In conclusion, we used the oesophageal explant system to study adenoviral transduction. Cultured biopsies from normal oesophageal squamous epithelium, intestinal metaplasia and squamous cell carcinoma have a good viability and can be used to study different vectors and different means of transduction. Cultured biopsies from oesophageal adenocarcinoma had a very poor viability and were less useful for transduction studies. Overall the transduction was poor and not specific for cancer cells, even when using the RGD-targeted adenovirus. These findings stress the importance to use primary material for adenoviral targeting studies. The targeting properties of the adenoviral vectors have to be improved to obtain selective transduction in (pre-) malignant tissue, before any clinical trials for oesophageal carcinoma or high grade dysplasia can be undertaken.
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