

Adenovirus-Mediated Gene Transduction of Pancreatic Tumour Cells Lines

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Abstract

Pancreatic cancer is often a fatal disease due to its poor response to existing therapies. Thus new therapeutic approaches must be developed. The aim of this study was to evaluate the susceptibility of pancreatic cell lines to adenovirus-mediated transduction and to determine the expression of surface coxsackie B and adenovirus receptor (CAR). Five human pancreatic cancer cell lines were transduced using a recombinant adenovirus type 5 expressing enhanced green fluorescent protein (Ad5EGFP) and the expression of EGFP was analysed by flow cytometry. The cell lines varied in transduction efficiency, ranging from less than 1% to more than 30% cells expressing EGFP and susceptibility of cell lines to Ad5EGFP transduction has a positive correlation with the level of surface CAR expression and its presence may be a limiting step for efficient adenovirus transduction. These results suggest that gene therapy of pancreatic tumours is feasible, but then the status of CAR expression in the tumour needs to be evaluated.

Keywords: Adenovirus; CAR; pancreatic tumour, gene therapy

1. Introduction

Adenovirus (Ad) vectors have been used widely in clinical trials for gene transfer [1], especially for the treatment of cystic fibrosis [2]. Recently, adenovirus gene therapy is being developed to treat cancer patients [3]. Since pancreatic cancer is a highly fatal disease with only 10% of patients surviving 5 years even when conventional treatment can be accomplished [4], the use of adenoviruses as therapeutic gene delivery systems may offer a new approach to the treatment of pancreatic tumours. However, this requires basic studies of the adenovirus entry process in pancreatic tumour cells.

Human adenoviruses, especially those of subgroup C such as Ad5, have gained increasing popularity over other vector systems as a gene therapy vector due to a

number of positive attributes of the virus [5] and considered to be relatively safe to be used as vectors for gene transfer in humans [6].

Subgroup C adenovirus infection is initiated by attachment of the knob domain of the viral fibre protein to the human coxsackie B and adenovirus receptor (CAR) on the cell surface [7] and internalisation of virus into host cell is mediated by secondary interaction between the Arg-Gly-Asp motif on penton base protein loops and $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Inside the cell cytoplasm, the virus escapes its vesicle through the lysis of the endosomal and the partially dismantled virus translocates to the nuclear pore complex, and releasing its genome into the nucleoplasm where subsequent steps of viral replication take place [8]. Completion of the cycle triggers cell death and the release of progeny viruses.

The capability of an Ad vector to infect a cell is depends crucially on the primary (CAR) [9] and secondary adenovirus receptors (integrins) expression [10]. Because CAR serves as the primary attachment molecule for most human adenoviruses, its presence may be a limiting step for efficient adenovirus infection. CAR expression levels seems to be a limiting step in Ad transduction of skeletal muscle [11], endothelial and smooth muscle cells [12], brain cells [13], bladder cancer cells [14], ovarian cancer [15], human melanoma cells cultures [16] and human leukocytes [17].

In the present study, we have investigated the transduction of five pancreatic tumour cell lines by a recombinant adenovirus containing green fluorescent protein gene (Ad5EGFP). The level of surface CAR expression in pancreatic tumour cell lines has also been assessed. We find a strong correlation between the level of surface CAR expression and the level of transduction of pancreatic tumour cell lines by Ad5EGFP. Furthermore, CAR expression level in pancreatic tumour cells might be the limiting step in Ad5 transduction. This suggests that, in future gene therapy studies of pancreatic cancer by adenoviruses, the level of CAR expression in the tumour needs to be assessed if adenovirus-mediated gene therapy is to be effective.

2. Materials and methods

2.1 Adenoviral vectors

Ad5EGFP virus is a replication-deficient recombinant Ad5 expressing the enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus (CMV) promoter, inserted in place of the E1 region.

2.2 Cell culture

Five established human pancreatic tumour cells: PSN-1, ASPC-1, Panc-1 and Capan-1 were obtained from ECACC, Salisbury, UK and recently-established human pancreatic tumour cells NP18 [18] were obtained from Dr Ramon Alemany (Institut Catala d'Oncologia, Barcelona, Spain). Human lung carcinoma cells (A549) and chinese hamster ovary cells (CHO) were obtained from ECACC, Salisbury, UK. All pancreatic and A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and CHO cells were grown in MEM alpha medium. All media were supplemented with 1% (v/v) penicillin/streptomycin (10,000 units/ml each), 2% (v/v) L-glutamine (2 mM) and 10% (v/v) foetal calf serum (FCS) and the cells were maintained at 37°C in a 5% CO₂ atmosphere. MEM alpha medium was purchased from Invitrogen Ltd (Paisley, UK), FCS was purchased from LabTech International (East Sussex, UK) and DMEM medium and supplements were from Sigma-Aldrich Ltd (Poole, UK).

2.3 Flow cytometry analysis

Cells were grown in T75 flasks, harvested by brief treatment with trypsin (Sigma-Aldrich Ltd, Poole, UK) and resuspended in phosphate-buffered saline (PBS) (Sigma-Aldrich Ltd, Poole, UK). Two hundred thousand cells were incubated with 50 µl (1:100 dilution) of rabbit anti-human CAR [19] or no primary antibody (negative control) for 30 minutes at 4°C. Cells were then washed with an excess of PBS and incubated with 50 µl (1:50 dilution) fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma-Aldrich Ltd, Poole, UK) for 30 min at 4°C before being washed again with PBS. Finally the cells were analysed on a FACScalibur flow cytometer (Becton Dickinson, Oxford, UK) using CellQuest™ software (Becton Dickinson, Oxford, UK).

2.4 Transduction of cells with Ad5EGFP

Cells (1x10⁵) were grown in 6-well dishes to approximately 80-90% confluency before transduction. Prior to transduction, the cells were washed twice with PBS and then treated with Ad5EGFP at a multiplicity of infection (MOI) of 100 focus forming unit (FFU) per cell. After incubation for 1 hour at 37°C, the cells were

washed twice with DMEM containing 2% (v/v) FCS and fresh DMEM containing 10% (v/v) FCS was added before the cells were further incubated for 24 hours at 37°C. The cells were then harvested and the EGFP expression was assessed by flow cytometry.

3. Results

3.1 Transduction of pancreatic tumour cells by recombinant adenovirus expressing green fluorescent protein

The recombinant Ad5EGFP virus was used to examine the susceptibility of pancreatic tumour cells to adenoviral transduction. Since the successfully transduced cells can be directly measured by the presence of the EGFP transgene, this enabled the determination of gene transfer efficiency without selection or antibody staining. Conversely, cells resistant to adenovirus-based transduction will not express the EGFP transgene product. CHO cells have been previously described as being resistant to adenovirus infection [19] and were used as an example of CAR-deficient cells (negative control). A549 cells are human lung carcinoma cells that are susceptible to adenovirus infection and were used to represent CAR-expressing cells (positive control).

A summary of the results of five independent analyses in Figure 1 showed that certain cells were resistant to adenovirus transduction, whereas others were susceptible. Transduction efficiencies ranged from less than 1% to more than 30% of cells. PSN-1 cell lines showed strong resistance to transduction by Ad5EGFP (< 1% transduction). In contrast, the Panc-1 cell line was most susceptible (35% transduction) to transduction by Ad5EGFP, followed by NP18 and ASPC-1 and Capan-1. The control cell line, A549 showed the highest levels of transduction while CHO cells exhibited poor transduction efficiency. Low levels of EGFP expression in Ad5EGFP transduced pancreatic tumour cells suggests a reduced efficiency of gene transfer which could be due to a number of reasons, including impaired binding of virus at the plasma membrane. This was investigated further by assaying levels of surface receptors for adenoviruses in pancreatic tumour cells.

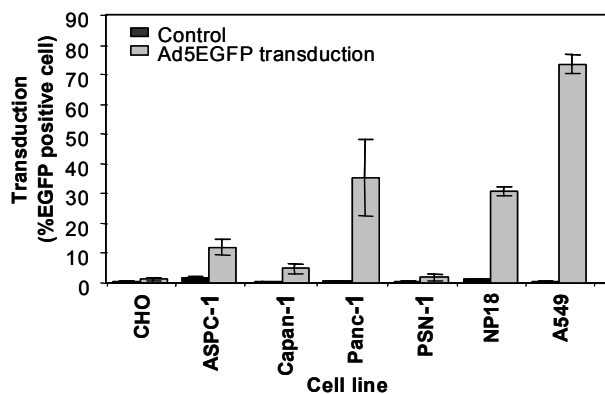


Figure 1: Ad5EGFP-mediated transduction in a panel of human pancreatic tumour cell lines. A series of pancreatic tumour, CHO and A549 cells were transduced by Ad5EGFP a recombinant adenovirus containing an enhanced green fluorescent protein (EGFP) transgene. Cells were exposed to virus at a multiplicity of 100 FFU Ad5EGFP per cell. The percentage of EGFP-positive cells was determined by flow cytometry at 24 hrs post-transduction.

3.2 Expression of the coxsackie B and adenovirus receptor (CAR) in pancreatic tumour cell lines

Five pancreatic tumour cell lines, CHO and A549 cells were analysed for cell surface expression of CAR by indirect immunofluorescence assay. Preliminary experiments were performed to ensure that a saturating level of anti-CAR antibody was used in all experiments (data not shown). Flow cytometric analysis of cell surface CAR expression (Figure 2) revealed that it was detectable in all of the pancreatic tumour cells.

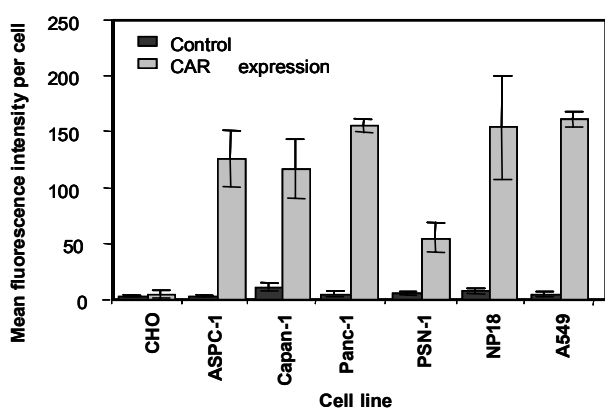


Figure 2: Expression of CAR cell surface protein in a panel of human pancreatic tumour cell lines. Individual pancreatic tumour cell lines were analysed by flow cytometry following treatment with polyclonal rabbit anti-CAR antibody (1:10 dilution) and then FITC-labeled goat anti-rabbit immunoglobulins secondary antibody (1:50 dilution). FITC-

labeled anti-rabbit IgG (in the absence of primary antibody) was used as a negative control.

However, expression of CAR varied between 50 to more than 150 mean fluorescence intensity (MFI). Panc-1 and NP 18 cells exhibited a relatively high CAR expression of more than 150 MFI per cells, which was comparable to A549 cells that formed the positive control (160 MFI per cell). In contrast, PSN-1 cells showed rather low CAR expression less than 60 MFI per cells. The remainder of the pancreatic tumour cell lines expressed intermediate levels of CAR on the cell surface. As expected, CHO cells had or almost no detectable surface CAR expression.

3.3 Correlation of CAR expression with adenovirus transduction of pancreatic tumour cell lines

To examine the correlation between the susceptibility of pancreatic tumour cell lines to transduction by Ad5EGFP (i.e. the percentage of EGFP-positive cells) and the expression level of surface CAR (i.e the number of adenovirus receptors per cell), the level of CAR expression versus the percentage of EGFP-positive cells was plotted (Figure 3 and Figure 4) and Pearson's statistical analysis was performed using SPSS 11.0 software [20]. As shown in Figure 3, it seems likely that the lower susceptibility of pancreatic tumour cell lines to adenovirus transduction might be related to the differences in expression level of CAR, because the cell lines with the highest level of CAR demonstrated an increase susceptibility to transduction with adenovirus and those cell lines with the lowest levels of surface CAR were refractory to adenovirus transduction. These data indicated that the level of CAR on the cell surface is an important factor in the efficacy of gene transfer.

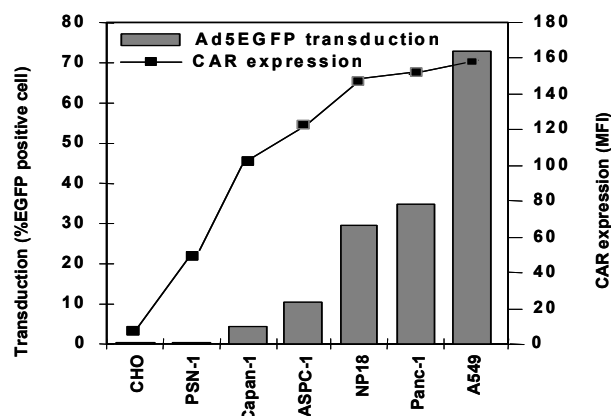


Figure 3: Relationship between Ad5EGFP-mediated transduction and CAR expression in a panel of human pancreatic tumour cell lines. CAR expression (expressed in mean fluorescence intensity units, MFI, per cell) was analysed using a polyclonal anti-human CAR rabbit antibody and FITC-

conjugated secondary antibody. All values were calculated after deduction of control antibody binding to each cell line.

Furthermore, if transduction of Ad5EGFP was plotted against log MFI of CAR expression it was clearly seen that the CAR has to be expressed up to a certain limit before a significant transduction could be achieved (Figure 4).

Statistical analysis also demonstrated that there was strong significant correlation ($r = 0.818$; $p < 0.01$) between CAR expression on the cell surface and susceptibility of pancreatic tumour cell lines to Ad5EGFP transduction.

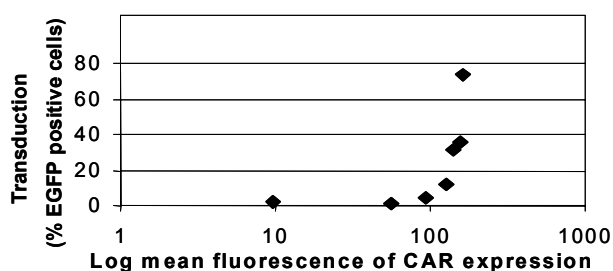


Figure 4: Relationship between percent transduction of Ad5EGFP and Log CAR expression in a panel of human pancreatic tumour cell lines. Each point represents one cell lines and the values were expressed after deduction of control antibody binding for each cell line. The expression level of CAR in pancreatic tumour cell lines might be a limiting factor in adenovirus-mediated transduction due to the fact that the CAR has to be expressed up to a certain limit before a significant Ad5EGFP transduction could be observed.

3.4 Transgene expression of pancreatic tumour cell lines after blocking of cell surface CAR

Following the statistical analysis and the fact that the expression level of CAR in pancreatic tumour cell lines might be a limiting factor in successful adenovirus-mediated gene transduction (Figure 3 and 4), further investigation was performed to evaluate the role of CAR in transduction of Ad5EGFP in pancreatic tumour cells by blocking experiment i.e blocking the attachment of Ad5EGFP to CAR using the polyclonal anti-CAR antibody.

Results in Figure 5 showed that pre-incubation of Panc-1 cells with antibody against CAR reduced transgene expression to almost 50% of that control (untreated with anti-CAR antibody). Thus, this blocking analysis showed that CAR clearly plays an important role in adenovirus-mediated gene transfer.

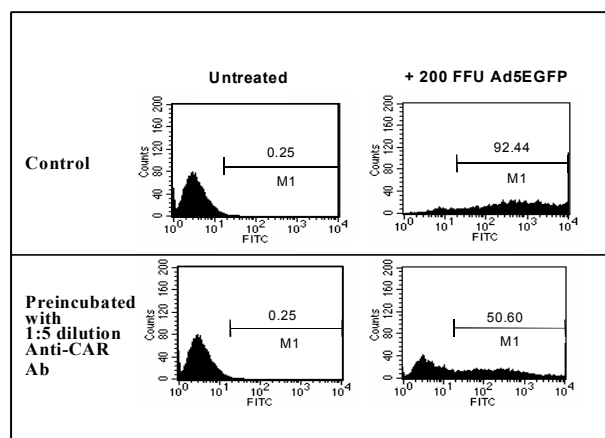


Figure 5: Pre-incubation of anti-CAR antibody reduced Ad5EGFP transduction of Panc-1 cells. Panc-1 cells were pre-treated with anti-CAR antibody (1:5 dilution) for 30 minutes at 4°C before exposed to 200 FFU per cell of Ad5EGFP virus. After 24 hrs of incubation, cells were harvested, and analysed by flow cytometry. Control cells were not treated with anti-CAR antibody. A frequency histogram of Panc-1 cells treated with anti-CAR antibody showed a strong reduction in percentage of EGFP-positive cells.

4. Discussion

Adenovirus gene therapy is an intense area of research and is being developed as a therapeutic modality for a variety of malignancies[1]. Since pancreatic cancer shows a poor response to existing treatment, thus there is a great need of new approach to overcome this problem.

Therefore, a study was performed by transducing pancreatic tumour cell lines with Ad5EGFP. The investigation on the susceptibility of five pancreatic tumour cell lines found that all the pancreatic tumour cell lines were relatively susceptible to transduction by Ad5EGFP (Figure 1). However, PSN-1 pancreatic tumour cell lines exhibited strong degree of resistant to adenovirus-mediated transduction. Previous studies have also shown that certain tumour types and established cell lines, for example in human bone marrow cells [21] were relatively resistant to adenovirus-mediated gene transfer. Furthermore, transduction of Ad5EGFP showed a strong correlation ($p < 0.01$) between transduction and the level of CAR surface expression in each of the cell lines, as determined by flow cytometry (Figure 3). Clearly, efficient transduction of pancreatic tumour cell lines by Ad5EGFP requires adequate CAR expression.

We also showed that cell surface CAR was detected in all of the pancreatic tumour cell lines but varied in the level of expression, as shown by flow cytometry analysis (Figure 1). This lead to the fact that CAR is the primary receptor for Ad5 in pancreatic cells in culture, highest level of CAR demonstrated an increase susceptibility to transduction with adenovirus

and those cell lines with the lowest levels of surface CAR were refractory to adenovirus.

Moreover, investigation on the relative importance of receptors as a primary attachment of Ad5EGFP, has shown that CAR expression plays a major role in limiting transgene expression in pancreatic tumour cell lines. In support of this we showed that when CAR expression in pancreatic tumour cell lines are blocked by anti-CAR antibody, their susceptibility to adenovirus-mediated gene transfer is markedly reduced as shown by blocking experiment (Figure 5). This study is in agreement with others studies ([9],[11],[16]).

5. Conclusion

The conclusion of this study is that gene therapy of pancreatic tumour cells is feasible using Ad-mediated gene transfer, and also suggesting that if adenovirus vectors are applied to pancreatic cells, the status of CAR expression in the tumour needs to be evaluated. In this respect, the particular treatment required for pancreatic cancer may need to be tailored to the individual patient.

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