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Gene therapy and cement injection for the treatment of hip prosthesis loosening in elderly patients

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Towards gene therapy in prosthesis loosening: Efficient killing of interface cells by Gene-Directed Enzyme Prodrug Therapy with nitroreductase and the prodrug CB1954

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Abstract

Background

Loosening is a major complication in prosthesis surgery. To stabilise loosened orthopaedic implants, the interface tissue surrounding the implant must be removed. As an alternative to manual removal, we explored the possibility of removing the tissue by gene-directed enzyme prodrug therapy. In the current study we investigated whether interface cells can be transduced by an HAdV-5 vector carrying the *E.coli*-derived nitroreductase gene and sensitised to the prodrug CB1954.

Methods

The gene transfer efficiency into cultures of diploid human interface cells was tested by exposing these cells to various concentrations of Ad.CMV.LacZ. Subsequently, we studied the susceptibility of cells to the Ntr/CB1954 combination.

Results

X-gal staining of the Ad.CMV.LacZ-transduced cell cultures revealed that at 200 plaque forming units (pfu)/cell, 74% of the cells expressed the LacZ gene. Infection with an Ntr construct in interface cell lines resulted in a 60-fold sensitisation to the prodrug CB1954. In addition we observed that iotrolan (Isovist) contrast medium had no effect on viability of the cells. However, the presence of the contrast medium completely inhibited adenovirus-mediated gene transfer.

Conclusions

From these data we conclude that HAdV-5-based vectors carrying nitroreductase can be used to sensitise interface tissue. Instead of contrast medium the clinical protocol will use an alternative visualisation procedure.

Introduction

Approximately 1 million total hip replacement operations are carried out worldwide annually for degenerative joint disease, mostly osteoarthritis and rheumatoid arthritis. Of these prostheses 7-13% will have loosening within 10 years, causing pain and difficulty in walking.^{54,78,112} The current treatment for prosthesis loosening is revision surgery, which has a high mortality and morbidity rate, especially in elderly patients with comorbidity.¹¹⁴ For these patients revision surgery is not an option and there remains a need for effective treatment of implant loosening in this patient population.

Loosening of orthopaedic implants is for the greater part caused by an inflammatory reaction to wear particles (mostly polyethylene).⁵ The inflammatory reaction causes a periprosthetic tissue to be formed, consisting of fibroblasts and macrophages, which is called interface tissue.^{61,62} Before a loosened prosthesis can be refixed, the interface tissue and the prosthesis need to be removed; thereafter a new prosthesis is implanted. This revision surgery can often be extensive (3-8 h surgery), due to the necessity of removing all interface tissue and the prosthesis, leading to high morbidity rates. Alternatively, the interface tissue could be removed by introduction of a toxic component.

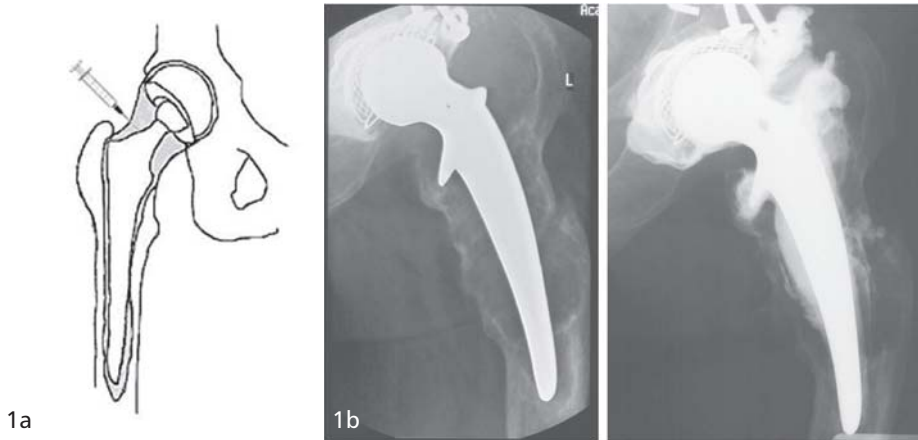
In the current study we tested whether interface cells can be sensitised by nitroreductase (Ntr) to the prodrug CB1954. The prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is a weak monofunctional alkylating agent, which is converted by the *Escherichia coli* enzyme Ntr to a cytotoxic derivative.⁶⁸ Cells containing Ntr convert CB1954 into a bifunctional alkylating agent which is capable of forming DNA inter-strand cross links, resulting in apoptosis or cell death.^{18,34}

Since there is no human homologue to Ntr²³, only cells expressing the nitroreductase gene are killed when exposed to CB1954. In the present study the therapeutic window for interface cells is determined to find out which CB1954 concentrations are safe to use in interface tissue, preventing killing of non-transduced cells.

To refix a loosened joint prosthesis, the interface cells in the periprosthetic space need to be eradicated. The interface cells are located in the joint space, which is a closed compartment. This has the advantage that with a high local concentration of vector the systemic exposure may be minimal (Figure 1a).

Contrast medium may be used to visualise the cavity by radiological images. To assure that the vector is administered in the joint space, the position of the needle in the joint space can be monitored by injecting a small amount of contrast medium into the cavity, while making fluoroscopic images (Figure 1b). Before the contrast medium can be used in a clinical study together with CTL102 and CB1954, its effect on the efficiency of transduction and killing should be tested.

Figure 1. Schematic representation of a hip joint with a loosened prosthesis (a). Arthro-gram of a hip joint with a loosened prosthesis (b).



(a) The gray area indicates the joint space, which is continuous with the periprosthetic space. When injecting a fluid into the joint space, this will spread through the area that is marked gray in the image.
 (b) The contrast medium is injected into the joint space under fluoroscopic guidance. The picture shows that a part of the area around the prosthesis (periprosthetic space) is filled with contrast medium. This proves that the prosthesis is loose in that area.

The effect of contrast medium on fibroblastic cells has been studied previously.^{69,131} In various studies it was shown that exposure of cells to low-osmolarity contrast media has no significant influence on cell proliferation and apoptosis. These studies show that exposure of the interface cells to contrast media with low osmolarity will probably have no effect on the cells themselves. However, the influence of contrast medium on viral transduction of cells by a HAdV-5-vector has not been reported. In this study the influence of low-osmolarity contrast medium on adenoviral transduction is tested.

Materials and Methods

Adenoviral vector

The adenoviral vector CTL102 was constructed by homologous recombination in PER. C6 helper cells,^{38,114} as described in previous studies.³⁴ CTL102 carries the *E. coli* Ntr gene under control of the cytomegalovirus (CMV) promoter/enhancer. The complete Ntr expression cassette is cloned into an E1-deleted HAdV-5 adenovirus transfer vector. The Ad.CMV.LacZ¹²² vector is identical to CTL102, but the *E. coli* lacZ gene replaces the

Ntr gene. In the rAd5F35.CMV.LacZ vector the shaft and knob of the HAdV-5 fiber were replaced by the homologous regions of the HAdV-35 fiber.¹⁰⁰

Interface tissue samples

For all experiments described, interface cells were used. Interface tissue was removed from the periprosthetic space during revision surgery by an orthopaedic surgeon and collected in sterile phosphate-buffered saline (PBS). Connective tissue and fat were removed thoroughly and the interface tissue was digested for at least 2 h at 37°C using collagenase 1A (1 mg/ml; Sigma, St Louis, MO, USA). Cells were then harvested by filtering the tissue/collagenase substance through a 200 µm filter (NPBI, Emmer-Compascuum, The Netherlands). The cells were cultured in 75 cm² flasks (Cellstar, Greiner, Alphen aan de Rijn, The Netherlands) with Iscove's modified Dulbecco's medium (IMDM; Biowittaker, Verviers, Belgium), supplemented with glutamax (GibcoBRL, Paisley, UK), penicillin and streptomycin (Boehringer Mannheim, Germany), and 10% fetal calf serum (FCS; GibcoBRL) at 37°C and 5% CO₂.

Before each experiment interface cells were detached from the flasks using 0.25% trypsin (GibcoBRL). The cells were counted in a Bürker counter and death cells were excluded by trypan blue. Cells were seeded in a 96-well plate (flat bottomed) at a density of 5000 cells/well. Cells were incubated overnight to allow attachment to the bottom. Before each experiment the wells were washed twice with IMDM. For the experiments passage 2 to 4 interface cells were used. Light microscopy indicated that more than 95% of the cells were interface cells.

The human hepatoma cell line HepG2⁶⁷ was used as a control for experiments with contrast medium and sodium iodide (NaI).

Potential of cell-killing by Ntr/CB1954 GDEPT (Gene-Directed Enzyme Prodrug Therapy)

To investigate cell-killing potential, interface cells were infected by CTL102 (Cobra Bio-manufacturing plc, Keele, UK) in different concentrations (0, 25, 100, and 200 plaque forming units (pfu)/cell) in IMDM/10% FCS for 24 h, washed twice with IMDM, and then incubated with the prodrug CB1954 (Oxford Asymmetry Ltd, Abingdon, UK) in different concentrations (0, 25, 50, and 100 µM) in IMDM/10% FCS for 24 h. The cells were washed again and then cultured in IMDM/10% FCS for 1 day, after which the cell viability was measured using cell proliferation reagent WST-1 (Roche, Mannheim, Germany) according to manufacturer's instructions with a 2 h incubation period.

To study the infectivity of interface cells by HAdV-5, interface cells were infected with Ad.CMV.LacZ vector (in concentrations of 0, 25, 50, 100, 200, 400 pfu/cell). Twen-

ty-four hours post-infection the cells were washed twice with IMDM, and cultured for 2 days. Medium was refreshed each day. On day 3, the monolayer cultures were washed twice with PBS and fixed with 0.2% glutaraldehyde and 2% formaldehyde in PBS for 10 minutes at 4°C. Subsequently, cells were washed once with PBS and stained for β -galactosidase activity in 50 μ l of reaction mix (1 mg/ml X-gal (Eurogentec, Seraing, Belgium), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS) for 2 h at 37 °C. The percentage of transduced cells was assessed by counting at least 100 interface cells, using light microscopy. All conditions were tested in duplicate.

CB1954 toxicity

To assess CB1954 toxicity in interface cells that do not express nitroreductase, interface cells were exposed to CB1954 in various concentrations (0-4000 μ M) in IMDM/10% FCS for 24 h, in duplicate. Cells were then washed twice with 100 μ l IMDM, and cultured in IMDM/10% FCS for a further 2 days. On the third day, cell viability was measured using cell proliferation reagent WST-1 (Roche) according to manufacturer's instructions with a 2 h incubation period.

Effect of contrast medium on interface cells

Interface cells and HepG2 cells were seeded in 96-well plates. Into each well 50 μ l of IMDM/ 20% FCS and 50 μ l of a solution containing contrast medium and 0.9% NaCl in various concentrations (0, 12.5, 25, and 50% contrast medium) were added. The contrast media used were the low-osmolarity, non-ionic dimer iotrolan (Isovist; Schering, Berlin, Germany) with an iodide concentration of 300 mg/mL and the low-osmolarity, ionic dimer ioxaglate meglumine – ioxaglate sodium (Hexabrix, Guerbet, Aulnay-sous-Bois, France) with an iodide concentration of 320 mg/mL. As a control, sodium-iodide (Nal) was used in concentrations of 0 to 150 mM. After 4 h exposure to the contrast medium, the cells were washed twice and incubated in IMDM/10% FCS. The cells were cultured for a further 2 days, changing the culture medium every day. On day 4, cell viability was determined with the WST-1 cell viability assay kit (Roche) according to the manufacturer's protocol.

Effect of contrast medium on HAdV-5 transduction of interface cells

Interface cells and HepG2 cells were seeded in 96-well plates. After overnight incubation cells were infected with Ad.CMV.LacZ (concentrations of 0, 25, 100, and 200 pfu/

cell) in IMDM/ 20% FCS, 25 μ l per well. Then 25 μ l of iotrolan (Isovist) or ioxaglate meglumine – ioxaglate sodium (Hexabrix) in 0.9% NaCl were added in concentrations of 0, 25, 50, and 100%. (When diluted in the culture medium these concentrations decreased to 0, 12.5, 25, and 50%). As a control for iodide activity, NaI was added in concentrations of 0 to 150 mM (0, 1, 2.5, 5, 10, 25, 50, 100 and 150 mM). Four hours after infection, the cells were washed twice with IMDM and incubated for the rest of the day in IMDM/10% FCS at 37°C and 5% CO₂. The Ad.CMV.LacZ transduced cells were cultured for 2 days after removal of the vector and contrast medium. Subsequently, the cells were fixed and stained for β -gal activity. The transduction rate was assessed as described above.

To investigate the transient effect of contrast medium on transduction of interface cells, cells were first exposed to iotrolan for different periods of time. Subsequently, the contrast medium was removed, the cells were washed twice, and the vector CTL102 was added. The interval between removal of the contrast medium and addition of the vector was varied. Twenty-four hours post-infection cells were washed twice and CB1954 was added. The medium with CB1954 was removed after 24 h, and replaced with fresh medium without CB1954. Cell viability was determined after addition of the fresh medium.

Statistical analysis

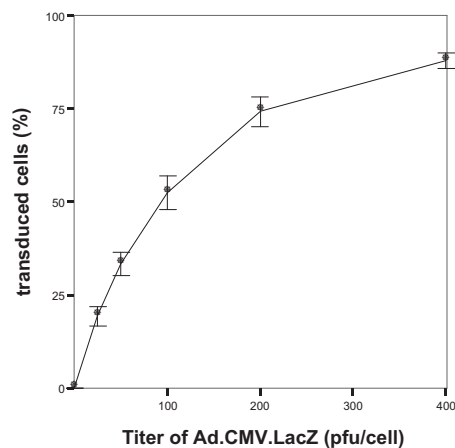
A univariate analysis of variance and Spearman's correlation was used to study the interaction between vector and prodrug and between vector and contrast medium and to study the effect of CB1954 on viability of the cells. A Mann-Whitney test for independent groups was performed to determine the difference in cell killing between the cells that were exposed to contrast medium and the non-exposed cells. In the experiment to study the effect of transient exposure to contrast medium on transduction of HAdV-5-vector Spearman's correlation between contact time and viability and between delay time and viability was tested. For all statistical analyses $p < 0.05$ was the level of statistical significance.

Results

Transduction and killing of interface cells by HAdV-5 vectors

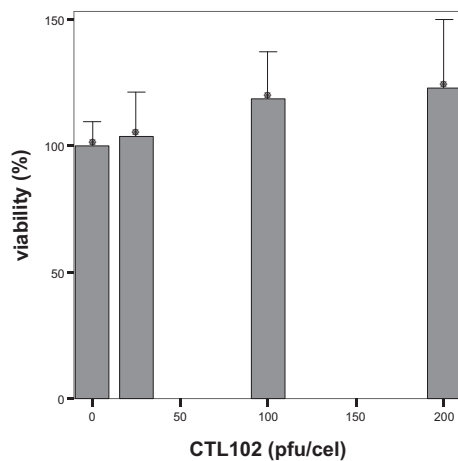
To test the susceptibility of interface cells to HAdV-5 vectors, primary cultures of interface cells were exposed to the HAdV-5 vector Ad.CMV.LacZ. Twenty-four hours post-infection the cells were stained with X-gal solution for β -galactosidase reporter

Figure 2. Transduction of interface cells by Ad.CMV.LacZ in six concentrations (0, 25, 50, 100, 200 and 400 pfu/ cell).



After 3 days, cells were fixed and stained with X-gal reaction mix. The percentage of transduced (blue) cells was counted. The figure shows the means and standard errors of 12 independent experiments

Figure 3. Toxicity of adenoviral vector CTL102 in interface cells.



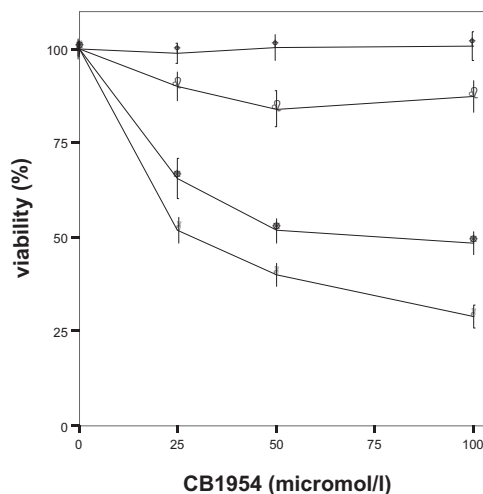
Cells were infected with CTL102 in concentrations of 0, 50, 100, and 200 pfu/ cell, without subsequent addition of prodrug. After 3 days cell viability was measured. The figure shows the means and standard deviations of 12 independent experiments.

gene expression. The transduction efficiency increased with increasing vector concentration. At 400 pfu/cell the percentage of cells expressing the reporter gene was 88% (standard deviation (sd) 4.0) (Figure 2). Thus HAdV-5 vectors can transduce interface cells.

To test whether the cells can be sensitised to the prodrug CB1954, interface cells were exposed to increasing concentrations of the HAdV-5 vector CTL102, which carries the *E.coli*-derived Ntr gene. After transduction, the cells were exposed to CB1954 and cell viability was assessed with the WST-1 viability assay kit. Cells that were transduced with the vector CTL102 without addition of prodrug showed a significant enhancement of cell viability with increasing vector concentration ($p < 0.001$; Figure 3).

In the calculations for the effect of CB1954 we corrected for this effect of CTL102 on viability. The level of sensitisation to CB1954 increased with increasing vector titer. At 200 pfu/cell the mean IC_{50} value was 25 μ M (Figure 4). There is a significant correlation (Spearman) between the viability of the cells and the concentration of CTL102 (corr. -0.444 , $p < 0.001$) and CB1954 (corr. -0.445 , $p < 0.001$; Figure 4). CTL102 and CB1954 showed interaction in their effect on viability ($p < 0.001$). These experiments show that interface cells can be killed using the Ntr/CB1954 approach.

Figure 4. Viability of interface cells after GDEPT using CTL102 and CB1954.



Cells were transduced with CTL102 and, after 24 h, CB1954 was added. Viability of interface cells was determined using WST-1 reagent. Cells were transduced with four concentrations of CTL102: (●) 0 pfu/cell; (×) 25 pfu/cell; (■) 100 pfu/cell; (○) 200 pfu/cell. (means and standard error of 12 independent experiments)

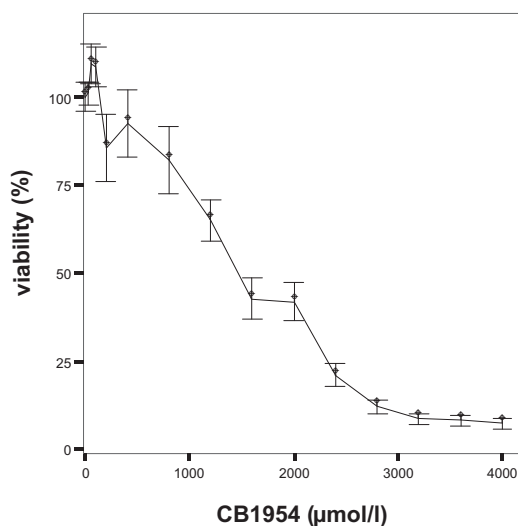
CB1954 toxicity

The toxicity of the prodrug for untransduced cells was tested by a dose ranging study. Interface cells were exposed to various concentrations of CB1954 and their viability was evaluated by WST-1 cell viability assay. In this assay, the absorbance was converted into viability by setting the absorbance of the wells containing interface cells without CB1954 as 100%, and the negative controls, in which no cells were present, as 0% viability. In these assays, the absorbance first increased at low concentrations of CB1954, reaching a maximum at 50-100 μM CB1954 (Figure 5). At higher concentrations, absorbance gradually decreased to a minimum of 0.211 (sd 0.011), corresponding to a cell viability of around 8.2% (sd 5.4). At a CB1954 concentration of 1500 μM 50% of the non-transduced cells were killed. The effect of CB1954 on cell viability was statistically significant ($p < 0.001$). In CTL102-transduced cells a CB1954 concentration of 25 μM revealed killing of 50% of the cells. Transduction of interface cells results in a 60-fold sensitisation to the prodrug CB1954.

Effect of contrast medium on interface cells

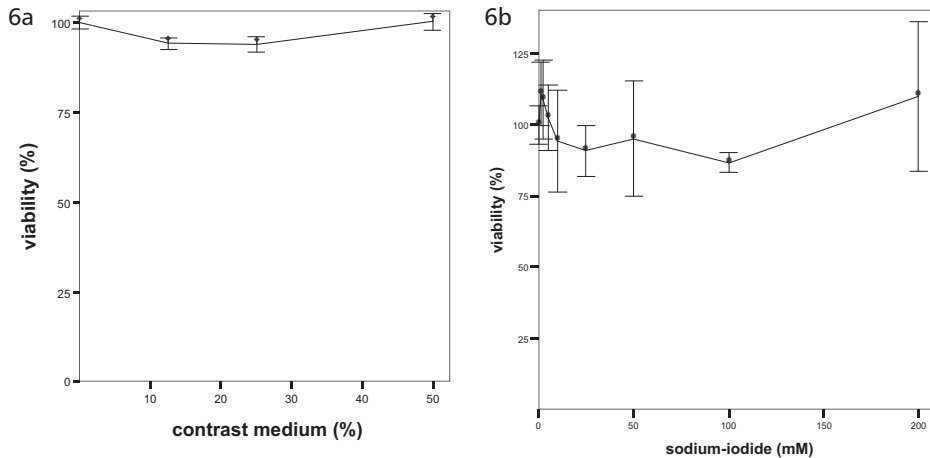
The toxicity of contrast medium (iotrolan) on interface cells was evaluated (Figure 6a). Iotrolan does not affect the viability of the cells at any concentration ($p = 0.563$). Ad-

Figure 5. Toxicity of CB1954 on interface cells.



Cells were exposed to a range of CB1954 concentrations for 24 h. Cell viability was measured using WST-1 reagent. (n=14)

Figure 6. Toxicity of (a) iotrolan (Isovist) contrast medium and (b) sodium iodide on interface cells.



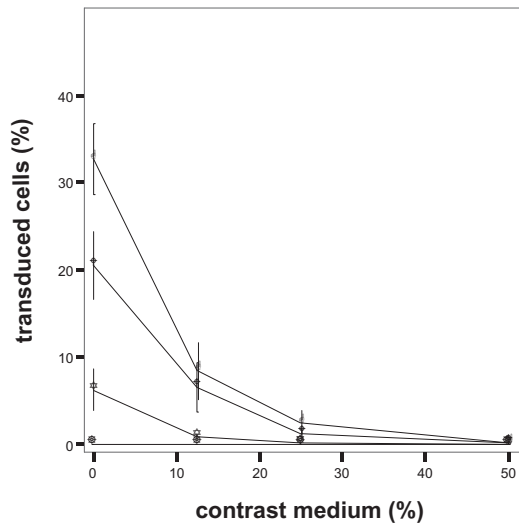
(a) Interface cells were exposed to contrast medium (iotrolan) for 4 h. After 3 days of cell culturing viability of the cells was measured. (n=12)

(b) Toxicity of sodium iodide (Nal) on interface cells and HepG2 cells. Cells were exposed to Nal for 4 h. Viability was tested after 3 days. (n=6)

dition of contrast medium to the interface cells for 4 h did not lead to killing of the cells. As a control for toxicity of possible iodide release the previous experiment was repeated with Nal instead of iotrolan (Figure 6b). There was no effect of Nal on viability of the cells ($p = 0.903$).

Effect of contrast medium on HAdV-5 transduction of interface cells

The effect of contrast medium (iotrolan) on HAdV-5 transduction of interface cells was investigated with Ad.CMV.LacZ. Infectivity of the cells increases with the concentration of HAdV-5 vector. However, the contrast medium has a restraining influence on the transduction efficiency. With higher concentrations of iotrolan, the HAdV-5 vector concentration has less effect on gene transfer efficiency. At a contrast medium concentration of 50% none of the cells were transduced (Figure 7). Between patients (n=6), interindividual differences were observed. The effect of iotrolan on the transduction is statistically significant ($p < 0.001$). Hexabrix was tested simultaneously with Isovist in two interface cell lines, and in HepG2 cells for both an Ad.CMV.LacZ and an rAd5F35. CMV.LacZ vector. Although the rAd5F35.CMV.LacZ vector was much more efficient, the results for Hexabrix and Isovist were similar for both viruses.

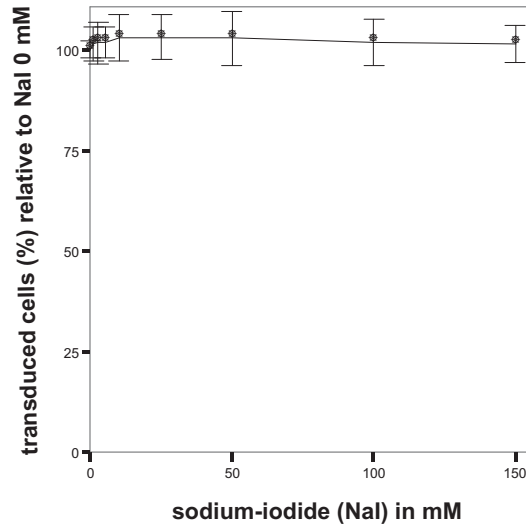
Figure 7. Effect of iotrolan on HAdV-5 transduction of interface cells.

Cells were exposed to a combination of different concentrations of Ad.CMV.LacZ and contrast medium for 4 h and then washed and cultured in IMDM/10% FCS. After 3 days the cells were fixed and stained with X-gal. Percentage of transduced cells was determined by counting blue cells. Ad.CMV.LacZ used: (■) 0 pfu/cell; (▼) 25 pfu/cell; (●) 100 pfu/cell; (◈) 200 pfu/cell. (n=12)

To investigate whether the effect of contrast medium on cell transduction by HAdV-5 vectors was caused by free iodide, NaI was used as a control. Sodium iodide had no effect on transduction by HAdV-5 vectors (Figure 8).

In summary, the results from these experiments suggest that iodide-containing contrast medium cannot be used simultaneously with the currently used HAdV-5 vectors. Therefore, the influence of transient exposure to contrast medium on the transduction of interface cells was investigated. Interface cells were exposed to contrast medium for 0 to 120 minutes and the period between washing away of the contrast medium and performing the Ntr/CB1954 cell killing approach was varied. Cell killing was not correlated with contact time (corr -0.033, $p = 0.691$) or length of the period between washing away of the contrast medium and addition of the vector (corr -0.004, $p = 0.962$). After removal of the contrast medium no influence on cell killing by Ntr/CB1954 was observed.

Figure 8. Effect of sodium-iodide (Nal) on transduction of two interface cell lines and HepG2 cells.



Cells were exposed to adenoviral vector and various concentrations of Nal in NaCl for 4 h. Cells were cultured for 3 days and fixed and stained with X-gal. Transduction rates are relative to percentage of transduced cells with Nal concentration of 0 mM. Ad.CMV.LacZ as well as rAd5F35.CMV.LacZ were used to infect the cells

Discussion

This study demonstrates that interface cells can be transduced by an HAdV-5 vector and killed by the Ntr/CB1954 approach.

Human adenovirus 5 is capable of infecting a broad range of dividing and non-dividing human cells including fibroblasts and macrophages.³⁴ To our knowledge periprosthetic interface cells have not been used previously as target cells for gene therapy. As interface tissue has the histological and histochemical characteristics of synovial tissue,⁴³ results of studies with synovial tissue could be an indicator of outcome in similar studies with interface tissue. The results of the current study are consistent with previous studies that have shown that synovial cells can be successfully transduced by HAdV-5 vectors.^{45,87}

Infection of interface cells with the vector CTL102 without subsequent addition of prodrug leads to an increase in cell viability. This anti-apoptotic effect has been described previously in lung epithelial cells.³⁹ As the cell viability increases with vector concentrations we conclude that the concentrations used in this experiment are not toxic for the interface cells.

Killing of cells by Gene-directed Enzyme Prodrug Therapy (GDEPT) has been studied before in various cell lines, using various approaches. The Ntr/CB1954 approach is attractive for clinical evaluation for several reasons: (1) it generates a toxic agent that can kill both dividing and non-dividing cells; (2) induction of cell death occurs by a p53-independent mechanism; and (3) CB1954 is well tolerated in humans.³⁴ Cell killing by the Ntr/CB1954 approach has been proved effective in a variety of human cancer cells,^{13,23,48,83,109,125,127} but has not been studied in synovial or interface cells. The current study shows that interface cells can be effectively killed by the Ntr/CB1954 approach. The doses used are assumed to represent concentrations that can be achieved in a clinical study.

For the current study passage 2 to 4 interface cells were used. These passages were used to maximally reduce culture artifacts. On the one hand, in very low passages (0 and 1) there is a risk of presence of contaminating cells (especially macrophages), which decreases with higher passages. On the other hand, at higher passages, the risk of substantial *in vitro* alteration/ growth selection exists (especially at passages higher than 4).¹³² In the current study, cultured interface cells of different patients were used. For the interpretation of the results the data of all patients were pooled. However, it must be noted that interindividual differences in infectivity were observed.

In this study the influence of contrast medium on transduction by an adenoviral vector was investigated in view of future clinical studies. Results show that the contrast medium does not seem to have any influence on viability of the interface cells. However, transduction of the cells by an adenoviral vector, in the presence of contrast medium, is almost negligible. The vector is assumed to be inactivated by the contrast medium. This effect was seen in an ionic (Hexabrix) and a non-ionic (Isovist) low-osmolarity contrast medium. The mechanism of transduction inactivation by iotrolan (Isovist) and ioxaglate meglumine – ioxaglate sodium (Hexabrix) remains unclear. The effect is not caused by a change of the cells themselves as transient exposure to contrast medium does not lead to inactivation of Ad.CMV.LacZ. The effect is independent of receptor since rAd5F35, an adenovirus that binds CD46 as receptor, is inhibited by Isovist and Hexabrix as well. The inactivation is not caused by iodide itself as exposure of the cells and vector to NaI did not lead to decrease in transduction efficacy. In a putative clinical study the viral vector will be injected in the joint space. Normally, contrast medium is used to verify the position of the needle in the joint. The results of this study however show that the use of contrast medium in combination with a viral vector is dissuaded. Thus, for a clinical study, we propose that alternative methods for the visualisation of the needle should be employed such as injection of air to create an "air-arthrogram".

In conclusion, the results of this study show that interface cells can be killed by the Ntr/CB1954 enzyme prodrug approach. These data are essential as preclinical work for the starting of a clinical study to kill interface tissue *in vivo*. In such a study, the currently employed contrast media cannot be used in one solution with an HAdV-5 vector given the effect on the transduction of cells.

Chapter 3
