Efficacy of Recombinant Adeno-Associated Viral Vectors Serotypes 1, 2, and 5 for the Transduction of Pancreatic and Colon Carcinoma Cells

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Abstract. *Background: The development of efficient and specific vector systems remains a central issue in gene therapy. Several different adeno-associated virus (AAV) serotypes have so far been characterized so far which show different tissue tropisms. Materials and Methods: The vectors used here contained AAV2 transgene cassette containing green fluorescent protein (GFP) in AAV1, AAV2, or AAV5 capsids, producing the recombinant pseudotypes rAAV2/1, rAAV2/2, and rAAV2/5. The transduction efficiency of the different pseudotyped AAV vectors was tested in vitro in pancreatic and colon cancer cells lines (HT-29, BXPC3, and Hs766T). Results: For all three serotypes, the percentage of GFP-positive cells was below 10% at multiplicities of infection (MOI) 100 rAAV vectors when used alone for infection. However, transduction efficiency for rAAV vectors increased dramatically when the cells were co-infected with wild-type adenovirus (wtAd). The percentage of GFP-positive cells ranged from 19.8-65.3% for AAV2/1 and 16.9-70.2% for AAV2/5, respectively. It was highest for rAAV2/2, at 40.9-88.4%. Variation between the cell lines was observed, with BXPC3 scoring the highest transduction rates and HT-29 the lowest. Conclusion: This study indicates that vectors based on distinct AAV serotypes 1, 2, and 5 all transduce pancreatic and colon cell lines poorly when used alone. Co-infection with wtAd increase transduction rates dramatically indicating that slow second-strand synthesis is a reason for the poor transduction efficiency. Due to the poor transduction rates, none of the rAAV serotypes tested here seem to be feasible for the treatment of malignant tumors.*

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Pancreatic carcinoma has a dismal prognosis with an overall 5-year survival below 3%, for colon carcinoma the overall rate is approximately 30%. Therefore, the development of new therapeutic options is warranted. Gene therapy approaches are an attractive alternative to standard treatments in cancer. Although some encouraging results have been achieved with *in vitro* and some phase I studies, the choice of the vector remains crucial. The vector system has to meet two goals: Efficient transduction of cells or tissue and high specificity in order to minimize toxicity. The most commonly employed viral vector for cancer gene therapy is recombinant adenovirus. Adenoviral vectors have the advantage of efficiently transducing a wide variety of cells (1). The main draw back of this viral vector is its high immunogenicity (2).

Recombinant AAV has been widely used as a vector in gene therapy. Its advantages include efficient transduction of many different tissues *in vivo*, leading to stable long-term expression of the transgene, and no or little toxicity (3). To date, the most commonly used vector has been based on AAV serotype 2, which was the first serotype to be described (4). It has already been used in clinical trials in humans (5, 6). So far the use recombinant AAV2 vectors for gene therapy of cancer has produced mixed results. Transduction of a variety of human tumor cells *e.g.* small cell lung carcinoma, ovarian carcinoma, melanoma, cervical carcinoma, and osteosarcoma, *in vitro* has been shown (7, 8). We have previously shown that transduction *in vitro* as well as *in vivo* was low (9). One reason for this was slow second-strand synthesis. However, other investigators demonstrated that rAAV2 can transduce tumor cells *in vivo* resulting in a therapeutic benefit (10). Penetration of the tumor tissue is also a factor that can hamper tumor cell

transduction *in vivo*. AAV is a small virus which can be used to advantage for infiltration of tumor tissue. In a human glioma model, rAAV of different serotypes were able to penetrate gliomas *in vivo* (11).

To date, more than 8 AAV serotypes have been described (12). Except for AAV 6, which differs in only 6 amino acids from AAV 1, all serotypes display considerable differences within the amino acid sequence of the viral capsid (13, 14). The serotypes also use different co-receptors for entrance into the cell (15), resulting in different tissue tropism. Serotypes AAV 1-8 have been used as recombinant viral vectors. Serotypes 1, 2 and 7 were most efficient for muscle gene transfer, AAVs 5 and 6 have advantages in the respiratory tract, and AAV2, AAV4, and AAV5 show superior efficiency in the CNS (16-19). For liver-directed gene transfer, AAV5 and AAV8 are most efficient (13, 20).

The purpose of this study was to evaluate the transduction efficiency of rAAV vectors based on serotypes 1 and 5, compared to the standard AAV2 in a panel of pancreatic and colon carcinoma cell lines *in vitro*. The CMV immediate early enhancer/promoter (CMV promoter) driving green fluorescent protein (GFP) was used as a reporter gene.

Materials and Methods

Cell lines. The human embryonic kidney cell line 293 and the human colon carcinoma cell line HT-29 (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 10% bovine calf serum (BCS) and antibiotics. The human pancreatic carcinoma cell lines BXPC3 (ATCC, Manassas, Viginia, USA), and Hs766T (Dr. Schwarte-Waldhoff, University of Bochum, Germany) were maintained in RPMI-1640 supplemented with 10% BCS and antibiotics.

Production of recombinant AAV vectors. The AAV recombinant genome, *p*TRUF5, contains the coding sequence for humanized GFP (21) under the control of the CMV immediate early enhancer/promoter flanked by AAV2 TRs. *p*DG (22), a plasmid that expresses the AAV2 rep and cap genes as well as the adenovirus E4, VA, and E2a helper regions, was co-transfected into 293 cells with *p*TRUF5 to produce rAAV2/2 . Recombinant virus was then purified as previously described (23). To produce rAAV2/1 and rAAV2/5, pDG was substituted with *p*XYZ1 and *p*XYZ5, respectively. *p*XYZ1 and *p*XYZ5, in addition to the adenovirus helper genes described above, express AAV2 rep and the AAV1 or AAV5 capsid genes, respectively. rAAV2/1 and rAAV2/5 were purified by iodixanol step gradients and Sepharose Q column chromatography as previously described (23). All three virus stocks were at least 99% pure as judged by silver-stained SDS acrylamide gel fractionation. Vector titers were determined by dot-blot assay as described (24) and the titers were rAAV2/1 6.2×10^{12} genome copies/ml; rAAV2/2 1.8×10^{12} genome copies/ml, rAAV2/5 1×10^{12} genome copies/ml. In the case of rAAV2/2, the infectivity of the virus preparation was determined by the green cell assay as described elsewhere (25) and the particle-to-infectivity ratio was approximately 50:1. Standard assays for the infectivity of rAAV1 and rAAV5 are not yet available.

Infection of cell lines with rAAV2 in vitro. Cells ($0.5\text{-}1\times10^6$) were incubated in suspension with the viruses at the specified multiplicity of infection (MOI) in a total volume of 200 μl of media without BCS or antibiotics at 37˚C. After 3 hours, 1000 μl of complete medium were added and cells were plated in a 6-well dish.

FACS analysis. Cells were assayed for GFP expression by FACS analysis 48 hours after infection (Becton Dickinson flow cytometer, University of Bochum, Department of Internal Medicine, Facility for Flow Cytometry). A cell was scored positive for GFP fluorescence when its fluorescence was higher than the threshold which was set in the control sample and which is determined by the background fluorescence.

Results

Infection of tumor cell lines with recombinant AAV serotypes 1, 2, and 5 in vitro. rAAV1 and rAAV5 vectors have been reported to show different cell tropism compared to the widely used rAAV2. This has been shown showen for tissues such as muscle, liver and brain when injected into the lateral ventricle (3). To investigate cell tropism and transduction efficiency in tumor cells, we produced pseudotyped rAAV vectors in which the same genome was packaged into three specific serotypes (1, 2, and 5). This eliminated the effect of *cis*-active genome components and allowed us to study soley the effects of the capsid-receptor interactions. The reporter gene, GFP, was under the control of the CMV promoter, which is active in a wide variety of cells (26, 27). We produced rAAV2/1, rAAV2/2, and rAAV2/5 vector stocks of similar titers.

The results for HT-29, a human colon carcinoma cell line, BXPC3, and Hs766T, two human pancreatic carcinoma cell lines, are summarized in Figure 1. None of the cell lines was efficiently transduced by the rAAV2/1, rAAV2/2, and rAAV2/5 vectors. At an MOI of 100, 5.7% of BXPC3 cells scored positive for GFP when infected with rAAV2/2. At the same MOI, only 0.5-3.4% of cells expressed GFP when rAAV2/1 or rAAV2/5 were used. This was true for all other tumor cell lines and all different AAV serotypes tested.

Co-infection of tumor cell lines with recombinant AAV serotypes 1, 2, and 5 and wtAd in vitro. Wild-type adenovirus is known to accelerate second-strand synthesis of AAV (28). Therefore, we co-infected all the tumor cell lines with the different AAV serotypes and wtAd in order to investigate whether viral entrance into the cells or second-strand synthesis was the reason for the low transduction efficiency.

In all human tumor cell lines, the transduction efficiency for all AAV serotypes was dramatically improved after co-infection with wtAd as shown in Figure 2. The percentage of GFP-positive cells following an infection with rAAV2/1 and wtAd increased to 19.8-65.3% at an MOI of 100. Infection with AAV2/2 and wtAd resulted in 40.9-88.4%

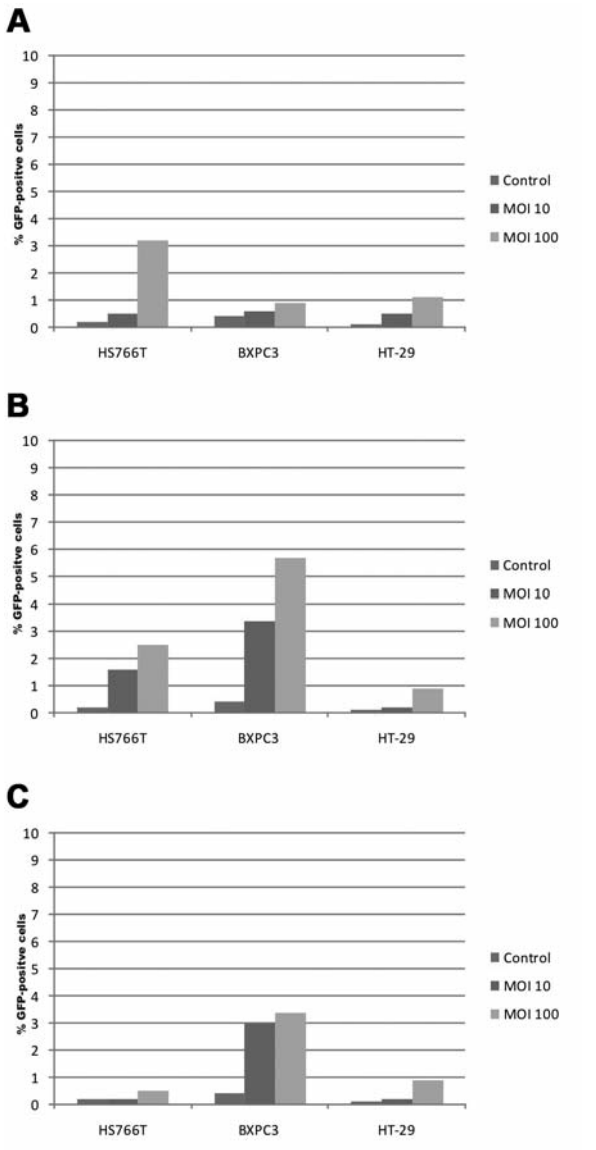

Figure 1. *In vitro infection of tumor cell lines with recombinant AAV serotypes 1, 2, and 5 alone. A: rAAV2/1; B: rAAV2/2; C: rAAV2/5. Infection was carried out at the MOIs indicated as described in the Materials and Methods. Cells were analyzed for GFP expression (percentage) by FACSscan.*

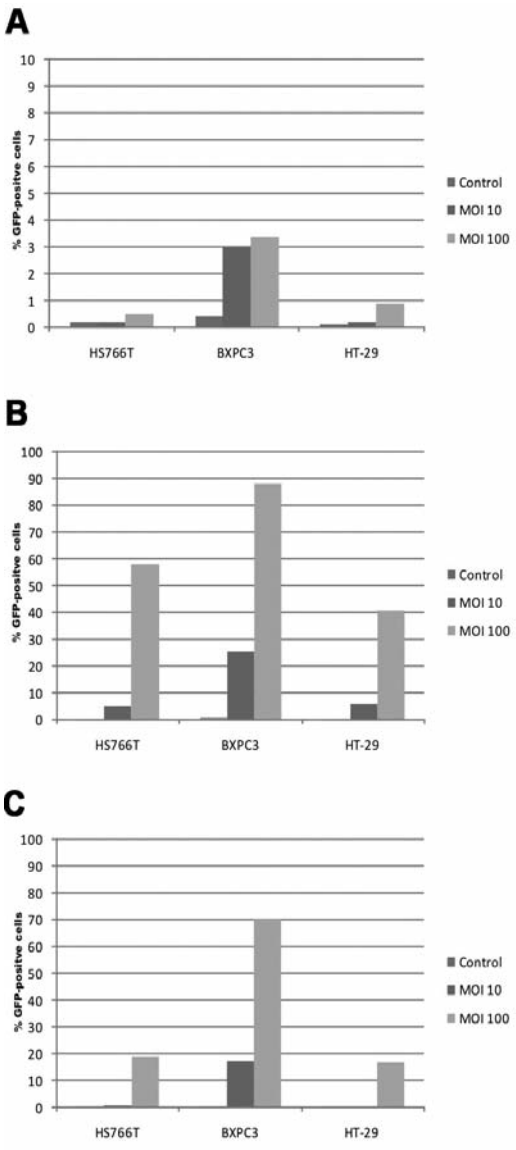

Figure 2. *In vitro infection of tumor cell lines with recombinant AAV serotypes 1, 2, and 5 together with wtAd at an MOI of 5. A: rAAV2/1; B: rAAV2/2; C: rAAV2/5. Infection was carried out at the MOIs indicated as described in the Materials and Methods. Cells were analyzed for GFP expression (percentage) by FACSscan.*

GFP-positive cells at an MOI of 100. A similar result was obtained for rAAV2/5: 16.9-70.2% of tumor cells scored positive by FACS analysis when infected with rAAV2/5 and wtAd at an MOI of 100. In general, the pancreatic tumor cell line BXPC3 was the easiest to transducer, whereas the colon cell line HT-29 showed the lowest results. Co-infection with wtAd not only resulted in a dramatic increase of the transduction rate but also in a manyfold higher mean expression level of GFP (data not shown).

Discussion

The choice of vector is crucial for all gene therapy approaches, including cancer gene therapy. A number of obstacles have to be overcome in an *in vivo* setting. Firstly, cells need to be efficiently infected with low toxicity, and secondly transgene expression has to be high enough for therapeutic effects. Thirdly, the extracellular matrix in tumors can inhibit penetration of the vector (29). Recombinat AAV2

has been used extensively and successfully in a number of gene therapy approaches including clinical phase I trials for metabolic diseases (3). The use of AAV2 for cancer gene therapy is very limited, although it seems well suited for *in vivo* application due to its small size. We had shown that AAV2 is not efficient for gene therapy approaches in pancreatic and colon carcinoma (9). To date eight different AAV serotypes have been used as viral vectors, displaying different tissue tropisms (30).

In the present study, the transduction efficiency of rAAV vector serotypes 1, 2, and 5 was tested using GFP as a transgene in a panel of human tumor cell lines *in vitro*. The results obtained for the 3 AAV serotypes were similar. The transduction efficiency when cells were infected with rAAV alone was low. When co-infected with wtAd, we were able to dramatically increase transduction efficiency of AAVs. Comparing the three serotypes used, AAV2 was slightly more effective than the other two. Similar results have been reported by other groups (31). The fact that wtAd substantially increases the transduction efficiency indicates that rAAV successfully enters the cell but is apparently lacking essential steps for transcription and translation of the transgene. This can be most likely attributed to slow second-strand synthesis in the absence of helper virus function which leads to a delayed expression of the transgene (32). Similar results were obtained for ovarian cancer cells and hepatocellular carcinoma (33-35). One way to circumvent the problem of second-strand synthesis is the use of self complementary AAV vectors (31, 36, 37).

We conclude that rAAV serotypes 1, 2, and 5 are able to infect human pancreatic and colon carcinoma cell lines *in vitro*. However, the efficiency remains low most likely due to slow second-strand synthesis. Therefore, an *in vivo* approach does not seem feasible, as we have in fact shown in earlier studies (9). In comparison, AAV serotype 2 was more efficient than the other two tested serotypes. A useful approach of rAAV2 in cancer gene therapy might be the infection of tumor cells *ex vivo* in order to generate cancer cell vaccines (26); similar approaches of rAAV in cancer gene therapy have been the stable expression of antiangiogenic factors after transduction of muscle or liver (38, 39).

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