Recombinant adeno-associated virus vector hybrids efficiently target different skeletal cells

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1. ABSTRACT

Finding the optimal recombinant adeno-associated virus (rAAV) serotypes for efficient as well as tissue specific transduction has become imperative for successful gene therapy. We used rat condylar chondrocytes, osteoblast-like cell line UMR106 and bone marrow stromal cells (BMSCs) to evaluate the transduction efficiency of different rAAV serotypes in vitro; hoping to establish an efficient in vivo rAAV mediated delivery system for gene therapy in craniofacial region. All of the selected rAAV serotypes were able to infect target cells and gave rise to eGFP expression and VEGF secretion. Quantified by fluorescence activated cell sorter (FACS) and ELISA analysis, rAAV2 was superior for efficient transduction of rat chondrocytes, rAAV1 was most efficient when introduced into UMR106 cell line and rAAV5 yielded the highest infection efficiency in BMSCs. Hence, differences in receptor binding in different oral tissues and transduction pathways suggest rAAV based hybrids have various transduction efficiencies and can efficiently target different oral tissues.

2. INTRODUCTION

Successful repair of bone deficiencies in the craniofacial region, whether arising from trauma, tumor resection or congenital disorders, continues to be a major concern to reconstructive surgeons. On the basis of recent insights into the growth, development and adaptation of bone, together with the significant advances in molecular biology, the development of novel approaches—the combination of matrix-based, cell-based, and factor-based therapies, gene therapy was recognized to maximally stimulate osteogenesis and reduce or ultimately overcome conventional growth factor delivery limitations.

The non-vial vector based systems involve either the physical or chemical transfer of genetic material, and are dependent on cellular transport mechanisms for uptake and expression in the host cell. They include naked DNA alone (1, 2), or DNA associated with carrier molecules (such as liposomes or a polymer matrix) (3). However, in Ohashi’s research (4), a naked DNA injection alone (25–50 µg) resulted in a very faint expression of transgene. This is
consistent with Yavandich's findings (5) that direct intra-articular administration of 100 µg naked DNA induced a very low level of marker gene (Lac z) expression in both the rat and rabbit synovium. Therefore, the poor transduction reached by most nonviral methods (10). However, retrovirus are incapable of infecting nondividing cells and may give rise to insertional mutagenesis, a fact that limits its clinical application (11). The great shortcoming of adenoviral vectors is the stimulation of a significant host immune response. Overall, among potential vector systems for gene therapy application in craniofacial region, AAV is a favourable choice as it has several major advantages. It can efficiently infect dividing as well as non-dividing cells with a broad host range, including human and murine embryonic stem cells (12), hematopoietic progenitor cells (13, 14), mesenchymal stem cells (15-18), chondrocytes (19-22), osteoblasts(23), myoblasts (24), brain cells (25), hepatic stellate cells (26), and epithelial cells (27). Delivery by rAAV vectors results in long-term expression of therapeutic genes as it persists mostly in episomal or concatameric form but not integration into host chromosomal DNA, does not result in destructive cellular immune responses against infected target cells, and has not been associated with any human disease (28-36).

Up to now, 11 serotypes were identified and they have different intrinsic properties. The sequence homology among the different serotypes is high. Sequence comparison revealed that the greatest divergence lies in the capsid proteins (37-39) leading to differences in both tropism and serological neutralisation (40). AAV2 has been the most widely utilized serotype, the first AAV adapted for therapeutic gene delivery, most thoroughly characterized and frequently employed (41). Moreover, vectors based on at least seven other AAV serotypes have also been developed and are being actively pursued as gene delivery vectors. Interest in these alternative serotypes has been driven by the fact that they exhibit different cellular tropisms and are often more efficient than AAV2 in vivo. To find the optimal AAV serotypes for efficient and tissue specific transduction has become imperative for successful gene therapy.

Therefore, the aim of our study is to compare the transduction efficiency of different target tissues in order to establish an efficient in vivo rAAV mediated gene delivery system for future craniofacial gene therapy.

3. MATERIALS & METHODS

3.1. Construct rAAV-VEGF vector

The full-length VEGF_{164} cDNA fragments were isolated by PCR using rat liver first-stranded cDNA as template, using the following two primers 5'-CGGTCTGCAGATGAACTTTCTGCTCTCT-3', and 5'-ATTCGAATTCTCACCGCCTTGGCTTGTC-3'. To attain a constitutive, high-level expression of VEGF, the cDNAs encoding VEGF_{164} was cloned into AAV-2 vector under the control of CAG promoter to obtain the pAAV-VEGF plasmid, in which the expression cassette was flanked by the AAV serotype-2 ITRs (Figure 1). The insertion of cDNA fragments into the vectors was confirmed by restriction enzyme digestion and DNA sequencing.

3.2. Generation of rAAV-VEGF_{164} and rAAV-eGFP particles

Different pseudotypes of rAAV were generated by standard production and purification protocols (42). The rAAV2 and pseudotyped rAAV1, rAAV5 rAAV6 and rAAV8 were generated by packaging identical AAV2-ITR recombinant genomes in AAV2, AAV1, AAV5, AAV6 and AAV8 capsids, respectively. They were generated by using a three-plasmid transfection protocol as previously described (43). Briefly, HEK293 cells were tritransfected by calcium phosphate precipitation with an adenovirus helper plasmid pFD6 (44), a AAV packaging helper plasmid expressing the rep and cap genes, and a plasmid bearing the recombinant pAAV-VEGF. All the
recombinant vectors were purified by an OptiPrep-based gradient ultracentrifugation (45, 46). The viral titer was quantified by quantitative real-time PCR (7700, Applied Biosystems) (47).

3.3. Isolation culture of chondrocytes, bone marrow stromal cells and UMR106 cell line

The use of animal tissues were approved by the University Ethics Committee and performed according to institutional guidelines. Primary rat chondrocytes were obtained from mandibular condylar cartilage removed under sterile conditions according to the previous report (46, 48). The chondrocytes used in this study were maintained as monolayer cultures for no more than two passages, to maintain the differentiated chondrocyte phenotype. BMSCs were obtained from 5-week-old Sprague-Dawley rats. Cell isolation from the femur and tibia was performed according to previously report (49). After marrow isolation and dispersion, cells were centrifuged at 1200 rpm for 5 minutes. The resulting cell pellet was washed and resuspended in the DMEM medium (low glucose). UMR106 cells (American Type Culture Collection) were grown in DMEM medium supplemented with 10% FBS.

3.4. In vitro transduction with rAAV-eGFP

The chondrocytes, BMSCs and UMR106 cells were allowed to adhere for at least 24 h before addition of AAV. The cells were washed once with 1×PBS, and then the different rAAV hybrids were added at the dose of 5×10^4 MOI (multiplicity of infection) in DMEM medium. After 5 hours, cells were incubated in complete DMEM medium with 10% FBS. After transduction of day 3, 5 and 7, the capacity of the rAAV-eGFP infection on these three cells was analyzed by fluorescence microscopy and the transduction efficiency was performed by a FACSCalibur (Becton Dickinson) respectively. As previously described (46, 50), 1x 10^5 cells were counted per acquisition. The percentage of live gfp-expressing cells in this population was evaluated. The data were further analyzed with CellQuest software (Becton Dickinson). A maximum level of 5% was set as the background autofluorescence in live, uninfected cells.

3.5. In vitro transduction with rAAV-VEGF

The rAAV1, rAAV2 and rAAV5 were used to infect these three cells according to the previous description. The culture media of the transduced cells with different serotypes of rAAV-VEGF were collected and the VEGF concentration in the media was evaluated by ELISA. Quantification of VEGF secretion in the conditioned media of day 7 was performed by mouse VEGF ELISA kit (R&D) following the manufacturer’s instructions. These experiments were repeated four times.

4. RESULTS

4.1. In vitro detection of transduction efficiency

The vectors contained AAV2 terminal repeats flanking transgene in AAV1, AAV2, AAV5, AAV6 or AAV8 capsid, producing the pseudotypes rAAV1, rAAV2, rAAV5, rAAV6 and rAAV8. Quantified by flow cytometric analysis, the infection efficiencies on these cells were time-dependent (Figure 2). On day 7, the transduction rates of chondrocytes to be 61.62 ± 3.42% for rAAV1, 80.86 ± 4.19% for rAAV2, 63.27 ± 4.04% for rAAV5, 48.79 ± 2.27% for rAAV6, and 50.49 ± 3.39% for rAAV8. For UMR106 cells, the infection efficiencies were 80.61±3.75%, 53.47±3.76%, 62.68±4.51%, 38.62 ± 4.35% and 56.74± 5.72% for rAAV1, rAAV2, rAAV5, rAAV6 and rAAV8 respectively. For BMSCs, the highest infection efficiency was 72.75 ± 4.99% for rAAV2/5.

4.2. In vitro detection of VEGF secretion

Furthermore, we tested whether the encoded VEGF could be secreted into the medium. Based on the previous comparison of transduction efficiency, rAAV1, rAAV2, rAAV5 mediated VEGF were selected to infect these three cells. VEGF secretion was quantified by ELISA and expressed as the mean of four independent experiments. Compared with the PBS treatment, all of the three serotypes significantly increased the VEGF expression after 5 days treatment (Figure 3). For primary chondrocytes, the highest level of 57.05±6.37ng/ml VEGF was identified in rAAV2-VEGF treatment, which was 3.14 and 2.85 fold increases than that of rAAV2-VEGF and rAAV5-VEGF treatment, respectively. The BMSCs treated by rAAV5-VEGF presented the highest secretion level of 46.45±6.18ng/ml,which was 2.09 and 1.94 fold more than that of rAAV1-VEGF and rAAV2-VEGF treatment, however, infected by rAAV1-VEGF, UMR 106 cell line yielded the highest VEGF expression as 68.66±4.10mg/ml, which was 2.88 and 2.14 fold enhance than that of rAAV2-VEGF and rAAV5-VEGF treatment.

5. DISCUSSION

This study successfully identified the ideal rAAV serotype for different tissues in the craniofacial region, which is of paramount importance for future gene therapy because it provides the basis onto which gene therapy in this area could be based. In the field of dentistry, these include genetic disorders, such as hemifacial microsomia, micrognathia, TMJ arthritis and acquired conditions, such as segmental craniofacial bone defects, cartilage damage and periodontal bone loss. The challenge now is to precisely define optimal cellular targets, therapeutic genes, and to develop safe and efficient ways to deliver such therapeutic genes to target cells. Furthermore, the efficiency of therapeutic genes to target cells in different skeletal tissues is a key step in the development of gene therapy in craniofacial-dental field. Recent advances in the technology of rAAV production facilitated its use in human clinical trials (51). Recombinant crosspackaging of AAV genome of one serotype into other AAV serotypes has opened the possibility to optimize tissue-specific gene transduction and expression. In an effort to compare the role of serotype-specific virion shells on vector transduction to different target cells, the gene transfer capability of rAAV1, rAAV2, rAAV5, rAAV6 and rAAV8 were evaluated in this study. The reporter gene of enhanced green fluorescent protein (eGFP), an exogenous intracellular molecule was used for in situ identification of the transduced cells. Furthermore, we tested whether the encoded VEGF could be secreted into the medium. Using
AAV serotypes infection on different skeletal tissues

Figure 2. Transduction efficiency of different rAAV serotypes on three cells. A: Chondrocytes, UMR106 cells and BMSCs were infected with different serotypes of AAV-eGFP at an MOI of 5 x 10^4 particles per cell were examined by fluorescent microscopy. B: FACS photos of different serotypes infection on each cell at day 7. C: Quantification analysis of transduction efficiency of different serotypes on day 3, 5 and 7.

VEGF as a therapeutic gene was important because it is a secreted homologous protein precisely measured by ELISA and allows us to monitor the kinetics and production level from the conditioned medium. Moreover, VEGF, the best-characterized angiogenic factor, has been shown to play an important role in long bone and mandibular condylar growth (52, 53). Some successful experiments were reported demonstrating the effect of recombinant VEGF and in vivo gene therapy on bone formation (54, 55).

Using fluorescence activated cell sorter (FACS), the transduction efficiency was identified in a time-dependent manner (Figure 2). The serotype 2 was found to be superior for efficient transduction of rat chondrocytes. In this study, chondrocytes were isolated from rat mandibular condyle which play integral role in mandibular growth (53). These cells were then transfected with rAAV2 and yielded the highest secretion level of VEGF (Fig 3). This was consistent with our recent study.
AAV serotypes infection on different skeletal tissues

where rAAV2 was shown to direct VEGF expression in mandibular condylar chondrocytes in vitro and in vivo and resulted in significant condylar growth (unpublished paper). The reason behind the superior transduction efficiency for chondrocyte is most likely due to the expression of cellular receptors and coreceptors of rAAV2 in the chondrocytes making them a target for rAAV2 delivery. The AAV2 capsid binds initially to heparin sulfate proteoglycan (HSPG) molecules on the cell surface, and then uses either fibroblast growth factor receptor type 1 (FGFR1) or integrin αVβ5 as a coreceptor for entry (56, 57). To identify such receptors in condylar cartilage, we carried out a microarray analysis that revealed the presence of cellular receptors and coreceptors of rAAV2 in mandibular condylar cartilage during natural growth (unpublished data). These receptors were also identified on a protein level in all the layers of cartilage, especially hypotrophic layer (58-60). These results presented our field with valuable tools to deliver any gene of interest to mandibular condylar cartilage for the purpose of manipulating condylar growth. Such manipulation of condylar growth could be of great interest benefit to future gene therapy of several craniofacial disorders such as micrognathia and mandibular hypoplasia or macrognathia and mandibular hyperplasia.

The Rat osteoblast-like cell line UMR 106 derived from osteosarcoma retain many markers of the osteoblast phenotype and is considered to be representative of a relatively mature osteoblast phenotype (61). The order of transduction efficiency is different from that of mandibular condrocytes. Type 1 was found to be the most efficient vector (Figure 2). Therefore, different serotypes have unique profiles of transduction and, as a consequence, vary in their tropism for target tissues. The mechanism of viral entry and binding to cells is known only for a few AAV serotypes, the receptor for AAV1 capsid has not yet been identified and AAV1 has not been characterized to the extent of AAV2 in terms of transduction pathway. Further studies are needed to identify the receptors that might lead to the superior efficiency of serotype 1 to bone cells. Our data provide our field with an effective and ideal vehicle to deliver potential therapeutic genes to bone tissues.

BMSCs represent a population of non-hematopoietic marrow-derived cells, a subset of which has multipotent capability to generate new cartilage, bone, tendon, muscle, nerve and adipose tissue and have recently attracted much attention for gene therapy and tissue-engineering purposes. However, the poor transduction efficiency of rAAV2 into BMSCs was identified in some studies (18). Luckily, in this study, BMSCs were found to be most sensitive to AAV5 infection, which was 1.94 fold more than that of AAV2 infection when delivering VEGF gene. Because AAV5 infection does not depend on HSPG, the capsid binds sialic acid through the platelet-derived growth factor receptor (PDGFR) for cell entry (62). A hierarchy has been established for efficient serotype-specific vector infection depending on the target tissues (37). Therefore, the serotype of AAV5 represents an ideal vehicle for in vivo and ex vivo gene therapy to modify the BMSCs for bone and cartilage repair.

Collectively, this was the first direct comparative study to evaluate the transduction efficiency of rAAVs in skeletal cells, and further indicate that rAAV2 is the best serotype to infect chondrocyte; rAAV1 was most efficient when was introduced into UMR 106 cell and rAAV5 yielded the highest infection efficiency in BMSCs, thus it will provide us with the basis for potential use of rAAV in future gene therapy targeting skeletal tissues or to understand mechanisms of action of different genes involved in condylar growth.

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7. REFERENCES

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