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Adeno-associated virus type 2 vectors: transduction and long-term expression in cerebellar Purkinje cells in vivo is mediated by the fibroblast growth factor receptor 1:

bFGFR-1 mediates AAV2 transduction of Purkinje cells

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Abstract

The receptor for adeno-associated virus sero-type 2 (AAV2) in Purkinje cells has not been identified, but based on work carried out in non-neuronal cell lines, heparan sulfate proteoglycan is thought to act as a primary receptor, with basic fibroblast growth factor receptor-1 being reported as a co-receptor. In this study, using antibody interference and protein competition strategies, we show specific reduction in Purkinje cell transduction by AAV2 vector in the presence of these inhibitors. We also demonstrate AAV2-mediated transgene expression in Purkinje cells in vivo that extends out to one year post-injection. These results provide new information on fibro-blast growth factor receptor-1 involvement in AAV2-mediated transduction of Purkinje cells and have important implications for AAV-mediated treatment of various cerebellar disorders.

Adeno-associated viruses (AAV) are members of the genus *Dependovirus* in the family *Parvoviridae* and are small (20–30 nm diameter), non-enveloped viruses. The virus is non-pathogenic in humans, is replication deficient in the absence of helper functions, and has a small single-stranded 4.7-kb DNA genome that is flanked by 145-base-pair palindromic

inverted terminal repeats. To date, 11 primary isolates of AAV have been identified, and their tropism has been studied in vitro using cultured cell lines and in vivo in murine tissues [1]. Vectors derived from AAV have been extensively explored for potential therapeutic application, due to their ability to be maintained episomally in the transduced cell, and due to effective transduction of both dividing and non-dividing cells. AAV serotype 2 has been the most commonly used serotype for gene delivery and has been shown to be effective in mediating gene transfer and expression in a wide variety of tissue types in vivo, especially in different regions of the central nervous system [2–6]. Transduction by AAV serotype 2 in the brain occurs in neurons, and long-term stable gene expression has been reported with very little accompanying toxicity [7]. Persistence of gene expression in the brain has ranged from 3 months up to a year, depending on the region of the brain and on the promoter type used [8]. Studies in non-neuronal cell lines have indicated that heparan sulfate proteoglycan (HSPG) is the primary receptor for AAV2 [9]. Other receptors or co-receptors for AAV2 reported in non-neuronal cells lines include fibroblast growth factor receptor-1 (FGFR-1), $\alpha V\beta 5$ integrin, and the hepatocyte growth factor receptor (c-Met) [10–12]. A novel 150-kD glycoprotein has also been proposed to be involved in virus binding, although the identity of this molecule is still unclear [13].

The cerebellum plays an essential role in motor control, balance and coordination, and motor learning. Purkinje cells are the largest of the neuronal types present in the cerebellum and are responsible for information transmitted from the cerebellum to other motor areas of the brain. Purkinje cell and cerebellar dysfunction are seen in a number of hereditary disorders, and correction or reversal of this Purkinje cell loss or dysfunction would be expected to have a major effect on human disease.

We have previously reported successful use of AAV2 vector to transduce Purkinje cells in the cerebellum in mice [14]. The mechanism of transduction in Purkinje cells is unknown, although there has been indirect evidence to suggest that the basic fibroblast growth factor receptor-1 (bFGFR-1) may be involved in AAV uptake into Purkinje cells [15, 16]. Here, we examine the role of bFGFR-1 in AAV2 transduction of cerebellar Purkinje cells in vivo.

In order to determine if bFGFR-1 is involved in AAV transduction, several antibodies and blocking agents were used to inhibit binding of the vector to the receptor. Three 9-week-old female FVB/N wild-type littermates were used in this experiment. Prior to vector injections, each mouse received antibody pre-treatment injections into the cerebellar hemispheres at the coordinates AP –3, ML $\pm$ 1.75, and DV 0.5 mm from lambda. Each mouse served as its own control, with the contralateral hemisphere injection site receiving a control pre-treatment. In the first mouse, the left hemisphere was pre-treated with 1 μ l rabbit anti-bFGFR-1 (Research Diagnostics, Inc., Flanders, New Jersey) at a 1:40 working dilution. The right hemisphere was pre-treated with the same amount of anti-bFGFR-1 antibody after in vitro blocking of that antibody with a blocking peptide (RDI-FGFR1-CP, Research Diagnostics). Briefly, a 1:40 working dilution of the anti-bFGFR1 antibody was mixed with an equal volume of blocking peptide (final concentrations of 5 μ g/ml antibody and 50 μ g/ml blocking peptide) and incubated at room temperature prior to use. In the second mouse, the left hemisphere site was pre-treated with 1 μ l rabbit anti-bFGFR-1 diluted 1:20 in sterile PBS. The right hemisphere was pre-treated with 1 μ l rabbit anti-tyrosine hydroxylase antibody (Pel-Freez,

Rogers, Arkansas) at a comparable working dilution in sterile PBS. This antibody served as a control for non-specific antibody effects and host immune reaction to the rabbit antibody. The third mouse received a pre-treatment of 1 μ l bFGF (Research Diagnostics) into the left hemisphere at a concentration of 0.1 ng/ μ l. The right hemisphere received a pre-treatment injection of 1 μ l sterile PBS. Thirty minutes after antibody pretreatment injection, the mice were injected with an AAV2 vector transducing GFP (vTR-UF11, University of Florida, Gainesville) into the coordinates detailed above. Prior to each injection, the tip of the Hamilton syringe was dipped in charcoal power to enable precise identification of the injection site when the tissue was later sectioned for histological analysis.

The mice were sacrificed for histological analysis seven days after surgery. The entire cerebellum of each mouse was cut in 30- μ m serial sections in the coronal plane, allowing side-by-side comparison of the hemispheres. Prior to sectioning, the right hemisphere was “painted” with powdered eosin to ensure positive identification of the left versus right hemisphere in the sectioned tissue. Transduced Purkinje cells were identified by morphology, location, and positive GFP signal under fluorescence microscopy, and exhaustively counted.

AAV vector vTR-UF11, depicted in Fig. 1, has been described previously [14]. Briefly, this vector transduces GFP under transcriptional regulation of the hybrid CMV enhancer and β -actin promoter. The highly efficient transduction of cerebellar Purkinje cells by AAV is shown in Fig. 2. The left and right hemisphere injection sites are not visible within a single coronal section because the sectioning plane did not perfectly correspond to the ML axis of the stereotactic coordinate system. If bFGFR-1 is key to AAV2 uptake into Purkinje cells, then each mouse should show reduced Purkinje cell transduction by vTR-UF11 in its left hemisphere compared to its right hemisphere. Mouse 1, which received anti bFGFR-1 antibody in the left hemisphere, showed a total of 22 GFP-positive cells (A), as compared to 288 GFP-positive Purkinje cells in the right hemisphere (B), suggesting that pretreatment with the bFGFR-1 antibody blocked AAV2 binding or entry into Purkinje cells. Mouse 2, which was also injected with anti-bFGFR-1 in the left hemisphere prior to AAV-GFP, shows inhibited transduction with a total of 7 GFP-positive cells (C), while the right hemisphere that was treated with an unrelated anti-tyrosine hydroxylase antibody shows 109 GFP-positive cells (D). This further demonstrates that transduction by the vector is due to binding or internalization that is mediated by the bFGFR-1, rather than by a non-specific effect or host reaction to the antibody. In mouse 3, there was reduced transduction observed when the left hemisphere was pre-treated with bFGF protein (E), resulting in 2 GFP-positive cells, compared to the right hemisphere, where there were 30 GFP-positive Purkinje cells (F). Although the total number of GFP-positive cells was much smaller than that observed in the other two mice, it nevertheless suggests that pre-treatment with bFGF protein prevented AAV2 transduction of Purkinje cells. These results clearly show that bFGFR-R plays an important role in AAV transduction in cerebellar Purkinje cells.

In order to study the duration of transgene expression, mice were injected with vTR-UF11 into the deep cerebellar nuclei of the right hemisphere at the coordinates AP -3, ML $\pm$ 2.25, and DV -0.5 mm from lambda. One mouse was sacrificed 7 days post-injection, and quantitation of the transduction frequency indicated that up to 3% of cerebellar Purkinje

cells were transduced after a single vector injection (Fig. 3a). The other animal was sacrificed exactly one year later and the brain examined for transgene expression (Fig. 3b). Sections of mouse cerebellum showed that the expression level and transduction frequency at 1 year was indistinguishable from that observed in the animal evaluated 1 week after injection.

Heparan sulfate proteoglycan is known to mediate binding of a number of viruses including AAV2 [9]. $\alpha V\beta 5$ integrin and FGFR-1 have also been implicated as co-receptors for AAV2 binding and internalization [10, 11]. There is evidence that each of these molecules is expressed in Purkinje cells [15, 17, 18]. For example, Matsuda et al. [16] have shown that Purkinje cells and neurons in the deep cerebellar nucleus are immunoreactive for bFGF. There is also evidence suggesting that if AAV2 enters Purkinje cells via a bFGF receptor-mediated route, internal mechanisms exist that might lead to efficient transport of the vector to the Purkinje cell nucleus [16]. This might account for the efficient delivery and expression that we have observed using vTR-UF11 in the cerebellum [14].

The results from this study do not assess the quantitative differences between receptor binding and internalization, or interactions with other co-receptors, but they do establish qualitative interactions between AAV serotype 2 and bFGFR-1. Since the cohort of animals used in this experiment was limited, the results must be interpreted with caution. However, the significant observation here is that blockade of the bFGF receptor type1 with antibody, as well as by pretreatment with bFGF receptor ligand, resulted in inhibition of vector transduction in Purkinje cells. Entry of AAV2 vector into Purkinje cells in vivo is dependent upon bFGFR-1, which leads to effective gene transfer and extended expression in Purkinje cells with no apparent observed toxicity. These results provide a better understanding of the mechanism mediating AAV2 transduction in the cerebellum and thereby application of AAV2 as a vector system in treatment of cerebellar disorders.

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Fig. 1.

Schematic of AAV vector vTR-UF11. pA, polyadenylation signal; Neo, neomycin phosphotransferase coding sequence under transcriptional regulation of the thymidine kinase (TK) promoter, GFP coding sequence under transcriptional regulation of the hybrid CMV enhancer and β -actin promoter. *Arrows* indicate transcriptional start sites. TR, *black boxes*

Fig. 2.

Role of the bFGF receptor in AAV transduction of cerebellar Purkinje cells. *Arrows* indicate the presence or absence of GFP-positive cells at the site of injection. Animals were injected with AAV vector vTR-UF11 after preinjection with: **a** Anti-bFGFR antibody; **b** Anti-bFGFR antibody plus bFGFR blocking peptide; **c** Anti-bFGFR antibody; **d** Anti-tyrosine hydroxylase antibody; **e** bFGF protein; **f** PBS treatment

Fig. 3.

Persistence of gene expression in mouse Purkinje cells 1 year post-injection. Sagittal sections of mouse cerebellum in animals injected with 2.7×10^8 functional units of vTRUF11. **a** GFP expression in Purkinje cells 1 week post-injection, 4 $\times$ magnification, *scale bar* 300 microns. **b** Transduction in Purkinje cells 1 year after injection, 10 $\times$ magnification