In vivo gene therapy of the equine distal extremity with recombinant adeno-associated viral vectors

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Equine laminitis is a crippling disease that affects all breeds of horses and results in intense suffering for those afflicted with its most serious forms. Severe laminitis is commonly a career-ending disease and is often life-threatening. Connections between laminae in the equine distal extremity facilitate the transfer of weight from the distal phalanx to the hoof wall. Laminitis is a failure of the connection between epidermal and dermal laminae. Treatment of laminitis continues to be a controversial topic in equine veterinary medicine because there is a lack of universally efficacious treatment modalities.

Failure of the weight-transferring lamellar tissues occurs within the confines of the keratinized hoof-wall. This 'enclosed' location makes non-destructive access to these tissues very difficult, but this location also makes laminitis a particularly good candidate for a gene therapy approach. To treat laminitis with gene therapy, we need to use a virus to
insert a therapeutic gene into the cells of the lamellar tissues without disrupting the native architecture of the foot. These transfected cells will then produce a therapeutic protein within the foot to block the degradation of the epidermal-dermal connection.

Recombinant adeno-associated viral vectors (rAAVs) are very proficient at infecting cells and tissues and possess specific cell and tissue tropisms: the ability to transduce different types of cells or tissues with a range of efficiencies [1, 2]. Attachment to and entry into susceptible cells is mediated by the viral serotype, the protein envelope on the surface of the virus. Several previously identified tropisms include: AAV1 (retina, pancreas), AAV2 (liver, kidney), AAV5 (lung, retina), AAV6 (heart, lung), AAV7 (retina), AAV8 (liver, retina, pancreas, heart) and AAV9 (liver, heart, brain, lungs, pancreas, kidney). Adeno-associated viruses are safe to use, are currently being used in human clinical trials and are predominantly a non-integrating vector; they rarely insert viral DNA into the chromosomal DNA of the host. Because of this, rAAVs are associated with a greatly reduced risk of causing insertional mutagenesis or disrupting the patient's DNA. These viruses are known to be less immunogenic than commonly used adenoviral vectors and can provide long-term gene expression in a variety of target tissues. Our initial motivation for this project was to avoid contralateral-limb laminitis in the orthopedic patient with major musculoskeletal injuries by developing a gene therapy approach for the prevention of laminitis in the contralateral foot of these patients. In addition, laminitis initiated by other physical or metabolic causes may benefit as well from this relatively non-invasive gene therapy treatment.

We recently demonstrated successful in vivo transduction of the equine distal extremity with a rAAV vector [3]. In most rAAV gene therapy experiments, select virus serotypes more efficiently transduce a specific tissue better than other serotypes, and this was the case in tissues of the equine distal extremity. Differences in transduction efficiency of tissues of the equine distal extremity between serotypes were minor among the serotypes used in vivo. This observation opens the possibility of successfully transducing of the equine distal extremity in vivo with at least three different rAAV serotypes, using three replicate or potentially different treatments.

An important consideration to keep in mind when planning to use experimental or therapeutic gene therapy is the potential existence of neutralizing antibodies to the injected virus. Many animals have pre-existing neutralizing antibodies to multiple AAV serotypes, which can prevent the effective use of that specific serotype for gene therapy treatment in a specific patient [4]. We found that 100% of the horses that we tested were positive for neutralizing antibody titers to AAV serotype 2/5 (one of the most commonly-used and most successful AAV serotypes used), which effectively prevented transduction with this serotype. This strongly suggests that all potential gene therapy candidates should be tested for neutralizing antibodies to the viral serotype planned for use.

Delivery of rAAV was necessarily through the extensive vascular plexus of the equine foot, and thus offered an advantage to the vector serotype best able to cross blood vessel barriers, specifically serotype 2/8. Regionally, transduction of the dorsal hoof wall region of the foot was low in all experiments, with the only exceptions seen at the very highest-dose experiments or when an increased amount of surfactant was included in the injection diluent (Figure 1).
Increasing surfactant levels in the injection fluid significantly increased the levels of gene expression as well as increasing the distribution of vector transduction within the foot. This capability makes the use of rAAV within the equine foot more economically and immunologically favorable as a treatment option for laminitis. A lower dose of virus can be significantly less expensive and is less likely to induce a significant immune response by the gene therapy patient to the injected vector. Surfactant is normally used during virus production to prevent the attachment and loss of virus during handling. The increased tissue distribution of viral transduction seen in these experiments is greater than what would be expected by minimizing virus loss alone and likely represents increased access to cells/tissues provided by an increased surfactant level.

As is the case with many tissues, equine lamellar tissue is constantly being regenerated and remodeled. In the equine distal extremity this 'new' tissue migrates distally to replace older tissues, similar to human finger nails. This study established the migration of β-galactosidase protein from the coronary band down the dorsal hoof wall and from the distal tip of the juncture of the sole and hoof wall toward the tip of the third phalanx (Figure 2). This observation provides strong support for the future therapeutic potential of gene therapy in the equine distal extremity.

In summary, our data demonstrate that transgenes can be successfully delivered to the equine distal extremity using rAAV vectors and that serotypes 2/8, 2/9 and 2/1 can successfully transduce tissues of the equine distal extremity. When these vectors were diluted with an increased concentration of surfactant in the saline diluent, the level of transduction increased dramatically. The increased level of transduction due to the addition of surfactant was coupled to an improved transduction distribution pattern. To move toward our long-term goal of providing a practical clinical therapy for the treatment/prevention of laminitis, a next logical step is to perfect rAAV vector injection in a standing horse with a procedure more similar to a standard regional limb perfusion and incorporate a therapeutic rAAV-delivered transgene. Our laboratory has cloned the equine tissue inhibitor of metalloproteinase-3 gene. The product of this gene, Timp-3, is known to specifically block several of the most destructive enzymes that destroy the distal phalanx-hoof wall connection [5]. While Timp-3 is a logical choice to address the devastating degradation associated with laminitis, it is possible that Timp-3 may not be the ultimate gene therapy ‘target’. The extensive work being done now by other researchers exploring the etiology of laminitis may identify additional therapeutic genes in the future, and this study should provide a strong basis for further investigations using gene therapy in the treatment of equine laminitis.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

J.B.M and D.W.R. conceived of the study, participated in the design of the study and carried out the experiments. J.B.M performed the statistical analysis, analyzed the results and drafted the manuscript. J.B.M and D.W.R. read and approved the final manuscript.

Abbreviations

rAAV: recombinant adeno-associated viral vector.

References


