Gene Therapy for Osteoarthritis: Pharmacokinetics of Intra-Articular Self-Complementary Adeno-Associated Virus Interleukin-1 Receptor Antagonist Delivery in an Equine Model

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Toward the treatment of osteoarthritis (OA), the authors have been investigating self-complementary adeno-associated virus (scAAV) for intra-articular delivery of therapeutic gene products. As OA frequently affects weight-bearing joints, pharmacokinetic studies of scAAV gene delivery were performed in the joints of the equine forelimb to identify parameters relevant to clinical translation in humans. Using interleukin-1 receptor antagonist (IL-1Ra) as a secreted therapeutic reporter, scAAV vector plasmids containing codon-optimized cDNA for equine *IL-1Ra* (*eqIL-1Ra*) were generated, which produced eqIL-1Ra at levels 30- to 50-fold higher than the native sequence. The most efficient cDNA was packaged in AAV2.5 capsid, and following characterization *in vitro*, the virus was injected into the carpal and metacarpophalangeal joints of horses over a 100-fold dose range. A putative ceiling dose of 5×10^{12} viral genomes was identified that elevated the steady-state eqIL-1Ra in the synovial fluids of injected joints by >40-fold over endogenous levels and was sustained for at least 6 months. No adverse effects were seen, and eqIL-1Ra in serum and urine remained at background levels throughout. Using the 5×10^{12} viral genome dose of scAAV, and green fluorescent protein as a cytologic marker, the local and systemic distribution of vector and transduced cells following intra-articular injection scAAV.GFP were compared in healthy equine joints and in those with late-stage, naturally occurring OA. In both cases, 99.7% of the vector remained within the injected joint. Strikingly, the pathologies characteristic of OA (synovitis, osteophyte formation, and cartilage erosion) were associated with a substantial increase in transgenic expression relative to tissues in healthy joints. This was most notable in regions of articular cartilage with visible damage, where foci of brilliantly fluorescent chondrocytes were observed. Overall, these data suggest that AAV-mediated gene transfer can provide relatively safe, sustained protein drug delivery to joints of human proportions.

Keywords: pharmacokinetics, osteoarthritis, gene therapy, arthritis, AAV, IL-1Ra

INTRODUCTION

OSTEOARTHRITIS (OA) IS A chronic, erosive, often debilitating condition common to large weight-bearing joints. An estimated 30–40 million people in the United States have symptomatic OA. Yet,

despite its prevalence, there are no pharmacologic agents capable of halting the underlying disease processes.¹ Although analgesics can alleviate joint pain, they cannot block the degenerative progression of OA, and many patients progress to end-stage

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disease, with joint replacement surgery as the only medical option.

Over the last decade, the understanding of OA pathogenesis at the cellular and molecular levels has advanced considerably,² and several naturally occurring proteins with potential as disease-modifying agents have been identified. Among the more promising candidates is interleukin-1 receptor antagonist (IL-1Ra), which functions as a competitive inhibitor of interleukin-1 (IL-1),³ a primary inflammatory cytokine and mediator of cartilage degradation.^{4,5} Unfortunately, the administration of the recombinant forms of proteins, such as IL-1Ra (Anakinra), has not met with clinical success in OA due to difficulties achieving and sustaining effective levels in joints with chronic disease.⁶

Gene-based treatment approaches may provide an effective alternative.⁷⁻⁹ By transferring the cDNAs encoding anti-arthritis proteins to cells resident in the tissues of diseased joints, and providing for high levels of independent expression, the biosynthetic machinery of the modified cells can be directed to overproduce and secrete the transgenic protein into the synovial fluid and surrounding tissue continuously.¹⁰ This provides the greatest concentration of therapeutic protein specifically at the site of disease, and eliminates the need for repeated application.⁹

Many vector systems have been evaluated for intra-articular gene transfer, but those based on adeno-associated virus (AAV) currently appear the most viable for clinical studies in OA. The benefits of this system include the low immunogenicity of transduced cell populations, the capacity for sustained transgene expression in the joints of laboratory animals,^{11,12} and a track record of safety in clinical trials for other diseases.¹³⁻¹⁵ Advances in AAV technology, including self-complementary (sc) vectors^{16,17} and improved methods of vector production, further enhance its potential for mainstream clinical use in joint disease.

Building from positive results with local scAAV.IL-1Ra delivery in rabbits with experimental arthritis,¹⁸ the authors have worked to investigate AAV gene transfer on a scale relevant to application in large human joints. In this regard, the horse offers several advantages as a model system. The intercarpal and metacarpophalangeal (MCP) joints of the equine forelimb are similar in size and function to the human knee, and as the forelimbs carry the majority of the horse's weight during locomotion, these joints are also highly vulnerable to OA secondary to trauma and excessive loading.¹⁹ Moreover, the diagnostic technologies used to assess joint

pathology in OA are the same for both humans and horses.

In pilot studies using green fluorescent protein (GFP) and human (h)IL-1Ra as reporter genes, several AAV capsid serotypes efficiently transduced fibroblasts in the synovial lining of the equine joint, and to a lesser extent chondrocytes in articular cartilage, were found.²⁰ Delivery of the human cDNA generated significant levels of hIL-1Ra in the equine synovial fluids, but following a peak at 1-2 weeks post injection, hIL-1Ra expression diminished and, after 7 weeks, was essentially undetectable by enzyme-linked immunosorbent assay (ELISA). This was attributed to immune recognition of the transduced cells expressing the xenogeneic (human) protein.²⁰

Using the equine model, this study worked to generate a detailed pharmacokinetic profile of homologous gene delivery of scAAV.IL-1Ra gene delivery in a large mammalian joint. Issues directly relevant to clinical translation, such as dosing, the levels and persistence of IL-1Ra expression, and the effect of the OA environment on intra-articular gene transfer and vector biodistribution, are addressed.

RESULTS AND DISCUSSION

Objectives and study design

Using a codon-optimized cDNA for the equine (*eq*) *IL-1Ra* orthologue as a homologous transgene and scAAV as a delivery vehicle, over a 100-fold range from 5×10^{10} to 5×10^{12} vector genomes (vg), first a series of dosing studies targeting the intercarpal and MCP joints of healthy horses were performed. eqIL-1Ra levels in synovial fluid, blood, and urine were measured periodically over a period of 6 months and compared to pre-injection levels. Then, to determine the impact of the pathologies characteristic of OA joints on intra-articular gene transfer, using scAAV.GFP as a cytologic marker, the local and systemic distribution of the vector and transduced cell populations following vector delivery into healthy joints and those with late-stage, naturally occurring OA were examined.

Summary of data

scAAV.eqIL-1Ra vector construction and characterization in vitro and in vivo. In a study performed >15 years ago, measurable levels of transgenic eqIL-1Ra production in equine joints were reported following intra-articular injection of a first-generation recombinant adenoviral vector.²¹ More recently, as validated equine-specific ELISA reagents became available, protein expression from the native cDNA

(NCBI: NM_001082525.2) was found to be somewhat modest. Thus, to minimize immune recognition of the IL-1Ra transgene product²² and maximize expression, the *eqIL-1Ra* cDNA codon was optimized²³ and synthesized both with and without a consensus Kozak sequence²⁴ leader immediately upstream of the translation start site. Following insertion of the cDNAs into the scAAV vector plasmid, pHpa-trs-SK,^{16,17} in transient transfection assays, both optimized constructs produced eqIL-1Ra at levels 30- to 50-fold higher than the native sequence (Fig. 1a). As the construct with the Kozak sequence consistently provided the highest eqIL-1Ra expression in these assays, it was selected for viral packaging and testing *in vivo*.

The previous data showed human synovial fibroblasts in culture to have a preference for infection with AAV2.²⁰ Considering possible translation to human testing, the eqIL-1Ra vector construct was packaged in the AAV2.5 capsid,²⁵ which maintains AAV2 tropism but shows reduced reactivity with AAV2 neutralizing antibody, prevalent among the human population.²⁶ Infection of equine synovial fibroblast cultures with a range of doses of the AAV2.5 vector resulted in exceptionally high expression of eqIL-1Ra, which exceeded 10 μ g/mL at 10⁵ DNase resistant viral genomes (vg)/cell (Fig. 1b). eqIL-1Ra production did not exceed background in parallel control cultures infected at 10⁵ vg/cell with an scAAV2.5 vector containing GFP (scAAV.GFP). The function of the transgenic eqIL-1Ra protein was confirmed in bioassays, where around a 90% reduction in prostaglandin E₂ (PGE₂) levels was seen in media conditioned by

equine synovial cells previously infected with AAV.eqIL-1Ra at 10⁵ vg/cell and subsequently challenged with increasing amounts of recombinant human IL-1 (Fig. 1c).

To determine the effect of vector dose on eqIL-1Ra expression following intra-articular delivery, an approach was designed to provide insight into the intra- and inter-animal variability while using a minimum number of experimental animals. In each of six horses, injections of scAAV.eqIL-1Ra were distributed in random order among both intercarpal and MCP joints of both forelimbs at three different doses: 5 $\times$ 10¹⁰, 5 $\times$ 10¹¹, and 5 $\times$ 10¹² vg; Fig. 2a). The remaining joint was injected with an equivalent volume of the saline delivery vehicle (Lactated Ringer's solution) and served as a negative control. Immediately prior to vector injection, peripheral blood was drawn, urine was collected, and synovial fluid was aspirated from each of the four joints. Periodically thereafter, over a predetermined interval of 6 months, synovial fluid, blood, and urine were similarly collected from each animal. The eqIL-1Ra content in the recovered fluids was measured using commercially available ELISA, and was assessed relative to pre-injection values.

Despite receiving AAV in three forelimb joints, no adverse effects were observed acutely or at any point during the protocol, and eqIL-1Ra levels in both serum and urine remained at baseline levels. Among the control joints injected with the fluid vehicle, synovial fluid eqIL-1Ra also remained at pre-injection levels (<1 ng/mL) throughout (Fig. 2b). In joints receiving the AAV vector, dose-related

Figure 1. Expression and function of the codon-optimized *eqIL-1Ra* transgene *in vitro*. **(a)** eqIL-1Ra in conditioned media at 48 h following transfection of equine synovial fibroblasts with the Hpa-trs-sk, scAAV vector plasmid containing the coding sequences for *GFP*, native *eqIL-1Ra*, codon-optimized *eqIL-1Ra* (*Opt*), and codon-optimized *eqIL-1Ra* with a Kozak sequence leader (*K + Opt*). $n=3$ transfections. **(b)** eqIL-1Ra in conditioned media following infection of equine synovial fibroblasts with increasing doses of scAAV.eqIL-1Ra packaged in serotype 2.5. Viral genomes (vg) per cell of scAAV.eqIL-1Ra are shown to the right of the respective expression curves. Parallel infection with 10⁵ vg/cell of scAAV.GFP was used as a negative control. $n=3$ infections. **(c)** PGE₂ production following IL-1 stimulation of cultured equine synovial fibroblasts that were uninfected (no virus), or transduced with 10⁵ vg/cell of scAAV.GFP or scAAV.eqIL-1Ra 48 h previously. Following the addition of IL-1 to the growth media to final concentrations of 0, 1, 5, or 10 ng/mL, the conditioned media were collected and assayed for PGE₂ by ELISA. Each bar represents the mean of $n=3$. Error bars represent $\pm$ SEM. eqIL-1Ra, equine interleukin-1 receptor antagonist; scAAV, self-complementary adeno-associated virus; GFP, green fluorescent protein; PGE₂, prostaglandin E₂; IL-1, interleukin-1; ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

Figure 2. scAAV administration and expression in the equine joint. **(a)** *Right:* Anatomic locations of the carpal (Ca) and metacarpophalangeal (MCP) joints of the equine forelimbs (*black arrows*). *Left:* Radiographic image of the equine carpus, showing the locations of the intercarpal (*white arrow*), carpometacarpal (*circle*), and antebrachioacarpal (*asterisk*) joints. The synovial cavities of the intercarpal and carpometacarpal joints communicate. The locations of the radiocarpal (RC) and third carpal (C3) bones discussed in Fig. 3a and b are shown as indicated. **(b)** eqIL-1Ra in synovial fluids of forelimb joints injected with either saline vehicle (Lactated Ringer's solution) or vehicle containing scAAV.eqIL-1Ra at vg doses shown to the right of the respective plots ($n=6$ joints). Error bars represent $\pm$ SEM. The mean IL-1Ra levels at each dose and time point were analyzed using independent sample t -tests. **(a)** IL-1Ra expression at 5×10^{12} and 5×10^{11} vg doses is greater than 5×10^{10} vg dose; $p < 0.05$. **(b)** IL-1Ra expression at 5×10^{12} vg dose is greater than 5×10^{11} vg dose; $p < 0.05$.

increases in synovial fluid eqIL-1Ra were observed within 2 weeks of injection, with mean levels ranging from around 6 ng/mL at 5×10^{10} vg to around 40 ng/mL at 5×10^{12} vg. Peak eqIL-1Ra production occurred between 4 and 8 weeks post injection, and these levels were maintained for the remainder of the 6-month study. In contrast to the near-linear relationship between vector dose and eqIL-1Ra expression seen in culture (Fig. 1b), over the dose range tested *in vivo*, a 100-fold increase in vector was required to produce around a sevenfold increase in synovial fluid eqIL-1Ra. Moreover, at the high end of the dose range, the 10-fold increase in vector dose from 5×10^{11} to 5×10^{12} vg, only increased the mean eqIL-1Ra level by around 50%, which was not statistically significant (Fig. 2b). These data suggest a possible ceiling effect²⁷ such that additional increases in vector dose would be unlikely to generate a meaningful increase in synovial fluid eqIL-1Ra, at least within the context of a healthy joint.

AAV2.5 transgene expression in healthy versus OA joints. As the 5×10^{12} vg dose of scAAV.eqIL-1Ra consistently provided the highest eqIL-1Ra production intra-articularly, seemed reasonably safe, and appeared to represent a maximum functional dose, it was selected for further characterization *in vivo*. To gain insight into the safety of intra-articular AAV gene delivery and thus its potential for clinical application in OA, the scAAV.GFP vector was first packaged in the AAV2.5 capsid. Then 5×10^{12} vg of scAAV.GFP was injected into one intercarpal joint of three healthy horses and three horses with advanced naturally occurring

OA (Fig. 3a and b). Two weeks post injection, the horses were euthanized, and tissues from the injected joints and nine additional sites throughout the body (*e.g.*, the brain, heart, liver, and spleen) were collected for analysis. GFP fluorescence was used as a cytologic marker to determine the influence of the characteristic OA pathologies (Fig. 3b) on the local and systemic distribution of the transduced cell populations relative to healthy joints. Quantitative polymerase chain reaction (qPCR) was used to quantify the scAAV genome copies in the various tissues to determine the vector biodistribution.

Similar to results with AAV serotypes tested previously,²⁰ in each of the healthy joints, the predominant site of GFP expression was the synovium (Fig. 3c–e). Examination of the freshly harvested tissues revealed abundant fluorescent cells throughout the lining of the joint capsules of the intercarpal and the carpometacarpal joints, which was expected, as the synovial cavities of these joints communicate (see Fig. 2a). In both joints, the majority of the fluorescently labeled cells appeared to be concentrated in the thicker villous regions (Fig. 3c). When viewed in cross-section under increased magnification, the modified cells were comprised primarily of non-reactive fibroblasts residing in a thin synovial membrane (Fig. 3d and e). GFP activity was also visible in articular cartilage shavings from both joints, but was generally faint and limited to scattered isolated cells typical of that seen in Fig. 4a and b. In striking contrast, GFP activity in the synovium of the OA joints was much higher than that seen in healthy joints. These samples were often brilliantly

Figure 3. GFP expression in equine synovium from healthy and osteoarthritis (OA) joints following intra-articular gene delivery with scAAV. One intercarpal joint of three healthy horses and three with advanced, naturally occurring OA was injected with 5×10^{12} vg of scAAV.GFP. Two weeks later, the joint tissues were collected and analyzed for fluorescence. **(a and b)** Opposing articulating surfaces from representative healthy **(a)** and OA **(b)** intercarpal joints are shown in the same orientation. The locations of the radiocarpal (RC) and third carpal (C3) bones are as indicated in **(a)**. Full-thickness cartilage erosions on the surfaces of both the RC and C3 bones are indicated by *black arrows*; osteophytes along the margins of the C3 bone are indicated with *asterisks*. Bone and cartilage damage from an osteochondral fracture is indicated by the *red arrow*. **(c and d)** scAAV.GFP expression in the synovium of a healthy joint. **(e)** Hematoxylin and eosin (H&E) stain of normal synovial membrane shows the surface (intimal) layer composed of single row of flat and nonreactive cells overlaying vascularized fibro-adipose tissue. **(f and g)** GFP expression across the synovial surface of an OA joint. **(c and f)** Direct fluorescence in freshly harvested tissues at $10\times$. **(d and g)** Paraffin sections immunohistochemically stained for GFP at $20\times$. **(h)** H&E stain of synovial membrane from an OA joint shows an irregular intimal lining and markedly increased cellularity due to hypertrophy and hyperplasia of the fibroblasts and a chronic inflammatory infiltrate (primarily lymphocytes). **(i)** Direct fluorescence microscopy of synovium from a joint that was not injected with scAAV.GFP and serves as a control for autofluorescence. **(j)** Control synovium following immunohistochemical stain for GFP expression reflects the specificity of the antibody and the absence of background staining. **(k)** Representative GFP expression (*arrows*) in fresh synovial tissue harvested from the antebrachicarpal joint of the carpus following injection of scAAV.GFP into the intercarpal joint. These compartments do not communicate within the carpal joint. (The images shown here and in Fig. 4 are composites from the six animals in both groups. As the fresh-tissue samples were of variable thickness and composition, a wide range of exposure times was used to capture the varying fluorescence intensities at the different magnifications indicated. The contrast and brightness of individual panels were adjusted linearly for uniformity of appearance and to reflect fluorescence intensities viewed by direct microscopy.)

fluorescent, even at low magnification (Fig. 3f). While the density of the fluorescent cells was visibly higher across the entire expanse of the lining of the OA joints, it was particularly so in regions with marked fibroblast hyperplasia and leukocytic infiltration typical of chronic inflammation (Fig. 3g and h). Similar to healthy joints, the fluorescent cells were almost exclusively delimited to the synovium and subsynovium, and only rarely seen in the supporting fibrous tissues.

Relative to the articular tissues in the healthy joints, the OA cartilage showed the most dramatic increase in GFP activity, as populations of brightly fluorescent cells were readily apparent in

all shavings recovered (Fig. 4d–f). The labeled chondrocytes included both elongated cells, consistent with superficial layer chondrocytes (Fig. 4e), and cells with more spherical morphology characteristic of chondrocytes in deeper layers (Fig. 4f). Shavings harvested near full-thickness erosions often contained focal regions with intense GFP fluorescence readily visible at low magnification (Fig. 4g and h). These areas frequently contained cells with spindle-shaped morphology, consistent with dedifferentiation to fibrochondrocytes (Fig. 4h and i).²⁸ GFP expression was particularly prominent in chondrocyte clusters characteristic of OA cartilage (Fig. 4j–l). Pockets of brightly fluorescent

Figure 4. GFP expression in equine cartilage from healthy and OA joints following intra-articular gene delivery with scAAV. The intercarpal joints of three healthy horses and three with late-stage, naturally occurring OA were injected with 5×10^{12} vg of scAAV.GFP. Two weeks later, the joint tissues were collected and analyzed for fluorescence. (a and b) GFP expression in fresh cartilage shavings immediately following harvest from a healthy joint. Arrows indicate representative fluorescent cells. (c) GFP activity in healthy cartilage shaving after 48 h in explant culture. (d–f) GFP activity in fresh cartilage shavings from an OA joint. (e and f) Magnified fields from (d) (d: 10 $\times$; e and f: 20 $\times$). (g and h) GFP activity in fresh OA cartilage from regions with visible erosion (g: 10 $\times$; h: 20 $\times$). (i) GFP+ chondrocytes changing morphology in degrading cartilage matrix (paraffin section; 40 $\times$). (j) GFP expression in chondrocyte clusters in fresh cartilage shavings OA joint (40 $\times$). (k) GFP expression in cartilage clusters from an OA joint (paraffin section). (l) H&E-stained section of OA cartilage similar to (k). (m) Cartilage section from a naïve joint immunostained for GFP (negative control for positive staining in i and k). (n and o) GFP activity in freshly harvested osteophyte (n: 10 $\times$; o: 20 $\times$).

cells were also visible across the surfaces of osteophytes (Fig. 4n and o) recovered from the margins of the OA joints (Fig. 3b).

For both healthy and OA joints receiving virus, the synovium of the antebrachiocarpal joint of the

carpus was the only tissue outside the injected joint noted to contain visible GFP fluorescence (Fig. 3k). Despite its location immediately proximal to the site of injection (Fig. 2a, *), only sparse fluorescent cells were seen and in only a few of the harvested samples.

Table 1. Distribution of AAV.GFP genomes following injection in the intercarpal joint of healthy horses and those with naturally occurring osteoarthritis

Tissue	Healthy			OA		
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
Synovium intercarpal joint	178,313 ± 559	66,022 ± 167	16,460 ± 114	212,262 ± 237	15,590 ± 280	142,033 ± 720
Cartilage intercarpal joint	3,349 ± 43	989 ± 109	1,726 ± 59	2,499 ± 69	887 ± 19	6,177 ± 55
Antebrachicarpal synovium	159 ± 86	20 ± 3	ND	ND	ND	84 ± 16
Peri-articular muscle	ND	ND	ND	ND	ND	ND
Contralateral intercarpal synovium	ND	ND	ND	ND	ND	ND
Contralateral quadriceps	ND	ND	ND	ND	ND	ND
Ipsilateral MCP	ND	ND	ND	ND	ND	ND
Brain	ND	ND	ND	ND	ND	ND
Heart	ND	ND	ND	ND	ND	ND
Liver	ND	ND	45 ± 4.8	380 ± 15	ND	ND
Lung	ND	ND	ND	ND	ND	ND
Spleen	ND	ND	ND	277 ± 64	ND	ND

Values represent vector genome copies per microgram of genomic DNA and are means of at least three replicates ± standard error of the mean. Tissues and locations are in reference to the injected joint.

MCP, metacarpophalangeal; ND, not detected.

In a related observation, it was found that the GFP expression in shavings of healthy cartilage (barely detectable at harvest; Fig. 4a and b) increased dramatically after 48 h of incubation in explant culture, such that dense populations of vividly fluorescent cells appeared throughout the matrix in each sample (Fig. 4c). This suggested that a large percentage of the chondrocytes had actually been transduced by the virus *in situ*, but failed to express the fluorescent reporter protein above the visible threshold in the context of the healthy joint.

AAV2.5 biodistribution. To assess the emigration of the AAV vector from the joint following intra-articular injection, total DNA was isolated from the articular and extra-articular tissue samples harvested from the animals injected with scAAV.GFP and assayed for vector genome content by qPCR. As shown in Table 1, the systemic distribution of vector genomes was largely consistent with visible GFP activity. For all animals, the tissues from the joints receiving virus showed the highest vector genome content. Though there was wide variation among individuals, on average, the vector DNA measured in the synovium was around 30- to 50-fold higher than in cartilage, with no significant differences between OA and normal joints. Detectable but considerably fewer vector genomes were found in the synovium of the adjacent antebrachicarpal joint in horses from both groups. Outside the carpus, one animal from the healthy group showed a low number of vector genomes in the liver, while the liver and spleen from one animal in the OA group were also positive for vector content. Altogether, under both healthy and

diseased conditions, >99.7% of the AAV vector genomes detected were in the joint tissues. These results indicate that the AAV vector primarily remains within the injected joint, and while the OA environment appears to substantially enhance transgene expression intra-articularly, it does not appear to impact extra-articular vector dispersion meaningfully.

CONCLUSIONS

These studies show that in a large mammalian joint, scAAV-mediated delivery of a homologous *IL-1Ra* cDNA is capable of elevating the steady state levels of IL-1Ra protein in the synovial fluid by >40-fold over the endogenous background. Distinct from the relatively brief expression seen previously following delivery of the xenogeneic human *IL-1Ra* cDNA in the equine joint,²⁰ in the absence of immune recognition and elimination, the articular cells transduced by the vector can sustain high level IL-1Ra synthesis for at least 6 months.

Favorable to the development of a gene-based therapy, the pathologic changes in tissue morphology and cellularity typical of the OA joint appear to increase viral transduction and transgenic expression substantially. Nonetheless, in both healthy and diseased environments, the vast majority of the vector DNA is retained in the injected joints. As the vector-mediated overproduction in the forelimb joints did not increase the circulating IL-1Ra levels in peripheral blood, it appears that AAV.*IL-1Ra* gene delivery in large-mammal joints provides little risk of immunosuppression from systemic IL-1 blockade, at least with the vector doses used here.

The expression data in healthy equine joints are largely consistent with those reported in an exploratory study by Goodrich *et al.*²⁹ In both cases, codon optimization of the native *eqIL-1Ra* sequence enhanced secretion of the encoded protein by at least 20-fold. In the Goodrich study, despite administering a similar vector dose, the transgenic IL-1Ra levels in the synovial fluid were around two- to threefold higher than observed in the current study. The basis for this is uncertain, but it may reflect the small sample size in the earlier study or possibly variations in methods for fluid collection and IL-1Ra quantification. Regardless of the discrepancies in absolute level, the overall patterns of expression are in good agreement, and independently serve to document the capacity of the AAV vector to enable sustained therapeutic transgene expression in a large synovial joint.

Local AAV-mediated gene transfer was explored previously in human joints for the treatment of rheumatoid arthritis³⁰ and entered Phase II study.³¹ Unfortunately, measurement of the transgenic protein (a tumor necrosis factor alpha antagonist) produced in the joints was not part of the protocol, and no information emerged from the study regarding the level and duration of expression that was achieved in human joints. This information, though, seems crucial to effective clinical application. To this end, the use of the equine system has proven particularly informative, allowing the capacity of an AAV vector for therapeutic gene delivery to be tested on a scale equivalent to the human knee. The ability to aspirate synovial fluid serially from the equine joint enables direct measurement of secreted transgene products from the same animal over time and the generation of reliable pharmacokinetic profiles. A particularly beneficial aspect of the horse as a model system is the ability to study gene delivery in the context of naturally occurring joint disease, rather than an experimental model.

The studies of vector biodistribution show similar results for both healthy and OA joints. The cells within the synovial lining are the primary targets for AAV gene transfer, and on average are infected around 40-fold more efficiently than chondrocytes in articular cartilage and thus are primarily responsible for the transgenic IL-1Ra content in the synovial fluid. In equine synovium, on average around 100,000 AAV vector genomes per microgram of cellular genomic DNA (gDNA), or around one vector genome per cell, was measured. This efficiency of infection is similar to the results in rats following injection in the stifle joints of weight-adjusted doses of 10^9 and 10^{10} vg of scAAV.hIL-1Ra, also packaged

in the AAV2.5 capsid.³² Recently, Bevaart *et al.*³³ described a safety study of AAV5-mediated human IFN β gene delivery in the joints of nonhuman primates with collagen-induced arthritis. In this case, infection levels in synovium >200 vg/cell were reported, but at vector doses >150 times that used in equine joints. Considering the data in rats and horses with the sc-vector and AAV2.5 capsid, the greater tissue and fluid volume of the equine joint does not appear to affect gene delivery to synovium adversely, such that dosing and infection efficiency scale upward proportionally.

Interestingly, the IL-1Ra expression studies point to a vector ceiling dose at around 5×10^{12} vg, and suggest that vector doses even 10-fold or more higher are unlikely to provide a significant increase in steady-state IL-1Ra levels in synovial fluid. This effect is likely a reflection of the rapid turnover of the synovial fluid and the continuous efflux of the fluid and solutes into the extra-synovial interstitium and lymphatics.^{34,35} In contrast to the static conditions in culture where secreted transgene products, such as IL-1Ra, can steadily accumulate in conditioned medium, small soluble molecules have a brief residence time in synovial fluid. Thus, the increase in synovial fluid IL-1Ra content following gene delivery represent homeostatic levels in the face of ongoing dilution. Since the 5×10^{12} vg dose was able to maintain a >40 -fold increase in synovial fluid IL-1Ra content for several months, this apparent ceiling effect is unlikely to impede the efficacy of therapeutic gene delivery.

Considering therapeutic application, markedly higher expression of the GFP reporter was seen in association with the characteristic cellular and morphologic changes in the joint found in advanced OA.³⁶ For example, in the synovium, chronic inflammatory stimulation frequently induces synovial hyperplasia, angiogenesis, leukocytic infiltration, and fibrotic thickening.^{37,38} While AAV-mediated GFP expression appeared consistently higher throughout the synovium of OA joints, fluorescence was especially pronounced in regions of infiltration and hyperplasia.

The most notable increase in GFP expression occurred in the OA cartilage. In stark contrast with cartilage from healthy joints where fluorescent cells were sparse and barely visible, shavings harvested from OA joints contained abundant brightly fluorescent cells throughout, with the most striking GFP activity focused at sites with obvious matrix erosion. While it would seem that physical damage to the matrix would facilitate vector diffusion into the OA cartilage, no difference in vector genome content was seen in the

chondrocytes from OA and healthy joints. Instead, the data suggest that the increased transgene expression in the cartilage (and synovium as well) arises from heightened inflammatory and stress-induced activation in OA tissues.³⁹

Chondrocytes residing in healthy articular cartilage exist in a non-distressed, resting state. In OA, however, damage to the cartilage matrix diminishes its protective properties and exposes the regional chondrocyte populations to excessive mechanical loading.⁴⁰ These abnormal, pathologic forces induce stress responses in the chondrocytes, which drive the metabolism of the normally quiescent cells into a highly activated state.^{41–45} The chondrocytes become proliferative, secrete high levels of inflammatory cytokines, and release proteolytic enzymes that further degrade the surrounding matrix.^{44–46}

AAV transduction efficiency is known to be enhanced (in some cases by several log orders) by molecular mechanisms associated with intracellular stress.^{47–51} Stimuli that cause subcellular stress frequently disrupt the ubiquitin–proteasome pathway and thereby inhibit targeted degradation of AAV vector particles that accumulate in the peri-nuclear cytoplasm of infected cells. Heightened vector stability in concert with increased nucleolar import and capsid uncoating in stressed cell populations serve to amplify the number of vector genomes in the nucleus available for transgene expression.^{49,51–55}

In its native viral context, transcription from the cytomegalovirus (CMV) immediate early promoter is induced in response to NF- κ B activation and signal transduction from p38 and other stress-activated protein kinases. Stress-related induction reactivates human CMV from latency, and is required for expression of genes necessary for DNA replication.^{56–60} Inflammation and cellular stress can likewise increase transcription and expression of transgenes under control of the CMV immediate early promoter.^{58,61–64} In this regard, OA cartilage is highly enriched with stress-activated chondrocytes,⁴³ especially at sites of cartilage degradation (where AAV.GFP expression was greatest). GFP fluorescence was also prominent at sites of chondrocyte proliferation and cluster formation and in osteophytes, which arise from persistent activation of chondro-osseous progenitors at the transition from cartilage to synovium.⁶⁵ Potent induction of GFP expression was also seen in cartilage shavings from healthy joints following incubation in explant culture. As no additional vector was added to the shavings, this sudden burst in GFP activity had to arise from vector DNA already

present in the chondrocytes. Therefore, it must reflect a dramatic change in metabolism in infected chondrocytes from the stress of cartilage harvest and/or change in growth conditions.

A number of reports describe the generation of synthetic inflammation-inducible promoter systems for gene therapy applications.^{66–68} The AAV vector discussed here, at least within the context of a large mammalian joint, appears to be highly responsive to the OA environment and innately disease activated. Moreover, the regional differences in GFP expression seen in OA cartilage indicate the potential to direct therapeutic transgene expression preferentially to cartilage regions under the greatest pathologic stress. This opens the door for development of derivative vectors for disease-targeted anabolic stimulation of cartilage repair and regeneration.

Altogether, the data from this study support consideration of scAAV.II-1Ra for human testing. These results validate the use of AAV gene transfer for sustained protein drug delivery in large mammalian joints, and the capacity of the transduced cells to sustain transgenic protein expression at high levels for several months. Fortuitously, transgene delivery and expression appear to be considerably more efficient in the disease environment of the OA joint. As no adverse effects of treatment were seen, and the vector and IL-1Ra production appear effectively contained within the treated joints, the procedure overall seems reasonably safe, with the potential to offer benefit in joints of human proportions. The Materials and Methods section can be found in the Supplementary Data (available online at www.liebertpub.com/humc).

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The optimized equine IL-1Ra cDNA can be obtained from the corresponding author through material transfer agreement with the University of Florida. The pXR.2.5, pXX6-80, and pHpa-tr-sk (ScAAV-CMV-GFP) plasmids for packaging self-complementary vectors in the AAV2.5 capsid are available through the National Gene Vector Bio-

repository and material transfer agreement with the University of North Carolina at Chapel Hill.

AUTHOR DISCLOSURE

C.H.E. and S.C.G. are inventors on several patents and patent applications describing cell- and gene-based therapies for arthritis and connective tissue disorders.

REFERENCES

- Zhang W, Ouyang H, Dass CR, et al. Current research on pharmacologic and regenerative therapies for osteoarthritis. *Bone Res* 2016;4:15040.
- Golding SR, Goldring MB. Clinical aspects, pathology and pathophysiology of osteoarthritis. *J Musculoskelet Neuronal Interact* 2006;6:376–378.
- Arend WP, Malyak M, Guthridge CJ, et al. Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 1998;16:27–55.
- Kapoor M, Martel-Pelletier J, Lajeunesse D, et al. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat Rev Rheumatol* 2011;7:33–42.
- Olson SA, Horne P, Furman B, et al. The role of cytokines in posttraumatic arthritis. *J Am Acad Orthop Surg* 2014;22:29–37.
- Chevalier X, Goupille P, Beaulieu AD, et al. Intraarticular injection of anakinra in osteoarthritis of the knee: a multicenter, randomized, double-blind, placebo-controlled study. *Arthritis Rheum* 2009;61:344–352.
- Ghivizzani SC, Gouze E, Gouze JN, et al. Perspectives on the use of gene therapy for chronic joint diseases. *Curr Gene Ther* 2008;8:273–286.
- Bellavia D, Veronesi F, Carina V, et al. Gene therapy for chondral and osteochondral regeneration: is the future now? *Cell Mol Life Sci* 2018; 75:649–667.
- Evans CH, Ghivizzani SC, Robbins PD. Gene delivery to joints by intra-articular injection. *Hum Gene Ther* 2018;29:2–14.
- Evans CH, Ghivizzani SC, Robbins PD. Gene therapy for arthritis: what next? *Arthritis Rheum* 2006; 54:1714–1729.
- Evans CH, Ghivizzani SC, Robbins PD. Arthritis gene therapy and its tortuous path into the clinic. *Transl Res* 2013;161:205–216.
- Madry H, Cucchiariini M. Advances and challenges in gene-based approaches for osteoarthritis. *J Gene Med* 2013;15:343–355.
- Smith BK, Collins SW, Conlon TJ, et al. Phase I/II trial of adeno-associated virus-mediated alpha-glucosidase gene therapy to the diaphragm for chronic respiratory failure in Pompe disease: initial safety and ventilatory outcomes. *Hum Gene Ther* 2013;24:630–640.
- Jacobson SG, Cideciyan AV, Ratnakaram R, et al. Gene therapy for Leber congenital amaurosis caused by *RPE65* mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch Ophthalmol* 2012;130:9–24.
- Flotte TR, Trapnell BC, Humphries M, et al. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing alpha1-antitrypsin: interim results. *Hum Gene Ther* 2011;22:1239–1247.
- McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* 2003;10:2112–2118.
- McCarty DM, Monahan PE, Samulski RJ. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 2001;8:1248–1254.
- Kay JD, Gouze E, Oligino TJ, et al. Intra-articular gene delivery and expression of interleukin-1Ra mediated by self-complementary adeno-associated virus. *J Gene Med* 2009;11:605–614.
- Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse—a review. *Vet J* 2006; 171:51–69.
- Watson RS, Broome TA, Levings PP, et al. scAAV-mediated gene transfer of interleukin-1-receptor antagonist to synovium and articular cartilage in large mammalian joints. *Gene Ther* 2013;20:670–677.
- Frisbie DD, Ghivizzani SC, Robbins PD, et al. Treatment of experimental equine osteoarthritis by *in vivo* delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 2002;9: 12–20.
- Gouze E, Gouze JN, Palmer GD, et al. Transgene persistence and cell turnover in the diarthrodial joint: implications for gene therapy of chronic joint diseases. *Mol Ther* 2007;15:1114–1120.
- Fath S, Bauer AP, Liss M, et al. Multiparameter RNA and codon optimization: a standardized tool to assess and enhance autologous mammalian gene expression. *PLoS One* 2011;6:e17596.
- Kozak M. Interpreting cDNA sequences: some insights from studies on translation. *Mamm Genome* 1996;7:563–574.
- Li C, Diprimio N, Bowles DE, et al. Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles. *J Virol* 2012;86:7752–7759.
- Bowles DE, McPhee SW, Li C, et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. *Mol Ther* 2012; 20:443–455.
- Walsh SL, Preston KL, Stitzer ML, et al. Clinical pharmacology of buprenorphine: ceiling effects at high doses. *Clin Pharmacol Ther* 1994;55: 569–580.
- Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 2001;3:107–113.
- Goodrich LR, Phillips JN, McIlwraith CW, et al. Optimization of scAAVIL-1ra *in vitro* and *in vivo* to deliver high levels of therapeutic protein for treatment of osteoarthritis. *Mol Ther Nucleic Acids* 2013;2:e70.
- Mease PJ, Hobbs K, Chalmers A, et al. Local delivery of a recombinant adeno-associated vector containing a tumor necrosis factor alpha antagonist gene in inflammatory arthritis: a Phase 1 dose-escalation safety and tolerability study. *Ann Rheum Dis* 2009;68:1247–1254.
- Mease PJ, Wei N, Fudman EJ, et al. Safety, tolerability, and clinical outcomes after intraarticular injection of a recombinant adeno-associated vector containing a tumor necrosis factor antagonist gene: results of a Phase 1/2 Study. *J Rheumatol* 2010;37: 692–703.
- Wang G, Evans CH, Benson JM, et al. Safety and biodistribution assessment of sc-rAAV2.5IL-1Ra administered via intra-articular injection in a mono-iodoacetate-induced osteoarthritis rat model. *Mol Ther Methods Clin Dev* 2016;3:15052.
- Bevaart L, Aalbers CC, Vierboom M, et al. Safety, biodistribution, and efficacy of an AAV-5 vector encoding human interferon-beta (Art-102) delivered

- via intra-articular injection in rhesus monkeys with collagen-induced arthritis. *Hum Gene Ther Clin Dev* 2015;26:103–112.
34. Evans CH, Kraus VB, Setton LA. Progress in intra-articular therapy. *Nat Rev Rheumatol* 2014;10:11–22.
 35. Gerwin N, Hops C, Lucke A. Intraarticular drug delivery in osteoarthritis. *Adv Drug Deliv Rev* 2006;58:226–242.
 36. Loeser RF, Goldring SR, Scanzello CR, et al. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum* 2012;64:1697–1707.
 37. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. *Bone* 2012;51:249–257.
 38. Rollín R, Marco F, Jover JA, et al. Early lymphocyte activation in the synovial microenvironment in patients with osteoarthritis: comparison with rheumatoid arthritis patients and healthy controls. *Rheumatol Int* 2008;28:757–764.
 39. Schmidt EV, Christoph G, Zeller R, et al. The cytomegalovirus enhancer: a pan-active control element in transgenic mice. *Mol Cell Biol* 1990;10:4406–4411.
 40. Lane Smith R, Trindade MC, Ikenoue T, et al. Effects of shear stress on articular chondrocyte metabolism. *Biorheology* 2000;37:95–107.
 41. Berenbaum F. Signaling transduction: target in osteoarthritis. *Curr Opin Rheumatol* 2004;16:616–622.
 42. Malemud CJ. Protein kinases in chondrocyte signaling and osteoarthritis. *Clin Orthop Relat Res* 2004;S145–S151.
 43. Rigoglou S, Papavassiliou AG. The NF- κ B signaling pathway in osteoarthritis. *Int J Biochem Cell Biol* 2013;45:2580–2584.
 44. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther* 2009;11:224.
 45. Goldring MB. Chondrogenesis, chondrocyte differentiation, and articular cartilage metabolism in health and osteoarthritis. *Ther Adv Musculoskelet Dis* 2012;4:269–285.
 46. Liu-Bryan R, Terkeltaub R. Emerging regulators of the inflammatory process in osteoarthritis. *Nat Rev Rheumatol* 2015;11:35–44.
 47. Wheeler MD, Kono H, Rusyn I, et al. Chronic ethanol increases adeno-associated viral transgene expression in rat liver via oxidant and NF κ B-dependent mechanisms. *Hepatology* 2000;32:1050–1059.
 48. Duan D, Yue Y, Yan Z, et al. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J Clin Invest* 2000;105:1573–1587.
 49. Johnson JS, Samulski RJ. Enhancement of adeno-associated virus infection by mobilizing capsids into and out of the nucleolus. *J Virol* 2009;83:2632–2644.
 50. Collaco RF, Bevington JM, Bhargava V, et al. Adeno-associated virus and adenovirus coinfection induces a cellular DNA damage and repair response via redundant phosphatidylinositol 3-like kinase pathways. *Virology* 2009;392:24–33.
 51. Johnson JS, Gentzsch M, Zhang L, et al. AAV exploits subcellular stress associated with inflammation, endoplasmic reticulum expansion, and misfolded proteins in models of cystic fibrosis. *PLoS Pathog* 2011;7:e1002053.
 52. Yan Z, Zak R, Zhang Y, et al. Distinct classes of proteasome-modulating agents cooperatively augment recombinant adeno-associated virus type 2 and type 5-mediated transduction from the apical surfaces of human airway epithelia. *J Virol* 2004;78:2863–2874.
 53. Zhong L, Li B, Mah CS, et al. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A* 2008;105:7827–7832.
 54. Mitchell AM, Li C, Samulski RJ. Arsenic trioxide stabilizes accumulations of adeno-associated virus virions at the perinuclear region, increasing transduction *in vitro* and *in vivo*. *J Virol* 2013;87:4571–4583.
 55. Mitchell AM, Samulski RJ. Mechanistic insights into the enhancement of adeno-associated virus transduction by proteasome inhibitors. *J Virol* 2013;87:13035–13041.
 56. Löser P, Jennings GS, Strauss M, et al. Re-activation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NF κ B. *J Virol* 1998;72:180–190.
 57. Johnson RA, Huong SM, Huang ES. Activation of the mitogen-activated protein kinase p38 by human cytomegalovirus infection through two distinct pathways: a novel mechanism for activation of p38. *J Virol* 2000;74:1158–1167.
 58. Svensson RU, Barnes JM, Rokhlin OW, et al. Chemotherapeutic agents up-regulate the cytomegalovirus promoter: implications for bioluminescence imaging of tumor response to therapy. *Cancer Res* 2007;67:10445–10454.
 59. Meier JL, Keller MJ, McCoy JJ. Requirement of multiple cis-acting elements in the human cytomegalovirus major immediate-early distal enhancer for viral gene expression and replication. *J Virol* 2002;76:313–326.
 60. Liu XF, Wang X, Yan S, et al. Epigenetic control of cytomegalovirus latency and reactivation. *Viruses* 2013;5:1325–1345.
 61. Bruening W, Giasson B, Mushynski W, et al. Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter. *Nucleic Acids Res* 1998;26:486–489.
 62. Ramanathan M, Haskó G, Leibovich SJ. Analysis of signal transduction pathways in macrophages using expression vectors with CMV promoters: a cautionary tale. *Inflammation* 2005;29:94–102.
 63. Simpson AJ, Cunningham GA, Porteous DJ, et al. Regulation of adenovirus-mediated elafin transgene expression by bacterial lipopolysaccharide. *Hum Gene Ther* 2001;12:1395–1406.
 64. Smith AD, Collaco RF, Trempe JP. Enhancement of recombinant adeno-associated virus type 2-mediated transgene expression in a lung epithelial cell line by inhibition of the epidermal growth factor receptor. *J Virol* 2003;77:6394–6404.
 65. Gelse K, Soder S, Eger W, et al. Osteophyte development—molecular characterization of differentiation stages. *Osteoarthritis Cartilage* 2003;11:141–148.
 66. Miagkov AV, Varley AW, Munford RS, et al. Endogenous regulation of a therapeutic transgene restores homeostasis in arthritic joints. *J Clin Invest* 2002;109:1223–1229.
 67. Khoury M, Adriaansen J, Vervoordeldonk MJ, et al. Inflammation-inducible anti-TNF gene expression mediated by intra-articular injection of serotype 5 adeno-associated virus reduces arthritis. *J Gene Med* 2007;9:596–604.
 68. van de Loo FA, de Hooge AS, Smeets RL, et al. An inflammation-inducible adenoviral expression system for local treatment of the arthritic joint. *Gene Ther* 2004;11:581–590.

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