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Chapter

Gene Therapy for the Treatment of Equine Osteoarthritis

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Abstract

Osteoarthritis (OA) is the predominant cause of lameness in horses. As in humans, the clinical symptoms of equine OA are persistent pain and dysfunction of the affected joint. Its pathology is similarly marked by progressive deterioration of the articular cartilage, subchondral bone sclerosis, marginal osteophytes, soft tissue inflammation and joint effusion. Disease pathogenesis is mediated by elevated levels of inflammatory cytokines and proteolytic enzymes in the articular tissues and synovial fluid. Existing pharmacologic agents can alleviate OA joint pain; none are able to inhibit erosive disease progression. As several gene-based treatments for human disease have received approval by the Food and Drug Administration (FDA), the transition to veterinary medicine will almost certainly follow. Several viral vector systems have demonstrated highly efficient gene transfer to the equine joint, enabling expression of therapeutic transgenes at efficacious levels for well over a year. Because of its large size, the equine joint is well suited to studies of gene-based therapies for arthritic disease. The forelimb joints are vulnerable to OA onset, and treatment and diagnostic modalities are the same in humans and horses. Here, we discuss the various gene-transfer approaches under investigation and the current progress toward the development an effective gene therapy for equine OA.

Keywords: osteoarthritis, lameness, interleukin-1, IL-1Ra, gene therapy, adeno-associated virus

1. Introduction

Osteoarthritis (OA) is a chronic, painful, degenerative, often debilitating condition common in weight-bearing joints of both humans and horses. In humans the knees and hips are predominately affected, while in the horse the metacarpophalangeal and carpal joints of the forelimb are the primary sites of onset. In both species, the pathology of OA is marked by the gradual, persistent erosion of the articular cartilage, development of osteophytes at the joint margins, sclerotic growth of subchondral bone, synovitis and joint effusion [1]. Biochemical analyses reveal that the signaling molecules and pathways that drive the inflammatory and degenerative processes in both species are identical [2]. OA is incurable, difficult to manage and often progresses to disabling joint failure. It is estimated that over 50 million people in the US alone have symptomatic OA. Spontaneous joint disease is a common

clinical problem in the horse as well where it is estimated that OA accounts for up to 60% of lameness [3], and is among the leading causes of debilitation and wastage of athletic horses. As with humans, the need for an effective treatment for equine OA is immense.

In this chapter we describe progress with an experimental gene-based therapy for OA in parallel development for both humans and horses. The concept of a genetic therapy was initially put forth as a method to replace defective genes and associated protein deficiencies from monogenic diseases, such as cystic fibrosis, severe combined immunodeficiency and hemophilia. In the present application direct intra-articular gene transfer is used as an improved system for sustained local delivery of biologic agents with anti-arthritic potential [4]. By providing for high-level, persistent production of therapeutic gene products in chronically diseased joints, long-standing obstacles impeding effective drug delivery are overcome to provide stable production of gene products with activities capable of inhibiting not only pain and inflammation, but also the progression underlying the degenerative process. We discuss various approaches for intra-articular gene delivery and promising gene products. We also discuss progress toward clinical application and remaining challenges.

2. Osteoarthritis pathogenesis

The pathogenesis of OA is complex and can be initiated by a wide range of factors. It is most commonly linked with aging and accumulating degradation of the cartilage matrix from the loss of cellularity and reduced metabolic activity of the chondrocytes [5–7]. In younger individuals, OA most frequently occurs as a secondary consequence of joint injury (post-traumatic OA: PTOA) either from repetitive trauma to the joint surfaces due to overloading and overuse, or acute damage to the structural tissues.

Although cartilage damage and traumatic loading are considered initiating factors, a consensus in the literature indicates that inflammatory cross-talk between the synovium and cartilage is instrumental in driving the erosive progression of OA [8, 9]. Under normal conditions, the chondrocytes, which inhabit the articular cartilage at low density, maintain the integrity and quality of the matrix through slow continual remodeling through degradation and new matrix protein synthesis. Disruption of this homeostasis from chondrocyte dysfunction or depletion from apoptosis or necrosis, leads to a reduction in matrix quality, damage to the articular surface and pathologic load distribution. The increased compressive forces among weight-bearing regions, activates stress signaling pathways in regional chondrocytes and a phenotypic shift to an activated phenotype. Stress-induced activation of nuclear factor-kappa B (NF-κB), and p38 MAPK and c-Jun N-terminal kinases and their downstream signaling cascades halts the synthesis of key extracellular matrix (ECM) proteins, stimulates the release of inflammatory cytokines and chemokines and expression of matrix metalloproteinases and aggrecanases [1, 10]. The release of cellular debris and matrix molecules from eroding cartilage stimulates cytokine and toll-like receptors in the synovial lining cells and an inflammatory response in the synovium [11, 12]. The resulting synovitis, marked by hyperplasia and hypertrophy of synovial fibroblasts, infiltrating macrophages, T cells, and mast cells, is a common feature of both early and late-stage disease. Inflammatory activation of the synovium stimulates production of enzymes and inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) that feeds back in a self-perpetuating cycle to further alter chondrocyte metabolism and the balance of cartilage matrix synthesis and degradation [9]. With increasing loss of the

protective cartilage cushion the increased mechanical forces stimulate a compensatory reaction in the calcified cartilage resulting in increased thickening and stiffness of the subchondral bone.

As an avascular tissue, injured cartilage has no mechanism for self-repair or regeneration. There is no influx of exogenous cells from ruptured blood vessels to generate space-filling tissue. Although local chondrocytes attempt proliferate and form chondrocyte clusters in an apparent regenerative, reparative response, the dense ECM limits the migration of the limited number of chondrocytes. In cases of significant damage, cartilage lesions are essentially permanent and progress to fibrillation, formation of fissures, and ultimately complete loss of the cartilage surface. Cumulatively, the slow insidious processes cause fibrillation, fissures, ulceration and over time the full thickness loss of cartilage and painful bone on bone articulation [13].

3. Treatment limitations

Existing medications for OA, such as analgesics and non-steroidal anti-inflammatory (NSAID) agents are palliative and only provide temporary relief of joint pain without significantly altering disease progression or restoring cartilage integrity. While there are a variety of biologic agents with activities known to inhibit pathologic signaling pathways, due to the unique anatomy and physiology of synovial joints, conventional methods of drug delivery are unable to achieve or maintain effective concentrations of therapeutic molecules in chronically diseased joints [14]. The synovial fluid which serves to lubricate the articulating surfaces and nourish the chondroctyes is a dialysate of blood plasma that enters the joint through fenestrated capillaries in the subsynovium. This "sieving effect" restricts the entry of proteins and other large molecules into the joint space from the circulation [14].

While intra-articular (IA) injection circumvents physical barriers to systemic delivery, elevated pressure causes rapid turnover of synovial fluid through the lymphatics. Continuous circulation of the synovial fluid causes injected molecules to be rapidly cleared from the joint, often with a half-life of less than 4–5 hours, depending on the size. Repeated intra-articular injection is not a useful clinically as frequent repeated needle sticks are painful, can exacerbate joint pathology and carry increased risk of infection [14].

Local intra-articular injection of corticosteroids can provide temporary relief of joint pain, but the broad spectrum anti-inflammatory effects are transient. Despite the short residence time of intra-articular therapies, studies frequently report positive effects from a number of patient-derived preparations, such as platelet rich plasma, autologous conditioned serum and various formulations of "mesenchymal stem cells" (MSCs). However due to inconsistent methods of preparation and characterization, conflicts of interest and investigator bias, the efficacy of these treatments in both human and equine medicine remains highly controversial. Indeed, the assertion that MSCs injected in suspension have an intrinsic capacity to sense and address whatever is needed for the repair and regeneration of cartilaginous tissue in the joint is not based on scientific evidence [15, 16].

4. OA gene therapy principles

Arthritis gene therapy was conceived as a novel protein-drug delivery system capable of exploiting the anti-arthritic properties of endogenous soluble gene products for treatment of chronic joint disease [17]. By delivering cDNAs encoding

therapeutic products to cells resident in the articular tissues, and providing for high levels of independent expression, the biosynthetic machinery of the modified cells is directed to overproduce and continuously secrete the transgenic protein into the synovial fluid and surrounding tissue. In this manner, the diseased joint becomes an endogenous site of sustained, elevated drug production, eliminating the need for repeated application, while providing the greatest concentration of the protein specifically at the site of disease. While originally envisaged for delivery of secreted proteins, similar principles can be applied to gene products that function intracellularly, including transcription factors and interfering RNAs among others. OA is an excellent candidate for a local gene-based therapy, as only one or two joints are affected in most patients, and there is an absence of significant extra-articular disease. Distinct from any existing treatment for OA, this approach has the capacity for continuous local delivery of therapeutic molecules that block painful symptoms and erosive progression of disease from a single intra-articular injection [4].

The distinct advantage of using a secreted protein is that overproduction from a relatively small number of cells can treat the entire joint. While at least in theory, gene delivery provides the opportunity to explore the application of cDNAs whose products that function intra-cellularly (e.g. transcription factors and interfering RNAs), practical application is far more challenging than it may initially appear. In order to alter the biology of a diseased tissue, a substantial portion of the cells must be modified, requiring extraordinarily high levels of gene transfer in vivo.

A variety of methods can be used deliver therapeutic gene products to joints. Once a candidate cDNA is identified, the delivery vehicle that provides efficient targeting and modification of the desired cell types in vivo and robust transgene expression that persists for a prolonged period of time. For chronic joint diseases, such as RA and OA, a minimum of 6 months to a year or more of benefit following a single injection would likely be the minimum standard for efficacy. Such a profile requires metabolically active target cells with limited turnover [18]. Additionally, the vector and genetically modified cells must avoid recognition and elimination by the immune system whose central function is to eradicate infectious viruses and virally infected cells expressing non-self, surface antigens and stress-induced signaling molecules. The immune stealth of the vector, transgene product and modified cells are essential for effective gene delivery and prolonged, functional transgene expression.

5. Parallel development of gene therapy for human and equine OA

Early preclinical studies showed that local intra-articular delivery of certain cDNAs could inhibit experimental arthritis in the joints of laboratory animals. Although rodents and rabbits are useful for proof-of-concept studies, their small size does not accurately reflect the environment of the human or equine OA joint. Following intra-articular injection of a gene delivery vehicle, ensuing patterns of transgene expression are dictated by the biophysical interactions between the vector and the target tissues. In the case of a recombinant virus for example, dispersion in the joint space through the viscous synovial fluid, and its subsequent penetration in the ECM of the various tissues, determines the locations, phenotype, number and density of the cells that are physically encountered by the vector and genetically modified. The composition of the cell population modified by the virus at the time of injection, determines the level and duration of therapeutic transgene expression -and, in turn, the efficacy of treatment. In this respect, the small joints of a 100–200 g quadruped rodent cannot duplicate the complex milieu of the knee of a 75 kg bipedal human, much less a 500 kg horse. The vastly greater size and internal fluid

volume, the differences in cellularity within the dramatically larger and thicker connective tissues, as well as the compressive forces generated during locomotion, have a profound influence on the biodistribution of the virus following injection [19].

To model the efficacy of gene delivery in joints of clinically relevant proportions and better assess its utility for treatment of OA, the carpal and metacarpophalangeal (MCP) joints of the equine forelimb provide highly useful targets. These joints are similar in size, function, and tissue composition to the human knee, and since they carry 60–65% of the horse's weight during locomotion, they are highly vulnerable to OA secondary to trauma and excessive training [2, 3].

Because of its large size, the equine system is particularly well suited for preclinical studies of joint disease. The horse can readily perform controlled exercise, and clinical treatment and diagnostic modalities are the same in humans and horses [2, 20]. The large joints facilitate joint function analyses, examination of internal structures using magnetic resonance imaging (MRI) and radiograph, and minimally invasive arthroscopy for visual assessment and biopsy of joint tissues. The capacity to aspirate undiluted synovial fluid permits analysis of transgenic protein content by enzyme linked immunosorbent assay (ELISA) [21], and since, joint fluids can be aspirated serially without adverse effect, patterns of transgenic expression can be monitored over time within the same animal.

By examining the efficacy of OA gene therapy in joints of similar proportion to the human knee and with similar disease, results representative of the human and equine response should be obtainable. Further, the use of the horse as an experimental subject allows practical experience with gene delivery in a relevant context and on an appropriate scale. This provides the ability to identify and troubleshoot technical and logistical problems in a clinical setting and refine working parameters for safety and efficacy prior to entering phase I human or field trials in client horses. Moreover, since OA is a significant health issue in both humans and horses, findings generated in this system can be applied to both species, allowing the development of human and equine medicines in parallel.

6. Ex vivo gene delivery

The initial proof-of-concept was demonstrated using an ex vivo method whereby autologous synovial fibroblasts isolated from surgically harvested joint tissues, were stably modified with recombinant oncoretroviral vector (Moloney murine leukemia virus) to overexpress a secreted IL-1 inhibitor (IL-1Ra) [22, 23]. After expansion in culture the cells were injected into the diseased joint where they engraft in the synovial lining and continuously secrete the transgene product. This method demonstrated the feasibility of intra-articular gene delivery and was used successfully in a phase I human trial [24]. However, the procedure proved to be labor intensive, time consuming and tedious; its exorbitant cost made the procedure impractical for widespread clinical application, especially for a common, non-fatal disease.

It is important to note that cell in suspension (regardless the tissue of origin), following injected into the joint space, consistently engraft in the synovial lining; they do not adhere to or colonize articular cartilage. Cells surgically implanted in cartilage defects within a support matrix will remain localized, but lacking a method of physical containment loose cells will disperse throughout the capsular lining. Along these lines, much has been made in the literature of the anti-arthritic potential of so-called "mesenchymal stem cells" or MSCs [15, 16]. These cells are amenable to genetic modification and can be used as a vehicle for ex vivo gene transfer. However, it is our experience that MSCs in and of themselves are not

immune privileged and have no more regenerative or anti-inflammatory value than any other cell type injected into the joint. Most investigators have found that allogeneic MSCs are cleared very rapidly from the joints of experimental animals, with few cells remaining beyond 1–2 weeks.

7. Direct intra-articular gene transfer

Relative to the ex vivo approach, direct injection into the joint of recombinant vectors dramatically streamlines the gene delivery procedure [4]. A broad range of vector systems, both viral and non-viral have been evaluated for their efficiency of gene transfer to the joint tissues in situ [25–30]. While the use of non-viral gene delivery vehicles has certain theoretical advantages (larger payload, increased perception of safety, straightforward vector production, reduced costs) extensive *in vivo* testing in our laboratory, as well as in others, has shown that non-viral delivery of nucleic acids is currently not suitable for treating chronic articular diseases; the efficiency of delivery is exceedingly low and typically persists in the joint cells for no greater than 2–3 days. While non-viral formulations are often effective transfection reagents in the context of monolayer cell culture, efficacy in vitro does not equate to performance in vivo [31]. Despite claims in the literature regarding the treatment of OA, the use of these systems should be avoided as their pharmacokinetic profile is incompatible with the pathologic progression of OA.

Similarly, there are dozens of published papers that report remarkable efficacy following intra-articular injection of shRNAs, miRNAs, and circRNAs into the joints of animals either in suspension or complexed in nano- or micro-particles. As mentioned above, for an intracellular approach to be effective in OA, an extraordinarily high efficiency of delivery is required to the cells in target tissues in vivo. Moreover as gene expression is an ongoing process, interfering RNAs must be maintained at exceptionally high levels in a large proportion of cells and be continuously replenished to sustain gene silencing. While achievable when delivered in an exogenous expression cassette, it is not possible with the delivery of soluble or complexed inhibitory RNAs. These reports should be regarded with a healthy degree of skepticism.

Viral-based vector systems exploit the natural ability of a virus to deliver its genetic payload to a target cell with high efficiency. For the generation of a viral-based vector system, the coding sequences for viral proteins essential for replication are removed from the viral genome and the products are supplied in *trans* during vector propagation in permissive engineered cell lines. Several recombinant viral vector systems have shown the capacity to deliver exogenous genes to joint tissues and enable expression of therapeutic transgene products at levels sufficient to inhibit arthritic pathologies in laboratory animals. Among these are recombinant adenovirus [27], herpes simplex virus [28], adeno-associated virus (AAV) [32, 33], and lentivirus [29, 34] among others. Each of these systems has inherent advantages and limitations that dictate the applications for which they are best suited. Currently only two viral vectors, recombinant adenovirus and AAV, are in serious preclinical development for equine or human OA.

7.1 Recombinant adenovirus

First generation recombinant adenovirus provides highly efficient transduction of target cells in various connective tissues both in culture and in vivo [25, 26]. Several years ago this system was the workhorse vector of the field of musculoskeletal gene therapy [35]. Adenoviral vectors showed that the concept of direct

intra-articular gene transfer was capable of providing functional levels of transgene expression in the joints of animal models. In the first generation vectors the E1 and E3 genes required for immediate early stage gene expression and initiation of viral replication were deleted from the genome to prohibit viral replication in cells infected by the vector. Their removal also provided room for the insertion of an exogenous expression cassette [36].

The relative ease of production reduced the barrier to entry and provided gene transfer technology to any laboratory with basic molecular biology capabilities. Although viral replication was crippled in non-permissive cells, the vector still retained the majority of the native coding sequences. Leaky expression of viral proteins by transduced cells caused them to be eliminated in 2–3 weeks by adaptive cellular immune responses [27, 37]. Despite its transient nature, adenoviral gene delivery provided a burst of high level transgene expression sufficient to examine the biological activity of a specific gene product in vivo. Adenovirus has the reputation of causing acute toxicity from innate inflammatory responses, but much of this is due to low quality, inconsistent vector preparations containing high levels of cellular debris. Advances in adenoviral technology include the development of helper dependent systems in which the coding sequences for all viral proteins have been removed and are supplied during propagation by a second "helper" adenoviral vector, which is removed by differential centrifugation during purification [38]. These modifications allow for increased immune avoidance and long-term transgene expression without significant reduction in infection efficiency [39–41].

7.2 Adeno-associated virus

Of the well-characterized viral systems, AAV offers many advantages that favor its use for the treatment of arthritis: (1) The wild type virus is not associated with any pathologic human condition. (2) The recombinant form does not contain native viral coding sequences, which reduces its immunogenicity. (3) AAV can infect both dividing and quiescent cells. (4) Persistent transgenic expression in vivo has been observed in many applications, and (5) the recombinant form does not integrate into the genome of the target cell with significant frequency [42].

A further potential advantage is the relative simplicity of the AAV vector, which is comprised of an ~5000 nucleotide single-stranded DNA genome packaged in a small (20–30 nm), non-enveloped icosahedral particle by three capsid proteins, differing only at their N termini [43]. The only required *cis* elements on the vector DNA are 145 nucleotide-long inverted terminal repeats (ITRs) that flank the transgene expression cassette.

Fortuitously, with regard to veterinary medicine, in head to head comparisons equine synovial fibroblasts in culture are significantly more receptive to AAV transduction than their human counterparts. Preliminary evidence suggests increased expression of surface receptors between the two species in culture. How this discrepancy translates to the in vivo situation is unclear, as phase I testing in humans has just begun, but transgene expression is robust in equine joints.

Additionally, humans are natural hosts to wild type AAV infection and often have high circulating titers of neutralizing antibodies (NAb) to AAV capsids of several serotypes (primarily AAV2, AAV1, and to lesser extents AAV5) from prior infections with wild type virus. Horses, however, are not common hosts for wild type AAV infection, and distinct from humans, have low circulating NAb titers to most AAV vector serotypes. While NAb to AAV5 appears relatively frequently among horses, and one report describes increased NAb titers to AAV2 capsid in a small test sample, pre-existing NAb do not appear to be prevalent in the equine population nor at sufficient titer to prohibit effective gene delivery [44, 45].

Typically, following intra-articular injection of a recombinant virus, the overwhelming majority of genetically modified cells are found in the synovium, subsynovium and supporting capsular and ligamentous tissues. Chondrocytes, while receptive to genetic modification in culture, are not efficiently transduced in vivo due to the inability of most vector particles to effectively penetrate the dense cartilage ECM. The only exception is AAV whose small particle size permits its entry and diffusion through the dense cartilage ECM enabling interaction and transduction of chondrocytes deep within the cartilage. As chondrocyte dysfunction and cartilage degeneration are the characteristic pathologies of OA, the capacity to deliver therapeutic genes to chondrocytes is a clear advantage to this vector technology. Moreover, since these cells are highly stable, their modification with AAV provides the prospect of enduring transgenic expression [46].

8. Therapeutic strategies

Two complementary gene-based strategies have been investigated for OA. The first is geared toward chondroprotection, and involves delivery of gene products that enhance joint lubrication or block the activities of specific inflammatory cytokines that stimulate inflammation and the subsequent degeneration of cartilage ECM by articular chondrocytes [47–49]. Most studies of OA gene therapy have involved the delivery of the cDNA for interleukin-1 receptor antagonist (IL-1Ra) a competitive inhibitor of IL-1 signaling [50–55].

The second strategy is directed toward cartilage repair or regeneration using various anabolic, proliferative or chondrogenic agents to stimulate regional chondrocytes to proliferate and elaborate cartilage ECM. While these strategies appear attractive at the outset, unfortunately, as vectors (and cells) injected into the joint primarily interact with synovial fibroblasts, many growth factors that may stimulate cartilage repair or matrix synthesis by chondrocytes will likewise stimulate the abundant fibroblast populations in the synovium to generate undesirable, often dramatic adverse side effects. For example, intra-articular delivery of adenovirus containing the cDNA for TGF-β1 induces an extraordinarily potent fibrotic, chondro-osseous response in the synovium and joint capsule [56, 57]. Systemic pathologies such as pulmonary fibrosis in rats and death in rabbits occurred when TGF-β1 was expressed intra-articularly at high levels. Overexpression of TGF-β1 and BMP-2 has also been shown to induce the formation of osteophytes, ectopic cartilage and bone formation [56, 58]. Of the growth factor genes tested thus far, only IGF-1 has not been associated with an overt pathologic response [45, 59], but it has not been evaluated extensively. Concerns over potential side effects have generally limited the use of growth factor genes to localized applications in tissue engineering for cartilage repair, whereby chondrocytes or MSCs are modified in culture to express a specific growth factor before surgical implantation into focal cartilage lesions, In this manner, the expressed protein is localized to the defect, reducing exposure to adjacent tissues.

8.1 Interleukin-1 receptor antagonist

A consensus in the literature indicates that IL-1, synthesized locally by chondrocytes and synovial cells, is instrumental in driving OA progression [60, 61]. Found at increased levels in OA joints, IL-1 is the most potent physiological inducer of chondrocytic chondrolysis (the major route to cartilage loss in OA) [62]. Even at trace levels, IL-1 strongly inhibits ECM production in cartilage by blocking collagen type II and proteoglycan synthesis and enhancing chondrocyte apoptosis. At

slightly higher concentrations proteolytic enzyme synthesis is induced in chondrocytes, driving enhanced production of matrix metalloproteinases (MMPs) and aggrecanases that degrade the cartilaginous matrix [63]. As a primary mediator of the inflammatory cascade, IL-1 stimulates articular cells to produce a full complement of OA effector molecules, including cyclooxygenases I and II, nitric oxide, phospholipase A2, prostaglandin E_2, reactive oxygen species as well as inflammatory cytokines and chemokines. Release of these molecules further stimulates cartilage matrix degradation, bone erosion, synovitis and fibrosis. IL-1 is also suspected to mediate pain in OA, the most common reason for consulting a physician [64–67].

Traditional pharmacologic approaches have failed to produce clinically useful molecules for inhibiting IL-1 activity intra-articularly [68]. However, two naturally occurring proteins exist specifically for this purpose: IL-1Ra and the soluble IL-1 type II receptor (sIL-1RII) [69, 70]. IL-1Ra functions as a competitive inhibitor by binding to the type I IL-1 signaling receptor and preventing subsequent interaction with IL-1. Once bound, IL-1Ra fails to recruit the IL-1R accessory protein (IL-1-AcP) to the complex and prevents intracellular activation and signaling. The sIL-1RII molecule, in contrast, titrates IL-1 activity by binding directly to soluble IL-1 molecules and blocking interaction with the type I receptor [71, 72]. Despite differences in their modes of action, the two molecules inhibit IL-1 signaling with equal potency. In the context of gene therapy, IL-1Ra is a smaller protein and easier to express as a transgene product. The recombinant protein (anakinra/Kineret®) is well characterized and is approved for clinical use in humans for RA and other conditions in which IL-1 is known to pay a significant role [71, 72]. As anakinra is administered daily by subcutaneous injections of 150 mg, the risk of adverse response from overproduction intra-articularly is extremely small.

Commercially available ELISAs with specificity for IL-1Ra orthologs in human, mouse and horse permit sensitive quantitation in culture media and biological fluids. Analysis of synovial fluid permits the use of IL-1Ra as a quantitative reporter of total gene transfer and therapeutic gene expression, allowing direct comparison of various delivery platforms. With respect to OA, IL-1Ra does not require sophisticated regulation. The goal is simply to express IL-1Ra at levels 10–100 fold over IL-1, where it completely inhibits IL-1 signaling activity. Once the threshold for efficacy has been achieved, expression beyond this has no adverse effect [73].

It is possible that a dual therapy combining elements of chondroprotection and regeneration could both inhibit degeneration and stimulate cartilage repair [48, 74]. Such a strategy, though, would likely require gene delivery via separate vectors to account for differences in their expression patterns for safe, effective application.

9. Preclinical studies

Following a series of preclinical successes in small laboratory animals demonstrating the proof of concept for direct viral-mediated gene delivery to joints, studies were performed to evaluate direct viral mediated gene delivery to equine joints using a first-generation adenoviral vector containing the cDNA for equine IL-1Ra. Administration of Ad.eqIL-1Ra in the joints of healthy horses, produced dose-dependent increases in IL-1Ra levels in synovial fluid aspirates. However, the highest viral dose tested, 5×10^{11} viral particles (vp) produced an acute synovitis [21].

To explore the capacity of Ad.eqIL-1Ra to inhibit OA pathologies, an osteochondral fragment (OCF) model of OA was used [21, 75]. In this system, a small osteochondral chip is surgically generated off the distal radial carpal bone of the midcarpal joint. Following a brief interval to recover from surgery, animals in the

treatment group are injected in the OCF joint with the vector, while control animals receive saline [21]. The horses are then exercised 5 days/week on a high-speed treadmill, which, in the context of the osteochondral fracture, generates predictable pathologic lesions that mimic the onset of equine disease [21]. ELISA analysis of joint aspirates showed a peak in eqIL-1Ra expression at 7 days post injection which gradually diminished over a period of 28 days. Clinical examinations indicated that the expression of IL-1Ra decreased joint pain and synovial effusion relative to untreated horses, and protected the cartilage from the loss of proteoglycans.

These findings provided strong support for local gene delivery of IL-1Ra in large mammalian joints. A central limitation, however, was the use of the first generation adenovirus. In later work, we found articular tissues to be highly immune sensitive to the expression of foreign proteins, such that cells expressing foreign non-homologous transgene products or viral proteins are recognized by cell-mediated immune responses which lead to abbreviated persistence of transgenic expression in vivo [37]. Thus while the results showed promise, they indicated an intense need for an improved, immune stealthy vector system.

9.1 AAV-mediated gene delivery to equine joints

The results of studies of other gene therapy applications, such as hemophilia, indicated that long-term transgene expression was achievable following direct delivery of AAV vectors. The results of exploratory experiments in joints were disappointing. Transgene expression from conventional single-strand AAV vectors required several days or weeks to onset with marginal levels of protein production intra-articularly, a pattern that prevented testing in experimental disease models.

AAV transduction efficiency is known to be enhanced by mechanisms associated with intracellular stress. Certain stimuli, such as UV radiation, which increase the production of DNA synthesis and repair enzymes, significantly enhance intra-articular transgene expression from conventional AAV vectors [33, 76, 77], which indicates that second strand DNA synthesis is rate-limiting in joint tissues. Accordingly, AAV vectors that are self-complementary (sc) (i.e. double stranded, containing both + and – DNA strands) generated through the use of half-genome sized vector plasmids, or those containing a mutation in one of the terminal resolution sequences of the AAV ITRs [78, 79], provided ~20-fold enhancement of gene expression, with rapid onset in synovial and capsular cells in vitro and in vivo [80]. This adaptation was found to provide transduction and transgene expression profiles comparable to that provided by adenovirus. The requirement for a half-sized genome, however, limits the size of the transgene to about 1000–1200 base pairs [79].

Following encouraging results with scAAV vectors in the joints of laboratory animals [80], studies of AAV gene transfer shifted to the equine model to assess more clearly its utility for therapeutic gene delivery in large OA joints [19, 81]. As before, the carpal and MCP joints of the equine forelimbs were targeted for injection. In pilot studies, AAV gene delivery to healthy joints was examined using vectors containing the cDNAs for human IL-1Ra (AAV.hIL-Ra) and green fluorescent protein (AAV.GFP) [19]. In the animals receiving AAV.hIL-1Ra, synovial fluids were aspirated periodically over a period of several weeks. Animals receiving AAV.GFP were euthanized 14 days after injection and the distribution of fluorescence in the joint tissues was used to determine the number and locations of the cells modified by the AAV virus following intra-articular injection.

AAV gene delivery in the equine joints was capable of elevating the steady state hIL-1Ra in synovial fluid to levels equivalent to or greater than observed previously in rodents [19]. Analysis of GFP fluorescence showed that the vast majority of the

transgene expression originated from the fibroblasts resident in the synovial lining. Fluorescent cells in the articular cartilage, though visible, were sparse, and GFP expression was faint. Peak levels of hIL-1Ra occurred at 1–2 weeks post-injection, but steadily declined over a period of 5 weeks. Studies in nude rats indicated that the abbreviated transgene expression was due to immune elimination of the cells expressing the xenogeneic human IL-1Ra protein [37].

9.2 Codon optimization of the equine IL-1Ra cDNA

The commercial release (R&D Systems, Minneapolis, MN) of an ELISA kit specific for the equine IL-1Ra ortholog in 2010 proved to an enabling technology, allowing for the first time definitive quantification of the equine transgene intra-articularly [82]. Prior to this point, expression data relied on inter-species cross-reactivity between human and murine ELISAs, results which were inconsistent and highly error prone.

To examine AAV-mediated transgene expression in the absence of immune interference, the human cDNA was replaced with the homologous equine IL-1Ra. To overcome initial problems with low production levels, codon-optimization resulted in >50-fold amplification in IL-1Ra secretion [18, 82]. For use in safety studies for the FDA, the cDNAs for human and rat IL-1Ra were also codon-optimized using the same algorithm [55]. After packaging in the AAV capsid, infection of synovial fibroblast cultures over a range of doses generated exceptionally high levels of IL-1Ra protein in conditioned medium, which exceeded 10 μg/ml at a vector dose of 10^5 vg/cell [18].

Using the optimized AAV.eqIL-1Ra vector, a series of pharmacokinetic studies were performed to establish vector dose expression profiles following intra-articular injection [18]. In each of six horses, the midcarpal and MCP joints of both forelimbs were injected with AAV.eqIL-1Ra at doses ranging from 5×10^{10} to 5×10^{12} vg; the remaining joint received an equivalent volume of saline and served as a negative control. Analysis of synovial fluid, peripheral blood and urine collected periodically over a period of 6 months showed dose-related increases in the eqIL-1Ra content in synovial fluid at 2 weeks of injection, with peak production between 4 and 8 weeks. At the highest vector dose synovial fluid IL-1Ra levels exceeded 40 ng/ml, ~400-fold higher than endogenous synthesis. Importantly IL-1Ra production remained at these levels for the duration of the 6-month study. Despite simultaneous injection of recombinant AAV in three forelimb joints, no adverse effects were observed. IL-1Ra in blood serum, urine and synovial fluid of control joints remained at pre-injection levels (<100 pg/ml) throughout [18].

9.3 AAV gene delivery in naturally occurring OA

As discussed previously, the pathologic progression of OA induces sweeping changes in the architecture, cellularity and activation of the articular tissues. In conjunction with vector dosing, a series of tracking studies were performed to examine the impact of the OA environment on transgene expression and the biodistribution of the vector DNA and transduced cells [18]. Using a dose of 5×10^{12} vg, AAV.GFP was injected into one forelimb joint of several healthy horses, and horses with advanced naturally-occurring OA. Analysis of tissue samples 2 weeks later showed GFP fluorescence in healthy joints was concentrated in the synovial lining, with only a handful of GFP+ cells visible in cartilage shavings (**Figure 1**). In joints with advanced OA, there was a striking increase in GFP expression in all joint tissues particularly in articular cartilage. In synovium, enhanced GFP expression was due to the increased cellularity from local inflammation. Although fluorescence

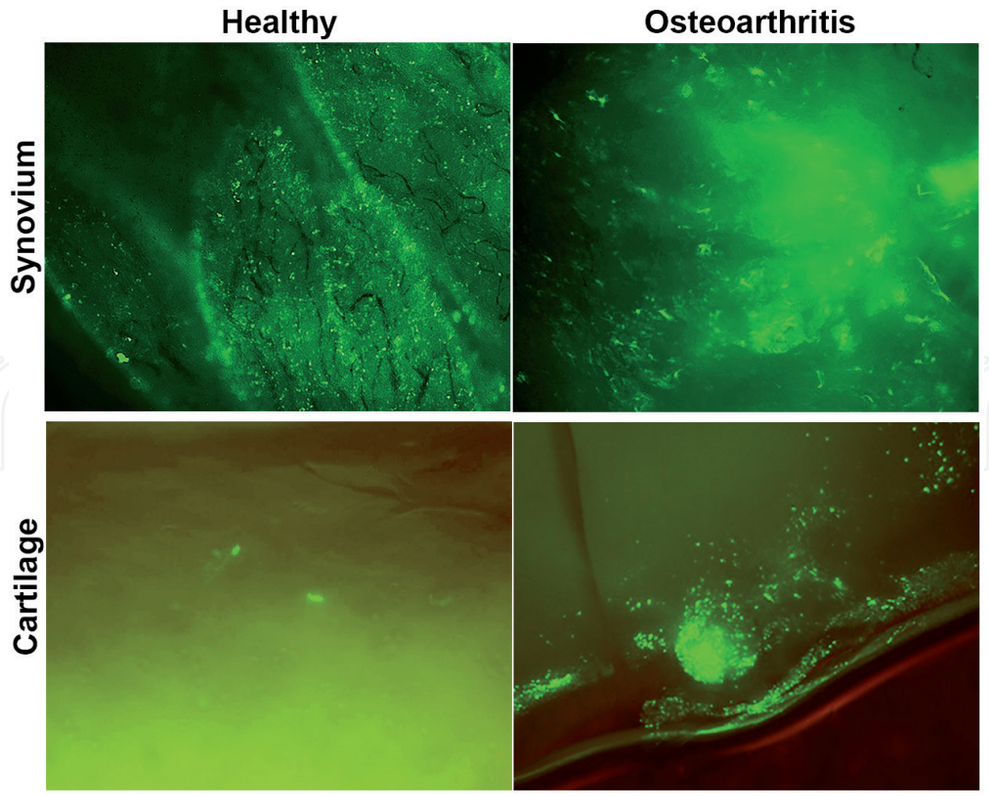

Figure 1.
Representative fluorescence activity in synovium (top row) and cartilage (bottom row) following intra-articular injection of an AAV vector containing the cDNA for green fluorescent protein (GFP) into healthy carpal joints or those with naturally-occurring OA.

was greater in all cartilage shavings, GFP expression in regions containing visibly damaged cartilage was markedly increased. GFP+ chondrocytes appeared throughout the damaged regions, particularly in clusters of proliferating chondrocytes, characteristic of OA cartilage.

In both healthy and OA joints receiving virus, no GFP+ cells were detected in extra-articular tissues, and qPCR analyses showed that 99.7% of the vector DNA was localized within the cartilage and synovium of the injected joint. Similar patterns of biodistribution were noted by Goodrich et al. [81]. While the enhanced transduction of OA chondrocytes might be presumed to arise from increased vector access from ECM degradation, qPCR analyses showed the vector DNA content in the chondrocytes of healthy and OA cartilage was essentially the same, suggesting that the increased transgene expression arose from increased metabolism due to inflammatory and stress induced activation [18].

Transcription from the CMV immediate early promoter, which drives the scAAV vector expression cassette, is induced in response to NF-κB activation and signal transduction from p38 and other stress-activated protein kinases. Similar stress-related induction of this promoter serves to re-activate human CMV from latency, and is required for expression of genes necessary for DNA replication [83–87]. In the same manner, inflammation and cellular stress can significantly increase the transcription and expression of transgenes under control of the CMV immediate early promoter [87–90]. In these respects, OA cartilage is highly enriched with stress-activated chondrocytes [91], especially at sites of cartilage degradation, where GFP expression was greatest.

Although several reports describe the generation of synthetic inflammation-inducible promoter systems for gene therapy applications, 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. The regional

differences in GFP expression seen in OA cartilage indicate the potential to direct therapeutic transgene expression preferentially to areas of articular cartilage under the greatest pathologic stress. This perhaps lays the groundwork for development of vectors for disease-targeted anabolic stimulation of cartilage repair and regeneration.

9.4 AAV.eqIL-1Ra delivery and expression in the OCF model

The OCF model was adapted to determine if the levels of equine IL-1Ra generated by AAV gene transfer were sufficient to mediate an appropriate biologic response [92]. Following creation of the osteochondral fracture, the OCF joints in the treated animals were injected with 5×10^{12} vg AAV.eqIL-1Ra, while controls animals received an equal volume of saline. One week later the animals were placed on a 5 day/week athletic training protocol for a period of 10 weeks. In this acute injury model, mean eqIL-1Ra expression was initially ~4-fold higher than observed in healthy joints at the same viral dose and correlated directly with the severity of joint pathology at the time of vector delivery. Over the 10 week training period, eqIL-1Ra expression gradually diminished to ~60 ng/ml, similar to that seen with AAV gene transfer in normal joints. Despite variable expression among animals the steady-state eqIL-1Ra in synovial fluids exceeded that of IL-1 by >500-fold in all animals. In agreement with increased IL-1Ra, the treated horses showed a reduction synovial fluid PGE_2 levels and a progressive reduction in joint pain. Improved joint function was accompanied by significant reduction in joint pathology by both arthroscopy and MRI. By both diagnostics, the treated animals showed significant reductions in synovial effusion and marrow edema, local protection of cartilage and enhanced repair of the osteochondral fragment [92].

Consistent with the findings of others [45, 93], we observed an increase in AAV capsid-specific neutralizing antibody (NAb) titer over time in both the blood serum and synovial fluid of the horses receiving the AAV vector [92]. The NAb titer in synovial fluids was consistently several-fold higher than in blood but had no obvious effect on transgene expression.

Humans are natural hosts to wild type AAV infection and often have high circulating titers of neutralizing antibodies (NAb) to AAV capsids of several serotypes (primarily AAV2, AAV1, and to lesser extents AAV5) from prior infections with wild type virus. Horses, however, are not common hosts for wild type AAV infection and distinct from humans, have low circulating titers to most AAV vector serotypes. While NAb to AAV5 appears to be relatively common among horses, and one report describes increased NAb titers to AAV2 capsid in a small test sample, pre-existing NAb do not appear to be prevalent in the equine population nor at sufficient titer to prohibit effective gene delivery. As indicated above, high levels of capsid serotype specific NAb will arise from prior treatment with an AAV vector, which can inhibit the efficacy of subsequent vector administration. There is evidence that vector neutralization can be averted through the use of an alternate capsid serotype.

9.5 Long-term efficacy

The primary advantage of a gene-based therapy for OA lies with the capacity for sustained local delivery of anti-arthritic agents and the promise long term therapeutic benefit. To address this, we recently completed a series of studies to assess the safety and efficacy of AAV.eqIL-1Ra delivery in a chronic model of joint disease over the course of a year. For the chronic model, the 10 weeks OCF protocol was used to induce joint pathology consistent with early symptomatic disease. At the

completion of the athletic training period, 5×10^{12} vg of the AAV.eqIL-1Ra vector was injected into the OCF joint, and a 3 day/week training regimen was instituted to maintain a slow but progressive degenerative condition for the following 12 months.

Immediately prior to injection, and then at 2 weeks and monthly thereafter, peripheral blood, urine and synovial fluids were collected, and joint pain and kinematic assessments were performed. Radiographic and MR imaging of both midcarpal joints was performed prior to injection and then at 6- and 12-month time points. Arthroscopic examination of the joints was performed at endpoint and digitally recorded, and synovial and articular cartilage biopsies were taken for histologic examination. At the conclusion of the year-long protocol all animals in the treatment group and five animals from the Control group were euthanized for biodistribution and toxicology analysis.

Analysis of synovial fluids showed that high IL-1Ra levels of 40–50 ng/ml were sustained over the 12-month course of the study. In the chronic OCF model transgenic IL-1Ra expression was far more consistent among individual animals than in joints with an acute osteochondral fracture. Relative to arthritic controls, the treated animals showed a ~40% reduction in lameness, indicative of reduced joint pain and improved mobility. By MRI assessment, joint pathology in the was reduced by ~28% relative to baseline disease, while in control joints the overall pathology was largely unchanged. Relative to pretreatment levels the treated group showed ~28% improvement in all major OA pathologies relative to baseline while in arthritic controls pathologic scores remained unchanged or increased in severity.

9.6 Toxicology and biodistribution

To establish a qualified biosafety profile for AAV.IL-1Ra gene transfer in a large mammalian joint, formal preclinical toxicology and biodistribution studies were performed addressing the acute and long-term phases of vector delivery. In the Acute Phase studies, early stage disease was induced in one midcarpal joint using the OCF protocol. Three horses each were injected with 5×10^{12} vg AAV.eqIL-1Ra, 1× anticipated clinical dose, and three horses with 5×10^{13}, 10× clinical dose, intended to represent a "worst case scenario." For the long-term toxicology studies, each of the 10 horses from the treated group in the 12-month study and 5 horses randomly selected from the control group were euthanized for necropsy. Following euthanasia, samples from more than 50 tissues were collected for histopathologic evaluation or DNA extraction and PCR analysis of vector genome content.

In all animals injected with AAV.eqIL-1Ra at the 1× dose, high vector genome copies (10^4–10^6) were detected by qPCR in the synovium and cartilage, which were equivalent in animals euthanized at 2 weeks and 12 months post-injection. No vector DNA was detected in extra-articular tissues. No pathologic response associated with vector injection was observed in any tissue.

The cumulative data from these pharmacokinetic, toxicology and efficacy studies in the equine model demonstrate that a gene-based therapy using recombinant AAV can provide safe, long-term, effective delivery of anti-arthritic proteins, such as IL-1Ra, in large mammalian joints. The results of these safety and efficacy studies in horses formed the bases for a successful IND application and the initiation of a phase I trial of AAV-IL-1Ra delivery for knee OA.

9.7 Gene delivery with high-capacity adenovirus

Recently, studies involving gene delivery with HD.Ad have begun to move toward clinical studies, at least in human OA. Initial studies in mice using

intra-articular HD.Ad-mediated delivery of the cDNA for lubricin (Prg4) showed a marked chondroprotective effect, maintaining matrix volume and prevention of degeneration in a cruciate ligament transection (CLT) PTOA model [39].

Progressing from these results, studies were performed to examine the therapeutic capacity of IL-1Ra gene delivery via HD.Ad in mice and the forelimb joints of horses [94]. In most gene therapy applications a strong, constitutively active promoter sequence, such as the CMV promoter/enhancer or the eukaryotic translation initiation factor 1α (EIF-1α) promoter is used. In this case, however, transgene expression was driven by an inflammation-inducible promoter comprised of a minimal endothelial cell leukocyte adhesion molecule (ELAM1) promoter linked to multiple upstream NF-κB recognition elements. The rationale being that therapeutic transgene expression would be delimited specifically to inflammatory flares.

Similar to prior studies with Prg4, HD.Ad delivery of the homologous murine IL-1Ra transgene was found to inhibit osteophyte formation and cartilage erosion in the CLT defect model [94]. Importantly, vector delivery was also associated with a significant reduction in pain sensitivity. Following preliminary dosing studies in healthy equine joints, HD.Ad was used to deliver the eqIL-1Ra cDNA into the carpal joints of horses following surgical generation of the osteochondral lesion in the OCF model. IL-1Ra levels in the injected joints rose to ~20 ng/ml within the first week, but then dropped about 10 fold by week 2 and were near endogenous background by week 3. Despite the relative brevity of expression, the treated animals showed significant improvement in lameness, reduced joint effusion, synovitis and osteocyte formation and improved cartilage matrix integrity [94].

Due its relatively large size, the adenoviral particle cannot penetrate the ECM of the synovium or cartilage and remains constrained to cells residing in superficial regions [39]. Interestingly in both reports where intra-articular transgene expression was quantified, a 90–99% loss in therapeutic transgene expression was observed within 1–2 weeks of vector injection, regardless of whether an inducible promoter was used [39, 94]. While transgene expression seems to persist long-term, therapeutic protein levels appear at trace levels. Given this profile, it will be interesting to see how this platform moves ahead in the future.

10. Conclusions

These data altogether show that in large mammalian joints, local gene transfer can provide persistent IL-1Ra transgene expression at therapeutically relevant levels. Despite variable expression among treated joints in the context of acute inflammation, sustained IL-1Ra expression provides meaningful benefit, such that a single injection reduces joint pain and intra-articular inflammation, and improves repair of the damaged bone and protects cartilage against degradation. No adverse response to the vectors or transgene have been observed with either AAV or HD.Ad, and at least within the equine system local overexpression of IL-1Ra provides no apparent risk of systemic immunosuppression.

Having established safety and efficacy of IL-1Ra gene delivery in the equine joint, the next stage in development would be the move into field testing with client animals. Given the limited resources available for equine research, such a large and costly undertaking is likely feasible only through support from partners in the veterinary pharmaceutical industry or private investors looking to advance the treatment methods toward commercialization. In this respect, questions of market size, cost of goods and profitability move to the forefront. Currently human gene-based therapies come with a substantial price tag, and range at the high end from $450,000 per eye for Luxterna® for congenital retinal degeneration to $2,100,000

for Zolgensma®, a gene correction therapy for spinal muscular atrophy in infants. Both of these "drugs" employ AAV as a vector. As the popularity of gene-based therapies continues to advance, production costs will likely fall considerably as the field grows and therapies with greater efficacy emerge. Among these business issues, questions regarding genetic enhancement in the racing industry will need to be addressed and resolved.

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Conflict of interest

Steve Ghivizzani is a founder and share-holder in Genascence Inc., a company pursuing development and commercialization of gene therapies for inflammatory conditions.

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