INTRODUCTION
Osteoarthritis (OA) is a common, debilitating joint condition marked by progressive deterioration of the articular cartilage, subchondral bone sclerosis, osteophyte formation and inflammation of the soft tissues. Over time, the chronic degenerative process often leads to total joint failure and loss of mobility, necessitating prosthetic replacement. Despite the high prevalence and impact of OA, its treatment remains inadequate due, in large part, to a limited understanding of the pathogenesis and the biological mediators that drive the disease processes. Over the last several years, increasing evidence has emerged of an important role for interleukin-1 (IL-1) as an intra-articular mediator of cartilage loss, joint pain and inflammation in OA. In this regard, a naturally occurring inhibitor of IL-1 signaling, the IL-1 receptor antagonist (IL-1Ra), holds promise as a biological drug for treatment. The recombinant form of IL-1Ra (anakinra) has been shown to be a useful anti-inflammatory agent in certain clinical applications; however, its efficacy in treating arthritic disease has been hindered by an inability to achieve and maintain therapeutic concentrations intra-articularly.

To overcome problems with effective administration of recombinant IL-1Ra, we have worked to develop technologies to deliver the IL-1Ra cDNA to cells in the synovium and capsular tissues of joints. These tissues are genetically modified to become endogenous sites of sustained, elevated IL-1Ra production, which, following secretion from the modified cells, diffuses throughout the articular tissues. This gene-based approach to protein–drug delivery removes the need for repeated application while providing the greatest concentration of the therapeutic agent specifically at the site of disease. We, and several others, have shown that direct intra-articular injection of certain recombinant viral vectors can provide expression of therapeutic transgenes at levels sufficient to halt experimental arthritis in the joints of laboratory animals. Further we have shown that with the use of immunologically compatible vectors and cDNAs, exogenous transgenes can be expressed in the joint tissues for at least 6 months.

In considering vector systems to move into clinical studies for the treatment of OA, adeno-associated virus (AAV) offers several advantages over other well-characterized systems. AAV is non-pathogenic; transduced cells have a low immunogenic profile and it enables persistent transgene expression in many applications. In early experiments, we found that conventional single-strand AAV vectors provided only marginal levels of transgene expression intra-articularly. More recently, we showed that self-complementary (sc; double-stranded) AAV vectors could provide >20-fold enhancement of gene expression, with rapid onset in synovial and capsular cells in vitro and in vivo. We found that scAAV transgene expression levels in the knees of rabbits were similar to those provided by adenovirus, and were sufficient to mediate therapeutic responses in these animals. Additional advances in recombinant AAV technology, including
methods for vector production, have overcome previous technical limitations such that AAV can now be realistically considered as a candidate for human application in OA.

In humans, OA most frequently develops in the knees and hips, large joints that support the weight of the body while standing and during locomotion. Although we have shown that scAAV.hIL-1Ra can effectively block experimental arthritis in the joints of small laboratory animals, the successful scale-up from rodents to humans is often a difficult challenge for gene-based treatments. In vivo, patterns of cellular transduction and ensuing transgenic expression are a function of the biophysical interaction of the vector with the specific target tissues. Critical but unpredictable variables include the following: volume, surface area and composition of the target tissues and extracellular matrices, the different resident cell populations and their densities, the vascularity of the tissues, and the volume and composition of extracellular fluids, among others. In this respect, the small joints of a 100–200 g rodent cannot duplicate the complex milieu of the knee of a 75 000 g human. Indeed, the greater joint size and internal volume, together with the dramatically larger and thicker connective tissues, as well as the increased fluid and compressive forces generated during human locomotion can have a profound influence on the biodistribution of the injected virus, the cell populations that are effectively transduced and the downstream, temporal patterns of transgene expression.

In an effort to model scAAV-mediated gene delivery in large human joints and, in turn, generate a more accurate depiction of its potential as a vector for use in the treatment of OA, we investigated its capacity for gene delivery to the forelimb joints of horses. The equine carpal and metacarpophalangeal (MCP) joints (located in anatomical positions analogous to the human knee and ankle, respectively) are similar in size, architecture and tissue composition to the human knee. Likewise, as these joints carry 60–65% of the horse’s weight during locomotion, they are highly vulnerable to the onset of OA as a consequence of excessive loading imposed by athletic training and racing. Moreover, current diagnostic modalities and clinical treatment for OA are the same in humans and horses. Also, the large joints of horses permit the aspiration of milliliter volumes of undiluted synovial fluid, enabling the direct measurement of therapeutic protein levels. Thus, patterns of therapeutic transgene expression in the treated joint can be directly monitored over time within the same animal.

In this study, we packaged the scAAV2 vector genome in several common capsid serotypes and compared their efficiency of transduction in equine and human synovial fibroblasts in culture. Following these in vitro studies we selected certain serotypes for analysis of gene transfer in vivo following their delivery into the equine joint. The levels and duration of therapeutic transgene expression were compared, as well as the tissues and cell types transduced intra-articularly.

**RESULTS**

Using scAAV vectors packaged in a battery of widely studied AAV capsid serotypes, we first compared the receptiveness to transduction of early passage synovial fibroblasts isolated from equine and human joint tissues. To enable quantitation of transgene expression, we employed the coding sequences for green fluorescent protein (GFP) and human IL-1Ra (hIL-1Ra). GFP was used to determine the percentage of cells effectively transduced with each serotype, and hIL-1Ra as a secreted, quantifiable reporter of therapeutic protein expression. Cells from each species were seeded in parallel into 12-well plates, and 24 h later were infected with scAAV.GFP or hIL-1Ra packaged in serotypes 1, 2, 5, 8 or 9 at doses in 10-fold increments ranging from 10^2 to 10^5 viral genomes (vg) per cell. The conditioned media from cultures infected with scAAV.hIL-1Ra were collected at days 3, 5, 7 and 10 for analysis by enzyme-linked immunosorbent assay (ELISA; Figure 1). Cultures infected with scAAV.GFP were analyzed daily for fluorescence by...
using inverted microscopy, and at day 5 a subset of the cultures was examined using flow cytometry (Figure 2).

Typical of the hIL-1Ra profile shown in Figure 1 for AAV1, we found the effective vector serotypes provided rapid onset of transgene expression in both human and equine cells. Peak levels of hIL-1Ra expression were achieved by days 3–5 post infection and were maintained through the remainder of the experiment. In both the equine and human cell cultures, serotypes 1, 2 and 5 showed the greatest production of hIL-1Ra and GFP (Figures 1b and 2), with serotype 2 providing the highest level of expression. Transgene expression from vectors packaged in serotypes 8 and 9, however, was significantly lower, at near background levels, even at the $10^4$ vg/cell dose.

Notably, for serotypes 1, 2 and 5, the levels of hIL-1Ra produced by the equine cells were dramatically higher (approximately 25, 9 and 50-fold, respectively) than the human cells infected in parallel (Figure 1b). Analysis of GFP expression supported these data and showed that for each of these serotypes, about 3–5 times more equine cells were effectively transduced and expressed GFP at much greater levels, resulting in increases in mean levels of fluorescence between 18- to 20-fold higher than the human cells (Figure 2b).

To gain insight into the biological basis for the disparity in transgene expression between the cells of the two species, we first compared each cell type for relative expression of AAV cell surface receptor molecules. Because of the availability of antibodies with cross-reactivity between equine and human proteins, we examined the cells for expression of receptor molecules for AAV2: heparan sulfate proteoglycan (HSPG; the primary binding receptor) and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (co-receptors involved in virus internalization). In parallel, equal numbers of cells from the equine and human synovial fibroblast cultures were trypsinized and incubated with each antibody individually, followed by a fluorescently tagged secondary antibody. The cells were then analyzed for fluorescence by flow cytometry. As shown in Figure 3, consistent with their heightened transduction with the AAV2 vector, HSPG expression was found to be considerably higher in the equine cells, which appeared uniformly positive for the HSPG content. HSPG expression on the surface of the human cells was not only reduced relative to the equine cells, but the absolute...
levels were strikingly low, just above background. Both cell types expressed high levels of $\alpha_v\beta_3$ integrin, and although $\alpha_v\beta_5$ expression was lower than $\alpha_v\beta_3$ in the equine cells, it was not detectable over the isotype controls in the human cells.

In previous work with synovial fibroblasts from rabbits, we found that the translocation of vector genomes to the nucleus was inefficient, such that following infection the vast majority of the viral DNA within the cells remained in the cytoplasm and less than 20% had translocated to nucleus.\textsuperscript{16} To determine if intracellular trafficking may also contribute to the differences in transduction, we performed similar vector genome trafficking studies in the human and equine cultures. As before, parallel cultures of human and equine cells were infected with $\sim 10^6$ vg/cell of scAAV.GFP packaged in serotype 5. Twenty-four hours post infection, the cells were collected, gently lysed, and the cytoplasmic and nuclear compartments were fractionated. Viral genomes in the respective cell fractions were then quantitated using quantitative PCR. As shown in Figure 4, greater than 20-fold more viral genomes were detected in the equine cells than in the human cells; however, the relative ratios of nuclear to cytoplasmic viral DNA were the same. These data indicate that the efficiency of nuclear importation in both cell types is similarly low (< 25%) and, together with the surface receptor data in Figure 3, indicate that the increased transgene expression observed in the equine cells is due to increased uptake of virions, likely attributable, at least in part, to increased levels of AAV surface receptor molecules.

Having characterized several AAV serotypes for their capacity to transduce equine articular fibroblasts in culture, we selected three of these (AAV2, 5 and 8) for analysis in vitro following intra-articular injection into equine joints. Serotypes 2 and 5 were chosen because they provided the most robust transgene expression in culture, and several reports have examined their utility for intra-articular gene delivery in small animal models. Although serotype 8 generated only trace levels of hIL-1Ra expression in culture in either cell type, it was included as a means to test the validity of the in vitro assays.

Approximately $2 \times 10^{11}$ vg of scAAV.hIL-1Ra of each serotype were injected into both front midcarpal and MCP joints of different groups of horses. As the synovial volumes of these joints (midcarpal, 14.9 ± 0.6 ml and MCP, 12.5 ± 1.0 ml) are nearly equivalent,\textsuperscript{7,20} this delivery strategy enabled us to increase our sample size per animal from two to four. Immediately before vector delivery and at periodic intervals thereafter, synovial fluid was aspirated directly from each of the joints. Recovered joint fluids were analyzed for human IL-1Ra content by ELISA, as well for leukocytic infiltration. Consistent with the in vitro results, scAAV.hIL-1Ra packaged in serotypes 2 and 5 generated meaningful levels of transgenic expression and produced about 1 and 1.5 ng ml$^{-1}$ of human IL-1Ra, respectively, during the first 3 weeks (Figure 5). The hIL-1Ra expression began to diminish by week 5 and returned to baseline by week 10. Somewhat surprisingly, in contrast to our in vitro findings, serotype 8 also produced measurable levels of human IL-1Ra intra-articularly, at levels comparable to serotype 2, and with a similar temporal pattern.

To visualize the relative number and distribution of the cells genetically modified by the different vector serotypes intra-articularly, $\sim 1 \times 10^{12}$ vg of scAAV.GFP of each serotype were injected into the midcarpal or MCP joints of one horse. At day 10 post injection, the animal was killed, and the capsular tissues and cartilage shavings were collected for analysis. Consistent with the results achieved with hIL-1Ra, no meaningful differences were seen among serotypes in the phenotype, number or locations of the cells transduced. Typical of that shown in Figure 6 for serotype 2, direct fluorescence microscopy of freshly collected synovial tissues revealed large numbers of GFP + cells across the entire
The modest differences are likely attributable to consistent with those reported previously for human and horse, relative transduction efficiencies of the serotypes tested are regardless of vector dose. In general, our results regarding the amenable to transduction with vectors packaged in the same joint as a model for AAV-mediated gene transfer to large, weight-bearing human joints, which are frequent sites of disease onset. With the long-term goal of developing an effective gene-based treatment for OA, we performed studies to evaluate the equine joint as a model for AAV-mediated gene transfer to large, weight-bearing human joints, which are frequent sites of disease onset. In vitro, we found that equine and human articular fibroblasts were amenable to transduction with vectors packaged in the same capsid serotypes and were similarly resistant to others. We saw high, dose-dependent transgene expression with serotypes 1, 2 and 5, with negligible transduction from serotypes 8 and 9, regardless of vector dose. In general, our results regarding the relative transduction efficiencies of the serotypes tested are consistent with those reported previously for human and horse, individually. The modest differences are likely attributable to variations in methods for measuring viral titer as well as experimental conditions, including cell source, passage number, and culture and infection conditions.

An intriguing finding of the present studies is the enhanced transduction efficiency of the equine synovial cells relative to those of human origin. During the process of infection, AAV vectors initially attach to the surface of target cells via specific cell surface glycans; serotypes 1 and 5 bind sialic acid variants, whereas AAV2 uses HSPG. After binding, interaction with specific protein co-receptor molecules is thought to stabilize binding and facilitate virus internalization via endocytosis in clathrin pits. Once inside the cell, the virus is transported in endosomes to the nucleus for uncoating and genome release. That synovial cells from both species are receptive and resistant to transduction with the same serotypes in culture indicates that they express similar types of surface antigens and is consistent with their common tissue origin and function. With respect to AAV2 receptors, our data show that equine synovial fibroblasts express moderate to high levels of HSPG and co-receptor molecules, integrins αvβ3 and αvβ5. The HSPG and αvβ5 expression on the surface of the human cells was noticeably reduced relative to the equine cells and was only marginally detectable over background. This is somewhat surprising as, among the variants tested, the human cells were most receptive to vectors packaged in AAV2. Our genome-tracking studies using AAV5 showed that following infection with the same viral dose, the equine cells contained > 20-fold more vector genomes intracellularly. For both cell types, though, only ∼25% of the viral genomes were found in the nucleus, whereas ∼75% remained cytoplasmic. As the intracellular trafficking and nuclear import appear to be similarly inefficient in the cells of both species, the differences in transduction appear to occur at the level of viral entry. Although we have not addressed expression of viral endocytosis, our data thus far suggest that increased transduction in the equine cells is due, at least in part, to heightened expression of primary binding receptor and co-receptor molecules. These phenotypic differences may be attributable to differences in the innate biology of human and equine synovial cells or, as discussed below, may possibly reflect artifact arising from adaptation to growth in monolayer.

In agreement with previous reports in mice, AAV serotypes 2 and 5 were capable of mediating effective intra-articular gene transfer in the equine joint. Following injection of 2 × 1011 vg, scAAV vectors packaged in both serotypes elevated the steady state levels of hIL-1Ra in the synovial fluids to ∼1 ng ml−1, a level shown to have beneficial effects in other model systems.6 Serotype 8, which generated only trace levels of hIL-1Ra expression in equine synovial cells in culture (500- to 1000-fold less than AAV2 or 5), was surprisingly effective and enabled transgene expression at levels comparable to these serotypes in vivo. Interestingly, despite the inability of AAV8 to transduce synovial fibroblasts in culture relative to AAV2 and 5, the locations and apparent cell types transduced by all three vectors intra-articularly were indistinguishable.

At present we have no definitive explanation for this result, but it is likely attributable to alterations in the expression of surface receptor molecules in synovial fibroblasts adapted to growth in monolayer relative to those in the natural context of the joint lining. For example, a known receptor for AAV8, the 67-kDa laminin receptor (67LR), binds with high affinity to laminin 1 found in the articular cartilage, synovial membranes of many tissues, promoting cell adhesion and interaction of laminin with integrin. Expression of 67LR, however, is noted to vary significantly with growth conditions in culture.25 Thus, although in vitro assays can be useful experimental tools, they are not necessarily reflective of the biology of cells in their native context. The data shown here emphasize that caution must be applied when using these methods to screen AAV serotypes for their potential as vectors for gene delivery to articular tissues. In this respect, the use of fresh tissue explants may provide a more representative depiction of the receptiveness of tissues and cell types to transduction with specific AAV capsid variants. In the equine joint, we saw a gradual drop-off in hIL-1Ra transgene expression over a 7-week time period. Similar to

Figure 5. hIL-1Ra levels in synovial fluid following intra-articular injection of scAAV vectors into the forelimb joints of horses. Approximately 2 × 1011 vg of scAAV/hIL-1Ra packaged in serotypes 2, 5 or 8 were injected in a random fashion into the midcarpal and MCP joints of both forelimbs of thoroughbred horses. One of the four joints was injected with saline and served as a negative control. At 1, 3, 5 and 10 weeks post injection, synovial fluid was collected from the joints via arthrocentesis, and the hIL-1Ra content was measured by ELISA. Data points reflect mean values of three injected joints, with error bars indicating ± s.e.m.
previous work in rats,\textsuperscript{11} we attribute this to the xenogenic human transgene we employed as a quantifiable reporter of therapeutic protein expression. In assessing different vectors for efficiency of intra-articular gene delivery, as well as the receptiveness of different animal systems, we have frequently used this cDNA as a reference standard for comparison. The reagents used to measure hIL-1Ra are species specific and provide unambiguous detection of the product against the background of endogenous proteins in animal models. The limitation of this transgene is that it stimulates a T-cell-mediated immune response to the transduced cells, which leads to their elimination in immune competent animals, and thus, abbreviated transgene expression. Since completing the work described here, reagents for sensitive detection of the equine IL-1Ra analog have become commercially available, and we have generated scAAV vectors containing the equine cDNA. We are currently in the process of evaluating expression of this transgene in the equine joint.

Our data show that scAAV vectors packaged in serotypes 2, 5 and 8 are capable of mediating sustained expression of exogenous transgenes at biologically relevant levels following delivery into the forelimb joints of horses. As the equine joint is of similar size and architecture to the human knee, these early results provide optimism that similar methods can be effectively applied to achieve sustained delivery of therapeutic gene products in large human joints frequently affected by OA. The enhanced receptiveness of the equine fibroblasts to AAV transduction in vitro relative to their human counterparts, though, suggests that it may be necessary to administer greater doses of virus to achieve equivalent levels of transgene expression clinically. In this respect, at a dose of $1 \times 10^{12}$ vg we saw no adverse response to treatment.

\textbf{Figure 6.} Locations and phenotypes of the cells transduced by scAAV vectors following intra-articular injection in the equine joint. Approximately $1 \times 10^{12}$ vg of scAAV.GFP packaged in serotypes 2, 5 or 8 were injected into the midcarpal or MCP joints of one horse. Following killing at day 10, joint tissues were collected and analyzed for GFP expression, either directly using inverted fluorescence microscopy or following paraffin section and immunohistochemical staining. The images shown are from a joint injected with serotype 2, but are representative of all three serotypes. (a) Arthroscopic images of the interior of a healthy equine midcarpal joint are shown to illustrate the anatomy and morphology of the articular tissues. The top image shows the smooth, rounded surfaces of articular cartilage with adjacent tissues of the synovial lining. The lower image illustrates the highly villous nature of the synovium. (b) scAAV.GFP expression in the synovium. (c) scAAV.GFP expression in articular cartilage. For both b and c, the images in the top row show the distribution of GFP expression across the surfaces of the freshly collected tissues using direct inverted fluorescence microscopy. The lower panels show GFP expression in each tissue in cross section, following paraffin section and immunohistochemical staining.
either at delivery or any time thereafter, which suggests that considerably greater doses of vector could be safely delivered if necessary. Alternatively, it may be possible to engineer the AAV capsid to generate novel variants that target alternate surface receptors more highly expressed on human synovial cells.

The purpose for moving away from rats to studies in the equine joint is to more closely simulate gene transfer in the setting of large human joints. In this respect, comparison of the transduced cell populations within the equine joint with those observed in the rat provides interesting differences that may have important functional implications. In earlier work we saw significant movement of gene transfer vectors beyond the synovium into the ligaments and tendons of the joint capsule and peri-articular muscle. The cells transduced in these non-synovial tissues appeared to provide more stable transgene expression than those in the synovium. In the larger equine joint, with its dramatically thicker tissues, we saw little apparent movement of the vector beyond the synovial lining. It will be interesting to see how this impacts the stability of expression from a homologous, equine transgene.

An additional consideration relative to the rat is the large volume of viscous synovial fluid in the equine joint (5–10 μl versus > 10 ml) and its potential to affect vector dispersion and ensuing transduction patterns. Moreover, as a result of natural prior infection from wild-type AAV, synovial fluid in humans has been found to contain elevated titers of neutralizing antibodies to several AAV serotypes.

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the cells were trypsinized, incubated in hypotonic buffer for 5 min on ice and lysed in non-ionic detergent. Centrifugation of the lysate allowed the nuclear fraction to be collected as the pellet, whereas the supernatant was reserved as the cytoplasmic fraction. Low molecular weight DNA from each fraction was isolated by Hirt extraction11 and then used for quantitative PCR. Primer pairs were designed to anneal to sequences within the cytomegalovirus promoter sequence. Viral genomes were detected using SYBR Green dye (Applied Biosystems, Carlsbad, CA, USA) in an Eppendorf Mastercycler Reaplex2 (Eppendorf, Hauppaugue, NY, USA). The results were standardized to a dilution series of vector plasmid DNA of known copy number. Three independent experiments were performed, yielding similar results. Values were then expressed as the mean of these experiments.

Intra-articular gene delivery

For intra-articular delivery of scAAV, approximately 2 × 10^11 vg of each vector were diluted into 5 ml of lactated ringers solution. Following surgical scrub of the forelimb joints, vector was injected directly into the midcarpal and MCP joints. Negative control joints received injection of lactated ringers alone. For quantitation of secreted transgene products, synovial fluid was collected from the joints by arthrocentesis at days 7, 21, 35 and 70 post injection, and the recovered fluids were analyzed for hIL-1Ra content by ELISA. Animals receiving vectors with GFP were killed at 35 and 70 post injection, and the recovered fluids were analyzed for hIL-

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CONFLICT OF INTEREST

Dr Ghivizzani has an equity interest in Molecular Orthopaedics Inc., a company pursuing gene-based treatments for arthritic disease. All other authors declare no conflict of interest.

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Gene Therapy (2013) 667 – 670

AAV-mediated gene transfer to the equine joint
RS Watson et al

677