npg
Gene Therapy (2002) 9, 12–20
© 2002 Nature Publishing Group All rights reserved 0969-7128/02 $25.00
www.nature.com/gt

RESEARCH ARTICLE

Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene

DD Frisbie[1], SC Ghivizzani[2], PD Robbins[3], CH Evans[2] and CW McIlwraith[1]

[1]*Equine Orthopaedic Research Laboratory, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA;* [2]*Center for Molecular Orthopedics, Harvard Medical School, Boston, MA, USA; and* [3]*Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*

Osteoarthritis in horses and in humans is a significant social and economic problem and continued research and improvements in therapy are needed. Because horses have naturally occurring osteoarthritis, which is similar to that of humans, the horse was chosen as a species with which to investigate gene transfer as a potential therapeutic modality for the clinical treatment of osteoarthritis. Using an established model of equine osteoarthritis that mimics clinical osteoarthritis, the therapeutic effects resulting from intra-articular overexpression of the equine interleukin-1 receptor antagonist gene through adenoviral-mediated gene transfer were investigated. In vivo delivery of the equine IL-IRa gene led to elevated intra-articular expression of interleukin-1 receptor antagonist for approximately 28 days, resulting in significant improvement in clinical parameters of pain and disease activity, preservation of articular cartilage, and beneficial effects on the histologic parameters of synovial membrane and articular cartilage. Based on these findings, gene transfer of interleukin-1 receptor antagonist is an attractive treatment modality for the equine patient and also offers future promise for human patients with osteoarthritis.
Gene Therapy (2002) **9,** 12–20. DOI: 10.1038/sj/gt/3301608

Keywords: *gene therapy; osteoarthritis; interleukin-1 receptor antagonist; in vivo; equine*

Introduction

The concept of using gene therapy for the treatment of arthritis is now well established,[1] but clinical evidence of its efficacy is lacking.[2] Horses, like humans, are often affected by musculoskeletal diseases including osteoarthritis (OA), but testing of novel therapeutic treatments in horses is less cumbersome than in humans. This makes the horse a favorable species with which to evaluate the clinical efficacy of gene therapy for the treatment of OA. Moreover, once affected by OA, the ability of horses to maintain athletic performance is greatly impeded such that musculoskeletal disease is the most economically important equine disorder.[3,4] Furthermore, similar limitations to those seen in the treatment of human OA also hamper equine treatment.

We have developed an equine model of OA that quickly mimics clinical disease.[5] In addition to having a large volume of tissue available for laboratory study, this equine model is unique in its ability to utilize current clinical parameters of disease monitoring, such as pain and joint effusion scoring, as well as modern imaging techniques. Moreover, unlike other veterinary species the pathophysiology of clinically relevant equine arthritis has been studied and reported for many years.[6] Numerous proof of principle experiments have been carried out using *ex vivo* and *in vivo* gene transfer with retroviral and adenoviral vectors carrying potential anti-arthritic genes.[7,8] Although *ex vivo* methodologies provide for extensive safety testing, and through the use of retroviral vectors, a potential for prolonged transgene expression, these approaches are laborious, have not led to long-term gene expression, and may be cost prohibitive for use in clinical arthritis. Adenoviral vectors provide good transduction efficiencies, but short transgene expression periods, and immune responses to the vectors have limited their widespread clinical use in chronic diseases. However, all studies to date have used heterologous gene sequences. Thus an adenoviral vector carrying a homologous gene sequence was developed for the current study.

Many genes have a therapeutic potential in OA, including IL-1Ra, TNF soluble receptors, IL-4 and IL-10.[9,10] Because several reports in laboratory animals have shown therapeutic benefits of both IL-1Ra protein and gene administration in OA, and because the equine IL-1Ra gene was available to the authors,[9–11] equine IL-1Ra was chosen as the therapeutic gene to be studied.

The purpose of the current study was to evaluate the clinical and laboratory utility of the equine IL-1Ra gene as a treatment for OA using an equine model. The study included: (1) the construction of an adenoviral vector expressing equine IL-1Ra DNA (Ad-EqIL-1Ra); (2) testing the utility of this vector and its anti-IL-1 activity *in vitro*; (3) determining a safe and potentially efficient dose of

Correspondence: DD Frisbie, Equine Orthopaedic Research Laboratory, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, 80523, USA
Received 26 June 2001; accepted 16 October 2001

the vector *in vivo* using normal equine joints; (4) *in vivo* testing of the vector using an established equine OA model. This investigation had several novel features including the use of an autologous gene, and the assessment of improvement using clinical as well as laboratory criteria.

Results

In vitro study

A dose-dependent increase in media concentrations of IL-1Ra was demonstrated 48 h after transduction of equine synoviocytes with Ad-EqIL-1Ra at 0, 1, 10, and 100 MOI, reaching a peak concentration of 39 ng/10^6 cells at 100 MOI (data not shown). Crossreactivity of the human IL-1Ra kit with equine IL-1Ra was confirmed using human and equine Ad-IL-1Ra constructs (data not shown). The biological activity of the IL-1Ra produced by the transduced cells was confirmed by its ability to inhibit PGE_2 production in response to human IL-1α (10 ng/ml) (Figure 1). Human IL-1α has recently been shown to have similar biologic function as equine IL-1α.[12,13]

In vivo dose titration study

Intra-articular injection of the Ad-EqIL-1Ra vector in normal equine joints lead to a dose-dependent increase in the concentration of IL-1Ra in synovial fluid aspirates (Figure 2a). Maximum duration of IL-1Ra production (28 days) occurred with 10 and 20 × 10^{10} particles/joint (Figure 2a).

The highest Ad-EqIL-1Ra concentration (50 × 10^{10} particles/joint) produced a marked, acute synovial fluid leukocytosis. This observation was not seen at any lower viral loads (Figure 2b).

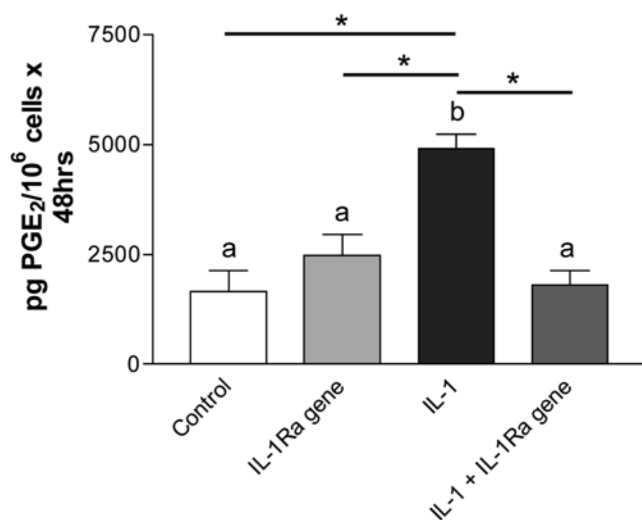

Figure 1 *Inhibition of PGE_2 production in response to IL-1 following cell transduction with Ad-EqIL-1Ra (10 MOI). Transduced and untransduced cells were incubated in vitro with human IL-1α (10 ng/ml) or the equivalent volume of saline. After 48 h, media were harvested and analyzed for PGE_2 by ELISA. Values represent the mean ± standard error (error bars), different letters indicate a statistical difference between groups (P value < 0.05), specifically in this graph the IL-1 treatment group (b) had a significantly higher concentration of PGE_2 as compared to any other treatment group (a). Lines with an asterisk (*) linking treatment groups also indicate a statistical difference between treatment groups.*

In vivo OA model

In this model OA was induced in a solitary joint while the contralateral joint served as a control (normal joint). In this study half of the horses were administered treatment (intra-articular Ad-EqIL-1Ra) in a joint with experimental OA while the contralateral joint and the other half of the horses were untreated.

Clinical examinations: Throughout the duration of the study no horse was observed with a lameness that prohibited humane participation in the study. Seventy days after the induction of OA and 56 days following IA administration of Ad-EqIL-1Ra, significant improvement in clinical pain was observed in treated compared to untreated horses (Figure 3a). The EqIL-1Ra gene also reduced the synovial effusion score (Figure 3b).

Significantly higher radiograph lesion scores were observed in all joints with OA compared to those without (1.8 ± 0.1 and 0.0 ± 0.1, respectively mean ± s.e.m.). Although treatment with the EqIL-1Ra gene lowered the average scores, it was not statistically significant when joints with OA were compared between treated and untreated horses (1.7 ± 0.2 and 1.9 ± 0.2, respectively).

Synovial fluid analysis: No significant changes in the synovial fluid color, mucin clot scores or total protein content were seen in any comparisons made. IL-1Ra concentrations were significantly increased in joints transduced with Ad-EqIL-1Ra on days 7, 14 and 21 after transduction (days 21, 28 and 35 after lesion creation) (Figure 4).

Postmortem examination: All joints where OA was induced contained some level of pathologic change in the form of partial or full-thickness articular cartilage erosions, mostly in a site remote to the osteochondral fragment used to induce the OA. Furthermore, the most pronounced full thickness erosions were observed independent of 'kissing' lesions adjacent to the osteochondral fragment, suggesting that these lesions were a result of OA secondary to the surgery. The most dramatic changes were noted in OA joints from untreated horses (Figure 5).

Histologic evaluation of synovial membrane: In this model, OA is accompanied by a slight synovitis. Transfer of the EqIL-1Ra failed to reduce the level of lymphocytic infiltration of the synovium; indeed, infiltration was higher in the presence of the EqIL-1Ra gene (Figure 6). The cellular infiltration was primarily characterized by perivascular lymphocytic infiltration (Figure 6). Osteoarthritic joints also had significantly more intimal hyperplasia compared with control joints (1.5 ± 0.3 *versus* 0.7 ± 0.3, P value 0.0498), and although the administration of Ad-EqIL-1Ra lessened these scores (0.9 ± 0.3), this difference was not statistically different (P value 0.1546). The scores for subintimal edema were significantly higher for OA joints (1.8 ± 0.4) compared with normal joints (0.9 ± 0.4) from horses receiving Ad-EqIL-1Ra, and although they were higher than those from OA joints of untreated horses (0.8 ± 0.4), the difference was not statistically different (P value 0.0762). No significant differences were noted for subintimal fibrosis, but all joints from Ad-EqIL-1Ra treated horses had significantly lower vascularity scores compared with those from untreated horses (Figure 6).

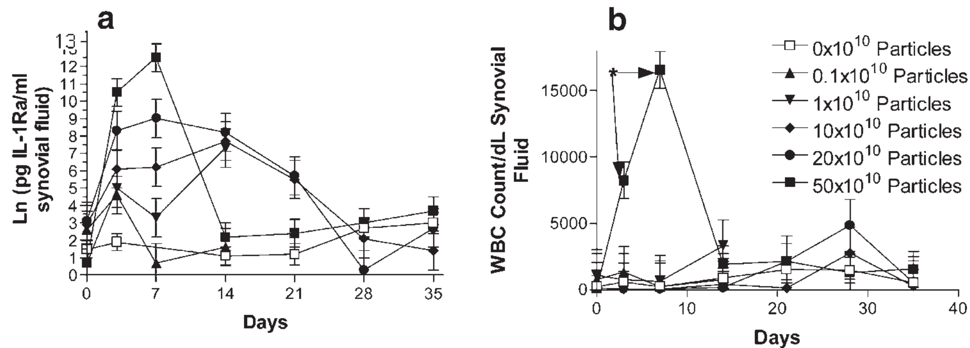

Figure 2 Production of EqIL-1Ra in vivo following intra-articular injection of various amount of vector (A). Effect of intra-articular injection of vector on synovial fluid leukocytosis (B). Asterisks (∗) denotes a statistical difference (P value < 0.05) in the data point compared to untreated joints at that time period.

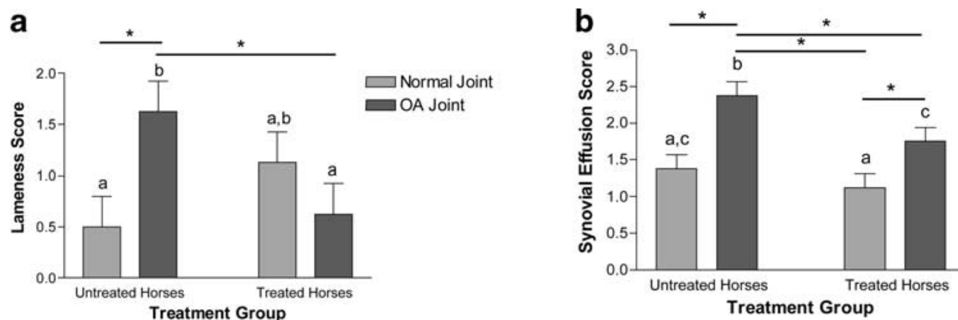

Figure 3 Effect of gene transfer 70 days after surgery on lameness (A) and the synovial effusion score (B). Different letters indicate a statistical difference (P value <0.05) between bars. Lines with an asterisk (∗) linking treatment groups also indicate a statistical difference between treatment groups.

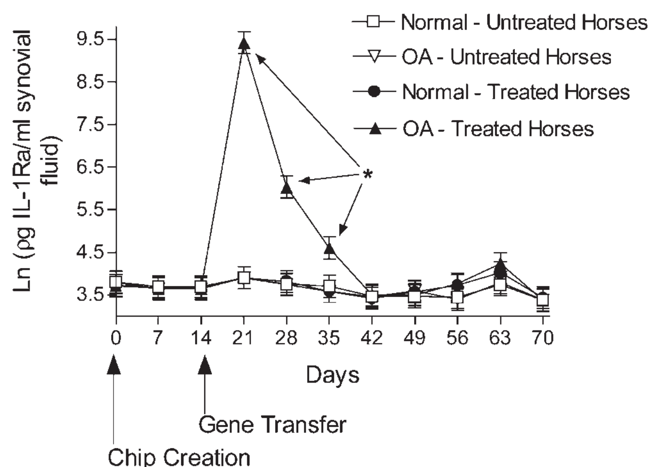

Figure 4 Expression of EqIL-1Ra in joints of treated and untreated horses. An asterisk (∗) denotes a statistical difference (P value < 0.05) in the data point compared to all other data points at that time period.

Histologic and histochemical evaluation of articular cartilage: Five-micron articular cartilage sections were evaluated from three different locations within each joint for fibrillation, chondrocyte necrosis, chondrocyte cloning (chondron formation), and focal cell loss. Because similar results were seen from each location the results for each category were totaled for all sections. The score for chondrone formation was significantly higher from OA joints independent of treatment as compared to normal joints; no other categories had statistically significant findings.

Articular cartilage sections stained with Safranin-O and fast green (SOFG) were also evaluated from 3 different areas within each joint. Similar results were obtained for the staining patterns from each zone, as well as from all locations within the joint. Therefore, for ease of reporting a total score for SOFG staining was determined (0–48 possible score). Osteoarthritis reduced the SOFG score, and this effect was reversed by treatment with the EqIL-1Ra gene (Figure 7).

Cartilage matrix synthesis: Articular cartilage was harvested from one location to evaluate proteoglycan synthesis at day 70 after surgery. Results of the $^{35}SO_4$ incorporation binding assay indicated a significantly higher proteoglycan synthesis overall in OA joints (independent of treatment) (3427 ± 360 c.p.m./mg dw) as compared with normal joints (2541 ± 369 c.p.m./mg dw). Specifically comparing Ad-EqIL-1Ra (3823 ± 522 c.p.m./mg dw) and untreated (3030 ± 522 c.p.m./mg dw) OA joints, there was a higher proteoglycan synthesis in Ad-EqIL-1Ra treated joints, although this difference was not statistically different (*P* value 0.2944).

Discussion

This work describes the construction of an adenoviral vector expressing a biologically active equine IL-1Ra transgene. The vector was then used to deliver this gene intra-articularly in horses and its efficacy as a treatment of an experimental model of OA was evaluated.

In vitro studies confirmed that transduction of articular cells with Ad-EqIL-1Ra led to the synthesis of large

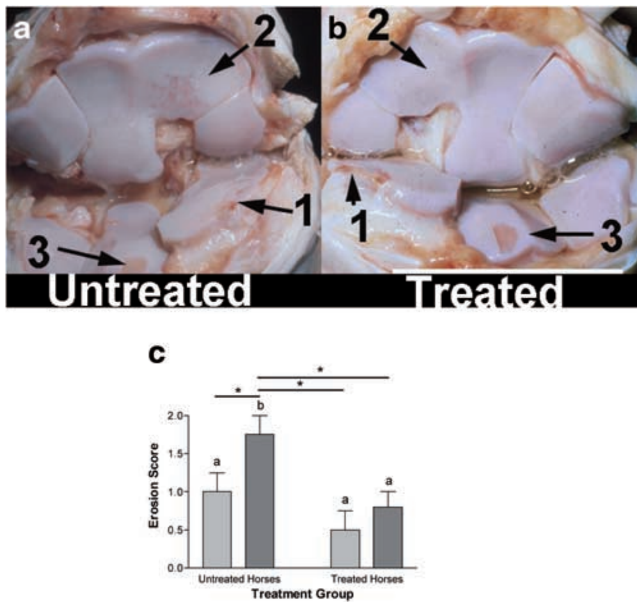

Figure 5 *Effect of osteoarthritis and gene transfer on cartilage erosion. Photographs of the intercarpal joint highlighting third carpal bone lesions in OA joints of both untreated (a) and Ad-EqIL-1Ra (b) treated horses. Note more extensive full-thickness articular cartilage erosions in the untreated joint especially in areas of the third carpal bone (2) bone not adjacent to the osteochondral fragment (1). Photos were taken after aseptic harvest of cartilage from the intermediate carpal bone (3). Plot of cartilage erosion scores by treatment group (c). Different letters associated with bars indicate a statistical difference (P value <0.05) between bars. Lines with an asterisk (∗) linking treatment groups also indicate a statistical difference between treatment groups.*

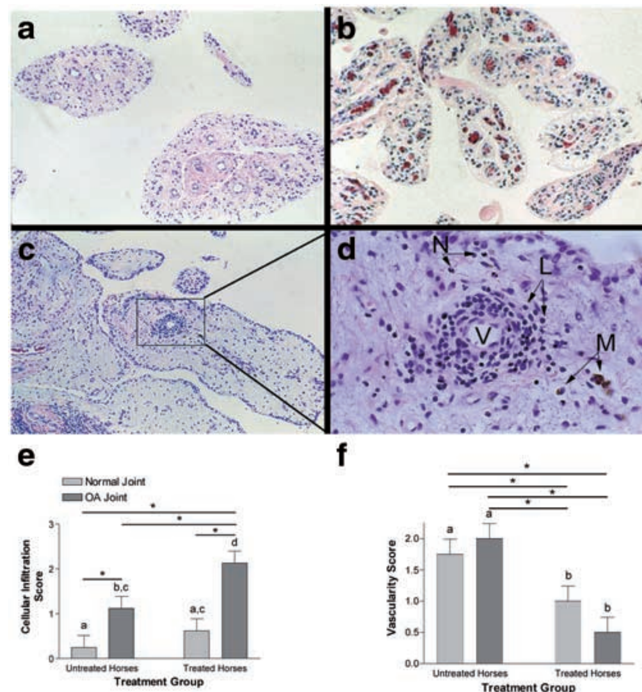

Figure 6 *Photomicrograph from 5-μm sections of synovial membrane stained with H&E. Plate (a) is a representative area of synovial membrane with slight-mild increased vascularity harvested from a normal joint of an Ad-EqIL-1Ra treated horse. Plate (b) is a representative area of synovial membrane with mild-moderated increased vascularity harvested from an OA joint of an untreated horse. Plate (c) is a representative area showing one of the most severe examples of perivascular lymphocytic infiltration (L), neutrophils (N) and macrophages (M). These can also be seen in the 40× magnification (D) of tissue from an OA joint treated with Ad-EqIL-1Ra. Plates are at 4× unless otherwise noted. Effects of OA and gene transfer on cellular infiltration of the synovial membrane are shown quantitatively in panel (e). Effect of gene transfer on synovial vascularity are shown in panel (f). Different letters associated with bars indicate a statistical difference (P value <0.05) between bars. Lines with an asterisk (∗) linking treatment groups also indicate a statistical difference between treatment groups.*

amounts of biologically active equine IL-1Ra. In the *in vivo* dose response study, peak IL-1Ra levels were observed at the highest concentration of vector. However, this concentration was also associated with the shortest transgene expression period. At 10 and 20 × 10^{10} Ad-EqIL-1Ra particles/joint, intraarticular IL-1Ra concentrations were similar to those previously reported to have anti-arthritic effects.[14–19] Moreover, the duration of detectable gene expression was three to four times longer than that seen by other investigators using intraarticular adenoviral vectors.[14,20] Differences in viral preparation or the reactions of equine joints to adenovirus and to viral protein products are possibly responsible for these findings. However, the current study is novel in the use of an autologous gene in an adenoviral vector. The expression of equine IL-1Ra in an equine joint is unlikely to provoke an immune reaction to the transgene product, and this may well attribute to the extended period of expression as compared to previously reported studies.

A number of studies have been conducted to assess the effects of IL-1Ra administration on normal and diseased joint tissues.[15–17,19–27] Furthermore, to circumvent the need for repeated protein administration, some of these studies utilized gene transfer to obtain sustained IL-1Ra levels.[15–17,19,20,25,27,28] However, the study presented here not only utilized a species with naturally occurring OA, but the IL-1Ra gene was expressed in the species from which it was obtained and is the first to use an autologous IL-1Ra gene in an adenoviral vector. Furthermore, use of horses allowed clinical examinations similar to those utilized by human physicians to be performed.

Clinical examinations of the horses indicated that the therapeutic expression of IL-1Ra significantly decreased signs of joint pain as measured by degree of lameness. The amount of synovial effusion associated with the osteochondral fragments was also significantly decreased in joints administered Ad-EqIL-1Ra. Although a relatively non-specific evaluation of joint pathology, the degree of synovial effusion is often a good indicator of disease activity in both humans and horses.[29–31]

Postmortem examinations indicated fewer gross pathologic changes in OA joints administered Ad-EqIL-1Ra compared to joints from untreated horses. Others have also reported less articular cartilage erosions associated with IL-1Ra administration both through direct IA administration of the protein[27] and gene transfer techniques.[17] In previous reports, IL-1Ra treatment was administered within 48 h of the experimentally induced joint pathology, unlike the current study where treatment was not instituted until 14 days after induction of pathology, a time frame more consistent with early OA in horses. These findings are very promising for the long-term health of joints that have already sustained trauma and are only subsequently treated with IL-1Ra.

The administration of Ad-EqIL-1Ra was associated

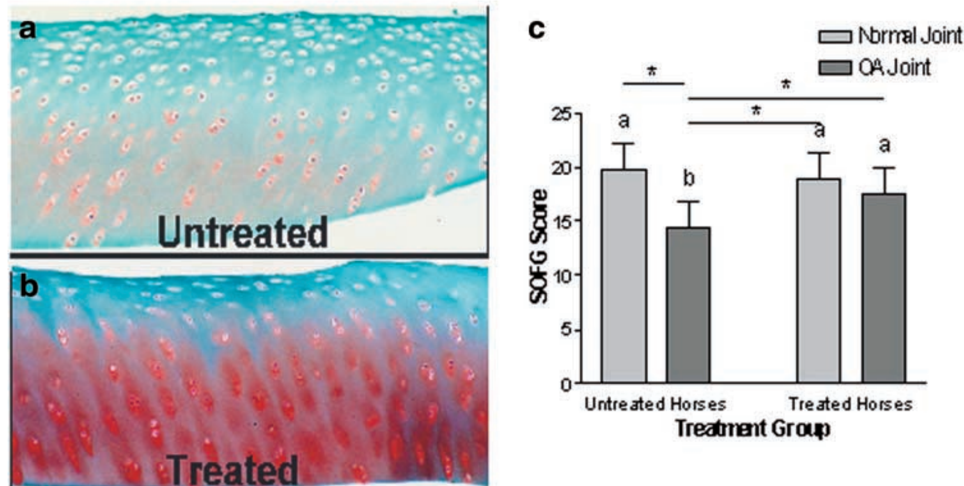

Figure 7 *Effect of osteoarthritis and gene transfer on cartilage histology. Photomicrographs from 5-μm sections of articular cartilage stained with SOFG are shown. Plate (a) is a representative area of OA cartilage showing little or no stain uptake in all areas. The sample was harvested from an OA joint of an untreated horse. Plate (b) is a representative area showing moderate stain uptake patterns in all areas. The tissue was harvested from an OA joint with Ad-EqIL-1Ra treated horse. Effect of OA and gene transfer on cartilage staining scores (c). Different letters associated with bars indicate a statistical difference (P value <0.05) between bars. Lines with an asterisk (*) linking treatment groups also indicate a statistical difference between treatment groups.*

with a mild synovial membrane perivascular lymphocytic infiltration and subintimal edema 54 days after administration. The creation of an osteochondral lesion within the joint was also associated with similar changes but not to the same extent. The administration of Ad-EqIL-1Ra had beneficial effects on the degree of synovial membrane vascularity in both joints of the Ad-EqIL-1Ra treated horses. An increase in synovial membrane vascularity is a hallmark of active synovitis and improvement in this parameter could be beneficial.[6,32,33]

Articular cartilage sections obtained from OA joints of untreated horses had significantly less SOFG staining as compared to all other evaluated sections. This finding suggests that IL-1Ra had a protective effect on proteoglycan loss and is consistent with SOFG data observed by others.[17] Proteoglycan synthesis was higher in OA joints and the highest levels existed in Ad-EqIL-1Ra treated joints. It is interesting that the IL-1Ra gene increased proteoglycan synthesis in non-arthritic joints, suggesting a role for IL-1 in the homeostatic regulation of proteoglycan metabolism and potential remote site effects. However, without a statistically significant difference, further speculation is unwarranted. Thus, these data are in agreement with a growing body of evidence to suggest that IL-1Ra has good chondroprotective properties, but is only partially active against synovitis.

In summary, the work presented here reports the therapeutic effect of IL-1Ra utilizing an established equine model of OA that not only evaluates histologic and biochemical parameters of OA but also allows clinical evaluation (Table 1). This is the first study to demonstrate a clinical improvement in OA as a result of gene therapy. Based on the significant improvements in clinical, gross and histologic examinations of OA joints treated with the EqIL-1Ra gene this therapy is practical and efficient for the equine OA patient and may offer promise for human patients in the future.

Table 1 Summary of the effects of equine IL-1Ra gene therapy on various aspects of equine experimental OA.

Variable	*Improved*	*Worsened*	*Unaffected*
Leukocytosis		+	
Edema			+
Fibrosis	+		
Macroscopic lesions	+		
Histologic Score	+		
GAG Loss (SOFG)	+		
Radiography			+[a]
Clinical lameness	+		
Effusion Scores	+		

[a]Trend towards improvement, but not statistically significant.

Materials and methods

In vitro study

Cell culture: Equine synovium was harvested, digested using 0.2% collagenase in Ham's F12 media for 4 h in a 37°C, 5% CO_2 environment with gentle agitation. Each liter of standard Ham's F12 was supplemented with 25 ml 1 M HEPES, 200 ml fetal calf serum, 50 mg ascorbic acid, 300 mg L-glutamine, 30 mg α-ketoglutaric acid, and 20 000 IU penicillin and streptomycin. After collagenase digestion, the cell suspensions were strained to remove undigested tissue using 4-ply gauze and cell numbers were quantified. Cells (5×10^5 cells/flask) were cultured in 25 cm^2 culture flasks containing 4 ml of supplemented media in a 37°C and 5% CO_2 environment. The media were changed at 7-day intervals unless otherwise noted.

Transductions: All cells were grown to approximately 60–70% confluency before transduction experiments were started. Flasks were rinsed twice with sterile phosphate

buffered saline (PBS) and 2 ml of serum-free medium were added to each flask. The appropriate volume of viral preparation was then added to each flask, followed by gentle rocking in a 37°C, 5% CO_2 environment for 4 h. Cells were then rinsed twice with PBS and serum containing supplemented media added to the flasks. The cells were maintained in a 37°C, 5% CO_2 environment unless otherwise noted.

Vector: Recombinant adenovirus containing the coding sequence of equine IL-1Ra (Ad-EqIL-1Ra) was constructed by *crelox* recombination.[34] Equine IL-1Ra (Accession, U92482) was engineered to contain *Bam*HI restriction sites on either side of the coding sequence (nt 14–548) using upstream (5′-GGTTGT**GGATCC**AGGATG GAAATCCGCAGG) and downstream (5′-GTCTCT**GGA TCC**ATCGACATGCTGGGAATAGG) PCR primers. Sequences containing a *Bam*HI site are shown in bold. The PCR products were digested with *Bam*HI, purified from a 1.5% agarose gel and ligated into the pAdlox adenoviral shuttle plasmid.[34] After restriction digestion of the pAdlox plasmid with *Sfi*I, recombinant virus was generated by co-transfection of the digested DNA with purified genomic DNA from the ψ5 adenoviral helper virus into CRE8 cells. After several days, plaques were isolated, expanded and characterized for insertion of the Eq-IL-1Ra cDNA and its expression. To generate stocks of virus, confluent flasks of CRE8 cells were infected with the Ad-EqIL-1Ra virus. After detection of significant cytopathic effects, the cells were harvested, pelleted, resuspended in 5 ml of saline and stored at −80°C. To purify the virus, the cell pellet was lysed by three rounds of freeze–thaw, cell debris was pelleted by centrifugation, and the cleared lysate was collected. Virus was banded three times over successive cesium chloride step gradients. After dialysis against buffer containing 4% sucrose, 10 mM Tris HCl, 150 mM NaCl, and 10 mM $mgCl_2$ at pH 7.8, the virus was collected, aliquoted and stored at −80°C until use.

IL-1Ra and PGE_2 detection: Concentrations of IL-1Ra and PGE_2 were estimated from collected media stored at −80°C. Media aliquots were used for determination of both IL-1Ra (Quantikine Human IL-1Ra immunoassay, R&D Systems, Minneapolis, MN, USA) and PGE_2 (TiterZyme PGE_2 enzyme immunoassay kit, PerSpetive Biosystems, Inc., Framingham, MA, USA) utilizing commercially available kits according to manufacturers' recommendations.

In vivo dose titration study

Experimental design: The institutional Animal Care and Use Committee approved all *in vivo* studies. Six horses (aged 2–5 years) were utilized for determining the optimal therapeutic range for Ad-EqIL-1Ra. One equine midcarpal and metacarpophalangeal joint (on opposite limbs) were transduced with one of six doses of virus (0.0, 0.1, 1.0, 10, 20, or 50 × 10^{10} particles/joint of Ad-EqIL-1Ra) while the contralateral joint served as the placebo-treated control, receiving a similar volume of Gey's balanced salt solution (GBSS). Synovial fluid was collected through routine arthrocentesis performed prior to transduction or placebo treatment, 3 and 7 days and weekly thereafter after or until IL-1Ra was no longer detected in the synovial fluid. Synovial fluid was analyzed through conventional methods and IL-1Ra quantified. Synovial volumes of both joints (midcarpal, 14.9 ± 0.6 ml and metacarpophalangeal, 12.5 ± 1.0 ml) were considered equilvalent for the purposes of this study.[35]

Conventional synovial fluid analysis: Synovial fluid color, clarity and mucin content were evaluated subjectively, and total protein and inflammatory cell (WBC) concentrations were determined by use of routine clinicopathologic methods. A previously reported numeric grading scale was used for each measured subjective parameter.[5]

Joint effusion and lameness examinations: Evaluation of joint effusion and pain (lameness) were conducted as previously described.[36,37] Briefly, before synovial fluid arthrocentesis, joints were subjectively graded on a scale of 0–4 as having normal, slight, mild, moderate, or severe synovial effusion. At similar time points each limb was assigned a numeric grade of pain (lameness) based on a standardized scale (grade 0 is normal and grade 5 the most severe degree of pain).[37] Carpal flexion was also performed for 45 s, after which time the horse was jogged at a consistent gait, and the response to flexion of the carpus was graded on a scale of 0 to 4 with 0 indicating no response and 4 a severe response. This test can be considered similar to human pain assessment during range of motion evaluation.

In vivo OA model

Experimental design: Sixteen skeletally mature horses, aged 2–5 years, were used in the study. Horses were in good health, without palpable effusions or radiographic abnormalities, and free of lameness before and after joint manipulation. The horses were divided into two equal groups, treated and untreated. All horses had an osteochondral fragment created in one randomly selected intercarpal joint, to produce an experimental OA, and the opposite joint served as the control (Figure 8). Fourteen days after surgery, treated horses received 20 × 10^{10} Ad-EqIL-1Ra viral particles/joint diluted to a total volume of 1 ml with GBSS in their joint with a lesion, while the opposite non-fragmented (normal) joint received a similar volume of GBSS. The 'untreated' group of horses received IA administration of 1ml GBSS in both intercarpal joints (Figure 8).

Induction of osteoarthritis: Briefly, the osteochondral fragments were created as follows. An 8 mm osteochondral fragment was created[5] in one randomly chosen intercarpal joint of each horse. Exposed subchondral bone between fragment and parent bone was debrided using a motorized arthroburr to form a 15 mm wide defect bed (Figure 8).[5,36] The size, location of the fragment, loss of bone, and subsequent synovitis mimic naturally occurring equine OA. Diagnostic arthroscopy was also performed on the contralateral intercarpal joint to confirm the absence of any significant lesions.

Exercise protocol and clinical examinations: After surgery, all horses were housed in 3.65 m by 3.65 m stalls unless otherwise noted. Exercise on a high-speed treadmill began on day 14 after fragment creation and continued 5 days per week until day 70, simulating athletic

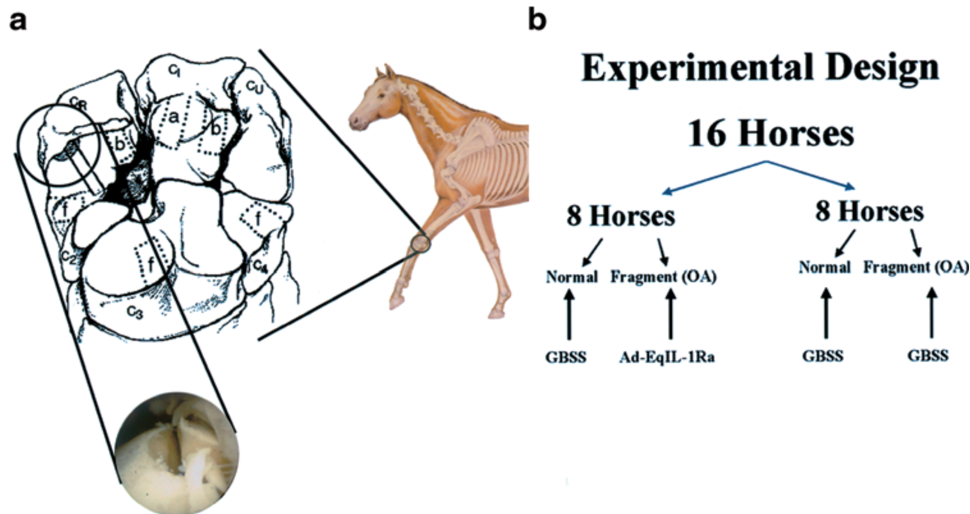

Figure 8 Dorsal view of the equine intercarpal joint depicting areas of specific tissue sampling and the osteochondral fragment (a). a, area from which articular cartilage was harvested for estimation of proteoglycan synthesis; b, areas from which articular cartilage was harvested for analysis of GAG content; f. areas from which articular cartilage was harvested for histopathology. The filled in area in the radiocarpal bone (CR) represents the osteochondral fragment, the solid lines running through this region represent the section of bone harvested for routine histopathology. Arthroscopic view of radiocarpal bone after fragment creation but prior to bone and cartilage debridment (c). Diagram outlining the experimental design of the in vivo OA model (b). CI, intermediate carpal bone, CU, ulnar carpal bone; C2, second carpal bone; C3, third carpal bone; and C4, fourth carpal bone.

conditions commonly seen in horses developing osteochondral fragmentation and subsequent OA.

Synovial effusion, pain (lameness) and joint flexion clinical examinations were repeated on days 14 and 70 after surgery and were conducted as previously described.[36] Bilateral carpal radiographic examinations (anterior–posterior and flexed lateral) were performed before, and 70 days after OA induction. Radiographs were graded by a radiologist unaware of treatment assignment, for changes in the subchondral bone and for periarticular osteophyte formation. A grading scale 0–3 was utilized to statistically evaluate radiographic changes (0 = no significant changes, 1 = focal subchondral bone lysis on either view, 2 = diffuse, shallow subchondral bone lysis, 3 = deep subchondral bone lysis with or without bone fragmentation).

Synovial fluid analysis: Synovial fluid was collected at the time of surgery and nine additional evenly spaced time periods between surgery and the termination of the experiment 70 days after surgery. An aliquot of synovial fluid was immediately evaluated and graded using routine clinicopathologic parameters, as previously described.[38] The remaining synovial fluid was stored at −80°C for measurement of IL-1Ra and PGE_2 concentrations as previously described

Postmortem examination: The horses were killed with an overdose of sodium pentobarbital. Each intercarpal joint was aseptically prepared and opened for harvest of cartilage and synovial membrane. Photographic documentation of joint tissue pathology was then performed. The extent and location of articular cartilage erosions were recorded as none, slight, mild, moderate or severe (numeric values assigned 0–4, respectively).

Histologic evaluation of synovial membrane: Synovial membrane and joint capsule were harvested dorsal to the osteochondral fragment, and placed in 10% buffered formalin for hematoxylin and eosin staining (H&E). Five micron sections were evaluated and graded by an evaluator unaware of treatment assignments for cellular infiltration, synovial intimal hyperplasia, subintimal edema, subintimal fibrosis and vascularity.[5,36] Each variable was graded and reported as a numeric value 0 to 4 (0 = normal, 1 = slight change, 2 = mild change, 3 = moderate change, and 4 = severe change).

Histological and histochemical evaluation of articular cartilage: Articular cartilage pieces, 5 mm^2, were obtained from each joint (Figure 8) and stored in 10% buffered formalin for 7 days followed by routine histologic processing. Half of the 5-μm sections obtained were stained with H&E, and the remainder with safranin-O fast green (SOFG).

Sections stained with H & E were evaluated blindly for articular cartilage fibrillation, chondrocyte necrosis, chondrone formation (chondrocyte division within a lacuna) and focal loss of cells.[5,36] Numeric values ranging from 0–4 were assigned to each measured parameter, as preciously described.[5] Total scores were determined and reported for each section with a total possible score of 16.

Articular cartilage sections stained with SOFG were evaluated blindly for intensity of staining in the tangential, intermediate, radiate territorial and radiate interterritorial zones.5, 36 Numeric values ranging from 0 indicating no stain uptake to a 4 for normal stain uptake were assigned to each measured parameter, with a possible total score of 16.[5]

Articular cartilage matrix evaluation: To estimate articular cartilage proteoglycan content the total articular cartilage glycosaminoglycan (GAG) content was measured using previously reported 1,9-dimethyl methylene blue (DMMB) technique.[5] Articular cartilage pieces were obtained aseptically from the locations within each joint (Figure 8), and each piece was stored at −80°C before further processing and analysis. Samples were run in

duplicate, and samples reported as μg GAG/mg cartilage dry weight (dw).

For analysis of cartilage matrix metabolism, articular cartilage samples were also aseptically collected from the weight-bearing surfaces within each joint (Figure 8) and $^{35}SO_4$ incorporation measured using previously reported methods.[39] Samples were run in duplicate and the results reported as counts per minute (c.p.m.)/mg dry weight.

Statistical analysis

Results were analyzed using a Mixed model analysis of variance.[40] Residual plots were constructed to test for fulfillment of model assumptions; if model assumptions were not met, data transformation was performed and reported. When individual comparisons were made a Least Squares Mean procedure was utilized[41] and a P value <0.05 was considered significant.

The *in vitro* study used the vector concentration as the independent variable. Synovial fluid samples collected in the *in vivo* dose titration study utilized the following independent variables: joint location (intercarpal or metacarpophalangeal joints), treatment (administration of Ad-EqIL-1Ra or GBSS), day of sample collection, and all interactions between main effect variables. The subject (horse) was considered a random variable.

In the *in vivo* OA model dependent variables measured at more than two time points were analyzed using the following independent variables; day of sample collection, presence or absence of a fragment within the joint/limb, and treatment, measuring if the horse was treated with (Ad-EqIL-1Ra) or untreated (GBSS). The subject within treatment variables, as well as, the interaction between chip and subject horse within treatment variables were used as random effect variables. The time (day of collection) variable was also utilized in the repeated statement with the interaction between and subject within treatment variables acting as the subject, using a type I auto-regressive covariance matrix. Dependent variables measured at two or less time points had a similar analysis performed. However, the model statement only utilized the presence of chip, treatment and the interaction between these variables, and the random statement only utilized the subject within treatment variables.

Acknowledgements

This work was supported by the American Association of Equine Practitioners; College Research Council, Colorado State University; and the Southern California Equine Foundation. The authors would like to acknowledge Dr Rick D Howard for kindly providing phagemid containing the EqIL-1Ra gene and Heather Colhoun, Emily Sandler, Jennifer Goodnight, and the Veterinary Teaching Hospital's Volunteer program for technical assistance, making this work possible.

References

1 Evans CH, Robbins PD. Gene therapy for arthritis, In: Wolff JA (ed.). *Gene Therapeutics: Methods and Applications of Direct Gene Transferm*. Birkhauser: Boston, 1994, pp 320–343.
2 Evans CH *et al*. Clinical trials in the gene therapy of arthritis. *Clin Orthop* 2000; **379**: 300–307 (Suppl.).
3 Auer J, Fackelman G. Treatment of degenerative joint disease of the horse a review and commentary. *Vet Sur* 1981; **2**: 80–90.
4 Vachon A, McIlwraith CW. Articular cartilage healing: Current concepts, In: White NA, Moore JN (eds). *Current Practice of Equine Surgery*, 1st edn. Lippincott: Philadelphia, 1990, pp 539–645.
5 Frisbie DD *et al*. The effects of 6-alpha methylprednisolone acetate on an in vivo equine osteochondral fragment exercise model. *Am J Vet Res* 1998; **12**: 1619–1628.
6 McIlwraith CW. General pathobiology of the joint and response to injury, In: McIlwraith CW, Trotter GW (eds). *Joint Disease in the Horse*, 1st edn. WB Saunders Company: Philadelphia, 1997, pp 40–70.
7 Evans CH *et al*. Gene therapy for rheumatic diseases. *Arthritis Rheum* 1999; **42**: (1) 1–16.
8 Evans CH, Robbins PD. Potential treatment of osteoarthritis by gene therapy. *Rheum Dis Clin North Am* 1999; **25**: 333–344.
9 Muzzonigro TS *et al*. The role of gene therapy. Fact or fiction? *Clin Sports Med* 1999; **18**: 223–239.
10 Evans CH, Robbins PD. Gene therapy of arthritis. *Intern Med* 1999; **38**: 233–239.
11 Howard RD *et al*. Cloning of equine interleukin 1 receptor antagonist and determination of its full-length cDNA sequence. *Am J Vet Res* 1998; **9**: 712–716.
12 Takafuji VA, McIlwraith CW, Howard RD. Effects of recombinant equine interleukin-1alpha and interleukin-1beta on proteoglycan metabolism and prostaglandin E2 synthesis in equine articular cartilage explants. *Am J Vet Res* 2001 (accepted).
13 Frisbie DD *et al*. Metabolic mitogentic activities of insulin-like growth factor-1 in interleukin-1-conditioned equine cartilage. *Am J Vet Res* 2000; **61**: 436–441.
14 Roessler BJ *et al*. Inhibition of interleukin-1 induced effects in synoviocytes transduced with the human IL-1 receptor antagonist cDNA using an adenoviral vector. *Hum Gene Ther* 1995; **6**: 307–316.
15 Bandara G *et al*. Intraarticular expression of biologically active interleukin-1 receptor antagonist protein by ex vivo gene transfer. *Proc Natl Acad Sci USA* 1993; **90**: 10764–10768.
16 Hung G *et al*. Suppression of the intraarticular response to interleukin-1 by transfer of the interleukin-1 receptor antagonists gene to synovium. *Gene Therapy* 1994; **1**: 64–69.
17 Pelletier JP *et al*. *In vivo* suppression of early experimental osteoarthritis by interleukin-1 receptor antagonist using gene therapy. *Arthritis Rheum* 1997; **40**: 1012–1019.
18 Muller-Ladner U *et al*. Human IL-1Ra gene transfer into human synovial fibroblasts is chondroprotective. *J Immunol* 1997; **158**: 3492–3498.
19 Otani K *et al*. Suppression of antigen-induced arthritis in rabbits by *ex vivo* gene therapy. *J Immunol* 1996; **156**: 3558–3562.
20 Roessler BJ *et al*. Adenoviral-mediated gene transfer to rabbit synovium *in vivo*. *J Clin Invest* 1993; **92**: 1085–1092.
21 Arend WP *et al*. Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest* 1990; **85**: 1694–1697.
22 Smith RJ *et al*. Biologic effects of an interleukin-1 receptor antagonist protein on interleukin-1 stimulated cartilage erosion and chondrocyte responsiveness. *Arthritis Rheum* 1991; **34**: 78–83.
23 Henderson B *et al*. Inhibition of interleukin-1 induced synovitis and articular cartilage proteoglycan loss in the rabbit knee by recombinant human, interleukin-1-receptor antagonist. *Cytokine* 1991; **3**: 246–249.
24 Van Lent PL *et al*. Major role for interleukin 1 but not for tumor necrosis factor in early cartilage damage in immune complex arthritis in mice. *J Rheumatol* 1995; **22**: 2250–2258.
25 Makarov SS *et al*. Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA. *Proc Natl Acad Sci USA* 1996; **93**: 402–406.
26 Bresnihan B *et al*. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* 1998; **41**: 2196–2204.
27 Caron JP *et al*. Chondroprotective effect of intraarticular injections of interleukin-1 receptor antagonist in experimental osteoarthritis. *Arthritis Rheum* 1996; **39**: 1535–1544.

28 Evans CH, Robbins PD. Clinical trial to assess the safety, feasibility, and efficacy, of transferring a potentially anti-arthritic cytokine gene to human joints with rheumatoid arthritis. *Hum Gene Ther* 1996; **7**: 1261–1280.
29 Todhunter R, Lust G. Pathophysiology of synovitis: Clinical signs and examination in horses. *The Compendium* 1990; **12**: 979–992.
30 McIlwraith CW. General pathobiology of the joint and response to injury, In: McIlwraith CW, Trotter GW (eds). *Joint Disease in the Horse*, 1st edn. Saunders Company: Philadelphia, PA, 1996, pp 40–69.
31 Pillemer SR *et al*. Meaningful improvement criteria sets in a rheumatoid arthritis clinical trial. MIRA Trial Group. Minocycline in Rheumatoid Arthritis. *Arthritis Rheum* 1997; **40**: 419–425.
32 Henderson B, Pettipher ER. The synovial lining cell: biology and pathobiology. *Semin Arthritis Rheum* 1985; **15**: 1–32.
33 Pelletier J *et al*. Role of synovial membrane inflammation in cartilage matrix breakdown in the Pond-Nuki dog model of osteoarthritis. *Arthritis Rheum* 1985; **28**: 554–561.
34 Hardy S *et al*. Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 1997; **71**: 1842–1849.
35 Ekman L *et al*. Volume of the synovia in certain joint vacities in the horse. *Acta Vet Scand* 1981; **22**: 23–31.
36 Frisbie DD *et al*. The effects of triamcinolone acetate on an in vivo equine osteochondral fragment exercise model. *Equine Vet J* 1997; **29**: 349–359.
37 Anonymous. Definition and classification of lameness. In: *Guide for Veterinary Service and Judging of Equestrian Events*. Lexington: AAEP; 1991, p 19.
38 Kawcak CE *et al*. Effects of intravenously administered sodium hyaluronate on equine carpal joints with osteochondral fragments under exercise. *Am J Vet Res* 1997; **58**: 1132–1140.
39 Frisbie DD *et al*. The assessment of chondrocyte proteoglycan metabolism using molecular sieve column chromatography as compared to three commonly utilized techniques. *Osteoarthr Cartilage* 1998; **6**: 137–145.
40 SAS/STAT user's guide, Version 6. 4 ed. Cary, NC: SAS Institute Inc; 1989.
41 SAS/STAT software; changes and enhancements through release 6.11. 6.11 ed. Cary, NC: SAS Institute Inc; 1996.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.