Early Inflammatory Arthritis in the Rabbit: The Influence of Intraarticular and Systemic Corticosteroids on mRNA Levels in Connective Tissues of the Knee

ALISON S. KYDD, CAROL R. RENO, HELEN W. TSAO, and DAVID A. HART

ABSTRACT. ***Objective.*** Using a rabbit model of inflammatory arthritis, to determine the influence of early disease on expression of specific genes and investigate the influence of intraarticular (IA) and intramuscular (IM) corticosteroids on the regulation of these genes in connective tissues of the rabbit knee.

Methods. Skeletally mature rabbits underwent induction of antigen-induced arthritis or remained untreated as control animals. Four days after disease induction, at an early stage of the disease, animals underwent either IA or IM treatment with glucocorticoids (GC) (5 mg/knee and 10 mg/kg methylprednisolone acetate, respectively). Twenty-four hours following treatment, synovium, menisci, and cartilage of the knee were collected and analyzed for changes in mRNA levels using reverse transcription-polymerase chain reaction for a number of relevant genes: collagen I, collagen II, biglycan, decorin, matrix metalloproteinases-3 and -13 (MMP-3 and MMP-13), cyclooxygenases-1 and -2 (COX-1 and COX-2), tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), inducible nitric oxide synthase (iNOS), hyaluronan synthase-2 (HAS-2), and the housekeeping gene ß-actin.

Results. Early inflammatory arthritis led to an overall upregulation of most genes assessed, but a downregulation of some genes (iNOS, HAS-2, COX-1) in some tissues. While genes such as collagen II, MMP-3, and MMP-13 were uniformly downregulated by GC treatment in both normal and arthritic tissues, other genes such as collagen I, biglycan, and decorin differed in their pattern of response depending on the tissue examined, the route of drug administration, and whether normal or arthritic tissue was studied.

Conclusion. Early mRNA changes in RA-like disease led to alterations in all tissues examined. The changes were uniquely altered by GC treatment. Route of GC administration influenced outcome. (First Release Nov 15 2006; J Rheumatol 2007;34:130–9)

Key Indexing Terms:
GLUCOCORTICOIDS mRNA CONNECTIVE TISSUE
INFLAMMATORY JOINT DISEASE ANIMAL MODEL

The potent antiinflammatory effects of glucocorticoids (GC) were first discovered over 55 years ago when it was reported that they had been successfully used in the treatment of patients with rheumatoid arthritis (RA)[1,2]. Despite their initial success and the promising nature of this discovery, GC treatments have been tempered by the significant side effects associated with their use including immunosuppression, hypertension, osteoporosis, and osteonecrosis, among others[3,4]. Since their initial discovery, GC have been used for the treatment of a number of inflammatory rheumatologic conditions including inflammatory arthritides, bursitis, and osteoarthritis. Despite the widespread application of GC in the treatment of such conditions, a clear understanding of their mechanism of action and effect on both normal/bystander and diseased connective tissues has not been determined in great detail. Moreover, there has been much controversy regarding the side effects of GC in connective tissues, which is likely the result of a complex relationship between the drugs and these tissues, and is also likely highly dependent on the dose of GC used and the duration of treatment[4]. For example, GC have been associated with both chondroprotective effects and chondrocyte apoptosis in cartilage, contradictory effects that appear to be dose-dependent (as reviewed[5]).

GC are believed to act primarily through regulation of cellular transcription via both direct interaction with promoter elements and indirect modulation of transcription factors and regulatory elements (as reviewed[6,7]). Direct interactions with promoter elements occur when a dimer forms between 2 receptor-bound GC complexes, allowing them to bind to consensus sequences such as the GC

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response element (GRE) or negative GC response element (nGRE). Binding to these GRE and nGRE elements leads to transcriptional upregulation (transactivation) or downregulation, respectively. The second main transcriptional mechanism of GC action is transrepression, which occurs when a GC receptor monomer binds to and interferes with components of other transcription factors such as apolipoprotein 1 (AP-1) or nuclear factor-κB (NF-κB); binding to the other transcription factor prevents those transcription factors from binding to their cognate promoter elements, thus leading to decreases in transcription of the associated genes.

Interestingly, it has been postulated that the antiinflammatory effects of GC occur via transrepression of inflammatory genes that are regulated by AP-1 and NF-κB, whereas many of the GC-mediated side effects are associated with transactivation of metabolic genes that are regulated by a GRE (as reviewed[6-8]).

In addition to their transcriptional regulation, it has recently been shown that GC can also act via a number of nontranscriptional and posttranscriptional mechanisms[9-13]. Further complicating the mechanisms of GC action are factors such as the phosphorylation state of the receptor that modulates its transcriptional activity[14], and purported membrane GC receptors (mGCR)[15] that appear to be responsible for a number of the nongenomic effects of the drugs[15].

The antigen-induced arthritis model is a well established model for inflammatory arthritis, first used by Dumonde and Glynn[16], that has been proposed as an appropriate model for the study for conditions such as RA[16,17]. The model is characterized by synovial histopathology like that in RA, immune complex deposition[18], pannus formation, and bone erosion (as reviewed[19]). Previous studies have shown that following the induction of arthritis by the intraarticular (IA) injection of antigen, there are a number of immediate effects, including swelling of the joint[20] and an increase in inflammatory mediators such as interleukin 1 (IL-1), cyclooxygenase-2 (COX-2), and prostaglandins (as reviewed[21]). A number of studies have examined the influence of various GC in this model system, measuring variables such as joint swelling and histology, with GC treatment being associated with a rapid decrease in joint swelling[22,23] but no associated reduction in cartilage erosions compared with saline treated controls[22]. Studies have not examined many of the molecular effects of GC in this model system, nor have they examined the effect of different routes of drug administration.

Our study was designed to further understand these mechanisms, an understanding that becomes more pertinent with the possibility of new corticosteroid pharmaceuticals on the horizon.

MATERIALS AND METHODS

Animals. Fifty-two skeletally mature 1-year-old female NZW rabbits were obtained from Riemans Furriers (St. Agatha, ON, Canada) and housed locally in the Medical Vivarium in accord with Canadian Council on Animal Care Guidelines and with the approval of the Faculty of Medicine Animal Care Committee. Animals were housed for 2 weeks prior to initiation of treatment to allow them to acclimate. An outline of animal use in the protocol of this study is provided in Figure 1.

Experimental protocol. Using an established antigen-induced arthritis model[23-26], 34 rabbits (average weight 5 kg) were preimmunized to ovalbumin [1 ml injection of 10 mg ovalbumin/ml in sterile phosphate buffered saline (PBS) and complete Freund's adjuvant]; 2 weeks later, a booster injection of ovalbumin was given (10 mg/ml ovalbumin in sterile PBS and incomplete Freund's adjuvant). Four weeks after the initial injection, 29 of the immunized rabbits underwent induction of arthritis using bilateral IA injections of ovalbumin (0.5 ml of 10 mg/ml ovalbumin in sterile PBS); the remaining 5 immunized rabbits were not induced to develop arthritis and were allotted to the 4-day immunized control group. Four days post-induction of arthritis, the experimental animals were divided into 3 groups: untreated early arthritis (6), IA treatment (12), and intramuscular (IM) treatment (11). An additional 18 nonimmunized rabbits were used as normal controls and were similarly divided into IA treatment (12) and IM treatment (6) groups.

IA GC treatment. Twelve control animals and 12 early arthritis animals were anesthetized and bilaterally injected with 5 mg methylprednisolone acetate (Depomedrol®)[27] or an equivalent volume of saline intraarticularly (6 per treatment group). As clinical doses of IA GC vary widely, this dose was chosen to fall in the middle range of clinically administered methylprednisolone acetate[28-31].

IM GC treatment. IM injections of GC were carried out as described[32,33]. Briefly, 6 control animals and 11 early arthritis animals were given bilateral injections of either methylprednisolone acetate (Depomedrol®) or an equivalent volume of saline into their flanks at a total dose of 10 mg/kg (Figure 1). Clinically, this systemic dose represents "pulse therapy"[34] of GC, which is a high dose of GC given once or on a short-term basis to terminate an active exacerbation of RA.

Molecular analysis. As indicated in Figure 1, animals were sacrificed with an overdose of Euthanyl (sodium pentobarbital) and connective tissues of the knees collected. Specifically, synovial tissue (Syn), medial and lateral menisci (MM and LM, respectively), and articular cartilage [femoral condyle (FC) and tibial plateau (TP)] were dissected, weighed, and frozen at –80ºC until processing. Total RNA was extracted using the TRIspin method[35] and quantified using the Sybr® Green reagent (Molecular Probes, Eugene, OR, USA) method[35-37].

The genes analyzed in this study were chosen as they represent inflammatory genes, connective tissue structural genes, and enzymes involved in homeostasis of connective tissues. COX-1 and COX-2 are a constitutively produced and an inducible proinflammatory enzyme, respectively, that are known to be modulated by GC and are targets of many antiinflammatory drugs used in the treatment of RA[38-40]. IL-1ß, tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS) represent proinflammatory molecules that are upregulated in RA. Collagens I and II are the primary fibrillar collagens of connective tissues, with collagen I being the predominant collagen of skin, synovium, and ligament and collagen II being more predominant in cartilaginous tissues such as the menisci and cartilage[41-44]. Biglycan and decorin are members of the small leucine-rich proteoglycan family and are prevalent in connective tissues and involved in the regulation of collagen fibrillogenesis[44,45]. Matrix metalloproteinases-3 (MMP-3) and -13 (MMP-13) are members of a large family of zinc-dependent endopeptidases that act to cleave proteins, including many in the extracellular matrix[46]. MMP-3 has a specificity for collagens III and V and many of the proteoglycans, and MMP-13 cleaves substrates including collagens I, II, and III, aggrecan, and versican (as reviewed[46,47]). Hyaluronan synthase-2 (HAS-2) is an enzyme that synthesizes hyaluronan, a component of synovial fluid.

Simultaneous reverse transcription (RT) reactions using 1 μg of RNA from all samples were carried out with the OmniScript kit (Qiagen, Hilden, Germany). Rigorously controlled semiquantitative polymerase chain reac-

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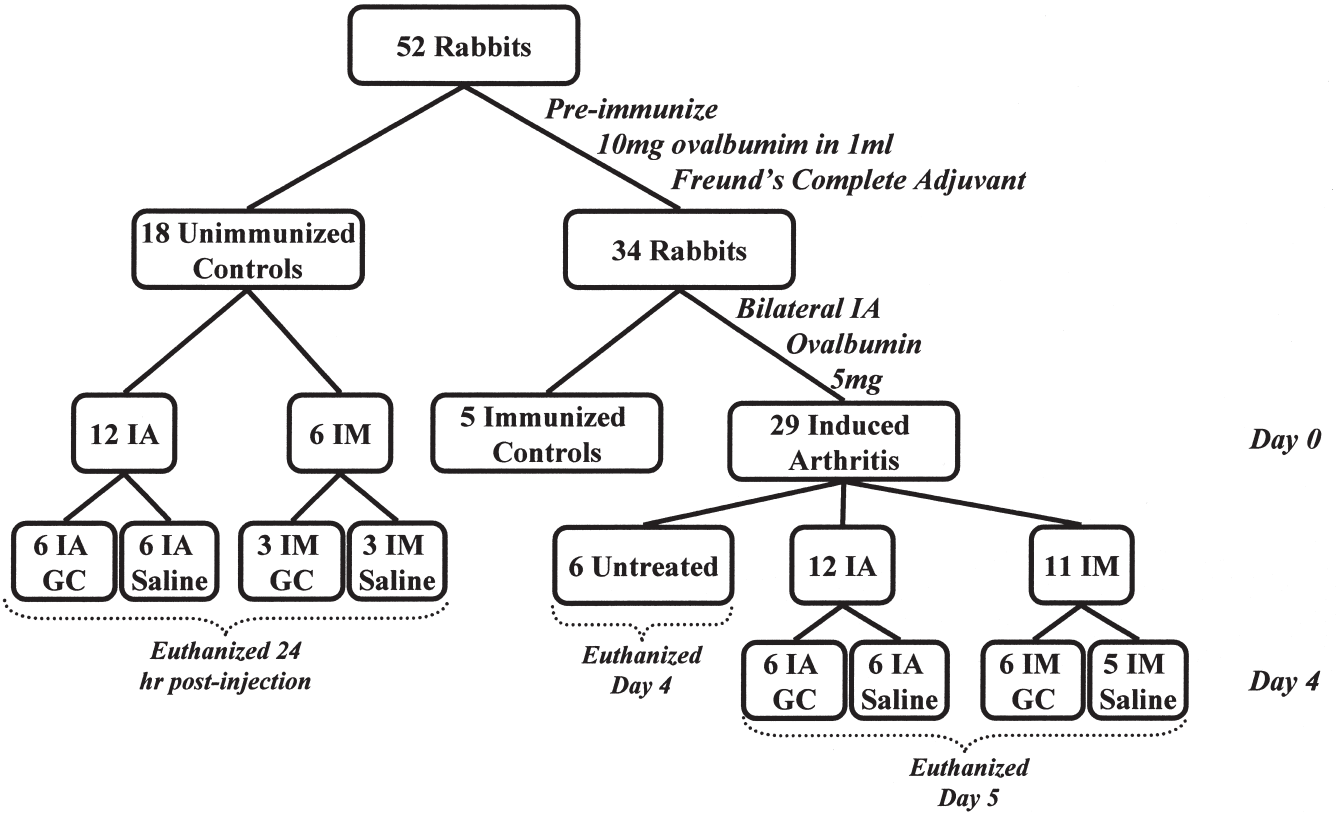

Figure 1. Summary of the experimental protocol. Treatment and control arms show n = number of animals. GC: glucocorticoid, IA: intraarticular, IM: intramuscular.

tion (PCR) was used to analyze results as described[32,33,35]. The validated, rabbit-specific primers used in these studies were as follows: collagen I (5'-GAT GCG TTC CAG TTC GAG TA-3' and 5'-GGT CTT CCG GTG GTC TTG TA-3'; (W.W. Kao, personal communication); 55°C, 24 cycles), collagen II (5'-GCA CCC ATG GAC ATT GGA GGG-3' and 5'-GAC ACG GAG TAG CAC CAT CG[48]; 65°C, 24 cycles), biglycan (5'-GAT GGC CTG AAG CTC AA-3' and 5'-GGT TGT TGA AGA GGC TG-3' from Genbank AF159382; 60°C, 28 cycles), decorin (5'-TGT GGA CAA TGG TTC TCT GG-3' and 5'-CCA CAT TGC AGT TAG GTT CC-3' from Genbank AF125537; 55°C, 20 cycles), MMP-3 (5'-GGC AAG AGA TGC TGT TGA TG -3' and 5'-AGG TCT GTG AAG GCG TTG TA-3' from Genbank M25664; 65°C, 34 cycles), MMP-13 (5'-TTC GGC TTA GAG GTG ACA GG-3' and 5'-ACT CTT GCC GGT GTA GGT GT-3'[36]; 65°C, 27 cycles), TNF-α (5'-AGC CCA CGT AGT AGC AAA CCC-3' and 5'-TTG ATG GCA GAG AGG AGG TGG A-3' from Genbank M60340; 65°C, 36 cycles), COX-1 (5'-CCT TGA CCG ATA CCA GTG TG-3' and 5'-GAT GAA CGT CCT CCT GAG CA-3' from Genbank AF026008; 60°C, 38 cycles), COX-2 (5'-TCA GCC ACG CAG CAA ATC CT-3' and 5'-GTG ATC TGG ATG TCA GCA CG-3' from Genbank U97696; 60°C, 32 cycles), IL-1ß (5'-ACA CCC CAC TGC CCT CCC TTG-3' and 5'-GCT GCT GCC TCT GGT CTC CTT-3' from Genbank M26295; 60°C, 34 cycles), iNOS (5'-CGC CCT TCC GCA GTT CT-3' and 5'-TCC AGG AGG ACA TGC AGC AC-3' from[49]; 65°C, 36 cycles), HAS-2 (5'-GGC CGG TCG TCT CAA ATT CA-3' and 5'-CCA CCC CAT TTT TGC ATG AT-3' from Genbank NM005328; 60°C, 30 cycles), and the housekeeping gene ß-actin (5'-TGC TTC TAG GCG GAC TGT TA-3' and 5'-CGT CAC ATG GCA TCT CAC GA-3' from Genbank U07786; 55°C, 21 cycles).

Agarose gel electrophoresis followed by staining with ethidium bromide was used for separation and detection of the PCR generated cDNA amplicons (Gel Doc XR System; BioRad, Hercules, CA, USA). The results were normalized by dividing values for each individual gene to the corresponding ß-actin values for the same sample. ß-actin levels did not change in any of the groups based on preliminary studies (Kydd, *et al*, unpublished data). As a check on the reliability of the RT-PCR methodology, a second aliquot of RNA from each experiment was again subjected to RT and PCR analysis for a subset of specific molecules. Results from such confirmatory analyses were indistinguishable from the reported values. A subset of genes for which appropriate probes have been validated were also analyzed using real-time PCR, with results indistinguishable from those presented.

Statistical analysis. Statistical analysis of differences between groups was performed with analysis of variance and the statistical package in Microsoft Excel 7.0 software. Differences with $p < 0.05$ were considered significant. Results are presented as significant increases or decreases of mRNA detected in experimental groups when compared to their respective controls.

RESULTS

Our results are divided into 3 main sections: (1) The effect of arthritis on connective tissues wherein control animals were compared with untreated 4-day arthritic animals (Table 1); (2) the effect of IA GC treatment on arthritis (Figure 3) compared with saline treatment; and (3) the effect of IM GC treatment in arthritic animals (Figure 4) that compared IM GC treatment to IM saline treatment.

Our results are largely presented as significant changes in mRNA levels for an experimental group compared to the respective control. This was done since the figures represent a compilation of mRNA analysis for numerous genes (collagen I, collagen II, biglycan, decorin, MMP-3, MMP-13,

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Table 1. Influence of early inflammatory arthritis on gene expression in tissues of the knee.

	Tissue				
Gene	**Syn**	**LM**	**MM**	**TP**	**FC**
Collagen I	↑↑↑	↑	—	↑	—
Collagen II	NA	↓↓↓	↓↓↓	↓↓↓	↓↓↓
Biglycan	↑↑	—	—	↓	—
Decorin	↓	↓↓↓	↓↓↓	↓↓↓	↓↓↓
MMP-3	↑↑↑	↑↑	↑↑	↑↑↑	↑↑
MMP-13	↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑
TNF-α	variable	variable	variable	variable	variable
COX-1	↓↓	ND	—	—	—
COX-2	—	↑↑	↑↑	↑↑↑	↑↑
IL-1β	↑	↑↑↑	—	↑	↑
iNOS	↓↓	—	—	ND	—
HAS-2	—	—	↓	↓↓↓	↓↓↓

LM: lateral meniscus, MM: medial meniscus, TP: tibial plateau, FC: femoral condyle, NA: not applicable, ND: not done. Arrows indicate significant changes in early arthritis compared with immunized controls: ↑ or ↓ $p \leq 0.05$, ↑↑ or ↓↓ $p \leq 0.01$, ↑↑↑ or ↓↓↓ $p \leq 0.001$.

COX-1, COX-2, TNF-α, IL-1ß, iNOS, HAS-2, and the housekeeping gene ß-actin). Figure 2 shows an example of the mRNA data collected for one of the genes, IL-1ß. In the first study, the effect of GC treatment (IA or IM) on normal tissue was examined (Panel A, Figure 2). The second part of the study examined the influence of the induction of inflammatory arthritis on mRNA levels for IL-1ß (Panel B, Figure 2). The final part of the study examined the effect of GC treatment (IA or IM) on IL-1ß mRNA levels during early inflammatory arthritis (Panel C, Figure 2). The remaining figures (Figures 3 and 4) and Table 1 present only the mRNA changes for the indicated panel of molecules that were significantly different relative to the respective control values.

Influence of IA vs IM GC on mRNA levels in normal connective tissues of the knee. Following GC treatment of normal animals via the IM or IA routes of administration, a number of different findings were observed (Figures 3 and 4). The constitutive COX-1 enzyme exhibited elevated mRNA levels in the FC cartilage following both drug treatment regimes but was unchanged in all other tissues. COX-2 mRNA levels, on the other hand, were largely unaffected by systemic drug treatment, but were seen to be significantly elevated in 3 of the 5 tissues examined (Syn, FC Cart, MM) following IA drug administration. IL-1ß and iNOS mRNA levels were largely unresponsive to GC treatment in the normal tissues, with iNOS exhibiting significant increases in mRNA level in intraarticularly treated LM and intramuscularly treated MM tissues only. The mRNA levels for matrix genes including collagen I and collagen II were largely decreased following GC treatment, primarily in those tissues exposed to IM GC (4/5 IM treated tissues examined vs 2/5 in the IA treatment). Such observations are consistent with findings from previous studies[32,33], which found mRNA levels for collagens I and III to be decreased in normal joints following systemic GC treatment. mRNA levels for the small leucine-rich proteoglycans, biglycan, and decorin were unchanged in synovial tissue, but were seen to be elevated in the meniscal tissues following IA GC treatment. In contrast, following IM GC treatment, decorin mRNA levels were elevated in the MM and decreased in TP cartilage, while those for biglycan were unaltered. MMP-3 and MMP-13 mRNA levels were largely depressed following GC treatment, more so in the menisci and synovium than in the articular cartilage. HAS-2 mRNA levels responded variably to drug treatment in the normal tissues, with elevated mRNA levels detected in both the IA and IM treated synovium and the IA treated menisci. In contrast, the articular cartilage showed significantly decreased levels of HAS-2 mRNA following drug treatment (Figures 3 and 4).

Influence of early inflammatory arthritis on gene expression in tissues of the knee. Analysis of specific gene mRNA levels in the early stage of inflammatory arthritis revealed differences in responses following disease induction both

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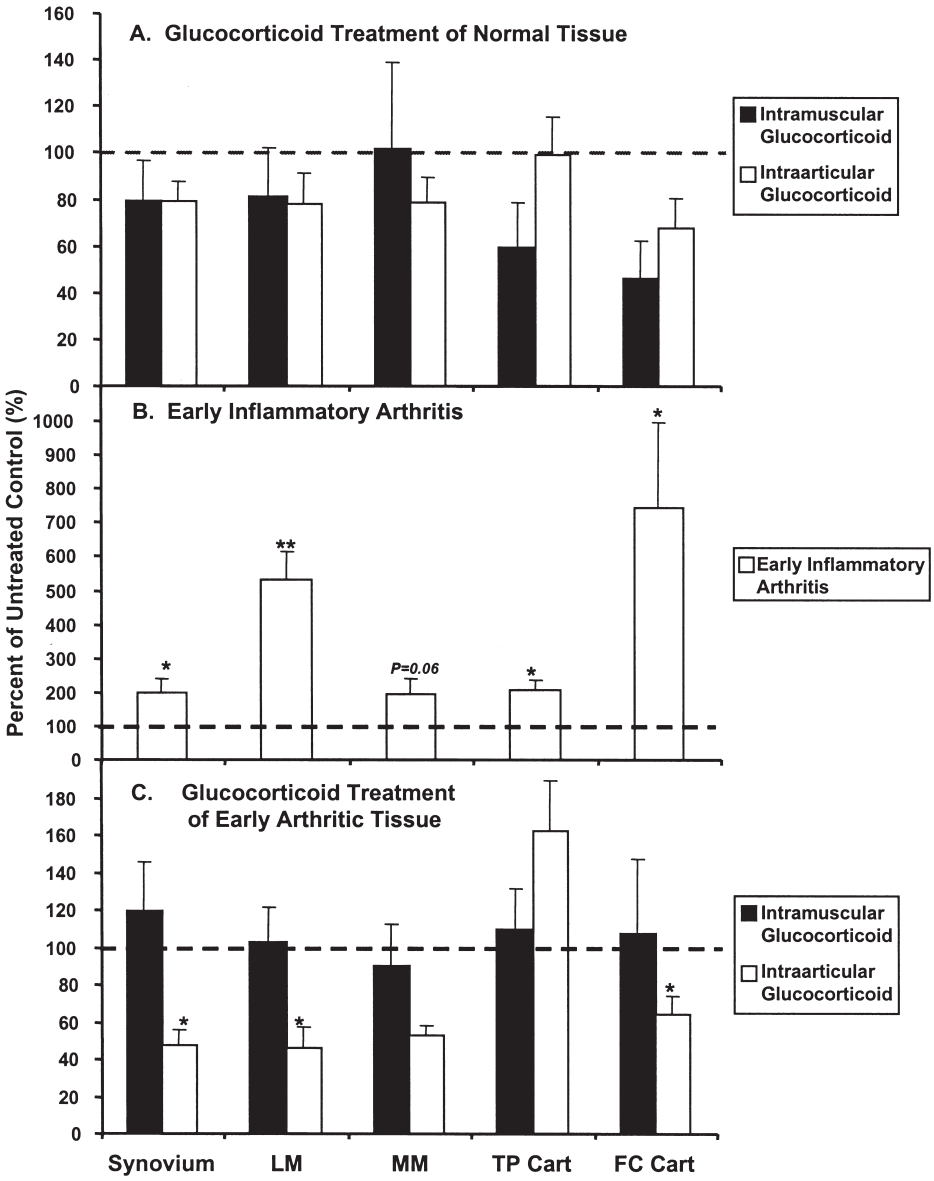

Figure 2. A. Mean IL-1ß mRNA levels in intramuscular vs intraarticular GC treatment of normal tissue (values plotted as percentage of IL-1ß mRNA in IM or IA saline controls). n = 6 IA saline controls (12 joints), 6 IA GC controls (12 joints), 3 IM saline controls (6 joints), 3 IM GC controls (6 joints). B. Effect of early inflammatory arthritis on IL-1ß (values plotted as percentage of IL-1ß mRNA in immunized controls). n = 5 immunized controls (10 joints), 6 untreated arthritic controls (12 joints). C. IL-1ß mRNA in IM vs IA GC treatment of early arthritic animals (values plotted as percentage of IL-1ß mRNA in IM or IA saline treated early arthritic animals). n = 6 IA saline arthritic animals (12 joints), 6 IA GC arthritic animals (12 joints), 5 IM saline arthritic animals (10 joints), 6 IM GC arthritic animals (12 joints). *Significant changes compared with respective controls: *$p \leq 0.05$, **$p \leq 0.001$. Broken lines represent control values (set to 100%).

between tissues and between gene families (Table 1). COX-2 exhibited increased mRNA levels in the meniscal and articular cartilage tissues of the early arthritic joints, but levels were unaltered in the synovial tissue. COX-1 mRNA levels, on the other hand, were largely unresponsive to disease induction in all tissues except the vascular synovium, where levels were seen to be depressed. Similarly, IL-1ß exhibited elevated mRNA levels in the synovium, lateral meniscus, and cartilage tissues, but levels were unaltered in the medial meniscal tissue. Assessments of TNF-α mRNA levels were quite variable at this early timepoint in the disease process, with some animals expressing high levels in tissues such as synovium, and others lower levels, so the differences between groups was not significant (data not

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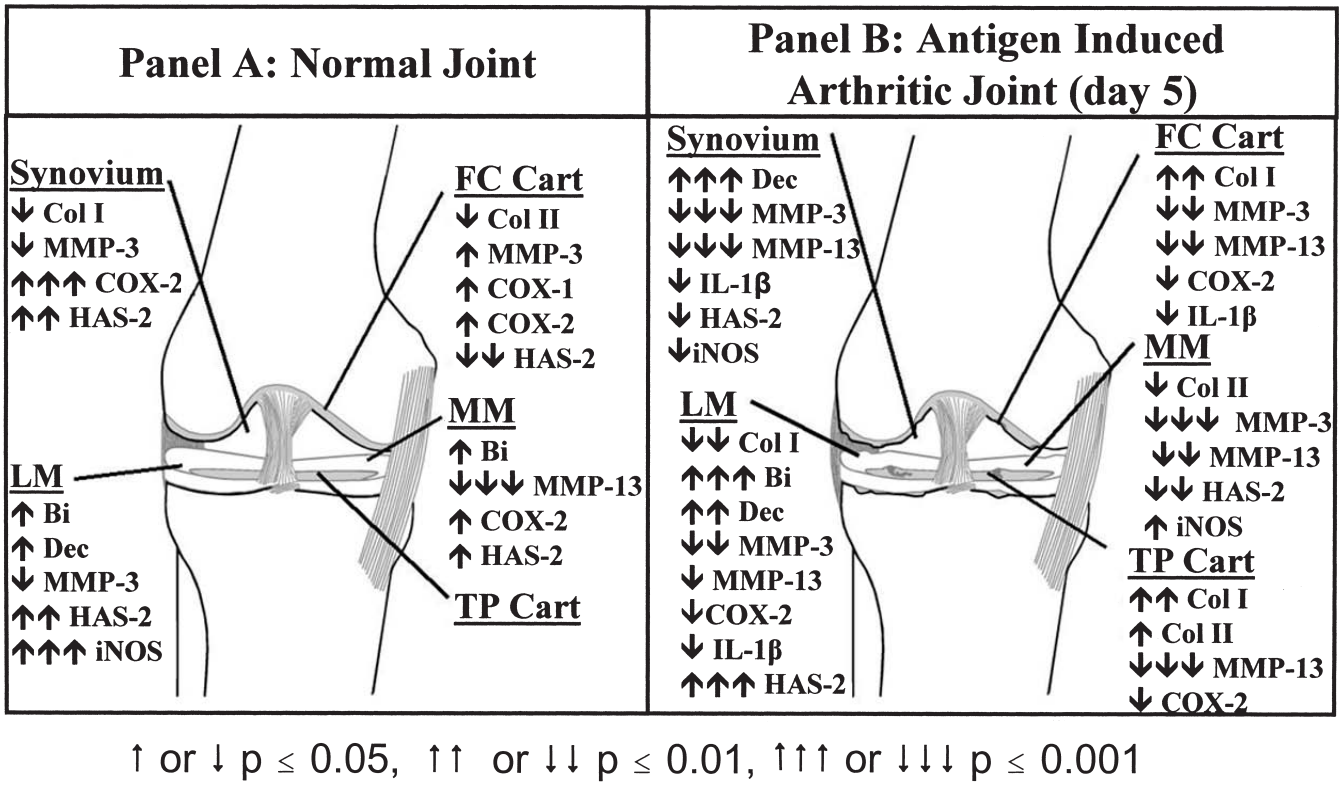

Figure 3. Influence of IA GC treatment on control vs early inflammatory arthritis: unique patterns of change in mRNA levels. Arrows indicate significant changes compared with saline treated controls. n = 6 IA saline controls (12 joints), 6 IA GC controls (12 joints), 6 IA saline arthritic animals (12 joints), 6 IA GC arthritic animals (12 joints). FC: femoral condyle, TP: tibial plateau, MM: medial meniscus, LM: lateral meniscus.

shown). Interestingly, injection of bacterial lipopolysaccharide into rabbit knees does lead to consistent elevations in TNF-α mRNA levels (Hart and Reno, unpublished data). iNOS mRNA levels, on the other hand, were decreased at 24 hours in the synovium, but unaltered in the cartilaginous tissues (menisci and cartilage). Following IA antigen challenge, collagen I and collagen II mRNA levels differed significantly by Day 4. Collagen I was shown to exhibit elevated mRNA levels in a number of the connective tissues (Syn, LM, TP Cart) examined in the early arthritic animals when compared to their respective controls. In contrast, collagen II had significantly depressed mRNA levels shortly after the induction of arthritis in both the meniscal and cartilage tissues. While mRNA levels for decorin, a member of the small leucine-rich proteoglycan family, were consistently depressed in the early arthritic animals, those for biglycan were less altered overall and exhibited significant elevations in mRNA levels in the synovial tissue. However, mRNA levels for this proteoglycan were significantly depressed in the TP cartilage tissue. Consistent with findings in the literature[50], induction of arthritis resulted in significant elevations in mRNA levels for MMP-3 and MMP-13 in all tissues examined. The HAS-2 mRNA levels were largely depressed in most of the cartilaginous tissues following disease induction (Table 1).

Influence of GC treatment on mRNA levels in inflammatory knee arthritis. A number of significant changes in mRNA levels were observed following GC treatment of animals with early antigen-induced arthritis, responses that appeared largely dependent on both the route of drug administration and the tissue examined (Figures 3 and 4). In the early-arthritic animals, mRNA levels for the inducible COX-2 were seen to be depressed following IA drug treatment, but not IM treatment. In contrast, COX-1 mRNA levels were unaltered following GC treatment in all of the tissues of the arthritic animals. Similar to COX-2, IL-1ß mRNA levels were found to be depressed in some tissues only following direct IA GC treatment, while they were largely unaltered in all connective tissues following IM drug administration. Interestingly, while collagen I mRNA levels were more responsive to IA treatment (showing a significant elevation in most tissues examined), collagen II levels were altered to a greater extent following IM drug treatment, where levels were seen to be depressed in the cartilaginous tissues. Similarly, MMP-3 mRNA levels were more affected in the IA treated animals, whereas MMP-13 was globally suppressed regardless of route of administration.

Evaluation of mRNA levels for TNF-α following GC treatment revealed significant interanimal variability that was not found for other genes evaluated. However, there

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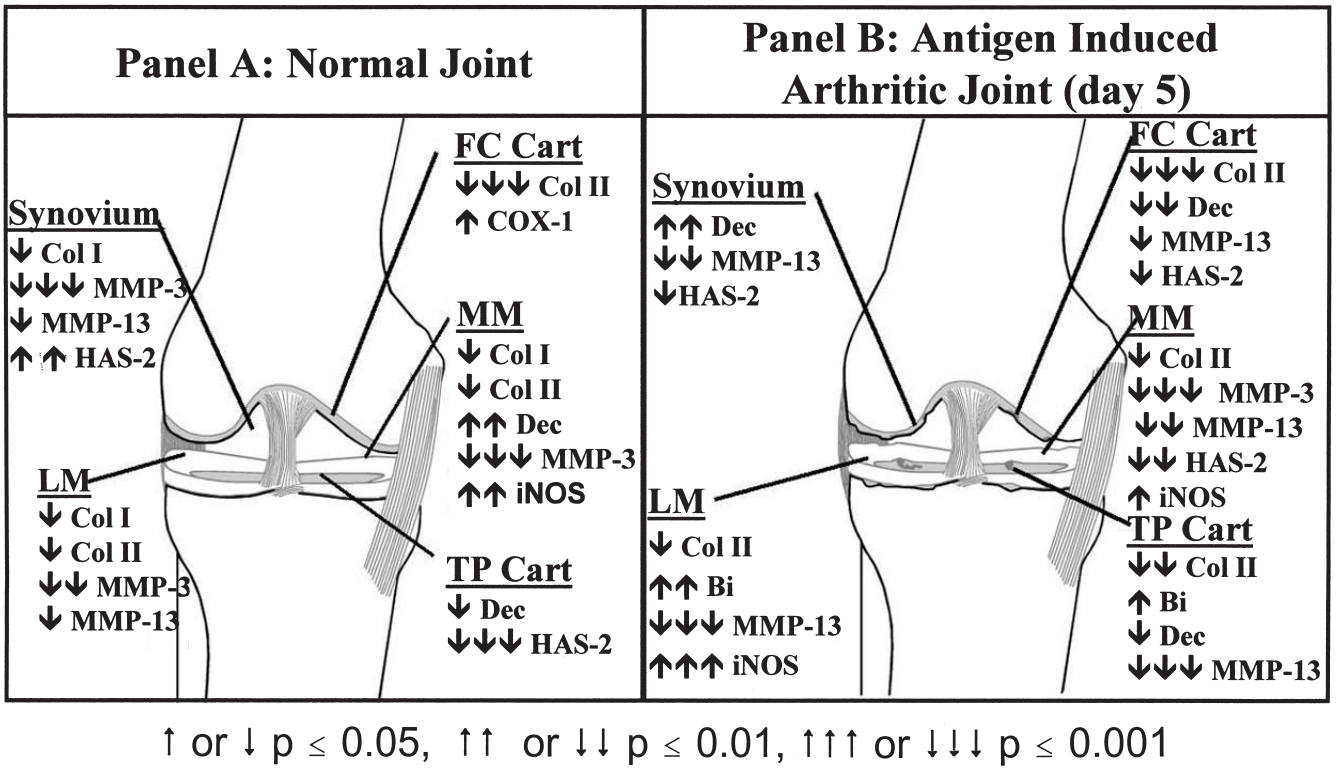

Figure 4. Influence of intramuscular GC treatment on control vs early inflammatory arthritis: unique patterns of change in mRNA levels. Arrows indicate significant changes following GC treatment of early arthritis compared with saline treated early arthritis. n = 3 IM saline controls (6 joints), 3 IM GC controls (6 joints), 5 IM saline arthritic animals (10 joints), 6 IM GC arthritic animals (12 joints). FC: femoral condyle, TP: tibial plateau, MM: medial meniscus, LM: lateral meniscus.

was a trend for lower levels of mRNA for this cytokine in the GC treated animals compared to the saline treated animals (data not shown). This finding may represent the highly variable induction of inflammation present early in this inflammatory arthritis model.

DISCUSSION

Inflammatory arthritis is a systemic disease or set of diseases that exhibits extensive synovial involvement, but also affects other tissues of the involved joints, as well as non-joint tissues and organs. This is true for human disease and experimental models. In our study, examining the effect of inflammatory arthritis and GC, a widely used treatment of inflammatory arthritis, on connective tissues of the knee has revealed a number of interesting and unexpected patterns of response. The early phase of inflammatory arthritis was reflected by both gene- and tissue-specific alterations in mRNA levels for some genes, whereas others exhibited more consistent changes in a tissue-independent manner. As an early phase of disease was investigated, other genes (e.g., TNF-α) were more variable in the changes observed following disease induction.

Similarly, GC did not confer uniform changes in mRNA levels in the different connective tissues examined early after disease induction. Moreover, the effect of GC varied greatly depending on the route of administration, be it a more localized IA injection or the more systemic IM injection. Interestingly, saline treatment of early arthritic animals resulted in variable decreases in mRNA for some inflammatory genes including COX-2, IL-1ß, iNOS, and the proteinase MMP-13. This may reflect either changes in the disease process between Day 4 and Day 5 or the induction of an endogenous stress response following injection, which would result in release of endogenous GC. This underscores the importance of using saline injections as a control for the GC treatment studies.

IA and IM routes of GC administration were not equivalent in their effect on the connective tissues of the knee. For example, COX-2 and IL-1ß mRNA levels were more responsive to GC treatment of early arthritic animals when the GC was given intraarticularly rather than intramuscularly. Similarly, while collagen I mRNA depression in normal tissues was more sensitive to systemic IM drug treatment, the mRNA elevations detected in the early arthritic tissues were more evident in response to localized IA treatment. There are a number of possible explanations for the differing responsiveness between IA and IM treatment. First, as connective tissues are relatively avascular, the 2 routes (IM and IA) of drug administration may result in a differential exposure of the various joint connective tissues. Second, the

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specific dosing experienced by cells of the knee would clearly be different between the IA and IM treatments, and, indeed, between different tissues, and therefore the findings may represent purely a dose-response relationship. Moreover, the pharmacokinetics and pharmacodynamics of IM vs IA GC treatments may differ with regard to factors such as the peak dose, bioavailability, and clearance of the steroid[51], factors that may result in the observed difference in response between IA and IM treatments. Third, the cell types (fibroblasts, chondrocytes, and synoviocytes) responsible for the paradoxical changes in mRNA in the normal and arthritic tissues may respond differently to IA or IM treatments. Finally, systemic IM drug treatment may also be acting on upstream cells such as immune or inflammatory cells that, in turn, exert secondary effects on tissues of the knee resulting in the contrasting response observed.

A second interesting finding of our study was that collagen I, the primary matrix protein of connective tissues, exhibited an altered pattern of responsiveness to GC treatment in normal and early arthritic tissues. In normal tissues, collagen I mRNA levels were more sensitive to systemic IM drug treatment, exhibiting decreased mRNA levels, which is consistent with previous findings in other normal connective tissues[32,33]. Conversely, collagen I mRNA levels were elevated following induction of inflammatory arthritis, and were further significantly elevated following GC treatment of the early arthritic joints, but only following localized IA drug treatment.

A more conventional response to GC treatment was detected for other connective tissue genes. Collagen II mRNA levels were uniformly depressed in tissues from arthritic joints and following systemic (IM) GC treatment of both normal and arthritic tissues. Similarly, consistent with the literature[50], MMP-3 and MMP-13 mRNA levels were increased rapidly following the induction of inflammatory arthritis, but were largely depressed following GC treatment of both arthritic and normal animals. This finding is particularly interesting with respect to MMP-13, which in previous studies[33] was shown both in *in vitro* transcriptional studies and in an *in vivo* animal model to exhibit the same paradoxical GC-mediated mRNA elevation as collagen I in injured ligament tissue.

The small leucine-rich proteoglycans revealed a third pattern of response, with significant tissue-specific changes being observed. Following GC treatment of both normal and arthritic animals, biglycan tended to exhibit increased mRNA levels in the meniscal and articular cartilage tissues examined, but levels were unaltered in the synovium. In contrast, decorin tended to exhibit elevated mRNA levels in meniscal tissues following drug treatment, but also tended towards decreased mRNA levels in the adjacent articular cartilage tissues, irrespective of whether the joint was normal or arthritic. These tissue-specific findings are similar to those of Kojima, *et al*[25], who found that following induction of ovalbumin-induced arthritis, the subsequent reduction in total cartilage proteoglycan content (primarily measuring aggrecan) occurred more in some regions of articular cartilage than others.

The complex patterns of change in mRNA levels following GC treatment observed in this study cannot be solely explained by the mechanisms of transactivation and transrepression, and thus likely represent the complex interaction of a number of mechanisms of GC action. The relevance of these seemingly complex results lies in their demonstration of a number of important themes in the action of GC, and how this may affect the treatment of inflammatory arthritis and ultimately influence outcomes. Both injury and arthritis represent situations of active inflammation, indicating a possible role of inflammatory cells or inflammatory mediators such as cytokines in modulating the GC-mediated response. For example, the literature would support a scenario in which inflammation would result in the activation of proinflammatory signal transduction pathways[9], leading to the phosphorylation of the GC receptor[14], and resulting in an altered response of the tissue following steroid treatment. Alternatively, the altered responsiveness could be related to the increased expression of membrane GC receptors (mGCR) that has been reported in patients with RA[15]. In other instances, the changes in mRNA levels were consistent in both arthritic and nonarthritic tissues and regardless of the route of administration (i.e., Col II, MMP-3, and MMP-13).

One additional interesting finding of our study was the degree of variability in TNF-α mRNA levels following early arthritis induction. The basis for the variability is unknown; however, as the timepoint sampled is that of early arthritis, it is reasonable to predict variability in the degree of inflammation. Interestingly, it should be pointed out that in human studies, there is a significant degree of variability with respect to response to anti-TNF-α therapies such that ~40% of patients exhibit unresponsiveness[52,53]. Clinically, these subsets may reflect a different genetic makeup or disease subtype. As the rabbits used in our study were not inbred, perhaps there is a genetic basis for the variation in animals, similar to that that may be occurring in humans.

It is important to point out that a number of factors may influence the interpretation of our results. First, the data collected in these studies could be specific for rabbit tissues and may represent a species-specific response. Second, RT-PCR is unable to distinguish between changes in rate of transcription and changes in transcript half-life. In addition, while RT-PCR results have shown good correlation between changes in mRNA levels and protein levels in previous studies for the assessment of MMP-13[36], the collagens[37,54], and more recently connective tissue growth factor[55], this still remains to be confirmed in the inflammatory arthritis model. Therefore, the detection of significant changes in mRNA levels by semiquantitative RT-PCR performed under

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rigorous conditions can still yield valid approximations of changes in mRNA levels for many molecules.

In humans, RA is a condition characterized by joint inflammation and the progressive loss of joint space, destruction of cartilage, and erosion of bone. The emphasis of RA treatment has become more aggressive, with early disease treatment to maximize responsiveness and prevent progressive joint destruction[56]. Radiological studies have revealed that joint destruction occurs rapidly following the onset of RA and that systemic GC treatment can slow the progression of joint erosions but not the joint space narrowing and cartilage loss associated with RA[57]. Antigen-induced arthritis is a rheumatoid-like experimental model that has been used for over 30 years to study the pathophysiology of this joint disease and to determine the response to various treatments[16-20]. Our results present interesting parallels that may potentially affect treatment of human disease in the future. First, the response of joint tissues to IA compared to IM treatment is not equivalent, with mRNA levels being modulated differently between the 2 routes of treatment. Second, different joint tissues (synovium vs meniscus vs cartilage) do not respond uniformly to GC treatment, and this response is further modulated by the presence of inflammation in the joint. Third, GC treatment alters mRNA levels for both genes involved in inflammation as well as those involved in connective tissue homeostasis. Better understanding of these issues may lead to improved use of GC for the optimal treatment of patients with RA.

Although GC have been used for the past 55 years in the treatment of inflammatory arthritis[1,2], it is clear that their mechanism of action and influence on connective tissues has yet to be fully elucidated. However, it is also clear that the actions of these drugs are complex and are influenced by factors such as the inflammatory state of the joint, the route of administration, and the tissue type involved, findings that need to be further examined.

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