IL-1 has no direct role in the IGF-1 non-responsive state during experimentally induced arthritis in mouse knee joints


Abstract
Objective—To investigate the involvement of interleukin-1 (IL-1) in the induction or maintenance of the insulin-like growth factor 1 (IGF-1) non-responsive state of chondrocytes during experimental arthritis in mouse knee joints.

Methods—To characterise IGF-1 non-responsiveness during arthritis, we measured chondrocyte proteoglycan (PG) synthesis by assaying incorporation of 35S-sulphate into mouse patellar cartilage, obtained from knee joints with experimentally induced arthritis and normal knee joints, cultured with IGF-1. We investigated whether suppressive mediators produced by the arthritic synovium or chondrocytes abolished the IGF-1 stimulation of normal cartilage, and used IL-1 primed cartilage to mimic the arthritic in vivo state. Specific inflammatory mediators responsible for the maintenance of the suppressed IGF-1 response were sought. We measured IGF-1 responsiveness in normal and arthritic patellae cultured with antibodies against tumour necrosis factor (TNF) or IL-1α/β, with IL-1 receptor antagonist (IL-1ra), and with several inhibitors of proteolytic enzymes or reactive oxygen species, and analysed the role of IL-1 in the development of IGF-1 non-responsiveness by studying IGF-1 responses in cartilage treated with IL-1β antibodies in vivo, at the onset of arthritis.

Results—Mediators from the surrounding tissue of both normal and arthritic cartilage suppressed chondrocyte IGF-1 responses. Priming the cartilage with IL-1 did not directly induce IGF-1 non-responsiveness, but enhanced the ability of suppressive mediators from synovium or chondrocytes to downregulate the IGF-1 responsive state. IL-1ra, IL-1α/β antibody, TNF antibody, or the inhibitors tested did not markedly improve the disturbed IGF-1 response, but treatment with anti-IL-1 at the onset of arthritis prevented the development of IGF-1 non-responsiveness.

Conclusion—IL-1 alone does not induce IGF-1 non-responsiveness and is not critical in the maintenance of this phenomenon. However, IL-1 does appear to be an important cofactor in the generation of the IGF-1 non-responsive state.

Materials and methods

ANIMALS
Sexually mature female C57 black/6 mice (8–12 weeks old, weights 20–25 g) were used. The animals were kept under routine laboratory conditions (21–22°C, relative humidity 60% and a 12 hour light-dark cycle), fed a standard commercial pellet diet (RHM, Hope Farms, The Netherlands), and given acidified tap water ad libitum.

INTRA-ARTICULAR IL-1 INJECTIONS
Mouse recombinant IL-1 10 ng (kindly supplied by Dr I G Otterness, Pfizer Central Research, Groten, CT, USA) was injected in a volume of 6 ml into the joint cavity of the right knee. The left contralateral joint received an equal volume of phosphate buffered saline (PBS). The biological activity of IL-1 was determined using the NOB1 assay.22 The IL-1 batch was stored at −20°C, and showed constant activity over the period studied. The cartilage was studied at day 1 after intra-articular injection of IL-1.

INDUCTION OF ARTHRITIS
Arthritis was induced in mouse right knee joints by intra-articular injection of yeast particles (180 μg sterilised Zymosan) (Sigma, St Louis, MO, USA) in pyrogen free saline. This experimental arthritis model is characterised by the development (two to three days after induction of arthritis) of an exudate containing large amounts of polymorphonuclear cells in the joint space, by loss of cartilage PGs, and by inhibition of chondrocyte PG synthesis. Inflammation subsides within a week and because the cartilage damage is reversible, the matrix is subsequently repaired.11 27 In our subsequent experiments, therefore, cartilage was examined two days after Zymosan induction of arthritis.

IN VIVO ANTI-IL-1 TREATMENT
Anti-IL-1 treatment before the onset of arthritis has been shown to normalise the inhibition of chondrocyte PG synthesis observed during arthritis, but to have no effect on joint swelling and cell influx.22 One hour before induction of arthritis, therefore, mice were injected intravenously with a standard dose of 0.6 mg/ml purified rabbit antimouse IL-1α and IL-1β antibodies (prepared in our own laboratory by A V de L; 1 μg anti-IL-1α/β consistently has a total neutralising capacity of 50–100 pg of both IL-1 subtypes tested in the NOB1 assay25). As a control, mice were injected intravenously with rabbit IgG.

CARTILAGE CULTURES
Mice were killed by cervical dislocation and whole patellae were dissected from the right arthritic and left control knee joints, leaving the cartilage fully intact.11 The patellae were isolated from the knee joints with minimal amounts of surrounding tissue. The fully intact cartilage11 25 was used to study radiosulphate incorporation, measured either directly at time zero (t = 0), reflecting the in vivo situation, or after culture for 24 or 48 hours. Patellae were cultured in RPMI 1640 medium (Flow Laboratories Irvine, Scotland) supplemented with 2 mmol/l glutamine, 40 μg/ml gentamycin, and 0·1% ultrapure bovine serum albumin (Sigma) in the presence or absence of recombinant human IGF-1 (Boehringer Mannheim, Germany) in a concentration of 0·25 μg/ml, which is close to those in mouse plasma.66 The incubations were performed in 24 well cluster dishes at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Suppressive mediators—The relative contribution of suppressive mediators produced by synovial tissue surrounding the patella specimen and by the patella specimen itself during culture was investigated by comparing cultures of patellae containing small amounts of surrounding tissue with those of patellae containing large amounts of surrounding tissue. In addition, cocultures of normal and arthritic patellae were performed in 6·5 mm transwell systems with a porous cell culture insert (Costar, Cambridge, UK) and the synthetic activity was measured in the cartilage of the normal patellae.

IL-1 and TNF in vitro—To identify which suppressive factors from the arthritic specimen suppressed the IGF-1 response, patellae were cultured in medium containing a mixture of rabbit antimouse antibodies against IL-1α and IL-1β (prepared in our own laboratory23), rat antimouse TNFα (V1q hybridoma cells, kindly given by Dr Krammer, German Cancer Research Center, Heidelberg, FRG). Recombinant human IL-1 receptor antagonist (IL-1ra) (Synergex, Boulder, Colorado, USA) was included in some cultures, to examine whether its smaller size enabled it to gain access to the chondrocytes and exert a greater influence than the larger antibodies capable of poorer penetration of the articular cartilage. The concentrations of IL-1ra, anti-IL-1, and anti-TNF used in the present study were based on those previously reported to exert positive effects.11 14 19 22 In the mixture of antibodies against IL-1α and IL-1β, both were used at concentrations of 0·06 mg/ml, while TNFα antibody was used at 30 000 U/ml (1 U of the antibody has a neutralising capacity of 90 pg TNFα in the L929 bioassay). IL-1ra was tested in a concentration range of 0·3–100 μg/ml. As control for the IL-1α, IL-1β, and TNFα antibodies, patellae were cultured with rabbit IgGs and rat IgGs.

Enzymes and reactive oxygen species may induce changes in chondrocyte signalling and thus interfere with IGF-1 responses, so patellae were also cultured in the presence or absence of the enzyme inhibitors leupetin (Boehringer) or recombinant human tissue inhibitor of metalloproteinases (TIMP) (gift from AMGEN, Thousand Oaks, CA, USA), the oxygen scavenger, catalase, together with horseradish peroxidase (Sigma) or the nitric oxide synthase inhibitor L-Nω-monomethyl arginine (l-NMMA) (Calbiochem, San Diego,
California, USA). The inhibitor concentrations we used (leupeptin 0.5 µg/ml; TIMP 20 µg/ml) were analogous to concentrations known to be effective. Catalase and horse-radish peroxidase were used together in concentrations of 3 mg/ml and 12 mg/ml, respectively, and L-NMMA was used at 100 or 1000 µmol/l.

**IN VITRO INCORPORATION OF RADIOSULPHATE**

Chondrocyte PG synthesis in patellar cartilage was measured by the incorporation of "S-sulphate as described previously. Briefly, cartilage was incubated in RPMI 1640 medium with 1.48 MBq "S-sulphate (Na₂"SO₄, Du Pont, 's-Hertogenbosch, The Netherlands) for three hours at 37°C and then washed three times in physiological saline to remove non-incorporated radiolabel. Patellae were then fixed in 4% buffered formaldehyde and decalcified in 5% formic acid for three hours, and the patellar cartilage removed from the surrounding tissue and digested in Lumasolve (Lumac, Landgraaf, The Netherlands) at 60°C. The quantity of radioactive sulphate incorporated was assayed by liquid scintillation counting.

**STATISTICS**

Statistical evaluation of the experiments was by one way analysis of variance in combination with Student's t test. A p value <0.05 was considered to be significant.

**Results**

**IGF-1 NON-RESPONSIVENESS IN ARTHRITIC CARTILAGE**

In keeping with previous observations, PG synthesis in patellar cartilage from normal knee joints declined to approximately 50% in 24 hour culture in the absence of IGF-1, whereas metabolic activity was maintained or even enhanced with physiological concentrations of IGF-1 (fig 1A). Cartilage from arthritic knee joints demonstrated suppressed chondrocyte PG synthesis and did not respond to further stimulation with IGF-1 (fig 1B). This non-responsive state of arthritic chondrocytes was maintained with supraphysiological concentrations of IGF-1 (2 µg/ml; data not shown).

**SUPPRESSIVE MEDIATORS**

When the contribution of suppressive mediators produced during culture by the synovial tissue surrounding the patella was investigated, it was found that IGF-1 responses were significantly lower in both normal and arthritic patellar cartilage embedded in large amounts of tissue (fig 1), indicating that substances in the surrounding tissue from both normal and arthritic cartilage suppressed IGF-1 stimulation of chondrocyte PG synthesis.

Coculture studies of normal and arthritic patellae, in which the synthetic activity was measured only in the normal cartilage showed that IGF-1 stimulation of normal cartilage was reduced in the presence of arthritic specimens, though a complete lack of IGF-1 responsiveness was not achieved (fig 2A).

In the cartilage of normal knee joints that was injected with IL-1 to mimic the in vivo state of arthritic cartilage, PG synthesis was significantly reduced, but IGF-1 was able to enhance the synthetic rate in a subsequent 24 hour culture period. However, when this culture was performed in the presence of arthritic patellae, a pronounced reduction of IGF-1 stimulation was noted. IGF-1 stimulation of IL-1 primed cartilage cultured with arthritic patellae was not significantly different when compared with the ex vivo PG synthetic rate of IL-1 primed cartilage (fig 2B).

**ROLE OF IL-1 AND TNF IN VITRO**

Figure 3 shows the effect of IL-1 and TNF modulation of the IGF-1 response. During 24 hours of culture, no significant effect was noted for either cytokine, whereas the synthetic rate was significantly greater when the IL-1 and TNF neutralising culture was extended to 48 hours, in both normal and arthritic specimens;
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However, the IGF-1 stimulation in arthritic cartilage was marginal and PG synthesis remained clearly reduced compared with that in normal cartilage.

Role of IL-1 in Vivo

Further to our findings indicating that the use of antibodies against IL-1, TNF, or several inhibitors did not interfere with the IGF-1 response during existing arthritis, we investigated the IGF-1 responses in mice treated with IL-1α/β antibodies at the onset of arthritis, and found that anti-IL-1 treatment almost normalised ex vivo chondrocyte PG synthesis (fig 4). In the absence of IGF-1, PG synthesis declined, but culturing arthritic cartilage from anti-IL-1 treated mice with IGF-1 resulted in a highly significant stimulation of chondrocyte PG synthesis, suggesting their defective IGF-1 signalling had been averted.

Involvement of Enzymes and Reactive Oxygen Species

The table summarises the effects of several inhibitory factors on the patellar cultures. Leupeptin, an inhibitor of cysteine and serine proteinases, and TIMP, a well known metalloproteinase inhibitor, did not affect IGF-1 responses in a 24 hour culture period. Similarly, a combination of the oxygen radical scavengers, catalase and horseradish peroxidase, did not alter IGF-1 stimulation in normal or arthritic cartilage. The potent nitric oxide synthase inhibitor, L-NMMA, in concentrations of 100 or 1000 μmol/l also failed to...
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**Discussion**

IGF-1 plays a key role in regulating chondrocyte biosynthetic functions. During experimentally induced arthritis, articular cartilage does not respond to IGF-1 stimulation of chondrocyte PG synthesis, and this IGF-1 non-responsiveness leads to the production of alternative PG monomers, which implies that IGF-1 is essential in the maintenance of cartilage integrity. Non-responsiveness of articular chondrocytes is not restricted to IGF-1, but extends to other growth factors, such as basic fibroblast growth factor, platelet derived growth factor, epidermal growth factor, or transforming growth factor β. This lack of anabolic signalling during arthritis may contribute to destruction of the cartilage. In the present study, we investigated the extent to which IL-1 is involved in the generation or maintenance of the IGF-1 non-responsive state during arthritis.

IL-1 is a potent inhibitor of chondrocyte PG synthesis and is thought to act as a key mediator in the pathogenesis of cartilage destruction in arthritic disorders. In experimental arthritis models, IL-1 has been shown to be a more potent mediator of cartilage destruction than TNF. The present study has shown that unidentified mediators from the surrounding tissue of both normal and arthritic cartilage were able to suppress IGF-1 responses. Treatment with IL-1ra, IL-1α/β antibodies, or TNF antibody did not abolish IGF-1 non-responsiveness, suggesting that IL-1 or TNF are not the key mediators that maintain IGF-1 non-responsiveness. The enhanced chondrocyte PG synthesis after culture of intact cartilage with anti-IL-1 and anti-TNF is probably specific for anticytokines, and may have reflected release of small amounts of cytokine from the tissue during its isolation. It is likely that the impact of the inflammatory process on the chondrocyte is complex and cannot be expected to be modulated by the elimination of one single factor. Although exposure of the cartilage to IL-1 did not itself cause IGF-1 non-responsiveness, the exposed cartilage became more susceptible to reduction of the IGF-1 response by other suppressive mediators in the synovium or the chondrocytes, when compared with the ex vivo PG synthetic rate of IL-1 primed cartilage. In addition, anti-IL-1 treatment at the onset of arthritis abolished generation of IGF-1 non-responsiveness. These data imply that IL-1 has an important role in the onset of IGF-1 non-responsiveness but that, once the IGF-1 non-responsiveness is initiated, the importance of IL-1 is lost.

Degradation enzymes and reactive oxygen species have been implicated in the apparent breakdown of articular cartilage during joint inflammation. It is still not understood whether these degradative effects are achieved partly via inhibition of growth factor signalling. In this context, the absence of an IGF-1 response could be the result of IGF-1 receptor stripping, or receptor damage caused by proteolytic enzymes or oxygen metabolites. Recent studies demonstrated positive effects of 1-NMMA and TIMP in patellae from arthritic knee joints. These inhibitors may be able to penetrate the cartilage matrix of diseased cartilage comparatively easily, in contrast to poor penetration to the chondrocytes in intact cartilage (A A Van de Loo, in preparation). In the present study, we found that the metalloproteinase inhibitor, TIMP, and the serine and cysteine proteinase inhibitors, leupeptin, were unable to influence IGF-1 responses in normal and arthritic cartilage. Moreover, the oxygen scavengers catalase and horseradish peroxidase, and the nitric oxide synthase inhibitor 1-NMMA did not affect IGF-1 responsiveness. These data suggest that enzymes or oxygen species cannot be indicated...
Figure 5  Schematic representation of the interplay of IL-1 and IGF-1 in chondrocyte metabolism. Cartilage chondrocyte proteoglycan synthesis is dependent on the balance of anabolic effects of IGF-1 and catabolic effects of IL-1. During arthritis, chondrocyte PG synthesis is inhibited as a result of excess IL-1, which is sustained by IGF-1 involved (together with the unknown factor, X) in generation of the phenomenon, as anti-IL-1 treatment can abolish development of IGF-1 non-responsiveness.

as key factors in IGF-1 non-responsiveness,—unless, perhaps, their inhibitors had been added to the culture system after irreversible damage had taken place. In keeping with the findings that enzymes or oxygen species do not have a key role in IGF non-responsiveness, we have shown previously that the transient chondrocyte IGF-1 non-responsiveness found after exposure to hydrogen peroxide was not related to IGF-1 receptor damage and that hydrogen peroxide mediated inhibition of chondrocyte PG synthesis was not associated with disturbed IGF-1 signalling.\(^{40,41}\) Moreover, we have also shown that IGF-1 receptors on the chondrocyte cell membrane were not decreased in amount during the stage of experimental arthritis in which IGF-1 non-responsiveness is manifest.\(^{42,43}\) Thus it seems likely that joint inflammation impaired the process of chondrocyte receptor mediated signal transduction, causing a modified biological response. It is of interest that IGF-1 non-responsiveness is not caused by total anergy of the chondrocytes, as forskolin—a stimulator of the adenylate cyclase pathway—is able to enhance chondrocyte PG synthesis in experimentally induced arthritic cartilage.\(^{44}\)

IGF-1 binding proteins have an affinity for IGF-1 similar to that of the IGF-1 receptor, and increased production of IGF-1 binding protein may therefore diminish IGF-1 bioavailability and affect IGF-1 non-responsiveness during arthritis.\(^{45-47}\) Dore et al\(^{47}\) demonstrated that the impaired biological response to IGF-1 by articular chondrocytes from human osteoarthritic cartilage was related to increased production of IGF binding proteins, though it should be noted that ex vivo PG synthesis in osteoarthritic cartilage is at a supranormal level, in contrast with the suppressed chondrocyte PG synthesis in arthritic cartilage. We investigated the non-responsiveness of articular chondrocytes in the presence of supraphysiological concentrations of IGF-1, to saturate all available IGF-1 binding proteins and thus exclude a role for excess binding proteins in our findings. The non-responsiveness of articular chondrocytes was maintained in the presence of these supraphysiological concentrations of IGF-1 (2 |g/ml), implicating inhibiting suppressive mediators produced by the cartilage or synovial tissue, or an intrinsic defect in IGF-1 signalling, rather than IGF-1 binding proteins, in the chondrocyte non-responsiveness. The possibility remains, however, that IGF binding proteins secreted in very high concentrations may be of importance.

In conclusion, lack of IGF-1 responses during arthritis are affected by a complex interaction of suppressive mediators (Fig. 5). Modulation of one individual mediator in the in vitro culture system does not influence the IGF-1 non-responsive state during arthritis. Priming of the cartilage with IL-1 is not fully responsible for IGF-1 non-responsiveness, but clearly makes the cartilage more susceptible to suppressive mediators. Moreover, anti-IL-1 treatment at the onset of arthritis averts IL-1-induced cartilage erosion and chondrocyte non-responsiveness. We propose that IL-1 does not induce IGF-1 non-responsiveness, but is involved indirectly in the generation of this phenomenon.

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