Investigation of the role of IL-1 and TNF in matrix degradation in the intervertebral disc

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Objective. To establish if IL-1 or TNF regulates matrix degradation in the non-degenerate or degenerate intervertebral disc (IVD).

Methods. In situ zymography (ISZ) has been used to investigate the role of IL-1 and TNF in the matrix degradation characterizing symptomatic IVD degeneration. ISZ employed three substrates (gelatin, collagen II, casein) and four different challenges, IL-1β, IL-1 receptor antagonist (IL-1Ra), TNF-α and anti-TNF.

Results. We have shown for the first time that whilst IL-1β will stimulate and IL-1 receptor antagonist will inhibit matrix degradation in intact human IVD tissue, neither TNF-α nor anti-TNF have any measurable effect on degradation of these matrices.

Conclusion. This study has addressed a current area of controversy in IVD biology, namely, whether either IL-1 or TNF or both are involved in driving matrix degradation. Our data indicate that IL-1 is a key cytokine mediating matrix degradation in the IVD and therefore a therapeutic target.

KEY WORDS: Intervertebral disc, Interleukin-1, Interleukin-1Ra, Tumour necrosis factor, In situ zymography, Matrix degradation.

Introduction

Chronic low back pain (CLBP) either alone, or in association with sciatica, is a common disorder causing considerable population morbidity (6% prevalence) and a £10 billion pound annual cost to the UK economy through social and health care expenditure and loss of work. Recent controlled studies have established a causal association between degeneration of the intervertebral disc (IVD) and CLBP [1, 2].

Individual IVDs are part of a complex of interdependent spinal structures, including the vertebrae, facet joints, ligaments, nerve root and spinal canals. The IVDs facilitate movement, but arguably even more important, they have a role as ‘spacers’ and ‘stabilizers’ maintaining the optimal anatomical and biomechanical relationship between the other structures locally in the spine. Simply, this is achieved by a balance between forces generated by two major structures within the IVD, the central nucleus pulposus (NP) and the outer annulus fibrosus (AF). The normal NP consists of type II collagen fibres and hydrophilic proteoglycans [3], which form a hydrophilic molecular complex that generates a swelling pressure sufficient to separate adjacent vertebrae, even under the loads operating within the erect human spine. Excessive vertebral separation is resisted by tension in the type I collagen fibre arrays of the AF.

In degeneration of the IVD (DIVD), there is loss of hydrophilic matrix molecules from the NP. This leads to reduced vertebral separation and local spinal instability, which probably initiates the processes that lead to CLBP through repetitive microtrauma [4], ingrowth of nociceptive nerves [5, 6] and disc bulging with encroachment of IVD tissue onto nerve roots [7]. Inhibiting degenerative processes would be a novel approach to managing CLBP and one in which modern molecular pathology should play a critical role through identifying key molecular targets. In this context, local production of matrix-degrading enzymes is seen as being pivotal to the progressive loss of matrix molecules in degeneration [8–11]. Although matrix-degrading enzyme production could be an attractive therapeutic target, the key upstream events driving enzyme production are largely unknown. The cytokines IL-1β [12] and TNF-α [13] are overexpressed in the degenerate IVD, and this has led to both being implicated in the matrix degradation that characterizes degeneration. There is some direct evidence that both IL-1 [14–16] and TNF [17] might be involved in matrix degradation, but there have been no definitive studies comparing the effect of inhibiting these cytokines on matrix degradation.

In this study, we investigate the effects of these cytokines and inhibitors on matrix-degrading enzyme activity in degenerate and non-degenerate IVD. In vivo enzyme activity is highly regulated at a number of different levels (e.g. gene expression, gene translation, proenzyme activation, cell–matrix and enzyme–matrix interactions through endogenous inhibitors, etc. [18, 19]). As such, conventional approaches (e.g. immunohistochemical study of enzyme expression, investigation of cells in artificial matrices, use of extraction zymography), which cannot account for the full spectrum of regulatory controls, cannot adequately reflect enzyme activity in vivo. This criticism has been directed at previous studies examining enzyme activity in the IVD [10]. One technique, in situ zymography (ISZ) [20, 21] recapitulates the in vivo situation more closely than any other and is becoming widely used in oncology [22, 23] and musculoskeletal research [24, 25] for localizing enzyme activity in human and animal tissues. The principle of the technique is that a tissue section made from snap-frozen, unfixed whole tissue is placed on a gel containing the molecular target of the enzyme to be investigated. The gel is rendered visible, most commonly by binding a fluorescent dye in the gel. If uninhibited enzyme is present, the gel is digested and fluorescence lost, the area appearing black under the fluorescence microscope (Fig. 1). Gel degradation can be quantified using image analysis to measure the proportion of the gel that has changed from fluorescent green to black.

The technique will only work if active, uninhibited enzyme is present, which means enzyme RNA must have been synthesized, translated to pro-enzyme and the pro-enzyme activated. Furthermore the reaction will only proceed if there is less ‘inhibitor potential’ than ‘enzyme potential’, and because the tissue is intact this will include not only soluble but also tissue-bound inhibitors.

ISZ is specific for the matrix molecule (e.g. type II collagen) but not the enzyme. Conventionally, several matrices are selected that...
cover the spectrum of enzymes being investigated (e.g. gelatinases, collagenases, stromelysins). The tissue can be pre-treated prior to freezing (which kills the cells in the tissue) to examine the effects of putative stimulators/inhibitors of enzyme activity.

In the experiments described here, we have examined enzyme activity directed at matrix degradation using three gels, collagen type II, gelatin and casein (considered to cover a broad spectrum of matrix-degrading enzymes) in normal and degenerate AF and NP, both with and without pre-treatment of the tissue with IL-1, TNF, IL-1Ra and anti-TNF.

**Materials and methods**

**Source of human IVD**

Non-degenerate and degenerate IVD were obtained from live patients (LREC: Salford and Trafford [01/049]; Central Manchester [C/01/008]) (Table 1), and cadavers within 12 h of death (Trent MREC: 05/MRE04/03; Central Manchester [C/01/008]) (Table 1). Nine non-degenerate (six cadaveric, three live patients) and 12 degenerate (scores 7–8) (five surgical, four cadaveric) IVDs were studied (see next section for IVD score). Six parallel, sagittally orientated, tissue blocks each \( \frac{L}{C} \times \frac{L}{C} \times \frac{L}{C} \) mm\(^3\) incorporating AF and NP were taken from each case.

**Tissue processing and histological assessment**

One block per IVD was processed for histological, and *in situ* hybridization (ISH) analysis, using formalin fixation and paraffin embedding [13]. All samples were checked for orientation and inclusion of AF and NP, then graded for degree of degeneration using a previously published histological 12-point scoring system [26] which scores the following parameters: demarcation between the AF and NP; the presence and extents of degenerate slits in the matrix of the IVD; the extent of metachromasia (i.e. the GAG content) in the NP matrix; and the presence and extent of chondrocyte clusters in the NP, and scores them from 0 to 3 giving a possible aggregate score between 0 and 12 where the lower the score the less degenerate the IVD. Non-degenerate IVDs were selected on the basis of their having a low score, that is, they showed: good demarcation between the AF and NP; no degenerate slits in the matrix of the NP; a matrix rich in metachromatic molecules and no chondrocyte cluster formation. IVD of scores 7–8 showed advanced matrix degradation, with slits in the NP, loss of demarcation between the AF and NP and little

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**TABLE 1. Details of the individuals and the tissue sources used in these studies**

<table>
<thead>
<tr>
<th>Age (yrs); sex</th>
<th>Spinal level</th>
<th>Score</th>
<th>Reason excised/cause of death</th>
</tr>
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<tbody>
<tr>
<td>Normal live tissue</td>
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<tr>
<td>58; M</td>
<td>L4/5</td>
<td>0</td>
<td>Spinal trauma</td>
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<tr>
<td>24; M</td>
<td>L3/4</td>
<td>1</td>
<td>Spinal trauma</td>
</tr>
<tr>
<td>48; F</td>
<td>L3/4</td>
<td>1</td>
<td>Spinal reconstruction for metastatic breast cancer</td>
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<tr>
<td>Normal cadaveric tissue</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>51; F</td>
<td>L3/4</td>
<td>1</td>
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<tr>
<td>52; M</td>
<td>L4/5</td>
<td>1</td>
<td>Myocardial infarction</td>
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<td>64; M</td>
<td>L5/S1</td>
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<td>L4/5</td>
<td>2</td>
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<td>CLBP and DIVD</td>
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<tr>
<td>59; M</td>
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<td>7</td>
<td>CLBP and DIVD</td>
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<td>L4/5</td>
<td>7</td>
<td>CLBP and DIVD</td>
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<td>L4/5</td>
<td>8</td>
<td>CLBP and DIVD</td>
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<tr>
<td>51; F</td>
<td>L4/5</td>
<td>8</td>
<td>Myocardial infarction</td>
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metachromasia in the NP matrix, together with cell cluster formation in the NP. The cells were all viable when probed using non-radioactive ISH with a poly T probe for polyadenylated mRNA [27].

In situ zymography

One block was incubated for 48 h at 35°C in Tris–HCl buffer, and another four in buffer supplemented with IL-1β [10 ng/ml recombinant human IL-1β (R&D Systems Europe Ltd., Abingdon, Oxford, UK)], IL-1Ra [100 ng/ml (R&D systems)] [16], TNF (10 ng/ml recombinant human TNF-α (Genzyme Diagnostics, West Malling Kent, UK)) [28]; or anti-TNF neutralizing antibody 10 μg/ml (R&D Systems).

After 48 h, the tissue blocks were snap frozen in isopentane and cooled with liquid nitrogen. Cryostat sections (15 μm thick) were mounted on slides pre-coated with a gel consisting of equal quantities of melted 1% agarose type IV and 1 mg/ml of FITC-labelled gelatin, casein or bovine type II collagen (Sigma, Poole, Dorset, UK). Inside a 100% humidity chamber, nine randomized step serial tissue sections (three for each matrix) were each covered by a large, self-supporting drop of liquid consisting of Tris–HCl buffer pH 7.4. The zymographic reaction was allowed to proceed for 48 h at 35°C, changing the medium every 12 h.

At the start and at 48 h, slides were viewed in a fluorescence microscope with image capture facility. The images were transferred to a Leica QWin image analyser. The area of gel digested was measured in the AF and NP using an area-measurement programme. For this, sequential images of the gel beneath the full area of NP and AF were captured and the degraded (black) areas outlined using a mouse-based drawing facility. The area of degradation was expressed as a proportion of the whole area of the NP or AF investigated. To minimize intra-observer measurement error, all measurements were repeated three times at intervals of at least 1 day and averaged. Full ethics committee approval and informed patient consent was obtained before carrying out this study.

Activity of biologicals

To ensure that the purchased cytokines were active, both were subjected to bioassay. TNF-α bioassay was based on cytotoxicity of L-929 cells [29]. The assay confirmed that the TNF purchased from Genzyme was bioactive. The efficacy of the anti-TNF antibodies was assessed by repeating the assay having first mixed the TNF-α and anti-TNF at the concentrations used in the ISZ studies. Anti-TNF completely inhibited the TNF induced cytotoxicity.

IL-1β bioactivity was assessed using a technique devised in our laboratories, which employed D10 cells as indicators [30]. Pre-incubating the cells with IL-1Ra at the concentrations used in the ISZ experiments eliminated all IL-1 bioactivity.

Statistical analysis

Differences were assessed using two-tailed analysis of variance.

Results

Data are shown in Figs 2 and 3. Figure 2 shows the results of experiments comparing matrix-degrading activity in non-degenerate and degenerate IVDs without cytokine or cytokine inhibitor challenge. Figure 3 demonstrates the effects of IL-1β, TNF-α, IL-1Ra and anti-TNF on enzyme activity in the AF and NP of degenerate and non-degenerate IVDs.

All matrix degradation occurred in areas of the gel beneath cells, rather than matrix alone.
activity using the one technique (ISZ) that allows active enzyme to be localized in intact human IVD tissue. Our investigation differs from previous studies in four major ways, by using:

1. Human tissue: challenging studies of IVDs are often conducted on animal tissue, animal cells in monolayer or animal cells in artificial biomatrices. It is recognized that animal systems do not necessarily recapitulate normal or diseased human connective tissues [39, 40], particularly when the animals and humans are not of comparable age, emphasizing the need to test all hypotheses based on observations of animal systems, in human tissue.

2. Intact tissue: cells change their phenotype during extraction from IVD tissue, and change their function and differentiation status in culture [41, 42]. This is in part related to the extraction treatment and in part to the very abnormal environment the cells experience in culture, where, unlike in tissue they make direct contact with other cells and are not encased in extracellular matrix.

3. Tissue from the NP and AF of IVD: many previous studies of human IVDs have employed herniated tissue; however, herniated tissue is not believed to be the seat of the processes of degeneration. By selecting cases for study in which whole IVDs have been excised, we have been able to investigate disease mechanisms within those IVD components primarily affected by the processes of degeneration.

4. ISZ: the advantages of using this technique over others to examine enzyme activity are discussed above, but relate largely to its ability to detect active, non-inhibited enzyme and to allow localization of enzyme activity.

The first evidence that IL-1 might be involved in matrix degradation in DIVD came in 1988 in in vitro experiments employing rabbit AF cells [14]. In 1997, a ground-breaking study [15] extended these observations into human IVDs, but did not fully localize enzymes within the AF and NP. More recently it has been shown that normal IVD cells express both the isoforms of IL-1 [12], and expression is matched by that of the natural inhibitor of IL-1, IL-1Ra. In degeneration, IL-1α/β are up-regulated without increased IL-1Ra. In 3D alginate culture, IL-1α and β up-regulate IVD cell expression of key matrix-degrading enzymes [16] and in monolayer/3D culture, co-culture of IVD cells from degenerate IVDs with cells engineered to over-express IL-1Ra, inhibits endogenous IL-1 synthesis. Our data add to this body of knowledge by showing that enzyme activity is up-regulated by IL-1 and reduced by its inhibitor, IL-1Ra, placing IL-1 as a key regulator of matrix enzyme activity in the normal and degenerate IVD.

TNF-α was first described in the IVD in association with sciatic pain (reviewed in [43]). Subsequent studies have shown TNF-α peptide to be widely expressed in normal and degenerate IVDs [13], and in NP-like in vitro engineered constructs of normal bovine NP cells on a porous calcium-based substrate, Seguin et al. [17] reported that TNF-α reduced synthesis of matrix molecules and increased gene expression of MMP1, 3, 13 and ADAMTS4, 5. Why our data should be different from this is debatable. For instance, although up-regulation of a gene does not imply up-regulation of gene product, Seguin et al.’s [17] study described loss of specific elements of the matrix over a time frame that indicated matrix degradation as well as decreased synthesis. It might be that young bovine cells behave differently to adult human cells [32, 33], particularly if they have been extracted from tissue and grown in monolayer in stimulatory medium, or on an unusual substrate. Our own work has shown that, by ISH and immunohistochemistry, native disc cells express very few TNF receptors and, if anything, the number declines in degeneration [44]. This would certainly explain why human tissue does not respond appreciably to exogenous TNF. Clearly, the extracted and cultured juvenile bovine cells do express TNF receptors, at
least when cultured on a ceramic-based medium and that is a major difference between the use of human tissue and the animal cell-based construct.

As all the gene and protein expression data indicate that TNF-α is up-regulated in the degenerate IVD, and assuming that TNF is active, the role for this cytokine needs to be elucidated. In the absence of receptor expression in disc tissue, the option of its working through other tissues, such as local nerves becomes even more attractive.

Matrix degradation is a key event in degeneration of the IVD. Prevention of the activity of matrix-degrading enzymes could be an important novel approach to preventing the progress of matrix loss in degeneration and in preparing the diseased IVDs for receipt of tissue-engineering constructs. Therefore, understanding the drivers of matrix degradation has practical therapeutic implications. Demonstrating that IL-1Ra almost completely eliminates matrix-degrading activity places IL-1 as a key regulator of matrix degradation in DIVD. This would explain why IL-1 has greater expression in those IVDs that are clinically demonstrated to be degenerated human intervertebral discs. Spine 2001;26:2666–72.

References

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References


