INTERLEUKIN-1β ENHANCES CARTILAGE-TO-CARTILAGE INTEGRATION

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Abstract

The failure of cartilages to fuse, particularly in the case of articular cartilage under conditions of repair is due to morphological and structural constraints of the tissue. Factors that impede integration include, non-vascularisation, low cellularity, and proteoglycan in the surrounding extracellular matrix acting as a natural barrier to cellular migration. We hypothesised that brief activation of a catabolic cascade by cytokines followed by culture under anabolic conditions would promote tissue fusion in a ring-disk model of cartilage integration. Our results show that transient exposure to 10 ng mL^{-1} interleukin-1β, followed by two weeks post-culture under anabolic conditions, enhanced cartilage-cartilage integration compared to untreated explants. Quantitative PCR analysis of catabolism-related genes ADAMTS4 and MMP13 showed both were transiently upregulated and these findings correlated with evidence of extracellular matrix remodelling. At the level of histology, we observed chondrocytes readily populated the interfacial matrix between fused explants in interleukin-1β treated explants, whereas in control explants this region was relatively acellular in comparison. Catabolic cytokine treated explants exhibited 29-fold greater adhesive strength (0.859 MPa *versus* 0.028 MPa, $P < 0.05$) than untreated counterparts. Collectively, our results demonstrate that a single short catabolic pulse followed by an anabolic response is sufficient to generate mechanically robust, integrative cartilage repair.

Keywords: Cartilage, integration, repair, interleukin-1β.

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Introduction

One of the problems associated with the repair of articular cartilage defects is the lack of lateral cartilage integration (Khan *et al*., 2008). Following wounding, or cartilage repair using autologous chondrocyte implantation (Brittberg *et al.*, 1994), the lack of cartilage fusion between host, or host and repair tissues may precipitate further degeneration (Shapiro *et al*., 1993). Numerous factors directly or indirectly influence tissue integration and there have been a number of strategies devised to overcome this particular obstacle to repair.

The fundamental intrinsic barriers to integration stem from the avascular and hypocellular nature of articular cartilage limiting the migration of chondrocytes or mesenchymal stem cells to an affected site. Additionally, the extracellular matrix (ECM) surrounding chondrocytes in articular cartilage, particularly the proteoglycan component, is inhibitory for cellular migration (Hunziker, 1999; Hunziker, 2002). Proteoglycans not only constitute a hydrophilic barrier to cell migration; but proteoglycan-4 (PRG4/lubricin/superficial zone protein), that provides boundary lubrication of congruent articular surfaces, also potently inhibits cartilage-cartilage integration (Schaefer *et al*., 2004). Enzymatic removal of proteoglycan using trypsin, hyaluronidase or chondroitinase ABC digestion has been used as a strategy to mobilise chondrocytes to enhance cartilage integration (Hunziker and Kapfinger, 1998). Other studies have shown that reconstitution of the collagen fibril network that is disrupted following wounding or cartilage repair is a major factor in initiating integrative cartilage repair. Integration of live and devitalised cartilage is dependent on collagen deposition (DiMicco and Sah, 2001). Additionally, inhibition of collagen fibril crosslinking using β-aminopropionitrile in cartilage explants, prior to fusion, increases the adhesive strength through the accumulation of crosslinking precursors (McGowan and Sah, 2005) that are then free to form bonds between collagen fibrils spontaneously when the opposing cartilages are fixed together and inhibitor washed out. Collagenase treatment of cartilage explants has also been shown quantitatively to increase cartilage integration – primarily, it has been hypothesised, by promoting an increase in cellularity at the lateral margins of the fused cartilages, triggered by collagen network (and proteoglycan) disruption and reformation (Bos *et al*., 2002).

Whilst many of the techniques described above are relevant to cartilage repair, they do have some disadvantages. For example, collagenase treatment of

articular cartilage can cause chondrocyte outgrowths from human explants at a rate ten times that of untreated cartilage (Qiu *et al.*, 2000), and, more importantly, disruption of the collagen fibril network is hypothesised to be an irreversible step on the pathway to cartilage degeneration (Stoop *et al*., 1999).

We hypothesised that by shifting the metabolic state of articular cartilage to a transient catabolic state, several of the desirable properties required for cartilage integration, such as increased proteoglycan turnover and disruption of the collagen network, could be induced. In this study, we used the well-characterised catabolic cytokine interleukin-1β (IL-1β) to transiently alter the metabolic state of wounded immature bovine articular cartilage. Then, we monitored the effect on cartilage integration, following recovery under anabolic conditions, using histological, molecular and mechanical analyses. IL-1 is a cytokine that is expressed by activated synoviocytes, mononuclear cells and chondrocytes (Ollivierre *et al.*, 1986; Smith *et al.*, 1997; Towle *et al.*, 1997). Levels of IL-1 are elevated in patients that have osteoarthritis (OA) and rheumatoid arthritis (RA) (Wood *et al.*, 1983; Farahat *et al.*, 1993) and studies where IL-1 has been injected into animal joints describe inflammation, joint swelling and degradation of collagen and proteoglycan (Saklatvala *et al.*, 1983; Pettipher *et al.*, 1986; Farahat *et al*., 1993; Billinghurst *et al.*, 2000). Studies have shown that short-term, 24 h, exposure of bovine articular cartilage to IL-1 does not affect chondrocyte viability though it does cause a 10 % reduction in proteoglycan content but this change has little impact on the overall ultrastructure of the tissue (Stabellini *et al.*, 2003). Critically, in terms of integration, following limited exposure to IL-1, and this has been shown to be true for other inflammatory mediators, such as carrageenin (Gillard and Lowther, 1976) the rate of proteoglycan synthesis in articular cartilage, which is initially depressed following exposure, increases above normal levels over a period of 28 d (Page Thomas *et al.*, 1991). Therefore, based upon our hypothesis, IL-1 fulfils the requirement for these studies in that a single pulse of this factor induces a transient, reversible metabolic disturbance resulting in remodelling of the ECM.

Materials and Methods

Materials

All chemicals, of analytical grade or above, were obtained from Sigma (Poole, UK) unless otherwise stated. Serum-free (anabolic) culture medium consisted of Dulbecco's modified Eagles medium (DMEM) plus GlutaMAX (4.5 g/L glucose, no pyruvate) containing 10 mM HEPES pH 7.4, 10 µg/mL gentamicin, 50 µg/mL ascorbate-2-phosphate and supplemented with with 10 µg/mL insulin, 5.5 µg/mL transferrin and 6.7 ng/mL selenium (ITS). ITS supplementation enables chondrocytes to maintain their normal metabolism and phenotype under serum-free conditions (Gilbert *et al*., 2006).

Cartilage explant preparation and culture

Articular cartilage was obtained from the metacarpophalangeal joint of 7 day old bovine steers. In order to simulate cartilage-cartilage integration in this study we used a combination of 6 mm and 3 mm diameter biopsy punches (Steifel, Maidenhead, UK) to excise composite cartilage explants that consisted of a 6 mm diameter outer annulus and 3 mm diameter inner disc core. Explants were only removed from the medial groove of the medial condyle where cartilage of a consistent depth was present. Explants were then washed in DMEM three times and randomly assigned to treatment groups. For catabolic cytokine stimulation, explants were incubated in the presence or absence of 10 ng/mL IL-1β (Peprotech, London, UK) in DMEM, 10 mM HEPES pH 7.4 and 10 µg/mL gentamicin for the indicated times. Explants were then cultured in serum-free (anabolic) medium supplemented with ITS at 37 °C in a humidified atmosphere of 5 % CO_2 – 95 % air.

Histological staining

Explants were fixed with 4 % paraformaldehyde for 12 h at 4 °C then processed for wax embedding. Eight µm sections were cut and then dewaxed, rehydrated and stained with Mayer's haemalum and 1 % eosin (H&E) to visualise the basic structure of tissue sections. To visualise proteoglycans in cartilage we used 1 % Safranin-O staining for 30 s of rehydrated sections; the intensity of Safranin-O staining is directly proportional to the proteoglycan content in normal cartilage (Camplejohn and Allard, 1988).

Quantitative polymerase chain reaction (qPCR)

Cartilage explants were washed in PBS and then frozen in liquid nitrogen. The frozen explants were then homogenised in the presence of 1 mL frozen TRI Reagent™ using a Mikro-Dismembrator U (B. Braun Biotech International, Melsungen, Germany) and RNA extracted using the RNAeasy extraction columns (Qiagen, Crawley, UK) with DNAse I treatment. Isolated RNA was

Table 1. Nucleotide sequences of primer sets used in this study.

18S rRNA
forward 5'GGCCTCACTAAACCATCCAA3'
reverse 5' GCAATTATTCCCCATGAACG3'
a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4)
forward 5' CTCCATGACAACTCGAAGCA 3'
reverse 5' CTAGGAGACAGTGCCCGAAG 3'
aggrecan (ACAN)
forward 5' GCTACCCTGACCCTTCAT 3'
reverse 5'AAGCTTTCTGGGATGTCCAC 3'.
metalloproteinase-13 (MMP13)
forward 5' TGGTGATGAAACCTGGACAA 3'
reverse 5' GGCGTTTTGGGATGTTTAGA 3'

resuspended in diethylpyrocarbonate-treated water and then quantified using a NanoDrop 2000c spectrophotometer (NanoDrop, Wilmington, DE, USA). qPCR analysis was performed using the GoTaq qPCR mastermix (Promega. Madison, WI, USA), 12.5 ng cDNA and 0.3 μM forward and reverse primers. Reactions were performed on a Stratagene Mx3000 real-time PCR analyser (Agilent Technologies, Santa Clara, CA, USA) with the following thermal cycling program; 95 ºC for 10 min – 1 cycle, 95 ºC 30 s, 55 ºC 60 s, 72 ºC 30 s – 40 cycles. Standard curves over the linear range of amplification were generated for all primer sets, and data were used where the efficiency of amplification was between 95-105 % and the melt curves generated a single product. The data shown are the ratio of the concentration of the gene of interest (in nanograms) and 18S rRNA (in nanograms). The nucleotide sequences of primer sets used in this study are given in Table 1.

Sulphated glycosaminoglycan (sGAG) concentration determination

40 μL of sample culture medium were added to a 96 well plate, followed by 200 mL of dimethylmethylene blue (DMMB) reagent (Farndale *et al.*, 1986). Shark chondroitin-6-sulphate (Sigma-Aldrich, St. Louis, MO, USA) dissolved at a concentration of 0-40 mg/mL in serum-free culture medium was used to produce a standard curve to determine experimental values. The final values for sGAG concentrations were calculated by dividing each value by the wet weight, in mg, of each respective explant. All assays were performed in triplicate and explants from three different animals were used.

Immunofluoresence detection of epitopes

Explants were treated with IL-1β 10 ng/mL for 24 h then flash frozen in n-hexane and 8 μm cryosections were generated. Sections were dried, fixed in 95 % alcohol, washed in Tris-buffered saline/0.1 % Tween-20, blocked with serum and then incubated with primary antibody mouse anti-col23/4m at a concentration of 5 mg/mL. Goat anti-mouse Alexa 488-conjugated secondary antibodies (Invitrogen, Paisley, UK) were used to visualise the location of primary antibodies using fluorescence microscopy (BX61; Olympus, Southend-on-Sea, UK). Negative controls used purified mouse IgG fractions.

Mechanical push-out testing

Explants were incubated in the presence or absence of 10 ng/mL IL-1β for 24 h, then cultured for a further 2-4 weeks in serum-free anabolic medium that was changed every other day. Mechanical testing was performed as previously described (Moretti *et al.*, 2005; Gilbert *et al.*, 2009). Briefly, a mechanical testing rig composed of a Lloyd LRX material testing machine (Lloyds Instruments, Hants, UK) with a push-out rod displaced the inner disc core of the explant from the outer annulus, placed in a custom made housing, at a rate of $1x10^{-18}$ N/mm^2/s using a computer controlled stepping motor. A load cell of 100 N coupled to the displacement rod measured the push-out force; the adhesive force was calculated from the maximum force measured at failure.

Statistical analysis

Data are presented as mean±standard deviation (SD; $n \geq 3$ replicates per treatment). All datasets were checked for normal distribution using the Shapiro-Wilk test and homogeneity of variances using Levene's test prior to parametric analysis. For analysis of multiple groups a one-way analysis of variance (ANOVA) test was used. Where treatment groups did not meet the assumptions for parametric analysis (indicated in the text) we conducted non-parametric analysis using the method of Kruskal-Wallis and pair-wise analysis of independent groups using the Mann-Whitney U test.

Results

The histological effect of IL-1β pre-treatment of cartilage explants was compared to that of collagenase pre-treatment, which has previously been shown to enhance integration (Fig. 1). Cartilage explants exposed to 30 U/mL of purified collagenase VII for 24 h, as previously described (Bos *et al.*, 2002), displayed almost total loss of proteoglycan from the ECM at the margins of explants as assessed histologically by the absence of Safranin-O labelling in these regions. Collagenase treatment also resulted in a reduction in size of the explant, presumably through an extensive loss of proteoglycan and collagen digestion, leading to an increased cell density especially at the peripheral aspects of the explants. Safranin-O staining in explants treated with 10 ng/mL IL-1β for 24 h was less intense but was present uniformly throughout the whole explant.

For our model of integrative cartilage repair we used two biopsy punches of 6 mm and 3 mm diameter to generate composite cartilage explants from the metacarpophalangeal joint of immature bovine articular cartilage composed of an outer annulus and inner disc core, Fig. 2 (Schaefer *et al.*, 2004; Moretti *et al.*, 2005; Gilbert *et al.*, 2009). We performed a preliminary examination of the effect of transient IL-1β stimulation on cartilage explant composites in DMEM for various times ranging from 0-48 h followed by *in vitro* culture in ITS-supplemented serum-free culture medium for 2 weeks. We had previously determined that the optimum concentration of IL-1β to induce a catabolic state in cartilage explants was 10 ng/mL (data not shown). The results of histological examination of tissue sections from ring-disk composites showed that incubation of explants with IL-1β for 12-48 h induced more complete fusion of cartilage compared to control untreated explants (Fig. 3a). Cartilage explants pre-treated for 24 h in 10 ng/mL IL-1β were used for all subsequent analyses.

When we sectioned IL-1β treated explants *en face*, we observed that both cartilages were fused and there was a region of reduced Safranin-O staining where the inner annulus met the outer disc core, indicating loss of proteoglycan (Fig. 3b). However, in the interfacial matrix between the disc and annulus of the explant cartilage there was a band of staining that was higher in intensity, and on inspection of higher power images we observed the presence of chondrocytes in this region of neo-cartilage.

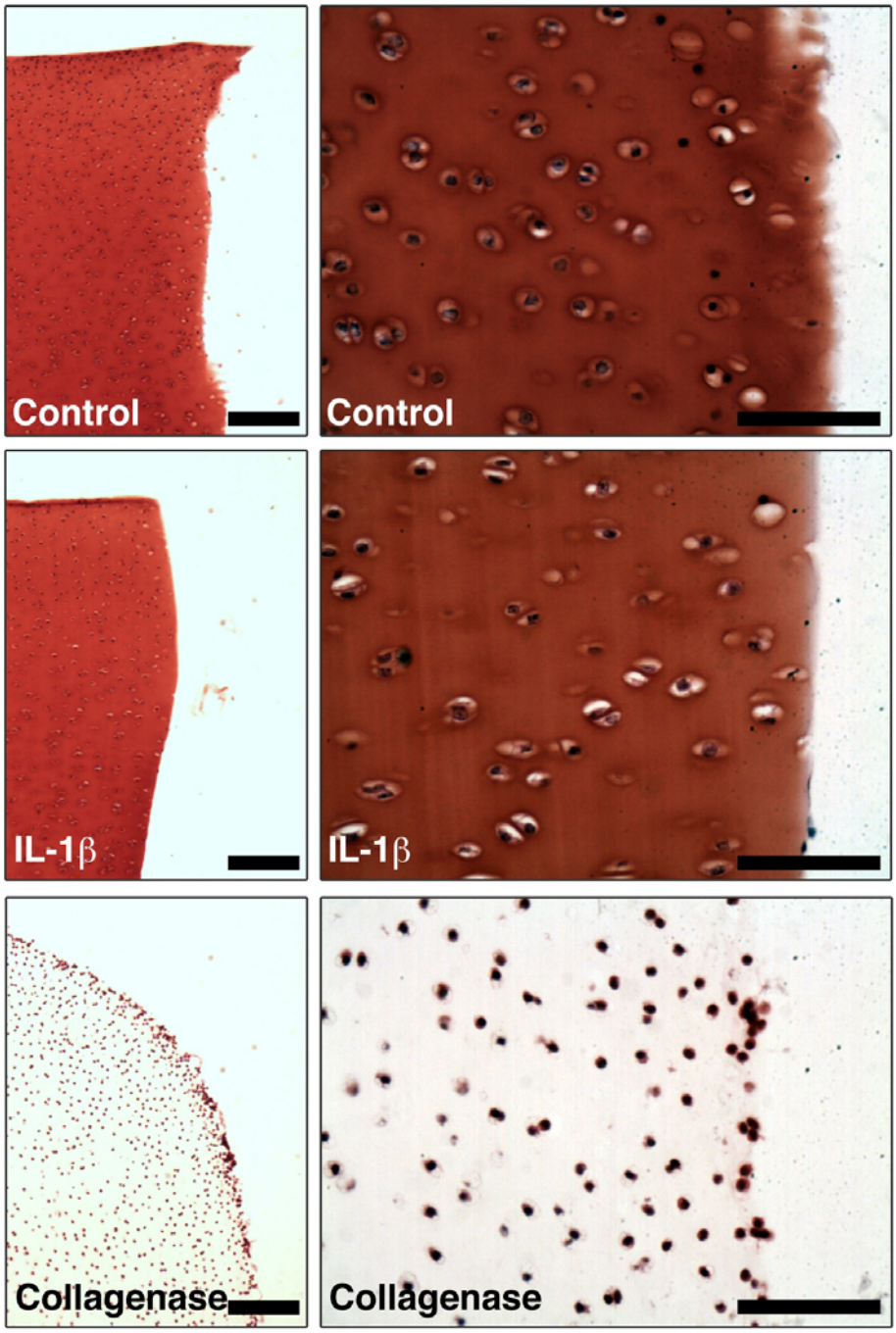

Fig. 1. Histology of IL-1β and collagenase treated immature articular cartilage explants. Cartilage explants from the metacarpophalangeal joint of immature (7-day-old) calves were equilibrated in 10 % foetal calf serum containing culture medium for 48 h then washed with DMEM and placed in DMEM with either 30 U/mL collagenase VII (Sigma-Aldrich) or 10 ng/mL IL-1β at 37 ºC for 24 h. Sections are representative of 3 independent experiments. Sections of cartilage were stained with Safranin-O to detect proteoglycan content of explants, low magnification (*left*: bar 100 μm) and high magnification (*right:* bar 50 μm) microscopy images of the explant edges are shown.

In contrast, in control explants, the interfacial matrix weakly stained with Safranin-O and was torn following histologic processing (Fig. 3b). In higher power images, the intervening matrix in control explants was acellular and this was true for the whole circumference of the wound.

Next, we analysed whether a 24 h incubation with IL-1β had a significant effect on the extracellular matrix of cartilage explants. We used qPCR to analyse the expression levels of two key catabolism-related genes, ADAMTS4 and MMP13 (Fig. 4a). ADAMTS4 gene expression was upregulated 27-fold ($P < 0.05$; K/W and Mann and Whitney U-test) from baseline day 0 (control) levels following IL-1β stimulation, then dropped to 4-fold by 2 d post-treatment to baseline levels by 5 d post-treatment (7.01E-4 ±5.46E-4 on day 1, 1.18E-4 ±2.76E-5 on day 3, 4.23E-5 ±1.24E-5 on day 6 *versus* 2.56E-5 ±1.13E-5 on day 0, all values ratio ADAMTS4/18S rRNA). MMP13 gene expression was similarly transiently upregulated 232-

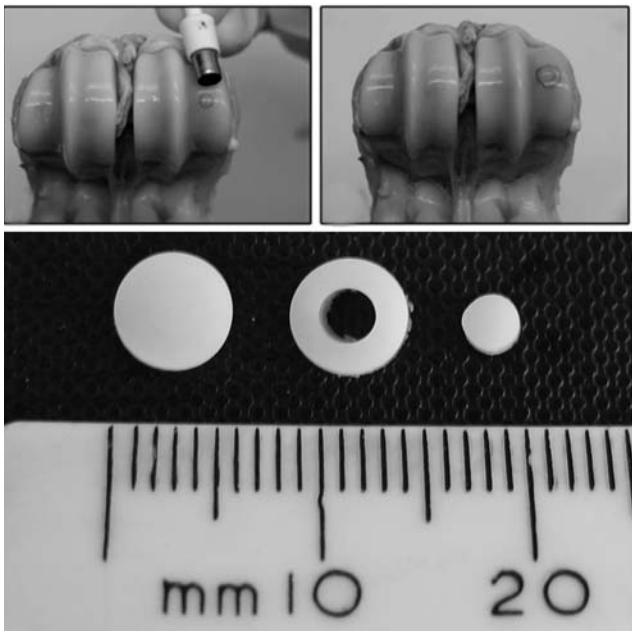

Fig. 2. Creation of disc-ring cartilage composites for studying cartilage integration. Biopsy punches of 6 mm and 3 mm diameter were used to form concentric rings in the medial groove of the medial condyle of the metacarpophalangeal joint of 7-day-old steers (*above*). The ring-disk cartilage composites were surgically excised and are shown intact (*below left*) and disassembled (*below right*). Ruler divisions are in mm. Experiments were performed only on the intact cartilage composites.

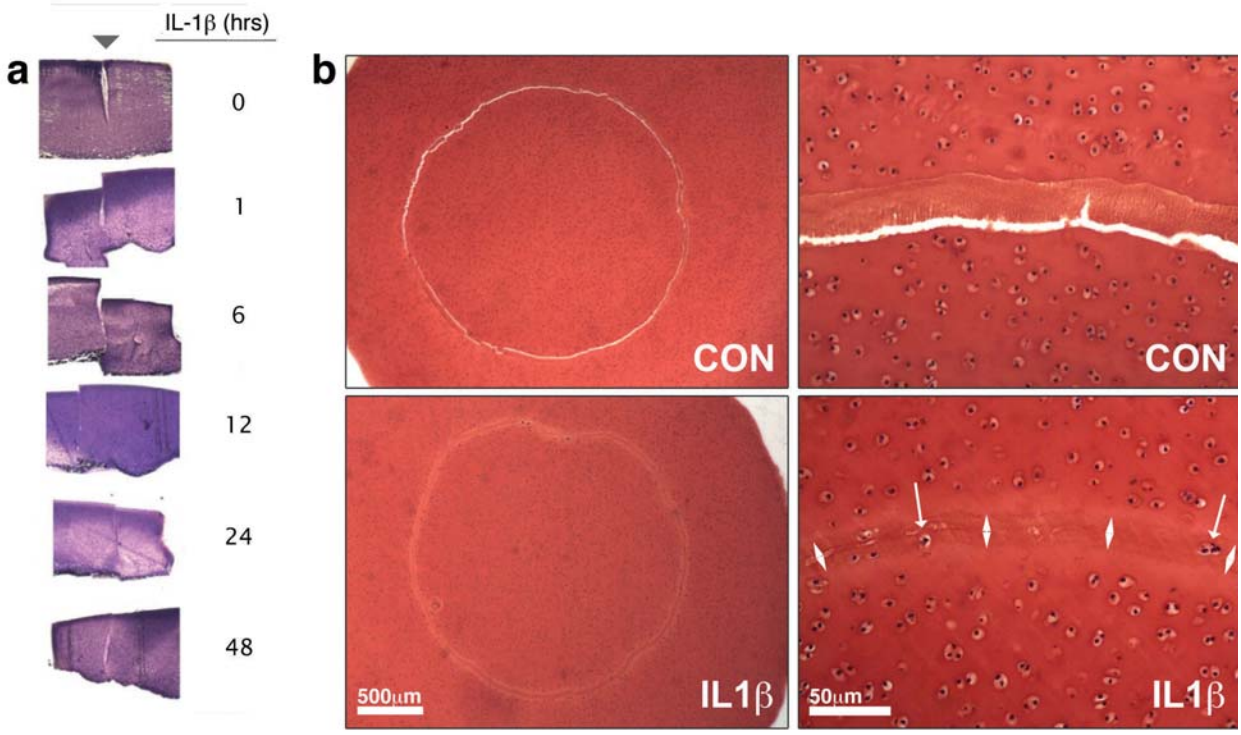

Fig. 3. Histologic analysis of integration in IL-1β treated wounded articular cartilage explants. (a) Wounded explants were exposed to 10 ng mL^{-1} IL-1β for 0-48 h then cultured in serum-free medium for 2 weeks. H &E stained sections are shown from representative samples from each treatment group. **(b)** Safranin-0 stained tissues are shown for explants sectioned *en face* incubated in the presence (IL-1β) or absence (con) catabolic cytokine for 24 h then cultured in serum-free, ITS-containing medium for 2 weeks (low power images, *left*). In the higher power images (*right*) the intervening matrix (delineated by opposing white arrowheads) between the wounded cartilages has been infiltrated by chondrocytes (*white arrows*) that are absent in control sections.

fold from baseline levels and fell to 18-fold and 4-fold, 2 and 5 d post-treatment, respectively (5.26E-2 ±1.76E-2 on day 1, 4.07E-3 ±2.33E-3 on day 3, 1.01E-3 ±5.61E-4 on day 6 *versus* 2.27E-4 ±8.86E-5 on day 0, all values ratio MMP13/18S rRNA).

A more direct measure of the effects of transient IL-1β treatment on the extracellular matrix of composite cartilage explants was analysis of proteoglycan release into the culture medium. The amount of sGAG released into the culture medium by IL-1β treated explants over the period of eight days (Fig. 4b, *left*), was significantly higher than that by untreated explant cultures for the first 6 d of measurement ($P < 0.05$; ANOVA). This separation in values continued until the period covered by days 6 and

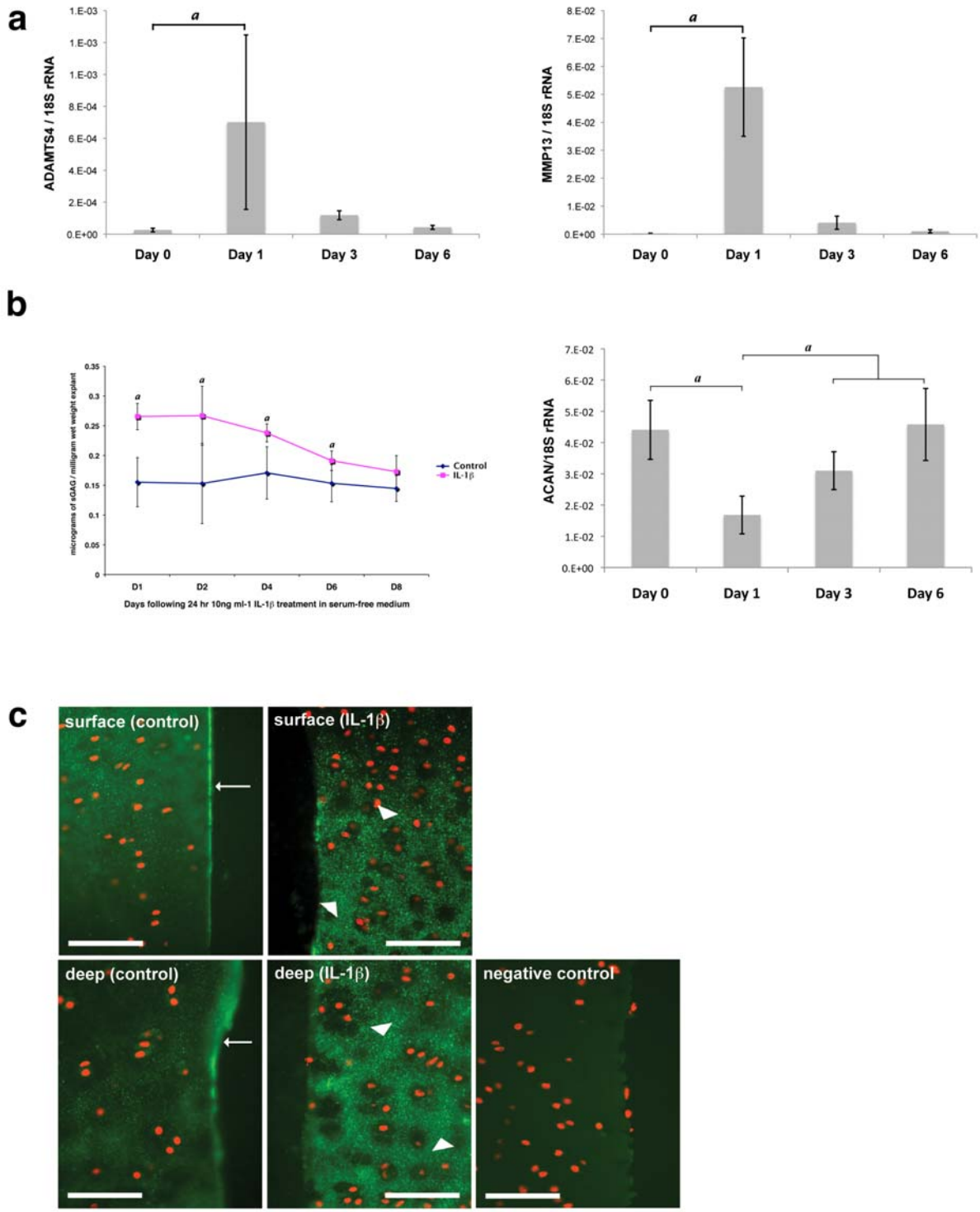

Fig. 4. The effects of IL-1β exposure are reversible. **(a)** Gene expression analysis by qPCR shows that ADAMTS4 and MMP13 gene expression are significantly upregulated following IL-1β treatment (Day 1) from baseline levels (Day 0) but that 5 d post-treatment transcript levels fall to near baseline levels. Gene expression data are shown mean±SD, ($n = 4$ for all groups) and analysed by non-parametric Kruskal-Wallis and Mann-Whitney pairwise analyses (a, $P < 0.05$). **(b)** Quantification of sulphated glycosaminoglycan (sGAG) content in the culture medium following 24 h, 10 ng/mL IL-1β treatment, then culture in anabolic medium ($n = 4$; a, $P < 0.05$) (*left*). Gene expression analysis of aggrecan (ACAN) prior (day 0) and following (days 1-6) IL-1β treatment ($n = 4$, a, $P < 0.05$) (*right*). **(c)** Col23/4m labelling was intense in explants stimulated with 10 ng/mL IL-1β for 24 h compared to control untreated explants. Labelling was visualised as bright, punctate dots distributed throughout the extracellular matrix (*middle panel*, *white arrowheads*). Labelling was present at a much lower level in control sections, a normal occurrence in immature cartilage that is constantly turning over matrix (*left panel*). The most intense labelling in control section emanated from collagen denaturation caused by mechanical disruption of collagen fibrils by biopsy punches (*left panel*, *white arrows*). Control sections labelled with mouse IgG exhibited no labelling with secondary antibody (*right panel*). Bar 50 μm.

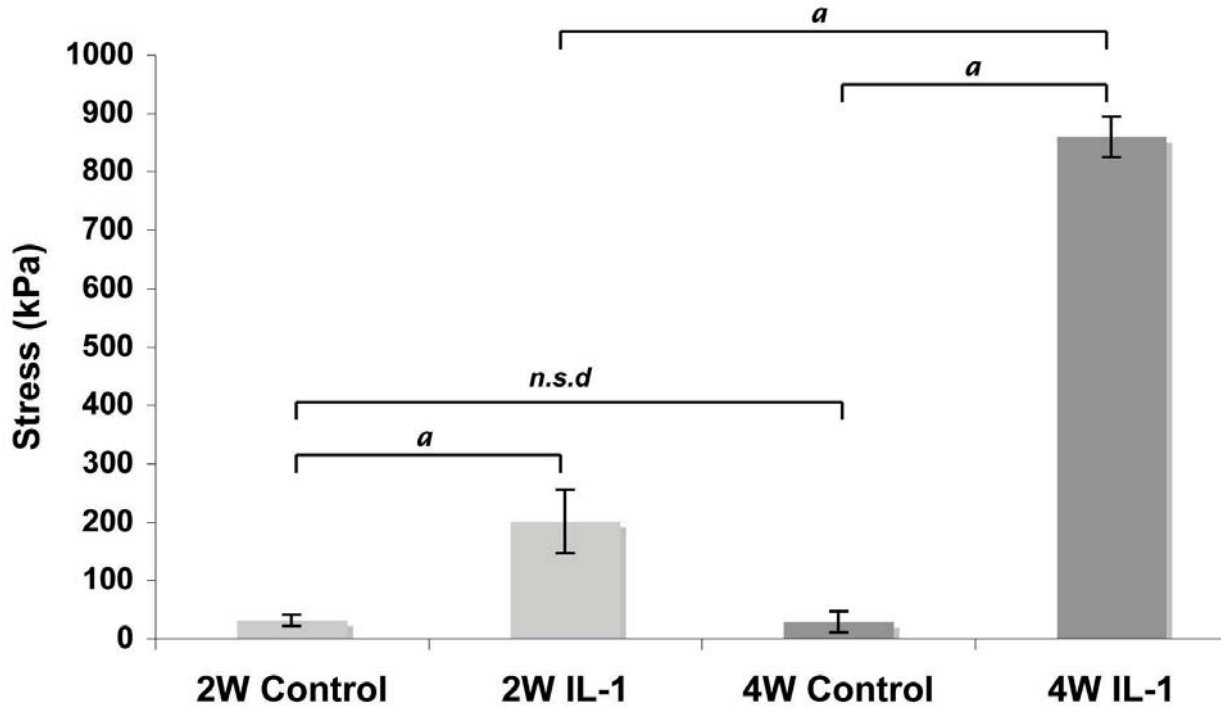

Fig. 5. Mechanical testing of IL-1β treated and untreated wounded cartilage explants for adhesive strength. Cartilage explants were treated with 10 ng/mL IL-1β for 24 h then cultured post-treatment for 2-4 weeks in serum-free medium supplemented with ITS. Explants were then tested for the adhesive strength of the inner disc core to the outer annulus using a mechanical testing rig (as described in Materials and Methods). Data are presented as mean±SD of 2 week control (n = 5), 2 week IL-1β (n = 4), 4 week control (n = 4) and 4 week IL-1β (n = 3) and analysed using ANOVA with post hoc testing using the method of Scheffe (a, $P < 0.05$).

8, at the end of which there was no significant difference in sGAG found in the culture medium between control and IL-1β treated composite explants. Using qPCR and primers for aggrecan (ACAN) we observed a 2.6-fold drop ($P < 0.01$; K/W and Mann and Whitney U Test) in transcript levels one day following IL-1β exposure. Between days 3-6 there was no significant difference between measured transcript levels compared to the time prior to treatment at day 0 (Fig. 4b, *right*).

Given the high-level induction of MMP13 gene expression we used monoclonal antibody col23/4m, which is a marker for collagen type II denaturation and recognises an epitope revealed upon enzymatic degradation (Hollander *et al*., 1994) to localise collagenase activity (Fig. 4c). In control explants, there was low level labelling throughout the matrix, consistent with constant collagen turnover associated with immature articular cartilage (Fig. 4c, *left panel*). The brightest signal in control sections emanated from the edge of explants where collagen denaturation had occurred through the process of wounding with the biopsy punch. In contrast, we observed a high intensity of labelling for col23/4m in 24 h IL-1β treated explants in both the surface and deep zones of cartilage. Labelling was throughout the extracellular matrix and visible as bright punctate dots (Fig. 4c, *middle panel*). The use of mouse IgG control antibodies elicited no non-specific labelling in sections (Fig. 4c, *right panel*).

The adhesive strength of the control and IL-1β treated explants cultured post-treatment for 2 or 4 weeks were measured using cartilage disc push-out testing apparatus. Our results show that following 2 weeks, an initial 24 h IL-1β pre-incubation period enhanced the mechanical strength of integration by approximately 6-fold ($P < 0.05$; ANOVA) compared to control explants (200.75 ±54.14 kPa *versus* 31.67 ±9.56 kPa, respectively) (Fig. 4). By 4 weeks, the difference in mechanical strength between treated and control explants increased by approximately 29-fold (859.21 ±34.24 kPa *versus* 28.67 ±18.09 kPa, respectively; $P < 0.05$; ANOVA). In the absence of IL-1β treatment there was no improvement over time of the mechanical strength scores for control explants.

Discussion

Integration of cartilages following articular cartilage repair has long been a problem due to factors intrinsic to its structure and function. We hypothesised that, by disturbing the metabolic balance of articular cartilage through induction of a transient catabolic state, it would be possible to promote integrative cartilage repair through controlled extracellular matrix remodelling. Our data demonstrated that IL-1β-induced transient changes in cartilage remodelling and anabolic gene synthesis correlated with enhanced adhesiveness of cartilages in an *in vitro* model of integration. Our method differs from previous techniques in that they were either designed to remove specific components of the ECM or bond

components of the ECM together to enhance cartilage to cartilage integration.

The transient nature of matrix remodelling was observed through assaying the gene expression levels of key catabolic, IL-1β inducible genes; ADAMTS4, encoding an aggrecanase, and MMP13, encoding a collagenase. The levels of transcriptional upregulation that we observed for ADAMTS4 and MMP13 (27-fold and 232-fold from baseline untreated day 0 values, respectively) correlated with data from Karsdal *et al.* (Karsdal *et al*., 2008) who showed 20-fold and 150-fold increases in proteoglycan and collagen metabolism, respectively, using biochemical assays of cytokine-stimulated cartilage explants. Additionally, our data show that ADAMTS4 and MMP13 returned to baseline, or near baseline, transcription levels 5 d post-treatment – demonstrating that transient catabolic effects are reversible at the level of gene expression. Karsdal *et al.* also showed that, following incubation with catabolic cytokines (7-17 d), subsequent culture in insulin growth factor-1 (IGF-1) containing medium completely reversed the effects of catabolism – as shown through the replenishment of proteoglycan levels in cartilage explants. Our experiments suggest that an IL-1β incubation period of 24 h only transiently decreased aggrecan gene synthesis – a 2.6-fold reduction, gene synthesis returning to non-IL-1β treated levels after day 3. We also demonstrated that proteoglycan secretion into the culture medium returned to baseline levels, after 7-8 d following IL-1β treatment, providing further evidence of the transient nature of the cytokine-induced catabolic effects.

Previous attempts at cartilage-cartilage integration have used techniques that include enzymatic modification of collagen or proteoglycan proteins in the extracellular matrix, or gluing of cartilage matrices. Tissue transglutaminase catalyses the calcium-dependent formation of covalent γ-glutamyl-ε-lysine isopeptide bonds between proteins, producing stable polymer networks, and has been used as a biological glue for cartilage-cartilage integration (Jurgensen *et al.*, 1997). The adhesive strength of cartilages bound by transglutaminase, approximately 36kPa, was shown to be 62 % higher than fibrin sealant that has also been used to seal and bind cartilages together (Kaplonyi *et al.*, 1988). Chemical methods to induce cartilage bonding have utilised crosslinking agents such as, glutaraldehyde, 1-ethyl-3-diaminopropyl-carbodiimide/N-hydroxysuccinimide (EDC/NHS) and chondroitin sulphate biopolymers that have been functionalised with methacrylate and aldehyde groups to bridge tissue proteins (Englert *et al.*, 2007; Wang *et al.*, 2007). Adhesive strengths of up to 60 kPa between cartilages were obtained using EDC/NHS, and uniaxial tensile and shear forces of approximately 45 kPa were measured between cartilage and hydrogels held together with chondroitin sulphate adhesive. In the latter cases, measurements were made immediately following crosslinking and cartilages were not tested following extended incubation and, therefore, it is not known if the adhesive strength increases with time (Englert *et al*., 2007).

Collagen type II is the major structural protein in articular cartilage and studies have shown that collagen deposition is a requirement for cartilage-cartilage integration (DiMicco and Sah, 2001). Inhibition of lysyl oxidase mediated collagen crosslinking of cartilage, using β-aminopropionitrile, negatively affects cartilage integration – providing further evidence of the role of collagen biosynthesis and maturation as crucial determinants of cartilage fusion (McGowan and Sah, 2005). Pretreatment of cartilage explants with β-aminopropionitrile was also found to accelerate integration of opposed explants, cultured for 14 d due to the build up of precursor crosslinks, and their subsequent maturation to form interfibrillar crosslinks. Enzymatic digestion of the collagenous component of the extracellular matrix of cartilage explants, using highly purified collagenase, has been shown to induce fusion of cartilage matrices. The mechanism through which fusion occurs was hypothesised to be, in part, through proliferation and mobilisation of chondrocytes, in addition to collagen fibrillogenesis (Bos *et al*., 2002). Further studies using collagenase digestion of cartilage explants to enhance integration showed that the adhesion strength, as measured using push-out testing, was on average 1.32 MPa – approximately 7-fold less than the intrinsic failure strength of 8.8 MPa for intact cartilage (van de Breevaart Bravenboer *et al.*, 2004). However, the concentrations of collagenase used in the latter studies caused profound reductions in proteoglycan content at the periphery of the cartilage explants and, by inference, a reduction in water content leading to a collapse of the ECM and the apparent increase in cellular density (Bos *et al*., 2002). The resultant accumulation of chondrocytes at the margin of collagenase treated explants correlated with an increase in adhesive strength of fused cartilages (Bos *et al*., 2002; van de Breevaart Bravenboer *et al*., 2004). However, treatment of cartilages with collagenases can be disadvantageous. The liberation of chondrocytes from the extracellular matrix induces outgrowths of chondrocytes that then express a contractile actin isoform, α-smooth muscle actin, that can modify the surrounding ECM (Qiu *et al*., 2000). Furthermore, a preliminary analysis of mechanical properties shows that the compressive stiffness of collagenase pre-treated cartilage is approximately 6-fold less than control cartilage and 3-fold less than IL-1β treated cartilages (data not shown). Therefore, IL-1β treated explants produce comparable levels of adhesive strength to collagenase treated explants (~0.86 MPa *versus* ~1.32 MPa, both measured using push-out testing), show greater compressive resistance, no gross morphological changes following pre-treatment and exhibit reversible effects on catabolic and anabolic gene synthesis; these data demonstrating that a transient catabolic pulse can enhance significantly cartilage-cartilage integration.

Chondrocytes were observed within the intervening matrixof IL-1β treated but not control explants, and it is possible that they account for the intense band of Safranin-O labelling causing the production and retention of proteoglycan within the interfacial matrix. The influence of chondrocyte cellularity and/or proliferation on promotion of integrative cartilage repair has been well documented (Hunziker and Kapfinger, 1998; Obradovic *et al.*, 2001; Bos *et al*., 2002; Quinn and Hunziker, 2002; van de Breevaart Bravenboer *et al*., 2004). Following

experimental wounding of articular cartilage, a zone of cell death (necrosis, resulting from the initial wounding, followed by apoptotic cell death) is induced that can penetrate the tissue up to 400 μm laterally (Tew *et al*., 2000). The associated lack of cellularity is thought to contribute to a failure of cartilage integration due to a lack of maintenance of the surrounding matrix (Shapiro *et al*., 1993). The latter phenomenon has also been noted in a study replicating surgical procedures used in humans, on rabbit and miniature pig knees, where it was shown that the maintenance of cartilage matrices close to wound edges was compromised due to lower than normal cellularity, indicating that 'repair' procedures were in fact contributing to further degenerative decline of cartilage (Hunziker and Quinn, 2003). Gilbert *et al.* have demonstrated the importance of cellular viability in repair of cartilages, through integration in experiments that caused inhibition of necrotic and/or apoptotic cell death using necrostain-1 or Z-VAD-FMK, respectively (Gilbert *et al*., 2009). In a ring-disc model of cartilage integration, inhibition of cell death resulted in qualitatively better integration using both cell death inhibitors, but only quantitatively increased adhesive strength using the apoptosis inhibitor alone. Bos *et al*. showed collagenase digestion of cartilage explants, prior to their fixation together, was accompanied by repopulation of a normally acellular zone, between the cartilages, following 14 d in culture (Bos *et al*., 2002). Further, it has been shown that isolated chondrocytes sandwiched, with fibrin glue, between pieces of devitalised ovine cartilage and implanted into nude mice induced bonding of the cartilages, an observation that did not occur in the absence of cells (Peretti *et al*., 1998). Whilst we have not performed a quantitative analysis of cellular density between IL-1β treated and untreated explants we rarely observed chondrocytes populating the interfacial matrix of untreated explants, whereas chondrocytes were always observed within the interfacial matrix of IL-1β treated explants. Therefore, we hypothesise that chondrocyte migration caused by transient IL-1β induced matrix remodelling, may be responsible to the presence of chondrocytes at the interfacial matrix. Alternatively, IL-1β induction of catabolic responses may cause a retraction of the matrix that is similar to the action of collagenase on cartilage explants that causes exposure and liberation of chondrocytes from the host matrix.

The presence of proteoglycans, in the ECM of articular cartilage, provide an intrinsic barrier to cellular migration and enzymatic digestion. Disruption of the core proteoglycan assembly, using chondroitinases, hyaluronidases and peptidases such as pepsin and trypsin, has been used in strategies to enhance cartilage repair through increased chondrocyte adhesion and/or cellular mobility (Hunziker and Kapfinger, 1998; Lee *et al.*, 2000). Because of difficulties in tracking chondrocyte migration *in situ* (Morales, 2007), many studies have only hypothesised the presence of this phenomenon – based on indirect experimental evidence. Enzymatic treatment of experimentally wounded surface cartilage of mature rabbit knees, with chondroitinase ABC, led to an increased coverage of the lesion after 1 month – although it was not possible to identify the origin of the repair cells (Hunziker and Kapfinger, 1998). Chondroitin sulphate inhibits movement of sub-populations of immature or mature chondrocytes into cartilage explants, but experiments have shown that pre-treatment of explants with chondroitinase ABC can induce exogenous chondrocyte migration into cartilage (Davies *et al.*, 2008). In addition to these latter studies, experiments using the Boyden chamber have shown that chondrocytes are chemotactic to foetal calf serum, IGF-1 and PDGF growth factors (Chang *et al.*, 2003; Mishima and Lotz, 2008). Therefore, although the critical elements for inducing chondrocyte migration, proteoglycan turnover and the presence of cells able to react to chemotactic stimuli are present, direct evidence of this phenomenon has yet to be demonstrated.

Similar experiments, studying integrative repair of fibrocartilaginous meniscal explants, but with *continual* exposure to IL-1 and tumour necrosis factor alpha (TNFα), showed no repair (Hennerbichler *et al.*, 2007). The latter experiment modelled situations such as degenerative joint conditions, where there is low level presence of cytokines, such that repair of meniscal tissue is severely compromised (McNulty and Guilak, 2008). However, experiments that included dynamic loading of tissue, that damp the pleiotropic effects of catabolic cytokinesin meniscal repair tissue, do have an effect of enhancing integrative fibrocartilaginous repair (McNulty *et al.*, 2010); a similar enhancement could be achieved using IL-1 receptor antagonist, IL-1ra and anti-TNFα antibodies (McNulty *et al.*, 2007). These data highlight how understanding the effect of cytokines on the biology of specific joint tissues has significance in predicting the course of integrative repair.

Whilst growth factors such as IGF-1 stimulate extracellular matrix synthesis, a purely anabolic response is not sufficient to repair experimental wounds in order to generate biomechanically robust integrative cartilage repair (unpublished observations). We hypothesised that integration could be promoted by a short period of extracellular remodelling brought about by increased rates of proteoglycan and collagen metabolism following transient exposure to IL-1β. The short and transient nature of induced catabolism did not affect subsequent gene synthesis of proteoglycan following a short 3 day period where transcript levels dropped 2.6-fold. During the process of extracellular matrix remodelling we hypothesise that chondrocytes at the periphery of the wound margins either increase in density through proliferation (Tew *et al.*, 2001; Redman *et al.*, 2004), or are able to migrate into an incipient matrix that emerges as a bridge between the wounded cartilages. These cells then secrete extracellular matrix, stabilising the junction between the cartilages. We observed that annulus and disc cartilage of IL-1β treated explants exhibit 29-fold greater mechanical strength than untreated explants, roughly one tenth the magnitude of normal cartilage.

Conclusion

The use of catabolic cytokines for therapeutic value is not unique; TNFα has been demonstrated to be potentially

useful for fragility fracture healing in osteoporosis. Localised application of TNFα, in a mouse model of slow healing fractures, accelerated bone healing and increased union strength through increased recruitment and differentiation of muscle derived stromal cells into osteoblasts (Glass *et al.*, 2011). This study merely provides a proof of principle, that a transient shift in the metabolic state of articular cartilage, using IL-1β to induce a catabolic response, significantly enhances cartilage-to-cartilage integration. Whether there is any practicality in using IL-1β as part of surgical interventions to repair cartilage lesions has to be weighed against the induction of an inflammatory response if the cytokine is presented to the whole joint. Therefore, localised and controlled delivery of the cytokine could be an option, and also, any molecule capable of eliciting a transient catabolic response in chondrocytes would potentially also be able to positively affect integration. It is interesting to note, nevertheless, that IL-1β is vital for normal homeostatic function of cartilage. Homozygous gene deletion not only accelerates the appearance OA lesions in knee joints of knockout mice subjected to partial medial meniscectomy but also in unoperated contralateral joints (Clements *et al.*, 2003). Where chronic inflammatory conditions are present, the use of IL-1β or TNFα antagonists may be appropriate to induce repair as previously described for meniscal cartilage lesions.

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Discussion with Reviewers

Reviewer II: It is not clear how IL-1β is superior to collagenase; in fact the disadvantages of collagenase use (mentioned in the Introduction) are shared by IL-1β treatment. Like collagenase, IL-1β disrupts the collagen network, which the authors state may lead to cartilage degeneration. A direct comparison of IL-1β to collagenase may be useful.

Authors: We appreciate that it is not possible to judge the merits of IL-1β *versus* collagenase pre-treatment of cartilage without a direct comparison. We have therefore provided Safranin-O sections of cartilage treated with either IL-1β (as described in our experiments) or collagenase (30 U/mL^{-1} collagenase type VII for 24 h, as described by Bos *et al.* (2002) (text reference). The changes in cartilage following collagenase treatment are quite profound and match an image shown by Bos *et al*. (2002) (text reference), Fig. 1B, that shows complete loss of proteoglycan labelling at the periphery of the cartilage explant as well as contraction of the extracellular matrix. From the histological perspective alone, IL-1β causes significantly less disruption to the ECM than collagenase treatment. A direct comparison of integration in terms of adhesive strength measurements for both pre-treatments was also possible from the published values for collagenase pretreatment (van de Breevaart Bravenbor *et al*., 2006) and the values generated from our experiments using IL-1β, as both studies used the same technique, push-out testing, to derive data.

Reviewer II: Inflammation (and inflammatory cytokines such as IL-1β) are often found in osteoarthritis (OA) joints and are part of the problem, therefore is further addition of IL-1β necessary or wise?

Authors: Generally, only small, isolated cartilage lesions are treated with techniques such as autologous chondrocyte implantation, where integration of repair and host tissue is critical and chronic inflammation is not often an issue. We do not, and have not advocated the use of IL-1β for cartilage repair or OA lesions. As we stated in the original manuscript, this study is merely a proof of principle that a transient shift in metabolic state correlates with enhanced integration. IL-1β was one factor we found that fitted the description of the molecule that was suitable for this study. Our approach differs from other studies, in that they have, in the main, sought to remove specific components of the ECM (GAGs, hyaluronic acid or collagen) or add specific components such as crosslinking chemicals to effect integration.

Reviewer II: Similarly, what were the compressive properties of the explants before and after IL-1β treatment? While the interface strength increased, the main mode of loading in joints is dynamic compression and this property is highly dependent on collagen. If the collagen network remains disrupted and function is compromised due to IL-1β, enhanced integration may not be very beneficial. Please comment.

Authors: An experiment to determine the compressive properties of explants immediately following IL-1β and collagenase pre-treatment was performed using atomic force microscopy, a technique we are using currently to assess the compressive properties of immature and mature cartilages (worked performed in collaboration with Drs Lewis Francis and R. Steve Conlan). The data showed that the Young's modulus decreased approximately 2-fold following IL-1β exposure and 6-fold following collagenase exposure (using the same conditions as described by Bos *et al.* (2002) (text reference), compared to untreated explants. Based on the limited morphological changes in cartilage explants elicited by IL-1β pre-treatment compared to collagenase pre-treatment, it would be easier to construct an argument claiming enhanced integration is not beneficial after collagenase pre-treatment.