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Zonal differences in meniscus matrix turnover and cytokine response

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SUMMARY

Objective: To determine the mechanisms of meniscal degeneration and whether this varied zonally and from articular cartilage.

Design: Normal ovine menisci were dissected into inner and outer zones and along with cartilage cultured ±IL-1α and TNFα. Glycosaminoglycan (GAG) and collagen release, and gene expression were quantified. Aggrecan proteolysis was analysed by Western blotting with neoepitope-specific antibodies. Matrix metalloproteinase (MMP)2, *MMP9* and *MMP13* activity was evaluated by gelatin zymography or fluorogenic assay.

Results: Inner meniscus was more cartilaginous containing more GAG and expressing more *ACAN* and *COL2A1* than outer zones. Higher expression of *VCAN* and *ADAMTS4* in medial outer and both zones of the lateral meniscus reflected their embryologic origin from cells outside the cartilage anlagen. All meniscal regions released a greater % GAG in response to cytokines; only outer zones had cytokine-stimulated collagenolysis. Cytokine-induced aggrecanolysis was primarily due to increased ADAMTS cleavage in cartilage and inner menisci but MMPs in the outer menisci. Outer menisci always released more active *MMP2* than other tissues and more active *MMP13* in basal and TNF-stimulated cultures. Expression of *ACAN*, *COL1A1* and *COL2A1* was decreased by both cytokines in all tissues, while *VCAN* was increased by IL-1α in cartilage and inner menisci. Metalloproteinase expression was differentially regulated by IL-1α and TNFα: *ADAMTS4*, *MMP1*, *MMP3* were upregulated more by IL-1α in inner zones whereas *ADAMTS5*, *MMP13* and *MMP9* were more upregulated by TNFα in outer zones.

Conclusions: Meniscal degeneration mechanisms are zonally-dependent, and may contribute to the enzymatic burden in the joint.

Introduction

The knee joint menisci provide joint congruity and are important weight bearing and stabilising structures, responsible for a 40–50% increase in contact and force transmission between the curved femoral condyle and flattened tibial plateau[1]. Type I collagen is the major fibrillar collagen with lesser amounts of type II, III and VI collagen constituting 60–70% of the dry weight of the meniscus[2–5]. The collagen fibres are arranged into complex radial, random and circumferential bundles constrained by radial tie fibres equipping the meniscus with its ability to withstand multi-directional tensile stresses[1]. The meniscus is a heterogeneous tissue, the outer third is vascular and fibrous, the inner third has structural and biochemical similarities to articular cartilage (AC)[1,6,7]. Fibrous, tensile connective tissues such as the outer meniscus typically contain versican; aggrecan is a major component of the inner third of the meniscus, conveying resistance to compressive loading[6].

Versican regulates cell–cell and cell–matrix interactions, cell migration and proliferation during development and may regulate lateral and medial meniscal development[8]. It is also associated with elastic and fibrous networks in connective tissues[9,10].

The importance of the meniscus to the knee joint is evident with early onset of osteoarthritic joint degeneration following complete or partial meniscectomy as well as procedures that interfere with the meniscus' ability to generate hoop stresses and resist compression[11]. This has a clinical corollary in humans, with partial or total meniscectomy resulting in premature onset of osteoarthritis (OA)[12]. There is a strong positive correlation between the severity of degenerative changes in the meniscus and the degree of cartilage degeneration in end-stage human OA, and the degree of

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meniscal degeneration on magnetic resonance imaging (MRI) is a significant predictor of OA progression[13–16]. Whether this association between meniscal and cartilage degeneration in OA is purely due to the mechanical role of the meniscus, or whether meniscal cells secrete enzymes and inflammatory mediators that contribute to degeneration of other joint tissues, is unclear. Nevertheless, the integrity of the meniscus is vital in maintaining healthy AC and prevention of degenerative changes in the knee joint[14,17–19].

Despite the clear link between meniscal deterioration and the development of OA, there have been relatively few studies investigating the molecular mechanisms of meniscal compared with AC degeneration[20–24]. As outlined above, the inner and outer regions of the menisci have distinct differences in extracellular matrix (ECM) composition, reflecting the discreet zonal phenotype of resident meniscal cells in adult tissues[25–30]. These differences may reflect the embryonic cellular origins in the medial meniscus, where cells in the inner zone originate from the cartilage anlagen while in the outer meniscus they are derived from cells that do not express *Col2a1* and invade the developing joint[31]. This pattern is quite different in the lateral meniscus where cells in all regions originate from outside the cartilage anlagen[31]. These distinct embryological cellular origins may partly explain the superior reparative potential of the outer meniscus, and together with joint biomechanics, the higher frequency of degeneration of the inner zone of the medial compared to lateral meniscus. Despite known regional differences, previous *in vitro* studies of the mechanisms of meniscal degeneration have used pooled medial and lateral menisci and explants derived from the central or outer zones, often with only the surface 1 mm thickness of tissue examined[21–24]. In the present study we have examined degeneration induced by IL-1α and TNFα in full-thickness explants from medial versus lateral and inner versus outer meniscus, and compared this with AC from the same joints. These studies have shown distinct differences in the mechanisms of AC, inner and outer meniscal degradation, that have important therapeutic implications for the treatment of OA.

Materials and methods

Explant cultures of meniscal tissues and AC

Full-depth 5 mm^2 plugs of AC (not including calcified cartilage) were harvested aseptically from the trochlear groove along with lateral (L) and medial (M) menisci from 6–12 month old ovine stifle joints. The menisci were trimmed of synovial and ligamentous attachments and dissected into outer (O), middle (discarded) and inner (I) regions, that were then dissected further into full-thickness explants ~5 mm^2. Explants were cultured in Dulbecco's Modified Eagles Medium (DMEM; Sigma, Castle Hill, NSW, Australia) buffered with sodium bicarbonate 3.7 g/L (Fronine, Riverstone, NSW, Australia) supplemented with 10% (v/v) Foetal Calf Serum (FCS; Trace Biosciences Pty. Ltd., Castle Hill, NSW, Australia), gentamicin (50 μg/mL), 2 mM L-glutamine (ICN Biochemicals Inc., Aurora, OH, USA) in an atmosphere of 90% humidity and 5% (v/v) CO_2 at 37°C for 48 h. The explants were then washed (3 × 5 min) in serum-free-DMEM and cultured individually for 4 days in 24-well culture plates in 1 ml of serum-free-DMEM ±10 ng/mL IL-1α or 100 ng/mL TNFα (PeproTech Inc., Rocky Hill, NJ, USA).

Quantitation of proteoglycan and collagen release from explant cultures

The proteoglycan sulphated glycosaminoglycan (GAG) content of the culture medium and papain digested tissues was measured with the metachromatic dye 1,9-dimethylmethylene blue using shark cartilage chondroitin sulphate-C as standard (Sigma–Aldrich, Castle Hill, NSW, Australia)[32]. The collagen hydroxyproline (hypro) content of culture medium and acid hydrolysed tissues was measured using the method of Stegemann and Stalder[33]. GAG and hypro release data were expressed as μg/mg of tissue wet weight or as a percentage of the total (media/[media + papain digested or acid hydrolysed tissue]).

mRNA extraction and real time reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA from samples of *ex-vivo* and cultured AC and meniscal zones (~100 mg) was extracted, quantitated and reverse-transcribed as described previously[34]. The quality of meniscal and AC RNA preparations was assessed using a Shimadzu MultiNA microchip electrophoretic system to determine the levels of 28S and 18S rRNA, and smaller RNA fragments. Only RNA samples with 28S:18S ratios >2.0, and minimal fragmentation were examined. Real time PCR was done using a Rotorgene 6000 (Corbett Life Science, NSW, Australia) and validated ovine-specific primers (Table I).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of aggrecan metabolites

Proteoglycans were extracted from the explants using 10 volumes 4 M guanidine hydrochloride (0.5 M sodium acetate pH 5.8 containing 10 mM EDTA, 20 mM benzamidine and 50 mM 6-aminohexanoic acid) using end-over end mixing for 48 h at 4°C. Replicate aliquots ($n \geq 6$) from each culture condition were pooled to provide representative samples from equivalent wet weights of tissue, precipitated with five volumes of absolute ethanol, resuspended in 0.1 M Tris acetate buffer (pH 6.5) then digested for 5 h at 37°C, with Chondroitinase ABC (0.05 U/ml) and Keratanase I (0.01 U/ml) (Seikagaku, Japan). Samples were re-precipitated and dissolved in NuPAGE 1× LDS sample buffer with 1× reducing agent (Invitrogen Australia Pty. Ltd) and boiled for 10 min. The samples (extract from 1 mg wet weight tissue in 25 μl) were electrophoresed in 10% (w/v) NuPAGE gels, blotted and subjected to Western blotting[35] using antibodies to the ADAMTS (a Disintegrin and Metalloproteinase with Thrombospondin Motifs) and matrix metalloproteinase (MMP) generated aggrecan neoepitope sequences[36], NITEGE (generously provided by Dr C. Flannery Pfizer Inc, Cambridge, MA, USA) and DIPEN[37] (generously provided by A/Prof AJ Fosang, University of Melbourne) respectively.

Gelatin zymography and MMP substrate assay

Conditioned medium from replicate cultures ($n = 6$) (AC, MO, MI, LO, LI) and treatment (basal, IL-1α, TNFα) were pooled to provide representative samples. *MMP2* secretion and activation in aliquots from an equal wet weight of tissue in each region and treatment were examined using gelatin zymography under non-reducing conditions. *MMP13* activity was measured ± activation by 4-aminophenylmercuric acetate (APMA, 1 mM for 15 min at 37°C) using a SensoLyte Plus 520 *MMP13* assay kit (AnaSpec, San Jose, CA) according to the manufacturer's instructions. *MMP13* activity was measured every 15 min over a 270 min time-course and reported as Δ fluorescence/h.

Statistical analyses

The quantitative data presented in Figs. 1–4 and Table II, is pooled from separate experiments (up to seven for GAG release data in Fig. 1). In each experiment, explants of different meniscal zones and cartilage were harvested from 3–10 individual sheep and

Table I
Ovine-specific cDNA primers used for qRT-PCR in this study

Gene	Species accession #	Sequence 5′–3′	T °C	Product (bp)
ACAN[a]	Bos taurus	F -TCA CCA TCC CCT GCT ACT TCA TC	58	105
	U76615	R -TCT CCT TGG AAA TGC GGC TC		
ADAMTS4[a]	Bos taurus	F -AAC TCG AAG CAA TGC ACT GGT	60	149
	NM181667	R -TGC CCG AAG CCA TTG TCT A		
ADAMTS5[a]	Bos taurus	F -GCA TTG ACG CAT CCA AAC CC	55	97
	AF192771	R -CGT GGT AGG TCC AGC AAA CAG TTA C		
TIMP1[b]	Ovis aries	F -GGT TCA GTG CCT TGA GAG ATG C	57	265
	S67450	R -GGG ATA GAT GAG CAG GGA AAC AC		
TIMP3[b]	Bos Taurus	F -CTT CCT TTG CCC TTC TCT ACC C	57	286
	NM174473	R -CT GGT CAA CCC AAG CAT CG		
VCAN[c]	Bos taurus	F -CAT CTC ACC AGT ATC CTG TCT CAC G	55	128
	NM181035	R -AGT GTG CTG CCA TCA GTC CAA C		
COL1A1[c]	Ovis aries	F -ATC CCT GGA CAA CCT GGA CTT C	57	107
	AF129287	R -TCA TCA TAG CCG TAA GAC AAC TGG		
COL2A1[b]	Bos taurus	F -TGA CCT GAC GCC CAT TCA TC	55	154
	X02420	R -TTT CCT GTC TCT GCC TTG ACC C		
MMP1[b]	Ovis aries	F -CAT TCT ACT GAC ATT GGG GCT CTG	55	122
	AF267156	R -TGA GTG GGA TTT TGG GAA GGT C		
MMP2[c]	Ovis aries	F -TGC TAC CAC CTC CAA CTA CGA TG	60	240
	AF267159	R -GTG CCA GTA TCA ATG TCA GGG G		
MMP3[b]	Bos taurus	F -TCC CCC AGT TTC CCC TAA TG	58	124
	AF135232	R -GAT TTC TCC CCT CAG TGT GCT G		
MMP9[c]	Bos taurus	F -AGG TGA ATC AGG TGG ACT ATG TGG	59	221
	X78324	R -AGA AAG GAA GGT GGG AAG AGA GG		
MMP13[a]	Ovis aries	F -GGT GAC AGG CAG ACT TGA TGA TAA C	58	349
	AY091604	R -ATT TGG TCC AGG AGG GAA AGC G		
MMP14[b]	Ovis aries	F -ACC AGG TGA TGG ATG GAT ACC C	56	126
	AF267160	R -CCC AGT GCT TGT CTC CTT TGAAG		

[a] From Smith *et al*[34].
[b] From Zreiqat *et al*[54].
[c] Designed by MacVector software (MacVector, Inc Cary, WC, USA).

pooled. Explants were then randomly assigned to replicate wells for different treatments and treated as independent observations. The minimum and maximum number of observations for each outcome is given in the respective figure legend. Pooling of tissues from multiple sheep was necessary as sufficient explants could not be obtained from individual menisci to enable all inter-animal comparisons of zonal differences (medial/lateral menisci; *ex-vivo*/control/IL-1/TNF). Furthermore, previous studies demonstrated that the *ex-vivo* variation between the inner and outer zones within a sheep was equal to or greater than the between-sheep variation within a given zone (e.g., within versus between sheep variation = 129% vs 106% for *ACAN* and 53% vs 54% for *COL1A1* expression). The potential for under-estimation of type-I error was further addressed by using Benjamini–Hochberg *post-hoc* correction for multiple analyses. The GAG and hydroxyproline contents of solubilised tissues and media samples from explant cultures (Fig. 1) were analysed using the Student's t test. Comparison of gene expression in different tissues *ex-vivo* (fold-change) expressed relative to AC (Table II), and mean fluorescence following culture under different conditions (Figs. 2–4), were analysed using the Kruskal–Wallis test for multiple groups and, if significance was found, Mann–Whitney U tests for between-group comparisons (StataSE software, Stata corporation, College Station, TX, USA). The means and confidence intervals (CIs) plotted in Figs. 2–4 were calculated on log-transformed data to ensure normalised distributions and non-negative expression levels. The α level was set at 0.05, and all graphical and tabulated data are expressed as mean ±95% CIs.

Results

Ex-vivo (un-cultured) AC, MI and LI contained more GAG per tissue wet weight than the LO or MO tissues [Fig. 1(A)]. In contrast, the hydroxyproline content of all meniscal zones was similar but higher than AC [Fig. 1(B)]. Under basal (control) culture meniscal zones released more GAG as a percentage of the total tissue GAG than AC cultures [Fig. 1(D), $P < 0.001$]. IL-1α and TNFα treatment resulted in significantly more GAG release than control cultures for AC and all meniscal zones except MO [Fig. 1(C)]. All meniscal zones released a greater % of their tissue GAG into the media compared with cartilage in response to IL-1α and TNFα [Fig. 1(D); $P < 0.001$]. Little or no hydroxyproline was released from control cultures, but in the MO only, IL-1α and TNFα significantly stimulated the release of hydroxyproline [Fig. 1(E and F)].

Consistent with zonal differences in composition, *ex-vivo* (un-cultured) *ACAN* and *COL2A1* expressions were higher and *COL1A1* lower in AC and inner meniscal tissue compared with outer meniscus (Table II and Supplemental Fig. 1). In contrast, *VCAN* expression was significantly higher in MO, LI and LO compared with AC. Tissue-specific differences in expression of MMPs were also apparent in un-cultured tissues (Table II). *Ex-vivo* expression of *ADAMTS4* in the MO, LI and LO zones was significantly greater than AC, while only the LI zone showed significantly greater *ADAMTS5* compared to AC. Interestingly, *MMP1 ex-vivo* gene expression was significantly lower in the outer compared to the inner meniscal zones and AC, while the expression of *MMP3* was significantly greater in the inner zones when compared to AC. The expression of a number of genes changed significantly in basal (un-stimulated control) culture compared with *ex-vivo* (un-cultured) (Figs. 2–4 and Supplementary Tables I–V). In particular, *ACAN* mRNA increased in AC but not meniscus; *COL1A1*, *COL2A1* and *VCAN* were down regulated especially in outer meniscus; and *MMP3* increased in inner and outer menisci, while *ADAMTS5* increased in MI, MO and LO.

In general both cytokines tended to decrease expression of the major ECM proteins (*ACAN*, *COL2A1* and *COL1A1*) in all tissues (Fig. 2). The exception to this was *VCAN* which was increased by IL-1α (AC, $P = 0.01$; MI, $P = 0.006$; LI, $P = 0.004$; LO, $P = 0.004$) but was either decreased (AC, $P = 0.04$) or unchanged (all meniscal tissues) by TNFα [Fig. 2(D)]. Expression of *TIMP1* was down

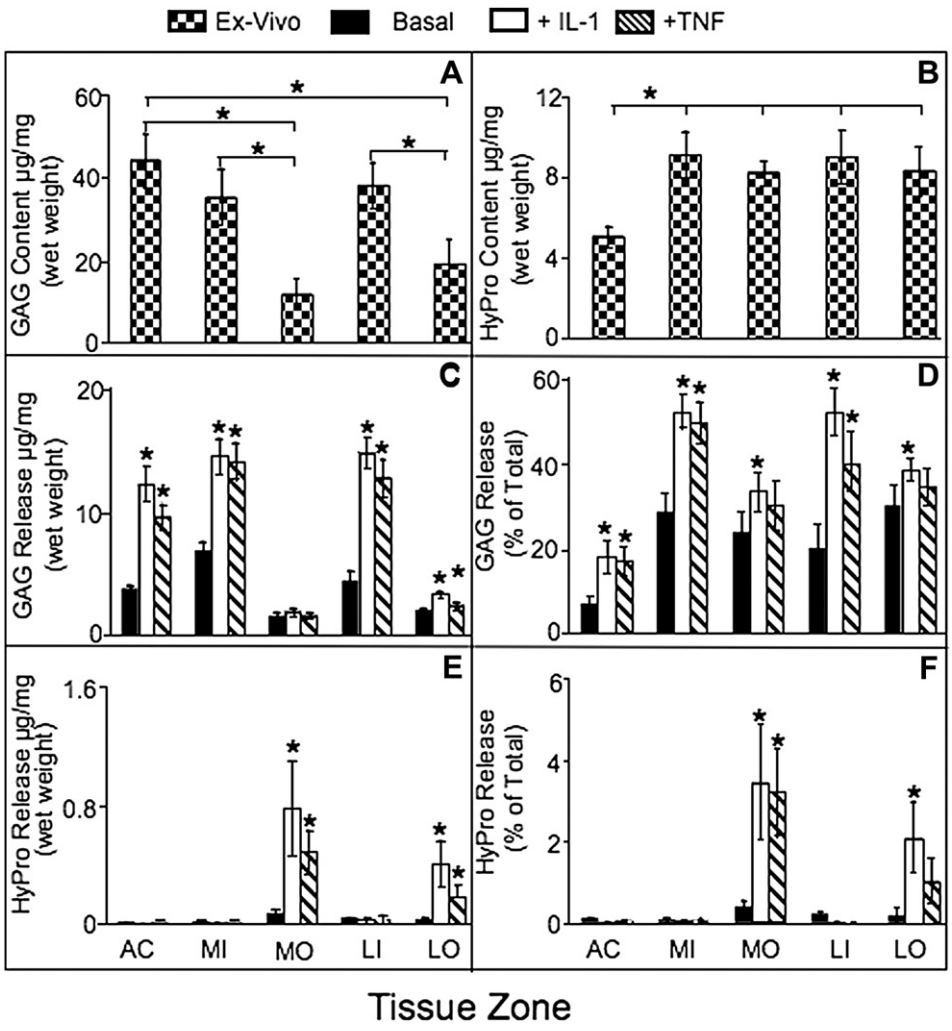

Fig. 1. Comparison of the (A) sulphated GAG and (B) hydroxyproline (hypro) content of ovine AC and meniscal zones *ex-vivo*. The release of GAG (C & D) and hypro (E & F) from explants stimulated with IL-1α (10 ng/ml) and TNFα (100 ng/ml) is expressed as µg/mg tissue wet weight (C & E) or as a percentage of the total in the media plus tissue (D & F). Graphs depict mean ±95% CI; $n = 6–10$ (A, B, E & F), $n = 42–106$ (C & D) replicate explants. Comparisons are between: A and B groups connected by bars, and C–F basal compared to IL-1α or TNFα treated cultures. * = $P < 0.001$ except the following: B where AC vs MI $P = 0.001$, AC vs LI $P = 0.002$ and AC vs LO $P = 0.002$; C where LO basal vs IL-1α $P = 0.01$; D where MO and LO basal vs IL-1α $P = 0.007$ and 0.005, respectively; E where MO and LO basal vs IL-1α $P = 0.002$ and LO basal vs TNFα $P = 0.004$ and F where MO and LO basal vs IL-1α $P = 0.009$ and MO basal vs TNFα $P = 0.003$, MI, MO: inner and outer medial meniscal zones; LI, LO: inner and outer lateral meniscal zones.

regulated by IL-1α and TNFα only in MO, LI and LO zones [Fig. 3(D)]. *TIMP3* was differentially regulated, being decreased by cytokine treatment in the AC and inner (MI by TNFα only) but not outer meniscal zones [Fig. 3(C)].

Expression of *ADAMTS4* was increased by both cytokines in all tissues, equally in meniscal outer zones but significantly more by IL-1α than TNFα in the cartilaginous tissues (AC, MI, LI) [Fig. 3(A)]. *ADAMTS5* was upregulated to a similar extent by both cytokines in AC, MI and LI, but only significantly increased by TNFα in the outer meniscal tissues. Regulation of MMP expression also showed distinct tissue and cytokine specific differences (Fig. 4). *MMP2* and *MMP14* expression showed limited regulation by cytokines in any tissue *(data not shown)*. *MMP9* expression was only significantly increased in the meniscal outer zones and only by TNFα. *MMP3* and *MMP1* were significantly increased in all tissues by both cytokines. *MMP13* mRNA was increased by IL-1α in AC and inner meniscus, and only by TNFα in the outer zones. The tissue pattern of differential regulation of MMPs by the two cytokines was quite similar for *MMP1*, *MMP3*, and *MMP13*, being less sensitive to TNFα in AC, and the MI and LI meniscal zones. In general, the expression of ADAMTS and MMPs was higher in meniscal cultures than AC.

Western blotting of aggrecan interglobular domain cleavage neoepitopes in pooled extracts showed distinct tissue differences (Fig. 5). In AC the MMP-generated DIPEN epitope was detected in tissue extracts prior to culture (*ex-vivo*) and showed only a slight increase in basal, IL-1α and TNFα cultures [Fig. 5(A)]. The ADAMTS-generated NITEGE epitope was not detected *ex-vivo* or in basal cultures in AC, but was increased by both cytokines [Fig. 5(B)]. There was little difference between medial and lateral menisci, but notable differences between the inner and outer zones. DIPEN was detected in all meniscal tissues prior to culture and did not increase in basal culture [Fig. 5(C and E)]. In the inner zones, the DIPEN neoepitope was elevated by IL-1α but not TNFα, while both cytokines increased DIPEN in the outer meniscal zones. There was little NITEGE detected in extracts of meniscal tissue *ex-vivo*, but elevated levels in all zones in basal cultures [Fig. 5(D and F)]. IL-1α and TNFα further increased NITEGE in inner but not outer meniscal zone cultures.

Very little active *MMP2* was detected in the basal AC cultures and it was only marginally changed by IL-1α or TNFα treatment [Fig. 6(A)]. Gelatin zymography yielded similar results for medial and lateral menisci but distinct differences between inner and outer menisci and AC. Very little pro- or active *MMP9* was detected

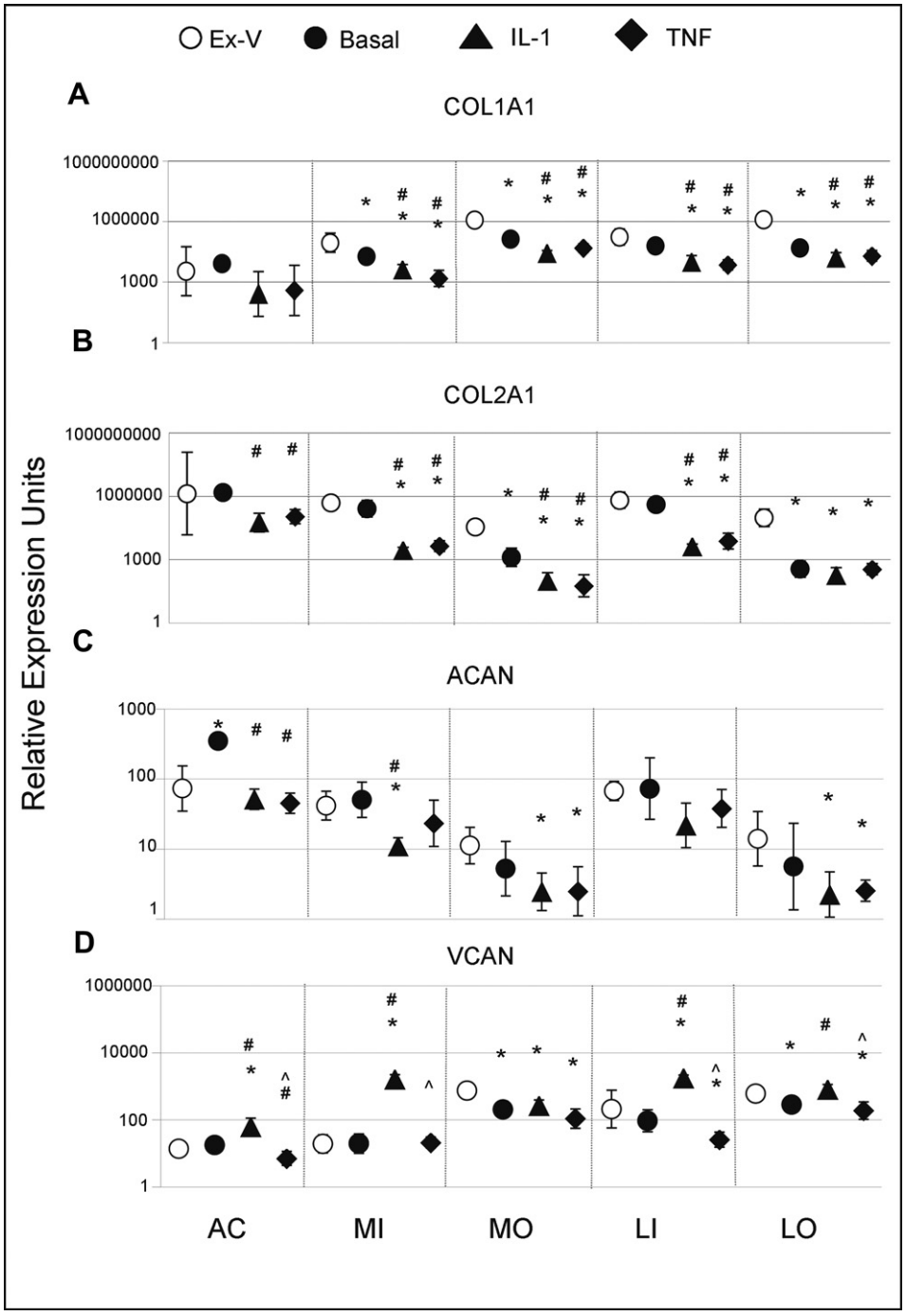

Fig. 2. Comparison of A: *COL1A1*, B: *COL2A1* C: *ACAN* and D: *VCAN* gene expression between *ex-vivo* ovine AC and meniscal zones and their response to stimulation with IL-1α (10 ng/ml) and TNFα (100 ng/ml) (expressed as relative expression units; REU). $P < 0.05$ for: * comparison to *ex-vivo* tissue; # comparison between basal cultured tissues and cytokine treated tissues; and ^ comparisons between IL-1α and TNFα treated tissues (the exact *P* values for each comparison are presented in Supplementary Tables I–V). Error bars represent 95% CI (calculated on log-transformed normalised data), $n = 5–6$ replicate cultures for each data point; refer to legend 1 for tissue zone abbreviations.

in any culture. In basal cultures of inner meniscus pro-MMP2 was predominant, while active *MMP2* was readily detected in basal cultures of outer meniscal zones. IL-1α but not TNFα stimulated the release of active *MMP2* in the inner zone meniscal cultures. In the outer meniscus both cytokines markedly increased active *MMP2*.

There was little or no spontaneously active *MMP13* detected in basal cultures in any tissue, although APMA pre-treatment revealed pro-MMP13 in outer meniscal zone basal cultures [Fig. 6(B)]. IL-1α stimulated low levels of active *MMP13* in all cultures (meniscus > AC), but the majority was present in pro-form with similar levels in all IL-1α stimulated cultures after APMA activation. Spontaneously active *MMP13* was detected in outer meniscal zone cultures in response to TNFα, although the majority was present as pro-MMP13 (APMA activated).

Discussion

The regional variation in ECM composition observed between the inner and outer menisci and AC is consistent with earlier

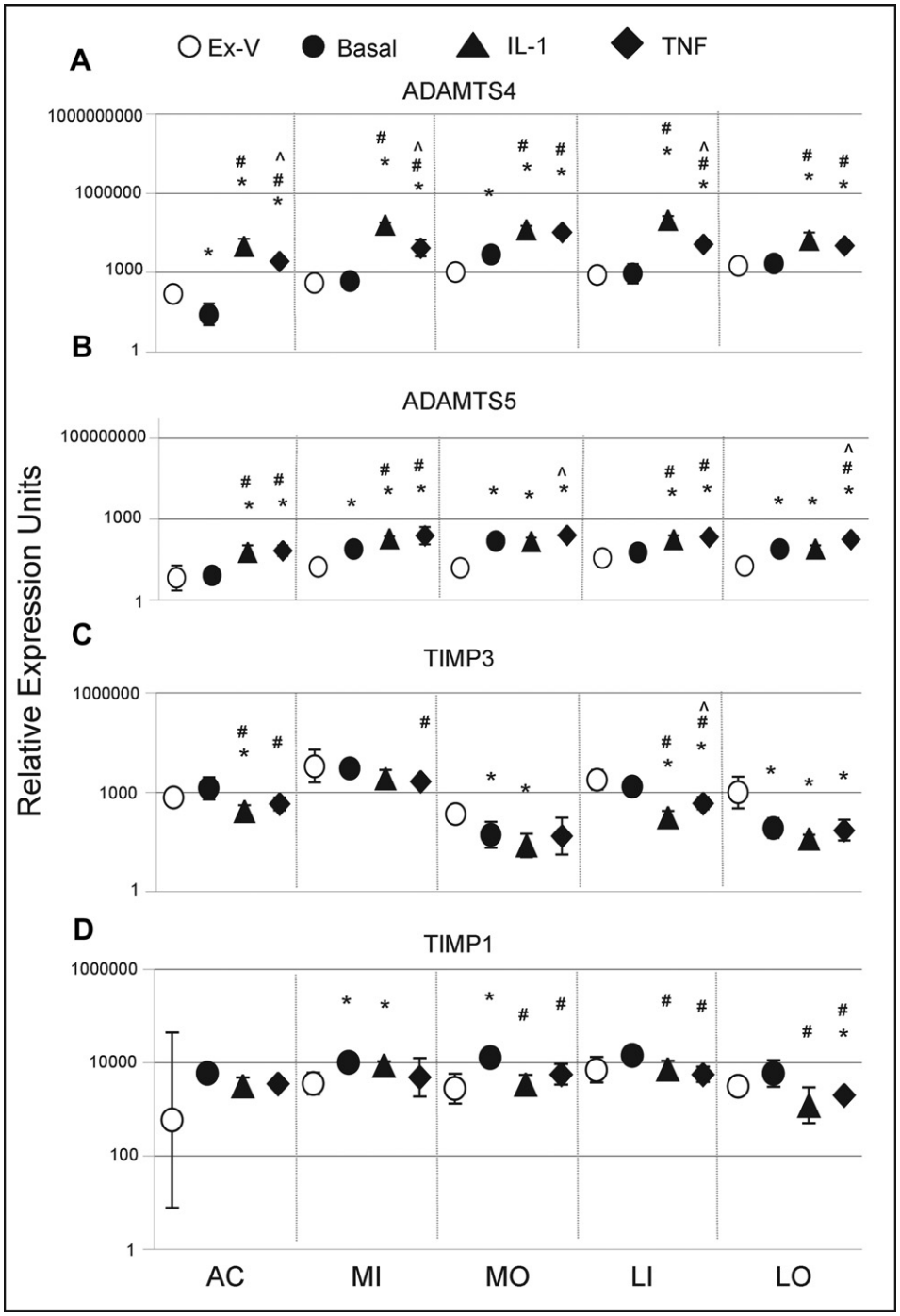

Fig. 3. Comparison of A: *ADAMTS4*, B: *ADAMTS5*, C: *TIMP3* and D: *TIMP1* gene expression between *ex-vivo* ovine AC and meniscal zones and their response to stimulation with IL-1α (10 ng/ml) and TNFα (100 ng/ml) (expressed as relative fluorescence; RF). $P < 0.05$ for: * comparison to *ex-vivo* tissue; # comparison between basal cultured tissues and cytokine treated tissues; and ^ comparisons between IL-1α and TNFα treated tissues (the exact *P* values for each comparison are presented in Supplementary Tables I–V). Error bars represent 95% CI (calculated on log-transformed normalised data), $n = 5–6$ replicate cultures for each data point; refer to legend 1 for tissue zone abbreviations.

reports[1,7,27], and *COL2A1* and *ACAN* expression followed a similar trend, confirming the chondrocyte-like nature of the meniscal inner zone cells. We have previously demonstrated that these zonal differences in aggrecan and type II collagen content of the ovine meniscus are evident as early as 2 days post-natally[28–30]. In the current study we found very similar zonal differences in GAG and collagen content and *ACAN* and *COL2A1* mRNA expression in medial and lateral menisci. Expression of *COL1A1* and *VCAN*, markers of more fibrous connective tissues[38], was higher in outer meniscal zones compared with AC. Together these results support a phenotypic difference between cells of the meniscal inner (chondrocytic) versus outer (fibroblastic) zones.

There were some expression patterns unique to the MO, LO and LI zones, consistent with their differing cellular origins compared to the MI and AC that are derived from the cartilage anlagen[31]. *VCAN* and *ADAMTS4* were more highly expressed *ex-vivo* in MO, LO and LI but not MI compared to AC. IL-1α and TNFα treatment inhibited *TIMP1* expression in the embryologically-related MO, LO and LI but not AC or MI tissue zones. The significance of these findings is unclear, although the presence of *VCAN* and its proteolysis by various

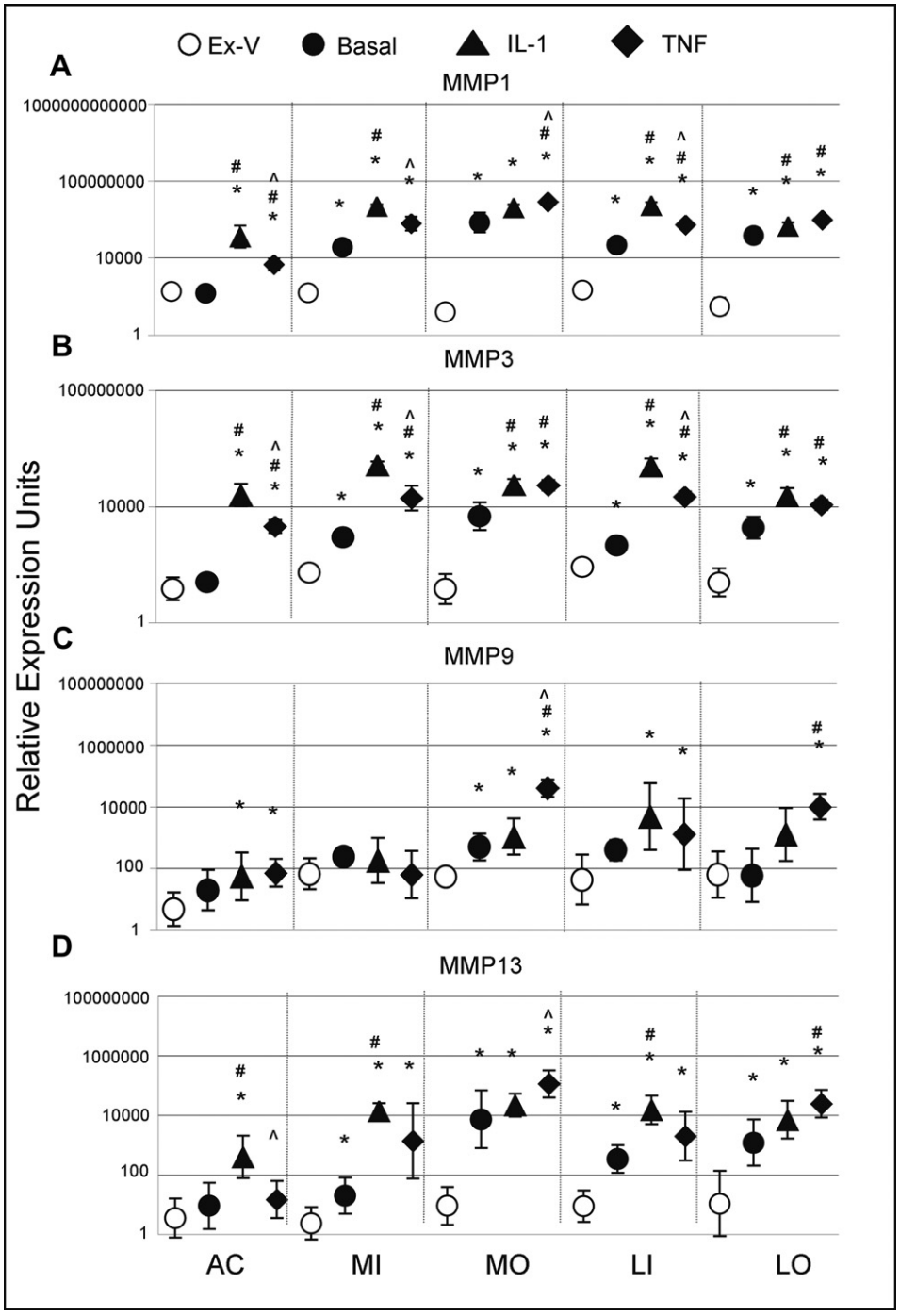

Fig. 4. Comparison of A: *MMP1*, B: *MMP3*, C: *MMP9* and D: *MMP13* gene expression between *ex-vivo* ovine AC and meniscal zones and their response to stimulation with IL-1α (10 ng/ml) and TNFα (100 ng/ml) (expressed as RF). $P < 0.05$ for: * comparison to *ex-vivo* tissue; # comparison between basal cultured tissues and cytokine treated tissues; and ^ comparisons between IL-1α and TNFα treated tissues (the exact *P* values for each comparison are presented in Supplementary Tables I–V). Error bars represent 95% CI (calculated on log-transformed normalised data), $n = 5–6$ replicate cultures for each data point; refer to legend 1 for tissue zone abbreviations.

ADAMTS including *ADAMTS-4*, is known to regulate cell apoptosis, cell–cell and cell–matrix interactions during development and remodelling of tissues[39–42]. These findings suggest that the developmental origins of the cells may have some bearing on their basal phenotype and the response of the resident cell populations to cytokines in the adult tissues.

Despite the specific examples given above, for the most part the composition, gene expression and response to IL-1α and TNFα of the resident cells of the inner versus outer lateral and medial menisci were similar. This suggests that post-natal regulation, by factors such as the compressive loading, likely drives the majority of phenotypic differences observed, such as higher *ex-vivo ACAN and COL2A1* expression in inner compared to outer zones. While the zonal differences in *ACAN*, *COL2A1*, *COL1A1*, *MMP2* and *MMP13* mRNA *ex-vivo* showed the same pattern as previously described, comparative expression of *MMP1* and *MMP3* in inner versus outer meniscus was different in our study to that reported by Upton *et al.*[25]. This may relate to differences in species (sheep versus pig)

Table II
Relative gene expression [Mean relative fluorescent units, ($n = 4$–6)] of regional (MI, medial inner; MO, medial outer; LI, lateral inner; LO, lateral outer) of *ex-vivo* un-cultured meniscal tissues expressed relative to *ex-vivo* un-cultured AC from the same joint

Gene	MI	MO	LI	LO
COL1A1	75.9 (−40.4, 192.2)	500.8 (118.6, 833.1)*#	104.1 (−31.8, 240)	500.05 (138.9, 861.2)*
COL2A1	0.43 (0.21, 0.66)	0.032 (0.015, 0.05)#	0.74 (0.15, 1.3)	0.11 (0.033, 0.19)*#
ACAN	0.65 (0.39, 0.90)	0.19 (0.087, 0.30)*#	0.98 (0.68, 1.3)	0.31 (0.034, 0.59)#
VCAN	1.7 (0.90, 2.5)	63.9 (34.5, 93.4)*#	36.4 (−2.8, 75.6)*	51.8 (23.1, 80.5)*
ADAMTS4	3.3 (1.4, 5.2)	8.2 (4.1, 12.2)*	8.3 (0.21, 16.4)*	14.1 (7.0, 21.2)*
ADAMTS5	3.9 (2.1, 5.7)	4.04 (1.9, 6.2)	12.6 (4.2, 21.1)*	4.5 (2.6, 6.4)
TIMP3	21.4 (−6.5, 49.3)*	0.34 (0.23, 0.46)*#	4.9 (0.37, 9.4)*	4.1 (−2.1, 10.2)
TIMP1	7.2 (3.9, 10.5)	6.6 (1.9, 11.3)	16.6 (1.9, 31.3)	5.8 (3.8, 7.8)
MMP1	0.89 (0.62, 1.2)	0.1 (0.062, 0.14)*#	2.0 (−0.14, 4.04)	0.26 (0.11, 0.41)*#
MMP2	8.3 (−5.6, 22.1)	4.6 (2.1, 7.1)	14.6 (−10.1, 39.4)	8.2 (2.1, 14.3)
MMP3	4.2 (2.3, 6.0)*	2.6 (−0.70, 5.8)	7.0 (3.4, 10.5)*	3.0 (0.82, 5.1)
MMP9	25.8 (7.5, 44.2)*	12.8 (7.0, 18.6)*	48.2 (−10.3, 106.3)	138.6 (−118.1, 395.4)*
MMP13	2.7 (−1.8, 7.1)	6.7 (−1.1, 14.5)	4.05 (0.51, 7.6)	27.3 (−23.3, 78.0)
MMP14	9.5 (5.8, 13.2)	8.3 (4.3, 12.2)	18.4 (4.9, 31.9)*	10.3 (8.2, 12.4)

Bracketed values represent ±95% confidence limits. Values labelled * or # were significantly different from AC and between inner and outer menisci respectively.

and/or age (< 1 versus > 2 years), with younger tissues in the current study having more active remodelling. Nevertheless the results of both studies emphasise the importance of zonal rather than global analysis of meniscus.

The cytokine response of the cartilaginous (AC, MI, LI) and fibrous (MO and LO) tissues also diverged. The former were more responsive to IL-1α than TNFα with regard to stimulation of *MMP1*, *MMP3*, and *ADAMTS4* whereas the fibrous outer zones were more responsive to TNFα for *MMP1*, *MMP9* and *MMP13*. Despite these regional differences, the meniscus was generally more responsive than AC from the same knee joints to cytokine stimulation in terms of the proportion of the total tissue GAG released into the media in explant culture and MMP mRNA levels induced. While our analyses have shown some striking similarities between the inner meniscus and cartilage, we have not directly compared the outer meniscus with another collagen-I rich fibrous tissue such as tendon. Such studies would be of interest to determine whether the response of cells in outer meniscus more closely mimics that of cells (tenocytes) from a tissue experiencing greater tensional loading.

Proteoglycan turnover in the meniscus was significantly higher compared to AC, perhaps suggesting a more profound physical disruption associated with cutting explants in meniscal tissues.

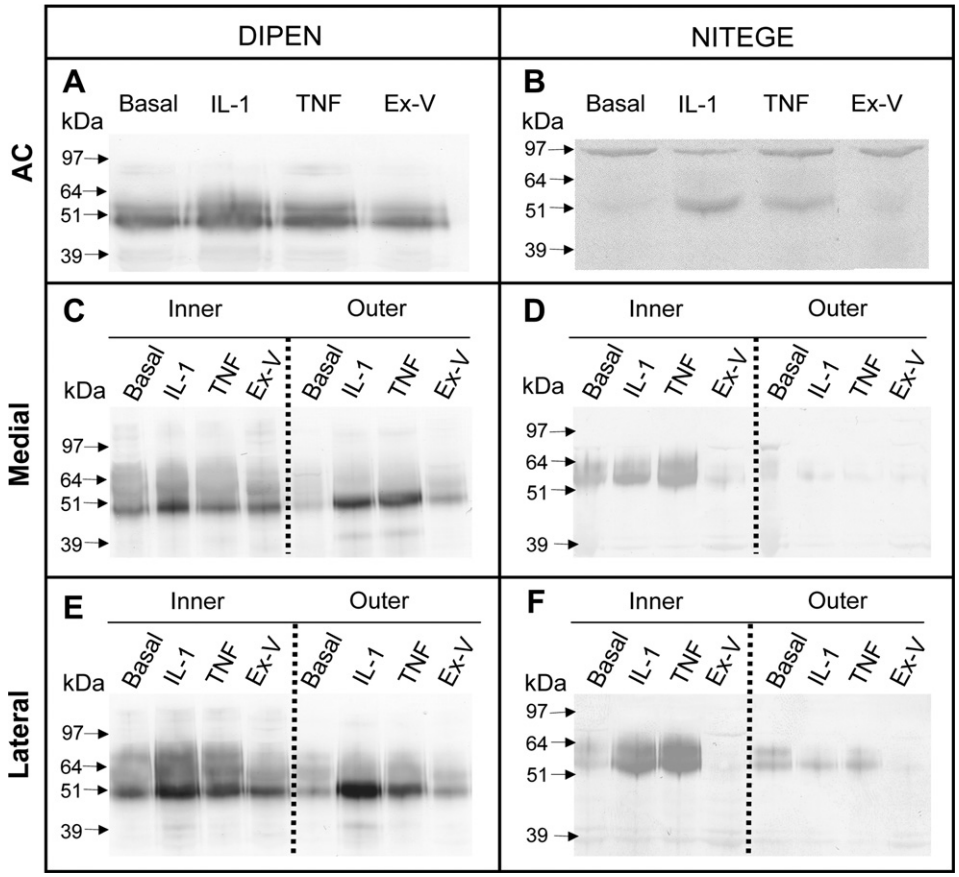

Fig. 5. Western blots of 4 M GuHCl tissue extracts, pool of $n = 6$, depicting aggrecanase and MMP-cleaved aggrecan neoepitopes (NITEGE and DIPEN, respectively) under basal conditions, treatment with IL-1α (10 ng/ml), TNFα (100 ng/ml), or in *ex-vivo* tissues of AC (A and B), medial meniscus (C and D) and lateral meniscus (E and F).

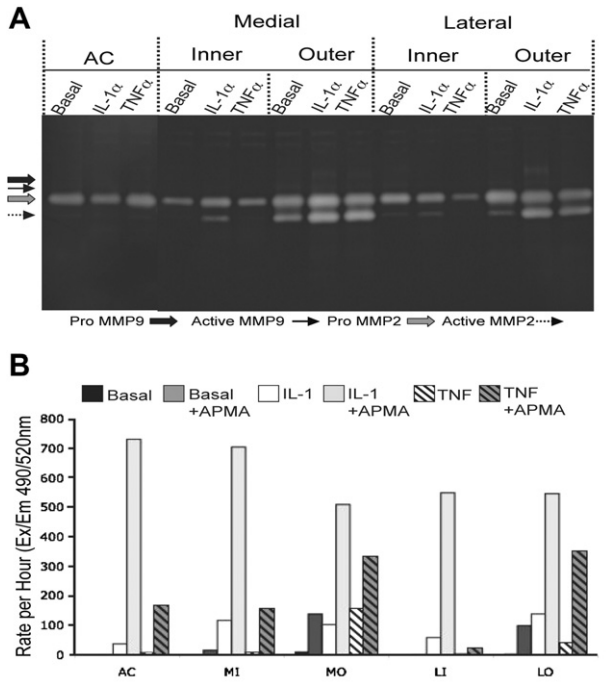

Fig. 6. A: *MMP2* and *MMP9* gelatin zymography of basal, IL-1α and TNFα stimulated ovine AC and meniscal explant culture media samples (pool of $n = 6$ individual cultures for each treatment). The migration positions of the pro- and active forms of *MMP2* and *MMP9* are shown on the left hand side of the figure. B: Assessment of *MMP13* activity of culture media samples from basal, IL-1α (10 ng/ml) and TNFα (100 ng/ml) stimulated ovine AC and meniscal explant cultures. Pooled media samples from $n = 6$ individual cultures from each treatment were tested once over a time-course. The samples were incubated ± APMA to activate MMPs. Tissue zone abbreviations are as indicated in the legend to Fig. 1.

GAG loss in basal cultures was associated with increased NITEGE retained in meniscus but not AC, suggesting that an increase in ADAMTS cleavage of aggrecan was at least partially responsible for higher GAG loss in basal meniscal cultures. Western blotting of media samples from basal cultures also demonstrated that high molecular mass (>97 kDa) ADAMTS-cleaved aggrecan fragments (initiating with the ARG neoepitope and recognised by the BC-3 antibody) were released from all meniscal zones but not AC (data not shown). Although there is not a simple relationship between mRNA levels for ADAMTS enzymes and aggrecanolytic activity[43], the increase in *ADAMTS5* mRNA in basal meniscal but not AC cultures, might implicate this enzyme in accelerated aggrecanolysis in the meniscus *in vitro*.

In contrast to basal cultures, pathological aggrecan degradation induced by cytokines in the MI and LI zones was similar to AC and distinct from the outer zones. Thus both cytokines stimulated cleavage of aggrecan by ADAMTS rather than MMPs only in the cartilaginous tissues (AC, LI, MI). This is largely consistent with previous reports where only the surface 1 mm of the central zone of the meniscus was studied[21,23,24]. Bearing in mind the same caveats previously discussed, our data suggest that in cartilaginous (AC, MI, LI) but not fibrous (MO, LO) tissues, *ADAMTS-4* plays a predominant role in IL-1α-stimulated aggrecanolysis given the marked increase in its mRNA expression compared with *ADAMTS-5*. On the other hand, the *ADAMTS5* gene is more strongly regulated by TNFα in the meniscus, particularly in its outer zones. Our topographical analyses have enabled us to demonstrate for the first time that, in marked contrast to the inner zones, aggrecan cleavage by MMPs may play a significant role in pathological/cytokine-stimulated aggrecanolysis in the outer meniscus. This conclusion was based on analysis of aggrecan neoepitopes retained in the tissue, which could be confounded by secondary cleavage of G1-NITEGE fragments by MMPs. However, high molecular mass (>97 kDa) MMP-generated aggrecan fragments (initiating with the FFG neoepitope, recognised by the BC-14 antibody) were detected in media from IL-1α and TNFα stimulated MO cultures only (data not shown). This is consistent with the increase in the DIPEN neoepitope seen in these same tissues, and supports a potential role for primary aggrecanolysis by MMPs leading to aggrecan loss in this meniscal region. It is noteworthy that in the cartilaginous (AC, MI, LI) but not fibrous (MO, LO) tissues, *TIMP3* mRNA expression was decreased by IL-1α and TNFα, and this could also account in part for the predominance of ADAMTS- over MMP-driven aggrecanolysis in cytokine-stimulated AC, MI and LI cultures.

The afore-noted zonal distinctions between degenerative mechanisms indicate that different therapeutic strategies (inhibition of ADAMTS versus MMPs) may be needed to inhibit the degeneration of the inner versus outer meniscus. Additionally, it suggests that where significant meniscal degeneration contributes to the overall enzymatic burden in the joint, both IL-1α and TNFα may need to be inhibited to regulate this process. A similar dual inhibition approach has been suggested for the regulation of macrophages in OA as opposed to rheumatoid arthritis where TNFα is the predominant cytokine[44]. Our data also implicates the outer meniscus, rather than or in addition to AC, as the source of the MMP-generated aggrecan fragments that have been detected in human OA knee joint synovial fluids[45–47]. To the best of our knowledge, such MMP-generated aggrecan catabolites have not been detected in synovial fluids from joints devoid of a meniscus. It would be interesting to determine whether the presence of the MMP-cleaved aggrecan neoepitope FFG correlates with the severity of meniscal degeneration in OA knee joints.

Consistent with the role of MMPs in the degradation of aggrecan in the outer meniscus, these same regions also had significant collagenolysis upon cytokine stimulation, released more active-MMP2 and more pro- and active *MMP13* in basal and TNFα-stimulated cultures. The meniscus may therefore represent a significant source of collagenolytic MMPs that could directly degrade other intra-articular structures including cartilage, and activate pro-enzymes within these tissues. Similarly, meniscal cells express significantly higher levels of *ADAMTS4* and *ADAMTS5* mRNA than AC from the same joint when stimulated by IL-1α and TNFα. Release of these degradative enzymes may not only contribute to meniscal remodelling and loss of function with attendant biomechanical consequences to the underlying AC, but their secretion into the knee joint synovial fluid may contribute directly to degradation of other joint tissues.

OA is increasingly being recognised a disease of the whole joint[48,49]. In post-traumatic OA in animals induced by transection/rupture of the anterior cruciate ligament, it is well recognised that similar cellular, matrix and molecular changes occur in cartilage, meniscus, synovium and ligaments[50–52]. It is unclear whether this represents parallel but independent pathological change driven by abnormal mechanical loading, or if the enzymes and cytokines generated in one tissue directly contribute to the breakdown of another. Tang *et al.*[53] described an acute coordinated up-regulation of MMPs in all intra-articular tissues 1–3 days after anterior cruciate ligament rupture, suggesting that cytokines released at the time of injury upregulated MMP production, and that all joint tissues contributed to the accumulation of MMP activity in the synovial fluid. While the acute post-traumatic synovitis may resolve, the meniscus may remain a sustained source of degradative enzymes in the knee joint[50]. Meniscal degeneration may therefore contribute to global joint pathology, not only through loss of its load-bearing and stabilising function, but also by contributing

to the enzymatic burden in the joint. Our data suggests that the mechanisms that regulate and drive meniscal degeneration may be zonally-dependent and distinct from AC, and may therefore require a targeted therapeutic approach.

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Contributions

ESF: acquisition of data, day to day running of the study, CBL and JM: conception and experimental design, analysis and data interpretation, critical revision of the manuscript. MMS: statistics, supervision of the gene expression studies. All authors approved the final version of the manuscript.

Conflict of interests
None.

Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.joca.2011.10.002.

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