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Cartilage

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Introduction and Summary.

Osteoarthritis (OA) is a common degenerative disorder affecting whole synovial joints. Characteristic features include progressive loss of mechanically-weakened cartilage which eventually exposes sub-chondral bone, and movement results in extreme pain and ultimately joint failure[1]. Understanding early extracellular matrix (ECM) damage is a key research objective with aggrecan degradation a hallmark of OA[2]. Early OA shows strong immunostaining for the major pro-inflammatory cytokines IL-1β and TNF-α suggesting an important role in OA[3]. These stimulate production of two groups of proteolytic enzymes, the MMPs (metalloproteinases) and the ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) family both which cause aggrecan degradation. ADAMTS-4 (aggrecanase-1[4]) and ADAMTS-5 have been implicated since aggrecan fragments in OA match those produced by these enzymes[5]. ADAMTS-4 is induced by IL-1β and TNF-α whereas ADAMTS-5 is constitutively expressed in normal cartilage[6]. Relative mRNA levels of ADAMTS-4 are ~4-fold higher in knee OA compared to normal cartilage and correlate with degenerative progression[7]. However, inhibitory elements may be upregulated and post-transcriptional modifications occur, so this does not necessarily constitute higher ADAMTS-4 protein levels. Although ADAMTS-4 knockouts showed no effect against cartilage degeneration in an animal instability model[8], the pathway(s) for cartilage loss in idiopathic human OA may be different.

Early microscopic changes to chondrocytes in otherwise normal cartilage, might herald the start of the vicious cycle of OA. Imaging fluorescently-labelled *in situ* human chondrocytes by confocal scanning laser microscopy (CLSM), identified a small, but potentially important population of abnormally-shaped chondrocytes[9.10]. They exhibit cytoplasmic processes, the cells resembling a fibroblastic rather than a chondrocytic phenotype[11] which might reflect or lead to deleterious matrix metabolism[12]. Abnormal chondrocyte morphology correlated with increased cell-associated IL-1β, decreased chondron-localised collagen type VI[13] and increased collagen type I[14]. It is possible that IL-1β stimulation results in localised aggrecan depletion, the development/acceleration of abnormal

morphology and a fibro-cartilageous phenotype. Thus in non-degenerate human cartilage, these peculiar chondrocytes may possess increased ADAMTS-4 levels in contrast to normal (elliptical/rounded) chondrocytes. Alterations to ADAMTS-4 levels associated with chondrocyte clusters is also of interest as proliferation is a characteristic of later OA and could cause additional ECM weakness.

Using CLSM imaging, fluorescently-labelled *in situ* chondrocytes were classified cell-by-cell into those with (a) normal morphology, (b) abnormal morphology (≥1 process/cell) and (c) cells (≥4) in a cluster, within grade 0 (G0;normal non-degenerate) and grade 1 (G1;mild OA) human cartilage. On the same cells, we semi-quantitatively assessed ADAMTS-4 immuno-fluorescence (IF) and determined its relationship with chondrocyte morphology. The results demonstrated marked heterogeneity in the morphology and ADAMTS-4 levels of human chondrocytes. In macroscopically-normal cartilage, a small but significant population of abnormal chondrocytes was present possessing higher levels of ADAMTS-4 compared to normal or clustered cells. These early changes to ADAMTS-4 levels might indicate ECM regions more sensitive to mechanical load and focal points for OA cartilage degeneration.

Methods, Results and Discussion

Femoral heads (FHs) were obtained from N=14 patients (8 females;6 males (range 44-89yrs; mean 71yrs)) undergoing surgery for femoral neck fracture. Ethical permission (Tissue Governance (NHS), Lothian) and consent were obtained. Cartilage areas were visually graded G0 or G1 using OARSI criteria and explants taken[11]. Chondrocyte morphology was identified using 5-chloromethylfluorescein-diacetate (CMFDA). Spheroidal/elliptical cells without cytoplasmic processes were classified as normal cells. A cytoplasmic process was defined as a CMFDA-labelled protrusion of ≥2μm. Chondrocyte clusters possessed ≥4 cells within the lacunar space. Sections were incubated with ADAMTS-4 antibody (PA1-1749A, Invitrogen;1:100), then incubated with secondary antibody (AlexaFluor™ ab175471, Abcam;1:200). Sections were mounted (propyl-gallate;Sigma-Aldrich, Dorset), imaged (Zeiss LSM800) and analysed (Imaris™, Oxford Instruments, U.K.). A section from each explant was used as a negative control with background secondary antibody fluorescence subtracted to identify specific labelling. A cell-by-cell mask selection allowed

analysis of cell-specific (punctate) immuno-fluorescence which localised with CMFDA labelling. For cell clusters, the selection encompassed the whole cluster and fluorescence assessed on a 'per cell' basis. Fluorescence intensity was presented in Relative Fluorescence Units (RFU). Asterisks represent $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***. Femoral heads (N) and cells (n) were presented as [N(n)].

There were clear differences between cells in G0 and G1 cartilage (Fig.1A(a,b)). At low magnification, the majority of cells in G0 were normal, however at high magnification, abnormal chondrocytes were observed (Fig.1A(a)(iii)). In G1, clustering and cytoplasmic processes were detected at low and high magnification (Fig.1A(b)(i-iv)). Chondrocyte morphology classified into normal, abnormal or clusters was performed by eye. There was a decrease (90% to 50%) in normal chondrocytes between G0 and G1 ($P<0.01$;Fig.1B(a)). However, there was ~2-fold increase in abnormal cells ($P<0.05$;Fig.1B(b)), and a >6-fold increase in cells within a cluster ($P<0.001$;Fig.1B(c)). Occasionally, these cells exhibited processes (Fig.1A(b)(ii)) and were classified as a cell in a cluster. The average length of processes increased from 3.81±0.67μm to 6.44±1.16μm (G0 *vs* G1 cartilage [4(77);$P=0.0125$)].

Having identified the chondrocyte morphologies, their association with ADAMTS-4 levels was assessed. There was clear ADAMTS-4 immuno-fluorescence (Fig.2A(ii&iv)) which matched the CMFDA-labelled cells, appearing as punctate labelling closely delineating the same size/morphology suggesting cell-associated labelling (Fig.2A(i&iii)). CLSM visualisation highlighted the heterogeneous nature of ADAMTS-4 labelling. Both diffuse and punctate labelling was evident with strong labelling in some cells close to weakly-labelled cells (Fig. 2A(ii&iv)). For all cell types assessed in either G0 or G1 cartilage, there was no difference between mean or punctate fluorescence levels ($P=0.5245$;$P=0.4080$ respectively). This indicated mean levels over a relatively large area or when measured as cell-associated protein did not change with early cartilage degeneration. However cell-associated (punctate) labelling was higher ($P<0.001$) in both G0 and G1 compared to mean levels and this was expected since it related to cell-specific labelling which covered smaller and more intense areas of labelling. Clearly, this generalised chondrocyte labelling could mask the heterogeneous distribution of ADAMTS-4 between the various morphologies (Fig.1A). Accordingly, >4000 cells within G0 and G1 cartilage were imaged, and morphology and ADAMTS-4

immunofluorescence analysed cell-by-cell. Despite the data spread, abnormal cells demonstrated higher intensity than normal (P=0.0440) or clustered chondrocytes (P<0.0001;Fig.2B(i)).

With cartilage degeneration, there was a marked increase in the proportion of abnormal cells and clustered cells (Fig.1B(a-c)). Thus in G1 cartilage it was possible increased numbers of abnormal cells with greater levels of ADAMTS-4 would be offset by the increased number of cell clusters which had lower levels of the enzyme. This could account for the lack of a difference between overall levels of the enzyme between G0 and G1 noted above. Accordingly, the percentages of chondrocytes in G0 and G1 cartilage with a given IF intensity were plotted (Fig.2B(ii)). While there was a broad distribution in both grades, the levels in G0 cartilage (with normal and abnormal cells, but negligible clusters) was higher and broader than that in G1 cartilage. For G1 cartilage, all three cell categories were present (Fig.1B(a-c)), but there was a higher proportion of cells in clusters possessing lower levels of ADAMTS-4 as reflected in the sharper lower IF intensity peak. There was no difference between ADAMTS-4 labelling for normal cells (P=0.9980), cells with processes (P>0.9999) or cells in a cluster between G0 and G1 (P=0.9744;[3(436)]) suggesting morphological/clustering properties of the cells determined ADAMTS-4 levels and not cartilage grade. Although we have graded cartilage, our description of a small population of morphologically-abnormal chondrocytes in apparently G0 cartilage (Fig.1B(a)) raises an interesting question about cartilage grading. Chondrocyte death and clustering in histological sections are important criteria for cartilage grading however observing subtle changes in chondrocyte morphology requires different approaches such as those described here. We accept that a limitation is that the G0 cartilage studied here might have microscopic characteristics of very early cartilage degeneration and as such might not be 'truly' non-degenerate. Nevertheless we feel that the identification of aberrant chondrocytes provides an important insight into initial changes associated with cartilage degeneration.

CLSM and imaging identified marked heterogeneity in ADAMTS-4 levels which depended on chondrocyte shape and clustering. This approach assesses the responses of single *in situ* cells and revealed subtle and local properties of chondrocytes obscured with whole tissue samples. Morphologically-abnormal chondrocytes labelled more strongly for ADAMTS-4 compared to normal or clustered chondrocytes (Fig.2B(i)). As the chondrocyte population changed with cartilage degeneration (i.e. with more abnormal cells and clusters) this was reflected in a shift to left of the

distribution curve as there was a large number of cells in clusters with lower levels of ADAMTS-4 (Fig.2(B)(ii). There was also heterogeneous labelling in each of the chondrocyte categories but there was no *a priori* reason that cells in each category should behave identically. Examples showed chondrocytes with no clear ADAMTS-4 labelling beside strongly-labelled cells, and even cells within the same lacuna exhibiting varying levels of labelling (Fig.2A(iv)). This could be due to variations in primary and/or secondary antibody penetration, however heterogeneity was observed in single sections and images where all cells would be exposed to identical antibody levels. A more likely explanation could be that access of factors controlling ADAMTS-4 production are variable and dependent e.g. on local ECM permeability. While IL-1β upregulates ADAMTS-4 gene expression in human chondrocytes and cartilage explants[3], cell-associated levels of IL-1β are very different between *in situ* human chondrocytes of varying morphology[13]. Thus IL-1β availability either from intracellular metabolism or an inflammatory response, could be variable accounting for the heterogeneity in ADAMTS-4 levels. The estimated $t_{½}$ of ADAMTS-4 exocytosis is ~220mins[15] and it is possible that there are varying levels of ADAMTS-4 depending on local IL-1β concentrations. We also do not know the aggrecan levels around chondrocytes, however as they may be reduced in the vicinity of morphologically-abnormal cells, this could lead to focal regions of mechanical weakness.

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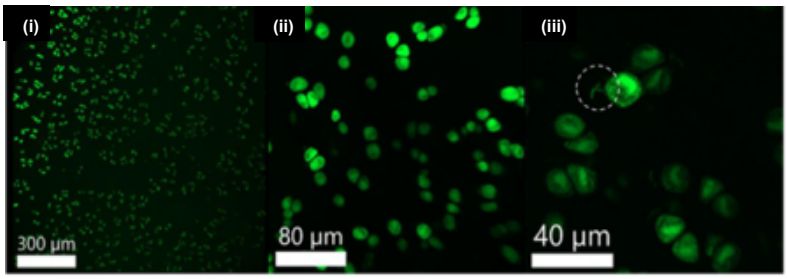
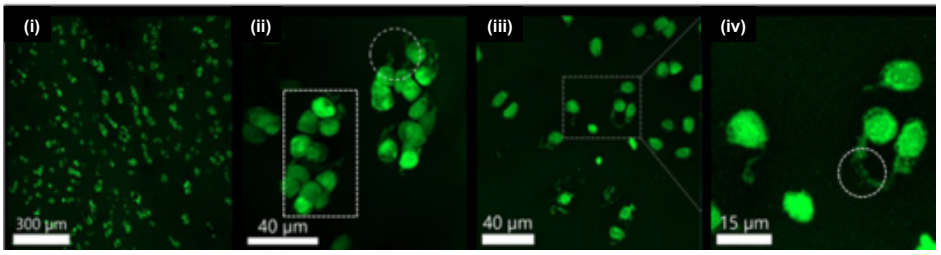
Herren et al (2023) Figure 1.

(A)

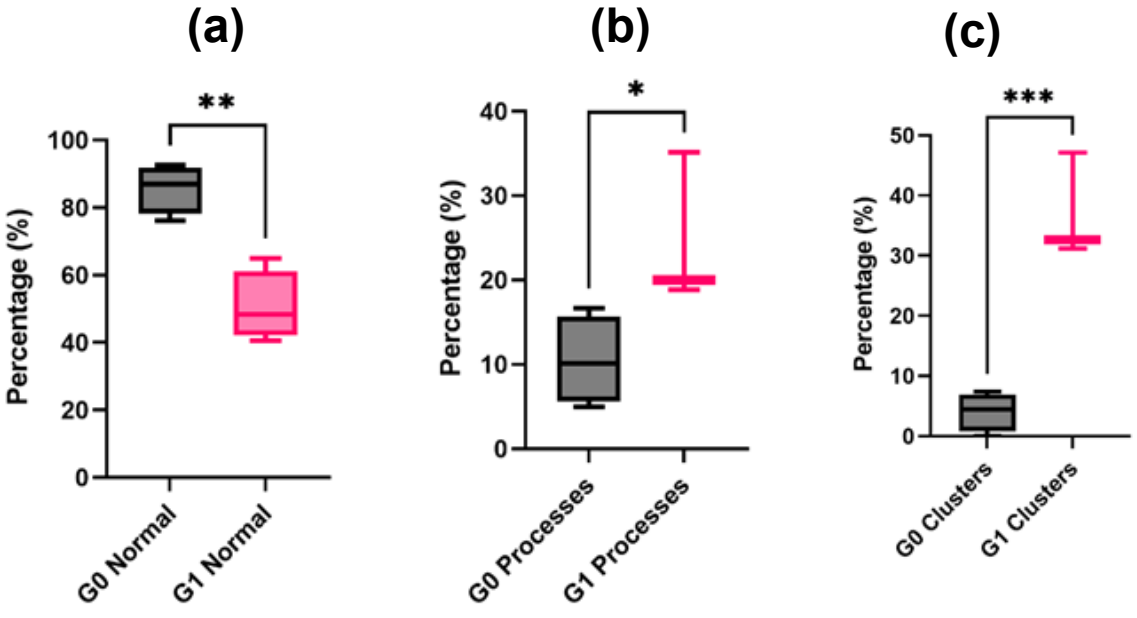
(a) (G0 cartilage)



(b) (G1 cartilage)

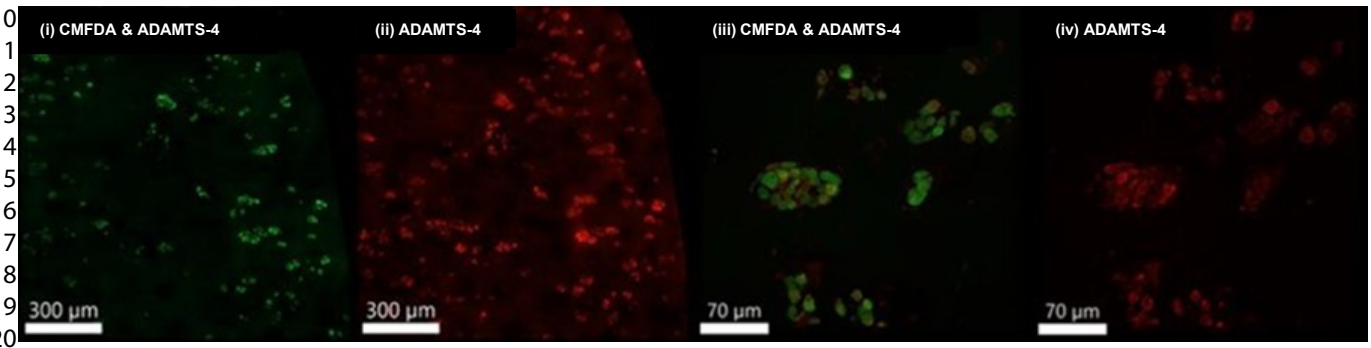


(B)



Herren et al (2023) Figure 2.

(A)



(B)