Chondrocytes within Normal and Osteoarthritic Adult Human Articular Cartilage can be Stimulated to Proliferate *in Situ* by Proteoglycan Depletion and Collagen Degradation

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INTRODUCTION: The proliferation of adult human articular chondrocytes may be desirable and beneficial, on the one hand, or pathognomonic, on the other. Normal (NL) adult articular cartilage (AC) includes widely dispersed chondrocytes that maintain the tissue but do not effectively repair damage. Articular defects can be effectively regenerated *in vivo* by ACI and MACI with culture-expanded autologous chondrocytes, amplified *in vitro* through extensive proliferation.[1,2] However, in osteoarthritis (OA), chondrocyte clusters are a hallmark of degenerated AC that does not repair effectively.[3] Thus, both for providing a source of regenerative cells and for elucidating OA pathogenesis mechanisms, being able to trigger and localize chondrocyte proliferation *in situ* would be of use. To isolate adult chondrocytes from cartilage, classical methods use sequential digestion, removing proteoglycan and then freeing cells from collagen (COL).[4,5] In OA, severe AC damage is due to a sequence of protease actions, first on proteoglycan and then COL.[6] Thus, we tested whether enzyme treatment of NL or OA human AC (hAC) with partial COL degradation could yield a cell-laden tissue with cells exhibiting a proliferative response.

METHODS: ***hAC Explant Model.*** hAC explants were isolated fresh, as half discs (2 mm diameter by 1 mm thick) from weight-bearing regions of femoral condyles of healthy allografts (hNL, n=3; 18,32 yr M, 23 yr F) and knee replacement tissue remnants (hOA, n=3; 67,71 yr M, 78 yr F). Discs were sequentially digested with 0.25% trypsin in medium for 1 hr, and then with 0.0002, 0.002, 0.007, or 0.02% collagenase-P in medium with 10% FBS for 16 hr, or incubated without enzymes as controls (CTRL). Some discs were incubated for 3 days in DMEM ± 5 μM EdU, 1 ng/mL TGF-β1, 5 ng/mL FGF-2, 10 ng/mL PDGF-BB, and 5% FBS.[7] ***Chondrocyte Viability and Proliferation.*** Chondrocyte viability was assessed in with Live/Dead™ and fluorescence microscopy. Proliferating cells were localized by reacting EdU with AlexaFluor™ 594 (red fluorescence). All cells were stained with Hoechst 33342 (blue fluorescence). Total and EdU/Total cells were quantified in *en face* and vertical orientations, the latter in Superficial, Middle, and Deep (0-250, 250-500, >500 μm) zones. ***GAG Depletion and COL Degradation.*** Digest media and tissues were assayed for proteoglycan as sulfated glycosaminoglycan (GAG) and COL as hydroxyproline. Matrix contents were normalized to initial tissue wet weight (ww). ***Statistics.*** For each tissue type (hNL or hOA), effects of digestion were assessed by ANOVA and Dunnett *post-hoc* comparison to CTRL, with $p<0.05$ taken as significant.

RESULTS: ***(1) GAG depletion and COL degradation.*** Trypsin induced substantial GAG release from AC, with little passive loss from CTRL (15%) and extensive loss from all treatment groups for both hNL and hOA (78% and 88%, each $p<0.0001$). In contrast, collagenase caused COL release from AC (**Fig 1**) in a dose-dependent manner, elevated from low levels in CTRL (0.3%) to moderate levels with 0.007% collagenase, 135 μg/mg, 75% in hNL (**Fig 1A**) and 95 μg/mg, 73% in hOA (**Fig 1B**). Higher (0.02%) collagenase caused complete tissue dissolution. ***(2) Chondrocyte viability and proliferation***. Chondrocyte viability remained high (averaging 81%, $p>0.8$) following all treatments. Chondrocytes in both hNL and hOA cartilage exhibited proliferative responses that also depended on collagenase dose (**Figs 2,3**). In *en face* images (**Fig 2 Bi, Ci-iii, Di-iii**), digestion with 0.25% trypsin and 0.007% collagenase elevated EdU+ cells from 0.5% (CTRL) to 10.7% in hNL and 0.3% (CTRL) to 6.7% in hOA explants. In vertical images (**Fig 2 Bii, Civ-vi, Div-vi, Fig 3AB**), a similar trend was evident in the S zone, and additional cells were variably stimulated to proliferate throughout in S, M and D zones.

DISCUSSION: This is the first report of using low concentrations of collagenase to trigger chondrocyte proliferation *in situ* in both NL and OA hAC. Without enzyme treatment, the near zero *in situ* proliferation of chondrocytes in CTRL groups is consistent with maintained phenotypes (cytokine-responsive but non-regenerative) of hAC explant culture.[5] Enzyme levels titrated in concentration and duration are important and likely tissue-specific, since in adult humans, the COL network is more dense and more crosslinked than that of many animal tissues. In addition, the partial digest solutions may contain intact cells or small tissue fragments. Finally, for effective cartilage regeneration, chondrocyte proliferation is one of several cell fates that will need to be controlled.

SIGNIFICANCE/CLINICAL RELEVANCE: Controlled digests of cartilage explants may have a variety of potential uses. Cartilage repair surgeries may use such tissue, either NL or OA hAC, as an effective source of proliferative and regenerative cells. Studies of OA may use such tissue explants to better understand mechanisms of cartilage deterioration with disease initiation and progression, as well as to test regenerative strategies.

REFERENCES: [1] Brittberg M+, *New Engl J Med* 1994. [2] Bartlett W+, *J Bone Joint Surg [Br]* 2005. [3] Lotz MK+, *Arthritis Rheum* 2010. [4] Kuettner KE+, *J Cell Biol* 1982. [5] Hauselmann HJ+, *Arthritis Rheum* 1996. [6] Poole AR+, *Ann Rheum Dis* 2002. [7] Hsieh-Bonassera ND, *Tissue Eng Part A* 2009.

FIGURES:

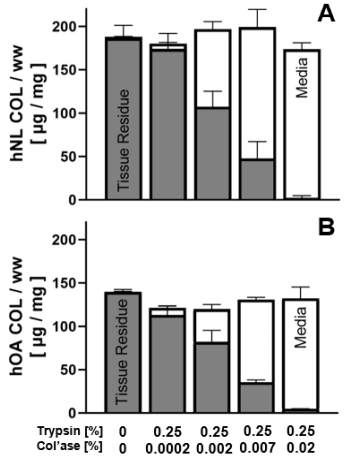

Fig 1. Effect of enzymes on collagen in tissue residue vs media. (A) hNL. (B) hOA. N=3/grp.

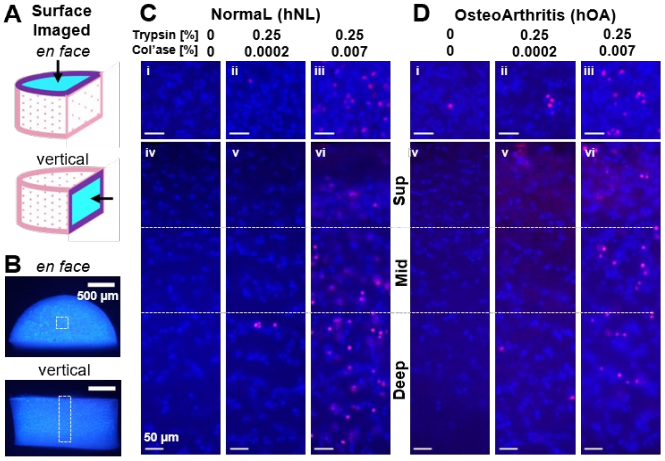

Fig. 2. Effect of enzymes on chondrocyte proliferation *in situ*. (A) Surfaces imaged. **(B-D)** EdU (red) and Hoechst (blue) cell fluorescence. **(C,D)** Zoom areas: **(i-iii)** *en face*, **(iv-vi)** vertical, in **Sup**erficial, **Mid**dle, and **Deep** zones.

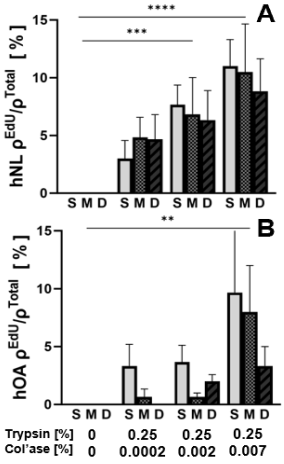

Fig 3. Chondrocyte proliferation in Sup, Mid, and Deep zones. (A) hNL. (B) hOA. N=3/grp.