Deformation Thresholds for Chondrocyte Death and the Protective Effect of the Pericellular Matrix

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In cartilage tissue engineering studies, the stimulatory effect of mechanical perturbation declines after the first 2 weeks of culture. Similarly, it is known that chondrocyte–agarose constructs should not be loaded within the first days after seeding, to prevent considerable cell death, suggesting a mechanical threshold. This study aims to establish a relationship between chondrocyte deformation and death, and to evaluate the protective effect of the pericellular matrix (PCM) that is formed in 3D cultures. Chondrocyte viability was monitored every hour for 24 h after applying a strain range of 0% to 25% to agarose constructs containing chondrocytes, cultured for 1, 3, 5, 7, or 10 days. At these culture time points, the PCM thickness and chondrocyte deformation were assessed by means of histology and assayed for biochemical contents. Inverse finite element (FE) simulations were used to evaluate the change of mechanical properties of the chondrocyte and PCM over the 10-day culture duration. Chondrocyte death was demonstrated to be dependent on both the magnitude and duration of straining. The highest cell death was observed at day 1 (43%), reducing over culture duration (15% at day 3 and 2.5% at day 10). Cell deformation at 25% compression decreased significantly over culture duration (aspect ratio of 2.24 ± 0.67 at day 1 and 1.45 ± 0.24 at day 3) and with increased matrix production. Inverse FE simulations showed an increasing PCM Young's modulus of 45 kPa at day 3 to 162 kPa at day 10. The current results provide evidence for a mechanical threshold for chondrocyte death and for the protective effect of the PCM. As such, these insights may help in establishing mechanical loading protocols for cartilage tissue engineering studies.

Introduction

ARTICULAR CARTILAGE (AC) is the thin layer of soft tissue that lines the articulating surfaces of bones in diarthrodial joints. It redistributes stresses and provides a low friction surface during joint motion. The chondrocyte is the only cell type residing in AC and is responsible for the maintenance and repair of the extracellular matrix (ECM), which is mainly composed of collagen type II and proteoglycans. Chondrocyte death may reduce AC repair capacity and can over time lead to the development of osteoarthritis (OA). Current methods that aim to repair OA cartilage have unsatisfactory success in the long term.^{1–3}

AC tissue engineering is a promising alternative, by which chondrocytes are typically seeded in a 3D scaffold. Such scaffolds can be made from various materials, including natural materials such as chitin, collagen, silk or agarose, and synthetic polymers, such as polylactic acid (PLA) or polyglycolic acid (PGA).^{4–6} Ideally, the matrix produced by the chondrocytes should over time obtain sufficient load-bearing

properties to function properly when implanted into the defected area. AC tissue engineering has, however, shown to be challenging, often yielding tissue with insufficient mechanical properties. Applying dynamic compression to chondrocyte-seeded agarose constructs has shown to increase matrix production.^{6,7} The stimulatory effect, however, declines over culture time. Furthermore, in our experience, loading chondrocyte–agarose constructs within the first few days after seeding causes considerable cell death, while viability remains high when mechanical stimulation starts a few days after seeding (unpublished results).

Both phenomena may be attributed to the formation of a pericellular matrix (PCM) that encapsulates the chondrocyte. The PCM contains relatively high concentrations of aggrecan and hyaluronan, as well as collagen type II, VI, and IX, of which collagen type VI is exclusive to the PCM.^{8,9} Due to the complex pericellular microenvironment, the chondron is classified as a micromechanical unit within the cartilage matrix. *In vivo*, the PCM is suggested to regulate local stresses and strains of the chondrocyte in a depth-

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dependent manner. The ECM compressive^{10,11} and tensile¹² stiffness increases with tissue depth, while PCM tensile stiffness remains constant.^{13,14} The PCM would therefore provide protection to chondrocytes in the surface region, while strains in the deeper AC region are more easily transferred to the chondrocytes. In a setting in which chondrocytes are cultured in a hydrogel, such as agarose, however, PCM is the stiffest element, followed by the surrounding agarose, which is in turn stiffer than the encapsulated chondrocyte. Therefore, the PCM may gain enough mechanical integrity during culture to provide a strain shielding effect on the chondrocyte, diminishing the stimulatory effect of mechanical loading during culture.

This study aims to establish a relationship between chondrocyte viability and chondrocyte deformation in a 3D agarose culture, and to determine whether the PCM that develops within a few days of culture may be sufficient to protect chondrocytes against excessive deformations. We hypothesize that there is a threshold of deformation, which when exceeded leads to chondrocyte death. We also hypothesize that the cell deformation as a result of construct loading is attenuated by the PCM. We approach this by correlating cell deformation with cell death during compression of chondrocyte-seeded agarose constructs during 10 days of culture. Maturation of the PCM, and therefore a change of mechanical properties over the culture period is expected, and this will be evaluated by means of inverse finite element (FE) simulations.

Materials and Methods

Chondrocyte isolation

Chondrocytes were obtained from bovine metacarpophalangeal joints. The joint was opened under aseptic conditions and cartilage was sliced from the joint using a scalpel, after which the pieces were digested overnight in 0.5% collagenase type II (Worthington, Lakewood, NJ) in the high glucose Dulbecco's modified Eagle's medium (hgDMEM; Gibco, Invitrogen, Carlsbad, CA) under constant stirring at 37°C and 5% CO₂. The cell suspension was filtered over a 70- μ m cell strainer and washed twice. An equal volume of medium containing 20 million cells/mL was mixed with a 4% agarose type VII (Sigma, Zwijndrecht, The Netherlands) solution. This was poured into a sterile stainless steel mold to make 2% agarose samples with a radius of 4 mm and a height of 400 μ m, containing 10 million cells/mL. Constructs were cultured in 1 mL of culture medium [hgDMEM supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Lonza, Basel, Switzerland), 50 mg/mL ascorbate 2-phosphate (Sigma), 0.5 $\times$ essential amino acids and 1 $\times$ nonessential amino acids (Gibco)] at 37°C and 5% CO₂. The medium was replaced three times per week.

Experimental setup

After 1, 3, 5, 7, and 10 days, a sample was taken out of culture and stained for 1 h with cell tracker green (CTG; Invitrogen) to visualize living cells and propidium iodide (PI; Invitrogen) to visualize dead cells. The stained sample was placed in 3 mL of culture medium in a compression setup.¹⁵ Medium temperature was kept at 37°C throughout the compression experiment. The setup was mounted on a microscope (LSM510 confocal microscope; Carl Zeiss,

Stuttgart, Germany). The setup is equipped with a rounded indenter with a radius of 2.5 mm and a height difference of 100 μ m from the center to the edge of the indenter. Therefore, lowering the indenter 100 μ m from the contact point results in a strain range of 25% at the center to 0% at the edge of the indenter (Fig. 1).

Starting from the center of the construct, either three positions with adjacent fields of view of 1303 $\times$ 1303 μ m or four positions with adjacent fields of view of 921.36 $\times$ 921.36 μ m were marked so that the construct was scanned over its complete radius. Fields of view consist of 512 $\times$ 512 pixels, and a 10 $\times$ objective with a numerical aperture of 0.3 was used. Before applying compression, a scan was made to retrieve initial cell death. The indenter was slowly lowered a 100 μ m from the contact point, and this displacement was kept constant for 24 h. After applying compression, at each position, a stack of 50 planes with a total height of 270 μ m was scanned every hour during the 24 h. Scans consisted of 50 planes with a total height of 270 μ m. The green (CTG, excitation: 488 nm, emission: 505–530 nm) and red (PI, excitation: 543 nm, emission: 617 nm) signals are recorded in separate channels. This experiment was repeated three times with different donors.

Chondrocyte death analysis

The stacks were analyzed for viability using a custom-made MATLAB script. The data were split in three compression areas: 20%–25%, 10%–20%, and 0%–10%. A Gaussian filter was applied to remove the major part of the noise. Manually, a size- and intensity-based threshold was set, and connected components were labeled in 3D. From each cell, the centroid coordinates were stored. To adjust for cells that appear both green and red, centroid coordinates from live and dead channels were compared. If agreement between the centroid coordinates was found (i.e., the distance between live and dead cell centroid was ≤ 5 μ m), the cell was assumed dead and deleted from the list of live cells. The list of dead cells was updated with each stack, and centroid coordinates of already listed dead cells were compared with the centroid coordinates of newly dead cells, to ensure dead cells were counted only once. For each culture time point and compression area, the percentage of dead chondrocytes over the 24-h compression time was calculated.

Chondrocyte deformation

For each donor, at each culture time point, one construct was formalin fixed overnight in an uncompressed state, and one construct was fixed in a 25% compressed state, by placing the 400- μ m-thick constructs between two glass

FIG. 1. Schematic representation of the agarose construct and the tip of the indenter.

slides, spaced with two 300- μm -thick metal plates. The slides were clamped together providing 25% compression to the constructs, and the setup was placed in formalin overnight. The constructs were paraffin sectioned in the direction of compression and stained with Alcian blue, to visualize the glycosaminoglycan (GAG) deposition. Pictures of 1936×2584 pixels (one pixel is $0.054 \times 0.054 \mu\text{m}$) were taken with a brightfield microscope (Observer Z1, objective: $100\times$, $\text{NA}=1.4$; Carl Zeiss). The chondrocyte height and width and PCM thickness were measured using a custom written MATLAB script. The PCM thickness was calculated as the average of three or four thickness measurements at different positions per cell.

Biochemical contents

For each donor, at each culture time point, five constructs were pooled together and stored at -80°C until they were assayed for biochemical contents. Constructs were lyophilized overnight, weighed dry, and digested in 1 mL of digestion buffer (100 mM phosphate buffer, 5 mM L-cystein, 5 mM ethylene diamine tetraacetic acid, and $140 \mu\text{g/mL}$ papain, all from Sigma) for 16 h at 60°C and stored at 4°C until they were assayed for GAG and DNA content. For analysis of sulfated GAG content, a modification of an earlier described assay¹⁶ was used, with a reference curve from shark cartilage chondroitin sulfate (Sigma). The amount of DNA was measured using the Hoechst dye method¹⁷ with a reference curve from calf thymus DNA (Sigma).

FE modeling

Using FEBio software¹⁸ (version 1.3; University of Utah, Salt Lake City, UT), a biphasic model was used to simulate 25% compression of chondrocyte-seeded agarose, corresponding to the 25% compression between the two glass plates in the experiment that was set up for assessing chondrocyte deformation. The first governing equation, the balance of momentum for the mixture is given as follows:

$$\nabla \cdot (-pl + T^e) = 0 \quad (\text{Eq.1})$$

where T^e represents the effective Cauchy stress, resulting from deformation of the solid matrix, and p is the fluid pressure. The second governing equation is the conservation of mass, given as follows:

$$\nabla \cdot (v^s - k\nabla p) = 0 \quad (\text{Eq.2})$$

where v^s represents the velocity of the solid matrix and k is the hydraulic permeability. In this formulation, the solid matrix of the various components was modeled as an isotropic neo-Hookean material, defined by Young's modulus (E) and Poisson's ratio (ν). Meshes were generated by a custom Salome (Open Cascade, Guyancourt, France) Python script. Meshes consisted of linear four-node tetrahedral elements, and the convergence analysis on meshes produced with this script was conducted in a previous study.¹⁹ For each culture time point, a specific mesh (a $100 \times 100 \times 100 \mu\text{m}$ cube containing a single chondrocyte), incorporating the experimentally obtained chondrocyte diameter and PCM thickness, was generated. Agarose parameters were obtained from literature.^{2,20} Agarose permeability, Young's modulus,

FIG. 2. Chondrocyte death in 0%–10%, 10%–20%, and 20%–25% compression areas over the 24-h compression period after a culture time of 1, 3, 5, 7, and 10 days.

and Poisson's ratio, and the chondrocyte's and PCM's permeability and Poisson's ratio were prescribed. Using the Nelder-Mead Simplex optimization method in MATLAB, the chondrocyte and PCM Young's moduli were fitted to experimental data, so that agreement between the experimental and numerical aspect ratios of chondrocytes under 25% compression was obtained. A compressive strain of 25% was simulated by a linearly ramped displacement boundary condition over a 1-s time interval. This displacement was kept constant until equilibrium was reached.

Statistics

Statistics were performed with GraphPad Prism. Chondrocyte viability data were tested for significant differences between strain levels at 24-h compression over the culture duration using a Friedman test and a *post hoc* Dunn's test. A Shapiro–Wilk test was used to test for normality of data sets for aspect ratios and matrix thickness. These data were tested for significant differences by ANOVA with a *post*

FIG. 3. Chondrocyte aspect ratios with (gray) and without (black) 25% compression at day 1, 3, 5, 7, and 10. * Indicates the difference from uncompressed controls ($p < 0.05$).

FIG. 4. Alcian blue staining of compressed chondrocytes at day 1, 3, 5, 7, and 10. Pictures are taken at a 100 $\times$ magnification.

hoc Tukey test for multiple comparisons. Statistical significance was assumed for $p < 0.05$. Data are represented as mean $\pm$ standard deviation.

Results

Chondrocyte death

The number-weighted average percentages of chondrocyte death over compression time (Fig. 2) show increased chondrocyte death with an increased magnitude and duration of straining. In addition, a reduction in chondrocyte death with culture time is observed. At day 1, chondrocyte death in the high compression area reaches 43%, while at day 3, cell death has reduced to 15% after 24 h of compression, and at day 10, it decreased to 2.5%. In the 20%–25% compression area, the viability after 24 h of compression at day 1 differed significantly from all other culture durations ($p < 0.05$). In addition, a significantly higher cell death is observed at day 3 after 24 h in the 20%–25% compression area compared with day 7 and 10. Although not significant, cell death was for each donor highest in high compression areas, and lowest in low compression areas up to day 5. Information regarding data variance can be found in (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

Chondrocyte deformation

Aspect ratios of uncompressed chondrocytes were all slightly above 1 for each culture time point (Fig. 3), with no significant differences between time points. Compression after 1 day of culture resulted in an average aspect ratio of 2.24 ± 0.67 . This decreases significantly over culture time to 1.45 ± 0.26 at day 3 and 1.16 ± 0.20 at day 5. At day 1 and 3, there is a significant difference in the aspect ratio between the compressed chondrocytes and their uncompressed controls. From day 5 on, this difference is not significant.

PCM thickness

The thickness of the PCM, measured manually from histological sections that were stained with Alcian blue (Fig.

4) increases with culture time (Fig. 5). At day 1, only some cells showed a small ring of PCM. For the majority of the cells, however, no sign of matrix production was observed. The average PCM thickness increased from $0.23 \pm 0.34 \mu\text{m}$ (average $\pm$ standard deviation) at day 1 to $0.88 \pm 0.37 \mu\text{m}$ at day 3. After 10 days of culture, the PCM thickness reached $1.64 \pm 0.39 \mu\text{m}$. The increase in PCM thickness was significant for successive culture time points up to day 7. The PCM thickness between day 7 and 10 was not significantly different, but the PCM thickness at day 5 differed significantly from the PCM thickness at day 10.

Association of chondrocyte death and deformation

To associate between cell death and deformation, chondrocyte death after 24 h in the 20%–25% compression area at each culture time point (day 1, 3, 5, 7, and 10) was plotted against the chondrocyte aspect ratios in 25% compressed samples from the same culture time points (Fig. 6A). Cell death increases linearly with the deformed aspect ratio. This plot is made for each hour of compression and the slope $s(t)$ and the offset $o(t)$ of the linear fits are calculated and plotted over the compression time (Fig. 6B).

FE modeling

Using a parameter fitting procedure, a similar chondrocyte deformation is attained as observed in histological images (Figs. 4 and 7). An increase in both the chondrocyte and PCM Young's modulus is found over the first 5 days of culture (Table 1). The maximum difference between the experimental aspect ratio and the aspect ratio from the fitting procedure was 1.3% of the experimental aspect ratio.

Discussion

One objective of this study was to explore the relationship between chondrocyte death and chondrocyte compression. Chondrocyte death was indeed found to increase with increased magnitude and duration of cell strain, and to decrease with increased culture times. The former is in agreement with

FIG. 5. (A) Average pericellular matrix (PCM) thickness at day 1, 3, 5, 7, and 10. (B) Glycosaminoglycan (GAG) per DNA content. * $p < 0.001$, ** $p < 0.01$, and *** $p < 0.05$.

FIG. 6. (A) The aspect ratio of compressed chondrocytes plotted against cell death after 24 h of compression. The linear fit ($\text{Cell Death} = 35.22 * \text{Aspect Ratio} - 35.78$) has an R^2 -value of 0.99. (B) Values of the slope (s) and offset (o) for every hour of compression.

previous studies, looking at chondrocytes in AC^{21,22} or other cell types.¹⁵ Interestingly, an inflection point is observed after 12 h of compression, after which the rate of cell death increases. This may be caused by the onset of apoptosis, which in other cell types, has shown to account for an increasing share in cell death starting after 10 h of compression.¹⁵ The decrease in chondrocyte deformation with increased culture time is also in agreement with former studies.^{23–26} These studies noted a developing matrix around the chondrocytes over a 6- or 7-day culture time. One study, however, associated an increasing GAG content directly to a decreasing chondrocyte deformation under compression, by showing an increased deformation after hyaluronidase treatment after 3 and 6 days of culture.²³ In the present study, a comparable decrease in cell deformation with increased GAG content over the culture time was observed.

Another objective of this study was to evaluate the hypothesis that PCM formation in chondrocyte-seeded agarose constructs would protect the cells from dying by shielding them from excessive strains. Over 10 days of culture, the amount of PCM increased significantly, as quantified with a GAG assay and by PCM thickness measurements from histological images. The low variance and the differences in PCM thickness, which were found for successive time points up to day 7 (Fig. 5A), indicate that synthesis of PCM was uniform for each time point. Experiments showed that chondrocyte aspect ratios in 25% compressed agarose constructs decreased from 2.2 at day 0 to 1.1 at day 10 of culture (Figs. 3 and 4), indicating that the formed PCM indeed prevented the chondrocytes from deforming.

By means of inverse FE simulations, using agarose stiffness, cell size, PCM thickness, and chondrocyte deformation obtained from histological images as inputs, similar stiffness for PCM (45–162 kPa) and cells (0.35–0.60 kPa) were computed as reported in literature (respectively, 24–240 kPa for PCM^{13,14,27,28} and 0.26–4.0 kPa for cells^{20,25,28,29}). Interestingly, the fitted PCM stiffness increased from 45 kPa at day 3 to 162 kPa at day 5 after which it remained constant, indicating matrix maturation with time of culture. Most FE studies investigated an *in situ* setting^{13,14,28,30} in which the

chondrocyte and PCM are embedded in a relatively stiff ECM. Although such a setting is very different from a setting in which chondrocytes are embedded in a soft hydrogel such as agarose, it was found that the PCM has a significant effect on the mechanical environment of the chondrocyte. One study, however, also looked at a setting in which the chondrocyte is embedded in a softer environment such as agarose.³⁰ Comparable to the present study, it was found that the presence of a PCM significantly reduces cellular strain magnitudes in such a setting. The experimental data as well as the simulations confirm the hypothesis that with increasing maturation of the PCM over culture time, the PCM becomes more effective in protecting the chondrocyte against deformation, and therefore, in preventing cell death following mechanical perturbations at the construct level.

The current results provide an explanation for the decreased cell viability that occurred when mechanical stimulation was applied in the first few days after seeding. In the absence of the PCM, the mechanical matrix strain was directly transferred to the chondrocyte, resulting in excessive cell deformation. Because cell death increases linearly with an increased aspect ratio (Fig. 6A), cell viability was compromised. For every compression time point, the relation between cell death and cell deformation can be written as follows:

$$\text{Chondrocyte Death}[\%] = s(t) * \text{Aspect Ratio} - o(t) \quad (\text{Eq.3})$$

Because the differences between $s(t)$ and $o(t)$ are small (Fig. 6B), Equation 3 may be written as follows:

$$\text{Chondrocyte Death}[\%] = (\text{Aspect Ratio} - 1)c(t) \quad (\text{Eq.4})$$

Where $c(t) = s(t) = o(t)$. Equation 4 shows that increased cell deformation and duration of deformation lead to increased chondrocyte death, where the factor $(\text{Aspect Ratio} - 1)$ indicates that no cell death occurs when cells remain spherical.

It can be speculated that the protective effect of the developing PCM not only reduces cell death, but also may

FIG. 7. Finite element representation of 25% compressed chondrocytes.

TABLE 1. FITTED CHONDROCYTE AND PERICELLULAR MATRIX YOUNG'S MODULI

Culture time	Day 1	Day 3	Day 5	Day 7	Day 10
$E_{\text{chondrocyte}}$ (kPa)	0.35	0.36	0.58	0.60	0.57
E_{PCM} (kPa)	—	45.26	161.91	179.25	161.93

PCM, pericellular matrix.

similarly shield cells from receiving mechanical stimuli. It is commonly observed in cartilage tissue engineering studies that the stimulatory effect of mechanical perturbation declines after the first 2 weeks of culture.^{31,32} This study suggests that this may be caused by an increasing protective effect of the PCM against mechanical deformation of the chondrocytes. Even a 25% compressive strain does not significantly deform cells after 10 days of culture in the present study. An alternative culture regimen may result in different matrix distributions, and therefore in different relationships between macroscopically applied loads and cell perturbations. Regardless, the present study shows the significant effect that tissue development has on the mechanical environment of chondrocytes in 3D constructs. This shows that the applied mechanical stimulus at the construct level would have to be varied over culture time to ensure continuous stimulation of cells in the construct. This insight is critical for developing culture regimens for cartilage tissue engineering.

Although the trends in chondrocyte viability are similar between donors, the small sample size may be regarded as a limitation to this study. The current method to count living and dead cells is labor intensive, due to the need for a manual threshold input. However, cell counts are believed to be accurate because only small variations in the number of total cells over the compression time in each experiment were observed. Furthermore, a biphasic model was initially used so that time-dependent effects could be taken into account. However, for the purpose of the current study, we only looked at the equilibrium state of the chondrocyte under compression, meaning that simulations could have been performed with a simpler model.

In conclusion, it was shown that chondrocyte death increased with the magnitude and duration of cell deformation, which changed as a function of culture time as a result of the development and maturation of PCM. The results reveal significant temporal variations in the relationship between the mechanical loading applied at the macroscopic level of tissue-engineered constructs and the eventual mechanical perturbation of cells. Such insight will be critical to improve the mechanical stimulation regimens for cartilage tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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