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The effect of chemically defined medium on spontaneous calcium signaling of *in situ* chondrocytes during long-term culture

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ABSTRACT

Chemically defined serum-free medium has been shown to better maintain the mechanical integrity of articular cartilage explants than serum-supplemented medium during long-term *in vitro* culture, but little is known about its effect on cellular mechanisms. We hypothesized that the chemically defined culture medium could regulate the spontaneous calcium signaling of *in situ* chondrocytes, which may modulate the cellular metabolic activities. Bovine cartilage explants were cultured in chemically defined serum-free or serum-supplemented medium for four weeks. The spontaneous intracellular calcium ($[Ca^{2+}]_i$) signaling of *in situ* chondrocytes was longitudinally measured together along with the biomechanical properties of the explants. The spontaneous $[Ca^{2+}]_i$ oscillations in chondrocytes were enhanced at the initial exposure of serum-supplemented medium, but were significantly dampened afterwards. In contrast, cartilage explants in chemically defined medium preserved the level of calcium signaling, and showed more responsive cells with higher and more frequent $[Ca^{2+}]_i$ peaks throughout the four week culture in comparison to those in serum medium. Regardless of the culture medium that the explants were exposed, a positive correlation was detected between the $[Ca^{2+}]_i$ responsive rate and the stiffness of cartilage (Spearman's rank correlation coefficient = 0.762). A stable pattern of $[Ca^{2+}]_i$ peaks was revealed for each chondrocyte, *i.e.*, the spatiotemporal features of $[Ca^{2+}]_i$ peaks from a cell were highly consistent during the observation period (15 min). This study showed that the beneficial effect of chemically defined culture of cartilage explants is associated with the spontaneous $[Ca^{2+}]_i$ signaling of chondrocytes in cartilage.

1. Introduction

Autologous tissue transplantation and tissue engineering-based techniques are often used for clinical cartilage lesion repairs (Guilak et al., 2014; Hung et al., 2004; Hunziker, 2002; Peterson et al., 2002). To increase the in-shelf time of osteochondral explants and to promote the extracellular matrix deposition of chondrocytes, the medium and supplements used for long-term *in vitro* culture are critical for the mechanical integrity of cartilage tissue and the viability, phenotype and metabolic activities of chondrocytes. Serum, a common supplement in culture medium, is used as a major source of nutrients during the *in vitro* culture of chondrocytes and cartilage. However, many recent studies have shown that exposing cartilage to blood or serum can induce extracellular matrix damage. The adverse effect was partially attributed to cytotoxic oxygen metabolites (Roosendaal et al., 1999; Valentino et al., 2007). Cytokines in serum, such as COMP, IL-1 and TNF-α, may also induce the inflammatory processes (Isomäki and Punnonen, 1997; Schuerwegh et al., 2003), which could further produce metalloproteinase and precipitate the degradation of cartilage matrix (Kapoor et al., 2011). Therefore, serum-supplemented medium may not always be an optimal choice for the *in vitro* culture of chondrocytes. Several studies showed that chemically defined serum-free medium can significantly benefit the biomechanical and biochemical properties of cartilage explants during long-term *in vitro* culture in comparison to traditional serum-supplemented medium (Bian et al., 2008, 2010; Byers et al., 2008; Garrity et al., 2012). Using the chemically defined medium, the mechanical properties and proteoglycan content of cartilage explants were increased after 2-week culture with minor loss of cell viability (Bian et al., 2008). However, little knowledge is available about the beneficial mechanisms of chemically defined medium at the cellular or molecular level.

Intracellular calcium ($[Ca^{2+}]_i$) signaling is one of the earliest responses in chondrocytes under many physical stimuli (Kono et al., 2006; O'Conor et al., 2014a, 2014b; Sanchez-Adams et al., 2014b). As an essential regulator of the mechanotransduction process, $[Ca^{2+}]_i$ signaling is the upstream of multiple signaling pathways in chondrocytes, which are ultimately involved in the regulation of various physiological processes such as secretion and gene expression

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(Grandolfo et al., 1998; Hung et al., 1997; Pritchard and Guilak, 2006; Pritchard et al., 2008; Sanchez-Adams et al., 2014a). Since chondrocytes are isolated in cartilage and lack direct cell-to-cell connection, the calcium wave propagation facilitated by the diffusion of messengers can be an essential intercellular communication pathway in cartilage (Kono et al., 2006). Besides the calcium signaling triggered by physical stimulation, both isolated and *in situ* chondrocytes have been found to release spontaneous $[Ca^{2+}]_i$ signaling (Fodor et al., 2013; Kono et al., 2006; O'Conor et al., 2014a, 2014b), *i.e.*, the $[Ca^{2+}]_i$ concentration oscillates in chondrocytes without the presence of any extraneous mechanical or chemical stimuli.

Due to the critical role of $[Ca^{2+}]_i$ signaling in chondrocyte mechanotransduction and cartilage remodeling, we hypothesize that the beneficial mechanisms of chemically defined medium during the *in vitro* culture of cartilage explants are associated with the spontaneous $[Ca^{2+}]_i$ signaling in chondrocytes. The objectives of this study are (1) to characterize the spatiotemporal features of spontaneous $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes, and (2) to compare the longitudinal $[Ca^{2+}]_i$ signaling of *in situ* chondrocytes cultured in two types of medium, a medium supplemented with serum and a chemically defined serum-free medium.

2. Method

2.1. Sample preparation and tissue culture

Cylindrical cartilage explants were harvested from the central region of femoral condyle heads of four 3–6 month-old fresh calf knee joints (Green Village, NJ) using a 3 mm biopsy punch. The superficial-to-middle zone of the cartilage (2 mm in thickness) was obtained with a custom designed cutting tool. Cartilage explants were randomly assigned to two groups and cultured at 37 °C and 100% humidity in either serum-supplemented medium (DMEM, 10% FBS, 1% P/S) or chemically defined medium (DMEM, 1% ITS+Premix, 50 μg/ml L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate and 50 μg/ml ascorbate 2-phosphate) for four weeks (Bian et al., 2008). Samples used for calcium signaling were cultured in 6-well plates with 8 explants in each well. The explants used for mechanical testing were cultured individually, one sample per well, in 24-well plates, so that their mechanical properties could be measured repeatedly at various time points. Culture medium was changed every other day for both groups, and the plate was changed every week to avoid the proliferation of migrating chondrocytes at plate bottom.

2.2. Calcium signaling of in-situ chondrocytes

Spontaneous $[Ca^{2+}]_i$ signaling of chondrocytes was recorded on days 2, 8, 15, and 29. Cartilage explant was halved axially with a cutting tool (ASI-Instruments, MI) and dyed with 5 μM Fluo-8 AM (AAT Bioquest, CA) and incubated for 40 min (Jing et al., 2014). The dyed cartilage was then washed three times with pure DMEM for five minutes each at 37 °C. Each half cylindrical cartilage sample, with the cutting surface facing down, was placed in an imaging chamber mounted on a confocal microscope (Zeiss LSM510) (Fig. 1A). The sample was allowed a 15 min resting period for the cells to recover from any agitation during preparation (Godin et al., 2007). The focal plane of the fluorescent image was 30 μm deep below the cutting surface to avoid viewing damaged cells. The imaging area was located 200 μm below the articular surface and on the center axis of the sample, as indicated in Fig. 1B. Fluorescent images of chondrocytes were recorded every 1.5 s for 15 min while the sample was undisturbed. $[Ca^{2+}]_i$ signaling of each individual cell was analyzed with an image processing technique as described previously (Lu et al., 2012). Due to the large number of cells in each video, only a band in the center of image across the thickness direction is processed, and 150–200 cells were analyzed in each sample. Oscillation of $[Ca^{2+}]_i$ concentration was measured by the average image intensity of each cell. A cell was defined as responsive if it shows a calcium peak with a magnitude four times higher than its maximum fluctuation along the baseline (Donahue et al., 2003). The responsive rate was defined as the fraction of responsive cells over total processed cells. For all responsive cells, the number of $[Ca^{2+}]_i$ peaks during 15-min period was counted. To further compare the calcium signaling between two groups, spatiotemporal features of the $[Ca^{2+}]_i$ peaks, including the magnitude of peaks, time to reach a peak, relaxation time from a peak and time interval between two neighboring peaks, were extracted (Fig. 1C) (Huo et al., 2010a, 2010b, 2008; Jing et al., 2014; Lu et al., 2012). All spatiotemporal parameters are defined in Fig. 1C using a typical curve of $[Ca^{2+}]_i$ signaling. Five cartilage explants were used for calcium signaling from each group at each time point. The number of total responding cells in the chemically defined group was 243, 169, 136 and 146 at days 2, 8, 15 and 29, respectively; and the number in serum group was 262, 120, 114 and 51 at days 2, 8, 15 and 29, respectively. Cell density of the cartilage samples at each time point, defined as the number of dyed cells divided by the image area, was also counted using the full calcium images of each sample.

2.3. Mechanical properties and GAG content

Mechanical properties of cartilage explants ($N=12$) were measured longitudinally by unconfined compression tests on days 2, 8, 15, and 29 of culture using a loading device in a biological safety cabinet (Lu and Mow, 2008). The original thickness of the cartilage explant was measured as the distance between the upper and lower loading platens with a 5-g compression force. During the test, 10% strain was applied on the tissue at a constant speed of 2 μm/s followed by a 15-min relaxation period. After the tissue reaction force reached an equilibrium state, sinusoidal dynamic loading was applied at 0.5 Hz with a magnitude of ± 1% strain. Equilibrium Young's modulus and dynamic modulus of the samples were determined from the recorded force history during the test.

At the end of culture, a quarter of each cartilage explant was weighed wet, and digested using papain solution at 60 °C for 16 h (Lu et al., 2004). The glycosaminoglycan (GAG) content was measured using dimethylmethylene blue (Biocolor, Life Science Assays) dye-binding assay with chondroitin 4-sulfate as standard (Lu et al., 2004).

2.4. Statistical analysis

Student's *t*-test was used to compare the mechanical properties between the two groups, and the data are shown as mean ± standard deviation. For the parameters related to $[Ca^{2+}]_i$ signaling, nonparametric Mann–Whitney *U* test was utilized to detect the significant difference between two groups, and the data are shown as mean ± SEM. *P*-values less than 0.05 are considered significant. Chi-square test was utilized to compare the responsive rate of $[Ca^{2+}]_i$ signaling in two groups. The Mann–Kendall test was performed to detect the temporal trend for parameters, confidence factor larger than 95% was considered significant. Spearman's rank

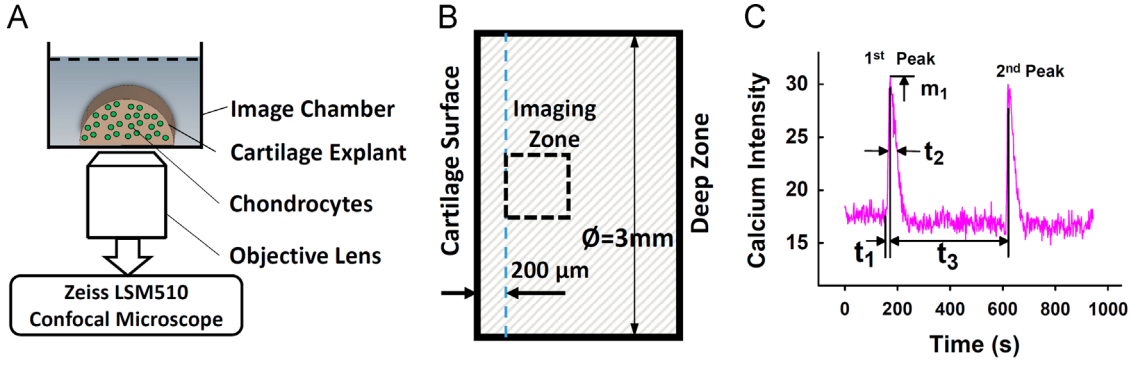

Fig. 1. (A) Illustration of calcium imaging for *in situ* chondrocytes in a cartilage explant. Half cylindrical cartilage explant (3 mm in diameter and 2 mm in thickness) in a glass-slide imaging chamber is mounted on a confocal microscope (Zeiss LSM510) and imaged with 20× objectives. (B) Imaging area is chosen to be 200 μm below the explant's articular surface and on the center axis of explant. (C) A typical $[Ca^{2+}]_i$ intensity oscillation curve of a chondrocyte over 15 min and the definitions of spatiotemporal parameters, including the number of $[Ca^{2+}]_i$ peaks, magnitude of 1st peak m_1, time to 1st peak t_1, relaxation time of 1st peak t_2, and time interval between two neighboring peaks t_3.