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Purinergic responses of chondrogenic stem cells to dynamic loading•

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Abstract: In habitually loaded tissues, dynamic loading can trigger ATP (adenosine-5′-triphosphate) release to the extracellular environment, and result in calcium signaling *via* ATP binding to purine P2 receptors.[1] In the current study, the purinergic responses (ATP release) of two types of cells: bovine chondrocytes (bCHs) and human mesenchymal stem cells (hMSCs) that were encapsulated in agarose and subjected to dynamic loading were compared. Both cell types were cultured under chondrogenic conditions, and their responses to loading were evaluated by an ATP release assay in combination with a connexin (Cx)-sensitive fluorescent dye (lucifer yellow – LY) and a Cx-hemichannel blocker (flufenamic acid – FFA). In response to dynamic loading, the chondrogenic hMSCs released significantly higher amounts of ATP (5-fold) in comparison to the bCHs early in culture (day 2). The triggering of LY uptake in the bCHs and hMSCs by dynamic loading implies opening of the Cx-hemichannels. However, the number of LY-positive cells in the hMSC-constructs was 2.5-fold lower compared to the loaded bCH-constructs, suggesting utilization of additional mechanisms of ATP release. Cx-reactive sites were detected in both the bCHs and hMSCs-constructs. FFA application led to reduced ATP release in both the bCHs and hMSCs, which confirmed the involvement of connexin hemichannels, with more prominent effects in the bCHs than in the hMSCs, further implying the existence of additional mechanisms of ATP release in chondrogenic hMSCs. Taken together, these results indicate a stronger purinergic response to dynamic loading of the chondrogenic hMSCs than that of primary chondrocytes, by activation of connexin hemichannels and additional mechanisms of ATP release.

Keywords: cartilage; loading; calcium signaling *via* ATP; ATP binding; Cx-hemichannels.

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• To Professor Branislav Nikolić, a great scientist, mentor and teacher, on the occasion of his 70th birthday.
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INTRODUCTION

Tissue engineering is a new discipline that has enormous potential to provide biological substitutes for damaged or dysfunctional human tissues. Tissues can be grown in the laboratory or regenerated in the body using a number of different strategies. In all cases, the presence of biosynthetically active cells is a fundamental requirement, as there is no way to grow a tissue except from cells, by mechanisms similar to those underlying normal development. In addition, biomaterial scaffolds are employed to provide cells with a structural and logistic template for tissue formation, and bioreactors are used to "instruct" the cells to form a specific tissue structure by providing molecular and physical regulatory factors. The designs of tissue engineering systems require guidance by biological requirements, which are in turn tissue-specific and "biomimetic" that is derived from the developmental principles.

One tissue of particularly high interest is articular cartilage, the lining of the surfaces of bones in human joints, due to the complete inability of this load-bearing tissue to regenerate itself following injury or disease. In the aging human population, the maintenance of healthy cartilage is an already significant and a constantly growing health concern. A number of new strategies for cartilage treatment and cartilage tissue engineering are being devised using different cell sources. Human mesenchymal stem cells (hMSCs) emerged as a clinically relevant cell source for regenerative medicine due to their multi-lineage differentiation potential and relative ease of isolation and expansion in culture.[2] Based on their ability to undergo chondrogenesis in a variety of natural and synthetic scaffold materials in the presence of the appropriate growth factors and mechanical loading, hMSCs are especially suitable for cartilage repair.[3] In addition, hMSCs are less likely to dedifferentiate when expanded *in vitro* in comparison to native chondrocytes.[4]

However, native chondrocytes (CHs) produce an extracellular matrix (ECM) with superior mechanical properties as compared to MSCs.[5] Achieving the physiological composition and functionality of hMSC-engineered cartilage remains a challenge. The focus of many current studies is set on the utilization of signaling molecules, scaffolds and mechanical stimulation towards achieving this goal.[6] In the context of mechanical stimulation and signaling molecules, it is crucial to identify and characterize the mechanotransduction pathways through which cells sense and respond to their mechanical environment. Potential mechanotransduction events include mechanical deformation of the nucleus,[7] changes in the membrane potential,[8] alterations in membrane transport and activation of ion channels.[9]

Several studies have shown that dynamic compression of native articular CHs activates intracellular calcium signaling.[10] This response is blocked by the addition of apyrase, which degrades extracellular adenosine-5′-triphosphate (ATP), or

by treatment with nonspecific blockers of purine P2 receptors.[11] Other studies report that in chondrocytes, ATP can induce calcium signaling,[12] which is released in response to compressive stress.[13] These results suggest that dynamic loading can trigger ATP release to the extracellular environment and induce calcium signaling in CHs *via* ATP binding to purine P2 receptors.[1]

At this time, the mechanisms of ATP release in CHs have not been defined. In other cell types, main physiological ATP release mechanisms have been elucidated: connexin and pannexin hemichannels, anion channels, and exocytosis of ATP-filled vesicles.[14] Recent studies report that native CHs release ATP *via* hemichannels composed of connexin 43 (Cx)[15,16] and/or pannexin 3.[17] However, the mechanisms of ATP release in chondrogenic hMSCs in response to dynamic loading remain largely unknown, despite being of great interest for the progression of cartilage formation in these cells. The purinergic responses to dynamic mechanical loading during chondrogenic differentiation of human mesenchymal stem cells were investigated in the present study, in order to better understand and potentially utilize this mechanism to enhance cartilage formation.

EXPERIMENTAL

Cell isolation and expansion

Two types of chondrogenic cells were studied: primary chondrocytes, and mesenchymal stem cells. For the primary chondrocytes, articular cartilage was harvested from fresh bovine carpometacarpal joints obtained from 4–6-month old calves. The cartilage was rinsed in phosphate buffered saline (PBS) and digested in Dulbecco Modified Eagle Medium (DMEM, Gibco, New York) with 0.5 mg mL^{-1} collagenase type IV (Sigma Chemicals, St. Louis, MO) for 10 h at 37 °C under stirring. The resulting cell suspension was filtered through a 70-μm pore size mesh to isolate the individual cells.[18] After rinsing the pellets, the chondrocytes were plated at high density (>1×10^5 cells cm^{-2}) in chondrocyte culture medium (high glucose DMEM – hgDMEM – supplemented with 10 % FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin). For the mesenchymal stem cells, commercially obtained human bone marrow aspirates were used to derive human mesenchymal stem cells (hMSCs). The cells were cultured to passage 3 in an expansion medium (hgDMEM supplemented with 10 % fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg mL^{-1} streptomycin and 1 ng mL^{-1} basic fibroblast growth factor) and used in the experiments.

Cell seeding in hydrogel

To produce cell-ladened agarose gels, type VII agarose (AG) (Sigma Chemicals, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) at a concentration of 4 %, w/v, autoclaved and cooled to 40 °C. The AG was combined with the cell suspension (40×10^6 cells mL^{-1}) in a 1:1 ratio to result in a seeding density of 20×10^6 cells ml^{-1}. The suspension was cast between two glass plates separated by 2.5 mm spacers. After cooling, cylindrical disks (4 mm in diameter×2.5 mm thick) were cored out using a biopsy punch as in previous studies,[18,19] resulting in 6.2×10^5 cells per scaffold.

Mechanical loading

The cell–agarose constructs were pre-cultured under static conditions for 48 h and then subjected to controlled dynamic deformational loading within a well-characterized loading

system used in previous studies.[20-22] The constructs are placed at the bottom of a 35×10 mm Petri dish and 3mL of sterile PBS was added into the Petri dish. A loading platen was positioned on top of the constructs inducing a tare strain of less than 0.5 %. Cyclic compression was applied for 40 min at a frequency of 1 Hz and a nominal 10 % strain. Sinusoidal deformation was applied *via* revolution of an eccentric cam calibrated to produce a defined displacement of a spring-loaded linear stage follower connected to the loading platen.[22] Unloaded constructs served as controls (Fig. 1).

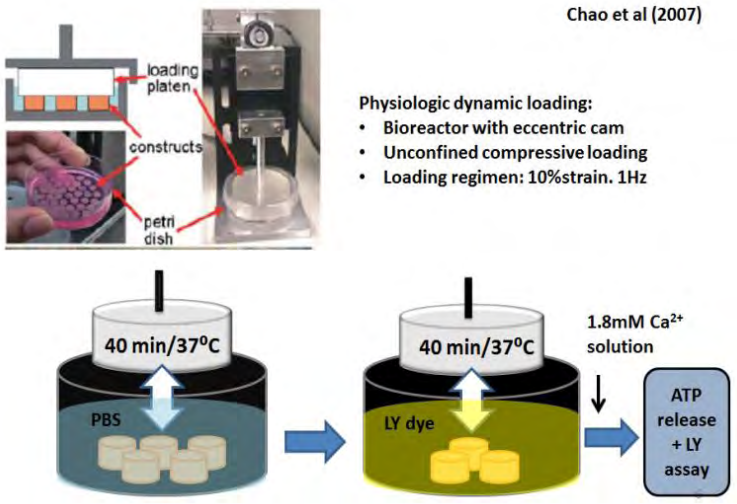

Fig. 1. Experimental setup. Cartilage constructs were prepared in the form of 4 mm in diameter×2.5 mm thick discs, as in previous studies[20] by encapsulation of primary chondrocytes and hMSCs in agarose. After 2 days of preculture, the constructs were subjected to 40 min of dynamic mechanical loading (with unloaded constructs serving as controls), and evaluated for ATP release and activation of connexin hemichannels.

ATP release

The effect of mechanical loading on the chondrogenic cells was first evaluated by measuring the release of ATP into the extracellular surrounding. The cell–agarose constructs were subjected to a 40-min period of mechanical loading or an equivalent unloaded period, after which the PBS bathing solution was removed and boiled for 1 min to deactivate any ATPases.[15] The solutions were kept individually frozen at –20 °C prior to determination of the ATP concentration, using a commercially available luciferin–luciferase assay kit (Sigma–Aldrich, St. Louis, MO) in conjunction with a standard luminescence plate reader.[1,15] For calibration purposes, ATP standards were freshly prepared using an ATP stock solution (Sigma–Aldrich, St. Louis, MO) in PBS. Additional cell–agarose constructs were treated prior to and during mechanical loading with flufenamic acid (500 μM FFA) – a widely used hemichannel blocker.[23] The ATP assay readout was in RLU (relative light units) proportional to the amount of ATP in the solution. The RLU values were used to calculate the ATP (as the RLU/DNA ratio).

Activation of hemichannels

To examine the influence of mechanical loading on the activation of connexin hemichannels, cell–agarose constructs were mechanically loaded as described above, in the presence of the hemichannel indicator lucifer yellow (LY). For the LY incorporation assay, a solution of 0.4 % LY, w/v (CH dilithium salt, Sigma–Aldrich, Poole, UK), was prepared in phosphate-buffered saline (PBS) with 5 mM ethidium homodimer-1 (Sigma–Aldrich, St. Louis, MO) to label the nuclei of dead cells. Control constructs remained unstrained in the presence of an identical LY solution. Separate groups of constructs were additionally treated with FFA, both prior to and during a 40-min period of mechanical loading. Unloaded control constructs were treated in the same manner.

The concentrations and incubation periods for each reagent were based on those used in previous studies.[15] Immediately after loading, the constructs were washed in normal DMEM supplemented with 20 % fetal calf serum (FCS, Gibco, New York) containing 1.8 mM Ca^{2+} to close any activated hemichannels, and then fixed in 3.7 % formaldehyde (37 °C, 15 min). The cell–agarose constructs were cut into 1-mm thick slices, perpendicular to the cylindrical axis, and the central section of each construct was mounted on a cover-slip. The samples were imaged using a color CCD camera mounted onto an inverted microscope (Olympus IX-81) and analyzed using MetaMorph (Molecular Devices, Sunnyvale, CA). Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter blocks were used to visualize the lucifer yellow and ethidium homodimer-1, respectively. The number of LY-positive viable cells was determined in 10 adjacent fields of view that covered the whole diameter of the construct cross section. Each field of view contained approximately 70 individual cells. The number of TRITC-positive cells was deduced from the total number of fluorescent cells. The procedure was repeated on 3–5 separate constructs under each condition.

Biochemical composition

The tissue constructs were blotted dry, weighed and lyophilized overnight. Dry samples were digested with proteinase K overnight at 56 °C, as described previously.[24] For quantification of the DNA content, aliquots were analyzed using the PicoGreen assay (Invitrogen, Carlsbad, CA).

Immunochemistry

For immunochemical staining, tissue sections were first deparaffinized in Citrisolv (Fisher) and rehydrated in a series of descending concentrations of aqueous solutions of ethanol. Antigen retrieval was performed by heating in 0.01 M citrate buffer, pH 6.0, for 10 min. Slides were incubated for 5 min with 0.5 % Triton-X-100 in PBS for permeabilization and for 30 min at room temperature (RT) in 5% goat serum for blocking. Next, primary antibody (rabbit anti-connexin 43 antibody, C-terminus, cytosolic – AB1728 in 1:200 dilution from Chemicon – Millipore, Billerica, MA) was added overnight at 4 °C. The secondary goat anti-rabbit-FITC antibody (65-6111, Invitrogen) was applied to sections for 1 h at RT in the dark. Sections were mounted onto slides using Vectashield (Vector, Burlingame, CA) mounting medium. Construct samples without the applied primary antibody were used as a negative control. The samples were imaged using a color CCD camera mounted onto an inverted microscope (Olympus IX-81) and analyzed using MetaMorph (Molecular Devices, Downingtown, PA).

Statistical analysis

Statistics were performed with GraphPadPrism 5.01 software (http://graphpad-prism.software.informer.com/5.0/). Each data point represents the average±*SEM* of $n = 5$ samples for the chondrocyte-groups, and n of 3–5 samples for the hMSCs-groups. Each group was examined for significant differences by the one-way ANOVA, with the ATP/DNA ratio and number of LY-positive cells as the dependent variables using the Tukey honest significant difference test.

RESULTS

ATP release in response to dynamic loading. Bovine chondrocytes (bCHs)

Unconfined compressive loading (1 Hz, 10 % strain, 40 min) induced an approximately 5-fold increase of the normalized ATP/DNA ratio in comparison to both the unloaded control group and the group treated with the hemichannel blocker – FFA prior to loading (Fig. 2).

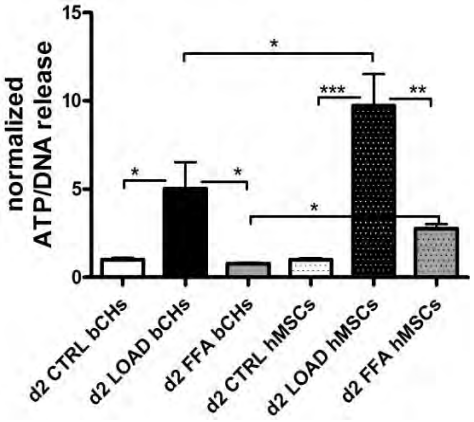

Fig. 2. ATP release in response to dynamic loading and connexin-blocking treatment. (d2: day two of culture; CTRL: unloaded; LOAD: loaded; bCHs: bovine chondrocytes; hMSCs: human mesenchymal stem cells; FFA: supplementation of flufenamic acid. Values indicate the mean ATP/DNA ratios normalized to the unloaded corresponding controls. ***$p < 0.001$, ** $p < 0.01$ and *$p < 0.05$.

Human MSCs (hMSCs). Notably, in the hMSC-ladened constructs, the ATP/DNA ratio was approximately 10-fold higher in the loaded group in comparison to unloaded control. FFA-treatment prior to loading induced a significant, 3-fold reduction of ATP release *vs*. the loaded group (Fig. 2). Moreover, the ATP/DNA ratio was 5-fold higher in the loaded group of hMSC-seeded constructs in comparison to the loaded bCHs constructs. In the FFA-treated hMSC-group, the normalized ATP/DNA ratio was also significantly higher than in the FFA-treated bCHs group (Fig. 2).

Hemichannel indicator (LY) uptake – bovine chondrocytes (bCHs). Fluorescence microscopy revealed a high number of LY-positive cells in the loaded group; about 3-fold higher than in the CTRL and FFA-treated groups (Fig. 3).

Human MSCs (hMSCs). The number of LY-positive cells was significantly higher in the loaded group than in both the CTRL and FFA groups (Fig. 3). The

loaded hMSC-constructs had about 2.5-fold lower number of LY-positive cells compared to the loaded bCH-constructs (Fig. 3).

Expression of connexin 43. Immunochemistry for connexin 43, the main hemichannel component, revealed positive reactive sites in both the bCHs- and hMSCs-constructs. The images in Fig. 4 show cross sections of paraffin-embedded bCHs- and hMSC-agarose constructs stained with primary anti-connexin 43 antibody and FITC-labeled secondary antibodies (green).

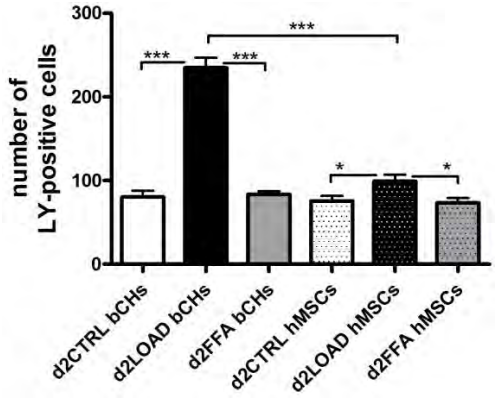

Fig. 3. Connexin indicator (LY) incurporation. Values indicate the average number of LY-positive cells in 10 fields-of-view that cover the whole diameter of the cross section. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$. Abbreviations as in Fig. 2.

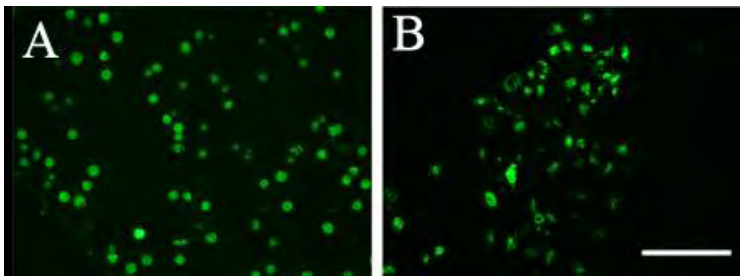

Fig. 4. Cross sections of paraffin-embedded bCHs- (A) and hMSC-agarose (B) constructs stained with primary anti-connexin 43 antibodies and FITC-labeled secondary antibodies (green).

DISCUSSION

The present study shows that chondrogenic cells, both primary chondrocytes and mesenchymal stem cells, respond to dynamic mechanical loading by purinergic signals that involve the activation of connexin hemichannels and additional mechanisms of ATP release. Under chondrogenic conditions, hMSCs released 5-fold higher amounts of ATP in response to dynamic loading than native bCHs. Dynamic loading triggered hemichannel-indicator (LY) uptake in both cell types, implying opening of the connexin hemichannels. However, the number of LY-positive cells in the hMSC-constructs was 2.5-fold lower com-

pared to the loaded bCH-constructs, suggesting that additional mechanisms of ATP release are utilized in the chondrogenic hMSCs.

Connexin-reactive sites were detected by immunochemistry in both the bCHs- and hMSCs-constructs. Hemichannel blocker (FFA) application led to reduced ATP release in both bCHs and hMSCs, which confirmed the involvement of connexin hemichannels, with more prominent effects in the bCHs than in the hMSCs, further implying additional mechanisms of ATP release in chondrogenic hMSCs.

Previous studies were mainly focused on undifferentiated bone-marrow derived hMSCs and detected expression of several subtypes of P2 receptors, which were activated by their natural ligand ATP, inducing fast changes in the intracellular ion homeostasis and modulating the molecular and functional properties of hMSCs.[25,26] Riddle *et al*. showed that exposure to fluid flow induced a flow rate--dependent release of ATP from undifferentiated hMSCs, and that ATP is unique, among nucleotides, in its ability to induce hMSC proliferation.[27] The same study reported a vesicular mechanism of ATP release. Hemichannels (connexin 43 and connexin 45) were also detected in MSCs,[28,29] which is consistent with the present results. In addition, shear stress modified the expression of connexin 43[30] in undifferentiated stromal hMSCs. However, there are no reports on the mechanisms of ATP secretion and expression of connexin 43 in chondrogenic MSCs. The study by Fodor *et al*. only reports that chondrifying micromass cultures of chicken mesenchymal cells secrete ATP as an autocrine factor,[31] but without identification of the ATP secretion mechanisms.

The present results indicate that the mechanism of purinergic response to dynamic loading of chondrogenic hMSCs is different to that reported for hMSCs subjected to fluid flow, and that this purinergic response involves activation of connexin hemichannels. Furthermore, chondrogenic hMSCs responded to dynamic loading more strongly than primary chondrocytes. The present study also implies that the hemichannel-mediated route is not the only mechanism of ATP release occurring in the chondrogenic hMSCs in response to dynamic loading. Further studies are necessary to identify other components of the purinergic signaling system in chondrogenic MSCs and to develop tissue-engineering strategies utilizing this important mechanism.

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ИЗВОД

ПУРИНЕРГИЧКИ ОДГОВОР ХОНДРОГЕНИХ МАТИЧНИХ ЋЕЛИЈА НА МЕХАНИЧКО ОПТЕРЕЋЕЊЕ

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У многим типовима ћелија, механичко оптерећење изазива секрецију аденозин-трифосфата (ATP) у екстрацелуларну средину, што даље може активирати сигнализацију путем калцијума преко везивања ATP-а за пуринске П2 рецепторе.[1] У овом раду смо поредили пуринергички одговор, тј. ATP секрецију под утицајем динамичког оптерећења код говеђих хондроцита (bCHs) и хуманих мезенхималних матичних ћелија (hMSCs) у агарозном хидрогелу, коришћењем теста за ATP секрецију у комбинацији са флуоресцентном бојом (луцифер жуто – LY) специфичном за конексине у оквиру хемиканала, као и са блокером конексинских хемиканала, флуфенаминском киселином (енгл. *flufenamic acid* – FFA). Резултати показују да хондрогене hMSCs ослобађају ATP у одговору на динамичко оптерећење. Хондрогене hMSCs су ослободиле значајно веће количине ATP-а (5× веће) у поређењу са bCHs после истог времена у култури (2 дана). Динамичко оптерећење је довело и до преузимања LY боје и код bCHs и код hMSCs, што наводи на закључак да је дошло до отварања конексинских хемиканала. Међутим, број LY-позитивних ћелија у hMSC култури је био 2,5× нижи у поређењу са bCH-културом. Тај резултат указује на то да код хондрогених hMSCs постоји и други механизам ATP секреције поред оног који се одвија путем конексинских хемиканала. Имунохемијска анализа је показала позитивну реакцију за конексин 43 и у bCHs и у hMSCs култури. Такође, примена блокера FFA је довела до смањене ATP секреције у bCHs и hMSCs, али је смањење било израженије у bCHs култури што потврђује да су у секреције укључени хемиканали, али и да у hMSCs то није једини механизам за ослобађање ATP-а.

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