Dynamic Mechanosignal Transduction in Chondrocytes

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INTRODUCTION

During weight bearing and joint loading on articular cartilage, the hydrostatic stress and distortional stress are integrated, interacting mechanobiological forces.[1] Pauwels hypothesized that characteristics such as compression (hydrostatic pressure: HP) and deformation (stretching) influence the formation of hyaline cartilage, fibrocartilage, and connective tissue derived from embryonic mesenchyme (**Figure 1**).[2] We reported that hydrostatic fluid pressure stimulated chondrogenesis in bovine articular chondrocytes *in vitro*.[3] Our data partially substantiate Pauwels' concept; therefore, our experiments are being extended to investigate other mechanical stresses, such as shear and tensile stress.

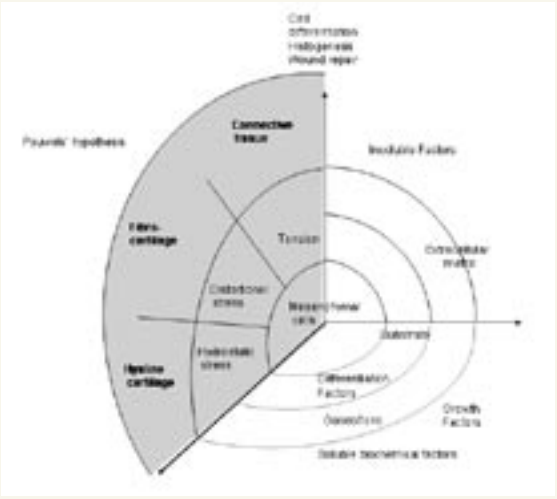

Figure 1. Conceptual cell differentiation due to mechanical stimuli. Based on Pouwels' concepts with other considerations added.

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Recently there has been much focus on the use of cell-based therapy to repair cartilage. Autologous expanded chondrocytes have been used clinically, but results have not always been optimal. If physical stimuli are useful in manipulating cellular phenotype, metabolic function and differentiation, then a greater understanding of the mechanism of mechanosignal transduction may provide opportunities to enhance cartilage repair.

Mechanosignal transduction examined in previous studies focused on cellular deformation but not pure HP in individual cells. Investigations have studied both direct mechanical stress induced by poking cells (involving deformation) with a glass needle[4,5] and indirect cell deformation induced by compressing cells either in agarose constructs[6] or in monolayer culture.[7,8] Effects on various concentrations of intracellular calcium ions ($[Ca^{2+}]i$), various signaling molecules and transport of other ions in a suspended mass of chondrocytes have been examined at extremely high HP (10 - 20 MPa).[9,10] Technically, it is difficult to distinguish between pure hydrostatic stress (pressure and tension) and pure distortional stress (shear stress) because compressive loading on a tissue or reconstituted tissue construct, e.g., agarose gel, involves gross tissue deformation.

Changes in $[Ca^{2+}]i$ or translocations from storage compartments are known to produce major effects on a cell's activity. Although deformation stimulates increase of $[Ca^{2+}]i$ *in vitro*, it is not clear that deformation is a major stimulus *in vivo*. It may appear contradictory that cartilage tissue is an incompressible material at physiological HP (~ 3 MPa).[11] Nevertheless, HP itself is transmitted both intracellularly and extracellularly. This is because the chondrocyte membrane is a selectively permeable partition separating extracellular and cytosolic fluid.

Isolated chondrocytes are used in current autologous repair methods. However, there may be an advantage to allowing the cells to accumulate some pericellular and extracellular matrix (ECM) to maintain phenotype prior to transplantation. In native articular cartilage, middle-zone (MZ) chondrocytes are surrounded by highly sulfated ECM and sandwiched between distinct surface (SZ) and deep zones. The MZ of native tissue contains more sulfated ECM than other zones. MZ cells *in vivo* are subjected to more HP than distortional stress during weight bearing and joint motion. These depth-dependent zones of tissue organization need to be understood because cell shape, matrix components, pathophysiology and stress distribution differ among the zones.[12,13] Zonal differences are very important to understanding the regeneration of engineered articular

cartilage, as regenerating tissue undergoes physical stress immediately upon transplantation.

To design procedures to enhance cartilage repair, we focused on the mechanism of dynamic mechanosignal transduction in chondrocytes due to pure HP. Our research used unique methodology to measure the effects of pure HP on dynamic $[Ca^{2+}]i$ in a single cell. We developed a pressure-proof optical chamber and apparatus to apply pure HP to individual cells and consequently allow for imaging of $[Ca^{2+}]i$. Our study tested the hypothesis that a change in $[Ca^{2+}]i$ due to HP varies with the depth of the tissue from which the cells were isolated.

METHODOLOGY DEVELOPED

The development of this new method was required to measure real-time changes in single cells in response to HP. Our methodology was recently presented at the Orthopedic Research Society meeting.[14] Other groups expressed an interest in performing similar experiments but encountered hardware limitations. Those other research groups seeded chondrocytes directly onto a glass window and used a lens with short working distance. That method was unsuccessful because the application of HP results in deformation of the glass windows, resulting in both deformation and HP effects.

Our results were obtained with a novel pressure-proof optical chamber with a 2 mm thick sapphire glass window resistant to pressures of up to 10 MPa. In this optical chamber, the cells were seeded onto a thin coverslip and suspended in a pressurized fluid. Therefore, the coverslip had no direct contact with the sapphire glass window. We could thereby avoid deformation due to application of HP and could measure fluorescent intensity with a Ca^{2+} indicator and a long working distance objective lens.

FINDINGS

Does HP stimulate an increase of [Ca2+] i in individual cells?

Application of 0.5 MPa HP for 5 min increased [Ca2+] i in articular chondrocytes (Figure 2).

Are there any differences in increase of [Ca2+]i between depth zones?

After 5 days' pre-culture there were morphological differences among cells and ECM isolated from different zones. MZ cells were polygonal and accumulated more ECM than SZ cells. The SZ cells acquired a more elongated shape. MZ- and SZ- derived cells showed a significant increase in $[Ca^{2+}]i$ due to the application of HP at 0.5 MPa for 5 min. Although both zones showed a significant increase in $[Ca^{2+}]i$, MZ-derived cells had a significantly greater response than SZ-derived cells. Thus, we formulated the working hypothesis that mechanosignal transduction of MZ-derived cells is stimulated predominantly by HP rather than by shear stress and that SZ-derived cells are stimulated predominantly by shear stress.

Where do the calcium ions come from?

To examine the source of the increased calcium, cells were cultured with a calcium channel inhibitor (gadolinium), a calcium-free buffer with EGTA and a calcium-storage inhibitor (dantrolene). Two possible sources of calcium were either cytosolic storage or extracellular space. Other mechanical stimuli e.g., deformation[5] and fluid flow,[15,16] seemed to result in significant calcium influx from the extracellular space. HP-stimulated increases in peak $[Ca^{2+}]i$ were inhibited in the presence of gadolinium or a calcium-free medium with EGTA. This means that influx of calcium ions from ECM were blocked. The application of HP did not increase peak $[Ca^{2+}]i$ when calcium ions from cytosolic storage were blocked with dantrolene. This means that the complete blocking of peak $[Ca^{2+}]i$ by either type of inhibition indicates interdependence of the membrane channel and a cytosolic storage mechanism involved with HP. Thus, we conclude that the calcium increase due to HP occurred as a result of the release of stored calcium in the cell and the subsequent calcium influx through membrane channels.

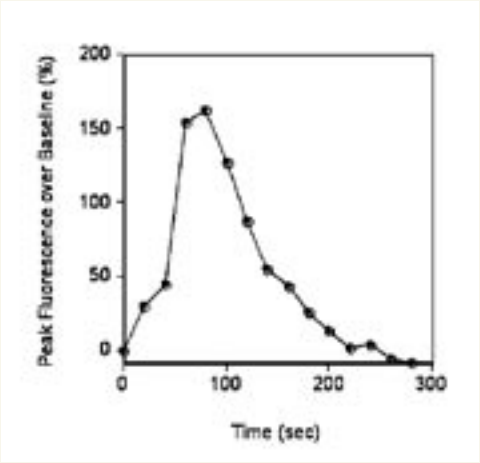

Figure 2.
Representative peak fluorescence of MZ-derived cells after constant HP at 0.5 MPa for 5 min. The cells were loaded with a calcium indicator, 5 μM X-rhod-1AM, for 40 to 60 min at room temperature. HP was applied with BSS for 5 min prior to image acquisition. Dynamic fluorescent imaging of $[Ca^{2+}]i$ at a single focal plane was acquired with a confocal microscope system at 800 nsec scan speed at 10-sec intervals for 5 min at 568 nm (excitation) and 590+ nm (emission).

FUTURE DIRECTIONS

Other pharmacological inhibitors are available to test for other signaling mechanisms. We plan to use verapamil to block cell membrane calcium channels and neomycin to block inositol-3-phosphate to further study the source of calcium in great detail. In addition, other signal pathways, such as extracellular signal regulated kinase (ERK), will be examined. Currently, protein kinase C (PKC)-green fluorescent protein vector is being utilized to visualize real-time localization of the signal molecule within a cell. These transiently transfected cells will allow illumination of transduction events as they happen *in vitro*. These methods are very useful because we can avoid artifacts such as extra-stimuli and injection of signal indicators with a needle prior to actual physical stimulation. It is our ongoing study to visualize dynamic relocation of those genetically labeled signal molecules in response to HP.

As an assumption, hydro-"static" pressure should not alter physical and physicochemical conditions (e.g., temperature, chemical concentration, molecule structure) at physiological magnitudes. However, something (e.g., chemical gradient, receptor affinity, membrane channel) has to be altered. Future studies will be directed towards discovering pressure-sensing molecules and new cascades. For this, we shall simulate *in vivo* conditions by testing *in vitro* effects of increasing, sustaining and decreasing HP. In addition, we found that zonal depth altered not only cell shape but also ECM components. For example, cells in the deep zone of tissue are surrounded with calcified ECM. On the other hand, chondrocytes at the SZ are surrounded with a greater amount of collagen type I. These ECM components are altered at pathophysiological conditions as well. We shall examine the effects of age and pathological conditions on responsiveness to HP.

How to apply the data from the bench to bedside?

Currently, the autologous chondrocyte procedure requires several steps: harvesting healthy chondrocytes from a non-weight bearing site (non-distinguished depth zones) in articular cartilage, expanding the number of cells *in vitro,* and injecting the cells into a pocket of damaged cartilage and periosteum. A fundamental question is whether the cells have the potential to expand yet maintain their chondrocytic phenotype or differentiate into chondrocytes including *de novo* regeneration process. If chondro-progenitor cells are identified non-invasively using physical stimuli, it will be very useful for isolating cells that are committed to becoming articular cartilage. Furthermore, we may be able to use physical stimuli to manipulate chondrogenesis and/or chondrocyte differentiation from stem cells. These developments may lead to further advances in cell-based treatment.

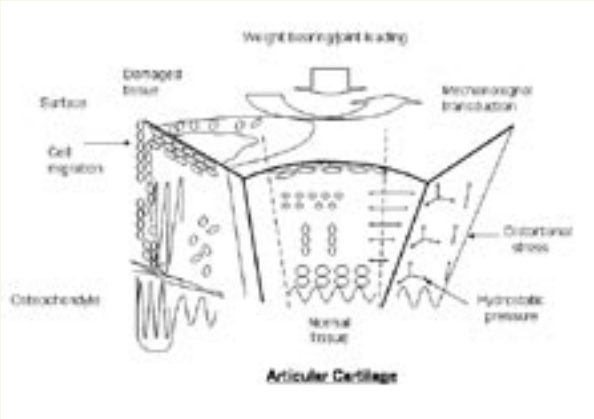

Figure 3. Clinical relevance of mechanical stimuli.

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