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Stepwise mechanical stretching inhibits chondrogenesis through cell–matrix adhesion mediated by integrins in embryonic rat limb-bud mesenchymal cells

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Abstract

Biomechanical forces are major epigenetic factors that determine the form and differentiation of skeletal tissues, and may be transduced through cell adhesion to the intracellular biochemical signaling pathway. To test the hypothesis that stepwise stretching is translated to molecular signals during early chondrogenesis, we developed a culture system to study the proliferation and differentiation of chondrocytes. Rat embryonic day-12 limb buds were microdissected and dissociated into cells, which were then micromass cultured on a silicone membrane and maintained for up to 7 days. Stepwise-increased stretching was applied to the silicone membrane, which exerted shearing stress on the cultures on day 4 after the initiation of chondrogenesis. Under stretched conditions, type II collagen expression was significantly inhibited by 44% on day 1 and by 67% on day 2, and this difference in type II collagen reached 80% after 3 days of culture. Accumulation of type II collagen protein and the size of the chondrogenic nodules had decreased by 50% on day 3. On the other hand, expression of the non-chondrogenic marker fibronectin was significantly up-regulated by 1.8-fold on day 3, while the up-regulation of type I collagen was minimal, even by day 3. The down-regulation in the expression of chondrogenic markers was completely recovered when cell–extracellular matrix attachment was inhibited by Gly–Arg–Gly–Asp–Ser–Pro–Lys peptide or by the application of blocking antibodies for $\alpha 2$, $\alpha 5$ or $\beta 1$ integrins. We conclude that shearing stress generated by stepwise stretching inhibits chondrogenesis

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EDTA, ethylene diamine tetra-acetic acid; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRGDSPK, glycine–arginine–glycine–aspartic acid–serine–proline–lysine; GRADSPK, glycine–arginine–alanine–aspartic acid–serine–proline–lysine. H&E, hematoxylin and eosin; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(ethanesulfonic acid); LIM, kinase (Lin-11/Isl-1/Mec-3)-domain-containing protein kinase; MAPK, mitogen-activated tyrosine kinase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PDVF, polyvinylidene difluoride; PMSF, phenylmethylsulfonylfluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; SD, standard deviation; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

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through integrins, and propose that signal transduction from biomechanical stimuli may be mediated by cell–extracellular matrix adhesion.

Keywords: Stretch; Chondrogenesis; Shear stress; Cell–extracellular matrix adhesion; Integrin

Introduction

The musculoskeletal system, which includes bone, cartilage, skeletal muscles and ligaments, responds to biomechanical stimulation with changes in metabolism, cytoskeletal organization, rate of proliferation, and state of differentiation during development. Chondrocytes also respond to mechanical forces by changing their metabolism (Millward-Sadler et al., 2000; Ragan et al., 2000; Moonsoo et al., 2001; Hunter et al., 2002; Quinn et al., 2002), their state of differentiation (Takahashi et al., 1996, 2003; Saitoh et al., 2000) and their proliferative activity (Hall., 1979; Wu et al., 2001). They respond differently to mechanical force, depending on the magnitude, frequency, and mode of mechanical stimulation. For example, heavy, continuous compressive force induces destructive changes in the mandibular condylar cartilage of the temporomandibular joint (Yamada et al., 2002), shearing stress exerted on the midpalatal suture cartilage inhibits chondrogenic differentiation (Saitoh et al., 2000), and, generally, cyclical mechanical compressive stimulation alters the metabolism of chondrocytes (Ragan et al., 2000; Grodzinsky et al., 2000). In particular, shearing stress has been considered to be one of the causes of pathological changes during the development of osteoarthritic diseases (van der Kraan et al. 1989; Beaupre et al., 2000; Ghosh and Smith, 2002). Interestingly, chondrocytes respond to biomechanical stimulation depending on the direction of the force exerted on them in vivo. In our previous studies (Takahashi et al., 1996; Saitoh et al., 2000), the differentiation of midpalatal suture cartilage cells was completely inhibited by expansive force but enhanced by compressive force when evaluated by type II collagen deposition. Therefore, we hypothesized that tensional stress could inhibit chondrogenic differentiation of mesenchymal cells and maintenance of the chondrogenic phenotype, which leads to destructive changes in cartilage tissue.

It is generally known that cell–extracellular matrix (ECM) adhesion through integrins is important for cellular motility, metabolism, and the proliferation of different kinds of mesenchymal cells. Differentiating chondrocytes express $\alpha5\beta1$ and $\alpha2\beta1$ integrins, and mature chondrocytes also express $\alpha5\beta1$, $\alpha2\beta1$, and $\alpha v\beta5$ integrins (Elices and Hemle, 1989; Shakibaei et al., 1995; Enomoto-Iwamoto et al., 1997; Loeser, 2000; Kurtis et al., 2003). Inhibition of integrin-mediated cell–ECM adhesion enhances the chondrogenic differentiation of embryonic mesenchymal cells in a pellet culture system (Yasuda et al., 1996). Generally, cell–ECM adhesion through integrins activates small GTPases and the mitogen-activated tyrosine kinase (MAPK) pathway and alters cytoskeletal structures. As seen in the mechano-response of fibroblasts, stress fiber formation accompanied by cytoskeletal restructuring is the predominant morphological change (Banes et al., 1995). In chondrocytes, the actin-based cytoskeleton is reorganized as stress fibers under tensile stimulation in vivo (Takahashi et al., 2003). Simultaneously, downstream signaling of integrins through these pathways could be a potent mechano-transduction pathway in chondrocytes (Grodzinsky et al., 2000). Indeed, different kinds of mechanical loading activate the extracellular-regulated kinase pathway (ERK-1/2) in chondrocytes, which is down-regulated with the progression of chondrogenesis (Yoon et al., 2002). Hung et al. (2000) indicated that fluid flow activated the ERK pathway, which is the dynamic component of loading on cartilage explants rather than free swelling (Li et al., 2003). Our previous study (Takahashi et al., 2003) indicated that an expansive force induced phosphorylation and nuclear translocation of ERK-1/2 in vivo. Therefore, cell-ECM adhesion could play a crucial role in mechano-transduction and the subsequent responses of chondrocytes to mechanical stimulation.

In the present study, we employed a modified micromass culture system to analyze the effect of stepwise stretching on chondrogenic cells (Solursh et al., 1982). Gene expression of chondrogenic markers (Silbermann et al., 1987; Ng et al., 1997; Sekiya et al., 2000), such as Sox9, type II collagen, and aggrecan, and protein deposition of type II collagen under shear stress were measured. Furthermore, to determine the role of cell–ECM adhesion via integrins in the mechano-response of chondrocytes, a blocking peptide or integrin subtype-specific blocking antibodies for integrin-mediated cell–ECM adhesion were applied to the culture system. We hypothesized that: (1) shear stress under stepwise stretching inhibits chondrogenesis in terms of gene expression of type II collagen, Sox9, and aggrecan, and also inhibits accumulation of type II collagen protein and glycosaminoglycans; and (2) the tension force is transferred by cell–ECM adhesion through integrins.