Effects of Cytoskeleton Modulation on the Phenotype of Articular Chondrocytes in Monolayer and Pellet Culture

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Previous studies have shown that chondrocytes in 2D culture are capable of keeping chondrocytic phenotype when formation of actin stress fiber is chemically restrained by cytochalasin; chondrocytes in 3D culture normally express better chondrogenic phenotype than in 2D, because formation of actin stress fiber is physically inhibited by cell aggregation instead of cell spreading. Besides actin, another cytoskeletal protein vimentin has also been shown to affect chondrocyte phenotype. These findings indicate that the chondrogenic expression of chondrocytes is associated with cytoskeleton organization. To systematically investigate the effects of cytoskeleton modulation on chondrocyte phenotype, here we cultured rabbit articular chondrocytes in 2D monolayer and 3D pellet, disrupted vimentin, tubulin, or actin network respectively, and measured the changes of chondrocyte phenotype. We found that vimentin disruption induced a reduction of type II collagen and aggrecan, and the extent was greater for chondrocytes in 3D than in 2D; tubulin disruption showed little effects on chondrocyte phenotype; actin disruption only promoted the expression of type II collagen for chondrocytes in 2D not in 3D. Our findings suggest a critical role of vimentin in the maintenance of chondrocyte phenotype, and further provide insights toward in vitro phenotype manipulation.

Keywords: Chondrocyte, Pellet Culture, Cytoskeleton, Type I Collagen, Type II Collagen, Aggrecan.

1. INTRODUCTION

Articular cartilage is avascular, aneural, and has a low cell density and metabolic activity. Therefore, damage to this tissue is unable to elicit an adequate healing response for even small defects, and surgical intervention is usually required. The cell based therapy utilizes implantation of mesenchymal stem cells (MSCs) or chondrocytes to the defect site to produce functional repair. However, both cell sources have limitations: MSCs require extensive in vitro manipulation to achieve the designated chondrogenic differentiation, and produce less matrix compared with chondrocytes under similar culture condition; chondrocytes are more frequently used than MSCs in clinical cartilage repair, but they undergo rapid dedifferentiation during ex vivo expansion, losing chondrocytic phenotype and producing inferior fibrocartilage. Therefore, the extent of cell phenotype maintenance largely determines the repair efficacy of the implanted chondrocytes.

It is found that chondrocytes progressively lose chondrogenic phenotype on traditional plastic culture dish. This is because 2D culture on hard surface promotes cell spreading and stress fiber formation, which in turn changes the chondrocyte phenotype into fibroblast-like. This effect is reversible, as disruption of actin stress fibers by cytochalasin restores the expression of chondrogenic marker genes, indicating an important role of actin cytoskeleton in the regulation of chondrocyte phenotype. To restrain actin stress fiber formation in chondrocyte expansion, some 3D culture methods have been developed as alternatives, including high-density micromass, pellet, and embedment of chondrocytes in compliant hydrogels. Besides actin, another major cytoskeleton component—vimentin intermediate filaments, has also been shown to affect chondrocyte phenotype. Disruption of vimentin in vitro results in reduction of type II collagen and aggrecan synthesis, and altered levels of vimentin are observed in situ in both human and rat osteoarthritic chondrocytes. All these observations indicate that modulation of cytoskeleton components is closely related to chondrocyte phenotype. However, owing to the different protocols used in previous...
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studies, it is difficult to determine the extent to which the modulation of each major cytoskeleton component (actin, vimentin, and tubulin) might influence the chondrocyte phenotype under the same cell culture condition.

In this study, we systematically analyzed the effects of cytoskeleton disruption on the chondrocyte phenotype in both 2D and 3D culture. High density monolayer culture and pellet culture of rabbit articular chondrocytes were selected as 2D and 3D culture model, respectively. Acrylamide, colchicines, and cytochalasin B were used to disrupt vimentin intermediate filaments, tubulin microtubules, and actin microfilaments, respectively. The expression of chondrogenic marker aggrekan and type II collagen, fibrotic marker type I collagen were measured by immunohistochemistry, immunofluorescence staining, and real-time PCR. Our results might provide new insights for the regulation of chondrocyte phenotype in cartilage tissue engineering and chondrocyte-based cartilage repair.

2. MATERIALS AND METHODS

2.1. Chondrocyte Isolation

Cartilage samples were obtained from the knees of 2-month-old New Zealand White rabbits. All experiments (Table I) were approved by the Animal Ethics Committee of Third Military Medical University. Cartilage specimens were removed from femoral condyles, washed three times with PBS supplemented with 1% (v/v) penicillin-streptomycin (P/S, Beyotime, China), and aseptically minced. Chondrocytes from the minced tissues were isolated by sequential digestion of the matrix in 0.25% pronase E (Sigma, USA) for 1 h and in 0.025% type II collagenase (Sigma, USA) for 9 h at 37 °C. The resulting cell suspension was filtered through a 40 μm cell strainer (BD Falcon, USA); collected cells were washed by centrifugation and resuspended in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, China) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, China) and 1% P/S. Chondrocytes were maintained at 37 °C and 5% CO2, and the growth medium was changed every 2 days until cells reached 90% confluence.

2.2. 2D Monolayer and 3D Pellet Culture of Chondrocytes

For high density 2D monolayer culture, chondrocytes at passage 1 (P1) were seeded onto a 24-well plate at the density of 1 × 10^5 cells in 1 mL chondrogenic medium per well; for 3D pellet culture, cell suspension containing 2.5 × 10^5 P1 chondrocytes were centrifuged at 1000 rpm for 5 min in 15-mL polypropylene conical tubes. Then, the supernatant was removed, and 2 mL chondrogenic medium was gently added into each tube without disturbing the cell sediment. For both 2D and 3D culture, the chondrogenic medium was high glucose DMEM supplemented with 2% FBS, 1% P/S, 10 ng/mL recombinant human transforming growth factor-β1 (TGF-β1; Peprotech, USA), 100 nM dexamethasone (Sigma, USA), 0.1 mM ascorbic acid 2-phosphate (Sigma, USA), and 1% Insulin-Transferrin-Selenium-A Supplement (1 g/L insulin, 0.55 g/L transferrin, 0.0067 g/L sodium selenite, and 11 g/L sodium pyruvate, Gibco, USA). Chondrocytes were maintained at 37 °C and 5% CO2, and the chondrogenic medium was replaced every 3 days.

2.3. Drug Intervention

From day 7 of chondrogenic culture, 10 μM cytochalasin B (Sigma, USA), or 4 mM acrylamide (Amresco, USA), or 2 μM colchicine (Sigma, USA) was added into culture medium every other 2 days to disrupt the actin microfilaments, or vimentin intermediate filaments, or tubulin microtubules of chondrocytes in experimental groups, respectively. The dose of these reagents was adopted from literature,16,19–21 and was confirmed by fluorescent staining of cytoskeleton. Briefly, in this preliminary test, 2D cultured chondrocytes were treated with the reagents for 3 days, then the cytoskeleton components were stained. For actin, Alexa 594 Phalloidin (Molecular Probes) was used; for vimentin and tubulin, mouse monoclonal anti-vimentin and anti-tubulin were used respectively as primary antibody (Abcam), and Alexa 488 donkey anti-mouse as used as secondary antibody (Molecular Probes). To quantify the fluorescent intensity, the contour of cells was drawn in ImageJ, then the intensity per unit area was automatically calculated. For final experiment, after 1 week of drug intervention, cells were fixed or collected for following measurements.

2.4. Histology, Immunohistochemistry, and Immunofluorescence Staining

One week after drug intervention, hematoxylin-eosin (H&E) staining for cell and matrix distribution, Alcian blue (SB, Sigma, USA), or 2% PAS, 5% picrosirius red (Sigma, USA), or 2% periodic acid Schiff (PAS, Sigma, USA), was used for histochemical detection. For immunohistochemistry, primary antibodies against type II collagen (Sigma, USA), or type I collagen (Novus, USA), or aggregan (Millipore, USA), or P2Y12 receptor (Millipore, USA), or COX-2 (Millipore, USA), or IL-1β (Millipore, USA), or IL-6 (Millipore, USA) were used; the secondary antibodies were Alexa 488 donkey anti-mouse (Molecular Probes) and Alexa 594 donkey anti-rabbit (Molecular Probes). For fluorescent staining of cytoskeleton, the reagents for 3 days, then the cytoskeleton components were stained. For actin, Alexa 594 Phalloidin (Molecular Probes) was used; for vimentin and tubulin, mouse monoclonal anti-vimentin and anti-tubulin were used respectively as primary antibody (Abcam), and Alexa 488 donkey anti-mouse as used as secondary antibody (Molecular Probes). To quantify the fluorescent intensity, the contour of cells was drawn in ImageJ, then the intensity per unit area was automatically calculated. For final experiment, after 1 week of drug intervention, cells were fixed or collected for following measurements.

Table I. Experiments involved in this study. Each single experiment was independent of one other.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Chondrocyte passage (P)</th>
<th>Culture model</th>
<th>Culture medium</th>
<th>Time (or time points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell expansion</td>
<td>Primary</td>
<td>2D</td>
<td>DMEM + 10% FBS</td>
<td>Till confluence</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>P1</td>
<td>2D, 3D</td>
<td>Chondrogenic medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Day 3, 7, 14</td>
</tr>
<tr>
<td>Cytoskeleton staining</td>
<td>P1</td>
<td>2D</td>
<td>Chondrogenic medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 Days treatment&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenotype measurements (IHC, IF, PCR, etc.)</td>
<td>P1</td>
<td>2D, 3D</td>
<td>Chondrogenic medium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7 Days culture + 7 Days treatment&lt;sup&gt;a&lt;/sup&gt;</td>
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Notes: 2D: indicates monolayer culture; 3D: indicates pellet culture; IHC: Immunohistochemistry; IF: Immunofluorescence; DMEM: Dulbecco’s modified Eagle’s medium; FBS: fetal bovine serum; <sup>a</sup>see Section 2.2 for ingredients of chondrogenic medium; <sup>b</sup>cytoskeleton modulation as described in Section 2.3.
blue staining for proteoglycan, immunohistochemistry and immunofluorescence for type I, II collagen were performed. For H&E staining, general protocol was used. For Alcian blue staining, samples were equilibrated in 3% glacial acetic acid for 30 min, stained with 0.1% Alcian blue dissolved in 3% glacial acetic acid (pH 2.5) for 30 min with constant agitation, and rinsed with 3% glacial acetic acid three times for 30 min each.

Prior to immunohistochemistry and immunofluorescence staining, chondrocyte monolayer was fixed in 4% paraformaldehyde for 30 min, and chondrocyte pellets were fixed in 4% paraformaldehyde for 3 h, serially dehydrated, paraffin embedded, and then sectioned at a thickness of 5 μm. For immunohistochemistry, samples were blocked with 5% bovine serum albumin (BSA; Sigma, USA) for 30 min, then incubated with polyclonal rabbit antibody targeting type I collagen (bs-0578R, Bioss, China) or type II collagen (bs-8859R, Bioss) overnight at 4 °C, followed by incubation with biotinylated goat anti-rabbit secondary immunoglobulin (Ig)G (bs-0295G-Bio, Bioss) for 30 min. The samples were then incubated with streptavidin-horseradish peroxidase (HRP) conjugate (BS-0437P-HRP, Bioss) for 30 min, and developed in DAB substrate reagents at our selected dosage, the three cytoskeleton components vimentin, tubulin, and actin were visualized respectively using immunofluorescence staining after 3 days of treatment in 2D culture model. The cells in modulation groups showed a slight disruption of the targeted cytoskeleton component with weaker staining intensity compared to control group (Fig. 2(a)). Quantification of fluorescent intensity by ImageJ (NIH) showed that the relative intensity of vimentin was 74 ± 13%, the relative intensity of tubulin was 65 ± 4%, and the relative intensity of actin was 78 ± 7%, respectively, all indicative of significant decrease after cytoskeleton modulation (Fig. 2(b)). Therefore, after 2 weeks of chondrogenic culture, the same dose of reagents was used for 1 week to modulate chondrocyte cytoskeleton in both 2D and 3D culture. For 2D monolayer culture, chondrocytes in all treatment groups maintained round shape as in control group; for 3D pellet culture, there was no apparent difference in macroscopic appearance of the pellet in all groups (Fig. 2(c)). In 2D culture, the cell number per field was counted by ImageJ, and results showed that there was no difference between groups except for the acrylamide-treated group (Fig. 2(d)). In 3D culture, the pellet size was measured, and no difference was observed between experiment groups and control group (Fig. 2(e)).

### 3. RESULTS

#### 3.1. Chondrocyte Morphology in 2D and 3D Culture

Three days after cell seeding, in 2D culture the chondrocytes adhered to the substrate and began to spread (Fig. 1(a)); while in 3D culture the chondrocyte pellet was clearly observed at the bottom of the 15 mL tube (Fig. 1(b)). At 1 week, the chondrocytes reached confluence in 2D culture, and the cells were round or polygonal, typical morphology of chondrocytes (Fig. 1(c)); while in 3D culture, the pellet was about 5 mm in diameter, and pink-red in color (Fig. 1(d)). At 2 weeks, the chondrocytes in 2D culture maintained typical morphology but were over confluent, and matrix deposition seemed apparent (Fig. 1(e)); in 3D culture, the pellet didn’t change much in size, and the color was more hyaline compared with that 1 week ago (Fig. 1(f)).

#### 3.2. General Observations After Cytoskeleton Modulation

To test the effectiveness of cytoskeleton modulation by reagents at our selected dosage, the three cytoskeleton components vimentin, tubulin, and actin were visualized respectively using immunofluorescence staining after 3 days of treatment in 2D culture model. The cells in modulation groups showed a slight disruption of the targeted cytoskeleton component with weaker staining intensity compared to control group (Fig. 2(a)). Quantification of fluorescent intensity by ImageJ (NIH) showed that the relative intensity of vimentin was 74 ± 13%, the relative intensity of tubulin was 65 ± 4%, and the relative intensity of actin was 78 ± 7%, respectively, all indicative of significant decrease after cytoskeleton modulation (Fig. 2(b)). Therefore, after 2 weeks of chondrogenic culture, the same dose of reagents was used for 1 week to modulate chondrocyte cytoskeleton in both 2D and 3D culture. For 2D monolayer culture, chondrocytes in all treatment groups maintained round shape as in control group; for 3D pellet culture, there was no apparent difference in macroscopic appearance of the pellet in all groups (Fig. 2(c)). In 2D culture, the cell number per field was counted by ImageJ, and results showed that there was no difference between groups except for the acrylamide-treated group (Fig. 2(d)). In 3D culture, the pellet size was measured, and no difference was observed between experiment groups and control group (Fig. 2(e)).
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Fig. 1. Morphology of chondrocytes in 2D and 3D culture. (a, c, e) Phase contrast images of chondrocytes after 3 days (a), 7 days (c), and 14 days (e) of 2D monolayer culture in chondrogenic medium. Insets: room-up of the cells in (a, c, e). (b, d, f) Macroscopic observations of chondrocyte pellets after 3 days (b), 7 days (d), and 14 days (f) of culture in chondrogenic medium. Arrow in b indicates the pellet (scale bars: 50 μm for a, c, e, and 5 mm for d, f).

3.3. H&E Staining and Alcian Blue Staining
To further test the effects of cytoskeleton modulation on chondrocyte phenotype, H&E staining and Alcian Blue staining were performed. In 2D monolayer culture, results of H&E staining showed that the experiment groups had similar cell shape and cell distribution to control group, except that both the cell size and the cell density were lower in acrylamide-treated group (Fig. 3(a)); Alcian blue assay indicated that the production of glycosaminoglycan (GAG) in cytochalasin B-treated group

Fig. 2. General observations of chondrocytes after cytoskeleton modulation. (a) Fluorescent images of vimentin, tubulin, and actin in chondrocytes after 3 days of acrylamide, colchicine, or cytochalasin B treatment, respectively (green: vimentin or tubulin, blue: nuclei, scale bars: 50 μm). (b) Quantified fluorescent level of vimentin, tubulin, and actin. Intensity of modulated protein was normalized to control (n = 3; *, p < 0.05). (c) Morphology of chondrocytes after 1 week of drug intervention. Upper panel: bright-field images of chondrocyte monolayer (scale bars: 50 μm). Lower panel: macroscopic images of chondrocyte micropellet (scale bars: 5 mm). (d) Cell numbers in chondrocyte monolayer after 7 days of acrylamide, colchicine, or cytochalasin-B treatment, respectively. Cells were counted by ImageJ, and cell numbers in each group were averaged from 5 bright-field images (n = 5; *, p < 0.05 vs. control). (e) Pellet size after 7 days of acrylamide, colchicines, or cytochalasin-B treatment, respectively (n = 5).

was close to control group, while cell-secreted GAG was lower in acrylamide-treated and colchicine-treated groups (Fig. 3(b)). In 3D pellet culture, H&E staining of sections showed similar pattern of colchicine-treated and cytochalasin B-treated groups to control group in cell distribution and matrix deposition, while fewer nuclei and less matrix were seen in acrylamide-treated group (Fig. 3(c)); Alcian blue staining revealed a decrease of GAG production in acrylamide treated group compared to other groups (Fig. 3(d)).

3.4. Immunohistochemistry and Immunofluorescence of Collagen Type I, II
To visualize the collagen matrix secreted by chondrocytes, immunohistochemistry and immunofluorescence of type I, II collagen were performed. In 2D monolayer culture, all groups showed little staining of type I collagen (Fig. 4(a)) but strong staining of type II collagen (Fig. 4(b)) in the matrix. The type II collagen staining in control group and cytochalasin B-treated group was more even than that in acrylamide-treated and colchicine-treated group. Immunohistochemical sections of chondrocyte pellet also showed bare Col-I staining (Fig. 4(c)) and abundant Col-II staining (Fig. 4(d)). Similarly, sections in acrylamide-treated group exhibited a sparser type II collagen network with weaker staining compared to other groups.

Fluorescent staining displayed a clear distribution of type I and type II collagen. In chondrocyte monolayer, only a few cells were observed with type I collagen staining (Fig. 5(a)), in contrast, most cells carried with type II collagen staining (Fig. 5(b)). For type I collagen, the cytochalasin B-treated group showed less staining than other groups; for type II collagen, there was no obvious difference between groups. However, there were gaps between the stained type II collagen, indicating the collagen matrix network was not formed. In sections of chondrocyte pellets, stained type I collagen was sporadically distributed (Fig. 5(c)), while stained type II collagen formed a dense network (Fig. 5(d)). For type I collagen, there was no apparent difference between groups; for type II collagen, the acrylamide-treated group showed sparser staining than other groups.

3.5. RT-PCR Results
Gene expression of aggrecan (ACAN), type I collagen (COL1A1), and type II collagen (COL2A1) was determined by quantitative PCR following normalization to the housekeeping gene GAPDH. The expression of COL1A1
was down-regulated (79 ± 10%) in cytochalasin B-treated chondrocyte monolayer (Fig. 6, p < 0.05); The expression of COL2A1 was down-regulated in acrylamide-treated chondrocyte monolayer (64 ± 9%) and pellet (40 ± 7%), and up-regulated in cytochalasin B-treated chondrocyte monolayer (Fig. 6, p < 0.05); The expression of ACAN was only down-regulated (75 ± 13%) in acrylamide-treated chondrocyte monolayer (Fig. 6, p < 0.05).

4. DISCUSSION
To date, major challenge still remains towards cartilage repair and regeneration, mainly because of the avascular, aneural nature of cartilage tissue. Chondrocytes, the only resident cells in articular cartilage, are responsible for signal transduction and maintenance of cartilage matrix.22 Consequently, chondrocyte dysfunction results in matrix loss and finally leads to cartilage degeneration. The cytoskeleton plays an important role in regulating the mechanical properties as well as the biological function of articular chondrocytes.23,24 The effects of cytoskeleton alteration on the mechanical properties of chondrocytes are clear, as it has been shown that disruption of actin microfilaments or vimentin intermediate filaments decreases the stiffness of chondrocytes in both 2D and 3D culture, while disruption of tubulin microtubules does not affect chondrocyte stiffness.19,20 However, the effects of cytoskeleton alteration on the biological function, especially matrix synthesis of chondrocytes remain unclear. Disruption of vimentin is shown to result in decreased expression of type II collagen and aggrecan;16 early studies demonstrated that disruption of actin or tubulin induces a decrease in the synthesis and secretion of both collagen and proteoglycan,25,26 while following studies showed that disruption of actin stress fibers promotes the production of type II collagen and aggrecan for chondrocytes undergoing dedifferentiation.11,27,28 In this study, we found that, vimentin disruption induced a reduction of type II collagen and aggrecan, and the extent of reduction was greater for chondrocytes in 3D culture than chondrocytes in 2D culture; tubulin disruption showed little effects on chondrocyte phenotype; actin disruption only suppressed the expression of type I collagen, promoted the expression of type II collagen for chondrocytes in 2D, but didn’t change the matrix production for chondrocytes in 3D.

In this study, both 2D monolayer and 3D pellet culture were used to test whether chondrocytes had different responses to cytoskeleton modulation under 2D and 3D culture. It has been shown that chondrocytes normally undergo rapid dedifferentiation in traditional 2D culture, moreover, passaging also progressively reduces chondrocytic phenotype.29 To overcome this problem,
Fig. 5. Images of type I (Col-I), type II (Col-II) collagen immunofluorescent (IF) staining after 7 days of acrylamide, colchicine, or cytochalasin-B treatment. (a) Col-I IF staining images of chondrocyte monolayer. (b) Col-II IF staining images of chondrocyte monolayer. (c) Col-I IF staining images of chondrocyte pellet sections. (d) Col-II IF staining images of chondrocyte pellet sections (scale bars: 50 μm).

For 2D monolayer culture here we expanded the primary chondrocytes and used exclusively P1 chondrocytes for experiments. The P1 chondrocytes were cultured in chondrogenic medium supplemented with 2% FBS. After 2 weeks, the cells maintained typical morphology of chondrocytes (Fig. 1(e)), and expressed abundant type II collagen but little type I collagen (Figs. 4(a, b); 5(a, b)), indicating dedifferentiation of chondrocyte monolayer was well controlled. For chondrocyte 3D culture, the pellet culture system we used provides a 3D environment similar to that found in precartilage condensation during embryonic development, which therefore helps the cells maintain the chondrogenic ability in vitro. Note that in this study 2% FBS was added into the standard chondrogenic medium with the purpose of compensating for the possible cell loss by drug-induced apoptosis.

To modulate vimentin, tubulin, and actin cytoskeleton, acrylamide, colchicine, and cytochalasin B were used respectively, all of which are typical reagents for disrupting the specified component without affecting other cytoskeletal organizations. The dose of these reagents was determined according to available literatures. After 7 days of treatment, the chondrocytes didn’t exhibit much difference in morphology (for pellet, appearance) (Fig. 2(c)) and cell numbers (for pellet, size) compared to untreated cells (Figs. 2(d, e)), except for the acrylamide-treated chondrocytes having a reduction in cell numbers. This observation was in accordance with a previous study, and is attributed to a pro-apoptotic response resulting from the absence of a functional vimentin network. The H&E staining of both monolayer and pellet sections further showed that the acrylamide-treated chondrocytes were more rounded, and appeared to lack of cell–cell junction compared to chondrocytes in other groups (Figs. 3(a, c)). It is worth noting that the Alcian blue staining of pellet sections was much stronger than that of cell monolayer (Figs. 3(b, d)), indicative of great advantage of GAG deposition in pellet culture.

To evaluate the extent of chondrocyte dedifferentiation, immunohistochemistry and immunofluorescence of type I collagen (a marker of fibrotic cartilage), type II collagen (a marker of hyaline cartilage) were performed. Interestingly, similar staining results were observed in all groups: the cells expressed predominantly type II collagen rather than type I collagen, indicating none of the reagents promoted the fibroblastic dedifferentiation of chondrocytes in the presence of chondrogenic medium. In addition, type II collagen network was observed in pellet sections (Figs. 4(d); 5(d)), even including the acrylamide-treated pellets, while the stained type II collagen in chondrocyte monolayer didn’t link with each other. This result of type II collagen staining together with Alcian blue staining proved the necessity of applying chondrocytes 3D culture in terms of cartilaginous matrix production. However, the difference between monolayers or between pellets was not
Fig. 6. Real-time quantitative RT-PCR analysis of COL1A1, COL2A1, and ACAN mRNA expressions in the monolayer and pellet after 7 days of acrylamide, colchicine, or cytochalasin-B treatment. COL1A1: type I collagen α-chain; COL2A1: type II collagen α-chain; ACAN: aggrecan. Relative expression levels of each gene were obtained by using the 2-ΔCt method (n = 3, *p < 0.05 compared to control).

obvious, which was possibly because the drug dose was not strong enough to reveal visible differences in the staining results.

Finally, RT-PCR was used to quantify the gene expression level of type I collagen, type II collagen, and aggrecan. For type I collagen, the different level of COL1A1 mRNA was only observed in cytochalasin B-treated chondrocyte monolayer, which might be associated with the disruption of stress fibers in 2D cultured chondrocytes. Since chondrocyte pellets had very few stress fibers, the cytochalasin treatment didn’t change much the COL1A1 expression. For type II collagen, the reduction of COL2A1 gene was shown in acrylamide-treated monolayer and pellets, with pellets having larger extent of decrease. This is possibly because the organization of the vimentin network in 3D cultured chondrocytes is more susceptible to acrylamide treatment than in 2D cultured chondrocytes. The collapse of vimentin and its induced changes in chondrocyte phenotype could be regulated by RhoA/ROCK signaling pathway.33,34 For aggrecan expression, there was no difference between drug treated groups and control group, except for the decrease of ACAN gene in acrylamide-treated monolayer. This indicates that chemical disruption of cytoskeleton has a relatively minor effect on aggrecan expression compared to collagen expression.

Interestingly, in this study, there was not much difference in both histochemistry and PCR results between tubulin-disrupted chondrocytes and nontreated chondrocytes. Previous studies show that disruption of tubulin by colchicine inhibits the synthesis of GAG,35,36 but little is known about the effects of tubulin disruption on type II collagen and aggrecan. Physiologically, tubulin microtubules do have essential roles in protein trafficking and secretion,37 as well as in withstanding cell-subjected compression.38 In this study, the chondrocytes were cultured in static environment, and maintained in chondrogenic medium with multiple supplements, thus the functions of tubulin might not be completely reflected here. For this reason, lacking of mechanical stimulation is thought to be the major limitation of this study, since it is unclear how such cells respond to cytoskeleton modulation in a physiologically relevant, loaded environment. Another limitation of this study is that only ECM markers were evaluated, other key markers such as SOX9 were not included. Nevertheless, our study for the first time provides data on the regulation of major ECM proteins in response to cytoskeleton disruption in 3D culture. Our following studies will be focused further on the interaction of chondrocyte cytoskeleton and ECM with the presence of mechanical stimulation, for instance, the role of chondrocyte integrins in the biological as well as the mechanical signal transduction from ECM to cytoskeleton network.

In summary, this study demonstrated the effects of cytoskeleton modulation on chondrocyte phenotype in vitro. In chondrogenic culture, disruption of vimentin significantly reduced the expression of type II collagen both in chondrocyte monolayer and pellet; disruption of tubulin almost didn’t affect chondrocyte phenotype either in 2D or in 3D culture; disruption of actin enhanced chondrocytic phenotype of 2D cultured chondrocytes by suppressing type I collagen while promoting type II collagen, but didn’t work for 3D cultured chondrocytes. Our findings not only indicate that an intact vimentin network plays a critical role in maintaining the phenotype of chondrocytes under static chondrogenic culture, but also suggest that cytoskeleton modulation could be an effective way to regulate chondrocyte phenotype.

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References and Notes


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