Cellular and molecular changes to chondrocytes in an in vitro model of developmental dysplasia of the hip—an experimental model of DDH with swaddling position

BO NING¹*, RUI JIN², LIN WAN³* and DAHUI WANG¹

¹Department of Pediatric Orthopedics, Children's Hospital of Fudan University, Shanghai 201102; ²Department of Pediatric Orthopedics, Children's Hospital of Anhui Medical University, Hefei, Anhui 230051; ³Department of Cardiothoracic Surgery, Shanghai Children's Hospital, Shanghai 230041, P.R. China

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Abstract. The aim of the present study was to assess the cellular and molecular changes to chondrocytes in a developmental dysplasia of the hip (DDH) model and to investigate the early metabolism of chondrocytes in DDH. Neonatal Wistar rats were used for the DDH model with swaddling position. Primary cultures of chondrocytes were prepared at serial interval stages (2, 4, 6 and 8 weeks) to investigate cellular proliferation. The expression of collagen II and aggrecan mRNA was detected to assess the anabolic ability of chondrocytes. The expression of matrix metallopeptidase (MMP) -13 and ADAM metallopeptidase with thrombospondin type 1 motif 5 (ADAMTS -5) mRNA was measured to investigate the degradation of collagen II and aggrecan, respectively. Morphological changes were observed in coronal dissection samples after the removal of fixation. Primary chondrocytes at serial intervals were assessed using a Cell Counting Kit-8 assay and the results revealed that DDH chondrocytes had more proliferative activity. The expression of collagen II mRNA was upregulated at 2 weeks and was more sensitive to mechanical loading compared with aggrecan. Similar changes occurred at 6 weeks. Furthermore, MMP-13 and ADAMTS-5 mRNA expression levels were upregulated at 2 weeks. It was also demonstrated that DDH chondrocytes exhibited high proliferative activity at the early stages and degeneration later.

Introduction

Developmental dysplasia of the hip (DDH) is one of the most common malformations affecting the lower extremities in children. If a stable and concentric reduction is maintained after close reduction, the acetabulum has the potential to remodel and resume normal growth and development. Furthermore, if younger patients are at the onset of treatment, this results in a greater potential for acetabular remodeling (1-5). The use of magnetic resonance imaging has resulted in enhanced consideration of the importance of acetabular cartilage in hip remodeling (6,7). Chondrocytes, one of the important components of cartilage, serve a critical role in maintaining the function and biological features of cartilage. However, it is difficult to obtain hip cartilage from DDH patients due to ethical constraints. Consequently, the function and pathophysiology of chondrocytes in the hips of patients with DDH cannot be investigated in vivo. In addition, it is not possible to obtain cultured human chondrocytes from patients with DDH. Due to these constraints, animal models are used to improve our understanding of chondrocytes in DDH in vivo. A rat model of unilateral DDH was established by Sijbrandij (8), and since then various experimental animals have been used to show that remodeling of the acetabulum is possible after the removal of fixation (9,10). However, previous studies mainly focused on the morphological and histological alterations of abnormal hips and the corresponding cartilage (11-13). Furthermore, since the swaddle position in infants is considered to be an important risk for the development of DDH, the model designed by the principle is thought to be an accurate model of cartilage in human DDH and may be suitable for further investigations (14). The authors previously developed a successful neonatal rat model of DDH corresponding to the swaddling position of the hip and early cartilage degeneration in DDH (10).

The aim of the present study was to assess the features of chondrocytes in DDH cartilage via primary cell culture in vitro. DDH models of neonatal Wistar rats were prepared in the present study and serial sections of hip cartilage were isolated and incubated primarily to investigate the cellular characteristics after the removal of fixation.
Materials and methods

Experimental animal models. All experimental protocols were approved by the Animal Ethical Committee of Fudan University (Shanghai, China). A total of 80 male specific pathogen free neonatal Wistar rats (5 g) were purchased from the Animal Research Institute of Medical College of Fudan University. Feeding environment was as follows: Temperature, 21-26°C; relative humidity 45-65%; ventilation for 8-12 times/h; 12-h light/dark cycle. The rats were fed with sterile pure water and adequate feed (HFK bio-technology, Beijing, China). Rats in the experimental DDH group (n=40) were immobilized, with the hip and knee fixed in an extended position with medical tapes for 10 days as described in our previous study (10,12). Following the removal of the fixation, rats were allowed to move freely in their cage for 2, 4, 6 or 8 weeks. Rats were sacrificed and the hips were isolated for macro-morphological examination and primary cell culture of the articular cartilage. Rats in the control group (n=40) were allowed to move freely throughout the study period.

Coronal histology and morphometry. Hips were isolated and fixed in 4% paraformaldehyde for 24 h in room temperature and decalcified with 10% EDTA, following which they were dissected through the longitudinal line from the ilium to the ischium of acetabulum. An abnormal association between the acetabulum and femoral head was observed in the experimental DDH group. The largest coronal that was selected from sections (5-µm) and acetabular index was measured. The acetabular depth ratio (ADR=depth/width x100%) was measured to assess changes in the acetabulum (Fig. 1A). The acetabular index (AI) was defined as the ratio of depth: Width measured using the reference line presented in Fig. 1A. The long line is identified as the width diameter of the longitudinal acetabulum from the upper edge to the distal border, excluding the rim of labrum. The short line is the perpendicular line to the width diameter of the acetabulum, and the curved line represents the acetabular shape following the removal of fixation.

Primary cell culture protocol. Primary cell culture was performed using the modified Manning method (15). Cartilage was obtained from the hips under sterile conditions, minced into 1 mm² pieces with scissors and digested using 0.25% Trypsin-EDTA and 2% collagenase II (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Tissues were passed through a 0.25 µm molecular filter and the cell suspension was centrifuged at 240 x g for 5 min in room temperature. The resulting pellet was resuspended in fresh Dulbecco’s modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 5 µg/ml penicillin and 5 µg/ml streptomycin. The final cell density was 5x10⁶ cells/ml and the suspension was incubated at 37°C in an atmosphere containing 5% CO₂ overnight, to allow primary chondrocytes to adhere. The medium was replaced with fresh DMEM every two days. Cells were then identified using collagen II immunofluorescence staining.

Chondrocyte identification using collagen II immunofluorescence. The medium was discarded and cells were rinsed three times with PBS for 5 min. Cell samples were fixed with 4% paraformaldehyde for 30 min at room temperature, following which, they were blocked in 0.2% Triton X-100 (PBST; Sigma-Aldrich; Merck KGaA) mixed with goat serum (Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Cells were immunoblotted and incubated overnight at 4°C with primary antibodies against collagen II (cat. no. ab34712; Abcam, Cambridge, UK; 1:100). The specimens were incubated for 1 h with secondary antibodies goat anti-rabbit IgG Alexa Fluor® 488 (cat. no.ab150077 Abcam; 1:200) at room temperature in the dark. Finally, specimens were stained with DAPI at room temperature and then viewed under an inverted fluorescence microscope (excitation wavelength 488 nm, magnification 200X) and images were captured. Collagen II staining at different time (2, 4, 6 and 8 weeks) was assessed and the number of cells was counted using Image Plus Pro 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Cell proliferation kinetics and growth curve. Primary cells were seeded in a 96-well plate at a density of 4,000 cells/200 µl and the number of chondrocytes was assessed using a Cell Counting Kit (CCK)-8 (DJD84000X; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) each day for 1 week. The optical density (OD) value of CCK-8 absorbance was measured at a wavelength of 450 nm using an ELISA reader and used to construct the growth curve.

Cell cycle analysis. Samples were centrifuged at 300 x g for 3 min at 4°C, then collected and rinsed with cold PBS. Cells were then fixed with 70% cold ethanol at 4°C overnight, then the cell were treated with Cell Cycle Detection kit (KGA512, Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cell cycle analysis was performed using flow cytometry equipment (FACS Aria II; BD Biosciences, Franklin Lakes, NJ, USA) and the data were collected using FlowJo analysis software (FlowJo-10.5.0; FlowJo LLC, Ashland, OR, USA). The number of cells in each phase of the cell cycle was recorded and the proportion of cells in S-phase was taken to be representative of proliferative activity.

mRNA expression levels of collagen II, aggrecan, matrix metallopeptidase (MMP)-13 and ADAM metallopeptidase with thrombospondin type I motif 5 (ADAMTS-5). Total RNA was extracted from the monolayer confluent chondrocytes using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and the purity and integrity were assayed using spectrophotometry and 10% agarose-gel electrophoresis respectively. The OD values of these mRNAs were determined between 1.8 and 2.0. A total of 1 µg RNA was transcribed to produce cDNA using a ReverTra Ace qPCR RT kit (Toyobo, Life Science, Osaka, Japan) according to the manufacturer’s protocol. The yield was quantified spectrophotometrically.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Denaturing 94°C, 60 sec; 40 cycles (Denaturing 94°C, 10 sec; annealing and extension 60°C, 30 sec) was performed using 5 µl cDNA (100 ng), 2 µl each primer (10 μM), 25 µl SYBR Green Real-time PCR Master Mix (Toyobo, Life Science) and 16 µl water to give a total volume of 50 µl. The RT-qPCR was programmed to an initial step of 10 min at 95°C for polymerase activity, followed by
40 cycles of 15 sec denaturation at 95°C, 15 sec annealing at 60°C, and 45 sec extension at 72°C. The expression levels of MMP-13, Collagen 2a1, ADAMTS-4 and ADAMTS-5 were normalized to β-actin. All primers used are listed in Table I. The results were quantified using the $2^{-\Delta\Delta C_q}$ method (16).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical significance was determined using one-way analysis of variance and paired t-tests. The bonferroni method was used for post hoc tests. Data were analyzed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Model and morphometry. Gross observations of the coronal dissection morphology of the hip are presented in Fig. 1A. A distinct DDH model was identified in the experimental DDH group, with dislocational hips observed (Fig. 1A). The acetabular index (AI) was significantly lower in the DDH rats compared with the control group at all time points (P<0.001; Fig. 1B). The results revealed increased proliferation in the experimental DDH group compared with the control group at all time points (P<0.001).

Cell proliferation was assessed by counting cells following staining with DAPI (Fig. 2B). The results revealed increased proliferation in the experimental DDH group compared with the control group at all time points (P<0.001).

Cell growth curve. At 2 weeks, the growth curve for the experimental DDH group began to enter the linear phase, the slope of which was greater than the control group, which indicated an increased trend of proliferation (Fig. 3A). However, no significant differences in the linear phase slope were observed between groups at 4 weeks. This suggested that proliferation in the experimental DDH group slowed over time (Fig. 3B). Furthermore, at week 6 the slope of the linear phase was reversed (Fig. 3C); this was maintained until 8 weeks with the experimental DDH group exhibiting a decreased slope (Fig. 3D).

Number of cells in S phase increases during DDH. Flow cytometry was performed to measure the proportion of cells in each phase of the cell cycle at different time points (Fig. 4). S-phase is when DNA synthesis occurs, and is therefore reflective of cell proliferation. At all time points, a greater number of cells in the experimental DDH group were in S-phase compared with the control group (P<0.001; Fig. 4A). Following the 2 week period, a gradual increase in the proportion of cells in S-phase was observed in the experimental DDH group over time (Fig. 4C). A reduced number of cells were observed in G1-phase in the experimental DDH group compared with the control, particularly at 2 and 8 weeks (Fig. 4B). The number of experimental DDH cells in G2-phase was also lower compared with the control group, particularly at 2 weeks (Fig. 4D). A
significant increase in experimental DDH cells in the S-phase was observed at 2 and 8 weeks (Fig. 4C).

mRNA expression levels of collagen II, aggrecan, MMP-13 and ADAMTS-5 are varied at different time-points in DDH. The mRNA expression levels were investigated using RT-qPCR (Fig. 5). Collagen II mRNA expression in the experimental DDH group was upregulated at 2 weeks after the removal of fixation, and downregulated at 4 weeks. However, a significant reversal was present at 6 weeks followed by a downregulation again at 8 weeks (Fig. 5A). The expression of MMP-13 mRNA was overexpressed in DDH cells compared with the control group at 2 weeks, decreased at 4 weeks and then this expression gradually increased during the following weeks (Fig. 5C). Aggrecan expression levels were significantly different between the experimental DDH and control groups at weeks 4 and 8; however, no significant differences were observed at weeks 2 and 6 (Fig. 5B). Conversely ADAMTS-5 mRNA in the DDH group was significantly different at weeks 2, 6 and 8 compared with the control (Fig. 5D).

Discussion

The DDH model of neonatal rats shows that the swaddling position is a mechanical risk factor that plays an important role in the pathogenesis of DDH. Previous studies of DDH models have revealed that the hip may be remodeled after the removal of fixation, which resembles close reduction treatment in human infants with DDH (17). Furthermore, Yamamoto (2) reported that shorter fixation duration gave better results. It has also been reported that the DDH is able to be completely reversed to prevent degeneration (18). Nevertheless, the results of the present study were not consistent with previous reports due to the failure to achieve close reduction; although remodeling of the macro-morphology was observed in the early stage, degeneration increased irreversibly with skeletal maturity (10,12). However, changes may be associated with cartilage content and resulting differences in mechanical features at different ages. In addition, the maintenance of subluxation resulted in no complete reduction, as the long duration of immobilization made the cartilage suffer from more abnormal weight-bearing. These kinds of changes leading to cartilage degeneration can be observed in clinical DDH X rays.

The histological and gross observation results revealed that remodeling occurred with proliferation at an early stage. Few studies focus on cellular proliferation of the chondrocytes, no matter whether or not the cells are loaded with abnormal mechanical forces. It is therefore important to investigate the proliferative ability at a cellular level (19). Chondrocytes in patients with DDH suffer from a variety of stresses, including shear, compression and tension loading, and so it is difficult to analyze the relationship between cell proliferation and mechanical loading. A number of studies have investigated the association between simple stress in vitro and cell proliferation and demonstrated that proliferation is associated with the type, intensity and mode of stress (20-25). In addition, a novel method for assessing articular cartilage chondrocytes in vivo has been described (26). However, the effects of different types of stress loading on the cartilage cannot be examined in vivo in a rat model, as the hip volume is too small. In the present study, cell proliferation was assessed using cell cycle analysis, and CCK-8 assays.

Cell cycle progression is the predominant means of regulating cell proliferation and differentiation, and so increasing our understanding of cell cycle progression in DDH chondrocytes may be beneficial. Changes in the number of cells in S-phase at 2 weeks may be due to an increase in proliferative ability following the transient removal of compress loading. The changes at weeks 4 and 6 may occur as a result of sustained complex loading due to mobilization, and accumulation of the proliferative cells may be a compensational reaction to maintain cartilage function. However, it is unclear why at 8 weeks cell numbers decreased while the proportion of cells in S-phase increased. Although an increase in S-phase cells indicated early proliferative activity, the proportion of cells in G0/G1-phase was significantly lower in the experimental DDH group compared with the control group, suggesting that proliferation and differentiation may be elevated in DDH chondrocytes. Nevertheless, the proliferation index regarding the S-phase accounted for the cell proliferative ability at the actual time points, while CCK-8 results indicated proliferative kinetics according to the slope of the growth curve. The results revealed that proliferation occurred faster soon after the removal of fixation in DDH chondrocytes.

The relationship between cartilage remodeling and cell proliferation has recently been reported by assessing the spatial reorganization of superficial chondrocytes in the early stages of osteoarthritis (27). Furthermore, a clear association has been reported between cell proliferation and ECM metabolism (28). Further investigation is required to discover further details regarding changes in ECM metabolism in DDH chondrocytes in response to changes in loading.
Collagen II and aggrecan provide the cartilage with tensile and compressive strength by forming a meshwork of collagen II in which the interstices are filled with aggrecans (29,30). MMPs and ADAMTSs secreted by chondrocytes are the two main groups of proteases in the ECM that mediate the degradation of collagen II and aggrecan. mRNA was extracted from primary cells and the expression of Col2a1 and aggrecan were assessed along with MMP-13 and ADAMTS-5 expression.

Homeostasis of the cellular environment is important for the function of the cartilage, maintaining a balance between the structural components and their proteolytic enzymes in response to dynamic loading (31). If chondrocyte metabolism is disrupted due to abnormal mechanical stresses and degradation of the ECM, the chondrocytes will initiate a compensational mechanism to counteract the inappropriate mechanical loading. Studies have revealed that cartilage regeneration and degeneration are dependent on the duration, quality and strength of abnormal loading (32,33). However, results have indicated that the metabolism of collagen in response to abnormal loading is different to that of aggrecan (20). As such, the upregulation of collagen II independent of aggrecan is considered to be a marker for early degeneration in DDH experiments as well as early osteoarthritis (11,34-36). In the present study, no significant differences in expression were observed between collagen II and aggrecan at 2 weeks. However, both were upregulated during the period after the transient removal of fixation (37). Collagen II rather than aggrecan demonstrated a difference between DDH and control group, while collagen II expression was downregulated at 8 weeks after modeling, which followed a gradual elevation until 6 weeks. Conversely, aggrecan mRNA expression was downregulated until 8 weeks post-modeling. The expression levels of MMP-13 and ADAMTS-5 were significantly affected by ECM synthesis and increased at 2 weeks, which was associated with the compression loading being released. Furthermore, following re-mobilization of the hip, the expression of both proteases was downregulated. MMP-13 and ADAMTS-5 have been reported to have an important effect in early degeneration during loading stress (38). Furthermore, ADAMTS-5 expression is higher than collagen II and aggrecan during the early stages of DDH and lower in the later stages, suggesting that ADAMTS-5 is more sensitive to changes in loading stress and serves a predominant role in hip remodeling in the early stages following load removal. If complete reduction is achieved, the expression of ADAMS-5 mRNA is reversible and cartilage degeneration may be prevented, as reported by Karsdal et al (39). A study by Breckon et al (40) involving a 14-year-old patient with DDH suggested that MMP-13 was not essential for the remodeling
Figure 3. Cell growth from 2 to 8 weeks. (A) At 2 weeks, the experimental DDH group entered the linear growth phase, the slope of which was greater than in the control group. This was indicative of increased proliferative ability. (B) No significant differences were observed between the slopes of the cell growth curve in each group at 4 weeks. (C) The slope of the linear phase reversed at week 6 and (D) these changes were maintained until 8 weeks, with a lower slope in the experimental DDH group. OD, optical density; DDH, developmental dysplasia of the hip.

Figure 4. Number of cells in S phase increases during DDH degeneration. (A) The proportion of cells in S-phase was assessed to indicate proliferative ability. The number of cells in S-phase increased gradually after the removal of the fixation from 4 weeks. Cell cycle distribution. The number of cells in (B) G1-phase was decreased in the experimental DDH group compared with the control group, especially at 2 and 8 weeks. (C) A significant increase was presented in the number of cells in the S phase in the experimental DDH group from 2 to 8. Furthermore, the number of cells in the (D) G2-phase was decreased in the experimental DDH group, compared with the control group, especially at 2 weeks. *P<0.05, **P<0.01 and ***P<0.001 vs. control. DDH, developmental dysplasia of the hip.
of cartilage growth and chondrocyte proliferation. Conversely, other studies have reported that MMP-13 serves an important role in cartilage development and ECM remodeling. Previous studies have revealed that MMP-13 and ADAMTS-5 expression levels are closely associated with stress activity (37,41,42), suggesting that several signal pathways could play complex roles in mediating chondrocyte metabolism in DDH cartilage. MMP-13 and ADAMTS-5 were upregulated during the early degeneration of cartilage and then downregulated sharply. This result suggests that DDH degeneration may be reversible during DDH degeneration.

It has previously been reported that collagen expression is associated with the longitudinal and transverse distribution and intensity of weight-bearing (43-47). A phenomenon known as dedifferentiation can occur during cell culture, and in the present study, early alternations in DDH cartilage at the cellular and molecular levels in vivo and in vitro resulted in no complete reduction and subluxation in consequence. This does not influence our results; although alternative findings in different tests were observed, the expression of target molecules was unaffected. Changes at the molecular level, which are the initial promoters of progressive degeneration, were not observed. Therefore, in future studies it will be interesting to focus on it whether the early degeneration occurs after the operations of DDH at molecular level and what time will be appropriate for the operation so that the degeneration could be prevented completely.

In conclusion, although complete reduction was not achieved after the removal of fixation, transient remodeling of the hip occurred over time, which was indicative of high proliferative activity in the chondrocytes as well as cell cycle progression at the early stage. However, degeneration occurred at the later stage. These results suggested that MMP-13 and ADAMTS-5 serve a dominant role not only in the remodeling phase but also in the degeneration stage.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.
Authors' contributions

DW designed the experiments and revised the paper. BN, RJ and LW performed the experiments and wrote the paper. BN and LW analyzed the data. BN and DW read and revised the paper.

Ethics approval and consent to participate

All methods in this study were approved by the Research Medical Ethics Committee of Fudan University. All experimental protocols were performed in accordance with the Institutional Ethics Committee of the Animal Ethical Committee of Fudan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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