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Original Article

Articular chondrocyte alignment in the rat after surgically induced osteoarthritis

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Abstract. [Purpose] Chondrocytes in articular cartilage are aligned as columns from the joint surface. Notably, loss of chondrocyte and abnormalities of differentiation factors give rise to osteoarthritis (OA). However, the relationship between chondrocyte alignment and OA progression remains unclear. This study was performed to investigate temporal alterations in surgically-induced OA rats. [Subjects and Methods] Thirteen-week-old Wistar rats (n=30) underwent destabilized medial meniscus surgery in their right knee and sham surgery in their left knee. Specimens (n=5) were collected at 0, 1, 2, 4 and 8 weeks after surgery. Histological analysis with Osteoarthritis Research Society International (OARSI) scores, cell density ratios, cell alignments and correlation between OARSI scores and cell density/alignment was performed. [Results] OARSI scores were significantly higher at 1, 2, 4 and 8 weeks in the DMM group than in the control. Cell density ratios were decreased significantly in the DMM group at 2, 4 and 8 weeks compared with the control. Chondrocyte alignment was decreased significantly in the DMM group at 4 and 8 weeks. There were negative correlations between OA severity and cell density / cell alignment. [Conclusion] The results suggest a relationship between chondrocyte alignment and cartilage homeostasis, which plays an important role in OA progression.

Key words: Chondrocyte alignment, Osteoarthritis knee, Spatial autocorrelation

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INTRODUCTION

Osteoarthritis (OA) is the most prevalent disease of arthritis which induces the joint pain and limits activity[1)]. It is multifactorial disease (e.g., genetic, aging, obesity and mechanical stimuli)[2–6)]. These factors contribute to OA progression by either direct or indirect regulation the anabolic and catabolic pathways of the cartilage extracellular matrix (ECM)[7–9)]. However, a fundamental treatment strategy has not been established to markedly alter OA progression because of our incomplete understanding of chondrocyte behavior.

To maintain the ECM in articular cartilage, the chondrocyte number is associated with anabolic and catabolic activities. An inappropriate activity balance leads to ECM degeneration and chondrocyte loss accompanied by apoptotic cell death during OA. Several previous studies show activation of the apoptosis cascade during OA[10–15)]. Consequently, cell numbers decrease with OA progression[16)].

Understanding chondrocytes localization will help characterize cartilage homeostasis. Chondrocytes are aligned as regularly formed columns that are perpendicular to the joint surface and organized into four zonal layers: superficial zone (SZ), middle zone (MZ), deep zone (DZ) and calcified zone (CZ)[17–19)]. The SZ includes numerous elongated and flattened

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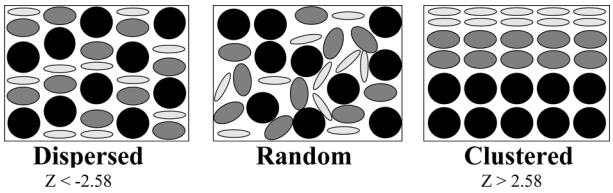

Fig. 1. Schematic diagram of spatial autocorrelation
Higher positive Z-scores indicate similar clustered attributes (cell size). The more cell alignment becomes random, the closer the Z-score is to zero.

chondrocytes. The MZ contains round chondrocytes called proliferative chondrocytes. The DZ and CZ contain hypertrophic chondrocytes. These zones are distinguished by a tide mark. Each chondrocyte grows in size with differentiation. Therefore, the zone-specific turnover relies on the differentiation phenotype and its localization.

Chondrocytes localization determines their differentiation that is influenced by multiple factors[9]. For instance, parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (IHH) signaling play pivotal roles in not only differentiation but also controlling chondrocyte localization[20, 21]. IHH released from hypertrophic chondrocytes in the DZ drives differentiation in the same region of cells and facilitates PTHrP production by proliferative chondrocytes in the MZ. PTHrP acts to both facilitate proliferative chondrocyte division in the MZ and inhibit the IHH production in hypertrophic chondrocytes. This negative feedback loop plays an important role in each zonal homeostasis and their arrangement. Lin and colleagues revealed that high levels of IHH are associated with OA pathogenesis in humans and animal model[22].

It remains unclear whether OA progression is related to chondrocyte alignment despite the possibility of losing differentiation control triggered by cell death. Studies have shown that chondrocytes are not aligned as columns in moderate OA according to qualitative observations[23, 24]. We have used a geographic information system (GIS) for quantitative assessment to determine the distribution of chondrocytes, which has been difficult to evaluate by common qualitative assessment. This useful system, which has been developed to analyze various aspects of the causal relationship between the position and space, can be applied to quantitative assessment. Furthermore, this geographic methodology has been widely used in several epidemiology studies[25–27]. In this study, we analyzed the relationship between OA progression and chondrocyte alignment using the GIS. Specifically, the GIS enabled evaluation of a cell alignment pattern whether similar cell sizes were clustered, dispersed or random (Fig. 1). To clarify the relationship, we examined temporal changes in the chondrocyte location as an important factor to maintain the articular cartilage in rats with surgically induced OA.

SUBJECTS AND METHODS

The study was approved by the Animal Committee of Niigata University of Health and Welfare. Thirty 10 weeks male Wistar rats (369 ± 21 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and randomly housed at two animals per standard cage at 25 ± 1 °C, 55 ± 5% humidity, and a 12:12 h light:dark cycle. The animals had free access to a standard diet and tap water.

At 13 weeks-old, the animals were anesthetized with sodium pentobarbital (50 mg/kg). The medial meniscus ligament was resected to destabilize the medial meniscus on the right knee joint as described previously for the destabilized medial meniscus (DMM) model[28]. A similar procedure was performed on left knee for the sham group but without dissecting the medial meniscus ligament. Each animal was maintained under the same conditions at two per cage.

The animals were sacrificed at day 0 for the control (n=6) and at 1, 2, 4, or 8 weeks (n=6 each) after surgery under anesthesia. Tissue was fixed by 30 min perfusion with a fixation solution (4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.35) via the abdominal aorta. Both knee joint were harvested from the hind limbs and placed in the fixation solution at 4 °C for 24 h. Each specimen was decalcified in 0.1 mol/l Ethylenediaminetetraacetic acid for 6wks at 4 °C and then bisected frontally. Specimens were gradually dehydrated with 70, 80, 90, 95 and 100% ethanol for 24 hrs at 4 °C. Subsequently, the samples embedded in paraffin and then dewaxed in xylene. Frontal 5 μm serial sections were prepared from the middle part of knee joint. The sections were stained with Safranin-O-fast green and hematoxylin and eosin (H-E). All histological images included medial tibial plateaus (MTPs) as observed by light microscopy (DM1000LED; Leica, Germany), and captured by a digital CCD camera (DFC425; Leica, Germany). Safranin-O-fast green images were used to evaluate OA severity. The evaluation was performed according to the Osteoarthritis Research Society International (OARSI) scoring system within grade and stage[29, 30]. The scoring was performed by a single trained observer (HT) in a blinded fashion.

H-E images stained images were used to measure the chondrocyte density and alignment. The MTP is divided into four weight-bearing areas, and the region of interest was the third area from the anterior cruciate ligament insertion. Chondrocytes and total area were traced manually at ×400 magnification using ImageJ (NIH, USA). Chondrocyte densities were calculated

as the number of cells divided by total articular cartilage area. To assess chondrocyte alignment, each chondrocyte coordinate from the center of mass and its size were measured using ImageJ. Thereafter, based on collected data of cell coordinates and sizes, the spatial autocorrelation were calculated using GIS software (Arc GIS 10.2.1; ESRI, CA, USA). We examined the spatial autocorrelation interaction between the cell location and associated attribute (cell size). Spatial autocorrelation has been proposed as a method to scale the distribution pattern. The data are shown as Z-score (Fig. 1). The more cell alignment becomes random, the closer the Z-score is to zero. Furthermore, the lager positive scores indicate a clustered cell alignment. Conversely, lager negative scores indicate a dispersed cell alignment. To determine the Z-score, we first calculated the Moran's I by the following formula:

$$I = \frac{n\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}(x_i - \bar{x})(x_j - \bar{x})}{\sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}\sum_{i=1}^{n}(xi - \bar{x})^2} \quad (1)$$

Subsequently, a standardized normal variable was calculated as the Z-score using the following formula:

$$z = \frac{I - E[I]}{\sqrt{V}[I]} \quad (2)$$

E[I] and V[I] was calculated as follows:

$$E[I] = -1/(n-1) \quad (3)$$

$$V[I] = E[I^2] - E[I]^2 \quad (4)$$

OARSI score are expressed as the median and iterquartile ranges. Cell density data of the control as the standard were converted to cell density ratios. Z-scores are expressed as mean ± standard deviation (SD). Data were analyzed by Kruskal-Wallis test followed by the post-hoc Steel test. Differences between groups were evaluated using the post-hoc Steel test. Differences were considered significant at $p<0.05$. Correlations between OARSI scores and chondrocyte density, and OARSI scores and Z-scores were examined by Spearman's partial rank correlation coefficient. $P<0.01$ was considered as a significant correlation. All statistical analyses were carried out using JMP® 12.2 (SAS Institute., Cary, NC, USA).

RESULTS

Figure 2 shows representative images of Safranin-O-fast green staining of MTPs at 0, 1, 2, 4 and 8 weeks after DMM surgery. Surface irregularity, and less safranin-O staining and cellularity from the articular cartilage surface were observed with longer morbidity. There was less staining and dissipation of surface chondrocytes from 2 weeks in the DMM group (Fig. 2I, L, O). In particular, at 4 and 8 weeks in the DMM group, less staining of more than three fourths and unequivocal dissipation of chondrocytes were observed at the lesion area (Fig. 2L, O). However, there was chondrocyte clustering in the proliferating zone only at 1 week in the DMM group (Fig. 2F). There were osteophytes at 4 and 8 weeks after the surgery (Fig. 2 J, M). Additionally, a subchondral cyst was confirmed immediately under the lesion at 4 weeks after surgery (Fig. 2 K). In contrast, control and sham groups showed no substantial differences.

Table 1 shows the alterations of OARSI scores, cell density ratios and Z-scores. OARSI scores were an exponential increase over time from day 0 to 4 weeks after DMM surgery. Thereafter, there was a slight increase from 4 to 8 weeks in the DMM group. OARSI scores were significantly higher at 1, 2, 4 and 8 weeks in the DMM group than in the control. In contrast, there were no significant differences between control and sham groups at any time point. The alteration of cell density ratios of MTPs using H-E stain were a slight increase at 1 week in the DMM group compared with the control but without significance. At 2 weeks in the DMM group, the cell density decreased suddenly and sharply at 1 week in the DMM group. Except at 1 week in the DMM group, comparisons between the control and DMM groups at 2, 4 and 8 weeks showed significant differences. To investigate the cell alignment, we applied H-E stained images to quantitative analysis using the spatial autocorrelation of GIS technology. Relative to the control, there was a significant decrease from 2 weeks to 8 weeks in the DMM group, respectively. In contrast, no significant differences were observed a between sham and control groups.

The relationship between OARSI scores and chondrocyte density ratio in control, sham and DMM groups was evaluated using the Z-score corrected Spearman's partial rank correlation coefficient that takes into account the third variable. Assessment of the relationship between OARSI scores and Z-scores was performed using the cell density ratio corrected to Spearman's partial rank correlation coefficient. It showed a poor negative correlation between the OARSI score and chondrocyte density ratio ($r=-0.41$) (Fig. 3A). Similarly, there was a negative correlation between the OARSI score and Z-score ($r=-0.62$) (Fig. 3B).

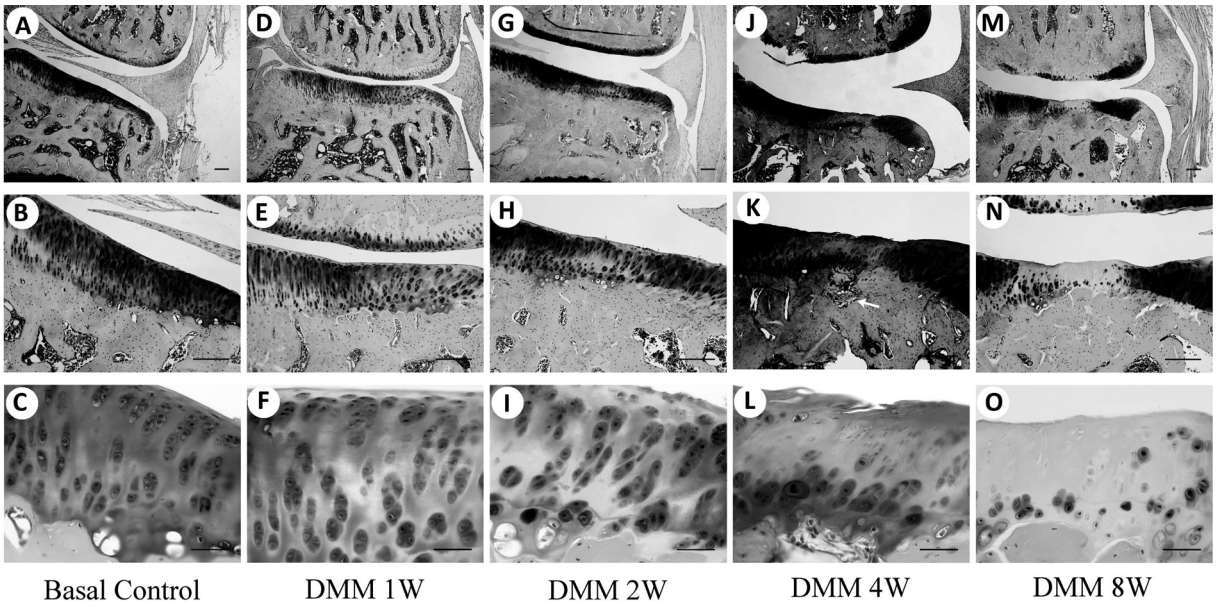

Fig. 2. Time course of histological section (medial tibial plateaus) in the control (A–C) and at 1 week (D–F), 2 weeks (G–I), 4 weeks (J–L), and 8 weeks (M–O) after DMM surgery: first row (×40, scale bars=200 μm); second row (×100, scale bars=200 μm); third row (×400, scale bars=50μm)

A representative safranin-O-stained section shows decreases in staining, the number of cells, and cell regularity from 2 weeks after surgery. White arrow shows subchondral bone cyst at 4 weeks after surgery.

Table 1. Temporal changes of OARSI scores, cell density ratio and Z-scores in medial tibial plateau

	0w	1w	2w	4w	8w
OARSI Score					
Control	0 (0–0)				
Sham		0 (0–0)	0 (0–0)	0.25 (0–0.5)	0 (0–0.75)
DMM		0.75 (0.5–1.38)*	3.31 (0.94–6.06)*	10.5 (9.38–11.1)**	13.25 (9.75–15.63)**
Cell Density Ratio (/10,000 μm^2)					
Control	100%				
Sham		114.4 ± 34.3%	117.2 ± 35.1%	110.2 ± 45.6%	109.2 ± 24.7%
DMM		129.8 ± 39.0%	61.0 ± 11.6%*	60.9 ± 16.9%*	43.4 ± 23.7%*
Z-Score					
Control	6.27 ± 1.04				
Sham		7.17 ± 1.04	6.93 ± 1.58	6.70 ± 2.90	6.86 ± 1.88
DMM		6.34 ± 2.80	3.32 ± 3.13*	2.89 ± 1.44*	3.16 ± 2.81*

OARSI scores are expressed as the median and iterquartile ranges. Cell density ratios are expressed as mean ± standard deviation compared with the control set at 100%. Z-scores are expressed as mean ± standard deviation. *p<0.05, **p<0.01 vs. control

DISCUSSION

This study demonstrated that the organization of chondrocytes deteriorates with OA progression in DMM rats. Chondrocyte disorder began at relatively early stages of OA. Similarly, the cell density decreased dramatically with OA progression. Interestingly, all timings of dramatic alterations, disordered the cell alignment, hypocellularity, and OA progression, were similar. While OA progression occurred over time, the chondrocyte alignment and cellularity were invariant from 4 to 8 weeks after surgery. In addition, chondrocyte alignment was highly associated with the severity of OA compared with cell density. Hence, these results support spatial autocorrelation for articular cartilage evaluation and the OARSI scoring system. Moreover, the chondrocyte alignment was principal factor to maintain the ECM in articular cartilage.

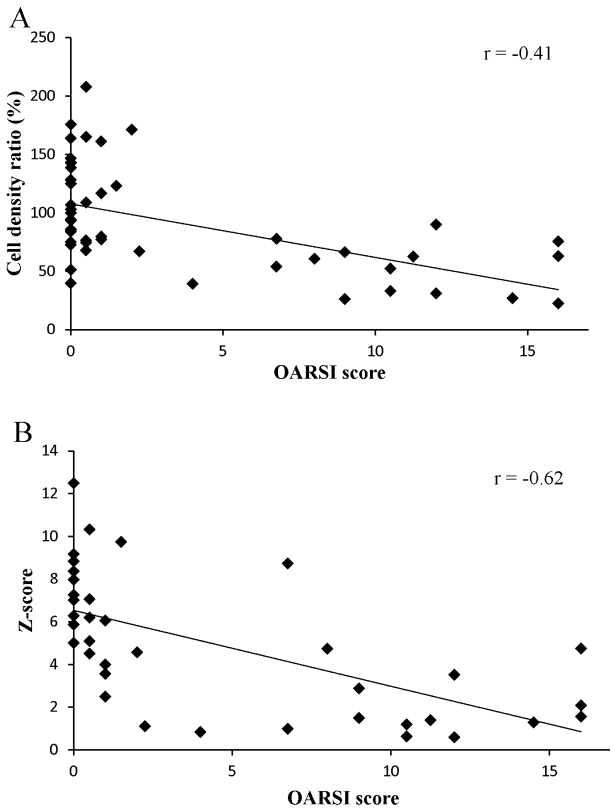

Fig. 3. Relationship between chondrocyte density, alignment and OARSI scores
(A) Adjusted correlation between the cell density ratio and OARSI score. OARSI score showed a poor negative correlation with the cell density ratio (r=−0.41). (B) Adjusted correlation between Z-scores and OARSI scores. Cell alignment decreased linearly with increasing OA severity (r=−0.62).

Accumulation of overloading at MTPs induces upregulation of inflammatory mediator such as interleukin (IL)-1β and IL-6 in knee joint immediately after DMM surgery[31]. Subsequently, apoptotic cell death occurs at the SZ and MZ[13, 15]. Studies have shown that OA severity is associated with apoptosis, cartilage thickness and ECM contents[13, 16, 32–34]. Our results also showed less chondrocytes at the surface lesion such as the SZ and MZ in early stages of OA. Considering these results, this phenomenon appears to be due to apoptosis. Nevertheless, our data showed no chondrocyte loss at 1 week after surgery while the degeneration progressed. There was a time difference between the degree of degeneration in articular cartilage and chondrocyte viability. Almonte-Becerril et al. revealed that both apoptosis and autophagy are co-localized with chondrocytes in the SZ and MZ during the early stages of OA[35]. Similarly, we also confirmed that mammalian target of rapamycin (m-TOR), which is upstream of autophagy inhibition, was not expressed in early stages of OA, whereas m-TOR was expressed in hypertrophic chondrocytes at the late stages of OA (unpublished data). Indeed, a time lag existed between ECM degeneration, such as the appearance of fibrillation, and chondrocyte viability in the early stages of OA.

Chondrocyte disorder was also highly related to OA progression. Chondrocyte differentiation is regulated by multiple signaling pathways[9, 36]. One of the pathways controlling chondrocyte differentiations is the negative feedback loop of PTHrP and IHH signaling[37]. It is certain that healthy chondrocytes of articular cartilage are arranged as columns owing to an orderly differentiation program. Studies suggest that aggravating OA may be caused by hypertrophic differentiation because of upregulating hypertrophic makers such as runt-related transcription factor 2 (RUNX2), collagen type X (Col X), matrix metalloproteinase 13 (MMP13) and alkaline phosphatase (ALP)[38]. It has also been reported that IHH signaling proteins are highly upregulated in both OA patients and surgically induced OA model animals, which is accompanied by upregulation of a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5), RUNX2, Col X and MMP13[22]. It appears that chondrocyte disorder in OA results from loss of cellularity in the SZ and MZ where it plays a role in inhibition of hypertrophic differentiation. Furthermore, the cause of chondrocyte disorder is considered to involve in gap junctions. Articular chondrocytes in the SZ communicate interactively though the gap junction protein connexin 43 for differentiation and metabolic homeostasis of the ECM[31, 39, 40]. The dynamic aggravation of OA coincides with chondrocyte disordering. Thus, loss of cellular location is presumed to be a driving factor in OA development. It also implies that ensuring crosstalk by maintaining each cell location is important to prevent OA development.

The initial major alteration occurred at 2 weeks after surgery. The decrease of cell density and alignment was −38.9% and −47% at 2 weeks after surgery compare with the control. Several studies have concluded that apoptosis is secondary to degeneration of articular cartilage in early stages of OA[12, 13, 41]. Other studies have shown that treatment of OA model animals with rapamycin, an inhibitor of m-TOR and activator of autophagy, reduces OA pathogenesis[42, 43]. In addition, hypertrophic chondrocytes activate degeneration of ECM and endochondral ossification[44]. Taken together, chondrocyte apoptosis in the SZ and MZ is a trigger of hypertrophic differentiation. The period of the onset was 2–4 weeks after surgery in our study. Note that reversing the differentiation after such a condition is impossible in vivo at present. Even though treatments delay the OA progression or symptoms, it is presumed to be difficult beyond this period. Therefore, we suggest that early detection and intervention are necessary with conservative treatment. We should focus on not only intervention method but also the period of appropriate therapeutic intervention. Three recent studies have demonstrated that pre or early potential preventive effect on human and animal model studies[45–47].

In conclusion, our findings suggest that chondrocyte alignment in articular cartilage due to catabolic and anabolic reactions plays an important role in the OA progression. Moreover, the changes in chondrocyte density and alignment were not consistent. Hence, to prevent the OA progression, we need to consider with appropriate therapeutic interventions.

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