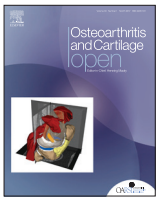
Osteoarthritis and Cartilage Open 5 (2023) 100409

Contents lists available at ScienceDirect

Osteoarthritis and Cartilage Open

journal homepage: www.elsevier.com/journals/osteoarthritis-and-cartilage-open/2665-9131



Establishment of a uniform histological evaluation method for early stage osteophytes in the destabilization of the medial meniscus mouse model

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ARTICLE INFO

Handling Editor: Professor H Madry

Keywords:
Osteoarthritis
Osteophyte
DMM mouse model
Synovium
Osteophyte maturity score

ABSTRACT

Background: Osteophyte formation is attracting attention as an early-stage pathology of knee osteoarthritis (OA). Although osteophyte formation is understood as a defense response to joint instability, its role and impact on OA remain largely unknown. Many studies have been conducted using the surgical destabilization of the medial meniscus (DMM) mouse model, but there are few standard evaluation methods, especially in the histological evaluation of early-stage osteophytes. The purpose of this study was to establish a reproducible and uniform method for histological evaluation of characteristics of early osteophyte formation in the DMM mouse model.
Methods: Male mice were operated with DMM at 12 weeks old and histologically evaluated at 4 days and 1, 2 and 4 weeks after DMM. Osteophyte Width, Osteophyte Area, and Original and Modified Maturity Scores were used to evaluate osteophytes for all sections.
Results: Osteophyte Width, Osteophyte Area and Maturity Scores were all greater anteriorly than posteriorly in the knee joint. The Modified Maturity Score was more strongly correlated with position than the Original Maturity Score, and could be used to evaluate early-stage osteophyte formation.
Conclusion: The Modified Maturity Score as well as Osteophyte Width and Area at the section of the anterior cruciate ligament (ACL) attachment site can provide a reproducible evaluation method to histologically assess the early-stage osteophyte formation in the DMM mouse model.

1. Introduction

Knee osteoarthritis (OA) is the most prevalent musculoskeletal disease in a super-aged society, and prevention and treatment of OA are very important to extend healthy life expectancy [1]. Currently, OA treatment includes conservative therapies such as hyaluronic acid injections and surgical treatments such as joint replacement. However, conservative treatment is mainly symptomatic therapy and has little effect on articular cartilage self-regeneration or repair [1]. Thus, the development of fundamental prevention and treatment methods is strongly desirable. To achieve these goals, it is necessary to elucidate the pathogenesis of OA. OA is a whole joint disease, involving structural changes in the articular cartilage, osteophyte, subchondral bone and periarticular muscles [2,3]. MRI-based studies suggested that Medial Meniscus Extrusion (MME) is one of the causes of knee OA [4,5]. It has been suggested that MME affects the onset and progression of knee OA due to loss of the load distribution function [6]. Recently, it has been reported that medial tibial osteophytes are strongly associated with MME [7]. It is known that periosteal synovial cells stimulated by mechanical stress or inflammation differentiate into chondrocytes and ossify to form osteophytes by a mechanism similar to endochondral ossification [8–12]. Although osteophytes are understood to be a body's protective response to abnormal mechanical stress [12,13], their actual role and effect on knee OA remains unclear [14]. Osteophytes are included in the Kellgren-Lawrence (KL) grading system, the gold standard for grading knee OA, and physicians worldwide recognize osteophytes on radiography as a characteristic of early knee OA [15]. However, considering the process of osteophyte formation, only ossified osteophytes are detected by radiography, and pre-ossified osteophytes are not evaluated. Recently, it has been reported that meniscus extrusion due to osteophyte formation, which is undetectable by radiography, is associated with the pathogenesis of early knee OA [16]. In this study, we confirmed periosteal synovial hyperplasia and osteophyte formation prior to articular cartilage degeneration in the destabilization

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https://doi.org/10.1016/j.ocarto.2023.100409
Received 17 April 2023; Accepted 12 September 2023

of the medial meniscus (DMM) mouse model. It was also observed that chondrogenesis occurs within the periosteal synovial hypertrophy, resulting in osteophyte formation. This phenomenon indicates that osteophytes are differentiated from the periosteal synovial hypertrophy. Furthermore, we found that there are differences in the size and maturity of osteophytes depending on the site of observation, even within the same knee. These differences may lead to overestimation or underestimation of osteophyte formation depending on the site of observation. The Osteoarthritis Research Society International (OARSI) provides recommendations for joint evaluation in the DMM model, but there is no consensus on the evaluation of osteophytes due to variations in observation methods among authors [17]. In addition, with regard to the assessment of osteophyte maturity, the Original Maturity Score reported by Little et al. did not include periosteal synovial hypertrophy, a feature of early osteophyte formation [18]. We therefore developed the Modified Maturity Score that added two additional stages of early characteristics of osteophyte formation to the Original Maturity Score: periosteal synovial hypertrophy and cartilage within the synovial hypertrophy. Additionally, to establish a reproducible and more detailed osteophyte evaluation method, we analyzed the detailed characteristics of osteophyte formation in the early stage DMM mouse model and recommend key features to use in the evaluation.

2. Material and methods

2.1. Animals

Male C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). They were housed in a specific pathogen-free facility under controlled conditions at room temperature (23 ± 2 °C) and a 12-h light/dark cycle and were provided with water and standard diet (MF: Solid [12 mmφpellet feed], Oriental Yeast, Tokyo, Japan) *ad libitum*. Animal experiments were approved by the Ehime University Animal Experiment Committee (Approval No. 37A1-1/16) and conducted in accordance with the Ehime University Animal Experiment Guidelines.

2.2. Surgical procedures of OA induction

DMM for induction of OA was performed on anesthetized 12-week-old mice, as previously reported [19]. Briefly, the medial menisco-tibial ligament (MMTL) was resected at the tibial attachment using 25G needle, and complete destabilization at the anterior angle of the meniscus was confirmed. Mice were euthanized 4 days, and 1, 2 and 4 weeks after DMM and knees were collected for histological analysis. In addition, to confirm the instability of the meniscus by DMM, the knee joints were obtained from 12-week-old male mice and the MMTL was excised to observe the displacement of the medial meniscus. A total of 23 mice were used in this experiment; 3 mice with patellar dislocation at the time of evaluation were excluded. Thus, data from 20 mice were analyzed, 5 mice in each group.

2.3. Histology and histomorphometry

The knee joints were collected and fixed with 4% paraformaldehyde (Nacalai Tespue, INC., Kyoto, Japan) at 90° of flexion, followed by decalcification with 0.5 M EDTA for 2 weeks and embedded in paraffin. These knee joints were cut into 10 μm coronal sections using a Leica RM2255 rotary microtome (Leica Biosystems, Nussloch, Germany). Sections were evaluated every 100 μm with the appearance of the medial tibial articular surface as the starting point (Fig. 1A and B) and the appearance of the posterior cruciate ligament attachment as the ending point (Fig. 1A, C). To evaluate osteophyte condition, sections were stained with Safranin O (Merck KGaA, Darmstadt, Germany.), Fast green (Wako, Osaka, Japan) and hematoxylin (MUTO PURE CHEMICALS CO.,LTD., Tokyo, Japan) [20]. Osteophyte Width and Area were measured using ImageJ [21] and maturity was assessed using Original and Modified Maturity Scores. Osteophyte Width was calculated by drawing a line parallel to the articular surface and measuring the maximum lateral diameter of the osteophyte (Fig. 2A). Osteophyte Area was measured by outlining the area of the osteophyte (Fig. 2B). In the early stages of osteophyte formation, only periosteal synovial hypertrophy was observed, in which case the upper border of the growth plate was used as the border (Fig. S1A). The border between chondrogenesis within the osteophyte and the growth plate was clear, and this border was used to measure Osteophyte Area. Periosteal synovial hypertrophy of the osteophyte surface was also included in the Osteophyte Area (Fig. S1B). Osteophyte maturity was scored using an Original Maturity Score reported by Little et al. [18] and our improved Modified Maturity Score (Table 1, Fig. 2C).

2.4. Statistical analysis

All statistical tests were analyzed using SPSS (IBM Corp., NY, USA). Spearman correlation coefficients were used to evaluate correlations between paired data. A correlation coefficient of 0.2–0.5 was considered weak, 0.5–0.7 was considered moderate, and 0.7 and greater indicated a strong correlation. One-way *ANOVA* followed by a post hoc Tukey's test was used for multi-group comparisons. $P < 0.05$ was considered statistically significant.

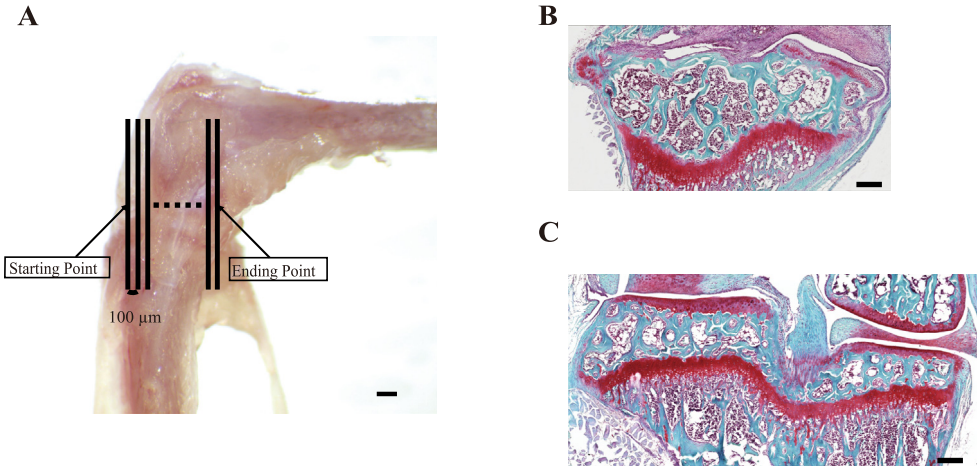

Fig. 1. Sectioning of the mouse knee. A, Sectioning range and width between each section. B, The starting point of sectioning stained with Safranin O/Fast green and hematoxylin. The starting point is where the medial articular surface appears. C, The ending point of sectioning stained with Safranin O/Fast green and hematoxylin. The ending point is where the tibial attachment of the posterior cruciate ligament appears. A. Scale bar: 500 μm. B, C. Scale bar: 200 μm.

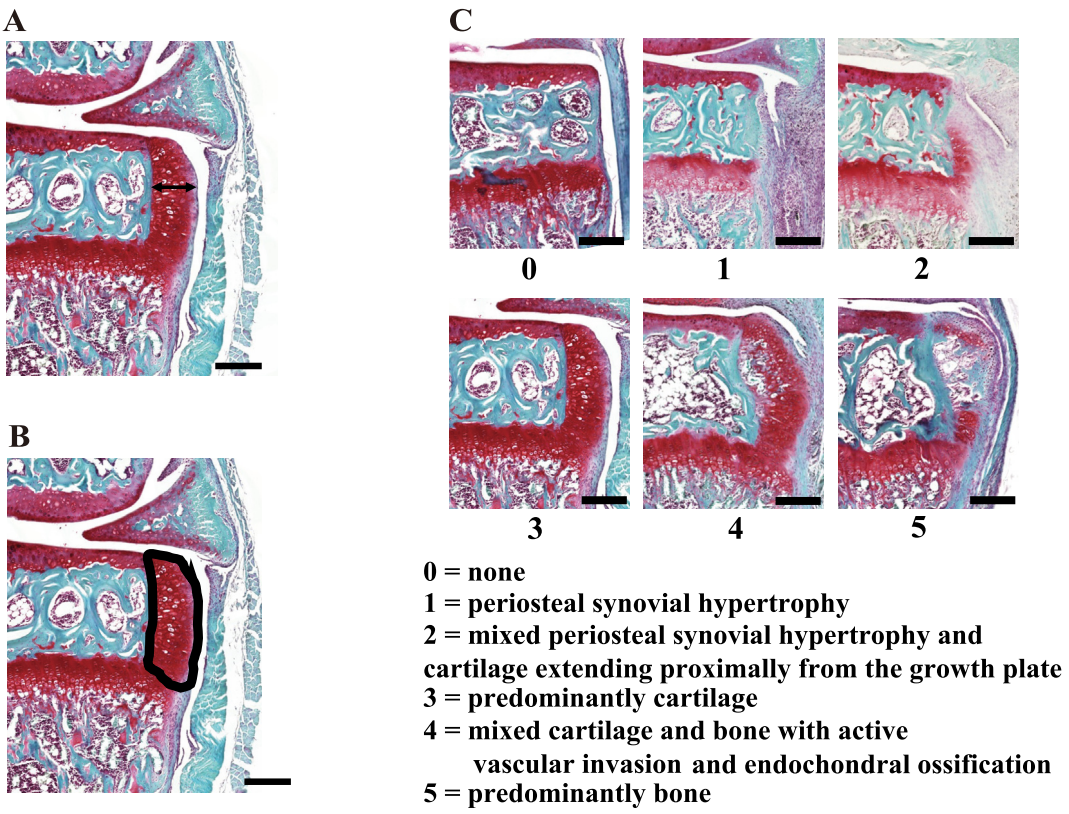

Fig. 2. Evaluation methods for osteophytes. A, Osteophyte Width was the maximum transverse diameter parallel to the articular surface of the osteophyte. B, Osteophyte Area was measured by demarcating the outer edge of the osteophyte using Image J. C, The measurement scheme and representative histology for the Modified Maturity Score. Scale bar: 200 μm.

To calculate Inter-rater reliability (ICC) of the Modified Maturity Score, 48 of the 230 images used in this study were randomly selected and scored by one experienced investigator (AJ) and two novices (YY, TK).

3. Results

3.1. Osteophyte Width and Area were greater at the anterior than posterior knee joint in the DMM mouse model

To confirm the details of osteophyte formation, knee joints were analyzed from anteriorly to posteriorly every 100 μm at 4 days, and 1, 2 and 4 weeks after DMM. There was a weak negative correlation between the distance from the starting point and Osteophyte Width 4 days and 1 week after DMM, a moderate negative correlation 2 weeks after DMM, and a strong negative correlation 4 weeks after DMM (Fig. 3A, Fig. S2). Osteophyte Area was negatively and moderately correlated with the distance from the starting point 1 and 2 weeks after DMM, but strongly correlated by 4 weeks (Fig. 3B, Fig. S2). These results indicate that the osteophytes tend to be larger anteriorly and smaller posteriorly at all time points.

Table 1
Comparison of Original Maturity Score and Modified Maturity Score.

Original Maturity Score (Little et al. [19])	
Grade	Osteophyte maturity
0	None
1	Predominantly cartilaginous
2	Mixed cartilage and bone with active vasccular invasion and endochondral ossification
3	Predominantly bone
Modified Maturity Score	
Grade	Osteophyte maturity
0	None
1	Periosteal synovial hypertrophy (synovial hypertrophy more than two rayer)
2	Mixed periosteal synovial hypertrophy and cartilage extending proximally from the growth plate
3	Predominantly cartilage
4	Mixed cartilage and bone with active vasccular invasion and endochondral ossification
5	Predominantly bone

3.2. 'Modified Maturity Score' can be used to evaluate early stage osteophyte formation

To appraise maturity of the osteophyte, we evaluated every section using the Original Maturity Score. Anterior location and Original Maturity Score were correlated only at 2 and 4 weeks after DMM (Fig. 4A). The reason for this may be that periosteal synovial hypertrophy is not included in this score although it is one of the characteristics of early-stage osteophyte formation. Therefore, we modified the maturity score to include periosteal synovial hypertrophy (Fig. 2C) and this modified score revealed a weak negative correlation 1 and 2 weeks after DMM and strong correlations 4 weeks following DMM (Fig. 4B, Fig. S2). Despite the obvious pathological condition of periosteal synovial hypertrophy beginning 4 days after DMM, it was not reflected in the original score until 2 weeks after DMM, but in the modified score within 1 week. The Modified Maturity Score had a good ICC of 0.84. It is a more useful scoring method that can easily be used to evaluate early osteophyte formation.

3.3. Osteophytes grow rapidly between 1 and 2 weeks after DMM

To understand the growth process of osteophytes, Osteophyte Width, Area, and Maturity Score from anterior to posterior were summed and plotted at each time point. Osteophyte size and maturity grew rapidly between 1 and 2 weeks after DMM (Fig. 5).

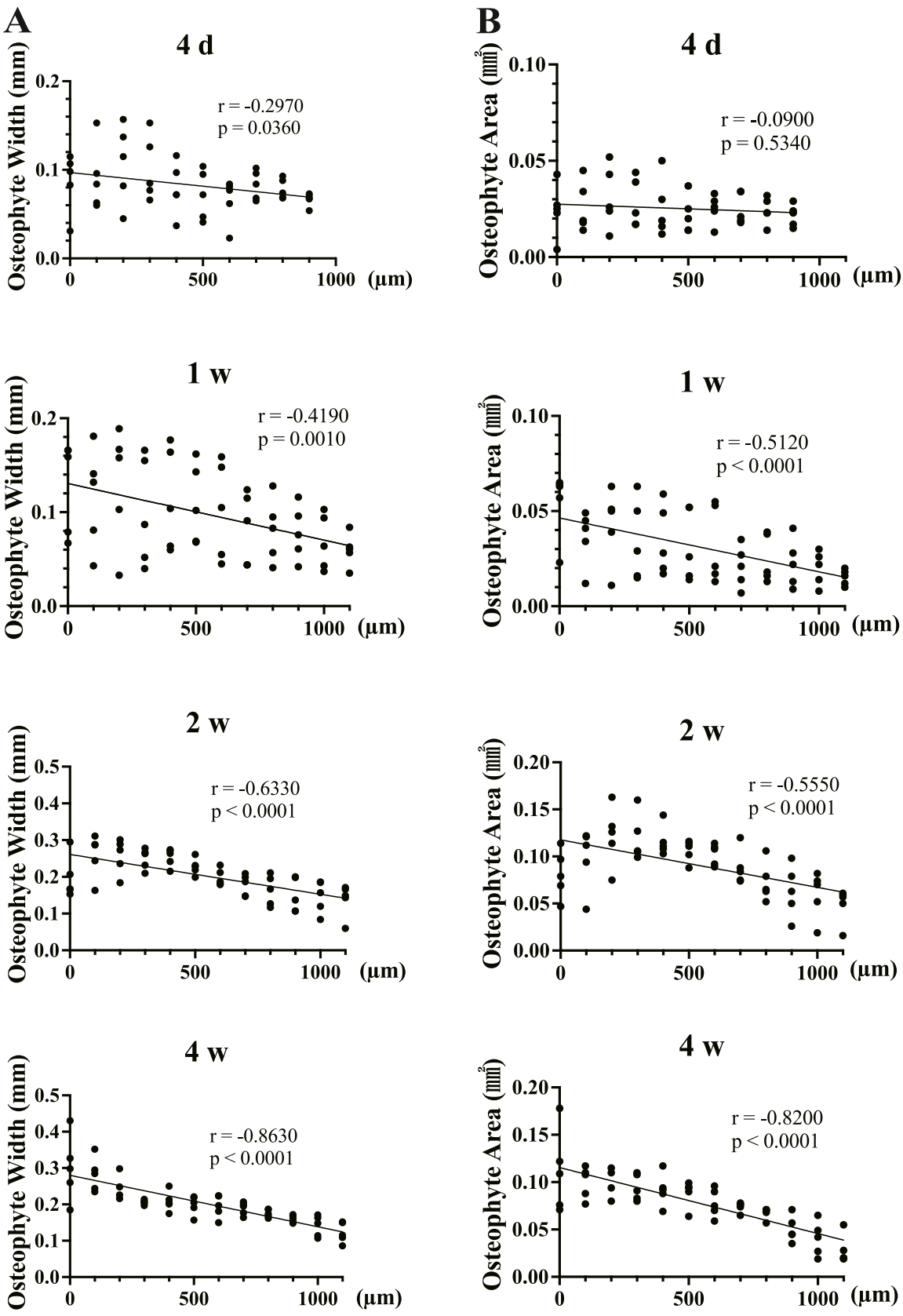

Fig. 3. Measurement results for Osteophyte Width and Area in histological coronal sections of the whole knee. A, Correlations between distance from the starting point and Osteophyte Width (n = 5). B, Correlations between distance from the starting point and Osteophyte Area (n = 5). Dots indicate data points for each animal at each distance. d: days, w: weeks.

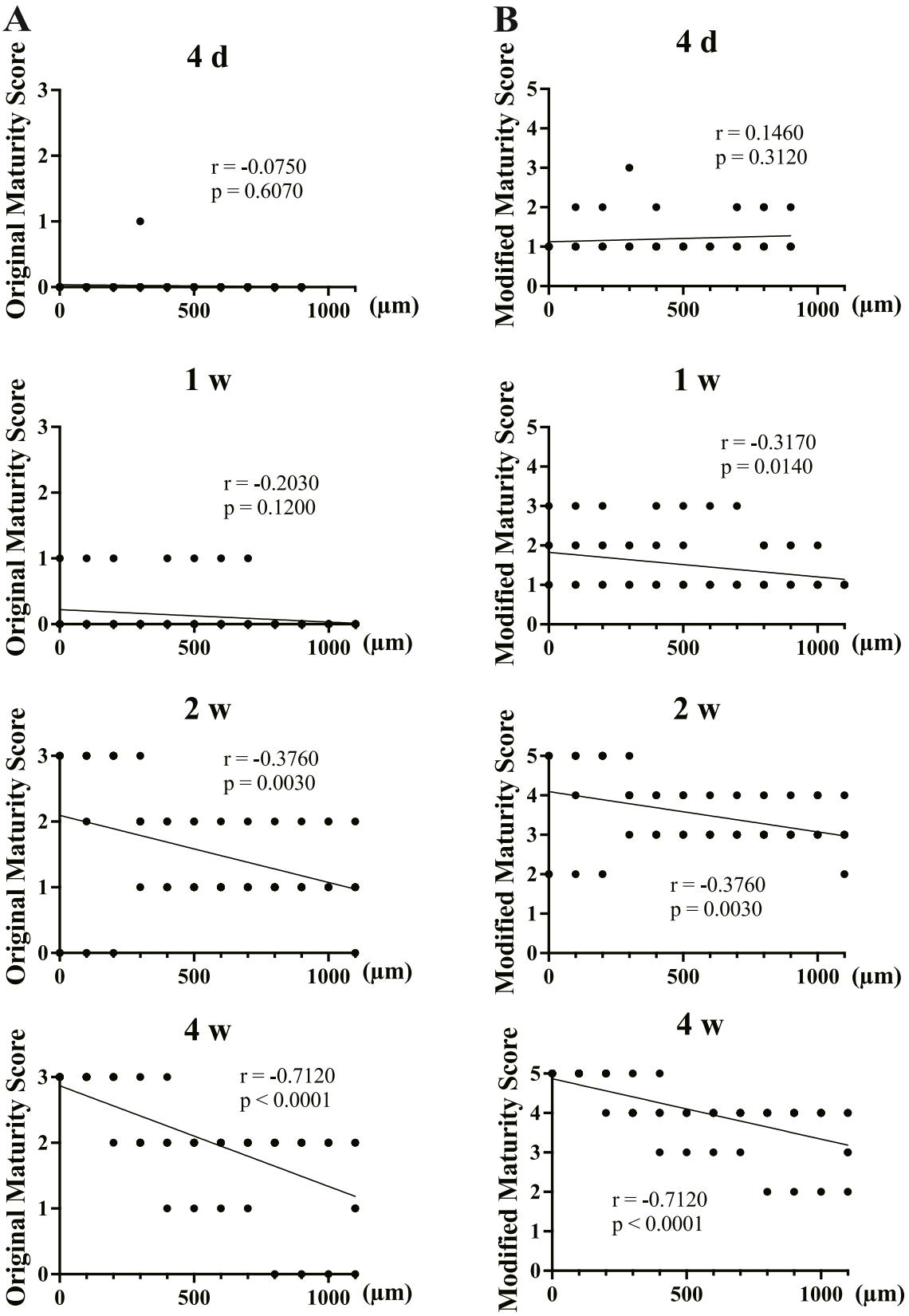

Fig. 4. Osteophyte maturity scores for histological coronal sections of the whole knee. A, Correlations between distance from the starting point and Original Maturity Score of the four grades reported by Little et al. (n = 5). B, Correlations between distance from the starting point and our Modified Maturity Score of the six grades (n = 5). Dots indicate data points for each animal at each distance. d: days, w: weeks.

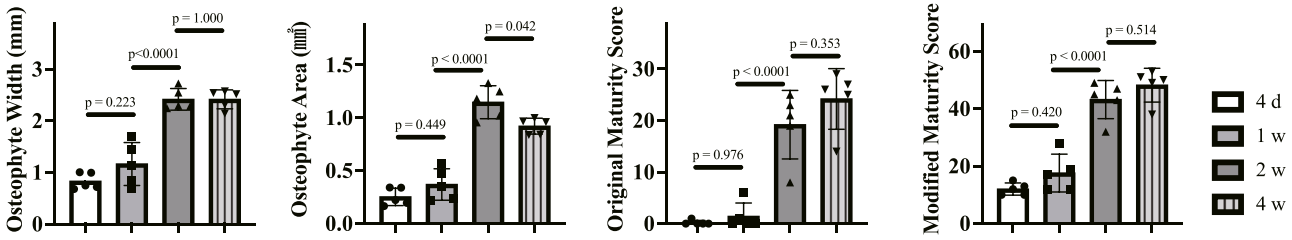

Fig. 5. Changes over time in Osteophyte Width, Area, Original Maturity Score, and Modified Maturity Score. The Original Maturity Score is the four grades score reported by Little et al. and the Modified Maturity Score is our six grades score. Dots indicate data points for each animal. The respective values from anterior to posterior were summed and plotted. The data are expressed as means ± SD. d: days, w: weeks.

4. Discussion

The most important finding of our study is that osteophyte formation and maturity in the DMM mouse model differ depending on the anatomical position. Osteophytes in each section were evaluated and found to be larger anteriorly and smaller posteriorly (Figs. 3, 4, 6, Fig. S2). The DMM surgery destabilized the medial meniscus by rupturing the medial menisco-tibial ligament, but meniscal instability was greater in the anterior segment and anterior horn and least after the midsection (Fig. S3). There are many reports that mechanical stress promotes osteophyte formation [12,22,23]. It has been suggested that the increased osteophyte formation in the anterior knee is caused by greater mechanical stress due to instability in the anterior knee.

Thus, osteophyte formation and maturity differ depending on the location of observation, so it is important to make measurements at specific locations for reproducible evaluation. The tibial attachment of the ACL is a reproducible landmark for evaluation. This observation point is not only easy to identify, but also located about 600–800 μm (mean value: 755 μm) from the starting point where the medial tibial articular surface appears. (Figs. 1, 3, 4 and 6).

Another new finding of our study is that our Modified Maturity Score is more useful than the previously reported Original Maturity Score in evaluating early osteophyte formation. Periosteal synovial hypertrophy occurred very early after DMM (Fig. 6). As reported by Kraan, osteophyte formation is known to begin with thickening of the synovium [11]. Huesa et al. observed DMM mice at the same time points as we did and reported that ossified osteophytes could be observed 7 days after DMM [24].

We observed chondrogenesis within the periosteal synovial hypertrophy, resulting in the formation of osteophytes (Fig. S4). However, there have been no reports of the detailed histological evaluation of periosteal synovial hypertrophy at an early stage of osteophyte formation. Many previous papers have not evaluated periosteal synovial hypertrophy and osteophytes together, but only separately [8,25]. Kaneko et al. reported a 5-grade scoring system modifying the Original Maturity Score of Kamekura et al. [26] and Little et al. [18] but it was considered insufficient to evaluate periosteal synovial hypertrophy [27]. In our study, we observed that chondrogenesis begins from the distal and deeper layers of the synovium on the medial tibial plateau, and we included this stage in our maturity score (Figs. 2C and 6, Fig. S4). When osteophytes were evaluated using the Original Maturity Score of Little et al., the evaluation score showed no significant correlation with location 1 week after DMM (Fig. 4A). When evaluated using the Modified Maturity Score, a significant mild correlation was observed (Fig. 4B). These results indicate that the Modified Maturity Score may be more useful for evaluating early osteophyte formation than the Original Maturity Score.

Our study has some limitations. First, we defined the observation area as starting at the appearance of the medial tibial articular surface and ending at the appearance of the posterior cruciate ligament attachment site. However, these starting and ending points may vary slightly among samples. Second, tissues were cut perpendicular to the articular surface at the center of the joint, but were not completely perpendicular to the articular surface away from the center because the tissue sections were made coronally. Furthermore, although no histological changes in articular cartilage, subchondral bone, or synovium were observed during

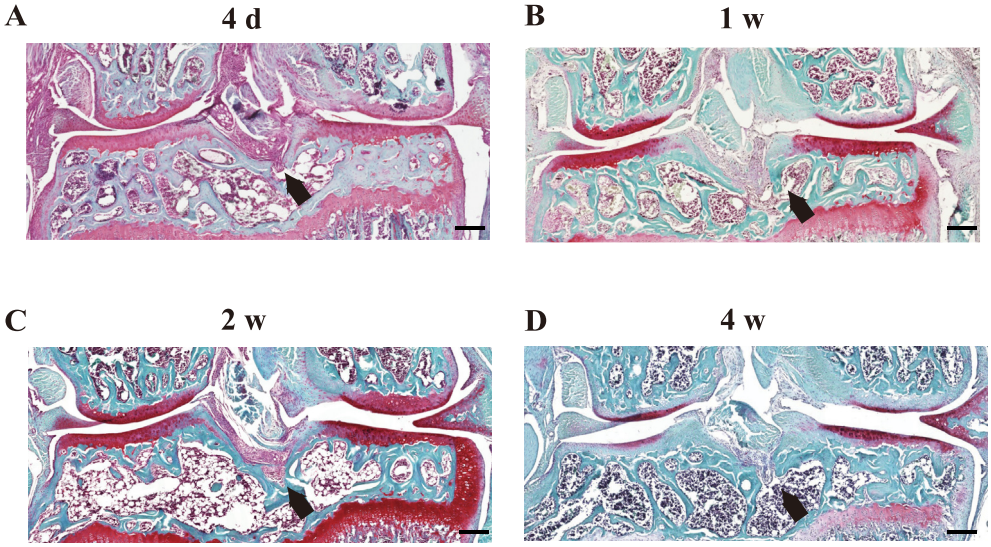

Fig. 6. Coronal section of mouse knee 4 days and 1, 2, and 4 weeks after DMM. Arrows indicate incisura of the tibial attachment of the anterior cruciate ligament. Scale bars: 200 μm. d: days, w: weeks, DMM: destabilization of the medial meniscus.

the observation period of this study, to assess the early to late stages of OA, it is necessary to consider a comprehensive evaluation method that includes the entire OA pathology such as subchondral bone, articular cartilage, synovium, and meniscus. For this purpose, long-term observation of the joints of OA model mice is necessary.

5. Conclusion and future perspective

Osteophyte formation in the DMM mouse model is greater anteriorly and smaller posteriorly, and therefore the site of observation is important to evaluate histology of OA model mice. A detailed examination of the features of osteophyte formation in the DMM mouse model revealed that the tibial attachment of the ACL is a reproducible site for evaluation. In addition, our proposed Modified Maturity Score is sufficiently sensitive to assess early-stage periosteal synovial hypertrophy in the DMM mouse model. Utilizing the Modified Maturity Score and known observation site, it will be possible to evaluate the early pathology of osteophyte formation with high reproducibility. This study may help to develop a unified understanding of osteophyte development in OA. In the future, combining osteophyte evaluation by this method with other assessments such as subchondral bone, cartilage, synovium and meniscus will contribute to the elucidation of the role of osteophytes in the development of OA.

Author contribution

AJ and YI conceived and designed this work. AJ conducted the experiments and assessed the outcomes. AJ, YY and TK performed the data analysis. All authors were involved in drafting the paper and revising it critically for intellectual content. All authors approved the final version for submission.

Declaration of competing interest

All authors declare that they have no conflict of interest.

Acknowledgement

The authors thank the staff of the Division of Analytical Bio-Medicine and the Division of Laboratory Animal Research, the Advanced Research Support Center (ADRES), the members of the Division of Integrative Pathophysiology, Proteo-Science Center (PROS), and Ehime University for their technical assistance and helpful support. This study was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI grants JP23689066, JP15H04961 and JP19H03786 (to YI).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2023.100409.

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