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Original Article

Immunohistochemical study on the distribution of telocytes in the knee joint components in a rat osteoarthritis model

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Abstract. [Purpose] Telocytes are stromal cells that participate in tissue homeostasis. Osteoarthritis is a common degenerative disorder of multiple joint components that causes inflammation; however, the distribution of telocytes in joint components and the impact of osteoarthritis on telocytes is unclear. Therefore, we aimed to clarify the distribution of the telocyte in the joint components and determine the effect of osteoarthritis on telocytes. [Participants and Methods] We divided 30 male rats into control and osteoarthritis groups and surgically induced osteoarthritis by destabilizing the medial meniscus. At two and eight weeks after surgery, we evaluated the changes in CD34-positive and CD31-negative area sizes in the joint components by immunohistochemistry. [Results] The results showed CD34-positive and CD31-negative areas in the loose connective tissue of the lateral meniscus attachment and the infrapatellar fat pad. However, it was not observed in the cartilage, subchondral bone, cruciate ligament, and meniscus. Moreover, there were no significant differences between the CD34-positive and CD31-negative area sizes in control and osteoarthritis groups at both time points. [Conclusion] CD34-positive and CD31-negative cells are distributed in multiple joint components; however, CD34-positive and CD31-negative areas are not affected by the progression of osteoarthritis. This result provides information on telocytes distribution in the knee joint and the impact of osteoarthritis on these cells.
Key words: Telocyte, Osteoarthritis, CD34-positive and CD31-negative cells

INTRODUCTION

Telocytes are a novel stromal (interstitial) cell type discovered by Popescu et al. in 2010[1]. They have unique ultrastructural features including extremely long, thin, dichotomously branched cytoplasmic processes called telopodes[2]. Telocytes are distributed in the stroma of various organs, especially just below the epithelium and beside blood vessels, nerves, and secretory glands of the heart, lungs, aorta, pulmonary arteries and veins, trachea and bronchi, meninges, pancreas, genitals, and mammary glands[3]. In the musculoskeletal system, telocytes are present in bones, skeletal muscles, fascia, and joint synoviums[4].

Telocytes seem to help control the local environment through the telopodes, which are involved in (1) cell-to-cell communication and signaling, (2) organ structure and mechanical sensing, and (3) the regulation of immune responses. Thus, they help maintain tissue homeostasis[3]. Telocytes are also implicated in the pathophysiology of many diseases. In pathological conditions characterized by chronic inflammation and connective tissue fibrosis, the telocyte stromal network is disturbed, reducing the number of telocytes[5]. For example, there are fewer telocytes in the synovium in rheumatoid arthritis[6]. Numer-

ous cellular and molecular studies have focused on the distribution of telocytes and their relationship to pathologies[1, 2]. The relevance of telocytes is also attracting attention in regenerative medicine[7].

Osteoarthritis (OA) is a common degenerative joint disorder affecting humans and animals, causing synovial inflammation and cartilage degeneration. The pain, disability, and cost associated with OA constitute a burden on society[8, 9]. Several treatments are currently used to alleviate OA symptoms[10]. However, our understanding the mechanisms involved in OA is necessary to develop more efficient therapies. Particularly, little is known regarding telocyte distribution in the joints and the impact of OA.

Immunohistochemical staining is frequently used for identifying telocytes. Telocytes are labeled by a variety of immunohistochemical markers in organs and tissues, and the antibodies positively recognizing telocytes vary according to the tissue stained. However, many studies identified stromal cells expressing the hematopoietic progenitor cell antigen CD34 but not the cluster of differentiation CD31 (also known as platelet cell adhesion factor, PECAM-1) observed by light microscopy as telocytes[4–6]. CD34 is a transmembrane sialomucin, and CD31 is a cell membrane glycoprotein. CD31 and CD34 are vascular endothelial markers.

To clarify the situation regarding OA impact on telocytes, this study aimed to investigate, for the first time, the distribution of CD34-positive and CD31-negative stromal cells in joint components, including the articular cartilage, subchondral bone, infrapatellar fat pad, cruciate ligament, meniscus, and loose connective tissue, and CD34-positive and CD31-negative stromal cells in OA, using surgically induced OA of the rat knee as model. As a result, CD34-positive and CD31-negative areas were observed in the connective tissue of the lateral meniscus attachment and infrapatellar fat pad. However, we showed that OA did not affect the size of the area containing CD34-positive and CD31-negative stromal cells, suggesting that it did not affect telocyte amounts.

PARTICIPANTS AND METHODS

The Animal Research Committee of the Graduate School of Medicine of Kanazawa University approved the study protocol (Kanazawa, Japan; approval no. 183933 and 204125), which complied with the ARRIVE guidelines[11, 12] and Guidelines for the Care and Use of Laboratory Animals of Kanazawa University. The specimens were obtained from the same animals sampled in previous studies under the 3Rs principle to limit the number of animals used. The specimens were freshly stained and evaluated, and we ensured none of the content is identical to that reported in our previous studies.

Fifteen male Wistar rats (8 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed under normal conditions for 5 weeks before the start of the experiments to acclimatize the animals to their new environment. One rat was housed per cage in a sanitary ventilated room under controlled temperature and humidity conditions and a 12-h light–dark cycle with ad libitum access to food and water. The experimental animals were monitored 2–3 times per week to control their health status, including general food and water intake, surgical wound condition, gait, and hindlimb suspension. The experimenter cleaned the cages once or twice every 2 weeks to keep the breeding environment clean.

The experimental protocol is shown in Fig. 1. The rats were divided into two groups: control group (CON, n=5) and surgical OA group (n=10, five rats for both time points analyzed).

As described previously, we induced OA by transecting the medial meniscotibial ligament (destabilization of the medial meniscus) in the left knee joint, and sham surgery was performed on the right knee[13, 14]. No further interventions, including no motion exercises, occurred during the experimental period, and no analgesics or anti-inflammatory drugs were administered. After the experimental period, the animals were euthanized via an intraperitoneal injection of a lethal dose of sodium secobarbital, and both hindlimbs were disarticulated at the hip joint.

Decalcified paraffin sections were prepared for histology as described previously[15, 16]. The knees were excised frontally to evaluate histological changes in the medial tibiofemoral joints. Serial sections of the cartilage from the medial tibiofemoral joint (3-μm thickness) were stained with hematoxylin–eosin and toluidine blue (0.05%, 15 min) and by immunohistochemistry. The staining was observed under a light microscope and imaged using a digital camera (BX51 and DP74; Olympus Corporation, Tokyo, Japan).
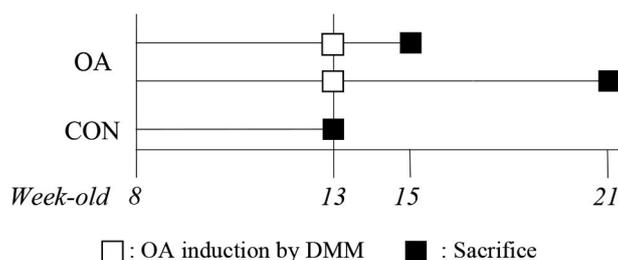

Fig. 1. Study design.
CON: Control group; OA: Osteoarthritis group.

We used the OA cartilage histopathology assessment system to determine the histological changes induced by OA in the articular cartilage of the tibia in the medial tibiofemoral joint[17]. The OARSI scoring system, consisting of six grades and four stages on a scale from 0 (normal) to 24 (severe cartilage lesion), was used[17]. Two blinded and trained independent observers (M.H., a pathologist, and I.T.) conducted this semiquantitative assessment of histological changes. In our previous study, interclass correlation coefficients for the intrarater and inter-rater reliabilities of the OARSI score assessment with 95% confidence intervals were excellent: 0.94 (0.92–0.95) and 0.91 (0.89–0.93), respectively[18].

Paraffin sections were immunohistochemically stained with antibodies recognizing CD31 (diluted 1:250, ab182981; Abcam, Tokyo, Japan) and CD34 (diluted 1:1,000, ab81289; Abcam, Tokyo, Japan). Between each step, sections were washed three times 5 min with 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4). After deparaffinization with xylene and dehydration with alcohol, the sections were heated in a hot water bath (citrate buffer, pH 6.0, 70 °C, 40 min) to activate CD31 and CD34 antigens. Then, endogenous peroxidase was inactivated using methanol with 3% H_2O_2 for 20 min and nonspecific binding of immunoglobulins was blocked using Protein Block Serum-Free (X0909; Dako Japan, Tokyo, Japan) for 15 min. The sections were incubated overnight with the primary antibody at 4 °C. Then, sections were washed in PBS and subsequently incubated Histofine Simple Stain Rat MAX PO (MULTI) (414191, Nichirei Biosciences Inc., Tokyo, Japan) for 1 h at room temperature following the immune–enzyme polymer technique. Immunoreactivity was visualized by incubating the sections with Histofine Simple Stain MAX PO (MULTI) (725191, Nichirei Biosciences Inc., Tokyo, Japan) for 5 min at room temperature. The sections were counterstained with hematoxylin for 1 min.

We analyzed the articular cartilage, subchondral bone, infrapatellar fat pad, meniscus, lateral meniscus, and loose fibrous connective tissue of the lateral meniscus attachment in the knee joint. We photographed serial sections of these tissues stained with CD34 and CD31, using a digital camera and Adobe Photoshop CC imaging software (Adobe Systems, Inc., San Jose, CA, USA) to measure the CD34- and CD31-positive areas. The CD31-positive area was subtracted from the CD34-positive area to determine the area occupied by cells presumed to be telocytes. We divided the obtained value by the CD34-positive area to account for individual differences among photographs. The resulting value was considered as the CD34-positive and CD31-negative area.

Statistical analyses were performed using JMP 14 software (SAS Institute, Cary, NC, USA). Data were statistically analyzed as parametric data. The sample size was five for each group. Descriptive statistics were calculated as the median with interquartile range for the OARSI scores and as the mean with standard deviation for the CD34-positive and CD31-negative area. For all data, we performed analysis of variance followed by the post-hoc Tukey's honest significant difference test. Differences were considered significant for $p < 0.05$. The exact p-values are shown in the figures.

RESULTS

No rat was discarded from the study or died during the experiment. Furthermore, the inflammation of the surgical wound was well controlled, with no serious infections. Therefore, all animals presented good general condition throughout the experiment as designed.

No histological changes of the cartilage were observed in the control group or the sham-operated limb of the OA rats. In the operated limb of the OA group, histological changes typical from OA, including fibrillation and decreased of the matrix, were observed. Moreover, the histological changes became significantly more severe with time (Fig. 2).

CD34-positive and CD31-negative areas were observed in the infrapatellar fad pad and and fibrous connective tissue (Table 1 and Figs. 3, 4). However, these areas were not observed in the articular cartilage, subchondral bone, cruciate liga-
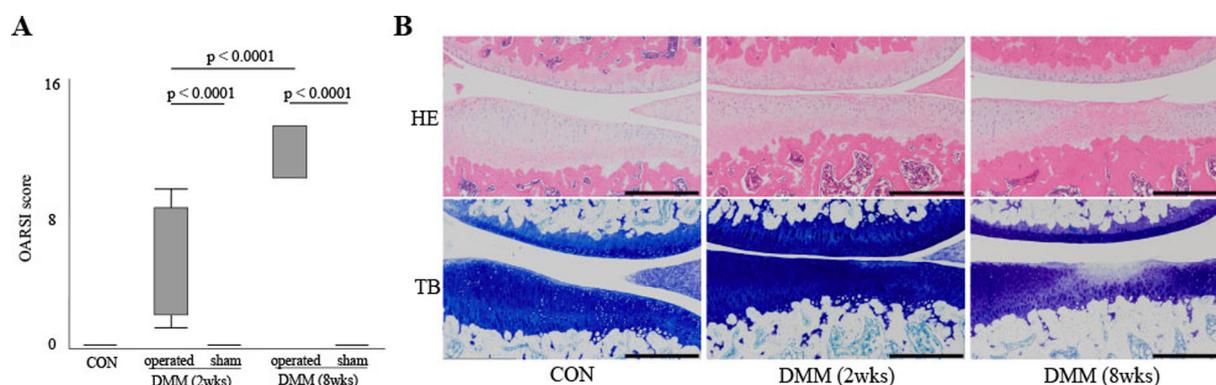

Fig. 2. Histological analysis of the articular cartilage.
OARSI: Osteoarthritis Research Society International; CON: Control; DMM: Destabilization of the medial meniscus.
Scale bar=500 μm.

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ment, and lateral meniscus. Since the specimen in this study was the anterior forehead, in most specimens, the synovium and joint capsule could not be observed sufficiently.

CD34-positive and CD31-negative areas were widely observed throughout the fibrous tissue in the control and OA groups (both in the operated and sham-operated limbs). The area ratio did not differ significantly between the groups (Fig. 4).

DISCUSSION

This study aimed to clarify where CD34-positive and CD31-negative cells are located in the joint components and how the presence of CD34-positive and CD31-negative cells changes with the progression of OA in a rat model. We obtained two new histological findings. First, CD34-positive and CD31-negative areas were found in the infrapatellar pad and fibrous connective tissue within the knee joint. Second, CD34-positive and CD31-negative areas were not affected by the progression of OA.

Table 1. Distribution of the CD34-positive and CD31-negative areas in knee components

	Control	OA2w (sham)	OA2w (operated)	OA8w (sham)	OA8w (operated)
Articular cartilage	0/5	0/5	0/5	0/5	0/5
Subchondral bone	0/5	0/5	0/5	0/5	0/5
Lateral meniscus	0/5	0/5	0/5	0/5	0/5
Cruciate ligament	0/5	0/5	0/5	0/5	0/5
Infrapatellar fad pad	3/3	3/3	3/3	2/2	1/1
Fibrous connective tissue	5/5	5/5	5/5	5/5	5/5

The denominator is the number of samples that could be evaluated, and the numerator is the number of samples that were confirmed to be either CD34-positive or CD31-negative.
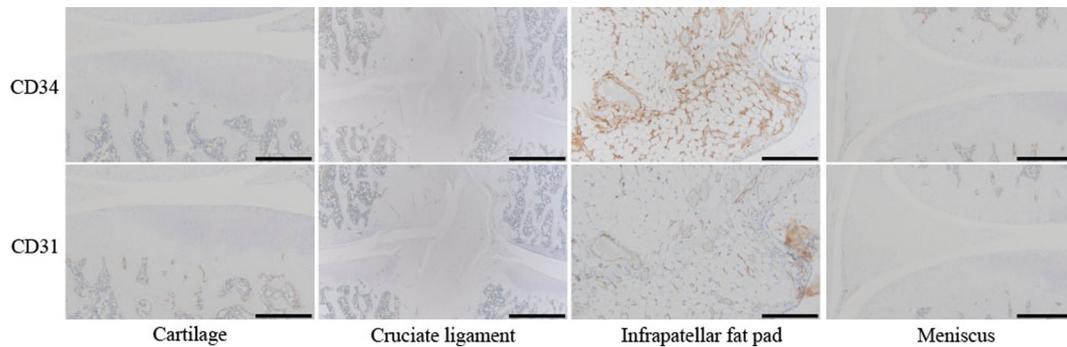

Fig. 3. Histology of CD34-positive and CD31-negative area for each joint component.
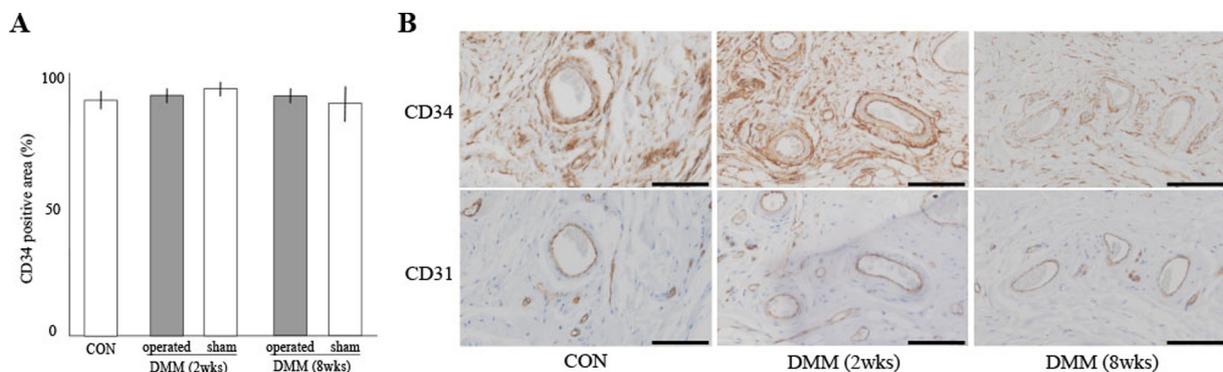Scale bar=500 μm (Cartilage and Meniscus), 1 mm (Cruciate ligament) and 200 μm (Infrapatellar fat pad).

Fig. 4. Immunohistochemical quantification of CD34-positive and CD31-negative areas.
CON: Control; DMM: Destabilization of the medial meniscus.
Scale bar=100 μm.

CD34-positive and CD31-negative cells were reportedly present in organs and stroma throughout the body[1, 3]. However, there have been few studies of telocytes in joint components, and telocytes did appear in skeletal muscle and synoviums[3, 4]. In the present study, we found CD34-positive and CD31-negative cells in the fibrous connective tissue in the knee joint and in the infrapatellar fat pad. Since CD34-positive and CD31-negative cells are reportedly present around blood vessels[1], it is reasonable to assume that CD34-positive and CD31-negative areas were not identified in the articular cartilage. However, the reason why CD34-positive and CD31-negative areas were not found in the meniscus and cruciate ligament, where blood flow exists, is unclear and requires further investigation.

Previous studies showed that onset and progression of certain pathologies affect the amount of CD34-positive and CD31-negative cells. For example, Manetti et al. reported a significant decrease in the number of CD34-positive and CD31-negative cells in systemic sclerosis[19]. Furthermore, Rosa et al. found a remarkable decrease in the number of CD34-positive and CD31-negative cells present in the synovium in rheumatoid arthritis[6]. These findings suggest perturbation of homeostasis at disease onset affects the amount of CD34-positive and CD31-negative cells. Here, we focused on OA, an age-related degenerative disease. However, the size of CD34-positive and CD31-negative areas was unchanged by OA onset or progression. It is possible that, due to the nature of the disease, the intra-articular homeostasis is not much disturbed by OA compared to the imbalance induced by systemic sclerosis or rheumatoid arthritis. To clarify this point, the relationship between OA and telocytes must be investigated in more detail in the future. For example, it might be necessary to use different OA models, such as gene knockout models in different species including pigs, cattle, and humans. We performed a similar study with a drug-induced OA model using monoiodoacetate[20]. We found OA had no impact on the number of CD34-positive and CD31-negative cells. However, our preliminary data obtained in a drug-induced OA model using monoiodoacetate revealed no impact of OA on the number of CD34-positive and CD31-negative cells.

The present study has three limitations. First, we used solely immunohistological analysis. Other methods for identifying telocytes include fluorescent immunostaining and electron microscopy[21–23]; they should be combined in the future to identify telocytes. Second, we used a relatively long-term model as the OA disease stage. However, in early stages, such as within a week after OA induction, inflammation may alter telocyte dynamics. Third, we chose the anterior section as the thin sectioning method for tissue specimens to evaluate cartilage degeneration caused by OA. As a result, we were able to properly evaluate the articular cartilage, meniscus, and cruciate ligament. On the other hand, the infrapatellar fat pad could not be identified in about half of the specimens, and the joint capsule and synovium could not be adequately observed in some specimens. When observing these joint components, it may be better to combine multiple observation methods, such as sagittal and horizontal sections, in addition to the anterior section.

In conclusion, our histopathological results suggested that CD34-positive CD31-negative cells are distributed in the infrapatellar fat pad and loose connective tissue, but its area is not affected by OA progression. This finding constitutes a first step in investigating the relationship between telocytes and intra-articular lesions, such as joint contractures, infectious arthritis, and crystal-induced arthritis. The link between telocytes in these lesions will be clarified in the future.

Conflict of interest

The authors have declared that no competing interests exist.

ACKNOWLEDGMENTS

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