Histological study on structural changes in attachment of medial collateral ligament in growing rats

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Summary

The medial collateral ligament (MCL) is susceptible to mechanical stimulation associated with weight-bearing, which affects the femur and tibial attachments of the enthesis. The weight on the body increases significantly with development. In this study, we observed the structural changes of the MCL of the Enthesis with development in rats aged 3, 7, and 13 weeks. The attachment area of MCL of the Enthesis increased with development, and this was more remarkable on the tibia side than on the femur side. The metachromatic response of the femur side of the enthesis decreased with age and showed a tendency toward calcification. In addition, the ligament fibers became perpendicular to the Enthesis instead of parallel to it, and were embedded in a complex intersection. In the tibia side of the Enthesis, many irregularities were formed at the interface between the fibers and bone as the weeks progressed, and an increase in fiber density was observed. These results suggest that the MCL of the Enthesis changes to a structure with enhanced bonding

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strength in response to increased mechanical stimulation during development.
Keywords：MCL, Enthesis, Development

Introduction

The medial collateral ligament (MCL) of the knee is known to be the most common sports injury in the knee joint[1]. It is particularly common in young athletes and is most likely to be injured in sports that involve pivoting movements, such as soccer, skiing, and ice hockey [2]. The pivoting motion exerts a large amount of external rotation, abduction, and shearing force on the knee joint, which can lead to ligament damage [3]. Thus, the MCL is an important tissue in the knee joint and is susceptible to mechanical stimulation caused by knee joint motion.

The ends of the MCL which is a dense connective tissue, attach to the femur and tibia respectively [4]. In general, the ligaments transmit mechanical stimuli to the bone by dispersing them at the bony attachments of both ends of the ligament (Since then, Enthesis)[5]. This is related to not only reduces of the mechanical stimulation of the ligament but also to induces structural remodeling of the enthesis [6]. This fact also supports the fact that the mechanical strength of enthesis is dependent on mechanical stimulation. It has been reported that there is a positive correlation between mechanical strength and body weight in rat MCL of the Enthesis [7]. In addition, a significant increase in body weight is observed during the so-called developmental period from birth to maturity [8]. This suggests that the increase in load associated with development improves the mechanical strength of enthesis.

Histological studies of MCL of the Enthesis have been reported in rabbits and rats [6,7,9-12], and the morphology and mechanical properties of MCL of the Enthesis have been clarified [7,9-12]. However, there are no reports on histological studies of structural changes during development. In this study, we aimed to compare and examine the structural changes of enthesis in the femur and tibia of the MCL by histological observation using developing rats of different ages.

Materials and Methods

Experimental animals

Twenty-four male Wistar rats (8 rats each) aged 3, 7, and 13 weeks-old were used in this study.

Specimen removal method

The rats were euthanized by carbon dioxide inhalation at the end of the experimental period. After confirming death, the hind limbs of the rats were removed and fixed in 4% paraformaldehyde solution(4℃, overnight), thereafter prepare various specimens.

Preparation of specimens for histological observation and observation

Polished non-demineralized resin specimens were prepared from the immersion-fixed specimens and stained with toluidine blue. And demineralized paraffin sections were prepared and stained with polychrome. Each specimen was observed under an optical microscope.

Results

Histological observation the enthesis of MCL by gross and weak magnification

The enthesis of the MCL was found to be located at the distal femur and proximal tibia at all ages (Fig. 1). The same was observed in the resin specimens. Both the femoral and tibial enthesis expanded their attachment areas in the direction of the long axis of the bone as they developed. In addition, the attachment area of the tibial enthesis was larger than that of the femoral enthesis, and this difference was more pronounced when comparing the 3 weeks and 13 weeks-old groups (Fig. 2).

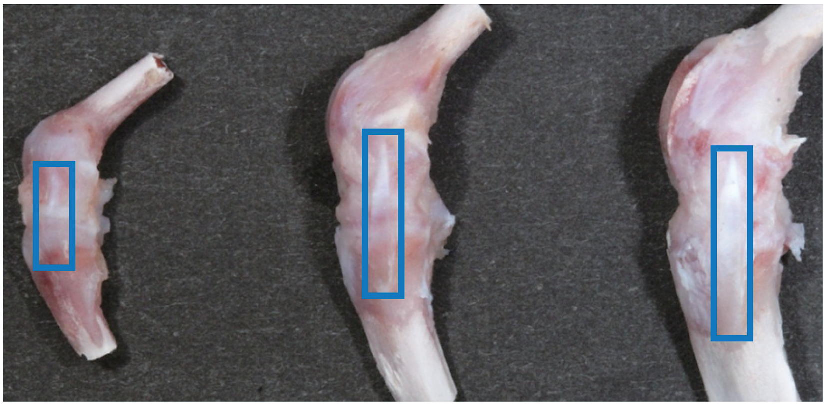

Fig. 1. Specimen for naked eye observation.
(Blue frame：MCL)
※From left to right：3, 7, and 13 weeks-old.

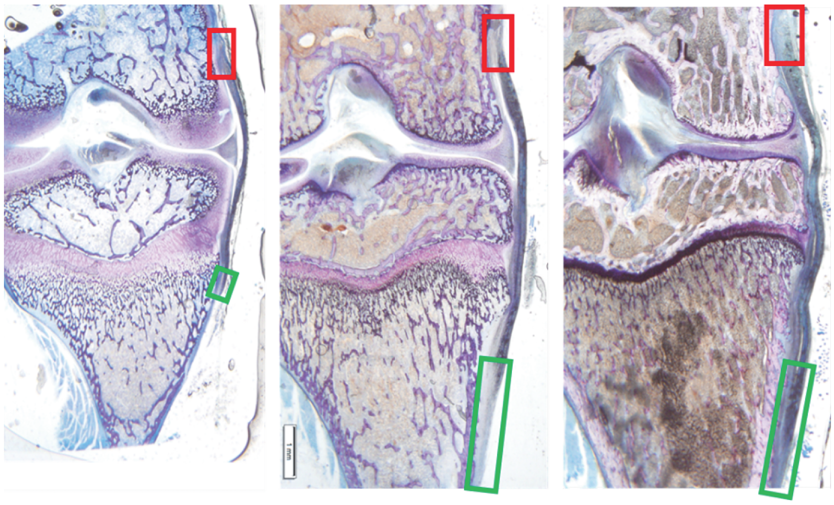

Fig. 2. Weakly enlarged forehead section of resin specimen.
(Red frame：Enthesis of femur , Green frame：Enthesis of tibia)

Histological observation of Femur side of the Enthesis

At the 3 weeks-old, ligamentous fibers, hyaline cartilage, and bone marrow were observed in order from the superficial layer, and metachromasia was observed between the cartilage matrix. At the 7 weeks-old, hyaline cartilage was not observed, but instead fibrocartilage was observed, and bone continuing from fibrocartilage was also observed. In addition, the metachromatic response which was clear at 3 weeks-old was slightly diminished. And ligament fibers were found to penetrate the enthesis in a slightly vertical direction with complex intersections. At 13 weeks-old, even smaller fibrocartilage was present which was more pronounced in paraffin specimens. At 13 weeks-old, smaller fibrocartilage was present which was more remarkable in the paraffin specimen, whereas no metachromasia was seen in the resin specimen. Ligament fibers which showed a tortuous tendency after 7 weeks-old became more complex and the implantation angle to the enthesis became more perpendicular. These facts suggest that the increase in load during development changes the orientation of the enthesis from parallel to perpendicular to the implantation angle (Fig. 3).

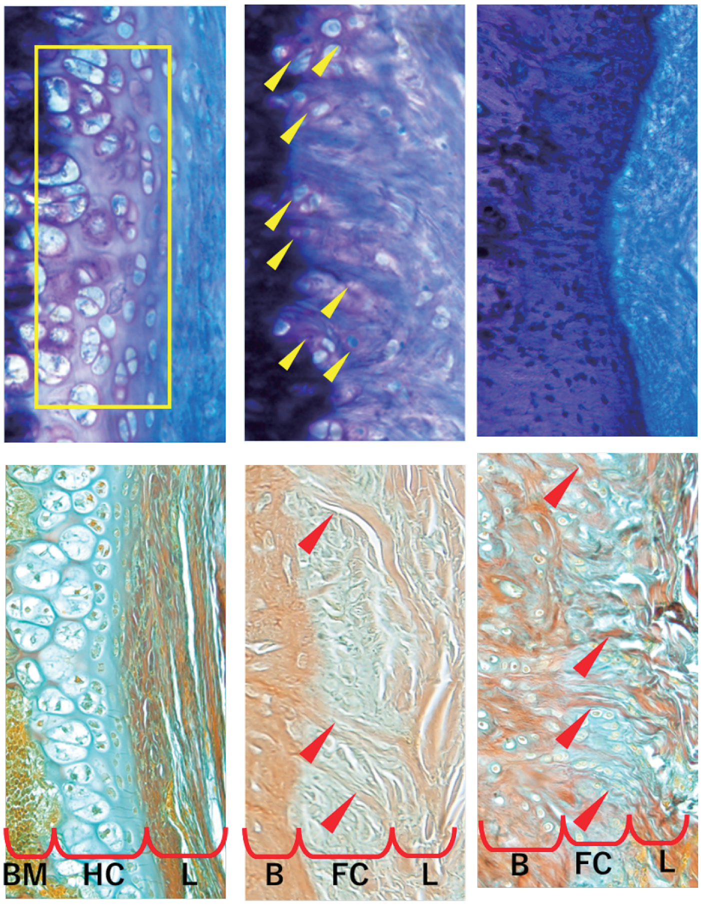

Fig. 3. Magnified images of femur side of the enthesis.
(Upper : Resin embedded sections, Lower : Paraffin embedded sections)
(Yellow frame, yellow arrowhead : Sites causing metachromasia,
Red arrowhead : Ligament fibers,
BM : Bone marrow, B : Bone, HC : Hyaline cartilage,
FC : Fibrocartilage, L : Ligament fibers)

Histological observation of Tibial side of the Enthesis

On the tibial side, the ligament fibers were directly attached to the bone through the periosteum. At the 3 weeks-old, the interface between the ligament and periosteum fibers and the bone was relatively smooth. At the 7 weeks-old, the interface was slightly uneven. At 13 weeks-old, the unevenness became more remarkable and corrugated. The paraffin specimens showed that the ligament fibers were pale orange at 3 weeks-old. On the other hand, the ligament fibers at 7 and 13 weeks-old showed a dark orange color. These results suggest that the staining of the ligament fibers becomes progressively darker with age, which may indicate an increase in the density of the ligament fibers (Fig. 4).

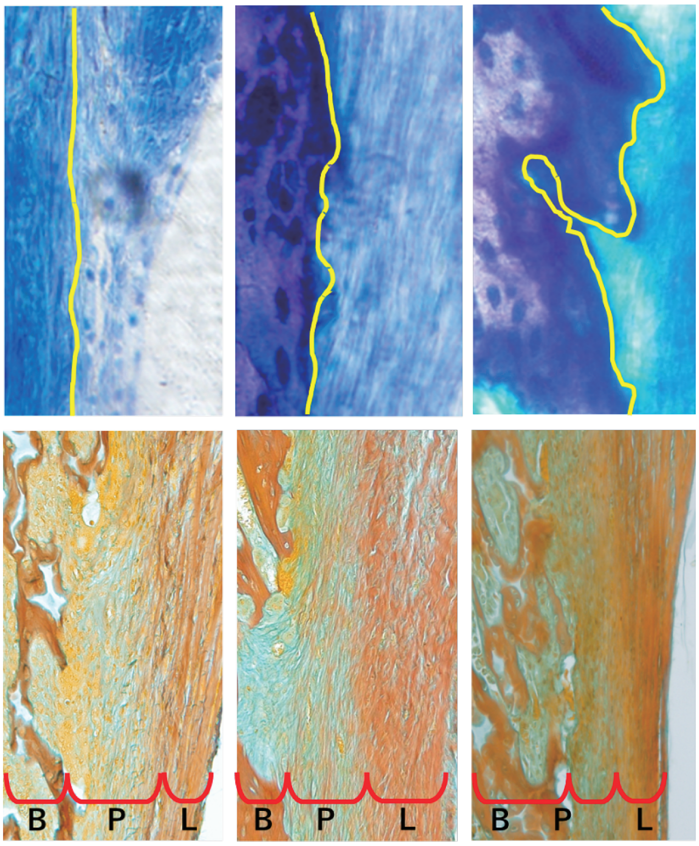

Fig. 4. Magnified images of tibial side of the enthesis.
(Upper：Resin embedded sections, Lower：Paraffin embedded sections)
(Yellow line：Boundary face between of the bone and the periosteum,
B：Bone, P：Periosteum, L：Ligament fibers)

Discussion

In this experiment, we used 3, 7, and 13 weeks-old developing rats to histologically observe the structural changes of enthesis in the femur and tibia of the MCL.

Functional integration of soft and hard tissues is essential for musculoskeletal locomotion [5]. Enthesis which is the attachment site of ligaments is composed of different tissues and these structural characteristics increase the resistance to mechanical stimuli applied to the enthesis. On the other hand, the anatomical structure of the enthesis can be roughly divided into direct insertion and indirect insertion [13]. The former is characterized by four layer structure consisting of ligament, uncalcified fibrocartilage, calcified fibrocartilage and bone [14]. On the other hand, the latter lacks fibrocartilage and the ligament is attached to the bone via the periosteum [6] (Fig. 5).

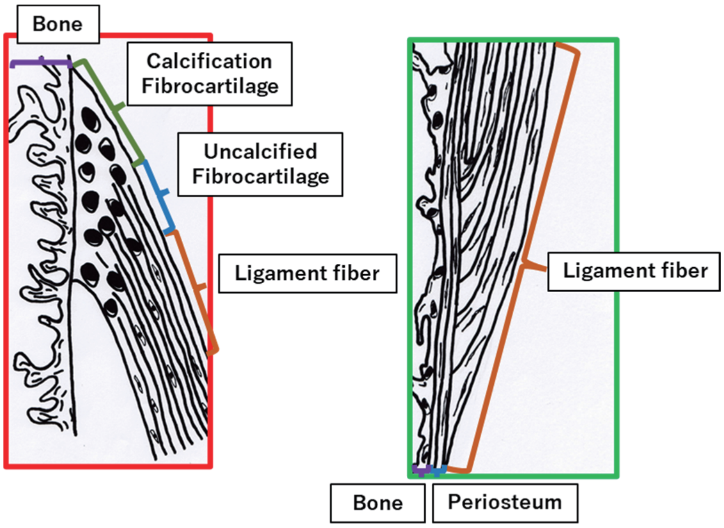

Fig. 5. Schematic diagram of portion of the enthesis of MCL.

Both ends of the rat MCL are attached to the medial aspect of the medial femur condyle and the upper part of the anterior medial aspect of the tibia, indicating that the enthesis of the MCL is located at the epiphysis and the diaphysis of each bone. This suggests that the origin and stop of the MCL are both long bones, but the structural characteristics of the enthesis are also different due to their different anatomical locations [6] (Fig. 6).

Fig. 6. Dried specimen of MCL.

The MCL supports the medial side of the knee joint, and together with the ligament tissue that also constitutes the knee joint is responsible for joint stability. In general, flexion and extension movements of the knee joint are pendulum movements with the femur side of enthesis as the fulcrum [11]. At this time, movement equivalent to the range of motion occurs in the vicinity of enthesis. On the other hand, the enthesis on the tibia side mainly pulls the tibia up to the femur side after fixing the tibia, and the area around the enthesis is slightly movable in the anterior-posterior direction compared to the range of motion [11]. Therefore, the femur side of the MCL must have structural characteristics that can resist mechanical stimulation associated with large movements, while the tibial side which is less mobile must have a fixation force to support the tibia securely. Thus, the two entheses at both ends of the MCL differ in their histological structure and functional aspects. In this study, we found that both entheses expanded their attachment area in the direction of the long axis of the bone during development. It was also more remarkable on the tibia side, although it was slight on the femur side. This was thought to be due to the difference in mobility around enthesis, and the enlargement of the attachment area was thought to be in conflict with the magnitude of mobility.

In vivo, it is said that hyaline cartilage exists in areas that are less receptive to mechanical stimuli, while fibrocartilage exists in areas that are more receptive [15]. In this study, hyaline cartilage was found in the femur side of the enthesis at the 3 weeks-old, but not at 7 and 13 weeks-old, and fibrocartilage was found instead. This suggests that the enthesis at 3 weeks-old is mechanically immature, but after 7 weeks-old, the fibrocartilage is resistant to mechanical loading and serves as a buffer between the ligament and the bone. The fibrocartilage fibers attached to the enthesis were parallel to the cartilage surface at 3 weeks-old, but tended to be embedded perpendicular to the cartilage surface at 7 and 13 weeks-old. This suggests that the miniaturization of the fibrocartilage with development indicates that the thickness of the ligament fibers around the fibrocartilage increased. In addition, the fibers that meandered perpendicular to the attachment surface could be assumed to increase the bonding strength with the fibrocartilage. The calcification of fibrocartilage on the femoral side of the enthesis increases with development [16]. In the present study, metachromasia in the intercellular matrix disappeared with development. Metachromasia is a heterochromatic staining unique to the intercellular matrix of cartilage, which is rich in acidic mucopolysaccharides [17]. This suggests that fibrocartilage tends to calcify with development, which also increases the strength of the enthesis. It has been suggested that the tibial side of the enthesis differentiates from periosteal cells to osteoclasts due to increased mechanical stimulation of the periosteum through the ligament [6]. Osteoclasts resorb bone and simultaneously induce bone formation by osteoblasts. In this study, we observed the enthesis on the tibia side of the resin specimen and found many irregularities at the interface of the enthesis as it developed. In the present study, we observed many irregularities at the interface of the enthesis on the tibial side of the resin specimen, suggesting that these irregularities are due to bone remodeling caused by increased mechanical stimulation. On the other hand, on the tibial side, a marked enlargement of the attachment occurs with development. Therefore, it can be speculated that the formation of irregularities on the tibial side is a structural change to increase the surface area of the attachment, leading to an increase in the bond strength between the ligament and periosteal fibers and bone. It is believed that the synthesis of the matrix fibers that make up the ligament is accelerated by increased mechanical stimulation due to exercise [18]. In this study, we showed that physical staining caused differences in the staining properties of ligament fibers at each week of age. This reflected an increase in the density of ligament fibers, suggesting that the enthesis of matrix fibers was activated by increased mechanical stimulation during development.

Conclusion

The structural changes in the MCL of the Enthesis during development suggest that it has acquired the ability to resist mechanical stimuli that are heightened by growth-induced increases in body weight and activity.

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発育期ラットにおける膝内側側副靭帯付着部の構造変化に関する組織学的研究

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要　旨

【背景】内側側副靭帯(MCL)は加重に伴う機械的刺激を受けやすく、それは大腿骨および脛骨付着部であるEnthesisに作用する。身体への加重は発育に伴って著しく増加する。
【目的】本研究では異なる週齢の発育期ラットを用いて、MCLの大腿骨および脛骨におけるEnthesisの構造変化を組織学的に観察し、比較、検討することを目的とした。
【材料および方法】3、7、13週齢のWistar系雄性ラット24匹(各8匹) を用い、種々の標本を作製して、大腿骨および脛骨のMCL付着部の構造を光学顕微鏡により観察した。
【結果】膝関節前額断の非脱灰樹脂研磨標本を弱拡大にて観察すると、大腿骨側に比べて脛骨側の付着部領域が顕著に拡大していた。同標本の大腿骨付着部を強拡大にて観察すると、増齢に伴ってメタクロマジーを起こす範囲が縮小しており、付着部が順次、石灰化することを認めた。また、同部位を脱灰パラフィン切片にポリクローム染色して観察すると、発育に従って、付着面に垂直かつ複雑に交差しながら深く埋入する線維を多く認めた。大腿骨付着部と同様に脛骨付着部も観察すると、週齢が進むのに従い、線維と骨との界面に多くの凹凸が形成されることを認めた。また、順次、線維密度が上昇することも認めた。
【考察】MCLのEnthesisは発育に伴って結合力を高めた構造に変化することが示唆された。
【結論】発育に伴うMCLのEnthesisの構造変化は、成長による自重や活動性の増加により高まる機械的刺激に抵抗する機能を獲得すると考えられた。