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ORIGINAL ARTICLE

Acute molecular biological responses during spontaneous anterior cruciate ligament healing in a rat model

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Abstract

Background Anterior cruciate ligament (ACL) injury is one of the most common injuries of the knee joint and is becoming more prevalent. While ACL reconstruction is considered the "gold standard" of treatment, some studies have demonstrated natural spontaneous healing of the ACL in humans, rabbits, and rats. At this time, the mechanism of ACL healing is poorly understood.

Aims The purpose of this study was to determine the process of ACL healing by examining the molecular and histological changes in the acute phases, using an ACL-Transection model and a spontaneous ACL healing model.

Methods Sixty adult male Wistar rats were randomly assigned to two groups: the ACL transection (ACLT) group and the controlled abnormal movement (CAM) group. Thirty rats were randomly assigned to three groups (day 1, day 3, and day 5). Then, all rats underwent an ACL transection procedure. The CAM group rats underwent controlled abnormal extra-articular tibial translation. Samples were harvested from rats and used for histological and biochemical analyses.

Results Both, the ACLT and the CAM groups exhibited ruptured ACLs. However, in the CAM group, the ends of the proximal remnants were not retracted. Expressions of MMP-3 and PDGF-α increased, and expression of TGF-β1 decreased in the CAM group on day 5 ($p < 0.01$); PDGF-β expression in the CAM group increased significantly at each time point ($p < 0.01$).

Conclusion Our results suggested that controlling abnormal movements changed intra-articular responses positively during the acute phase both histologically and biochemically.

Keywords Anterior cruciate ligament · Anterior cruciate ligament injury · Wound healing · Rehabilitation · Acute-phase reaction

Abbreviations

ACL	Anterior cruciate ligament
ACLT	Anterior cruciate ligament transection
CAM	Controlled abnormal movement
MCL	Medial collateral ligament
PTOA	Post-traumatic osteoarthritis

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Introduction

The anterior cruciate ligament (ACL) functions to control the static and dynamic stability of the knee. ACL injury is a common injury of the knee joint that is becoming more prevalent. After ACL injury, the knee joint has abnormal kinematics during all movements. Therefore, articular cartilage, meniscus, and subchondral bone undergo irreversible changes and post-traumatic osteoarthritis (PTOA) often develops [1–4].

Most of the current research regarding the ACL has focused on intra-articular responses after ACL injury and has reported that the ACL has poor healing capabilities due to blood supply [5], molecular responses [6], scaffold formation [7, 8], and synovial fluids [9]. Therefore, effective treatment of any ACL injury is required. Currently, ACL reconstruction is the "gold standard" method of treatment for the ACL. However, Streich et al. [10] suggested that it is impossible to prove that ACL reconstruction reduces the incidence of PTOA or improves the long-term symptomatic outcome. Further, ACL reconstruction is effective in treating ACL injury but is restricted for use due to various factors (e.g., subject's age). Bigoni et al. strongly recommend surgical treatment for pediatric ACL tears [11] and reported that arthroscopic ACL repair showed favorable results in skeletally immature patients with proximal ACL avulsion tears [12].

However, some studies have reported natural spontaneous healing of the ACL with conservative treatment [13–15]. Ihara et al. focused on the abnormal joint movement that occurs after ACL injury and reported that the ACL could heal spontaneously with dynamic stabilization and control of abnormal movement [16, 17]. Furthermore, our laboratory studied the controlled abnormal movement model exhibiting spontaneous ACL healing [18]. The histological results showed spontaneous ACL healing 2 weeks after ACL injury, and the biomechanical results demonstrated that the mechanical properties at 8 weeks after injury was < 50% of the intact ACL strength. Moreover, we suggested that controlled abnormal joint movement of the knee with an ACL injury in a rat model changes the biomolecular response, enhances the healing response, and leads to spontaneous healing. Thus, conservative treatment that brings about spontaneous ACL healing has the potential to be a new effective treatment for ACL injury.

To increase the incidence of spontaneous ACL healing with conservative treatment, it is necessary for the clinician to be aware of its mechanism; however, it is poorly understood. Many factors including mechanotransduction mechanism [19], inflammatory cytokines [20], matrix metalloproteinases (MMPs) [6, 7, 21], and growth factors [22, 23] were reported to be related to the ligament healing, while no study has clearly shown the influence of a specific factor. Murray et al. [8] also indicated that the human ACL undergoes four histological phases after injury, consisting of inflammation, epiligamentous regeneration, proliferation, and remodeling. In general, long-term chronic inflammation delays the transition to proliferation and remodeling after inflammation, resulting in delayed wound healing. Hence, the role of inflammatory phase is important. Nishikawa et al. studied molecular biological response in intra-articular tissues during the acute phase of ACL injury [24]. They suggested that controlling abnormal movement inhibited the inflammatory reaction in intra-articular tissues after ACL injury. However, factors that they evaluated were anabolic and catabolic factor. To understand the acute phase of ACL healing in detail, it is necessary to analyze cytokine and collagen synthesis factors. Bigoni et al. report on cytokine fluctuation in synovial fluid in the acute phase after human ACL injury. They show that IL-1ra, IL-6 and IL-10 increased in the acute phase, 0–48 h after ACL injury, and IL-1β and IL-8 increased in the sub-acute phase, 3–15 days [25]. According to preoperative follow-up observation, IL-6, IL-8 and IL-10 increased in the acute phase after injury, then decreased at the time of pre-operation, about 1 m after injury, and then increased again after surgery [26].

The purpose of this study was to determine the process of ACL healing by examining the molecular and histological changes in the acute phases, using a CAM model demonstrating spontaneous ACL healing and an ACL-Transection (ACLT) model demonstrating the failure of ACL healing. We hypothesized that these molecular and histological changes are observed during the acute phases in vivo.

Methods

Experimental design

All experiments were approved by the University Animal Experiment Ethics Committee (authorization number 26-5). Sixty male adult Wistar rats (age 11 weeks) were obtained for use in this study (Japan SLC, Hamamatsu, Japan). These 60 rats were randomly assigned to two groups (thirty in each group): the ACL transection (ACLT) group and the controlled abnormal movement (CAM) group. Thirty rats were randomly assigned to three groups (day 1, day 3, and day 5; 10 rats per group) (Fig. 1). Of these 10 rats, three were used for histological analysis and the other seven were used for biochemical analysis. The Intact group was used as a control, using the contralateral limbs of the ACLT group. The rats were allowed free access to water and food, while the room temperature was kept at 23 ± 1 °C with a humidity of 55 ± 5% and a 12-hour light–dark cycle.

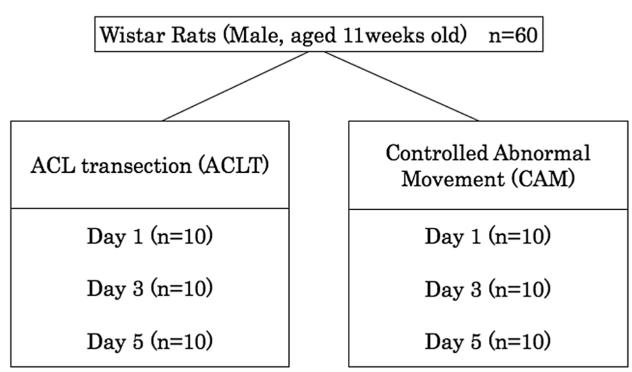

Fig. 1 Assignment of the subjects. Sixty rats were randomly assigned to two groups: the ACLT group or the CAM group. The 30 rats in each group were then randomly assigned to three groups (day 1, day 3, or day 5). Then, of the 10 rats in each sub-group, three were used for histological analysis and the remaining seven were used for biochemical analysis

Surgical procedure

All rats underwent an ACL transection procedure. Additionally, rats in the CAM group underwent a controlled abnormal movement of the right knee, as previously described [18]. Animals were anesthetized by intraperitoneal injections, and medial parapatellar arthrotomy was performed to transect the ACL horizontally at the mid-portion. Functional loss of the ACL was verified using the Lachman test. Subjects were fixed in a supine position with the knee passively flexed. The femur was fixed with one hand by the examiner, and the tibia was pulled forward with the other hand. The examiner then observed anterior displacement of the tibia. The joint capsule was then closed with a running suture. In the ACL-T group, the skin was closed. In the CAM group, a 1 mm diameter steelhead was drilled into the medial aspect of the tibial tuberosity in the mediolateral direction to control anterior tibial translation. A double 3–0 nylon suture was tied tightly through the tibial bone hole posterior to the condyle of the distal end of the femur. It was placed outside the joint capsule (Fig. 2). The Lachman test was performed to check the prevention of anterior tibial translation. The skin of the CAM group rats was then closed with running and interrupted suture. Animals were allowed unrestricted cage movement following surgery during the experimental period. All surgical procedures and the Lachman test were performed by the same operator and examiner.

Tissue collection

At the time of euthanasia, samples of the knee joints and ACLs were harvested at specified time points from both limbs of rodents in the ACLT group and from the right limbs of rodents in the CAM group (day 1, day 3, and day 5). Samples from the left limbs of the rats in the ACLT group were used for control as the Intact group. Knee joints were used for histological analysis. The muscle from each knee joint was removed. Then, the tibia and femur were cut 2 cm from the articular surface and washed with phosphate-buffered saline (PBS) to remove blood components. After that, the knee joints were fixed in 4% paraformaldehyde for 48 h. After fixation, the knee joints were washed three times with PBS and then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in PBS for 60 days. The 10% EDTA was changed after the first 3 days and then once a week afterwards. After decalcification, the knee joints were washed three times with PBS and soaked in serial dilutions of 10% sucrose/PBS for 4 h, 15% sucrose/PBS for 4 h, 20% sucrose/PBS overnight at 4 °C. Afterwards, the knee joints were immediately embedded and frozen in OCT compound (Sakura Finetek Japan Co., Ltd, Tokyo, Japan) and then stored at − 80 °C in a deep freezer. Later, these were cut into 14 µm longitudinal sections using the Leica CM3050S (Leica, Wetzlar, Germany) and stored at − 25 °C until histological analysis. ACLs that were previously harvested were used for biochemical analysis. Each ACL was washed with PBS to remove the blood components and stabilized in Allprotect Tissue Reagent (QIAGEN, Hilden, Germany) at 4 °C

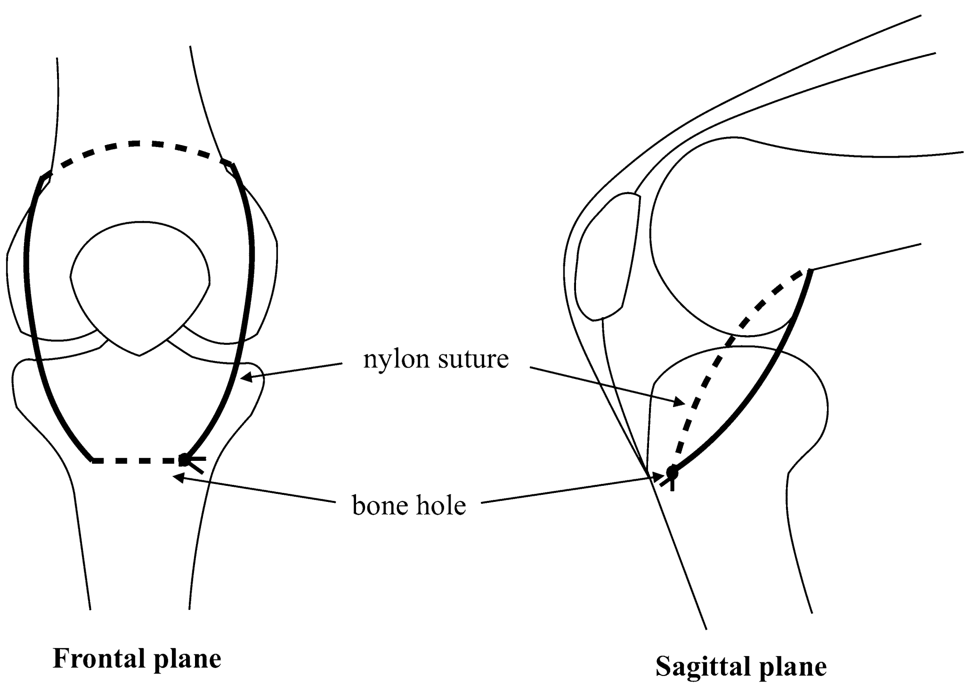

Fig. 2 Surgical procedure for the CAM group. The CAM group rats controlled abnormal tibial translation from the outside of the joint capsule

for 48 h. After stabilization, the Allprotect Tissue Reagent was removed from the ACLs and stored at − 80 °C.

Hematoxylin and eosin staining

Longitudinal sections were dried for 30 min at room temperature and then stained with Hematoxylin and Eosin (Sakura Finetek Japan Co., Ltd, Tokyo, Japan). Each section was enclosed with EXCEL mount, and the section was observed under a microscope (Leica, Wetzlar, Germany).

Quantitative real-time PCR

ACLs were examined for messenger RNA (mRNA) expression of several genes, which indicated the relevance for ACL healing, using real-time polymerase chain reaction (PCR). Tissue samples were homogenized by TissueLyser (Qiagen, Venlo, Netherlands), and RNA was extracted using a RNA extraction kit (Allprep DNA/RNA/Protein mini kit, Qiagen). Total RNA was transcribed into cDNA using a High Capacity Transcription Kit (Applied Biosystems, Foster City, CA, United States). Real-time PCR was performed using the Step One Plus System (Applied Biosystems, Foster City, CA, United States) with Taqman Gene Expression Assay probes for the following transcription factors according to the manufacturer's instructions: nuclear factor-kappa B (NF-κB) (Rn01399583_m1), interleukin-1β (IL-1β) (Rn00580432_m1), tumor necrosis factor α (TNF-α) (Rn01525860_g1), MMP-13 (Rn01448194_m1), tissue inhibitor of metalloproteinase-1 (TIMP-1) (Rn01430873_g1), MMP-3 (Rn00591740_m1), transforming growth factor-1β (TGF-β1) (Rn00572010_m1), platelet-derived growth factor (PDGF-α) (Rn00709363_m1), and PDGF-β (Rn01502596_m1). Gapdh (Rn01775763_g1) was used as a housekeeping control. The transcript level of the target genes was calculated and normalized to Gapdh using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Statistical tests on gene expression were performed using SPSS 23 for Windows (APSS Japan Inc, Tokyo, Japan). Gene expression data are presented as the mean ± standard error. One-way analysis of variance was used to assess differences in the group and the Tukey method was used to perform multiple comparisons among groups if variances were equal, or the Games–Howell methods were used to perform multiple comparisons among groups if variances were not equal. $p < 0.05$ was assumed to indicate statistical significant for all gene expression.

Results

Histological findings

In the intact group, ACLs were observed on day 1, day 3, and day 5 (Fig. 3). Collagen fibers were arranged in one direction from the femur to the tibia. In the ACLT group, the ruptured ACLs were observed, the ends of proximal remnants were displaced backwards, and the remnant gaps were expanded at each time point (Fig. 3d–f). On day 5, the destruction of the distal remnant was observed (Fig. 3f). In the CAM group, ruptured ACLs were also observed (Fig. 3g–i). However, backwards displacement of the proximal remnants, extension of the remnant gaps, and destruction of the distal remnants were not observed. Moreover, healing tissue was observed in the remnant gaps (Fig. 3h, i).

Results of transcriptional analysis

Gene expressions of NF-κβ, IL-1β, TNF-α, MMP-13, and TIMP-1 were not significantly different between the ACLT group and the CAM group at each time point (Fig. 4a–e). However, the gene expression of MMP-3 and PDGF-α in the CAM group increased significantly compared to that of the ACL-T group at day 5 ($p < 0.01$) (Fig. 4f, h). The gene expression of TGF-β1 in the CAM group decreased significantly compared to that of the ACLT group on day 5 ($p < 0.01$) (Fig. 4g). The gene expression of PDGF-β in the CAM group increased significantly compared to that of the ACLT group at each time point ($p < 0.01$) (Fig. 4i).

Discussion

In this study, we determined the process of ACL healing by examining the molecular and histological changes in the acute phases, using a CAM model demonstrating spontaneous ACL healing and ACLT model demonstrating the failure of ACL healing. There were significant differences between the ACLT group and CAM groups.

Hematoxylin and eosin staining that was performed to observe cell morphology showed collagen fibers arranged in a certain direction from the femur to the tibia in the intact group. This result agrees with the histological theory that collagen fibers are regularly arranged in a parallel manner [27, 28]. In the ACLT group, ruptured ACLs were observed and the ends of the proximal remnants were displaced backwards. With time, we observed the expansion of the remnant gaps and destruction of the distal remnants. Similar results have been reported by studies of the human ACL injury by Murray et al. [8]. However, in the CAM group, the

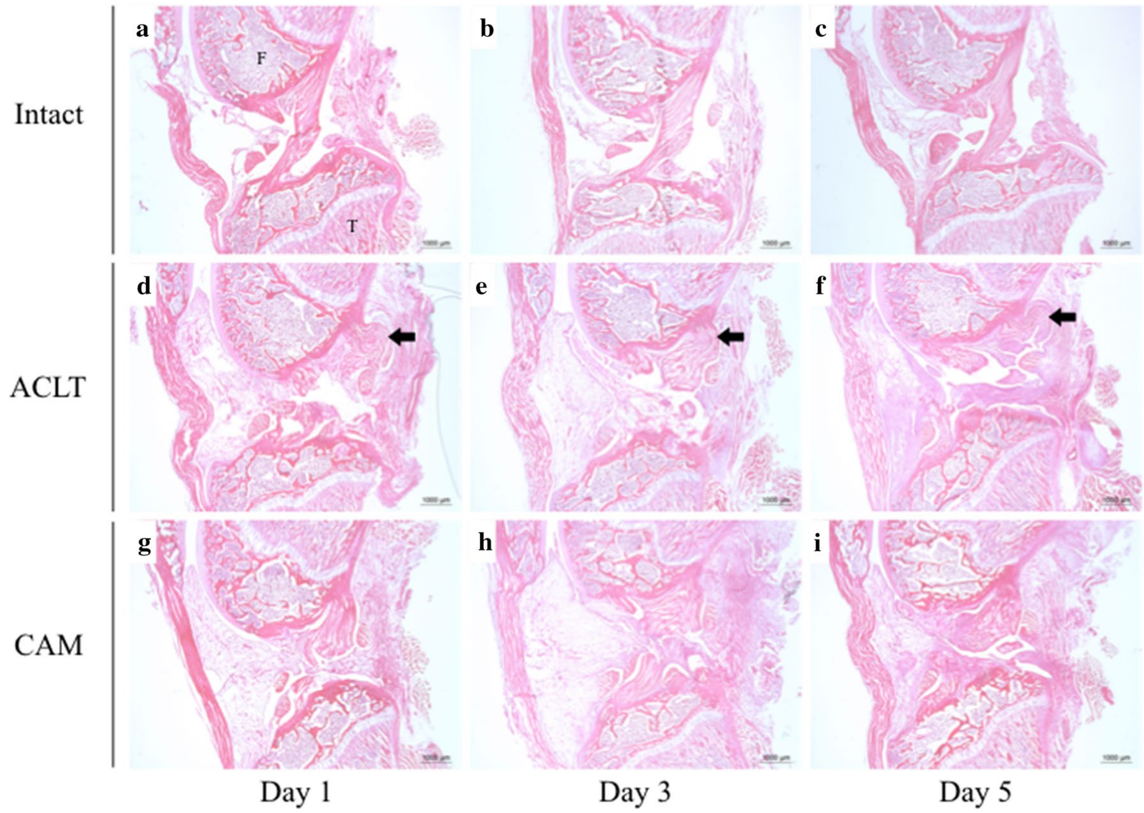

Fig. 3 Histological differences in each group at 1, 3, and 5 days post-operatively. In the Intact group, collagen fibers were arranged in unidirectionally from the femur to the tibia. In the anterior cruciate ligament transection (ACLT) and controlled abnormal movement (CAM) groups, the ruptured ACLs were observed. However, the backward displacement of the proximal remnants, the extension of the remnant gaps, and the destruction of the distal remnant were not observed in the CAM group. *F* Femur, *T* Tibia, Arrows mark displaced backwards of proximal remnants. Scale bar represents 1600 µm

backwards displacement of proximal remnants, extension of the remnant gaps, and destruction of the distal remnant were not observed. Moreover, during healing, the healing tissue was observed in the end of remnants. These results support the results of a previous study [18]. The time-dependent changes in the CAM group were similar to the process of the medial collateral ligament (MCL) healing that was reported by Chamberlain [29]. There is a difference in the mechanical environment between the ACL and the MCL, but it was found that the healing process is similar.

While destruction of the distal remnant was observed in the ACLT group, morphological destruction of remnants was not confirmed in the CAM group. Rather, we observed narrowing of the gaps with healing tissue in the CAM group. The only difference between the two groups was whether abnormal joint movement could be controlled. Therefore, it is suggested that abnormal joint movement induces destruction of the ACL remnant and that destruction of intra-articular tissue could be restrained by controlling abnormal joint movement. In general, distraction of intra-articular tissue, as had occurred in this ACLT group, delays inflammation and results in wound healing failure. Thus, the correction of abnormal kinetics, including controlling abnormal joint movement after ACL injury, resulted in changes in mechanical stress and prevented not only the distraction of intra-articular tissue but also chronic inflammation.

As a result of using real-time PCR analysis, significant differences between the ACLT group and the CAM group were observed with respect to gene expressions of PDGF-β on day 3 as well as MMP-3, TGF-β1, PDGF-α, and PDGF-β on day 5. The gene expression of MMP-3 increased significantly on day 5 in the CAM group compared to the ACLT group. The function of MMP-3 varies [30]. During the wound healing process, MMP-3 degrades the collagen and fibronectin of the extracellular matrix [31]. Beye et al. [6] and Xie et al. [32] reported that a significant increase in the expression of the MMP-3 in the ACL was observed from the early stages of ACL injury and that high expression of MMPs could inhibit ACL healing. However, post-injury day 5 is the beginning of the transition period from the inflammatory phase to the proliferative phase in the rat model. The expression of MMP-3 at 2 weeks after injury using the same model was higher in the ACLT group compared to the CAM group [18]. In addition, it has been reported that

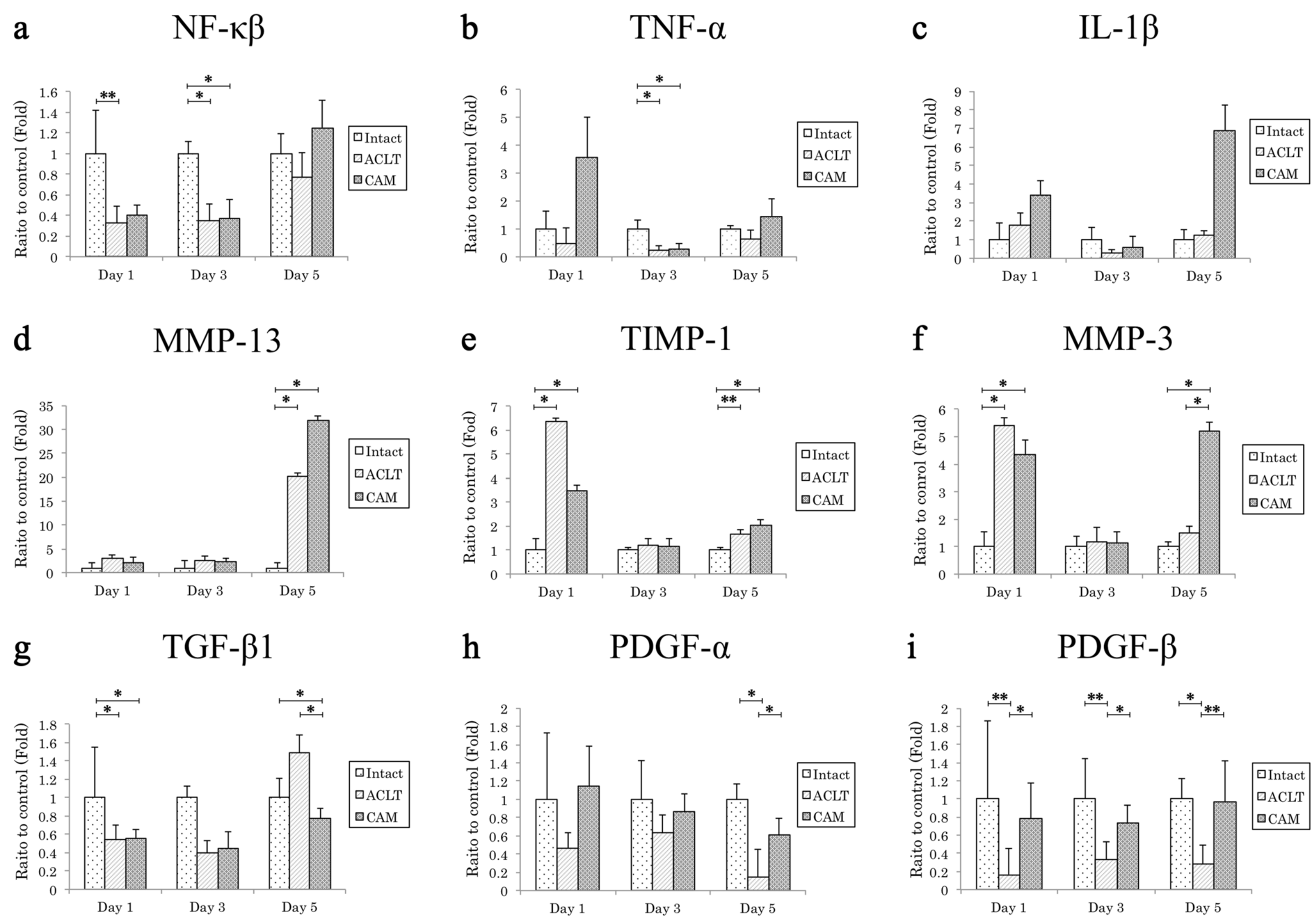

Fig. 4 Gene expression at 1, 3, and 5 days postoperatively. **a** Nuclear factor-κβ (NF-κβ), **b** interleukin-1β (IL-1β), **c** tumor necrosis factor-α (TNF-α), **d** matrix metalloproteinase-13 (MMP-13) and **e** tissue inhibitor of metalloproteinase-1 (TIMP-1) were not significantly different between rodents in the anterior cruciate ligament transection (ACLT) and controlled abnormal movement (CAM) groups. **f** MMP-3 expression was increased in the CAM group compared to the ACLT group at 5 days postoperatively ($p < 0.01$). **g** Transforming growth factor-β1 (TGF-β1) expression was decreased in the CAM group compared to the ACLT group at 5 days postoperatively. **h** Platelet-derived growth factor-α (PDGF-α) expression was increased in the CAM group compared to the ACLT group at 5 days postoperatively and **i** PDGF-β was increased in the CAM group than the ACLT group at all time points. $*p < 0.01$, $**p < 0.05$

MMP-3 has a role in the remodeling of the extracellular matrix, cell proliferation, and angiogenesis [33]. Therefore, it is suggested that a significant increase in the expression of the MMP-3 in the CAM group on post-injury day 5 would not inhibit ACL healing.

TGF-β1 was significantly decreased in the CAM group compared to the ACLT group on day 5. PDGF-α was significantly increased in the CAM group compared to the ACLT group on day 5. PDGF-β was significantly increased in the CAM group relative to the ACLT group on day 1, day 3, and day 5. TGF-β1, PDGF-α, and PDGF-β are healing-related factors, and these factors stimulate fibroblast proliferation and collagen production by fibroblasts. In a previous report, Beye et al. [6] reported that the expression level of TGF-β1 significantly increased after MCL injury, whereas the expression level of TGF-β1 did not show a significant change after ACL injury. Similarly, in histological study, Murray et al. [22] and Lee et al. [23] showed elevated levels of Pdgf and TGF-β1 expression at the MCL injury site and its surrounding area, whereas expressions of Pdgf and TGF-β1 at the ACL injury site and its surrounding area was poor. The expression of healing-related factors has been observed in the MCL, which is thought to have high self-healing abilities, and the expression of healing-related factors is likely to be important for ligament repair and remodeling. Based on the expression levels in the intact group, the expression levels in the ACLT group tended to fluctuate more than did those of the CAM group. Therefore, the large variation in healing-related gene expression level after ACL injury in the ACLT group, such as the decrease in PDGF-β on day 1 and day 3, and the increase in TGF-β1 and decrease in PDGF-α and PDGF-β on day 5, compared to that of the intact group, might inhibit healing. However, in the CAM group, there was no similar significant change in the levels of expression of healing-related factors; this may be the basis for ACL healing.

The number of ACL injury patients has increased across broadly since more people now participate in sports. Notably, the majority of patients who have undergone ACL reconstruction are young, who often seek to return to aggressive sports that resulted in the original injury. In these patients, surgery may be appropriate. Conversely, children, middle-aged patients, and the elderly often are not suitable candidates for surgical treatment. Historically, ACL reconstruction has been avoided for skeletally immature children due to the risk of growth plate injury and growth disturbances. Furthermore, orthopedists do not recommend that middle-aged and aged patients undergo ACL reconstruction because of poor results after ACL reconstruction. In addition to the subject's ages, it is important to consider the timing of intervention. The spontaneous ACL healing that has been documented in previous studies involved intervention being performed immediately after ACL injury [16, 17]. Similarly, in this study, early intervention, which involves controlling abnormal movement, was performed after ACL injury and showed positive effects both histologically and biochemically. Thus, correcting abnormal kinetics during the early stages of ACL injury is critical for spontaneous ACL healing. If the mechanism by which ACL healing can be elucidated, thus allowing conservative treatment for spontaneous ACL healing, as reported by Ihara [16, 17], it is possible to offer better treatment options for children, middle-aged patients, and the elderly with ACL injuries. This change would greatly improve the standards for patients with an ACL injury.

There are several limitations to the present study. First, this study is not shown the ACL healing in the histological analysis during the inflammatory phase. Second, the mechanism of ACL injury differs from that of humans. Third, we examined only gene expression as biochemical analysis. In the future, further research using the proliferative and remodeling phase and other methods of ACL injury, and protein levels analysis of factors considered in this study should be conducted to understand the process of ACL healing in detail.

Conclusion

Though natural spontaneous healing of the ACL was shown, the gold standard treatment after ACL injury is clinical ACL reconstruction. The reason for this is because traditional conservative treatment could not expect ligament healing, due to aftereffects such as PTOA, and decline the quality of life. Our laboratory performed some studies about the ligament's morphology and function, which heal naturally and spontaneously, and the mechanism of ligament healing. We have focused on abnormal joint movement, and it is unique characteristics compared from other studies.

In this study, controlling abnormal movement immediately after ACL injury leads to changes in mechanical stress and could prevent the destruction of intra-articular tissues; we could thus provide knowledge about the changes in intra-articular tissue during the acute phase of ACL healing.

It is important to control abnormal joint movement with ACL injury from a kinematic viewpoint. Conservative therapy would be useful in children, middle-aged, and aged patients with ACL injury to maintain joint stability. It is possible to elucidate the mechanism of ACL healing, develop new therapeutic methods including orthotic therapy, and present new findings to improve treatment outcomes by continuing histological and biochemical analyses in the future.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest directly relevant to the contents of this article.

Human and animal rights This study is an animal experimental study. All experiments were approved by the University Animal Experiment Ethics Committee (authorization number 26-5). In addition, in the actual experiments, the authors undertook to minimize the number of animals and stress on the animals.

Informed consent Informed consent is not applicable for this type of study.

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