Promotion of rabbit ligament healing by local delivery of hepatocyte growth factor

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Running title:

Effects of HGF on ligament healing

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Introduction

Extracapsular ligament injuries of the knee and ankle are common in sports activities. For example, isolated medial collateral ligament (MCL) injuries of the knee joint have been found to heal with conservative treatment with bracing and activity restriction for a few months required for sufficient healing of the ligament.[1] Yet, the mechanical properties, collagen types and histological appearance are not restored to pre-injury levels even after 1 to 2 years.[2,3] Clinically, severe isolated MCL injuries take several months for return pre-injury activity level, and they may require MCL surgery because of residual valgus laxity. Controversy remains regarding the best treatment for the MCL in combined ligamentous injuries.[1] Thus, enhancement of ligament healing would increase the stability of the joint and allow patients to return to athletic activities earlier and with greater safety.

Several different approaches, including growth factor treatment, hyaluronan treatment, hyperbaric oxygen treatment, gene transfer, cell therapy, mechanical stimulation, and the use of scaffolds have been studied in an attempt to accelerate and improve the healing of ligaments.[4,5,6] Among growth factors, hepatocyte growth factor (HGF) was originally identified and cloned as a growth-promoting factor for mature hepatocytes.[7,8] The c-Met membrane-spanning tyrosine kinase is functional receptor for HGF. The c-Met receptor is expressed in a wide variety of cells and HGF-Met coupling exhibits biological activities, such as promotion of cell proliferation and migration, prevention of cell death, and induction of morphogenesis. Based on these biological activities, HGF plays roles in tissue repair and protection. Administration of HGF or expression of the HGF gene promotes tissue regeneration or improves pathology in various disease models in different tissues. In the field of orthopedic surgery, local expression of the HGF gene promotes healing of bone fractures.[9] In addition, recombinant HGF administration enhances tendon healing in a bone tunnel and leads to improved biomechanical fixation in a shorter period of time.[10] HGF

prevents fibrosis or scar formation in different tissues after injury and facilitates tissue regeneration without fibrotic change.[11,12,13,14] The advantages of ligament healing without excessive fibrosis and scar formation are considerable for the recovery of a functional MCL.

Based on these previous studies, we hypothesized that HGF administration would enhance healing of a ligament and lead to better structural properties in a shorter period of time. To test this hypothesis, we locally administered recombinant HGF in a MCL healing model in rabbits.

Materials and methods

HGF

Recombinant human HGF (the 5 amino acid-deleted type) used in this study was obtained from Kringle Pharma, Inc. (Osaka, Japan).

The MCL model in rabbits

A total of 33 mature female Japanese white rabbits weighing between 2.5 and 3.0 kg were used. The animal experiments were carried out in strict accordance with the regulations of the Institutional Animal Care and Use Committee, Kanazawa University, Japan. The rabbits were anesthetized with sodium pentobarbital (Kyorin Pharmaceutical Co., Tokyo, Japan). Using aseptic techniques, each rabbit was subjected to surgical transection of the midsubstance of the MCL of both hind limbs, with one limb serving as the experimental limb and the contralateral limb serving as the control. The MCL in a rabbit is an easily identifiable structure on the medial side of the knee. In this study, the MCLs were exposed by a longitudinal incision through the overlying fascia, sharply transected just distal to the joint line, but not sutured. A small gap was thus formed between the

opposing ligament ends without repair or immobilization. For all rabbits, the wound was then irrigated and the fascia and skin were closed with 4-0 nylon sutures. These rabbits were allowed to move around unrestricted in individual cages and received a standard diet and water. During the immediate post-operative period and at post-operative days 1 to 6, the rabbits were injected with 100 μL of 100 μg/mL human recombinant HGF solution into the right MCL (HGF-treated group), while the left MCL was injected with 100 μL saline (control group). We chose this dose and dosing period based on a previous study in a tendon-bone healing model.[10] HGF was injected into the MCL gap or scar tissue percutaneously using a 29 gauge needle. Before the experimental series, a preliminary study was carried out in which 100 μL of aqueous methylene blue solution was injected using the same procedure. Because the rabbit knee had been sheared, the MCL gap was clearly observed. We confirmed that the injected solution stayed at the gap or scar tissue with minimal diffusion to surrounding tissues in the reproducible manner. Rabbits were sacrificed with an overdose of sodium pentobarbital as follows: 1 week after surgery, n = 3; 3, 6 and 12 weeks after surgery, n = 8. The femur-MCL-tibia complexes were extracted from both hind limbs. At 1 week after surgery, we performed histological evaluations only. At later time points, the 8 rabbits were allocated to either histological evaluation (n = 3) or biomechanical evaluation (n = 5). For immunohistochemical analysis of c-Met expression, one rabbit was sacrificed after 1, 3, 7, 21, 42 and 84 days.

Immunohistochemical analysis

Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C and dehydrated through graded alcohols (80% then 100%). They were decalcified using a formic acid-sodium citrate solution. Specimens were embedded in paraffin (Histprep568, Wako Junyaku, Osaka, Japan), and then they were sectioned

coronally with respect to the MCL. The sections were 2 μm thick and subjected to indirect immunofluorescence staining. Paraffin sections were treated with anti-Met (B-2) monoclonal antibody (SC-8057, Santa Cruz Biotechnology Inc., California, USA; dilution 1:100) and peroxidase-conjugated goat anti-mouse immunoglobulin (EnVision, DAKO, Carpinteria, CA, USA) was used as the secondary antibody. To develop the color, a DAB kit (EnVision, DAKO) was used. The sections were counterstained with Mayer's hematoxylin.

Histological evaluation

Femur-MCL-tibia complexes were excised immediately after sacrifice of the rabbit and the specimens were rapidly fixed in 10% formalin. Conventional paraffin-embedded sections were prepared. The specimens were sectioned at 2 μm thickness coronally with respect to the MCL and stained with Masson's trichrome. Cellularity, vascularity, and alignment of collagen fibers were qualitatively assessed during histological evaluation.

Biomechanical evaluation

The hind limbs of each rabbit were disarticulated at the hip, wrapped in saline-soaked gauze, immediately packed in plastic bags and stored at -80°C. The night before biomechanical testing, the specimens were thawed at 4°C.[15] All soft tissue, including muscles and fascia, was removed from the femur and tibia except the MCL. Bones were transected 4 to 5 cm from the joint line. The specimens were kept moist with 0.9% saline solution during testing. Both the HGF-treated and control femur-MCL-tibia complexes from each rabbit were mechanically tested using an electromechanical testing machine (AUTO GRAPH DCS25T, Shimadzu Co., Kyoto, Japan). Each femur-MCL-tibia complex was mounted in a custom jig to ensure that the tensile load could be applied along the

longitudinal axis of the ligament. A tensile force was applied at a constant elongation rate of 10 mm/min.[15] The structural properties of the femur-MCL-tibia complex (maximum load at failure, stiffness and energy absorbed at failure) were determined from the load-displacement curves of the complex by material testing software (TRAPEZIUM2, Shimadzu CO., Kyoto, Japan). Furthermore, the site of failure for each ligament was recorded.

Statistical analysis

Statistical significance was calculated using a Student's t-test to compare results between two groups. A p value < 0.05 was considered significant.

Results

Gross examination

Careful examination of the ligaments after 1 week revealed that the gaps in the MCLs were not completely filled with reparative tissue. However, by 3 weeks, the MCLs had clearly healed and showed a continuous neo-ligamentous tissue. In the HGF-treated and control groups, the appearances were similar in terms of scar mass and adhesion. The healing tissue was yellowish-white and translucent at the injury site. The boundaries of the healing MCL mass were difficult to define because they blended into the surrounding tissue. There was no evidence of infection, the decrease in range of motion of the knee joint and excessive inhabitation of scar formation in both groups.

Immunohistochemical evaluation

To see the distribution of cells that express c-Met receptor, tissues at the injury sites were subjected to immunohistochemistry. In control limbs, clear c-Met expression was not

observed (Fig. 1). In contrast, c-Met expression was observed in cells, presumably fibroblasts or fibroblastic cells, particularly at opposing ligament ends in the HGF-treated limbs 7 days after surgery (Fig. 1).

Histological changes

One week after surgery, inflammatory cells and fibroblasts infiltrated the edematous granulation tissue of the ligament gap in both groups. Neovascularization was observed more abundantly in the HGF-treated limbs compared to the reparative tissues in control limbs. After 3 weeks, the gaps in the MCLs were filled with granulation tissue, which was hypercellular and hypervascular in both groups. Although cells and collagen fibers were oriented irregularly in the control limbs, directionally aligned cells and collagen fibers were observed in regions in HGF-treated limbs (Fig. 2).

Six weeks after surgery, cellularity in the reparative tissues in both groups was still high, but lower than that observed in specimens obtained at 3 weeks (Fig. 2). The collagen fibers in HGF-treated limbs appeared more oriented in the longitudinal direction and tended to become parallel to the longitudinal axis of the ligament. Thus, the histological maturation in HGF-treated tissues occurred earlier than that in control tissues. After 12 weeks, the reparative tissues showed decreased cellularity and vascularity compared with earlier specimens and displayed improved collagen alignment and cellular organization similar to that of a normal MCL (Fig. 2). Although no clear differences between the two groups were identified at this point, no histological evidence of a negative effect of HGF was observed over the experimental period.

Biomechanical changes

To see whether the histological improvement in HGF-treated limbs was associated

with functional improvement, specimens were subjected to biomechanical evaluation. Load-displacement curves were obtained for all specimens. Five rabbits were used in each experimental groups, however, the number of specimens available for the biomechanical evaluation were as follows: at 3 weeks, 5 specimens in both groups; at 6 weeks, 2 specimens in the HGF-treated group and 3 control specimens; at 12 weeks, 4 specimens in both groups. Because avulsion of the ligament at the tibial insertion site during biomechanical test did not reflect the effect of HGF on reparative tissues directly, several specimens at 6 weeks and 12 weeks could not be evaluated, Thus, the data at 6 and 12 weeks after surgery were insufficient for the statistical analysis. Modes of failure included midsubstance failure and avulsion from the tibia, with similarity in failure modes noted between the two groups.

Three weeks following surgery, the HGF-treated limbs had a higher maximum load at failure, increased stiffness and higher energy absorbed at failure than did the paired control limbs. These parameters were significantly greater in the HGF-treated group compared to the control group ($P < 0.05$) (Fig. 3).

Discussion

HGF exerts a variety of biological activities in a variety of cells through the activation of the c-Met receptor tyrosine kinase. Our immunohistochemical results suggest that c-Met expression is inducible in response to administration of HGF to injured ligaments and that the percutaneously injected HGF would have a negligible effect on control limbs as a systemic effect.

In the healing process after ligament injuries, various kinds of cells, such as fibroblasts, platelets, white blood cells, and bone marrow stromal cells, enter injury sites and play

important roles. Bone marrow stromal cells act as progenitor cells in the healing process of ligament injuries as well as other tissue or organ injuries.[16] Previous studies have shown that HGF enhanced recruitment and proliferation of bone marrow-derived progenitor cells in the alveolar wall.[17] Likewise, it is possible that HGF may recruit mesenchymal stem cells and induce proliferation of those cells in the early stages of ligament healing.

HGF is one of the potent angiogenic growth factors that stimulates endothelial cell motility and growth.[18] Administration of HGF induced therapeutic angiogenesis in the rabbit ischemic hind limb model.[19,20] Neovascularization has an important role in ligament healing because neutrophils, macrophages, fibroblasts, nutrients, and growth factors gain access to the site of injury.[21] Therefore, our histological observations of abundant neovascularization and fibroblast proliferation indicate enhanced ligament healing in the early postoperative phase. Moreover, in the rabbit MCL injury model, the increase in blood flow by promoted angiogenesis was associated with its greater healing potential compared to the anterior cruciate ligament.[22] Likewise, nerve growth factor, which directly or indirectly promotes angiogenesis, improved ligament healing in the rat MCL injury model.[23] These reports support our observations because neovasculization contributed to a subsequent improved or accelerated healing response.

It has been speculated that alignment of collagen is an important component of ligament strength. In fact, a previous report proposed that collagen alignment and cross-linking of collagen fibers is more critical in determining scar strength than the volume of collagen produced.[24] We here observed that HGF treatment facilitated alignment of collagen fibers and facilitated ligament healing in the early postoperative phase. Mechanical loading, which stimulates the healing process and alignment of collagen fibers, occurs early in the process when a sufficient number of fibroblasts are present and a matrix capable of transmitting some load is produced.[25] Therefore, it is important to fill the

ligament gap with reparative tissue as early as possible. A previous in vitro study demonstrated that HGF stimulated the migration and proliferation of ligament fibroblasts.[26] Thus, local delivery of recombinant HGF may play a role in the recruitment/migration and proliferation of ligament fibroblasts during the early stages of healing when reparative tissues are subject to mechanical stress. Enhanced biomechanical recovery by HGF seems to be supported by histological observations that HGF-treated limbs possess well-aligned collagen fibers during the early stages.

In our study, HGF-treated limbs had significantly improved structural properties and more mature histological findings than the paired control limbs at 3 weeks after surgery. At 6 and 12 weeks after surgery, the differences between the HGF-treated and control groups in histological findings had gradually decreased. There are several possible explanations for these results. First, we administrated HGF only during the immediate post-operative period and on post-operative days 1 to 6. Second, the bioactivities of HGF, such as enhancement of migration and proliferation of ligament fibroblasts, promotion of neovascularization and recruitment of bone marrow stromal cells, seem to work in the early inflammatory and proliferation stages of ligament healing.

There are some limitations to this study. First, we did not investigate how long the administered HGF remained and retained in active at the ligament gap. Second, present study did not include further analysis to determine change in gene expression for type I and type III collagen. Third, the evaluation was limited to a 12 weeks period. The animal model may not represent the clinical situation on many aspects such as injury type, post injury rehabilitation. However, the positive results in this study suggest that the approach of using HGF to accelerate early ligament healing is promising. As to clinical relevance, the evaluation of the optimal dosage and its duration will be an important issue for future studies. It is an advantage that administration of HGF by percutaneous injection is simple

and does not require a surgery or a technically complex procedure, because extracapsular ligament injuries are typically treated with conservative therapy. For future treatment, if combined with appropriate post injury rehabilitation protocols, the clinical outcomes of the patients could be better.

In this study, we demonstrated that percutaneous injection of human recombinant HGF into an injured MCL in rabbits effectively promoted early ligament healing and had no side effects in the areas surrounding the injection. This is the first in vivo study to address the therapeutic effects of human recombinant HGF on ligament healing. The present data indicate the potential of HGF therapy for the treatment of ligament injuries. Local administration of recombinant HGF soon after ligament damage should shorten the time required for healing. Subsequent rapid recovery of biomechanical properties may accelerate efficiency of rehabilitation and result ultimately in the early return of patients to athletic activities.

Conclusion

In a rabbit model, local administration of recombinant HGF promotes early ligament healing. Local administration of HGF may represent a new therapeutic approach to accelerate histological and biomechanical healing and rehabilitation after ligament injury.

Conflict of Interest

Dr. Matsumoto is a co-founder and shareholder of Kringle Pharma (Toyonaka, Japan). He has served as a Chief Scientific Officer of Kringle Pharma and received payment for this role. The other authors did not receive and will not receive any benefits and funding from any commercial party related directly or indirectly to the subject of this article.

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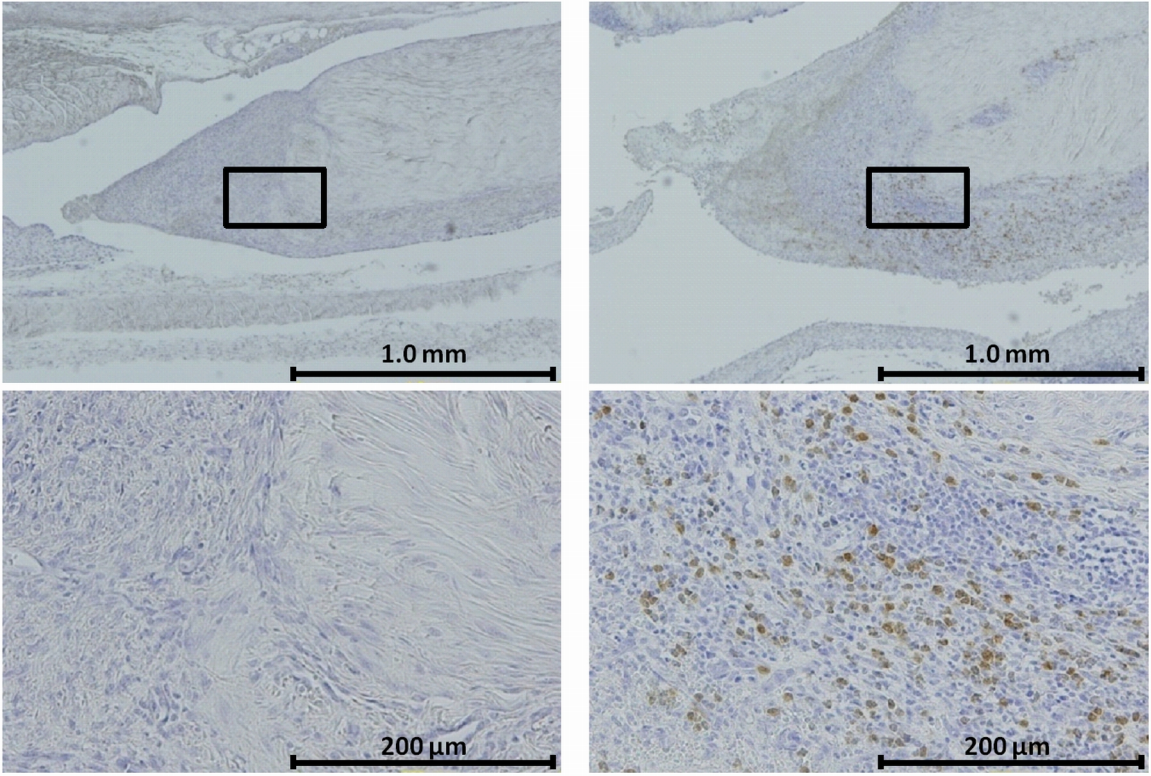
migration and growth kinetics of canine knee ligament fibroblasts. J Orthop Res 1999;17:398-404.

Figure Legends

Figure 1. Expression and distribution of c-Met/Hepatocyte growth factor (*HGF*) receptor in the healing site. They were analyzed by immunohistochemistry in HGF-treated and control limbs at 7 days after surgery. The boxed areas are shown at higher magnification in lower panels.

Figure 2. Changes in histological findings of the healing site by hepatocyte growth factor-treatment (Masson's trichrome staining). The double-headed arrows indicate the longitudinal axis of the ligament. The boxed area at higher magnification is shown in the right to show neovascularity.

Figure 3. Changes of the maximum load at failure, stiffness and the energy absorbed at failure of the femur-medial collateral ligament-tibia complex by hepatocyte growth factor-treatment at 3 weeks after surgery. Bars represent mean ± SEM (* $p < 0.05$).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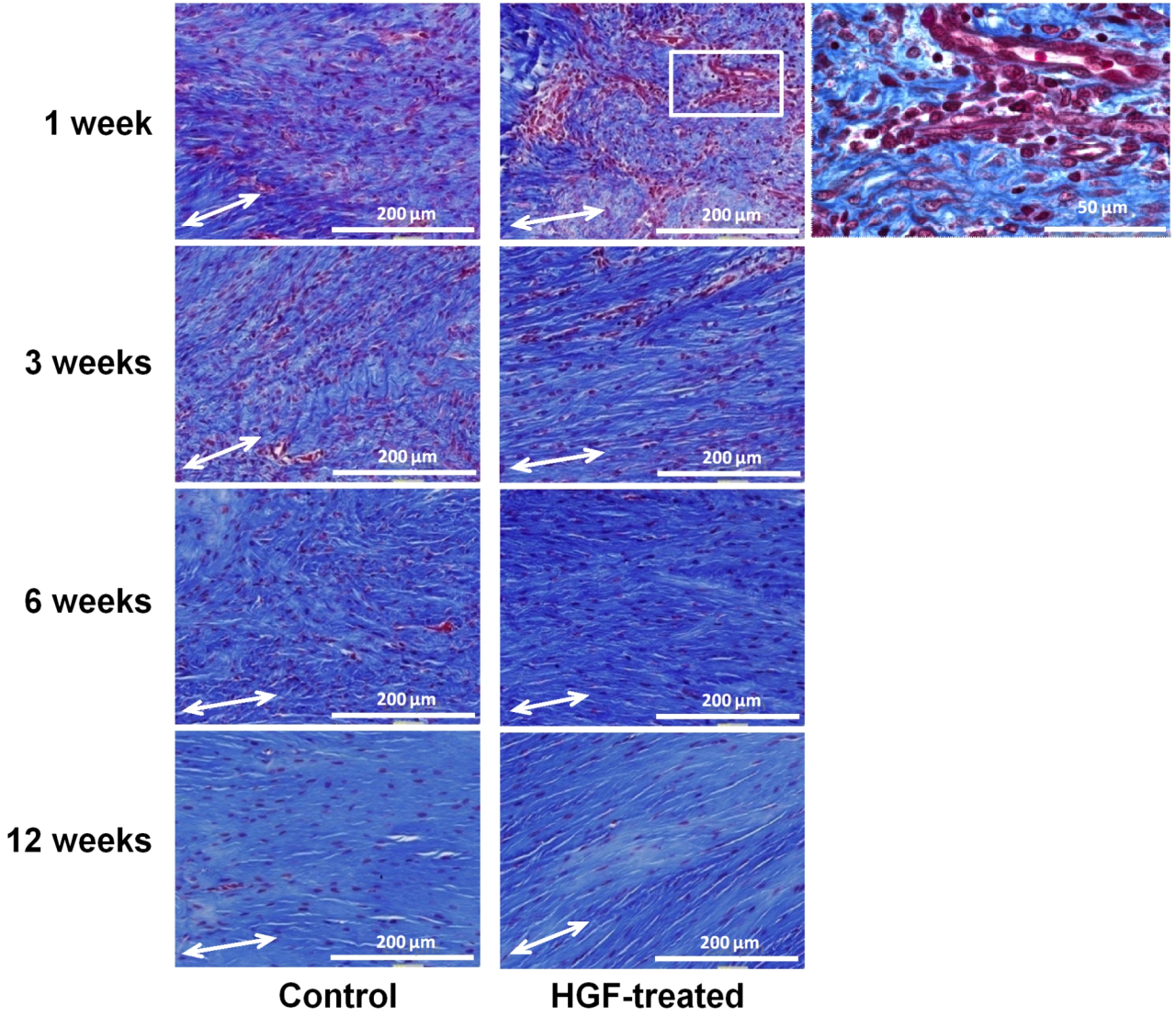
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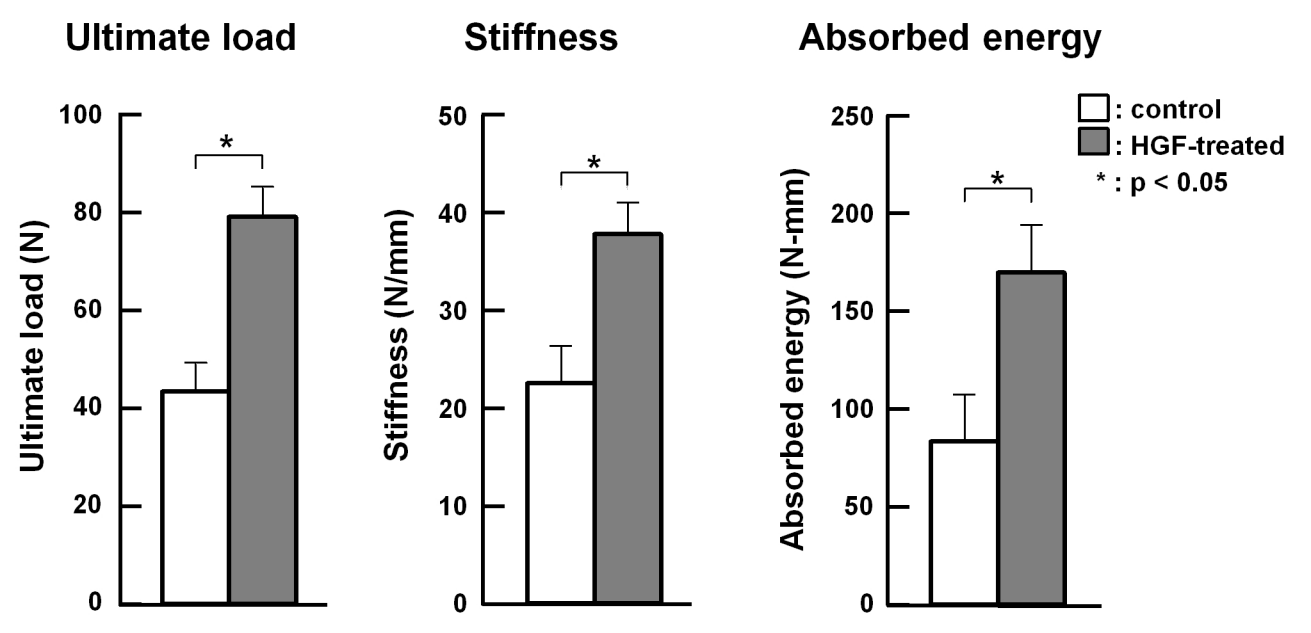
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