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Original Article

Local Upregulation of Stromal Cell–Derived Factor-1 After Ligament Injuries Enhances Homing Rate of Bone Marrow Stromal Cells in Rats

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In the present study the local expression of stromal cell–derived factor-1 (SDF-1) after ligament injury and correlated change in the homing rate of systemically induced bone marrow stromal cells (BMSCs) to the injured site were clarified using a rat medial collateral ligament (MCL) injury model. SDF-1 was temporarily upregulated peaking at 2 weeks after injury. Correlated with the alteration in SDF-1 expression, the homing rate of systemically induced BMSCs was temporarily upregulated peaking at 2–4 weeks after injury. The SDF-1 expression in the MCL seems to play a crucial role in cell homing. In addition, SDF-1 did not influence the BMSCs behavior *in vitro* in terms of the proliferation, adhesivity, and expression of ligament fibroblast markers. The cell-based therapy for ligament and tendon injury with reference to local expression of SDF-1 may be one of the available applications.

Introduction

ALTHOUGH IT IS WELL KNOWN that most cases of ligament and tendon injuries heal naturally in a living body, natural healing cannot be achieved in severe cases. To enhance the healing process, alternative strategies, including cell therapy and tissue engineering techniques using mesenchymal stem cells, have been developed.[1,2]

The healing process after tendon and ligament injuries is generally considered to be separated into four phases—hemorrhage, inflammation (within a week), proliferation (from 1 to 3 weeks), and remodeling or maturation (after 3 weeks).[3] In the inflammatory phase, various kinds of cells, such as fibroblasts, platelets, white blood cells, or bone marrow stromal cells (BMSCs), enter injury sites. These cells themselves and their cellular interactions play a crucial role in the healing process. Although BMSCs act as stem cells in the healing process of ligament and tendon injuries as mentioned above, the detailed mechanism influencing the healing process remains unclear.[4–6]

Many authors have demonstrated potential clinical applications of BMSCs in the field of tissue engineering and cell therapy.[1,2,7–20] Further, BMSCs are thought to migrate to injury sites and accelerate the healing of injured tissue or organs via differentiation into mature cells,[10,13,14,16–19] or through cell fusion.[21,22] Based on this phenomenon, the systemic infusion of BMSCs is considered to be one of the available pathways for cell therapy. Previous studies have demonstrated that functional improvement is achieved in the heart,[10] brain,[13,14,17,18] liver,[16] and kidney[19] as a result of systemic infusion of stem cells in animal organ injured models.

Recent studies have indicated that stromal cell–derived factor-1 (SDF-1 or CXCL12) acts as a key chemokine of stem cell homing to bone marrow.[23] These studies showed that the expression of SDF-1 mRNA was upregulated in injured sites, including the heart, brain, liver, and kidney.[10,13,14,16–19] However, to our knowledge, no studies have clarified the role of SDF-1 in the homing of BMSCs to injury sites in musculoskeletal soft tissues, such as tendons or ligaments. This elucidation will lead to a novel treatment strategy using BMSCs for ligament and tendon injuries.

In this study, we hypothesized that the temporary upregulation of SDF-1 would occur at the injury site of tendons and ligaments and that this upregulation could influence stem cell homing to the injury site. To test these hypotheses, we created an injury model of the knee medial collateral ligament (MCL) using rats and analyzed the SDF-1 mRNA expression and homing rate of systemic induced BMSCs in the MCLs. The specific objectives of this study were to clarify the changes in the SDF-1 mRNA expression and homing of systemic induced BMSCs at the injury site of the MCL, and to determine the effective timing of systemic administration of BMSCs for homing to the injury site.

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Materials and Methods

Animals and surgical procedures

Animals used for the current injury model were mature male Wister rats weighing 200–230 g. Surgical procedure for MCL transection was according to previous report with some modifications.[24] In brief, the animals were anesthetized with inhalation of isoflurane (0.5–2%, Abbott Laboratories, Mumbai, India; http://www.abbott.com) and oxygen by means of a coaxial nose cone. An approximate 10 mm skin incision was made on the medial aspect of the knee joint, and the overlying connective tissue was dissected to expose the MCL. Then, a 1 mm gap in the mid-substance of the MCL was created, and the gap was left without suturing (MCLT group). As a control, the MCL was treated with only exposure (sham group). Finally, the fascia and the skin were closed using a 6–0 nylon suture. After surgery, the rats were allowed cage activities. All procedures in this study involving animals were according to established ethical guidelines approved by the local animal care committee.

Preparation of BMSCs

Bone marrow stromal cells were isolated from green fluorescent protein (GFP) transgenic or wild-type Wister rats using the following procedure. Bone marrow tissues were obtained from the femurs and tibias by flushing the shaft using a syringe with no. 22-gauge needle under sterile conditions. The cells were disaggregated by gentle pipetting several times and passed through a 70 μm cell strainer (BD Biosciences, San Jose, CA; http://www.bdbiosciences.com) to remove remaining clumps of the tissues. Finally, the collected cells were seeded on culture dishes, and the adhesive cells were used as BMSCs. The BMSCs at second passage were used for further investigations.[25–27]

General culture condition

The culture medium used here was Dulbecco's modified Eagle's medium (D5796; Sigma Chemical Co., St. Louis, MO; http://www.sigmaaldrich.com), with or without 10% fetal calf serum (FCS, 812072; Invitrogen Corp., Carlsbad, CA; http://www.invitrogen.com), 10 μL/mL penicillin, strep- streptomycin, and fungizone (17-745H; BioWhittaker Inc., Walkersville, MD; www.biowhittaker.com). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After trypsinization, cells were counted using a hemocytometer.

Expression of SDF-1 after MCL injury

The MCLs were harvested at 1 h and 1, 2, 4, and 6 weeks after surgery in the MCLT and the sham groups ($n = 6$ in each period, 5 for real-time PCR, and 1 for immunohistochemistry). To detect the mRNA expression of SDF-1 in the MCL, total mRNA was extracted from bilateral MCLs according to the TRIzol Reagent protocol (Invitrogen). One microgram of RNA was converted to cDNA, and then quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed on 20 ng of cDNA in a 20 μL reaction volume. Quantitative RT-PCR was performed using the SYBR green PCR kit in Opticon II (Bio Rad Laboratories, Hercules, CA; http://www.bio-rad.com) with a gene-specific primer of SDF-1 designed previously.[10] Average threshold cycle (Ct) values of SDF-1 were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels. The oligonucleotide primers of GAPDH were designed using OLIGO (Molecular Biology Insights, Cascade, CO; http://www.oligo.net).

To determine the localization of SDF-1 on injured MCL, immunohistochemical stains of the MCL from the MCLT and the sham groups were also performed with an antibody to SDF-1 (14-7991, eBioscience, San Diego, CA; http://www.ebioscience.com) as described previously.[19]

Administration of donor BMSCs

Bone marrow stromal cells suspended with physiologic saline (3×10^6 cells/mL) were injected via a vein in the bulb of the penis under inhalation anesthesia, and an immunosuppressive agent FK506 (provided by Astellas Pharma, Tokyo, Japan; http://www.astellas.com) was administered to rats treated with BMSCs from GFP transgenic rats. The cell number was determined according to the report by Lagasse *et al.*[16] FK506 was dissolved in 10 mg/mL saline and administered by intramuscular injection at 5.0 mg/kg of body weight.

Change in the homing rate of systemic induced BMSCs

Thirty rats in each group were used in this evaluation. The BMSCs (1×10^6 cells) were injected intravenously to rats in both experimental groups at 1 h and 1, 2, 4, and 6 weeks after surgery. At 2 weeks after injection, the MCLs were harvested after euthanasia of the rats. Fluorescent microscopy of cryosection and anti-GFP immunohistostain with anti-GFP (BD Biosciences) were performed to detect the delivered GFP (+) BMSCs. Quantitative real-time RT-PCR ($n = 5$ in each period; protocol is same as above) was also performed with a gene-specific primer of GFP designed using DNASIS (Hitachi Software Engineering, Yokohama, Japan; http://hitachisoft.jp) to quantify the delivered GFP (+) BMSCs.

Homing blockage assay

To determine the role of SDF-1 in the homing of BMSCs to the injured MCL, an *in vivo* homing blockage assay was performed according to the protocol of Togel *et al.*[19] with some modifications. Briefly, BMSCs from GFP transgenic rats were preincubated with 5 μg/mL anti-CXCR4, a specific receptor of SDF-1, blocking antibody (bicyclam AMD3100, Sigma) or physiologic saline for 30 min as the control ($n = 5$ in each group). Pretreated BMSCs (3×10^6 cells) were then injected intravenously to rats after washing and centrifugation using the most effective timing according to the results of former assay. Animals were euthanized at 24 h after the injection, and MCLs were obtained for further analysis. Quantitative real-time RT-PCR was also performed with a gene-specific primer of GFP.

Expression of Transforming Growth Factor-Beta 1 on BMSCs administered for MCL injury

The functional influence of administered BMSCs on injured MCL was evaluated by quantifying the local expression of transforming growth factor-beta1 (TGF-β1), which is a chemoattractant for fibroblasts to accelerate wound healing.[28–30]

TABLE 1. LIST OF PRIMERS USED FOR POLYMERASE CHAIN REACTION

	Primer sequence	
Gene name	*Forward*	*Reverse*
SDF-1	5′-TTGCCAGCACAAAGACACTCC-3′	5′-CTCCAAAGCAAACCGAATACAG-3′
GFP	5′-AGAAGAACGGCATCAAGGTG-3′	5′-GAACTCCAGCAGGACCATGT-3′
GAPDH	5′-CACCACCCTGTTGCTGTA-3′	5′-TATGATGACATCAAGAAGCTGG-3′
TGF-β1	5′-CAACAATTCCTGGCGTTACC-3′	5′-AAGCCCTGTATTCCGTCTCC-3′
Collagen type I	5′-TCAAGATGGTGGCCGTTAC-3′	5′-CTGCGGATGTTCTCAATCTG-3′
Collagen type III	5′-GGCAAGGGTGATCGTGGTG-3′	5′-GACCAGCAGGACCCGTTTCT-3′
Tenascin-C	5′-TCGGGCCTTCCACCTAGT-3′	5′-GGTTGTCAACTTCCGGTTCA-3′

SDF-1, stromal cell–derived factor-1; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β1, transforming growth factor-beta 1.

MCL injury was created on bilateral knees in 10 rats. Bone marrow stromal cells (3×10^6 cells) from Wister rats (BMSC i.v. group, $n=5$) or physiologic saline (control group, $n=5$) were injected intravenously using the most effective timing after creating the same MCL injury as in the former assay. All animals were euthanized at 2 weeks after injection, and then quantitative real-time RT-PCR was performed with a gene-specific primer of TGF-β1 designed using OLIGO (Molecular Biology Insights) for harvested MCLs.

In vitro *effects of SDF-1 on BMSCs behaviors*

To clarify the direct influence of SDF-1 on the behaviors of BMSCs, including the proliferation, adhesivity, and expression of ligament fibroblast markers, the cells from Wister rats cultured *in vitro* with additional 200, 100, and 0 ng/mL (control) concentration of SDF-1 in culture medium were investigated ($n=5$ in each assay).

Cell proliferation assay was performed using a WST-8 (Dojindo Laboratories, Kumamoto, Japan; http://www.dojindo.co.jp). Briefly, BMSCs were seeded on 96-well plates (Falcon Microtest Plates, BD Biosciences) at 1×10^4 cells/well with or without SDF-1. After 48 h incubation, 10 μL of the working solution of WST-8 was added to each well, and the cells were incubated for an additional 4 h. The optic density (OD) of each well was measured at 450 nm against the reference absorbance at 600 nm with a Microplate Reader (Benchmark Plus, Bio Rad Laboratories).

To test the cell adhesivity of BMSCs, a cell adhesion assay was performed according to the method described previously.[31] Wells of 96-well plates (Falcon Microtest Plates; BD Biosciences) were used in this study. Nonspecific binding sites were blocked by incubating the plates with 100 μL of 2% bovine serum albumin for 1 h at 37°C. Then, the wells were washed three times with PBS. Bone marrow stromal cells (1×10^5 cells/well) in 100 μL of serum-free medium with or without SDF-1 were added to the wells and incubated for 1 h at 37°C. The unbound cells were removed by gently rinsing the wells three times with PBS. Then, the BMSCs attached to the plate were quantified with WST-8 (protocol is same as above).

To quantify the ligament fibroblast markers, including collagen types I and III and tenascin-C,[9] quantitative real-time RT-PCR was performed with gene-specific primers of collagen types I and III and tenascin-C designed using OLIGO (Molecular Biology Insights) for cultured BMSCs (3×10^5 cells/well) with or without SDF-1 for 3 days after seeding on six-well plates (Falcon Multiwell Plates; BD Biosciences).

Primer sequences

The primer sequences used in this study are listed in Table 1.

Data analysis

Statistical comparisons were performed using one-way ANOVA for transition of mRNA transcript level. To compare the level of mRNA level and absorbance between the groups, unpaired *t*-tests were used. Results are presented as mean ± standard error (SE). The differences were considered to be significant at a probability level of $p<0.05$. To avoid observer bias, slides were coded before all analysis.

Results

All the MCLs transected with surgery healed with continuity of ligament like tissue 1 week after surgery with or without administration of BMSCs and matured with time dependency. No macroscopic findings indicating postoperative infection were found in any rats.

Expression of SDF-1 after MCL injury

In the MCLT group, the mRNA of SDF-1 in the MCL was significantly gradually upregulated until 2 weeks postoperatively ($^*p<0.01$ vs. 1 h, $^\#p<0.05$ vs. 1 week; Fig. 1). Then, the expression was gradually downregulated from 2 to 6 weeks postoperatively ($^*p<0.01$ vs. 4 and 6 weeks; Fig. 1). On the other hand, the expression level of SDF-1 mRNA in the sham group did not alter after surgery. At 2 weeks postoperatively, the expression level of SDF-1 was significantly higher in the MCLT group than in the sham group (0.52 ± 0.31 vs. 0.13 ± 0.04, $p<0.01$). Immunohistochemical evaluation of SDF-1 at 2 weeks postoperatively revealed that there were apparently more cells expressing the SDF-1 in the injured MCL (Fig. 2A), whereas the expression was relatively lower throughout the ligament in the sham group (Fig. 2B). The photomicrographs were selected from the mid-section of the surgical site in each group.

Change in homing rate of systemic induced BMSCs

Fluorescent microscopic and immunohistochemical observation of the GFP revealed that exogenous BMSCs homed to the MCLs in the MCLT group injected at 2 weeks after surgery. The photomicrographs were selected from the mid-section of the surgical site in each group. On the other hand, there were less BMSCs homed to the MCLs injected at

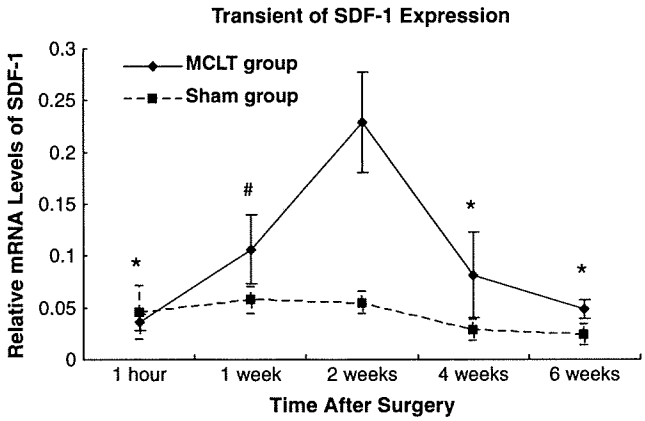

FIG. 1. In the MCLT group, the mRNA of SDF-1 in the MCL was significantly gradually upregulated until 2 weeks postoperatively. On the other hand, the expression level of SDF-1 mRNA in the sham group did not alter after surgery. $^{*}p < 0.01$ and $^{\#}p < 0.05$ compared with 2 weeks after surgery in the MCLT group. The results represent means ± SE ($n = 5$ in each time period). Groups are defined in Materials and Methods, "Animals and surgical procedures."

2 weeks after sham surgery and at 6 weeks after surgery (Fig. 3). Quantitative real-time PCR showed that the homing rate of BMSCs to the MCL was significantly gradually upregulated until 4 weeks postoperatively in the MCLT group ($^{*}p < 0.01$ vs. 1 h, 1 week, and 2 weeks; Fig. 4). Then, the rate was gradually downregulated from 4 to 6 weeks postoperatively ($^{*}p < 0.01$ vs. 6 weeks; Fig. 4). On the other hand, the homing rate of BMSCs in the sham group did not alter after surgery. There were significantly higher homing rates of BMSCs injected at 2 weeks (0.000124 ± 0.000023 vs. 0.000018 ± 0.000004, $p < 0.01$) and at 4 weeks (0.000434 ± 0.000117 vs. 0.000037 ± 0.000008, $p < 0.05$) after surgery in the MCLT group, compared to that in the sham group. There was no significant difference in the homing rate of BMSCs injected at 6 weeks after surgery between the MCLT group and the sham group.

Homing blockage assay

The results mentioned above indicate that the temporary upregulation of SDF-1 peaking at 2 weeks after MCL injury may increase the homing rate of systemically induced BMSCs. To confirm this direct correlation, we then blocked the effect of SDF-1 by injection of pretreating BMSCs with AMD3100 (CXCR-4 antagonist) at 2 weeks after surgery. The homing rate of BMSCs to injured MCLs significantly decreased, compared to that of BMSCs without pretreatment (0.0009 ± 0.0002 vs. 0.0042 ± 0.0010, $p < 0.01$).

Expression of TGF-β1 on BMSCs administered for MCL injury

To confirm the function of BMSCs induced to the MCLs, we then examined the local expression of TGF-β1 in the injured MCLs after injection of BMSCs 2 weeks after creating the MCL injury. The mRNA levels of TGF-β1 in the MCL were significantly higher in the BMSC i.v. group than in the control group (0.20 ± 0.02 vs. 0.09 ± 0.01, $p < 0.01$).

In vitro *effect of SDF-1 on BMSC behaviors*

All the assays including the proliferation, adhesivity, and expression of ligament fibroblast markers of BMSCs showed no significant differences in each assessment between the SDF-1 group and the control group (Fig. 5). The results obtained here indicate that SDF-1 has no direct effects on these cellular behaviors of BMSCs.

Discussion

The first purpose of this study was to clarify the changes in the SDF-1 mRNA expression after MCL injury. The current study showed that the local upregulation of SDF-1 occurred transiently after MCL injuries and that it peaked not imme-

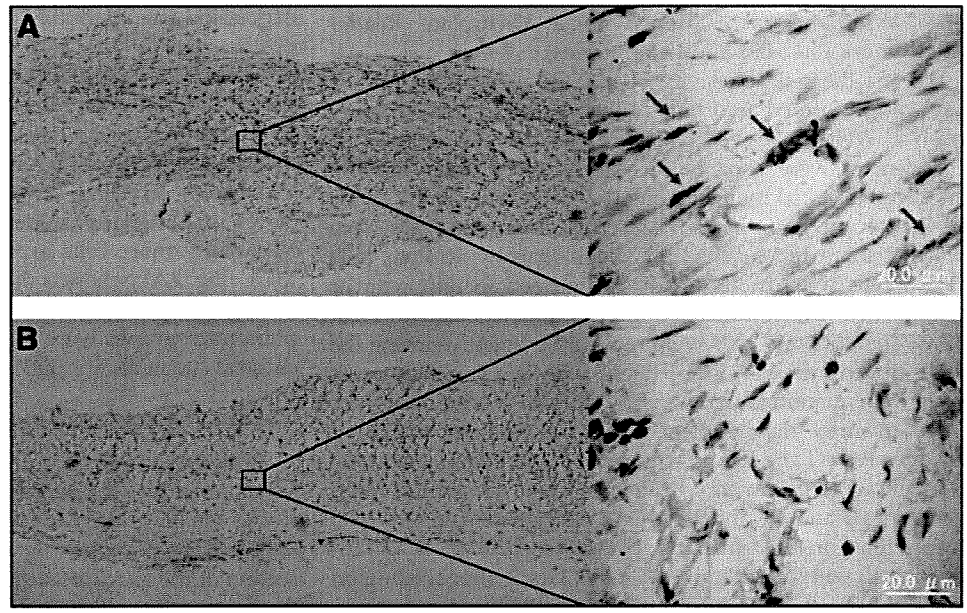

FIG. 2. (**A**) Immunohistochemistry of transected medial collateral ligament (MCL) at 2 weeks after ligament injury for SDF-1. (**B**) Immunohistochemistry of transected MCL at 2 weeks after sham surgery for SDF-1. Scale bars: 20 μm. Color images available online at www.liebertonline.com/ten.

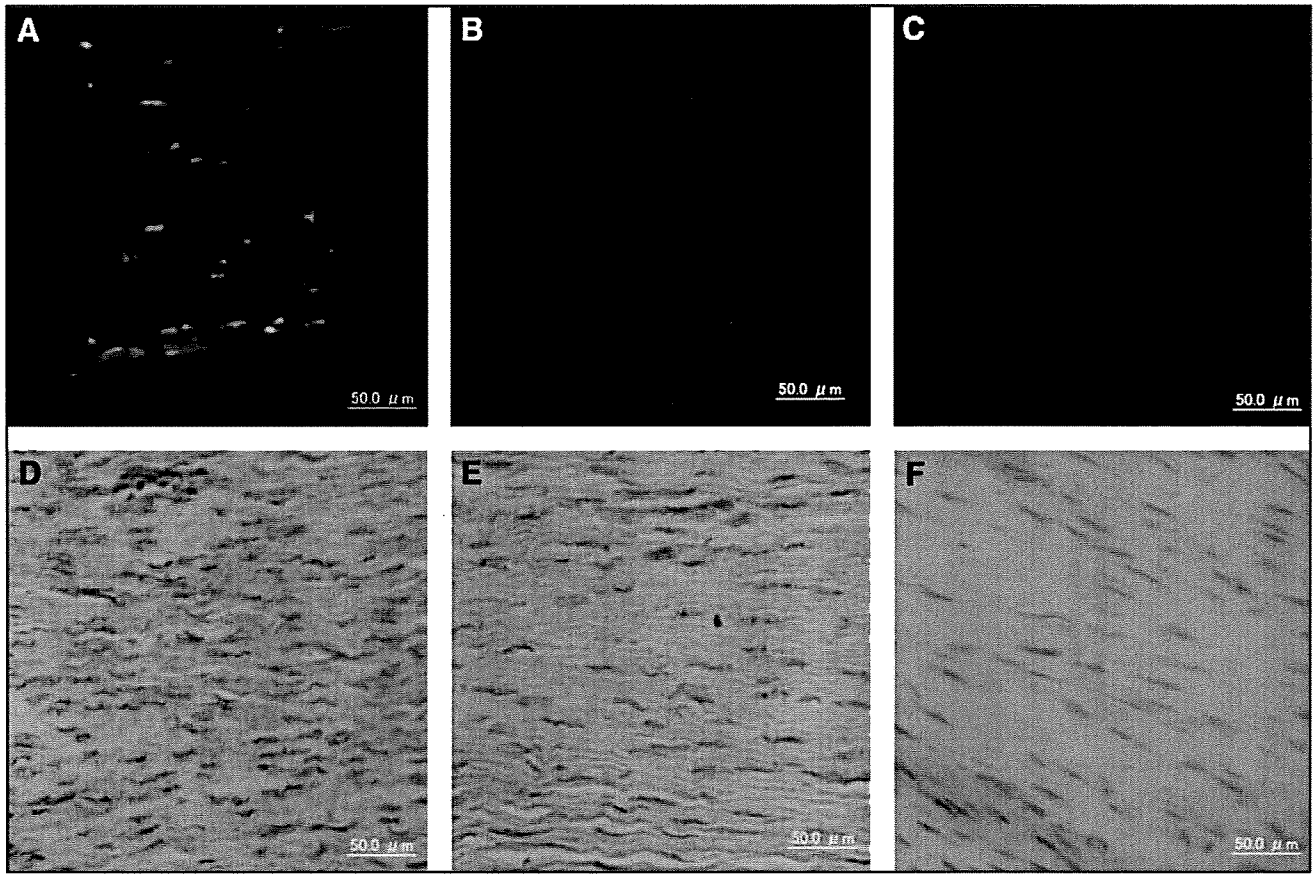

FIG. 3. Histological appearance of fluorescent microscopy of cryosection (**A–C**) and immunohistostain of GFP (**D–F**) in the MCLs. The GFP Tg bone marrow stromal cells (BMSCs) were injected at 2 weeks after MCL injury (**A, D**), at 2 weeks after sham surgery (**B, E**), and at 6 weeks after MCL injury (**C, F**). Scale bars: 50 μm. Color images available online at www.liebertonline.com/ten.

diately but 2 weeks after the injury. To date, the expression of SDF-1 after ligament and tendon injuries remains unclear throughout the natural healing process of these injuries. Dissimilar to other chemokines, SDF-1 is thought to be upregulated under the condition of tissue ischemia[8,10,13,17–19] or DNA damage.[32] Previous studies have shown the local upregulation of SDF-1 in relation to stem cells homing to injured organs such as myocardial infarction,[8,10] brain injury,[13,14,17,18] liver injury,[15] or renal infarction.[19] The current study clarified that the local expression of SDF-1 was upregulated after ligament injuries as well as other tissue or organ injuries. Askari *et al.* reported that the local upregulation of SDF-1 in myocardial muscle was found immediately after the damage.[10] On the other hand, Shen *et al.* showed that the expression of SDF-1 was upregulated until 4 months after a stroke.[17] Otherwise, in the kidney or liver, the local expression level of SDF-1 is higher within several days after the organ injury.[15,19] This inconsistency among these tissues and organs may be attributable to the difference of cellularity and types of cells. Therefore, a significance of this study is to first elucidate the changes in local expression of SDF-1 in ligament injuries.

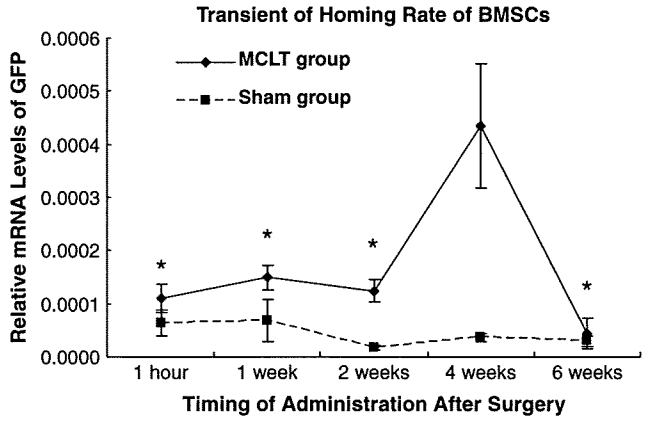

FIG. 4. The homing rate of BMSCs to the MCL was significantly gradually upregulated until 4 weeks postoperatively in the MCLT group. On the other hand, the homing rate of BMSCs in the sham group did not alter after surgery. *$p < 0.01$ compared with 4 weeks after surgery in MCLT group. Values given are means ± SE ($n = 5$ in each time period).

The second objective of this study was to determine the effective timing of systemic administration of BMSCs for homing to the injury site. The result obtained in the current study clearly indicated that effective timing is not immediately, but from 2 to 4 weeks after the injury as well as the transient of the local expression of SDF-1 in injured MCL. To our knowledge, this is the first attempt to clarify the effects of SDF-1 in relation to the systemic administration of BMSCs to tendon or ligament injuries. The present study demonstrated

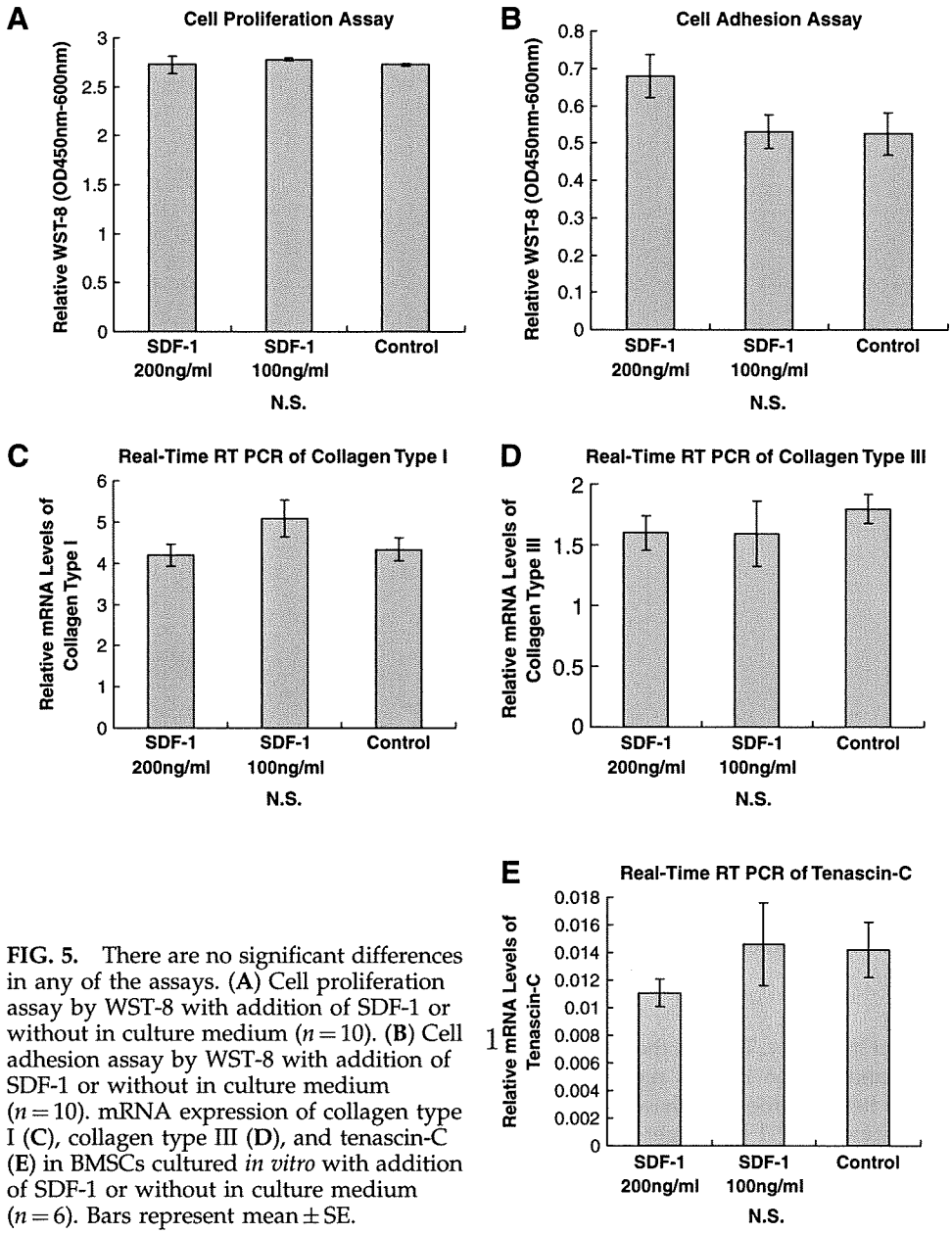

FIG. 5. There are no significant differences in any of the assays. (**A**) Cell proliferation assay by WST-8 with addition of SDF-1 or without in culture medium ($n = 10$). (**B**) Cell adhesion assay by WST-8 with addition of SDF-1 or without in culture medium ($n = 10$). mRNA expression of collagen type I (**C**), collagen type III (**D**), and tenascin-C (**E**) in BMSCs cultured *in vitro* with addition of SDF-1 or without in culture medium ($n = 6$). Bars represent mean ± SE.

that the homing rate was higher at 2–4 weeks after injury, when the expression of SDF-1 was upregulated in the proliferation phase as the natural reaction of the healing process.[3] These results hinted to us the relationship between upregulation of SDF-1 and the homing of BMSCs to injury sites. To confirm the direct relationship between upregulation of SDF-1 and the homing rate of BMSCs to the site of injury, we then attempted to block the effect of SDF-1 in our rat MCL injury model. The present results clearly demonstrated that BMSCs pretreated with CXCR-4 antagonist are not able to home to injured ligament any longer. This result indicates that the temporary upregulation of SDF-1 in injured MCL may lead to enhanced delivery of exogenous BMSCs to the injured site. Although ligament and tendon tissues are relatively hypovascular tissue compared to other tissues mentioned above, our results were consistent with other reports on the point that there was a correlation between upregulation of SDF-1 and an increase in the homing rate of BMSCs to the injured tissue.

In the present study, the expression of TGF-β1 was upregulated in the injured MCLs treated with BMSCs. This indicates that the BMSCs homing to injured ligament may have therapeutic effects on its healing, even if an administration pathway is systemic. Stem cells play an important role in repairing injured tissues in living bodies. Bone marrow stromal cells are one of the most capable stem cells for repairing injured ligament and tendon.[5,6] To date, there have been several reports on the local use of BMSCs for the treatment of ligament or tendon injuries in *in vivo* animal models.[1,2] However, no attempts have been made to apply the systemic use of BMSCs to those injuries. Consequently, the influence of systemic induced stem cells on ligament or tendon tissue remains unclear. The current study first indicates that the systemically delivered exogenous BMSCs have potential for

enhancing the MCL healing process like that occurs in other tissues. Further, it can be said that not only local application but also systemic infusion of stem cells with the combination of the management of local expression of SDF-1 may be a promising treatment for severe ligament or tendon injuries that require surgery.

We also tried to clarify through *in vitro* studies the direct relationship between the locally expressed SDF-1 and the behavior of homed BMSCs to the injury site. Our present results indicate that SDF-1 has no direct effects on the cellular behavior of BMSCs in terms of the proliferation, adhesivity, and expression of ligament fibroblast markers. These results suggest that SDF-1 may have no direst effect on BMSCs except homing to injury sites. There have been several reports on the action of SDF-1 on the cellular behavior of some kinds of cultured cells.[33–35] Gong *et al.* demonstrated that SDF-1 enhanced the proliferation of neural progenitor cells.[33] Lisignoli *et al.* showed that SDF-1 enhanced the proliferation and the collagen production of osteoblasts.[34] To the contrary, Jung *et al.* reported that SDF-1 had no significant effects on the proliferation of BMSCs.[35] This previous study supports the current result regarding the cellular proliferation. Based on the results obtained here, we speculate that SDF-1 affects the healing process of these injuries not through direct effect on BMSCs behavior, but through positive effect on the homing of BMSCs to the injured site.

There are further considerations to the clinical application of the current results. First, we did not investigate the transition of homing rates of intrinsic BMSCs originating from bone marrow. The intrinsic BMSCs might have more influence on the healing process of injured MCL. Next, the histological and biomechanical properties of healing MCL after systemic administration of BMSCs should be clarified. After elucidation of these points, we will establish a strategy based on the current results for the acceleration of the healing process of ligament or tendon injury.

In conclusion, we first clarified the change in local expression of SDF-1 after ligament injury of rats. The obtained results also showed that the increase in local SDF-1 expression enhanced the homing rate of exogenous BMSCs to the injured site. Additionally, the most effective timing of systemic administration of BMSCs to MCL injury may not be immediately after injury, but several weeks after the injury. These results may lead to the conclusion that a cell-based therapy with reference to local expression of SDF-1 can be applied to ligament and tendon injuries as well as other types of tissues.

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Disclosure Statement

No competing financial interests exist.

References

1. Young, R.G., Butler, D.L., Weber, W., Caplan, A.I., Gordon, S.L., and Fink, D.J. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res **16,** 406, 1998.
2. Watanabe, N., Woo, S.L., Papageorgiou, C., Celechovsky, C., and Takai, S. Fate of donor bone marrow cells in medial collateral ligament after simulated autologous transplantation. Microsc Res Technol **58,** 39, 2002.
3. Kannus, P., Parkkari, J., Jarvinen, T.L., Jarvinen, T.A., and Jarvinen, M. Basic science and clinical studies coincide: active treatment approach is needed after a sports injury. Scand J Med Sci Sports **13,** 150, 2003.
4. Andriacchi, T., Sabiston, P., DeHaven, K., Dahners, L., Woo, S.L., Frank, C., Oakes, B., Brand, R., and Lewis, J. Ligament: injury and repair. In: Woo, S.L., Buckwalter, J.A., eds. Injury and Repair of the Musculoskeletal Soft Tissues. IL: American academy of Orthopaedic Surgeons, 1988, p. 103.
5. Ross, R. The fibroblast and wound repair. Biol Rev Camb Philos Soc **43,** 51, 1968.
6. Woo, S.L., Hildebrand, K., Watanabe, N., Fenwick, J.A., Papageorgiou, C.D., and Wang, J.H. Tissue engineering of ligament and tendon healing. Clin Orthop Relat Res **S367,** S312, 1999.
7. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
8. Abbott, J.D., Huang, Y., Liu, D., Hickey, R., Krause, D.S., and Giordano, F.J. Stromal cell-derived factor-1 alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation **110,** 3300, 2004.
9. Altman, G.H., Horan, R.L., Martin, I., Farhadi, J., Stark, P.R., Volloch, V., Richmond, J.C., Vunjak-Novakovic, G., and Kaplan, D.L. Cell differentiation by mechanical stress. FASEB J **16,** 270, 2002.
10. Askari, A.T., Unzek, S., Popovic, Z.B., Goldman, C.K., Forudi, F., Kiedrowski, M., Rovner, A., Ellis, S.G., Thomas, J.D., DiCorleto, P.E., Topol, E.J., and Penn, M.S. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet **362,** 697, 2003.
11. Bruder, S.P., Fink, D.J., and Caplan, A.I. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem **56,** 283, 1994.
12. Cuevas, P., Carceller, F., Garcia-Gomez, I., Yan, M., and Dujovny, M. Bone marrow stromal cell implantation for peripheral nerve repair. Neurol Res **26,** 230, 2004.
13. Hoehn, M., Kustermann, E., Blunk, J., Wiedermann, D., Trapp, T., Wecker, S., Focking, M., Arnold, H., Hescheler, J., Fleischmann, B.K., Schwindt, W., and Buhrle, C. Monitoring of implanted stem cell migration *in vivo*: a highly resolved *in vivo* magnetic resonance imaging investigation of experimental stroke in rat. Proc Natl Acad Sci USA **99,** 16267, 2002.
14. Ji, J.F., He, B.P., Dheen, S.T., and Tay, S.S. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells **22,** 415, 2004.
15. Kollet, O., Shivtiel, S., Chen, Y.Q., Suriawinata, J., Thung, S.N., Dabeva, M.D., Kahn, J., Spiegel, A., Dar, A., Samira, S., Goichberg, P., Kalinkovich, A., Arenzana-Seisdedos, F., Nagler, A., Hardan, I., Revel, M., Shafritz, D.A., and Lapidot, T. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J Clin Invest **112,** 160, 2003.

16. Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., and Grompe, M. Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. Nat Med **6,** 1229, 2000.
17. Shen, L.H., Li, Y., Chen, J., Zacharek, A., Gao, Q., Kapke, A., Lu, M., Raginski, K., Vanguri, P., Smith, A., and Chopp, M. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. J Cereb Blood Flow Metab **27,** 6, 2007.
18. Shen, L.H., Li, Y., Chen, J., Zhang, J., Vanguri, P., Borneman, J., and Chopp, M. Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. Neuroscience **137,** 393, 2006.
19. Togel, F., Isaac, J., Hu, Z., Weiss, K., and Westenfelder, C. Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. Kidney Int **67,** 1772, 2005.
20. Wakitani, S., Goto, T., Pineda, S.J., Young, R.G., Mansour, J.M., Caplan, A.I., and Goldberg, V.M. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am **76,** 579, 1994.
21. Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S., and Grompe, M. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature **422,** 897, 2003.
22. Medvinsky, A., and Smith, A. Stem cells: fusion brings down barriers. Nature **422,** 823, 2003.
23. Ara, T., Tokoyoda, K., Sugiyama, T., Egawa, T., Kawabata, K., and Nagasawa, T. Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. Immunity **19,** 257, 2003.
24. Chen, J., Iosifidis, M., Zhu, J., Tatarintsev, I., and Wang, J.H. Vanadate ingestion enhances the organization and collagen fibril diameters of rat healing medical collateral ligaments. Knee Surg Sports Traumatol Arthrosc **14,** 750, 2006.
25. Shimode, K., Iwasaki, N., Majima, T., Funakoshi, T., Sawaguchi, N., Onodera, T., and Minami, A. Bone marrow stromal cells act as feeder cells for tendon fibroblasts through soluble factors. Tissue Eng **13,** 333, 2007.
26. Richards, M., Huibregtse, B.A., Caplan, A.I., Goulet, J.A., and Goldstein, S.A. Marrow-derived progenitor cell injections enhance new bone formation during distraction. J Orthop Res **17,** 900, 1999.
27. Fukada, S., Miyagoe-Suzuki, Y., Tsukihara, H., Yuasa, K., Higuchi, S., Ono, S., Tsujikawa, K., Takeda, S., and Yamamoto, H. Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein-gene transgenic mice. J Cell Sci **115,** 1285, 2002.
28. Mason, C.P.L., Gabriel, Y.F.N., and Yip, K.K. Therapeutic ultrasound enhances medial collateral ligament repair in rats. Ultrasound Med Biol **32,** 449, 2006.
29. Postlethwaite, A.E., Keski-Oja, J., Moses, H.L., and Kang, A.H. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. J Exp Med **165,** 251, 1987.
30. Beck, L.S., Deguzman, L., Lee, W.P., Xu, Y., McFatridge, L.A., and Amento, E.P. TGF-beta 1 accelerates wound healing: reversal of steroid-impaired healing in rats and rabbits. Growth Factors **5,** 295, 1991.
31. Riss, T. Comparison of attachment factors using the CellTiter 96^{TM} Assay. Promega Notes Mag **38,** 18, 1992.
32. Shen, K.C., Miller, F., Tait, L., Santner, S.J., Pauley, R., Raz, A., Tainsky, M., Brooks, S.C., and Wang, Y.A. Isolation and characterization of a breast progenitor epithelial cell line with robust DNA damage responses. Breast Cancer Res Treat **98,** 357, 2006.
33. Gong, X., He, X., Qi, L., Zuo, H., and Xie, Z. Stromal cell derived factor-1 acutely promotes neural progenitor cell proliferation *in vitro* by a mechanism involving the ERK1/2 and PI-3K signal pathways. Cell Biol Int **30,** 466, 2006.
34. Lisignoli, G., Toneguzzi, S., Piacentini, A., Cristino, S., Grassi, F., Cavallo, C., and Facchini, A. CXCL12 (SDF-1) and CXCL13 (BCA-1) chemokines significantly induce proliferation and collagen type I expression in osteoblasts from osteoarthritis patients. J Cell Physiol **206,** 78, 2006.
35. Jung, Y., Wang, J., Schneider, A., Sun, Y.X., Koh-Paige, A.J., Osman, N.I., McCauley, L.K., and Taichman, R.S. Regulation of SDF-1 (CXCL12) production by osteoblasts; a possible mechanism for stem cell homing. Bone **38,** 497, 2006.

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Effect of Selective Estrogen Receptor Modulator/Raloxifene Analogue on Proliferation and Collagen Metabolism of Tendon Fibroblast

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The selective estrogen receptor modulator raloxifene is therapeutically beneficial for postmenopausal connective tissue degradation, such as osteoporosis, vascular sclerosis, and dermal degradation; however, the effects of raloxifene on postmenopausal tendon metabolism have not been clarified. In this study, we investigated the effects of raloxifene analogue (LY117018) on cell proliferation and collagen metabolism using cultured rat Achilles tendon fibroblasts. 17β-Estradiol (E_2; 10^{-11}–10^{-9} M) and LY117018 (10^{-9}–10^{-7} M) had no significant effects on tendon fibroblast proliferation, based on a BrdU (5-bromo-2′-deoxyuridine) incorporation assay (24 hr) and a WST-8 colorimetric assay (2 or 6 days). Neither E_2 nor LY117018 significantly altered the expression of type I collagen, which is a main component of the tendon extracellular matrix (ECM), whereas both E_2 and LY117018 significantly increased the expression of matrix metalloproteinase (MMP)-13, which is responsible for tendon collagen degradation in rat. Also, both E_2 and LY117018 increased the expression of type III collagen and elastin, which are minor components of tendon ECM, but are considered to govern the elastic properties of tendons. These changes in collagen and MMP induced by either E_2 or LY117018 were attenuated by the estrogen receptor alpha blocker ICI 182,780. The results of this study suggest that postmenopausal estrogen deficiency might downregulate tendon collagen turnover and decrease tendon elasticity. Further, raloxifene treatment might restore these changes to premenopausal levels.

Keywords Raloxifene; Selective Estrogen Receptor Modulator; Estrogen; Tendon Fibroblast; Collagen

INTRODUCTION

Raloxifene is a nonsteroidal benzothiophene that is classified as a selective estrogen receptor modulator (SERM) based on the fact that it displays an unusual tissue-selective pharmacology: it acts as an agonist in some tissues such as bone, liver, and the cardiovascular system, and as an antagonist in the breast and uterine endometrium [1]. Raloxifene mimics the beneficial effects of estrogens without inducing estrogenic adverse events that increase the risk of breast and ovarian cancer. In postmenopausal women, raloxifene is approved for the treatment of osteoporosis and for reduction of breast cancer risk [2, 3].

Raloxifene analogue also has positive effects on other postmenopausal disorders such as dermal degenerative changes [4] and vascular sclerosis [5, 6]. Hormonal alterations after menopause lead to profound changes in various connective tissues, including bone, skin, vascular tissues, pelvic organs, and muscles [7–10]; raloxifene analogue has anabolic effects on most of these tissues. Collagen metabolism alterations mediated by estrogens are considered to influence dermal tissue structure and function, and raloxifene analogue increases collagen synthesis in skin fibroblasts [11]. It remains controversial, however, whether estrogen and other estrogen analogues such as SERM affect metabolism in these tissues.

Tendons and ligaments are important connective tissues that govern motion as well as share the load in diarthrodial joints. Homeostasis of these tissues depends on the balance between

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the synthetic and degradative activities of tendon fibroblasts [12]. The mechanical properties of these tissues change due to vascular, cellular, and collagen-related alterations, and the loss of elasticity and decrease in the strength of these tissues deteriorate motor function, leading to a decrease in the activities of daily living, especially in senile or postmenopausal people. Various factors, including skeletal maturity, aging, daily activity, exercise, sex, and hormonal factors, influence the properties of these tissues [13, 14]. It is not known if estrogen affects tendons and ligaments [15–17]. Previous studies, however, indicate the presence of estrogen receptors (ER) in tendons and ligaments [18, 19]. The identification of ER-positive fibroblasts in tendons and ligaments strongly suggests that female sex hormones may affect the metabolism of tendon and ligament fibroblasts and the maintenance and remodeling of these tissues.

The findings of some clinical studies suggest that estrogen affects the metabolism and properties of these tissues: female athletes are more susceptible to ligament injuries compared to male athletes, and the mechanical properties of the ligaments are influenced by the menstrual cycle [20–22]. To our knowledge, however, there is little data about the effect of SERM/raloxifene analogue on tendon fibroblasts. In the present study, we aimed to elucidate how estrogen and SERM/raloxifene analogue affect the proliferation and extracellular matrix (ECM) metabolism of tendon fibroblasts.

MATERIALS AND METHODS

17β-Estradiol (E_2) was purchased from Sigma Chemical Co. (St Louis, MO, USA). The raloxifene analogue (LY117018) was provided by Eli Lilly Research Laboratories (Indianapolis, IN, USA). ICI 182,780 was purchased from Tocris Cookson Co. (Bristol, MO, USA). Female Sprague-Dawley rats were purchased from CLEA Japan (Tokyo, Japan). Antibodies against type I and type III collagen and matrix metalloproteinase (MMP)-13 were purchased from Abcam (Cambridge, UK). Antibodies against tropoelastin were purchased from Elastin Products Company, (Owensville, MO, USA). Dextran-coated charcoal-treated fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA) and used in all the experiments in this study to minimize the influence of sex hormones contained in untreated FBS.

Cell Culture

Primary cultures of female rat Achilles tendon cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Fibroblasts cultured for, at most, 4 passages were used for the following studies.

Cell Proliferation Assay

Cell proliferation was measured using a bromodeoxyuridine (BrdU) incorporation assay (24 hr) and a WST-8 colorimetric assay (2, 6 days). Cells (5.0×10^3 per well) were seeded in a 48-well plate, and incubated in DMEM (200 μl/well) with 10% FBS. Cells were allowed to adhere to the plate overnight, and then the medium was changed to medium containing E_2 (10^{-13}, 10^{-11}, 10^{-9}, 10^{-7} M) or LY117018 (10^{-11}, 10^{-9}, 10^{-7} M) for the BrdU incorporation assay, and medium containing E_2 (10^{-11}, 10^{-10}, 10^{-9} M) or LY117018 (10^{-9}, 10^{-8}, 10^{-7} M) for the WST-8 colorimetric assay, and either vehicle or ICI 182,780 (100 nM). The medium was replaced by fresh medium containing E_2 or LY117018 every 48 hr for a maximum of 6 days for the WST-8 colorimetric assay. All experiments were performed in sextuplicate and proliferation was expressed as the ratio of the absorbance of treated cells to that of untreated control cells cultured in the same conditions.

BrdU Incorporation Assay

DNA synthesis at 24 hr was measured by a BrdU incorporation assay using a BrdU enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics K.K., Basel, Switzerland). Briefly, the cells were incubated for 2 hr at 37°C with 20 μl/well of BrdU. The supernatant was then removed and the cells were fixed with 400 μl/well of the FixDenat for 30 min at ambient temperature. After removal of the solution, 200 μl/well of anti-BrdU mouse monoclonal antibody conjugated with peroxidase was added. The immune complex was detected by the substrate reaction (tetramethylbenzidine). Absorbance of the samples was measured at 370 nm with the microplate spectrophotometer (Benchmark Plus™, Bio-Rad Laboratories, Hercules, CA, USA).

WST-8 Colorimetric Assay

Cell proliferation at culture days 2 and 6 was assessed by the WST-8 cell counting kit (Dojindo Co., Kumamoto, Japan). The WST-8 cell proliferation assay is a colorimetric method using tetrazolium salt in which the dye intensity is proportional to the number of viable cells. WST-8 solution (20 μl) was added to each well of a 48-well plate and the plate was incubated for 1 hr. Absorbance at 450 nm was measured using the microplate spectrophotometer.

Quantification of Gene Expression

RNA Extraction

Cells (2.5×10^5 per well) were seeded per well in a 6-well plate and incubated in DMEM with 5% FBS. Then 16 hrs later, E_2 (10^{-11}, 10^{-10}, 10^{-9} M) or LY117018 (10^{-9}, 10^{-8}, 10^{-7} M) and either vehicle or ICI 182,780 (10^{-7} M) were added to the cells and incubated for 8 hr. Total RNAs were extracted from

TABLE 1
Sequences of primers used in quantitative RT-PCR

Gene	Forward primers (5′–3′)	Reverse primers (5′–3′)	Amplicon (bp)	Accession number
COL1A1	TTACTACCGGGCCGATGA	CTGCGGATGTTCTCAATCTG	99	XM_213440
COL3A1	TCA AAGGCCCAGCTGGTATC	ACCCTTCTCTCCATTGCGTC	80	NM_032085
MMP-13	GCTCCCAGATGATGACGTTC	GGATAGGGCTGGGTCACAC	109	XM_001072242
TIMP-1	TGGGCTCTGAGAAGGGCTAC	GGAAGGCTTCGGGTCATC	106	NM_053819
ELASTIN	GACACCTTGAAGTCCGACGA	ACGGATGGATGGATGACG	121	NM_012722
GAPDH	GCTGGTCATCAACGGGAAA	ACGCCAGTAGACTCCACGAC	143	NM_017008

the samples using standard TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA).

Reverse Transcription and Real-Time Polymerase Chain Reaction Analysis

For cDNA synthesis, 1 μg of RNA was reverse-transcribed using random hexamer primers (Promega, Madison, WI, USA) and Improm-II reverse transcriptase (Promega). Real-time PCR was performed using Opticon II (Bio-Rad Laboratories) in a 20-μL reaction volume. Signals were detected using the SYBR Green qPCR Kit (Finzymes, Espoo, Finland) with gene-specific primers designed using OLIGO (Molecular Biology Insights, Cascade, CO, USA). Gene-specific primers of type I and III collagens (COL1A1, COL3A1), MMP-13, tissue inhibitors of metalloproteinases (TIMP)-1, ELASTIN, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene are shown in Table 1. The relative mRNA expression level of each targeted gene was normalized by the cycle threshold values of GAPDH.

Immunocytochemistry

Tendon fibroblasts were cultured on glass coverslips with or without 10^{-9} M E_2 or 10^{-7} M LY117018. The 48 hr later, cells were fixed for 5 min with 4% paraformaldehyde in phosphate buffered saline (PBS) and treated with 0.1% Triton X-100 to permeate the cell membranes. The cells were then treated with 0.3% H_2O_2 in PBS to quench the endogenous peroxidase activity, and nonspecific antibody binding was blocked with diluted normal blocking serum for 20 min. Samples were incubated for 1 hr with rabbit antirat polyclonal antibodies against type I or type III collagens and tropoelastin diluted in buffer. For the negative control, the same procedure was followed, excluding the primary antibody.

Bound antibodies were observed by the peroxidase detection system Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). According to the manufacturer's protocol, samples were incubated for 30 min with a biotinylated secondary antibody and avidin/biotinylated horseradish peroxidase. The signal was detected with diaminobenzidine substrate kit (Vector Laboratories) and the specimens were observed under light microscopy.

Measurement of Total Soluble Collagen

Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor, Belfast, Northern Ireland). For these experiments, cells (1.0×10^5 per well) were seeded in a 12-well plate and incubated in DMEM with 10% FBS. This high cell density was used to ensure that the cells were confluent, so that cell proliferation was minimized, whereas collagen synthesis was maximized. Cells were allowed to adhere to the plate overnight, and then the medium was changed to DMEM (500 μl/well) containing E_2 (10^{-9} M) or LY117018 (10^{-7} M), and either vehicle or ICI 182,780 (100 nM) with 5% FBS. Also, 50 μg/ml ascorbic acid was added to this medium for collagen maturation and secretion. The cells were then incubated in this medium for 48 hr or 72 hr. Then, the medium in each well was collected to measure total collagen levels. Next 1 ml of Sirius red dye, an anionic dye that reacts specifically with basic side-chain groups of collagens under assay conditions, was added to 200 μl supernatant, followed by incubation under gentle rotation for 30 min at room temperature. After centrifugation at 12,000 *g* for 10 min, the collagen-bound dye was redissolved with 1 ml of 0.5M NaOH, and the absorbance at 540 nm was measured using the microplate spectrophotometer. Five samples for each treatment group were used for this collagen synthesis assay

Western Blotting

Tendon fibroblasts (1.0×10^5 per well) were seeded in a 12-well plate and incubated in DMEM with 10% FBS and 50 μg/ml ascorbic acid. After 12 hr, the medium was exchanged with 500 μl of fresh medium containing 10^{-9} M E_2 or 10^{-7} M LY117018, and either vehicle or ICI 182,780 (10^{-7} M). After incubation for 48 hr, the supernatants were collected and 10 μl of each supernatant was electrophoresed on a 10% sodium dodecyl sulphate polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The polyvinylidene fluoride membranes were blocked with 5% bovine serum albumin in rinse buffer for 1 hr and then incubated for 12 hr at 4°C with rabbit antirat type I or type III collagens antibodies, or MMP-13 antibody, respectively, diluted 1/10,000 or 1/1000, or 1/1000 in rinse buffer. The membranes were then incubated with the secondary antibody (goat antirabbit IgG antibody conjugated

horseradish peroxidase, diluted 1/2500 in the rinse buffer) for 1 hr.

The bands for type I and III collagens and MMP-13 were detected by the chemiluminescent method using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). After exposing to Fuji X-ray film for 0.5–10 min as necessary to detect the signals, the intensity of immunoreactive band was analyzed using Photoshop (Adobe Systems, San Jose, CA, USA).

Statistical Analysis

Statistical analysis of cell proliferation assay results and quantitative PCR results were performed by analysis of variance using Dunn's method for multiple comparisons with a p value of 0.05 defined as significant. All results are presented as mean and standard deviation.

RESULTS

Effect of E_2 and Raloxifene Analogue on Tendon Fibroblast Proliferation

Fibroblasts never reached full confluence by day 6 of culture under the conditions of this experiment and cell proliferation was observed during this period. In the BrdU cell proliferation assay, neither E_2 nor LY117018 altered the amount of BrdU incorporation at 24 hr compared with untreated controls (Figure 1A). The estrogen antagonist ICI 182,780 also had no significant effect on the amount of BrdU incorporation, despite the presence of E_2 or LY117018. These results suggest that neither E_2 nor LY117018 affects the proliferation of tendon fibroblasts. We confirmed this finding by another proliferation assay using the WST-8 cell counting kit to investigate the effect of longer exposure of fibroblasts to E_2 or LY117018 on cell proliferation. At days 2 and 6, neither E_2 nor LY117018 significantly affected the proliferation of tendon fibroblasts (Figures 1B, 1C).

mRNA Expression of Tendon ECM-Related Genes

Both E_2 and LY117018 tended to upregulate mRNA expression level of type I collagen compared to the untreated controls, but the difference was not significant (Figure 2A). E_2 and LY117018 significantly upregulated the mRNA expression level of type III collagen and elastin, and the estrogen antagonist ICI 182,780 attenuated these changes (Figures 2B, 2E).

Both E_2 and LY117018 upregulated the mRNA expression level of MMP-13 in a dose-dependent manner (Figure 2C). The upregulation of MMP-13 mRNA expression induced by E_2 or LY117018 was attenuated by ICI 182,780. E2 did not significantly increase the mRNA expression of TIMP-1, but ICI 182,780 treatment significantly downregulated the mRNA expression levels of TIMP-1 in the presence of 10^{-9} M E2 (Figure 2D). LY117018 at a concentration of 10^{-8} M

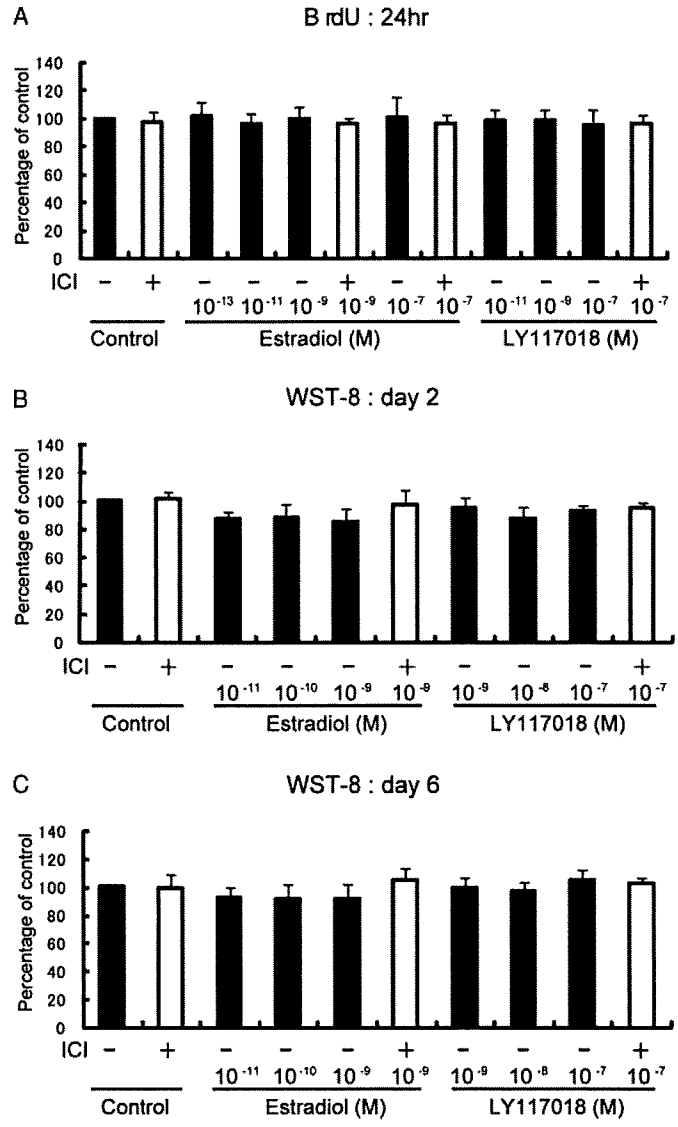

FIG. 1. Proliferative response of rat tendon fibroblasts to various concentrations of E_2 or LY117018 in the presence or absence of ICI 182,780. (A) BrdU incorporation assay results show DNA synthesis of fibroblasts at 24 hr after the treatment. Neither E_2 nor LY117018 had a significant effect on DNA synthesis of fibroblasts. (B) and (C) WST-8 assay results show the proportional value to the number of viable cells. At day 2 (B) and day 6 (C), neither E2 nor LY117018 significantly changed the number of viable cells compared to nonstimulated control cells. Data are expressed as mean ± SD of 6 separate experiments.

significantly upregulated the mRNA expression of TIMP-1, and ICI 182,780 treatment attenuated this upregulation.

Immunocytochemistry of Type I and Type III Collagens and Tropoelastin

No immunostaining was visible in the negative control cells, but the majority of tendon cells in the experimental groups exhibited brown staining for type I and type III collagens and tropoelastin. The stained material was localized exclusively in the cytoplasm. Especially, type I collagen was highly expressed

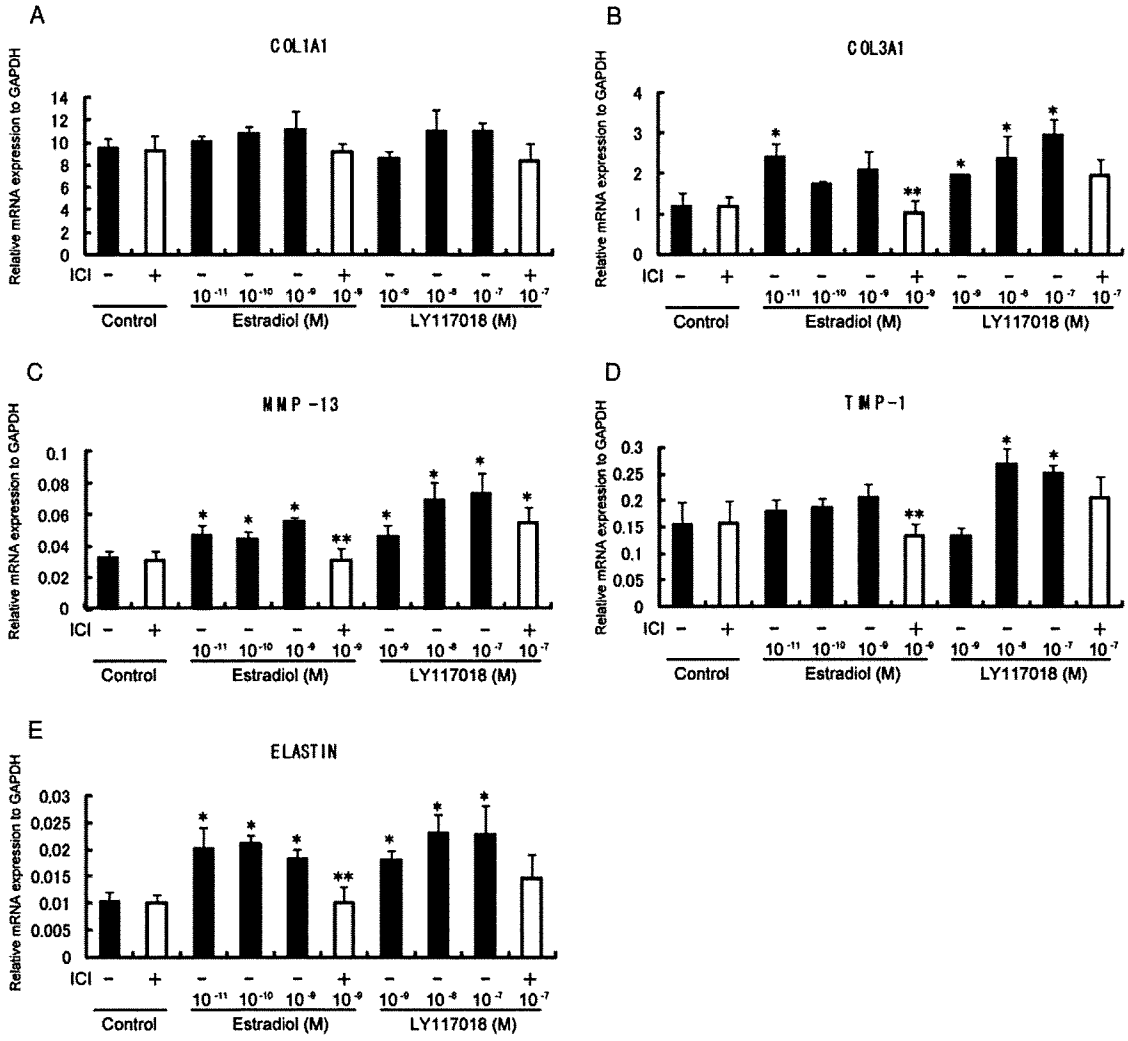

FIG. 2. mRNA expression of collagen metabolism related genes. (A) COL1A1, (B) COL3A1, (C) MMP-13, (D) TIMP-1, and (E) Elastin. Neither E_2 nor LY117018 significantly changed mRNA expression of type I collagen, whereas mRNA expression of type III collagen, elastin, MMP-13, and TIMP-1 was increased by treatment of E_2 and LY117018. These increases in mRNA expression induced by E_2 and LY117018 were attenuated by treatment with ICI 182,780. Data are expressed as mean ± SD of 3 separate experiments. Statistical difference between groups is indicated by * vs. control, ** vs. absence of ICI 182,780.

in the fibroblasts and there was no significant difference in the area or density of brown staining among controls, E_2-treated, and LY117018-treated groups (Figure 3A). In contrast, E_2- and LY117018-treated cells exhibited much stronger staining for type III collagen and tropoelastin compared to the untreated controls (Figure 3A).

Amount of Collagens and MMP Secreted in the Culture Medium

With regard to the amount of total soluble collagen in cell culture supernatants, neither E_2 nor LY117018 significantly affected collagen levels for 48 hr and 72 hr in a Sircol collagen assay (Figure 3B). In Western blotting experiments, exposure of fibroblasts to E_2 or LY117018 did not change the intensity of the band for type I collagen (Figure 3C). The band intensity for type III collagen and MMP-13, however, tended to increase with 10^{-9} M E_2 or 10^{-7} M LY117018 treatment and this effect was attenuated by 10^{-7} M ICI 182,780 (Figure 3C).

DISCUSSION

The results of the present study suggest that both estrogen and SERM/raloxifene analogue affect collagen metabolism in tendon fibroblasts. Although neither E_2 nor LY117018 significantly changed the expression of type I collagen, which is a main component of the tendon ECM, both E_2 and LY117018 increased the expression of MMP-13, which is implicated in collagen degradation of the rat tendon ECM [23]. Because structural and material properties of the tendons and ligaments are maintained by the balance between collagen synthesis and

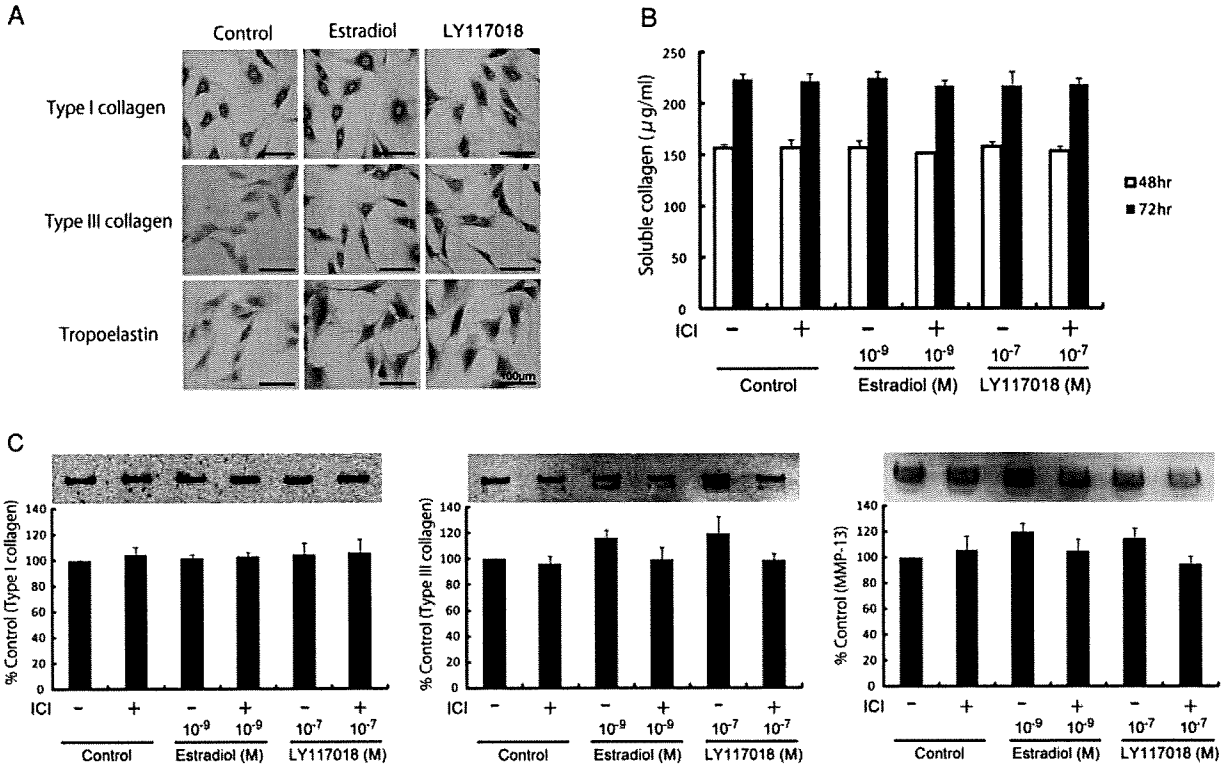

FIG. 3. (A) Immunocytochemistry of type I collagen, type III collagen, and tropoelastin in mock-treated rat tendon fibroblasts and E_2- or LY117018-treated fibroblasts. E_2- and LY117018-treated cells displayed stronger staining for type III collagen and tropoelastin compared to mock-treated cells, whereas there was no apparent difference in staining intensity for type I collagen among mock-treated cells and those treated with E_2 or LY117018. (B) Total soluble collagen levels in cell culture supernatants quantified using the Sircol collagen assay. Neither E_2 nor LY117018 significantly affected collagen levels. (C) Semiquantitative analyses of Western blots of collagens and matrix metalloproteinase secreted in the fibroblast culture medium. The intensity was compared relative to the intensity of the control. Date are expressed as mean ± SD of 3 separate experiments. Exposure of fibroblasts to 10^{-9} M E_2 and 10^{-7} M LY117018 tended to increase the amount of type III collagen and MMP-13 secreted in the culture medium and these effects of E_2 and LY117018 were attenuated by treatment with ICI 182,780 (10^{-7} M).

degradation [12], alterations in the expression of MMP-13 might indicate that estrogen deficiency retards collagen turnover and that treatment with either E_2 or SERM recovers MMP-13 expression to the premenopausal level. If this is the case in vivo, hormonal alterations after menopause might induce a breakdown in the balance of collagen metabolism and retard remodeling of the tendon ECM, leading to the degradation of tendon properties and structure. A recent clinical study reported that postmenopausal women on hormone replacement therapy had fewer tendon abnormalities and thinner tendons than those not on hormone replacement therapy, lending support to the idea that tendon remodeling is retarded in patients with estrogen deficiency [24].

Despite the finding that MMP-13 expression is altered by treatment with E_2 and LY117018 in cultured fibroblasts, caution must be used before applying these findings in vivo. Contrary to our expectation, there was no significant decrease in the amount of type I collagen contained in the E_2- or LY117018-treated cell culture medium compared to the control cell culture medium. There are two possible reasons for this finding: the Western blots may not have been sensitive enough to detect small changes, and the increased expression of TIMP-1 induced by E_2 and LY117018 treatment may have protected against a decrease in type I collagen.

TIMPs, which are synthesized and secreted by fibroblasts, are implicated in collagen metabolism by binding and inhibiting MMP activity [25]. Our finding that changes in the mRNA expression of TIMP-1 induced by E_2 and LY117018 paralleled changes in the expression of MMP-13 suggest that there is a mechanism that keeps collagen metabolism constant despite changing estrogen levels.

An interesting finding in the present study was that both E_2 and LY117018 increased the expression of type III collagen and elastin, which account for less than 10% of the tendon ECM but are considered to govern tendon elastic properties. This is consistent with the finding reported by Sullivan et al. that expression of type III collagen in the tendons was greater in women compared to men [26], suggesting that sex hormones affect type III collagen production. Sullivan et al. [26] also mentioned the possibility that increased expression of type III collagen relates to the increased risk of tendon injury in women compared to men. Based on the fact that type III collagen fibers are thinner and

more extensile than type I collagen fibers [27], and that increased type III collagen content is thought to weaken the tensile strength of tendons, it is likely that estrogen deficiency after menopause and treatment with raloxifene analogue alter the elastic properties of the tendons by modulating the type III collagen content.

Interactions between type I and III collagens are essential for normal fibrillogenesis and regulation of collagen fibril diameter [28–30], and type III collagen is thought to help modulate the size of type I collagen fibers in the tendons [30]. We could not conclude from the results of this study whether the increased expression of type III collagen and elastin in the presence of E_2 or raloxifene analogue deteriorates tendon properties.

The proliferative activity of tendon fibroblasts is also an important factor for tendon metabolism; however, in this study E_2 and LY117018 had no significant effect on the proliferative capacity of tendon fibroblasts. The results of both the cell proliferation assay and the BrdU DNA synthesis assay support this idea. Considering the variety of responses to estrogen in fibroblasts, such as enhanced cell proliferation of gingival fibroblasts [31] and the dose-dependent decrease in the proliferation of the E_2-treated anterior cruciate ligament fibroblasts [32, 33], the responsiveness of fibroblasts to E_2 or raloxifene analogue might differ according to the organ or tissue type from which the fibroblasts are derived. Because tendons originally comprise a small number of cells and excessive cell growth may make the tendon fibrotic such as in Dupuytren's disease [34], cell proliferation of tendon fibroblasts might be regulated by complex mechanisms independent of estrogen or SERM.

The reactions of tendon fibroblasts to LY117018 and E_2 were identical and the reaction to LY117018 was reversed with ICI 182,780, which is an ER alpha blocker [35]. Because SERM has agonist activity in some tissues and antagonistic activity in others, the actions of raloxifene analogue in tendons are of great interest to both physicians and researchers. The fact that the response to LY117018 is reversed with ICI 182,780 indicates that E_2 and LY117018 act on ECM metabolism of tendon fibroblasts through ER alpha.

A limitation of this study is that the results were obtained in an in vitro cell culture system. Collagen metabolism in the tendons and ligaments is regulated by various factors [36–40] and ECM metabolism of tendon fibroblasts might differ in vitro and in vivo. For example, mechanical stress has a crucial role in maintaining tendon homeostasis [40]. Lee et al. reported that E_2 alone increased the mRNA expression of type III collagen, but the combined administration of E_2 and mechanical stimulation inhibited the upregulation in cultured porcine anterior cruciate ligament fibroblasts [41, 42]. Given that the effect of mechanical stress overcomes the effect of estrogen on collagen metabolism, the effect of raloxifene analogue on tendon collagen metabolism might also be reversed by mechanical stress. Ascorbic acid, which is an antioxidant vitamin, is also reported to affect collagen metabolism in fibroblasts [43–45]. The amount of collagen secreted, however, was evaluated in the presence of ascorbic acid since ascorbic acid is essential for collagen maturation and secretion [46].

To distinguish the influence of ER agonists on collagen metabolism from that of ascorbic acid, we assessed the mRNA expression and intracellular protein expression of collagen in the absence of ascorbic acid. In this case, however, we could not exclude the possibility that intracellular accumulation of collagen induced by the absence of ascorbic acid might lead feedback regression of transcriptional level. Even though we used two different culture conditions—one was in the presence of ascorbic acid and the other in the absence of ascorbic acid—the fact that consistent results were obtained through mRNA expression, intracellular protein expression, and the secretion of collagen encourages our findings. Additionally, many cytokines affect tendon and ligament properties during activities of daily living [47] and these various influences might interact with the effects of estrogen, leading to conflicting results regarding whether estrogen affects tendon properties. Strickland et al. recently reported that the administration of raloxifene analogue does not significantly affect the mechanical properties of sheep knee ligaments [48]. For these reasons, care must be taken to apply these findings in the clinical setting.

CONCLUSIONS

In this in vitro study, estrogen and raloxifene analogue did not alter the expression of type I collagen but increased MMP-13 expression, which is involved in rat tendon ECM degradation. Estrogen and raloxifene analogue also increased the expression of type III collagen, which relates to fibrillogenesis as well as tendon elastic properties. These findings indicate that estrogen deficiency might retard tendon ECM remodeling and alter tendon elasticity. Treatment with raloxifene may help to recover these changes to premenopausal levels, but further in vivo studies are required.

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Declaration of Interest

Raloxifene analogue LY117018 was provided Eli Lilly and Company. The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Kim, I.Y., Kim, B.C., Seong, D.H., Lee, D.K., Seo, J.M., Hong, Y.J., Kim, H.T., Morton, R.A., and Kim, S.J. (2002). Raloxifene, a mixed estrogen agonist/antagonist, induces apoptosis in androgen-independent human prostate cancer cell lines. *Cancer Res.*, 62, 5365–5369.
2. Body, J.J. (2008). [Update on treatment of postmenopausal osteoporosis]. *Rev. Med. Brux.*, 29, 301–309.
3. Goldstein, S.R., Duvernoy, C.S., Calaf, J., Adachi, J.D., Mershon, J.L., Dowsett, S.A., Agnusdei, D., and Stuenkel, C.A. (2008). Raloxifene use in clinical practice: efficacy and safety. *Menopause*, Epub ahead of print, Dec. 13, 2008.

4. Sumino, H., Ichikawa, S., Kasama, S., Takahashi, T., Kumakura, H., Takayama, Y., Kanda, T., Murakami, M., and Kurabayashi, M. (2009). Effects of raloxifene and hormone replacement therapy on forearm skin elasticity in postmenopausal women. *Maturitas*, 20, 53–57.
5. Choi, B.G., Vilahur, G., Zafar, M.U., Cardoso, L., Yadegar, D., Ibanez, B., Tunstead, J., Viles-Gonzalez, J.F., Schaffler, M.B., Fuster, V., and Badimon, J.J. (2008). Selective estrogen receptor modulation influences atherosclerotic plaque composition in a rabbit menopause model. *Atherosclerosis*, Epub ahead of print, Feb. 21, 2008.
6. Abu-Fanne, R., Brzezinski, A., Golomb, M., Grad, E., Foldes, A.J., Shufaro, Y., Varon, D., Brill, A., Lotan, C., and Danenberg, H.D. (2008). Effects of estradiol and raloxifene on arterial thrombosis in ovariectomized mice. *Menopause*, 15, 98–104.
7. Verdier-Sevrain, S., Bonte, F., and Gilchrest, B. (2006). Biology of estrogens in skin: implications for skin aging. *Exp. Dermatol.*, 15, 83–94.
8. Stevenson, J.C. (2009). HRT and cardiovascular disease. *Best Pract. Res. Clin. Obstet. Gynaecol.*, 23, 109–120.
9. Pal, L., Hailpern, S.M., Santoro, N.F., Freeman, R., Barad, D., Kipersztok, S., Barnabei, V.M., and Wassertheil-Smoller, S. (2008). Association of pelvic organ prolapse and fractures in postmenopausal women: analysis of baseline data from the Women's Health Initiative Estrogen Plus Progestin trial. *Menopause*, 15, 59–66.
10. Orsatti, F.L., Nahas, E.A., Maesta, N., Nahas-Neto, J., and Burini, R.C. (2008). Plasma hormones, muscle mass and strength in resistance-trained postmenopausal women. *Maturitas*, 59, 394–404.
11. Surazynski, A., Jarzabek, K., Haczynski, J., Laudanski, P., Palka, J., and Wolczynski, S. (2003). Differential effects of estradiol and raloxifene on collagen biosynthesis in cultured human skin fibroblasts. *Int. J. Mol. Med.*, 12, 803–809.
12. Takeyama, N., Sakai, H., Ohtake, H., Mashitori, H., Tamai, K., and Saotome, K. (2007). Effects of hyperbaric oxygen on gene expressions of procollagen, matrix metalloproteinase and tissue inhibitor of metalloproteinase in injured medial collateral ligament and anterior cruciate ligament. *Knee Surg. Sports Traumatol. Arthrosc.*, 15, 443–452.
13. Miller, B.F., Hansen, M., Olesen, J.L., Schwarz, P., Babraj, J.A., Smith, K., Rennie, M.J., and Kjaer, M. (2007). Tendon collagen synthesis at rest and after exercise in women. *J. Appl. Physiol.*, 102, 541–546.
14. Wentorf, F.A., Sudoh, K., Moses, C., Arendt, E.A., and Carlson, C.S. (2006). The effects of estrogen on material and mechanical properties of the intra- and extra-articular knee structures. *Am. J. Sports Med.*, 34, 1948–1952.
15. Warden, S.J., Saxon, L.K., Castillo, A.B., and Turner, C.H. (2006). Knee ligament mechanical properties are not influenced by estrogen or its receptors. *Am. J. Physiol. Endocrinol. Metab.*, 290, E1034–E1040.
16. Komatsuda, T., Sugita, T., Sano, H., Kusakabe, T., Watanuki, M., Yoshizumi, Y., Murakami, T., Hashimoto, M., and Kokubun, S. (2006). Does estrogen alter the mechanical properties of the anterior cruciate ligament? An experimental study in rabbits. *Acta Orthop.*, 77, 973–980.
17. Slauterbeck, J., Clevenger, C., Lundberg, W., and Burchfield, D.M. (1999). Estrogen level alters the failure load of the rabbit anterior cruciate ligament. *J. Orthop. Res.*, 17, 405–408.
18. Sciore, P., Frank, C.B., and Hart, D.A. (1998). Identification of sex hormone receptors in human and rabbit ligaments of the knee by reverse transcription-polymerase chain reaction: evidence that receptors are present in tissue from both male and female subjects. *J. Orthop. Res.*, 16, 604–610.
19. Faryniarz, D.A., Bhargava, M., Lajam, C., Attia, E.T., and Hannafin, J.A. (2006). Quantitation of estrogen receptors and relaxin binding in human anterior cruciate ligament fibroblasts. *In Vitro Cell Dev. Biol. Anim.*, 42, 176–181.
20. Wojtys, E.M., Huston, L.J., Lindenfeld, T.N., Hewett, T.E., and Greenfield, M.L. (1998). Association between the menstrual cycle and anterior cruciate ligament injuries in female athletes. *Am. J. Sports Med.*, 26, 614–619.
21. Slauterbeck, J.R., Fuzie, S.F., Smith, M.P., Clark, R.J., Xu, K., Starch, D.W., and Hardy, D.M. (2002). The menstrual cycle, sex hormones, and anterior cruciate ligament injury. *J. Athl. Train.*, 37, 275–278.
22. Wojtys, E.M., Huston, L.J., Boynton, M.D., Spindler, K.P., and Lindenfeld, T.N. (2002). The effect of the menstrual cycle on anterior cruciate ligament injuries in women as determined by hormone levels. *Am. J. Sports Med.*, 30, 182–188.
23. Sun, H.B., Li, Y., Fung, D.T., Majeska, R.J., Schaffler, M.B., and Flatow, E.L. (2008). Coordinate regulation of IL-1beta and MMP-13 in rat tendons following subrupture fatigue damage. *Clin. Orthop. Relat. Res.*, 466, 1555–1561.
24. Cook, J.L., Bass, S.L., and Black, J.E. (2007). Hormone therapy is associated with smaller Achilles tendon diameter in active post-menopausal women. *Scand. J. Med. Sci. Sports*, 17, 128–132.
25. Nahm, D.S., Kim, H.J., Mah, J., and Baek, S.H. (2004). In vitro expression of matrix metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and transforming growth factor-beta1 in human periodontal ligament fibroblasts. *Eur. J. Orthod.*, 26, 129–135.
26. Sullivan, B.E., Carroll, C.C., Jemiolo, B., Trappe, S.W., Magnusson, S.P., Dossing, S., Kjaer, M., and Trappe, T.A. (2009). Effect of acute resistance exercise and sex on human patellar tendon structural and regulatory mRNA expression. *J. Appl. Physiol.*, 106, 468–475.
27. Gazoti Debessa, C.R., Mesiano Maifrino, L.B., and Rodrigues de Souza, R. (2001). Age related changes of the collagen network of the human heart. *Mech. Ageing Dev.*, 122, 1049–1058.
28. Liu, X., Wu, H., Byrne, M., Krane, S., and Jaenisch, R. (1997). Type III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. *Proc. Natl. Acad. Sci. USA*, 94, 1852–1856.
29. Birk, D.E., and Mayne, R. (1997). Localization of collagen types I, III and V during tendon development. Changes in collagen types I and III are correlated with changes in fibril diameter. *Eur. J. Cell Biol.*, 72, 352–361.
30. Banos, C.C., Thomas, A.H., and Kuo, C.K. (2008). Collagen fibrillogenesis in tendon development: current models and regulation of fibril assembly. *Birth Defects. Res. C Embryo Today*, 84, 228–244.
31. Mariotti, A.J. (2005). Estrogen and extracellular matrix influence human gingival fibroblast proliferation and protein production. *J. Periodontol.*, 76, 1391–1397.
32. Liu, S.H., Al-Shaikh, R.A., Panossian, V., Finerman, G.A., and Lane, J.M. (1997). Estrogen affects the cellular metabolism of the anterior cruciate ligament. A potential explanation for female athletic injury. *Am. J. Sports Med.*, 25, 704–709.
33. Yu, W.D., Liu, S.H., Hatch, J.D., Panossian, V., and Finerman, G.A. (1999). Effect of estrogen on cellular metabolism of the human anterior cruciate ligament. *Clin. Orthop. Relat. Res.*, 336, 229–238.
34. Townley, W.A., Cambrey, A.D., Khaw, P.T., and Grobbelaar, A.O. (2008). Matrix metalloproteinase inhibition reduces contraction by dupuytren fibroblasts. *J. Hand Surg. [Am]*, 33, 1608–1616.
35. Pearce, S.T., and Jordan, V.C. (2004). The biological role of estrogen receptors alpha and beta in cancer. *Crit. Rev. Oncol. Hematol.*, 50, 3–22.
36. Thornton, G.M., Shrive, N.G., and Frank, C.B. (2003). Healing ligaments have decreased cyclic modulus compared to normal ligaments and immobilization further compromises healing ligament response to cyclic loading. *J. Orthop. Res.*, 21, 716–722.
37. Kim, S.G., Akaike, T., Sasagaw, T., Atomi, Y., and Kurosawa, H. (2002). Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. *Cell Struct. Funct.*, 27, 139–144.
38. Park, S.A., Kim, I.A., Lee, Y.J., Shin, J.W., Kim, C.R., Kim, J.K., Yang, Y.I., and Shin, J.W. (2006). Biological responses of ligament fibroblasts and gene expression profiling on micropatterned silicone substrates subjected to mechanical stimuli. *J. Biosci. Bioeng.*, 102, 402–412.
39. Majima, T., Marchuk, L.L., Sciore, P., Shrive, N.G., Frank, C.B., and Hart, D.A. (2000). Compressive compared with tensile loading of medial collateral ligament scar in vitro uniquely influences mRNA levels for aggrecan, collagen type II, and collagenase. *J. Orthop. Res.*, 18, 524–531.

40. Majima, T., Yasuda, K., Yamamoto, N., Kaneda, K., and Hayashi, K. (1994). Deterioration of mechanical properties of the autograft in controlled stress-shielded augmentation procedures. An experimental study with rabbit patellar tendon. *Am. J. Sports Med.*, 22, 821–829.
41. Lee, C.Y., Liu, X., Smith, C.L., Zhang, X., Hsu, H.C., Wang, D.Y., and Luo, Z.P. (2004). The combined regulation of estrogen and cyclic tension on fibroblast biosynthesis derived from anterior cruciate ligament. *Matrix Biol.*, 23, 323–329.
42. Lee, C.Y., Smith, C.L., Zhang, X., Hsu, H.C., Wang, D.Y., and Luo, Z.P. (2004). Tensile forces attenuate estrogen-stimulated collagen synthesis in the ACL. *Biochem. Biophys. Res. Commun.*, 317, 1221–1225.
43. Shibayama, H., Hisama, M., Matsuda, S., Kawase, A., Ohtsuki, M., Hanada, K., and Iwaki, M. (2008). Effect of a novel ascorbic derivative, disodium isostearyl 2-O-L-ascorbyl phosphate, on normal human dermal fibroblasts against reactive oxygen species. *Biosci. Biotechnol. Biochem.*, 72, 1015–1022.
44. Amano, S., Ogura, Y., Akutsu, N., Nishiyama, and T. (2007). Quantitative analysis of the synthesis and secretion of type VII colagen in cultured human dermal fibroblast with a sensitive sandwich enzyme-linked immunoassay. *Exp. Dermatol.*, 16, 151–155.
45. Shiga, M., Kapila, Y.L., Zhang, Q., Hayashi, T., and Kapila, S. (2003). Ascorbic acid induces collagenase-1 in human periodontal ligament cells but not in MC3T3-E1 osteoblast-like cells: potential association between collagenase expression and changes in alkaline phosphatase phenotype. *J. Bone Miner. Res.*, 18, 67–77.
46. Qiao, H., Bell, J., Juliao, S., Li, L., and May, J.M. (2009) Ascorbic acid uptake and regulation of type I collagen synthesis in cultured vascular smooth muscle cells. *J. Vasc. Res.*, 46, 15–24.
47. Azuma, H., Yasuda, K., Tohyama, H., Sakai, T., Majima, T., Aoki, Y., and Minami, A. (2003). Timing of administration of transforming growth factor-beta and epidermal growth factor influences the effect on material properties of the in situ frozen-thawed anterior cruciate ligament. *J. Biomech.*, 36, 373–381.
48. Strickland, S.M., Belknap, T.W., Turner, S.A., Wright, T.M., and Hannafin, J.A. (2003). Lack of hormonal influences on mechanical properties of sheep knee ligaments. *Am. J. Sports Med.*, 31, 210–215.

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ORIGINAL ARTICLE

Treatment of juxta-articular intraosseous cystic lesions in rheumatoid arthritis patients with interconnected porous calcium hydroxyapatite ceramic

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Abstract In patients with rheumatoid arthritis (RA), juxta-articular intraosseous cystic lesions may cause spontaneous pathological fractures. The outcome of curettage and the packing of such lesions with interconnected porous calcium hydroxyapatite ceramic (IP-CHA) was investigated. Twelve lesions were treated in ten RA patients (three men and seven women with a mean age of 59 years). Ten lesions were associated with impending pathological fracture involving the articular surface. In all patients, curettage and packing of the bone cavity with IP-CHA were done. Assessment was based on final radiographs obtained an average of 30 months after surgery (range 10–47 months). Absorption of the implanted IP-CHA, expansion of the lesion, implant incorporation into host bone, and postoperative fractures were investigated. At final follow-up, there was no absorption of the implanted IP-CHA in any of the lesions. Expansion of the radiolucent area was only noted in one lesion. Seven of the other 11 lesions showed major incorporation of IP-CHA into host bone, while minor incorporation was seen in four lesions. There were no postoperative fractures. In conclusion, curettage and packing with IP-CHA is a feasible method of preventing pathological fracture due to juxta-articular intraosseous cystic lesions in RA patients.

Keywords Interconnected porous calcium hydroxyapatite ceramic · Juxta-articular intraosseous cystic lesions · Rheumatoid arthritis · Surgical treatment

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Introduction

Juxta-articular intraosseous cystic lesions in patients with rheumatoid arthritis (RA) have been variously termed synovial cysts [1], subchondral cysts [2], subarticular pseudocysts [3], or geodes [4]. These lesions are often found during the course of RA, but aggressive surgical treatment is not usually performed because the patient has no symptoms unless spontaneous or traumatic intra-articular fracture occurs. Once fracture occurs, however, joint destruction progresses rapidly due to mechanical stress rather than disease-related inflammation, and daily activities can be severely affected [5–8]. It is important to maintain the subchondral bone of RA patients in good condition, not only to prevent pathological fractures but also because of the possible need to perform arthroplasty in the future.

We have treated juxta-articular intraosseous cystic lesions in RA patients by curettage and packing with a hydroxyapatite filler (interconnected porous calcium hydroxyapatite ceramic, IP-CHA) in order to prevent subchondral fractures. In our experience, IP-CHA undergoes

extensive incorporation into host bone more rapidly than other types of porous calcium hydroxyapatite ceramic [9]. The purpose of the present study was to evaluate the preliminary results obtained with this treatment.

Materials and methods

Between September 2003 and March 2005, we treated 12 juxta-articular cystic lesions in ten RA patients (Table 1). Ten of the 12 lesions were associated with a risk of impending intra-articular fracture, judging from the fragile appearance of the subchondral bone on radiographs. The other two lesions were treated as an additional procedure during synovectomy and matched ulnar resection, respectively. All patients fulfilled the American Rheumatism Association diagnostic criteria for RA [10]. Three patients were men and seven were women, with an average age of 59 years (range 49–72 years) at the time of operation. The location and size of the lesions were determined by examination of preoperative anteroposterior plain X-ray films. Eight lesions were located in the distal radius (four involved most of the subarticular surface, two were located in the radial styloid process, and the remaining two were at the center of the subarticular bone), while one lesion was located in the head of the ulna, one in the center of the proximal tibial condyle, one in the medial malleolus of the tibia, and the lateral malleolus of the fibula. The lesions were classified into three groups on the basis of the transverse diameter: large lesions had a diameter of greater than two-thirds of the articular surface ($n = 6$), medium lesions had a diameter of one-third to two-thirds of the articular surface ($n = 4$), and small lesions had a diameter of less than one-third ($n = 2$). Disease activity was assessed preoperatively from the C-reactive protein (CRP) level and the tenderness and swelling of the involved joint. The Steinbrocker functional class and stage [11] and the Larsen grade [12] of the joint were also assessed. Each patient's medications were recorded (Table 2).

Operative technique

Surgery was performed under regional or general anesthesia. A pneumatic tourniquet was applied. Under fluoroscopic guidance as needed, each juxta-articular lesion was exposed via an extra-articular approach. For lesions of the distal radius, the second or fourth extensor retinaculum was opened through a dorsal skin incision about 2–3 cm long. For lesions of the knee, ankle, and ulna, a skin incision was made just over the target. A small window (about 8 mm square) was made in the wall of the lesion, taking care to preserve the periosteum of the resected bone. After performing intralesional curettage and complete resection of the capsule of the cyst, the residual bone defect was filled with blocks and granules of sterilized IP-CHA (Stryker Co., Tokyo, Japan). Then the small bone section was replaced in order to close the cortical window. After surgery, a splint was not applied and range-of-movement exercises were commenced immediately. Together with the above procedure, synovectomy of the wrist and knee joint was also done in two patients who had wrist synovitis (case 2) and a huge synovial cyst (case 9), respectively. Matched ulnar head resection was also performed in one patient (case 5) who presented with disability of the distal radioulnar joint.

Table 1 Details of the 12 lesions

Case no.	Age	Gender	Location	Size (mm)	Follow-up (months)	Expansion	Absorption	Incorporation (grade)	Combined operation
1	55	M	R distal radius	35 × 20 (large)	42	–	–	2	
			L distal radius	35 × 12 (large)		–	–	3	
			Distal ulna	16 × 9 (large)		–	–	3	
2	52	F	Proximal tibia	50 × 32 (medium)	10	–	–	2	#1
3	61	F	Distal fibula	33 × 21 (large)	40	–	–	3	
4	53	F	Distal radius	19 × 15 (medium)	47	–	–	3	
5	72	F	Distal radius	11 × 10 (small)	22	–	–	3	#2
6	63	M	Distal radius	25 × 12 (medium)	37	–	–	3	
7	49	F	Distal radius	35 × 20 (large)	16	+	–	–	
8	59	M	Distal radius	16 × 9 (medium)	19	–	–	2	
9	55	F	Distal radius	7 × 4 (small)	36	–	–	3	#1
10	71	F	Distal tibia	29 × 17 (large)	29	–	–	2	

Expansion expansion of the radiolucent area, *Absorption* absorption of implanted IP-CHA, *Combined operation* other procedures performed simultaneously, *#1* synovectomy, *#2* matched ulnar resection

Table 2 Details of the ten patients

Case no.	Preoperative CRP (mg/dl)	Location	Class	Stage	Larsen grade	Local tenderness	Local swelling	Medications (daily doses except for MTX)
1	0.9	R distal radius	3	4	4	Mild	Mild	Predonine 10 mg, MTX 6 mg
		L distal radius			4	Mild	Mild	
		Distal ulna			4	Mild	Mild	
2	2.9	Proximal tibia	2	3	2	Moderate	Severe	Predonine 10 mg, MTX 6 mg, bucillamine 300 mg
3	0.2	Distal fibula	2	3	4	Non	Non	MTX 4 mg, metronidazole, salazosulfapyridine 1,000 mg
4	0.2	Distal radius	2	3	4	Mild	Mild	Predonine 7.5 mg, MTX 6 mg
5	1.3	Distal radius	3	2	4	Moderate	Moderate	Predonine 5 mg, salazosulfapyridine 1,000 mg
6	0.9	Distal radius	2	3	3	Mild	Moderate	Predonine 5 mg, bucillamine 200 mg
7	2.5	Distal radius	3	3	5	Moderate	Moderate	Predonine 10 mg
8	0.8	Distal radius	2	3	5	Mild	Mild	Salazosulfapyridine 1,000 mg
9	0.4	Distal radius	1	2	3	Moderate	Moderate	Predonine 1 mg, salazosulfapyridine 1,000 mg
10	1.2	Distal tibia	2	4	2	Non	Non	Salazosulfapyridine 1,000 mg

Class Steinbrocker functional class, *Stage* Steinbrocker stage, *MTX* methotrexate (weekly doses)

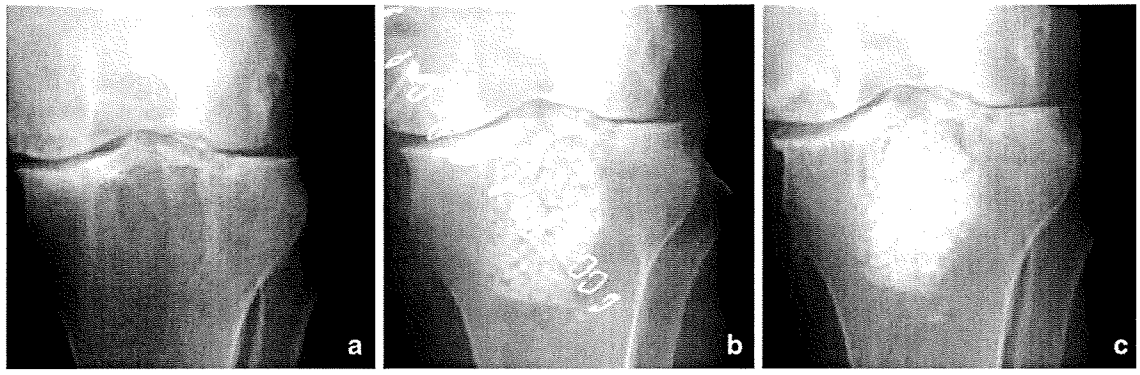

Fig. 1 Case 2: **a** preoperative radiograph reveals a large radiolucent area in the proximal tibia. **b** Radiograph obtained just after surgery. **c** At 10 months after surgery, there is no absorption of IP-CHA or expansion of the cystic lesion

Radiological assessment

Radiological assessment was performed by comparing radiographs obtained just after surgery with those obtained at final follow-up at an average of 30 months (range 10–47 months). The following four points were assessed: absorption of the implanted IP-CHA, expansion of the cystic lesion, incorporation of the implanted IP-CHA into host bone, and occurrence of postoperative fracture. The extent of incorporation of the implanted IP-CHA by host bone was graded according to the previously reported method [9]. In brief, Grade 1 was no incorporation, Grade 2 was minor incorporation (a slight increase in the density of the implanted IP-CHA granules and partial disappearance of the radiolucent lines between implant and host bone), and Grade 3 was major incorporation (a marked increase of density and/or disappearance of the spaces between IP-CHA granules).

Clinical assessment

Complications such as fracture, infection, and joint contracture related to surgery were assessed by clinical review.

Results

Absorption of the implanted IP-CHA did not occur in any patient and there were no postoperative fractures. Eleven of the 12 lesions showed no expansion at final follow-up. In these 11 lesions, the density of the implanted IP-CHA increased over time, and the granules appeared to become fused and incorporated into the surrounding host bone. Four of the 11 lesions showed grade 2 incorporation and seven lesions showed grade 3 incorporation at final follow-up (Figs. 1, 2). One patient (case 7) had poorly controlled RA due to concomitant hepatic and pancreatic dysfunction,