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Serum-dependent osteoblastic changes in cultured tenocytes isolated from rat Achilles tendon

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Abstract

Objective : Tendinopathy such as chronic tendon pain and tendon rupture is often accompanied by ectopic ossification and vascularization in elderly individuals. However, the exact mechanisms of the tendon degeneration including ossification have not been clarified yet. In the present study, we demonstrated the serum-dependent changes in the phenotype of rat tenocytes.

Methods : Tenocytes isolated from rat Achilles tendon were cultured in medium containing 2% fetal bovine serum (FBS) until confluence followed by continuous culture in medium containing various concentrations (2%, 5%, and 10%) of FBS for two weeks. Then, cells were subjected to alkaline phosphatase-dependent staining. Gene expression analysis was performed by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Results : Alkaline phosphatase-positive osteoblast-like cells were increased in a serum concentration-dependent manner. Quantitative real-time RT-PCR analysis also revealed that serum significantly increased the gene expression of alkaline phosphatase as well as osteopontin. In addition, the gene expression of collagen type I α1 chain, decorin, and tenomodulin was lowered in a serum concentration-dependent manner. On the other hand, collagen type II α1 and Sox-9 mRNA were not affected by serum.

Conclusion : These results indicate that the phenotype of rat tenocytes is readily changed to osteoblast-like cells in a serum-dependent manner accompanying with an increase of alkaline phosphatase and osteopontin as well as a decrease of decorin and tenomodulin. These findings in the present study are informative for the understanding of the degenerative mechanism of Achilles tendon and development of novel therapeutic methods of tendon injuries to prevent undesired ectopic ossification.

Introduction

Achilles tendinopathy such as chronic tendon pain and tendon rupture is relatively common in among especially men over 30 years of ages[1-4)]. Minor trauma and repetitive overuse in addition to individual genetic background are thought to be the cause of tendinopathy[5-7)], and degenerative changes including calcification and neovas-

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cularisation are often observed in injured tendons[8]. Tendons are predominantly comprised of aligned collagen fibrils[9-11]. The tensile strength of the collagen fibrils is mainly composed of type I collagen and other minor components including type III and V collagens and a small proteoglycan, decorin[9-11]. Decorin is recognized as a regulator for the assembly of collagen fibrils and acquisition of biomechanical properties in tendon[12]. Based on the structures and the properties of extracellular matrix macromolecules, tendons and ligaments have high mechanical strength and elasticity necessary to perform their functions. On the other hand, the dominant cell species in tendons is tenocytes that are embedded in the extensive collagen fibrils in the tendon[9-11]. Tenocytes are a source of the extracellular matrix components in tendon tissue[9-11], and produce the aligned collagen fibrils as fibripositors[13]. In addition to the aligned collagen fibrils of tendon, these are characterized as hypovascular tissues as well as cartilage[9-11]. Tenomodulin is an anti-angiogenic protein predominantly expressed in tendons, ligaments, and eyes[14][15]. Thus, the resident cells in tendon are in a place away from the blood components. However, neocvascularization has been shown in Achilles tendon with painful tendinopathy, resulting in influencing tenocytes by the blood component[8].

Tenocytes have been shown to be phenotypically altered in damaged tendon tissues, and phenotypic drift in tenocyte culture has been reported[16]. In addition, fibrocartilagenous changes of an injured region of a tendon with the increasing of carilagenous matrix components including type II collagen have been reported[17-19]. Fibrocartilagenous change is suggested to be induced by compressive loading[17-19]. Therefore, phenotypic changes of tenocytes are considered to be crucially involved in ectopic ossification. However, the cellular changes of tenocytes in regard to ossification have yet to be clarified so far.

In the present study, we present novel evidence that the phenotype of tenocytes derived from rat Achilles tendon is modified by serum, and the cells are changed to alkaline phosphatase-positive osteoblastic ones in a serum-related fashion.

Materials and Methods

Cell culture

Rat tenocytes were prepared from the Achilles tendons of male Sprague Dawley rats (six weeks old, Charles River Japan, Yokohama, Japan) by an outgrowth method as previously described[20]. In brief, minced Achilles tendon tissues (1-2 mm^3) were maintained on the culture dish in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Co., Carlsbad, CA) containing 2% fetal bovine serum (FBS) (Thermo ELECTRON Co., Melbourne, Australia) with antibiotics [100 units/ml of penicillin G (MP Biomedicals Inc, OH) and 100 μg/ml of streptomycin sulfate (Meiji Seika Ltd., Tokyo, Japan)] for 1-2 weeks, and the cells growing out around the tissue were collected by 0.25% trypsin in phosphate buffered saline (PBS) with 0.02% EDTA. For the studies on the effect of the serum, the collected cells were cultured in DMEM containing 2% FBS with antibiotics until confluence. Then, cells were maintained in DMEM containing 2%, 5%, or 10% FBS. The procedures described here were approved by the Committee on the Care and Use of Experimental Animals at Tokyo University of Pharmacy and Life Sciences.

Alkaline phosphatase staining

Cells in culture dishes were washed with PBS, and fixed with neutral 3.7% formaldehyde solution for 10 min. Then, cells were incubated with 100 mM Tris-HCl (pH 8.5)/2 mM $MgCl_2$/0.01% Naphtol AS-MX phosphate/0.06% Fast blue BB salt for 15 min under light protected condition[21]. Cells were washed with running water, and the stained cells were observed under microscopy.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from the cells cultured in 35-mm diameter dishes using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The total RNA (0.5 μg) was subjected to RT reaction using a PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. A portion (an equivalent of 2.5 ng of total RNA) of the products of RT reaction was subjected to real-time PCR using SYBR Premix EX Taq II. PCR primers used were as follows, rat alkaline phosphatase

(ALPase) (GenBank Accession No. NM_013059), forward primer, CGGACACAACTACCAGGCCCAGTCCG, reverse primer, GGCACAGTGGTCAAGGTTGG ; rat collagen type I α1 (GenBank Accession No. XM_213440), forward primer CGGCAGAAGTCTCAAGATGGTGGCCG, reverse primer, CTCTCCGCTCTTCCAGTCAGA ; rat collagen type II α1 (GenBank Accession No. NM_012929), forward primer, TCAGGAATTTGGTGTGGACATA, reverse primer, CAGCCATTCAGTGCAGATCCTA ; rat decorin (GenBank Accession No. NM_024129), forward primer, GACAACAACAAACTCCTCA, reverse primer, AGAAGTCATGCTCCCAAA ; rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Accession No. NM_012881), forward primer, GGCACAGT CAAGGCTGAGAATG, reverse primer, ATGGTG GTGAAGACGCCAGTA ; rat Sox-9 (GenBank Accession No. XM_001081628), forward primer, CTGGTT TCGTTCTCTGTTT, reverse primer, GCGAGCACT TAGCAGAGG ; and rat tenomodulin (GenBank Accession No. NM_022290), forward primer, AAGACCTAT GGCATGGAGCACA, reverse primer, CGGATCAAA GTAGATGCCAGTGTATCCG. These primers were synthesized by Operon Biotechnologies (Tokyo, Japan). Rat osteopontin (GenBank Accession No. NM_012881) primers were QuantiTect Primer Assays (Cat. No. QT00199101, Qiagen). PCR was performed using Thermal Cycler Dice TP-800 (Takara Bio Inc.) under the following conditions, denature at 94°C for 5 sec and annealing and extension at 60°C for 30 sec. Relative expression levels were calculated with $\Delta\Delta C_T$ method normalized by GAPDH. Data were expressed, taking mRNA level with the cells cultured under presence of 2% FBS as 1.

Statistical analysis

One-way ANOVA was performed using computer software StatView version 5.0 (SAS Institute, Inc, SAS Campus Drive Cary, NC) for the data analysis. Independent Student's *t*-test was applied for pair comparisons, and Fisher's PLSD *post-hoc* test was performed for multiple comparisons. The level of statistically significant difference was set at $p<0.05$.

Results

Serum-dependent augmentation of alkaline phosphatase activity in tenocytes derived from rat Achilles tendon

Changes in the phenotype of cultured human tenocytes with progressive passage have been reported[16]. First of all, we isolated the tenocytes from rat Achilles tendon as previously described[20]. The tenocytes cultured in DMEM containing 10% FBS showed a fibroblastic spindle-shape until passage 5 as shown in Figure 1. However, the cells at passage 8 were spread and diverse in shape as a result of repeated subculturing every 4 to 5 days (Fig. 1). Thus, the tenocytes in culture were found to be changed in morphology by repeated subculturing.

Since tendon is a typical avascular tissue[9-11], the morphological changes of the rat tenocytes are considered to

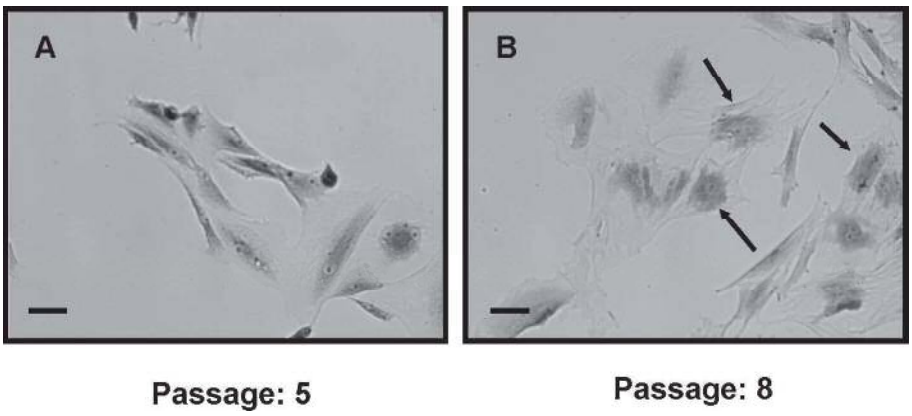

Fig. 1 Microscopic observation of tenocytes derived from rat Achilles tendon. Tenocytes at the passages 5(A) and 8(B) were cultured in DMEM/10% FBS and were stained with Giemsa's solution. Original magnification is ×100. Arrows indicate the morphologically changed tenocytes. Bars indicate 100 μm.

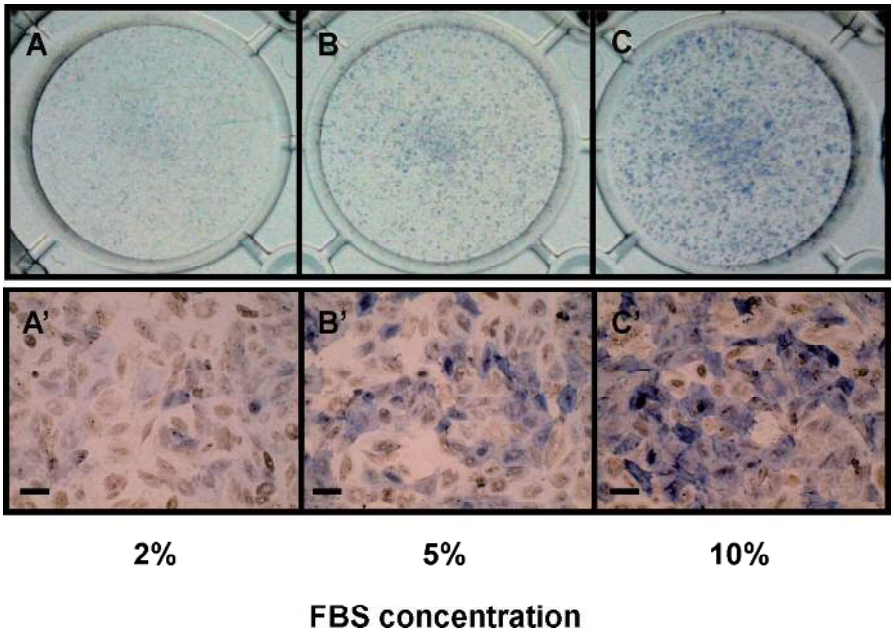

Fig. 2 Serum-dependent augmentation of alkaline phosphatase (ALPase) activity in tenocytes isolated from rat Achilles tendon. Tenocytes at passage 5 were cultured in DMEM containing 2% FBS until confluence, and then were maintained in DMEM containing 2%, 5%, and 10% FBS for another two weeks. ALPase activity was assessed by monitoring the enzyme reaction resulting in an insoluble blue product. Panels A, B, and C : macroscopic photographs of cells stained by ALPase activity, and panels A′, B′, and C′ : microscopic photographs corresponding to panels A, B, and C, respectively. Objective lens magnification is ×10. Bars indicate 100 μm.

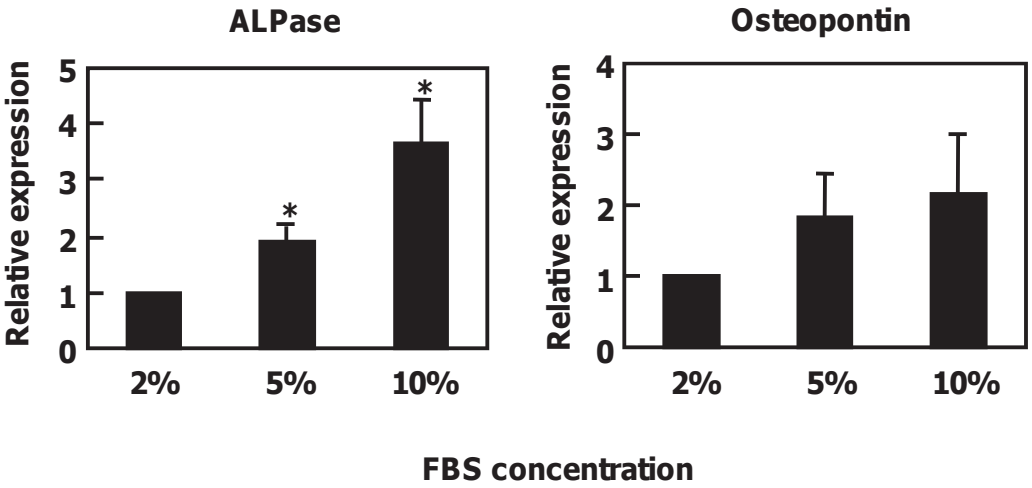

Fig. 3 Effects of serum on the expression of alkaline phosphatase (ALPase) and osteopontin mRNA in tenocytes derived from rat Achilles tendon. Confluent tenocytes at passage 5 were cultured in DMEM containing 2%, 5%, or 10% FBS for two weeks. Total RNA (2.5 ng) was subjected to real-time RT-PCR for ALPase and osteopontin mRNA as described in the text. Data are the mean ± SEM of three independent experiments. *, significantly different from the cells cultured in DMEM containing 2% FBS (p<0.05).

be due to the use of serum in the culture. To examine the effects of serum on the morphological changes of rat tenocytes, cells at passage 5 were cultured in DMEM containing 2% FBS until confluence. Then, confluent cells were continuously cultured in DMEM containing 2%, 5%, or 10% FBS changing the medium on alternate days for two weeks. As shown in Fig. 2, serum-dependent augmentation of alkaline phosphatase activity, a typical osteoblastic marker[22] was observed. These results were highly reproducible with other FBS from a

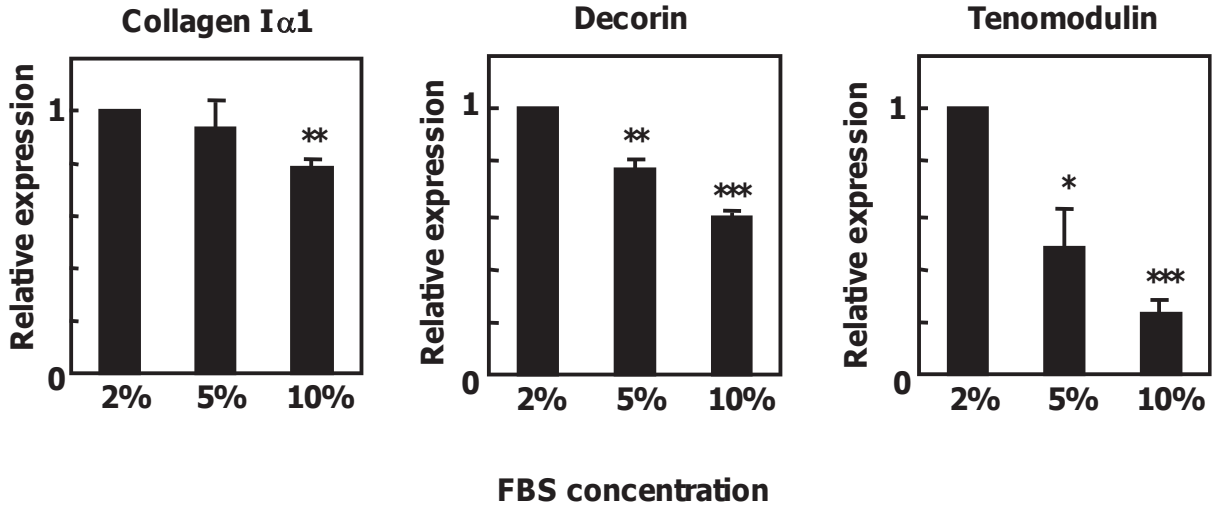

Fig. 4 Effects of serum on the expression of type I collagen, decorin, and tenomodulin mRNA in tenocytes derived from rat Achilles tendon. Tenocytes at passage 5 were treated as described in Fig. 3. Isolated total RNA (2.5 ng) was subjected to real-time RT-PCR for collagen type I α1 chain (Collagen I α1), decorin, and tenomodulin mRNA as described in the text. Data are the mean ± SEM of three independent experiments. *, **, and ***, significantly different from the cells cultured in DMEM containing 2% FBS (p<0.05, 0.01, and 0.001, respectively).

different origin (data not shown). Quantitative real-time RT-PCR analysis revealed that gene expression of alkaline phosphatase was significantly increased by serum (Fig. 3). The expression of osteopontin, a factor for bone formation[23] was also increased in serum-dependency. These results strongly indicate that rat tenocytes derived from Achilles tendon are serum-dependently transferred to be osteoblast-like cells.

Phenotype of tenocytes is changed to osteoblast-like cells, but not chondrogenic cells by serum

Tenocytes constitutively produce tendon extracellular matrix components, type I collagen and decorin. In addition, tenocytes express tenomodulin, a tenocyte specific angiogenesis inhibitor, and this has been found to contribute to tenocyte proliferation and tendon maturation[14)15)]. We examined whether these expressions are affected by serum or not. As shown in Fig. 4, gene expression of decorin and tenomodulin was extremely lowered by serum. On the other hand, the gene expression of collagen type I α1 chain was only slightly decreased. Thus, the phenotype of tenocytes is likely to be changed by serum.

Phenotypic alterations of tenocytes have been found in damaged tendon[19)]. Furthermore, fibrocartilagenous changes are frequently observed in damaged tendon tissue[17-19)]. Considered to be chondrogenic differentiation, changes in the gene expression of collagen type II α1 chain and the master transcriptional factor Sox-9[24)] were monitored by real-time RT-PCR. As shown in Fig. 5, their mRNAs were not affected by serum, suggesting the tenocytes are differentiated to osteoblast-like cells, but not to chondrogenic cells.

Discussion

Heterotopic calcification in tendon is common in athletes, and frequently affects the original tissue function. However, the cellular and molecular events in the tendon tissues have not been well understood. In this regard, Yao et al.[16)] have previously reported that the phenotype of cultured human tenocytes rapidly drifts with progressive passage. The characters of the drifted cells and the factors which induce the phenotypic drifting, however, have been clarified yet. In the present study, we have provided novel evidence that the phenotype of tenocytes isolated from rat Achilles tendon is readily altered to an alkaline phosphatase-positive osteoblast-like cells in serum-related fashion. It is noteworthy that the confluent tenocytes were changed to alkaline phosphatase-positive cells for two weeks according to the increase in serum concentrations. Furthermore, we found that the tenocytes differentiate to the osteoblast-like cells that show high alkaline phosphatase activity on

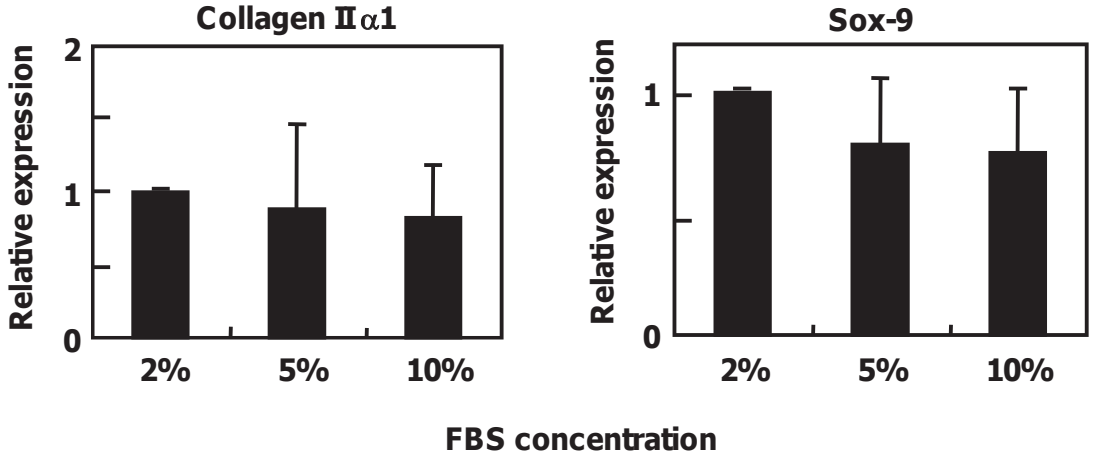

Fig. 5 Serum did not modulate the expression of type II collagen and Sox-9 mRNA in tenocytes derived from rat Achilles tendon. Tenocytes at passage 5 were treated as described in Fig. 3. Isolated total RNA (2.5 ng) was subjected to real-time RT-PCR for collagen type II α1 chain (Collagen II α1) and Sox-9 mRNA as described in the text. Data are the mean ± SEM of three independent experiments.

the cell surface. Differentiation of tenocytes was accompanied with a decrease in the gene expression of collagen type I α1 chain and decorin. Tendon tissues are mostly collagen fibril composed of type I collagen[9)10)]. Decorin is also an important factor for the assembly of collagen fibrils[12)]. Therefore, the serum-dependent down regulation of the collagen type I α1 chain and decorin expression in tenocytes leads to the incomplete rearrangement of tendon tissue, resulting in its dysfunction. Fibrocartilagenous changes in the injured region of tendon have also been reported[19)]. However, gene expression of collagen type II α1 chain and Sox-9, a transcriptional factor that promotes the transcription of type II collagen and aggrecan core protein was not changed by serum, suggesting that the ossification factors included in serum do not change the tenocytes to chondrogenic cells.

Tendon is a hypovascular tissue as well as cartilage, and tenocytes constitutively produce the anti-angiogenic factors including tenomodulin to maintain the avascular environment. In this study, we showed the tenomodulin gene expression in tenocytes was also reduced in a serum-dependent fashion. The down-regulation of tenomodulin is considered to induce the neovascularization into tendon tissues, resulting in an acceleration of tendon ossification. Thus, serum-dependent changes in phenotype of tenocytes may lead to the loss of tendon structure and function. Price et al.[25)] have reported the serum factors directly induce the re-calcification of demineralized bone and tendon. They have shown the significance of serum on the ossification of bone and tendon, but the calcification factor in serum is not yet clear. Bone morphogenetic proteins (BMPs) are well known as the factors that induce the osteoblastic differentiation of the cells[26)27)]. BMPs, especially BMPs-2 and-4 are mostly found in serum[26)27)]. In addition, sulfated oligosaccarides such as heparan sulfate have been shown to enhance the activity of BMPs[28)]. Further studies promise to be clear the precise mechanisms of serum-dependent phenotypic changes of tenocytes. Taken together with our results in this study, serum is a key factor for the ectopic ossification in tendons. Heterotopic ossification in tendon is frequently observed after minor trauma[5-7)] and blood flow is increased by neovascularization in Achilles tendon with painful tendinopathy[8)]. Possible progressive mechanisms for tendon ossification may be due to the induction of angiogenesis in tendon tissues.

In the present study, we have shown novel evidence that the phenotype of tenocytes derived from rat Achilles tendon is altered to alkaline phosphatase-positive osteoblast-like cells in a serum-dependent manner. These findings in the present study are informative for the understanding of the degenerative mechanism of Achilles tendon and development of novel therapeutic methods of tendon injuries to prevent undesired ectopic ossifica-

tion. We look forward to clarifying what serum factors induce the phenotypic changes of tenocytes.

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ラットアキレス腱より単離した培養アキレス腱細胞の血清依存的な骨芽細胞様変化

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高齢者に見られる慢性アキレス腱痛などのアキレス腱障害には、しばしば血管新生や異所性骨化などを伴う。しかしながら、このような腱の器質的変化のメカニズムについては未だ不明な点が多い。そこで、本研究では培養ラット腱細胞の表現型の変化について検討した。コンフルエントのラットアキレス腱細胞を2%、5%、10%ウシ胎児血清（FBS）濃度で2週間培養した結果、FBSの濃度依存的にアルカリ性ホスファターゼ陽性骨芽細胞様細胞への変化が認められた。この時、アルカリ性ホスファターゼとともにオステオポンチンの遺伝子発現も促進することが定量リアルタイムPCR法にて明らかとなった。また、アキレス腱細胞において恒常的に発現しているI型コラーゲンα1鎖、デコリン、テノモデュリンの遺伝子発現は、血清濃度依存的に低下した。一方、軟骨細胞特有のII型コラーゲンα1鎖、およびSox-9遺伝子発現には変化が見られなかった。以上の結果より、腱細胞がI型コラーゲン、デコリン、テノモデュリンなどの腱細胞特異的な遺伝子発現の抑制とアルカリ性ホスファターゼやオステオポンチンといった骨芽細胞特異的な遺伝子発現の増加を伴って血清濃度依存的に骨芽細胞様細胞に変化することが明らかとなった。本研究成果は腱変性メカニズムの理解のみならず、新たな異所性骨化予防策の開発に向けて有用な情報を提供するものである。

〈キーワード〉　腱、腱障害、腱細胞、異所性骨化、骨芽細胞