Distinguishing Tendon and Ligament Fibroblasts Based on ^1H Nuclear Magnetic Resonance Spectroscopy

Hun Yeong Ban¹, Ji Won Shin¹, Song-I Chun¹, Yun Gyeong Kang¹, Yanru Wu², Ji Eun Kim¹, Eun Jin Lee¹, Mi Jin Kim¹, Chi-Woong Mun¹, Jung-Woog Shin^{1,2,3*}

¹Department of Biomedical Engineering, Inje University, Gimhae, Korea

²Department of Health Science and Technology, Inje University, Gimhae, Korea

³Cardiovascular and Metabolic Disease Center, Institute of Aged Life Redesign, UHARC, Inje University, Gimhae, Korea

Tendon and ligament (T/L) have been known to be obviously different from each other in tissue level. However, due to the overlapping gene markers, distinction in cellular level has not been clearly verified yet. Recently, the use of nuclear magnetic resonance (NMR) spectroscopy has shown the potential to detect biological markers in cellular level. Therefore, in this study we applied a non-invasive technique based on NMR spectroscopy to establish biomarkers to distinguish between T/L fibroblasts. In addition the cellular morphologies and gene expression patterns were also investigated for comparison through optical microscopy and real-time polymerase chain reaction (PCR). No difference was observed from morphology and real-time PCR results, either as expected. However, we found clear differences in their metabolomic spectra using ^1H NMR spectroscopy. The calculated integral values of fatty acids (with chemical shifts at ~0.9, 1.26, 1.59, 2.05, 2.25, and 2.81 ppm), lactate (~1.33 ppm), and leucine (~2.72 ppm) were significantly different between the two types of fibroblasts. To be specific tendon group exhibited higher level of the metabolite than ligament group. In conclusion, in-cell metabolomic evaluation by NMR technique used in this study is believed to provide a promising tool in distinguishing cell types, especially T/L cells, which cannot be classified by conventional biological assays.

Tissue Eng Regen Med 2016;13(6):677-683

Key Words: Biomarkers; Ligament; Metabolomic evaluation; Nuclear magnetic resonance spectroscopy; Tendon

INTRODUCTION

Tendon and ligament (T/L) tissues, the connective tissues between bone and muscle and between bone and bone, respectively, withstand continuous tensile forces during daily activities. Therefore, they are susceptible to various types of damage. To repair T/L tissues that are partially damaged or torn, cell therapy rather than total replacement may be considered. For such cell therapy repair, it is essential to distinguish tendon from ligament fibroblasts [1,2].

Although T/L tissues appear similar to each other, they have many different characteristics including their mechanical properties, the content of their extracellular matrix (ECM), the size of their collagen fibers and the amount of cross-linking in the fibers [3-6]. Despite these differences in T/L tissues, few reports have distinguished T/L tissues at the cellular level. Most studies

regarding differentiation of stem cells into tendon or ligament fibroblasts have reported only that their stem cells differentiated into T/L-like cells [7,8]. In fact, several studies have reported that T/L fibroblasts have the same gene markers [9,10]. One investigation identified genes common to both T/L fibroblasts; however, the study failed to distinguish one type from the other [11].

Recently, metabolomic evaluation using nuclear magnetic resonance (NMR) spectroscopy to detect biological markers has emerged. This technique has been used in various applications including early detection of cancer cells and identification of neuronal regeneration. NMR spectroscopy is a widely used non-invasive technique that provides valuable information without sacrificing or damaging the specimens [12,13].

In this study, metabolomic evaluation utilizing ^1H NMR spectroscopy on primary T/L fibroblasts was employed to establish parameters that distinguish tendon from ligament fibroblasts at the cellular level.

Received: September 2, 2016

Revised: September 13, 2016

Accepted: September 15, 2016

***Corresponding author:** Jung-Woog Shin, Department of Biomedical Engineering, Inje University, 197 Inje-ro, Gimhae 50834, Korea.

Tel: 82-55-320-3317, Fax: 82-55-327-3292, E-mail: biomechshin@gmail.com

MATERIALS AND METHODS

Preparation of primary T/L fibroblasts

All of animal experiments in this study were approved by Institutional Animal Care and Use Committee at Inje University (IRB no. 2015302-2). Primary T/L fibroblasts were acquired from 5-week-old New Zealand white rabbits from achilles tendon, patella tendon, medial collateral ligament, and lateral collateral ligament tissues. Briefly, the dissected tissues were rinsed using Hank's balanced salts solution (Gibco BRL, Grand Island, NY, USA) and incubated at 37°C and 5% CO₂ for 6 h in Dulbecco's modified Eagle medium with high glucose (DMEM-HG; Gibco BRL). The samples were incubated in DMEM-HG containing 0.25% collagenase (Gibco BRL), 1% penicillin-streptomycin (P/S; Hyclone, Logan, UT, USA) and 10% fetal bovine serum (FBS; Gibco BRL) and then passed through a 70- μ m cell strainer (SPL, Seoul, Korea). The acquired cells were used for real-time polymerase chain reaction (PCR) and NMR spectroscopy within 24 h.

Optical microscopy

Primary T/L fibroblasts were cultured for up to 3 days in DMEM-HG containing 1% P/S and 10% FBS. To confirm fibroblastic morphology, the cells were observed daily using an optical microscope (Olympus, Tokyo, Japan).

Real-time PCR

Real-time PCR was performed to evaluate gene expression patterns in primary T/L fibroblasts. Tenascin-C (TNC), tenomodulin (TNMD), collagen type-1 (COL-1) and decorin (DCN) were used as T/L gene markers, and glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene (Table 1). Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed into using a high-

capacity cDNA reverse transcription kit (Applied Biosystems, Foster, CA, USA). Then, real-time PCR was performed under the following conditions: 10 min incubation at 95°C, 15 s denaturation at 95°C, 30 s annealing at the primer-specific melting temperature, and 30 s extension at 72°C for 40 cycles. The reaction was carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems), and StepOne software (Applied Biosystems) was used to analyze the data.

Preparation of mesenchymal stem cells

Mesenchymal stem cells (MSCs) were harvested from the femur and tibia bones of 5-week-old New Zealand white rabbits and used as the negative control in real-time PCR. The isolation procedure was conducted as described previously [14]. Isolated MSCs were incubated at 37°C and 5% CO₂ with Dulbecco's modified Eagle medium with low glucose (Gibco BRL) containing 1% P/S (Hyclone) and 10% FBS (Gibco BRL). To perform real-time PCR, cultured MSCs were trypsinized at passage.

NMR spectroscopy

Sample preparation

To prepare samples for NMR spectroscopy, 1×10^6 cells were pelleted and rinsed with D₂O normal saline solution (99% D₂O + 0.9% NaCl) to remove residual medium. This process aimed to eliminate background signals from H₂O. Then, each pellet sample was transferred to a 4 mm Ghx nanoprobe and suspended in the same solution (D₂O) with 25 μ M trimethylsilyl propionic acid (TSP; Sigma, St. Louis, MO, USA) as the external reference for the chemical shift. All measurements were conducted within 4 hours immediately after the preparation to minimize any damages to live cells *in vitro*.

Table 1. Summary of primers used in real-time PCR

Primer	Sequence (5'-3')	Product size (bp)	Annealing temperature	Accession number
TNC	(F): gatcacctacgtgccctgt (R): gatgagtgacccggagagag	166	59°C	FJ480400
TNMD	(F): aaaatgagcagtgggtggtc (R): ctagcaaaggttcgcagaca	201	59°C	NM_001109818
COL-1	(F): gagaaaggaaccaaggacc (R): cccttctttaccagcagcac	234	59°C	NM_001195668
DCN	(F): accctgtgcaatactgggag (R): aacatgaggggtgtcttggg	108	59°C	NM_001082330
GAPDH	(F): gtcgtctcctgcgactcaa (R): ccaccacctgttctgtag	136	59°C	NM_001082253

PCR: polymerase chain reaction, TNC: tenascin-C, TNMD: tenomodulin, COL-1: collagen type-1, DCN: decorin, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Data acquisition

^1H NMR spectroscopy was performed using a 600 MHz NMR spectrometer (VNMRS 500, Varian®, Palo Alto, CA, USA). To eliminate water signals, a pre-saturation technique was used for each sample with a PRESAT pulse sequence before the acquisition of pulses provided by Varian® before the acquisition of pulses. The pulse length was calibrated and adjusted to 90° . The parameter conditions used in the experiments were as follows: delay time=2 s; number of data points=16000; spectral width=9615.4 Hz; NEX=512.

Data analysis

Data was processed by NUTS commercial software (Acorn NMR, Livermore, CA, USA). Briefly, Fourier transformation, phase/baseline correction, peak picking and area integration were performed on the acquired raw spectra. The spectral peaks of interest were assigned to the assumed metabolites based on the literature [15,16]. The comparative analysis between groups were performed using the following equation suggested by a previous study [17].

$$C_m = C_{\text{TSP}} \times \frac{N_{\text{TSP}}}{N_m} \times \frac{I_m}{I_{\text{TSP}}} \times \frac{V}{W}$$

where,

C_m =metabolite concentration

C_{TSP} =TSP concentration

N_{TSP} =number of protons in the resonance peak of TSP (=9)

N_m =number of protons in the resonance peak of the metabolite

I_m =integral of selected ^1H resonance peak from the metabolite of interest

I_{TSP} =integral of TSP peak at 0 ppm

V =volume of TSP added in the NMR sample

W =weight of cell pellet (assuming it is proportional to the number of cells)

In this study, C_{TSP} , N_{TSP} , N_m , V , and W were kept constant in all samples. Therefore the part of $\frac{I_m}{I_{\text{TSP}}}$ was only calculated and used as an integral value of each metabolite. Finally, the integral value was normalized by the DNA contents of each sample and presented.

DNA assay

To normalize integral values of the assigned metabolites in the NMR spectra, the DNA contents of each sample was quantified using Quant-iT PicoGreen dsDNA Reagent (Molecular Probes, Eugene, OR, USA). Each cell pellet used for NMR spectroscopy was dissolved in Triton X-100 (USB, Cleveland, OH, USA) and permeabilized by a Sonics Vibra Cell CV18 tip sonicator (Sonics & Materials Inc., Newtown, CT, USA). After centrifugation at 13000 rpm (4°C for 5 min), the sample was reacted with PicoGreen reagent, which was diluted in TE buffer (1:200). The fluo-

Figure 1. Optical micrographs of primary T/L fibroblasts during 3 days of culture. Scale bar, 10 μm . T/L: tendon and ligament, AT: achilles tendon, PT: patella tendon, MCL: medial collateral ligament, LCL: lateral collateral ligament.

rescent intensity of each sample was detected at excited/emission wavelengths of 485/525 nm using a multi-detection microplate reader (Synergy HT, BioTek, Winooski, VT, USA).

Statistical analysis

Statistical analyses were performed utilizing SPSS (SPSS Inc., Chicago, IL, USA). Multiple comparisons were conducted by one-way analysis of variance and Fisher's least significant difference method. All results are expressed as means $\pm$ standard deviation, with statistical significance set at $p < 0.05$.

RESULTS

Observation of cellular morphology

The morphological observations detected by optical microscopy are presented in Figure 1. On day 1, most of the seeded cells displayed slightly thin and elongated shapes, but some remained unattached. On day 2, however, typical fibroblastic morphologies were confirmed in both T/L groups, which were maintained until day 3 when no morphological differences were found between the two groups.

Gene marker expression

The characterization of gene expression levels in primary T/L fibroblasts was conducted by real-time PCR, and the results are presented in Figure 2. Regardless of the anatomical origins, primary T/L fibroblasts showed a marked upregulation of gene marker expression compared with MSCs (control). Both T/L groups displayed significantly higher expression of mature markers, such as TNMD and COL-1, than early markers, such as TNC. However, the tendon groups exhibited distinctly higher levels of TNMD expression than that in the ligament groups. Expression of the other gene markers, including TNC, COL-1, and DCN, showed no specific tendencies that distinguished one from the other.

Metabolomic evaluation

The metabolomic information of primary T/L fibroblasts were acquired using ^1H NMR spectroscopy and were compared with each other. The spectral patterns in each group were nearly identical in the number and location of the metabolomic peaks (Fig. 3). However, differences between T/L fibroblasts were observed in the normalized integral values of several metabolites related to fatty acids (FAs) [~ 0.9 (FA-1), 1.26

Figure 2. Relative expression of common gene markers in primary T/L fibroblasts. (A) TNC, (B) TNMD, (C) COL-1, and (D) DCN. Expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($n=3$, $*p<0.05$). TNC: tenascin-C, TNMD: tenomodulin, COL-1: collagen type-1, DCN: decorin, MSC: mesenchymal stem cell, AT: achilles tendon, PT: patella tendon, MCL: medial collateral ligament, LCL: lateral collateral ligament.

Figure 3. NMR spectra of primary tendon fibroblasts using TSP as the reference. (A) AT, (B) PT, (C) MCL, and (D) LCL fibroblasts. NMR: nuclear magnetic resonance, TSP: trimethylsilyl propionic acid, AT: achilles tendon, PT: patella tendon, MCL: medial collateral ligament, LCL: lateral collateral ligament.

Figure 4. Integral values of the metabolites normalized by DNA contents. (A) FA-1, (B) FA-2, (C) FA-3, (D) FA-4, (E) FA-5, (F) FA-6, (G) lactate, and (H) leucine (n=3, * $p < 0.05$). FA: fatty acids, AT: achilles tendon, PT: patella tendon, MCL: medial collateral ligament, LCL: lateral collateral ligament.

(FA-2), 1.59 (FA-3), 2.05 (FA-4), 2.25 (FA-5), and 2.81 (FA-6) ppm], lactate (~1.33 ppm), and leucine (~2.72 ppm). As shown in Figure 4, the tendon group displayed significantly higher integral values of the metabolites than did the ligament group when there were no other significant differences between T/L fibroblasts. The chemical shifts and groups of the metabolites are presented in Table 2.

DISCUSSION

This study aimed to discern differences in T/L tissues at the cellular level. While establishing markers or parameters to distinguish fibroblasts in T/L tissues is an obvious fundamental goal, no conventional biological assessment has provided concrete methods or techniques. Most previous studies on stem cell differentiation into tendon or ligament fibroblasts have failed to detect whether the differentiated cells were tendon or ligament fibroblasts [7,8], only reporting that the differentiated cells were tendon/ligament-like fibroblasts. Therefore, a method to identify certain cell types that cannot be distinguished by gene expression profiles is essential.

Collagen, one of the major ECM components in T/L tissues, plays important roles in modulating the mechanical properties of T/Ls [18]. Collagen originates from the corresponding cell type, and many previous reports have addressed the differences in collagen contents between T/L tissues [4-6]. Therefore, investigating the secretion or generation of collagen at the cellular level is a worthwhile endeavor, especially in relation to tissue engineering or regenerative medicine. Many studies have adopted collagen as one parameter to evaluate T/L regeneration [7,8]. However, no differences in gene expression have been identified between T/L fibroblasts [11]. Likewise, we found no significant differences in gene expression, as shown in Figure 1. Therefore, other methods are required to discern the two types of cells at the cellular level.

We used NMR spectroscopy to evaluate various metabolites

related to collagen secretion among T/L fibroblasts. One advantage to this technique is that it is non-invasive.

Lactate is involved in T/L development, and many studies have shown that it acts as a mediator promoting collagen secretion from T/L fibroblasts. The metabolomic evaluation in this study showed a higher level of lactate in the tendon group than the ligament group, which was supported by previous studies [19,20].

Other metabolites in the lipid family that are unsaturated have been reported to play roles in collagen secretion [21]. FA-4 and FA-6, which have unsaturated bonds in their structures, showed higher integral values in the tendon group than in the ligament group in our results. This result is supported by the fact that tendon tissue has more collagen contents than that of ligament tissue. However, we recommend further investigation of saturated FAs, which showed comparable tendencies in this study.

Although leucine is not directly involved in collagen secretion, it plays critical roles in collagen fibrogenesis by leucine-rich proteins [22]. Our leucine measurements showed a significant difference between T/L fibroblasts. This result is related to the fact that the average diameters of collagen fibers in T/L differ between the two types of tissues [5].

To evaluate T/L tissues, it is necessary to evaluate both the metabolite and gene expression levels. We measured metabolites at the cellular level with a focus on collagen, which is a major component of T/L tissues. In this manner, we identified differences in the major metabolites related to collagen secretion in T/L fibroblasts, not tissues.

Most studies aiming to differentiate stem cells into tendon or ligament fibroblasts have failed to determine whether the differentiated cells were tendon or ligament fibroblasts, even though various methods were used to induce differentiation [7,8]. Most of these analyses were performed based on measurements of gene expression, rather than metabolites. Therefore, the non-invasive NMR spectroscopic approach described in this study

Table 2. Metabolites identified in ^1H nuclear magnetic resonance spectra, along with their chemical shifts and chemical groups

No.	Metabolites	Chemical shift (ppm)	Chemical group
1	FA-1	0.9	-CH ₃
2	FA-2	1.26	-(CH ₂) _n -
3	FA-3	1.59	-OOC-CH ₂ + -O-CH ₂ -CH ₂ -+ -CH ₂ -CH-(CH ₃) ₂
4	FA-4	2.05	=CH-CH ₂ -CH ₂ =CH-CH ₂ -CH ₃
5	FA-5	2.25	-COO-CH ₂ -CH ₂ -
6	FA-6	2.81	=CH-CH ₂ -CH=
7	Lactate	1.33	CH ₃
8	Leucine	1.72	β CH ₂ + γ CH

FA: fatty acids

provides a useful technique to distinguish various cell types, especially when they cannot be distinguished by gene expression methods alone.

This study confirmed the ability of NMR spectroscopy to distinguish T/L fibroblasts. Therefore, the methods and analyses adopted in this study provide a promising approach that could be utilized in various research areas such as regenerative medicine or other biological studies.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) Grant (NRF-2014K2A2A7066637) and by the Human Resource Training Program for Regional Innovation and Creativity through the Ministry of Education and National Research Foundation of Korea (NRF-2014H1C1A1073148).

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

All of animal experiments in this study were approved by Institutional Animal Care and Use Committee at Inje University (IRB no. 2015302-2).

REFERENCES

1. Lomas AJ, Ryan CN, Sorushanova A, Shologu N, Sideri AI, Tsioli V, et al. The past, present and future in scaffold-based tendon treatments. *Adv Drug Deliv Rev* 2015;84:257-277.
2. Chen B, Ding J, Zhang W, Zhou G, Cao Y, Liu W, et al. Tissue engineering of tendons: a comparison of muscle-derived cells, tenocytes, and dermal fibroblasts as cell sources. *Plast Reconstr Surg* 2016;137:536e-544e.
3. Jung HJ, Fisher MB, Woo SL. Role of biomechanics in the understanding of normal, injured, and healing ligaments and tendons. *Sports Med Arthrosc Rehabil Ther Technol* 2009;1:9.
4. Rodrigues MT, Reis RL, Gomes ME. Engineering tendon and ligament tissues: present developments towards successful clinical products. *J Tissue Eng Regen Med* 2013;7:673-686.
5. Rumian AP, Wallace AL, Birch HL. Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features--a comparative study in an ovine model. *J Orthop Res* 2007;25:458-464.
6. Amiel D, Frank C, Harwood F, Fronck J, Akeson W. Tendons and ligaments: a morphological and biochemical comparison. *J Orthop Res* 1984;1:257-265.
7. Cardwell RD, Dahlgren LA, Goldstein AS. Electrospun fibre diameter, not alignment, affects mesenchymal stem cell differentiation into the tendon/ligament lineage. *J Tissue Eng Regen Med* 2014;8:937-945.
8. Qiu Y, Lei J, Koob TJ, Temenoff JS. Cyclic tension promotes fibroblastic differentiation of human MSCs cultured on collagen-fibre scaffolds. *J Tissue Eng Regen Med* 2014 Feb 11 [Epub]. <http://dx.doi.org/10.1002/term.1880>.
9. Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. *Development* 2001;128:3855-3866.
10. Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. *Dev Biol* 2006;298:234-247.
11. Cooper JA Jr, Bailey LO, Carter JN, Castiglioni CE, Kofron MD, Ko FK, et al. Evaluation of the anterior cruciate ligament, medial collateral ligament, achilles tendon and patellar tendon as cell sources for tissue-engineered ligament. *Biomaterials* 2006;27:2747-2754.
12. Wen H, An YJ, Xu WJ, Kang KW, Park S. Real-time monitoring of cancer cell metabolism and effects of an anticancer agent using 2D in-cell NMR spectroscopy. *Angew Chem Int Ed Engl* 2015;54:5374-5377.
13. Ramm P, Couillard-Despres S, Plötz S, Rivera FJ, Krampert M, Lehner B, et al. A nuclear magnetic resonance biomarker for neural progenitor cells: is it all neurogenesis? *Stem Cells* 2009;27:420-423.
14. Kim DH, Kim SH, Heo SJ, Shin JW, Lee SW, Park SA, et al. Enhanced differentiation of mesenchymal stem cells into NP-like cells via 3D co-culturing with mechanical stimulation. *J Biosci Bioeng* 2009;108:63-67.
15. Chun SI, Cho JH, Yang YI, Shin JW, Shin WJ, Mun CW. Proton (^1H) nuclear magnetic resonance spectroscopy to define metabolomic changes as a biomarker of adipogenic differentiation in human mesenchymal stem cells. *Tissue Eng Regen Med* 2012;9:101-108.
16. Jue T, Arias-Mendoza F, Gonnella NC, Shulman GI, Shulman RG. A ^1H NMR technique for observing metabolite signals in the spectrum of perfused liver. *Proc Natl Acad Sci U S A* 1985;82:5246-5249.
17. Shi C, Wang X, Wu S, Zhu Y, Chung LW, Mao H. HRMAS ^1H -NMR measured changes of the metabolite profile as mesenchymal stem cells differentiate to targeted fat cells in vitro: implications for non-invasive monitoring of stem cell differentiation in vivo. *J Tissue Eng Regen Med* 2008;2:482-490.
18. Liu HY. In vivo evaluation of the stiffness of the patellar tendon. *Miami: ProQuest*; 2008.
19. Klein MB, Pham H, Yalamanchi N, Chang J. Flexor tendon wound healing in vitro: the effect of lactate on tendon cell proliferation and collagen production. *J Hand Surg Am* 2001;26:847-854.
20. Greve K, Domeij-Arverud E, Labruto E, Edman G, Bring D, Nilsson G, et al. Metabolic activity in early tendon repair can be enhanced by intermittent pneumatic compression. *Scand J Med Sci Sports* 2012;22:e55-e63.
21. Jia Y, Turek JJ. Inducible nitric oxide synthase links NF-kappaB to PGE2 in polyunsaturated fatty acid altered fibroblast in-vitro wound healing. *Lipids Health Dis* 2005;4:14.
22. Kalamajski S, Oldberg A. The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix Biol* 2010;29:248-253.