Comparative study of the properties of tendinocytes derived from three different sites in the equine superficial digital flexor tendon

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ABSTRACT

This aim of this study was to determine the characteristic differences in tendinocytes derived from three sites of the equine superficial digital flexor tendon (SDFT)—proximally the myotendinous junction (MTJ), mid-metacarpal (mM) and osteotendinous junction (OTJ)—in morphology, proliferation, and ability for synthesis of collagen and matrix metalloproteinases (MMPs). Little difference was observed in cell proliferation. Addition of tumor necrosis factor (TNF) α to the culture medium resulted in increased collagen synthesis by tendinocytes from all three sites. The amount of collagen synthesized by tendinocytes derived from the mM and OTJ was much larger than that synthesized by untreated tendinocytes. A collagen zymogram revealed that proMMP-13 synthesis was increased towards the distal site. However, TNFα treatment resulted in a significant decrease in the amount of proMMP-13 synthesized by tendinocytes from all three sites. On the other hand, a gelatin zymogram showed that the synthesis level of proMMP-9 tended to decrease towards the distal site, but there was little difference between synthesis levels of proMMP-9 before and after TNFα treatment. These results indicated that tendinocytes in the same tendon have different characteristics and that these characterisities would reflect the function of tendinocytes in vivo. Also, the isolated tendinocytes provided much information on the characteristics and properties of tendons for the ECM turnover system and on the responsiveness of tendinocytes to complex inflammatory responses in a tendinopathy condition.

Tendon injury, especially in the superficial digital flexor tendon (SDFT), has proved to be a major problem for racehorses. Recent studies have shown that about 10–30% of racehorses suffer from tendonitis (8, 30). Interestingly, tendonitis occurs more frequently in the forelimb, especially in the SDFT rather than the deep digital flexor tendon (DDFT), and rarely occurs in the common digital extensor tendon (CDET) (1, 30). The vulnerability of the SDFT is related to its functions in weight supporting and in accumulation and transmission of elastic force for effective movement. Damage to the SDFT usually occurs during high-speed work or racing competition in which the forelimbs are pulled up to the highest position to bear maximum load or straining power (14, 35). Moreover, tendonitis in the SDFT occurs in the mid-metacarpal (mM) site in most cases (1). Therefore, structural properties of the tendons might reflect the occurrence ratio of tendonitis (10). The etiology of tendonitis has been discussed in many reports; however, there is little information regarding the mechanism of tendon degradation.

Extracellular matrix (ECM) turnover in a tendon might occur repeatedly and routinely as in other connective tissues (13), and matrix metalloprotein-
ases (MMPs) play a major role in the turnover of tendon tissues (2, 35). Most MMPs are secreted into the extracellular space in an inactivated form (proMMP) and are activated indirectly by cytokines (15, 28, 31), and many other endogenous factors, such as tissue inhibitor of metalloproteinases and endogenous inhibitor of MMPs, have an effect on the activity of MMPs. MMPs are members of an endopeptidase family that require a zinc ion in their active site for catalytic activity and a calcium ion for the stability and the expression of enzymic activity (25, 26). On the basis of data in the Genbank database, the human MMP family consists of at least 23 enzymes, and nucleotide sequences of equine MMPs have been identified for 11 enzymes and predicted for another 7 enzymes. MMPs show different substrate specificities, though there is some overlapping (9), and they can be divided into five groups of enzymes based on substrate specificity: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins, membrane-type MMPs and other types of MMPs (2, 35). Human and equine studies have suggested that MMPs are involved in musculoskeletal diseases (3, 5–7, 11, 20, 22, 25).

Synthesis levels of MMPs in human tendons change drastically in inflammatory conditions (7, 20, 22). It is therefore likely that MMPs are involved in the occurrence and healing process of tendonitis in horses. Synthesis of proinflammatory cytokines has generally been observed at the time of inflammatory responses in humans and rats (23, 36, 37). An immunohistochemical study has shown that cells immunopositive for proinflammatory cytokines—interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF) α and interferon γ—were greatly increased in an inflamed tendon of horses compared to those in a normal tendon (17). Moreover it has been shown that some MMPs were stimulated by proinflammatory cytokines, including IL-1β and TNFα, and down-regulated by anti-TNFα antibody in human tendosynovitis. These findings suggest that MMPs and proinflammatory cytokines play an important role in tendon injuries, including horse tendonitis.

In our recent study, we determined the characteristic differences in tendinocytes derived from mid-metacarpal (mM) of three tendons in equine forelimbs—SDFT, deep digital flexor tendon and common digital flexor tendon—in morphology, proliferation, and ability for synthesis of collagen and MMPs. The SDFT, which had a large number of cells, high level of cellular proliferation, and ability for a high level of synthesis of collagen and MMPs, showed the highest turnover potential (19). Our other previous studies on three sites of the SDFT—the myotendinous junction (MTJ), mM and osteotendinous junction (OTJ)—revealed morphological and biochemical regional differences in vivo (33, 39, 40). We speculated that morphological structure corresponds to biomechanical function in some sites of the SDFT.

We have recently succeeded in establishing a method for isolating tendinocytes from equine tendons, and cultivation of isolated tendinocytes has provided much information on the properties of these cells types (16, 18). In this study, we examined and compared the properties of tendinocytes obtained from MTJ, mM and OTJ sites of the SDFT, including morphology, proliferation, and production of collagen and MMPs. The influence of TNFα on synthesis of collagen and MMPs was also investigated.

MATERIALS AND METHODS

All experiments followed the protocols approved by the Ethics Committee of Rakuno Gakuen University, Japan and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals [DHHS (DHHS) Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NH, Bethesda, MD 20205]. Tendon samples were obtained from 2 male horses and 1 female horse that had been euthanized because of problems other than a tendon or the musculoskeletal system.

Isolating tendinocytes and characteristics of the cells. Tendinocytes were isolated by the previously described outgrowth method (4). Briefly, SDFTs were divided into three equal 6-cm-long parts that were named MTJ, mM and OTJ sites. Each tendon specimen was cut into cubes of approximately 5 × 5 × 5 mm in size and incubated in a growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) with 5% fetal bovine serum (Gibco, Carlsbad, CA, USA) and antibiotic-antimycotic solution (100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; LifeTechnologies, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO₂ in a 100-mm culture dish for 7–10 days. During the harvested period, all tendon tissue blocks were mounted on cover glass slides to promote outgrowth of tendinocytes. The growth medium was replaced with fresh medium every three days and non-adhering cells were decanted. To confirm that the cells were confluent, the tendon tissue cube was removed from the flask and, the attached cells were harvested.
with growth medium and stored in liquid nitrogen until use. All experiments were performed during passage 3 or 4. At each passage, cells were seeded at a density of $5 \times 10^4$ cells/mL on plastic plates.

**Cell proliferation assay.** For quantification, cells were seeded at a density of $5 \times 10^4$ cells/mL in 96-well plastic plates (100 μL/well, TPP) and pre-cultured in standard conditions for 24 h. The medium was then replaced and the cells were cultured for 24, 48, 72 and 96 h. Cellular quantification was performed with a Cell Counting Kit (Dojindo, Kumamoto, Japan) following the manufacturer’s instructions. Briefly, dead cells were decanted and fresh medium containing the reagent in the counting kit was added. The plates were re-incubated at 37°C for 4 h. Absorbance of the medium was read at 405 nm and 690 nm with a spectrometer (ImmunoMini NJ-2300; System Instrument, Tokyo, Japan).

**Proinflammatory response to tendinocytes.** To evaluate the cellular response to TNFα, some cultured tendinocytes were incubated with 10 ng/mL purified equine TNFα (kindly provided by Dr. Rikio Kirisawa, Rakuno Gakuen University, Japan). The value was optimized prior to experiments (18, 19).

**Total collagen assay.** Total collagen content was determined by a method partially modified from the method of Walsh et al. (38). Briefly, tendinocytes were seeded at a density of $5 \times 10^5$ cells/mL in 96-well plastic plates (100 μL/well, TPP) and pre-cultured in standard conditions for 24 h. After replacing with a new medium, the seeds were re-cultured for another 24 h. In addition, a TNFα

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### Table 1 The primer of mRNA used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genbank ID</th>
<th>Product Size (kDa)</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procollagen Type 1</td>
<td>AF034691</td>
<td>387</td>
<td>GACGACAAACCACAGGAGTG ACCATCATCTCCGGTTGTCG</td>
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<tr>
<td>Procollagen Type 3</td>
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<tr>
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<tr>
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<td>NM002424</td>
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<tr>
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</tr>
<tr>
<td>MMP-2</td>
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</tr>
<tr>
<td>MMP-9</td>
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<td>62</td>
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</tr>
<tr>
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</tr>
<tr>
<td>β-actin</td>
<td>AF035774</td>
<td>488</td>
<td>TGCCTGACATCAAAGGAGAAG ACAGGTCCCTACGGATGTCG</td>
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</table>
Gelatin and collagen zymography. For the cellular MMP assay, tendinocytes were seeded at a density of $5 \times 10^5$ cells/mL in 6-well plastic plates (2 mL/well, TPP) and pre-cultured in standard conditions for 24 h. After replacing with a new culture medium, the cells were re-cultured for another 24 h. Moreover, a TNFα treatment group was set up and the cells were cultured under similar conditions. To obtain standard curves, type I collagen (Cellmatrix Type I-A; Nitta gelatin, Osaka, Japan) and type II collagen (Type II; Nitta gelatin) were diluted from 20 to 1.25 μg/100 μL in the culture medium. Collagen standards and cell culture supernatants were dried except for the lid of the plate at 37°C for 24 h. After rinsing the plates in distilled water three times, the wells were filled with 100 μL of 0.1% Sirius Red (Wako, Osaka, Japan) in saturated picric acid (w/v) to stain the samples for 1 h at room temperature. The plates were washed with 200 μL of 10 mM HCl five times for 10 s each time. The stained collagen was washed with 200 μL of 0.1 M NaOH for 5 min. The eluted stain was drawn up and down several times in a pipette and placed into a second plate. Absorbance was then read at 540 nm in a spectrometer (ImmunoMini NJ-2300). Finally, a standard curve was plotted as estimated collagen content of the samples in micrograms versus absorbance at 540 nm. Figures 3a and 3b show the correlation of amount of collagen versus absorbance at 540 nm by using type I collagen and type II collagen solutions. Similar standard curves were obtained for the two types of collagen. From these results, we obtained the value of collagen by use of the standard curve in the graph of type I collagen.

**Statistical analysis.** All assays were performed in at least triplicate. Obtained data were compared using one-way analysis of variance (ANOVA). All values are presented as means ± S.D. $P < 0.05$ indicated a significant difference.

**RESULTS**

**Gene expression**

All samples of tendinocytes isolated from the different sites of SDFT expressed mRNA of *tenomodulin* (*TeM*), a specific marker of tendon tissue, and mRNAs of procollagen type I, procollagen type III, and MMP-1, -2, -9 and -13 (Fig. 1). However, MMP-8 mRNA was not detectable. Since the sequence of equine MMP-8 has not yet been clarified, we substituted the primer of human MMP-8 mRNA in this study, and we therefore could not conclude whether equine tendinocytes express MMP-8.

**Morphology and quantification of tendinocytes**

Little morphological difference was observed among cells from the three sites of the tendon (Fig. 2a). All three types of tendinocytes had the characteristic appearance of fibroblasts: cells protruded in star-like processes in sparse cell cultures, and when confluence was reached the cells became spindle-shaped, producing a parallel array. Proliferation of cells derived from the MTJ tended to be greater than that of cells derived from the mM and OTJ, and proliferation of cells from the mM was greater than that of cells from the OTJ throughout the experimental period; however, there was no significant difference in the proliferation ratio between these tendinocytes (Fig. 2b).
Collagen synthesis

Figures 3a and b show the correlation of amount of collagen versus absorbance by using type I collagen and type II collagen solutions as standards, respectively. Similar standard curves were obtained for the two types of collagen. From these results, we calculated the amount of synthesized collagen by use of the standard curve in the graph of type I collagen. The tendinocytes derived from the MTJ tended to show a high level of collagen synthesis per well in both conditions (control and TNFα treatment), but there was no site-specific significant difference. Treatment with TNFα increased the synthesis of collagen and a significant difference was found between control and TNFα-treated tendinocytes derived from the mM and OTJ sites (Fig. 3c).

Activity of proMMPs in vitro

Activities of proMMP-1 and -13 were detected in a type I collagen zymogram (Fig. 4a) and proMMP-13 was the sole enzyme showing activity in a type II collagen zymogram (Fig. 4b). Activated forms of MMP-1 and -13 were hardly detectable in a type I or type II collagen zymogram (Fig. 4a and 4b), indicating that auto-activation of these proenzymes did not occur in vitro. EDTA inhibited all collagenase activities, confirming that the bands on the zymogram represent MMPs (data not shown). ProMMP-13 synthesis level was lowest in tendino-
cytes derived from the MTJ site and was increased most in both control and TNFα-treated tendinocytes from the OTJ site. Moreover, the amount of proMMP-13 tended to decrease in all tendinocytes with the addition of TNFα, and significant differences were found between control and TNFα-treated tendinocytes (Fig. 4c). The amount of synthesized proMMP-1 was extremely small and analysis by the NIH image was difficult. The gelatin zymogram showed significant activities for proMMP-9, but the activated form of MMP-9 was hardly detectable (Fig. 5a). EDTA also inhibited all gelatinase activities, confirming that the bands on the zymogram represent MMPs (data not shown). ProMMP-9 synthesis level was highest in tendinocytes derived from the MTJ and was lowest in tendinocytes derived from the OTJ site. TNFα had no effect on proMMP-9 synthesis in tendinocytes from all sites. Neither the pro- nor activated form of MMP-2 was detected in the gelatin zymogram.

DISCUSSION

In the present study, it was difficult to detect distinct differences in cellular morphology, proliferation rate, and amount of collagen synthesis according to cell collection site in the SDFT. On the other hand, MMPs, especially proMMP-13 and -9, showed different activities in the cultured tendinocytes. In a normal condition (without TNFα), the activity levels of proMMP-13 were lowest in tendinocytes from the MTJ and highest in tendinocytes from the OTJ (Fig. 4a-c). Type II collagen, as well as type I collagen, is a substrate of MMP-13, and it has been reported that MMP-13 is highly expressed and synthesized in cartilage and bone tissues, which contain a large amount of type II collagen. The OTJ is a junction region between tendon and bone, and tendinocytes around the OTJ are routinely exposed to high tension at muscle contraction (21). We previously observed tendinocytes around the distal site (near the site of OTJ) of the SDFT by electron microscopy and found that the cellular morphology was similar to that of chondrocytes. We have also analyzed contents of glycosaminoglycans in the SDFT and have shown that the ratios of chondroitin sulfate, an important structural component of cartilage, in the distal site was much higher than the ratio in other sites of the SDFT (our unpublished data). Therefore, it is likely that tendinocytes near the OTJ have properties of chondrocyte-like cells.

After treatment with TNFα, proMMP-13 synthesis decreased in tendinocytes from all sites. We have recently reported similar results using tendinocytes derived from three tendons in forelimbs—SDFT, DDFT and CDET (19). Therefore, TNFα has a stimulatory effect on production of proMMP-13 in cultured tendinocytes. Proinflammatory cytokines, such as IL-1β and TNFα, stimulate synthesis of MMPs in various types of cells (12, 18, 24, 42). In a normal tendon, restricted MMP-13 expression was observed in some fibroblastic cells and vascular component in the endotenon, suggesting the participation of MMP-13 in maintenance of homeostasis in matrix turnover in a tendon. Moreover, MMP-13 has been shown to be highly expressed in fibroelastic tissue in tendonitis in the horse (29) and to participate in collagen degradation during healing of the SDFT in the rat (32). These findings suggest
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frequently in the MTJ when extension load is added to muscle fibers. The MTJ has the characteristic property of being able to move over a wide range due to its elasticity. It has been reported that the temperature of the equine SDFT while running at full speed increases up to 39–45°C in a deep site of the tendon (41, 43). The temperature in the MTJ is likely to increase easily compared with the temperature in other sites due to its wide movable region, and heat induced-gelatinization would therefore progress more rapidly in the MTJ than in other sites. In support of this speculation, our previous study revealed that a hyperthermia environment accelerated MMP-9 synthesis (18). Tendinocytes might adjust to the environment surrounding the tendon.

MMP-13 plays an important role in tendon injury and its healing process. However, MMP-13-expressing cells have not been detected in tendonitis so far. Based on these observations, it is likely that MMP-13-expressing cells in tendonitis are not only tendinocytes but also other cells and/or that TNFα negatively regulates MMP-13 synthesis in tendinocytes.

The amount of proMMP-9 was largest in tendinocytes from the MTJ and tended to decrease toward the distal site (Fig. 5a and 5b), indicating that tendinocytes derived from the MTJ synthesized a large amount of proMMP-9. Tension load on the tendon at muscle contraction increases in the direction of the MTJ, and collagen degradation therefore occurs frequently in the MTJ when extension load is added to muscle fibers. The MTJ has the characteristic property of being able to move over a wide range due to its elasticity. It has been reported that the temperature of the equine SDFT while running at full speed increases up to 39–45°C in a deep site of the tendon (41, 43). The temperature in the MTJ is likely to increase easily compared with the temperature in other sites due to its wide movable region, and heat induced-gelatinization would therefore progress more rapidly in the MTJ than in other sites. In support of this speculation, our previous study revealed that a hyperthermia environment accelerated MMP-9 synthesis (18). Tendinocytes might adjust to the environment surrounding the tendon.
Our previous studies showed that tendon samples from the mM site of the equine EDFT have strong enzyme activity of pro- and activated MMP-2 and -9. However, in culture medium samples, a gelatin zymogram showed significant activity for pro-MMP-9 and very weak activity for proMMP-2, and the activated forms of both MMP-2 and -9 were hardly detectable (18, 19). Moreover, proMMP-9 showed positive immunohistochemical reaction, but proMMP-2 reaction was very weak in tendinocytes derived from the mM site, and proMMP-2 synthesis by tendinocytes did not change regardless of TNFα treatment (18). In the present study, we could not detect proMMP-2 in the gelatin zymogram in any tendinocytes derived from the three different sites (Fig. 5a). Therefore, it is thought that TNFα has a stimulatory effect on MMP-2 synthesis in tendinocytes and that MMP-2-expressing cells in the tendon are not tendinocytes but other types of cells.

It should be noted that the results of this study for collagen and MMP synthesis do not reflect the whole matrix turnover phenomenon in tendon tissue. However, the cells derived from the three sites of the equine SDFT were maintained in vitro to a certain extent, and the isolated tendinocytes provided much information on the characteristics of tendons for the ECM turnover system and on the responsiveness of tendinocytes to complex inflammatory responses in tendinopathy. Above all, it became clear that properties of tendinocytes in vivo could be maintained in vitro. Moreover, the results suggested that tendinocytes of the SDFT adapted to the surrounding environment and changed their cellular properties to form a more suitable tendinous structure.

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REFERENCES

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