Effect of heat on synthesis of gelatinases and pro-inflammatory cytokines in equine tendinocytes

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ABSTRACT
The aim of this study was to clarify whether matrix metalloproteinases (MMP-2 and -9: gelatinases) and pro-inflammatory cytokines [tumor necrosis factor (TNF) α and interleukin (IL)-1β] are induced by heat in tendon tissue in vitro and to test the hypothesis that heat exposure causes tendinocytes to synthesize pro-inflammatory cytokines and that synthesis of these cytokines, in turn, leads to up-regulation of synthesis of gelatinases. Isolated tendinocytes from equine superficial digital flexor tendons were cultured and all experiments were performed on cells passaged 3 or 4 times. In the cells exposed to heat (37 to 45°C, 0 to 60 min), the survival rate decreased sharply in a temperature- and time-dependent manner, especially at 42 and 45°C. Cells exposed at 40°C, however, showed little change in survival rate and morphology. Gelatin zymograms revealed that proMMP-2 and -9 were the only two MMPs remaining in the supernatant of the cultured tendinocytes, including that of untreated cells. Addition of TNFα and IL-1β to the culture medium of tendinocytes accelerated proMMP-9 synthesis considerably. Heating the tendinocytes (40°C) led to a three-fold increase in proMMP-9 synthesis in a short time. Only TNFα was detected in tendinocytes after heat exposure for 30 and 60 min. In contrast, IL-1β was under the detectable level in ELISA. Cooling of heat-exposed cells from 40°C to 37°C considerably down-regulated cellular proMMP-9 synthesis. Furthermore, proMMP-9 level was greatly reduced in cells treated at lower temperatures, 20°C and 5°C. These findings support our hypothesis that hyperthermia in the horse tendon induces tendinocytes to synthesize pro-inflammatory cytokines and that the synthesis of these cytokines results in the up-regulation of gelatinases.

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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 (29). Although the etiology of tendonitis has been discussed in many reports (2, 19, 25), there is little information on the mechanism of tendon degradation. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) α and interleukin (IL)-1β, and gelatinases in matrix metalloproteinases (MMPs) are involved in degradation of connective tissues (30, 33, 34, 40), and these pro-inflammatory cytokines and gelatinases are synthesized in tendinocytes (15, 18, 33). Additionally, TNF α and IL-1β are known to be initiators of the synthesis of gelatinases and of the synthesis and release of cytokines (3, 7, 8, 10, 24, 28).
MMPs are a family of zinc-dependent endopeptidases that selectively degrade the extracellular matrix (ECM). The MMP family consists of at least 20 enzymes divided into five different groups: collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs (36, 37, 43). The gelatinase group, MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa), appear to play an important role in tendinopathy, especially in degradation of the ECM, collagen fibers and glycosaminoglycans, such as decorin and biglycan (6, 24). The mechanism underlying the onset of tendonitis has not been fully clarified; however, exercise-induced heat is a highly plausible factor.

Tendons provide strong, pliable connections between muscles and their points of insertion into bones (19). This tissue exhibits powerful resistance to tensile force and is also known to be hypovascular. This strength appears to be closely associated with the characteristic properties of their ECMs, which have few capillary networks. During exercise, the tendon transmits muscular kinetic energy to the bone, and heat is also generated in the tendon as it extends and contracts repeatedly at the same time (1, 20, 32). The temperature in the core region of the tendon reaches 40 to 45°C when a horse is allowed to gallop (44). The central core of the tendon, which is the site of most marked temperature increases, is also the site of degradation and subsequent injury in both the equine SDFT (42) and human Achilles tendon (4). Such a high temperature may not only elicit tendon degradation but may also prompt the onset of tendonitis (5).

The aim of this study was to clarify whether MMPs (gelatinases) and pro-inflammatory cytokines are induced by heat in tendon tissue in vitro and to test the hypothesis that hyperthermia in the tendon causes tendinocytes to synthesize pro-inflammatory cytokines and that synthesis of these cytokines, in turn, leads to up-regulation of synthesis of gelatinases.

MATERIALS AND METHODS

This study was performed in accordance with the Guidelines for Animal Experimentation of Rakuno Gakuen University, Japan.

Method for isolating tendinocytes and characteristics of the cells. SDFTs were collected from two healthy female racehorses (1 and 2 years of age) that had been euthanized for reasons unrelated to the tendon or to the musculoskeletal system. Cells were isolated by the outgrowth method. Isolated cells were cultured at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO, USA) with 5% fetal bovine serum (Gibco, Carlsbad, CA, USA) and antibiotics (Sigma). Isolated cells had the characteristic appearance of fibroblasts; cell processes protruded in a star-like shape in sparse cell cultures, and as semi-confluence was reached cells became spindle-shaped, producing a parallel array (Fig. 1a).

All experiments were performed on cells from cultures that had undergone 3 or 4 passages (P3 or 4). Moreover, to test the tendinocytic character, tenomodulin, a specific marker of tendon tissue (14, 35), was detected by reverse transcriptional-polymerase chain reaction (RT-PCR). Sequences for primers (Sigma Genosys, Hokkaido, Japan) were as follows: tenomodulin (GenBank ID: AF059407, product size: 251 bp) forward: 5’-GTC CCT CAA GTG AAG GTG GA-3’, reverse: 5’-GTT GCA AGG CAT GAT GAC AC-3’; β-actin (GenBank ID: AF035774, product size: 488 bp) forward: 5’-TGC GTG ACA TCA AGG AGA AG-3’, reverse: 5’-ACA GGT CCT TAC GGA TGT CG-3’. The isolat-
ed cells of both P3 and P4 expressed tenomodulin mRNA the same as SDFT tissue (Fig. 1b).

Gelatin zymography. To compare the expression levels of MMPs in vivo and in vitro, SDFT tissue samples that had been cultured for 48 h and supernatant were used. SDFT samples and supernatant were each mixed at ratio of 1 : 4 and 1 : 1 (v/v), respectively, with sample buffer. The sample buffer consisted of 40 mM Tris, pH 6.8, 5% SDS, 20% glycerol, and 0.03% bromphenol blue without reducing agent or heating. Five ml of SDFT tissue- or supernatant-sample buffer mixture was loaded and electrophoresed through an 8% polyacrylamide gel containing 0.3% gelatin using an electrophoresis unit. After electrophoresis of the sample, the gel was washed and incubated in a reaction buffer (50 mM Tris-HCl and 10 mM CaCl2, pH 7.4) for 16 h at 37°C. The gels were stained with 0.2% Coomassie Brilliant Blue R (Sigma) and destained with 7% acetic acid and 4% methanol to visualize the unstained proteolytic band. In order to determine the type of gelatinases observed on the zymograms, 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the buffer during the incubation period. All procedures were performed as aforementioned for a gel without EDTA in the incubation buffer. The molecular weights of gelatinous bands were estimated by comparing their electrophoretic migration to that of protein standards (BioRad, Hercules, CA, USA).

For the cellular MMP assay, cells were incubated in 35-mm plastic plates (TPP, Trasadingen, Switzerland) with pro-inflammatory cytokines, purified horse TNFα (10 ng/mL) and IL-1β (10 ng/mL), at 37°C for 6 to 72 h, and another batch of cells was incubated at 40°C for 5 to 60 min. Additionally, some heat-exposed cells were incubated for 20 min at various temperatures after 30 min of incubation at 40°C (see below). After pro-inflammatory cytokine treatment and heat exposure, supernatants were collected and the gelatin zymogram procedure was carried out as described above. Densities of the bands were calculated using NIH image.

Immunofluorescent staining of proMMP-2 and -9. Tendinocytes were grown on coverslips (Fisher Scientific, Pittsburgh, PA, USA) for 48 h for immunostaining. The cells were rinsed with 10 mM phosphate-buffered saline (PBS), pH 7.4, and then fixed with methanol-acetone (1 : 1) at 4°C for 30 min and washed with PBS several times. After blocking for 30 min with 3% bovine serum albumin in PBS at room temperature, cells were incubated with goat anti-human proMMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 50 dilution or goat anti-human proMMP-9 antibody (Santa Cruz Biotechnology) at 1 : 100 dilution for 1 h at room temperature. After washing again with PBS, they were incubated with fluorescein isothiocyanate-conjugated rabbit anti-goat IgG (Molecular Probes, Eugene, OR, USA) at 1 : 100 dilution for 30 min at room temperature in a dark room. Cells on the coverslips were mounted on glass slides using an aqueous mounting medium (Permafluor, Immunotech, Marseille, France) and observed under a laser scan confocal microscope (Fluoview, Olympus, Tokyo, Japan).

Exposure to hyperthermia of different temperatures. Tendinocytes from P3 and P4 passages were seeded on 35-mm plastic plate (TPP) for viability and morphological analyses. For the counting of viable cells, cells were washed with PBS (-) (Nissui, Tokyo, Japan) and suspended in trypsin-EDTA (Gibco). Cell suspensions were adjusted to contain 1 × 10⁶ cells/mL, and the suspended cells were warmed in a water bath for 5, 10, 20, 30 and 60 min at 37, 40, 42 and 45°C. After heat exposure, the cells were incubated for 20 min at 37°C and transferred to 96-well plates (TPP) and then incubated for 24 h, during which time viable cells were able to re-adhere to the surface of the 96-well plate.

Quantification of cell survival. Viable cells that had adhered to the bottoms of 96-well plates were quantified with a cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, the medium with dead cells was decanted, and fresh medium containing the reagent equipped in the commercialized kit was added. The plates were returned to the incubator at 37°C for 4 h. After this time, absorbance of the medium was read at 405 and 690 nm with a spectrometer (ImmunoMini NJ-2300, System Instruments, Tokyo, Japan). Results were expressed as the percentage cell survival relative to that of cells kept at 37°C. All data are given as means ± standard error.

Scanning electron microscopy (SEM). Tendinocytes were also incubated for 60 min at various temperatures (37, 40, 42 and 45°C) and examined by SEM. After heat exposure, cells were washed with phosphate buffer and post-fixed with OsO₄ at room temperature for 1 h. Conductive staining was carried out by 1% thiocarboxydradize-1% OsO₄ treatment (11).
The samples were dehydrated and processed for freeze-drying with t-butyl alcohol substitution (17). The specimens were examined with a field emission scanning electron microscope (JSM-6000F, JEOL, Tokyo, Japan) at an acceleration voltage of 3 kV.

Assay for pro-inflammatory cytokines after heat exposure. Concentrations of pro-inflammatory cytokines in the supernatant were measured by using a self-produced sandwich enzyme-linked immunosorbent assay (ELISA) for equine TNF-α or IL-1β by a standard method. Mouse monoclonal antibodies against equine TNF-α or IL-1β (21) were used for capture antibodies. Rabbit polyclonal antibodies against equine TNF-α or IL-1β were used for detecting antibodies. Secondary peroxidase-conjugated goat anti-rabbit IgG was obtained from Zymed Laboratories (San Francisco, CA, USA). Minimum detectable concentration limit was 1 ng/mL for both TNF-α and IL-1β in the supernatant with the ELISA kits used in this study.

Effect of heat on synthesis of pro-inflammatory cytokines by tendinocytes. Due to the very low activity of proMMP-2, we determined only proMMP-9 (See Figs. 2 and 4). Heat-exposed cells were incubated for 20 min at 5, 20 and 37°C after incubation at 40°C for 30 min on ice or in a water bath. Cells that had not been exposed to heat (maintain at 37°C during the experiment) were used as control cells. Then the supernatant was collected and analyzed by gelatin zymography to determine the activity of proMMP-9. Densities of the bands were analyzed using NIH image software, and the relative ratio of proMMP-9 activities was calculated.

Statistical analysis. A computer program (Stat View for Windows, version 5.0) was used for the determination of means and standard errors and for one-way analysis of variance (ANOVA). Sheffe’s test was used to compare differences among mean relative ratios of MMP activities at a significant level of \( P < 0.05 \).

RESULTS

Activity and distribution of gelatinases (MMP-2 and -9) in vitro
Gelatin zymograms revealed that equine tendon samples have strong activity of pro- and activated MMP-2 and -9 (Fig. 2a). In the culture medium sample, the gelatin zymogram also showed significant activities for proMMP-2 and -9, but the activated forms of both MMPs were hardly detectable, indicating that auto-activation of these proenzymes did not occur in vitro (Fig. 2a). EDTA inhibited all gelatinase activities, confirming that the bands on the zymogram represent MMPs (Fig. 2a). Immunohistochemical analyses showed both proMMP-2 and -9 to be distributed in tendinocytes. ProMMP-9 showed positive immunohistochemical reaction, but proMMP-2 reaction was very weak in the tendinocytes (Fig. 2b).

Viability and morphological alteration of tendinocytes in hyperthermia model
Percentages of cells surviving after exposure to temperatures of 37 to 45°C for 0 to 60 min are shown in Fig. 3a. In the cells exposed to heat, the survival rate decreased sharply in temperature- and time-dependent manners, especially at 42 and 45°C. The
Heat effect on gelatinases and cytokines synthesis

percentages of tendinocytes that remained viable after exposure for 10 min to temperatures of 40°C, 42°C and 45°C were 75.3 ± 2.5%, 52.2 ± 4.4% and 7.7 ± 5.2%, respectively. At the end of this experiment, after 60 min of heating at 40°C, the survival rate of tendinocytes was 62.5 ± 2.8%, whereas heating for 60 min at 42°C and 45°C resulted in a drop in the cell survival rate to 5.3 ± 3.2% and 1.9 ± 0.6%, respectively. Electron microscopy revealed that the cellular structure collapsed in cells exposed to the highest temperature (45°C) for 60 min, with many holes in the cellular membrane (Fig. 3d). Cells exposed to 40°C for 60 min, however, showed only a slight change in survival rate, and multiple cellular projections were found (Fig. 3c), as if the cells had been activated by heat.

Effect of pro-inflammatory cytokines and heat on the gelatinase activities of tendinocytes

ProMMP-9 synthesis in tendinocytes is strongly induced by pro-inflammatory cytokines and by heating. Gelatin zymograms revealed that proMMP-2 and -9 were the only two MMPs remaining in the supernatant of the cultured tendinocytes, including that of untreated cells. Neither activated MMP-2 nor -9 showed change in activity level. Addition of TNFα and IL-1β to the culture medium of tendinocytes accelerated proMMP-9 synthesis considerably (Fig. 4a, b). On the other hand, the effect of TNFα or IL-1β on proMMP-2 synthesis was moderate (Fig. 4a, b). ProMMP-9 synthesis in tendinocytes was induced by heat in a short time (Fig. 4c, d). The band density of proMMP-9 at 60 min was three-fold stronger than the density of the control cells (0 min).

Effect of heat on pro-inflammatory cytokine synthesis by tendinocytes

Heated tendinocytes can produce TNFα in a short time. TNFα was detected in tendinocytes after heat exposure for 30 and 60 min. In contrast, the concentration of IL-1β was under the detectable level (1 ng/mL) throughout the experimental period (Fig. 5).

Effect of cooling treatment on proMMP-9 synthesis by heated tendinocytes

Cooling of heat-exposed tendinocytes reduced the proMMP-9 level. The proMMP-9 level of heat-exposed cells remaining at 40°C was 2.87-times higher than that of the control cells (relative ratio). On the other hand, cooling of heat-exposed cells from 40°C to 37°C resulted in a considerable decrease in cellular proMMP-9 synthesis (relative ratio: 2.22 times) compared with that in the cells remaining at 40°C. Furthermore, the proMMP-9 level was reduced more in cells cooled to 20°C (1.17 times) and 5°C (1.22 times) than in the control cells (Fig. 6). Significant differences were found among treatment temperatures.

DISCUSSION

The results presented in this study suggest that heat induces tendinocytes to synthesize TNFα and that synthesis of pro-inflammatory cytokines (TNFα and IL-1β) results in up-regulation of proMMP-9.

Severe alterations in the survival rate and morphology of tendinocytes were evident after exposure to a temperature of 45°C. The cells within tendons play a leading role in maintaining the ECM through the synthesis of collagen and other matrix components (19). The central core of the tendon, which is the site of the most marked temperature increases during exercise, is also the site of degradation and
subsequent tendon injury (4, 42, 44). Therefore, a sharp decrease in cell number caused by heat would result in a reduced synthetic capability, and alteration of cell metabolism may also endanger the integrity of the ECM. Heating cells in an in vitro model as carried out in this study is not completely analogous to the situation in vivo; however, the re-
results of this study suggest that exercise-induced hyperthermia might play a role in the pathogenesis of degenerative core lesions of the tendon.

ProMMP-9 synthesis in tendinocytes was induced by pro-inflammatory cytokines and by heating, and heated cells could produce pro-inflammatory cytokines. In the present study, the effects of two pro-inflammatory cytokines on activities of proMMP-2 and -9 in the culture of equine tendinocytes were examined at P3 or P4. This is the first report on the stimulatory effects of TNFα and IL-1β on production of MMPs in cultured tendinocytes. The gelatinases MMP-2 and -9 are known to be synthesized and secreted by several types of cells such as macrophages, mast cells, neutrophils and epidermal keratinocytes, and fibroblasts (36, 37, 43). Pro-inflammatory cytokines such as TNFα and IL-1β play a major role in the process of degradation and wound healing in connective tissue (13, 41). TNFα is produced by various cells and enhances many biological events, including production of MMPs and stimulation or inhibition of cellular proliferation, and secreted TNFα stimulates cells to synthesize IL-1β in a para/autocrine manner (12, 27, 28). Even in the tendon, these gelatinases and pro-inflammatory cytokines are synthesized by tendinocytes and are involved in the turnover of tendon tissue and in the maintenance of homeostasis (9, 15, 16, 23). In several types of fibroblast-like cells, pro-inflammatory cytokines can activate both MMP-2 and -9 (45). In the present study, both TNFα and IL-1β up-regulated the production of proMMP-9 but had little effect on the production of proMMP-2. These observations suggest that MMP-2 and -9 are differentially regulated in tendinocytes.

This study also showed that heated cells produced TNFα in a short time. A number of previous studies have shown that hyperthermia can modulate TNFα synthesis in many cells (31, 38, 39). Augmentation of TNFα synthesis has been thought to occur through several mechanisms, including increased transcription or translation of TNFα mRNA, altered secretory events or stability, or heat damage-induced release of membrane-bound TNFα (22, 38). We have limited the scope of this study to reveal the mechanism of induction of TNFα by heat, but clearly there are interactions between hyperthermia and TNFα secretion by tendinocytes.

Cooling of heat-exposed tendinocytes reduced the level of proMMP-9. Lowering the temperature from 40°C to 37°C resulted in considerable down-regulation of proMMP-9 activity in heat-exposed cells. Furthermore, proMMP-9 synthesis level was greatly reduced in cells treated at lower temperatures, 20°C and 5°C. In hypovascular tissues such as the tendon, ligament and epimysium of skeletal muscles, the rate of movement of materials is thought to be much slower than in other vascular tissues because materials move by diffusion (19, 46). This means that efflux of some biological factors synthesized in these tissues is difficult, and the factors easily accumulate in the tissue in a short time. Similarly, once heat is produced in a tendon, a hyperthermic condition is maintained for a long time because thermo-diffusion is hardly ever mediated by the bloodstream. The hypovascular nature of the tendon might facilitate the continuance of a hyperthermic condition after exercise and induction of pro-inflammatory cytokines by heat. Therefore, cooling of the tendon after exercise might allow heat-induced synthesis of pro-inflammatory cytokines to be controlled and might inhibit the occurrence or progression of tendonitis.

In conclusion, 1) heat influences the survival rate of cells and cellular morphology, 2) proMMP-9 synthesis in tendinocytes is induced by pro-inflammatory cytokines and by heating, and heated cells can produce TNFα in a short time, and 3) cooling of heat-exposed tendinocytes reduces the proMMP-9 level. Together, these findings support our hypotheses that hyperthermia in the horse tendon induces synthesis of pro-inflammatory cytokines by tendinocytes and that synthesis of these cytokines results in up-regulation of synthesis of gelatinases. The results of this study are of particular clinical importance for the prevention of tendon degradation possibly by control of the tendon temperature in the animal. It is conceivable that cooling the legs of horses after training is a simple but effective means of preventing tendinopathy.

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