

Active Hyaluronidase 2 Expression in the Granulation Tissue Formed in the Healing Process of Equine Superficial Digital Flexor Tendonitis

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ABSTRACT. Upregulation of hyaluronidase 2 (HYAL2), one of somatic hyaluronidase (HAase), was demonstrated in granulation tissue during the healing of equine superficial digital flexor tendon injuries. The activity of HAase was assessed by hyaluronan (HA)-containing gel zymography and *in situ* zymography using frozen sections obtained from normal and injured tendon tissues. Elevated HAase activity was identified in the extract from the tendinopathic tissues, with lower levels of the activity in normal tendons. *In situ* zymography using fluorescently-labeled HA demonstrated HAase activity in the granulation tissue formed in the injured region. In addition, *in situ* hybridization analysis indicated that fibroblastic cells in the granulation tissue of the injured tendon actively expressed HYAL2 but not HYAL1. Quantitative RT-PCR further confirmed a higher level of amplicons corresponding to HYAL2 in tendonitis-derived samples. These results suggest that elevated HYAL2 activity in the granulation tissue could participate in controlling the healing process in equine tendonitis.

KEY WORDS: horse, hyaluronidase, tendonitis, wound healing, zymography.

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It is known that matrix-related polysaccharides are degraded by hyaluronidase (HAase), and there are at least eight different forms of HAase, hyaluronidase 1 (HYAL1), HYAL2, HYAL3, HYAL4, HYAL5, hyaluronoglucosaminidase pseudogene 1 (HYALP1), sperm adhesion molecule 1 (SPAM1, also known as PH-20) and meningioma expressed antigen 5 (MGEA5) [3, 4, 8, 21]. Among these, HYAL1 and HYAL2 usually found in serum are considered as major somatic HAase [4], while SPAM1 is well known as testicular HAase [21]. HYAL1 has acidic pH optima, and its upregulation is found in several metastatic carcinomas, suggesting a potential prognostic marker and therapeutic target [10, 11, 23]. Another somatic HAase, HYAL2, shows high activity under acidic to neutral pH condition and has unique substrate specificity, cleaving high molecular mass hyaluronan (HA) polymers to intermediate size fragments of 20 kDa [9]. Recently, it was reported that platelet-derived HYAL2 cleaves HA into fragments that are specific for inflammatory and angiogenic signaling [5]. Additionally, HYALP1 is recognized as a pseudogene in the human, but the degrading activity for HA was found in the mouse [21]. Presence of all HAase except for HYALP1 in the horse has been predicted from corresponding genomic sequences, but there is no report concerning the analysis of these activities.

Equine tendonitis, that is, injury to the superficial digital flexor (SDF) tendon, commonly occurs in racehorses by excessive mechanical force loaded onto the tendon tissue and is known to be an intractable condition that tends to be recurrent [1, 20]. SDF tendon injury is characteristically results in partial rupture of the collagen bundle and consequent hemorrhage. We previously reported that MMP (matrix metalloproteinase)-13 (collagenase-3) and MMP-2 (gelatinase A) in addition to MMP-3 (stromelysin-1) and MMP-10 (stromelysin-2) were upregulated in the granulation tissue of equine tendonitis [17]. These results suggested that metabolism of collagen in SDF tendon injury was mediated by these MMPs, however, degradation mechanism of glycosaminoglycan has not been elucidated until now. Thus, in this study, we tried to characterize HA-degrading activity in the granulation tissue raised in equine SDF tendonitis.

Normal SDF tendon tissues were obtained from two adult (3 years old, N1 and N2) male thoroughbreds. Injured lesions were dissected from the SDF tendons of two adult male thoroughbreds (T1; 4 years 6 months old and T2; 6 years 3 months old) suffering from acute tendonitis. All Thoroughbred horses were kept in the Equine Research Institute of Japan Racing Association (JRA), and the euthanasia and sample-procurement procedures were approved by the Animal Welfare and Ethics Committee of the Equine Research Institute of JRA. Formalin-fixed paraffin-embedded thin sections of tendon tissues were stained with hematoxylin-eosin. Other parts of tissues were embedded in O.C.T compound (Sakura Finetek Japan Co., Tokyo, Japan), and 6 μ m frozen sections were cut and used for enzymatic and molecular biological analyses. HAase-containing frac-

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tions were prepared by extraction with 0.15 M NaCl/50 mM Tris-HCl, pH 7.6 (Tris-saline) from frozen sections, and then 20 μ g of protein was applied to substrate gel zymography containing 0.5% HA as previously reported [14, 15]. Commercially available testicular HAase (Sigma-Aldrich, St. Louis, MO, U.S.A.) was used as a control HAase sample. HA *in situ* zymography was performed as follows; FITC-labeled 5% HA dissolved with formate buffer, pH 3.5 was applied to air-dried frozen sections, and then covered with parafilm and incubated for 18 hr at 37°C in a humid chamber. After a brief rinse in Tris-saline to eliminate degraded FITC-HA, the sections were counterstained with propidium iodide and analyzed using a laser-scanning microscope (LSM710, Carl Zeiss Co., Ltd., Munich, Germany). To prepare the probe for *in situ* hybridization (ISH), PCR fragments corresponding to equine HYAL1 (143 bp; accession No. XM_001493665) and HYAL2 (123 bp; accession No. XM_001493640) were amplified with the appropriate primer sets (HYAL1 upper; GGGGCTTCTATGGCTTC, lower; AATGCTGGGGTAGAGGGC and HYAL2 upper; CATTGACTGGGAGGACTG, lower; ACTACACGGTCTGATGGC) and a tendon-derived cDNA. The amplicons were subcloned into pGEM-T easy plasmid vector (Promega Co., Madison, WI, U.S.A.), and then sense and antisense RNA probes were synthesized using SP6 or T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany) in the presence of digoxigenin (DIG)-UTP (Roche) according to the manufacturer's protocol. Frozen sections were fixed with 4% paraformaldehyde for 10 min, washed with diethylpyrocarbonate-treated water, followed by dehydration with ethanol and then incubated with hybridization mixture as previously reported [17]. Finally, slides were then incubated with FITC-labeled anti-DIG antibody (Roche), counterstained with propidium iodide and then analyzed using a laser-scanning microscope. Quantitative RT-PCR analysis was performed using the iCycler Real Time Detection System (Bio-Rad Laboratories, Hercules, CA, U.S.A.) as previously reported [22]. Primer sets corresponding to HYAL1 and HYAL2 were described above and equine β -actin (accession No. AF035774) was used as an internal control and amplified with the following primer set (upper; AGAGGGAAATCGTGCGTGAC, lower; AGGAAGGAGGGCTGGAAGAG). Statistical differences between the values obtained from normal and tendonitis were determined by Mann-Whitney's U test, and values of $P < 0.05$ were considered significant.

HA gel zymography indicated that tendinopathic tissues showed an apparent 60 kDa and acidic-active single band corresponding to HAase (Fig. 1). Estimation of activities with Image J (National Institutes of Health, Bethesda, MD, U.S.A.) indicated a 10-fold higher level in the injured tendon compared to the normal tendon (data not shown). As shown in Fig. 2a, tenocytes were scattered throughout the tendon bundles of normal tendons. By contrast, many fibroblastic cells and capillary vessels were found in the granulation tissue of the injured tendon (Fig. 2b), and a thickened endotenon had separated the normal area from the granulation tissue. Collagenous fibers observed in the granulation tissue were fragmented, while normal tendon bundle showed

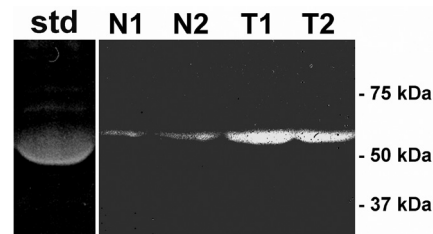


Fig. 1. Gel zymographs of acidic HAase in normal (N1, N2) and injured (T1, T2) tendons. Commercially available testicular HAase is applied as a standard enzyme (std). Approximately 60 kDa band representing HAase is found in 0.5% alcian blue-stained gel.

homogenous structure. Next, HA *in situ* zymography at pH 3.5 was performed using frozen sections. Normal tendon showed that the activity of acidic HAase was restricted to the surrounding area of the tendon bundle (Fig. 2c), and high power view revealed the activity was also detected in the endotenon (Fig. 2c, inset). In the granulation tissue formed in the tendinopathic region, the strong activity of acidic HAase was apparent in the center of the granulation tissue (Fig. 2d), and enzyme activity was also detected in the thickened endotenon. Additionally, HAase activity in the peripheral region of the granulation tissue decreased and disappeared (Fig. 2d, inset). To identify molecular species of HAase expressed in the granulation tissue, the following experiments were performed. ISH revealed that HYAL1 mRNA was found to be localized to cells in the endotenon in the tissues of both normal (Fig. 3a) and injured tendons (Fig. 3b). Although there was no positive signal corresponding to HYAL2 mRNA throughout the normal tendon (Fig. 3c), active expression of HYAL2 was evident in the cytoplasm of fibroblastic cells in the granulation tissue of the injured tendon (Fig. 3d). No positive signal was obtained by the use of the sense probe corresponding to HYAL1 and HYAL2 (data not shown). Furthermore, quantitative RT-PCR analysis for HYAL1 and HYAL2 was performed. Expression of HYAL1 mRNA in normal tissue was about 2-fold higher than that in tendinopathic tissue (Fig. 4a). On the other hand, mRNA level corresponding to HYAL2 in normal tendon was very low, and the level in the injured tendon was about 20-fold higher than that in the normal tendon (Fig. 4b). These results indicated that HYAL2 rather than HYAL1 could contribute to degradation of HA in the granulation tissue.

Taken together, these enzymatic and molecular biological analyses revealed high HYAL2 activity in the granulation tissue formed in equine tendonitis. ISH observation further indicated that HYAL1 is produced in the endotenon of normal tendon and may participate basic turnover of HA, whereas HYAL2 could be strongly upregulated in the healing process and HA-degrading activity in injured tendon could be brought by HYAL2. In this study, we provide the first evidence that shows higher activity of HYAL2 in granulation tissues formed in equine injured tendons. It is generally accepted that accumulation of HA in wound tissue

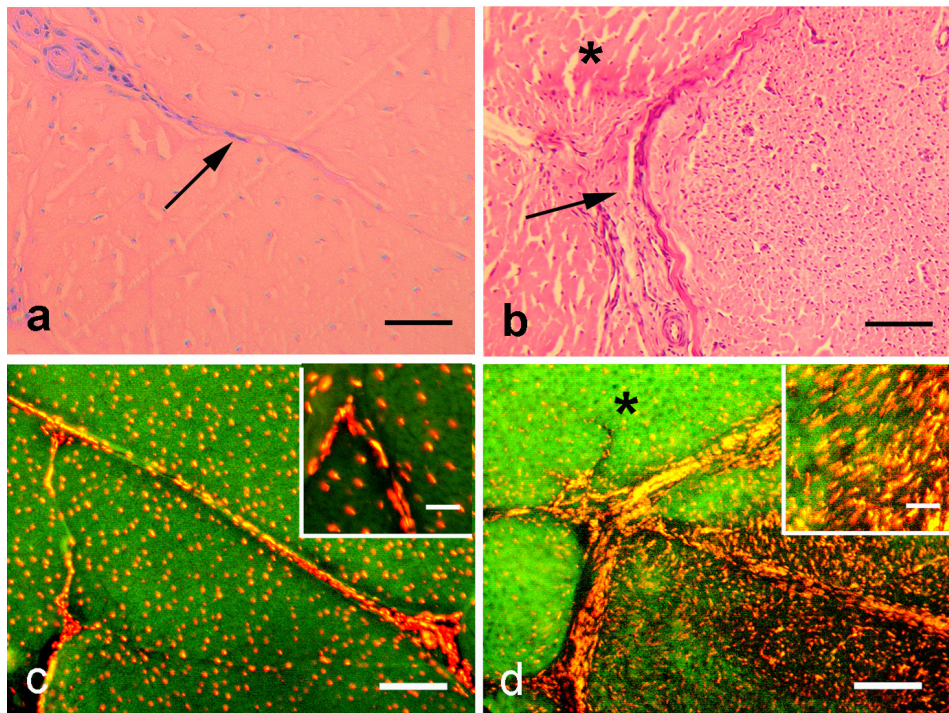


Fig. 2. Hematoxylin and eosin staining of normal (a) and injured tendon (b). The arrow indicates the endotenon. *In situ* zymography under acidic conditions in normal (case N1; c) and injured (case T1; d) tendons. While FITC-labeled HA is deposited throughout normal tendon bundles, green fluorescent signal decreases in the area where HAase was activated. The asterisk (b, d) indicates the normal area. Counterstainings are performed with propidium iodide. Bar=100 μ m (inset Bar=20 μ m).

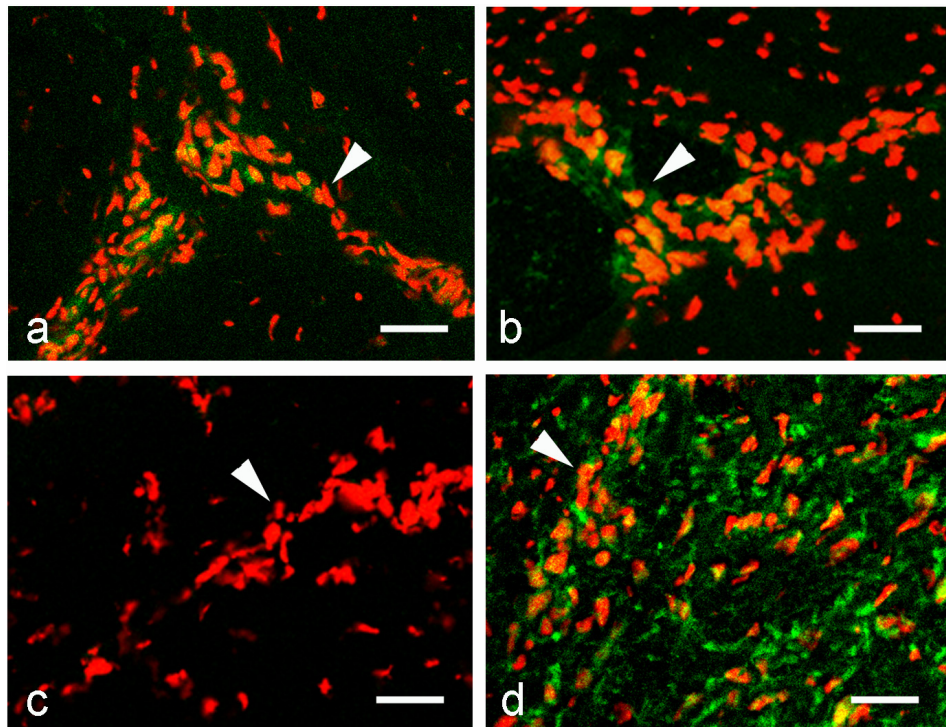


Fig. 3. *In situ* hybridization by antisense cRNA probe corresponding to HYAL1 (a, b) or HYAL2 (c, d) mRNA in normal (case N1; a, c) and injured (case T1; b, d) tendons. The corresponding mRNA signals are visualized as green fluorescence in the cytoplasm. Arrowheads indicate the endotenon. Counterstainings with propidium iodide are performed in all sections. Bar=20 μ m.

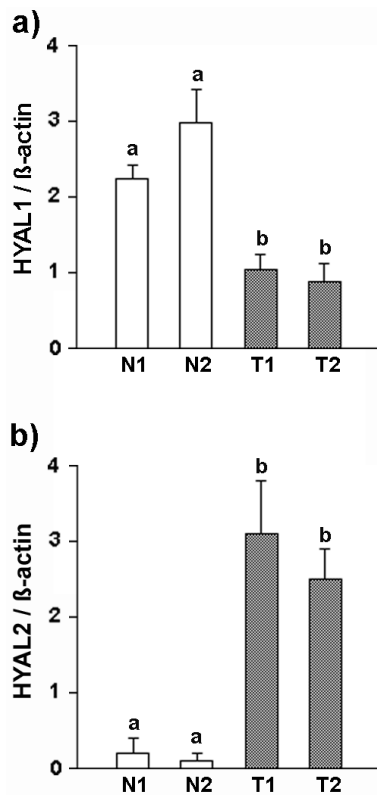


Fig. 4 Comparison of mRNA levels for HYAL1 (a) and HYAL2 (b) in normal and injured tendon tissues by quantitative PCR analysis. The mRNA level of each HAase is normalized against β -actin mRNA. Normal (open column; N1 & N2) and injured (shaded column; T1 & T2) tendons. The values are the mean \pm SD and are representative of three independent experiments. Different superscripts indicate significant differences, $P < 0.05$.

modulates the inflammation response to stabilize the granulation tissue [2, 16, 19]. In early stages of wound healing, it has been reported that HA accumulated in newly generated granulation tissue binds with fibrinogen for clot formation and inhibits angiogenesis [6, 7], and then HA is degraded by HYAL2 into smaller fragments endowed with specific biological activities such as inflammation and angiogenesis [5]. In human respiratory epithelial cells [15] and periodontal ligament fibroblasts [18], proinflammatory cytokines such as interleukin-1 β and tumor necrosis factor- α stimulated HAase expression, indicating cytokine-mediated HAase regulation. On the other hand, tendon is usually loaded with mechanical stress, and it was thought that the force affected several phenotype expression including matrix-degrading enzymes and subsequent healing process in injured tendon. It was recently reported that upregulation of transforming growth factor- β 1 and MMP-2 expression [12] in addition to the expression of scleraxis, a transcription factor specific for tenocytes and their progenitors in mouse model for tendon injury [13], was induced by mechanical loading. Taking the above into account, a further study about mechanical force-mediated

regulatory mechanism of HYAL2 expression in equine injured tendon will be needed.

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