

## Active Expression of Matrix Metalloproteinase-13 mRNA in the Granulation Tissue of Equine Superficial Digital Flexor Tendinitis

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**ABSTRACT.** The DNA microarray analysis for matrix metalloproteinase (MMP)-related mRNA expression in equine superficial digital flexor tendinitis indicated that mRNA level of MMP-13 was apparently up-regulated in the tendinitis as compared to normal tendon. *In situ* hybridization also revealed that fibroblastic cells proliferated in the granulation tissue generated in the tendinitis actively expressed MMP-13 mRNA. On the other hand, in normal tendon, a few fibroblastic cells and vascular components lied in the endotenon barely expressed its mRNA, but other cellular components in the tendon bundle were not positively hybridized. As mentioned above, MMP-13 but not other collagenases or gelatinases, may play an important role in tendon injuries in the racehorses.

**KEY WORDS:** equine, MMP-13, tendinitis.

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Matrix metalloproteinases (MMPs) are a family of structurally related zinc/calcium-dependent proteinases that play a central role in the degradation of the extracellular matrix (ECM) during both normal and pathological tissue remodeling processes [16, 21, 24]. The MMP family consists of several subfamily such as collagenase, gelatinase, stromelysin, membrane-type (MT-MMPs) and others, and MT-MMPs is known to be the activators for gelatinases. Collagenase subfamily further classified into MMP-1 (fibroblast collagenase), -8 (neutrophil collagenase) and -13 (collagenase-3) and mainly degraded fibrous collagen, e.g. type I, II, III, V and XI. Among these, MMP-13 was first identified in human breast carcinoma [9] and its increased expression in several pathological stages including arthritic synovium [25, 26] and osteoarthritic cartilage [7, 8, 15] have been demonstrated. Additionally, MMP-13 is a potential marker for a kind of joint diseases including cartilage degradation [20, 22]. This type of MMP could also act in several tendon injuries [13, 17–19] and up-regulation of MMP-13 mRNA level by interleukin-1 $\beta$  in human tendon-derived cells was demonstrated [2]. On the other hand, equine tendinitis, injury to the superficial digital flexor (SDF) tendon (T) commonly occurs in racehorses [1]. However, the molecular basis of pathology of equine SDF tendinitis was not fully characterized. In this report, we tried to characterize ECM-related mRNA expression profile in equine tendinitis and suggested participation of MMP-13 in the granulation tissue generated in this injury.

Normal SDFT was obtained from the fetus in late pregnancy (gestational days 296) and adult male thoroughbred. Two cases of injured lesions were dissected from SDFT in

adult male thoroughbreds suffering from acute tendinitis. Using total RNA extracted from adult normal and tendinitis tissues, MMP-related mRNA expression was surveyed using human ECM and adhesion molecules cDNA GEarray (SuperArray Bioscience Co., Fredrick, MD) according to the manufacture's protocol. Briefly, biotinylated cDNA was prepared by reverse transcription of tendon-derived total RNA in the presence of biotin-16-dUTP (SuperArray) and was hybridized with the nylon membrane array, then

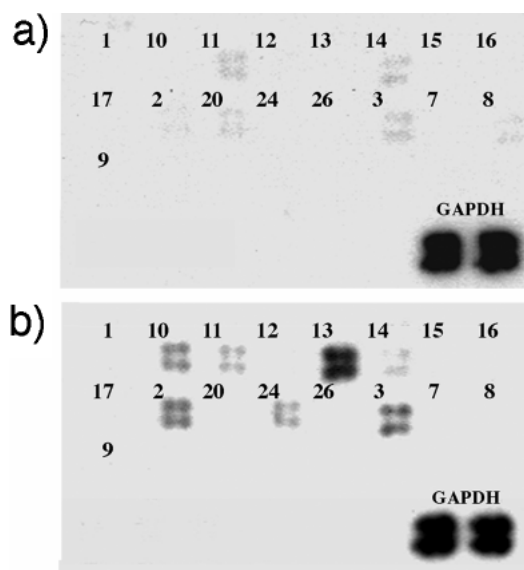


Fig. 1. Raw images of mRNA expression pattern of seventeen matrix metalloproteinase (MMP) species. Arabic numerals correspond to MMP numbers. a) normal superficial digital flexor (SDF) tendon, b) SDF tendinitis. GAPDH; internal control for mRNA expression.

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positive signals were chemiluminescently detected with alkaline phosphatase-conjugated streptavidin (SuperArray) and CDP-Star (SuperArray). Next, to prepare the probe for *in situ* hybridization (ISH), DNA fragment of equine MMP-13 (GenBank accession No. AF034087) was amplified by PCR with a primer set (upper; 5'-GCTGCCTATGAGCATCCTTC-3' and lower; 5'-ACCTCCAGACCTGGTTTCCT-3') and a tenocyte-derived cDNA template as previously reported [11]. The 208 bp fragment was subcloned into pGEM-T easy plasmid vector (Promega Co., Madison, WI) and the sequence was confirmed by a DSQ-2000L autosequencer (SHIMADZU, Kyoto, Japan), then sense and antisense RNA probes were synthesized by SP6 or T7 RNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany) in the presence of digoxigenin (DIG)-UTP (Roche Diagnostics) according to the manufacture's protocol. Formalin-fixed tissues were dehydrated, embedded in paraffin, and sectioned at 6  $\mu$ m. Deparaffinized thin sections were pretreated as previously reported [11], then hybridized with the probes appropriately diluted with 50% formamide/0.15 M Tris-HCl, pH 7.4/1x Denhardt's /0.6 M

NaCl/0.25% SDS/0.005% salmon sperm DNA/1 mM EDTA, pH 8.0/10% dextran sulfate for 18 hr at 50°C. After washing with  $0.2 \times$  SSC twice for 20 min at 50°C, slides were incubated with alkaline phosphatase-labeled anti-DIG antibody (Roche Diagnostics), then positive signals were visualized with nitro blue tetrazolium (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl-phosphate (Roche Diagnostics).

In general, nucleotide sequence of each MMP was known to be highly conserved across vertebrate species [6, 24] and the DNA sequence of PCR fragment corresponding to equine MMP-13 used in this study showed more than 90% homology with that of human MMP-13, but did not significantly agree with other MMPs' sequences (data not shown). Thus, human DNA microarray thought to be used for rough profiling for expression of equine MMPs. DNA microarray indicated increased level of MMP-13 mRNA in the tendinitis, however, other two collagenase species, MMP-1 and MMP-8, were not detected. Levels of mRNA corresponding to MMP-2 (gelatinase A), -3 (stromelysin-1) and MMP-10 (stromelysin-2) in addition to MMP-24 (MT5-MMP)

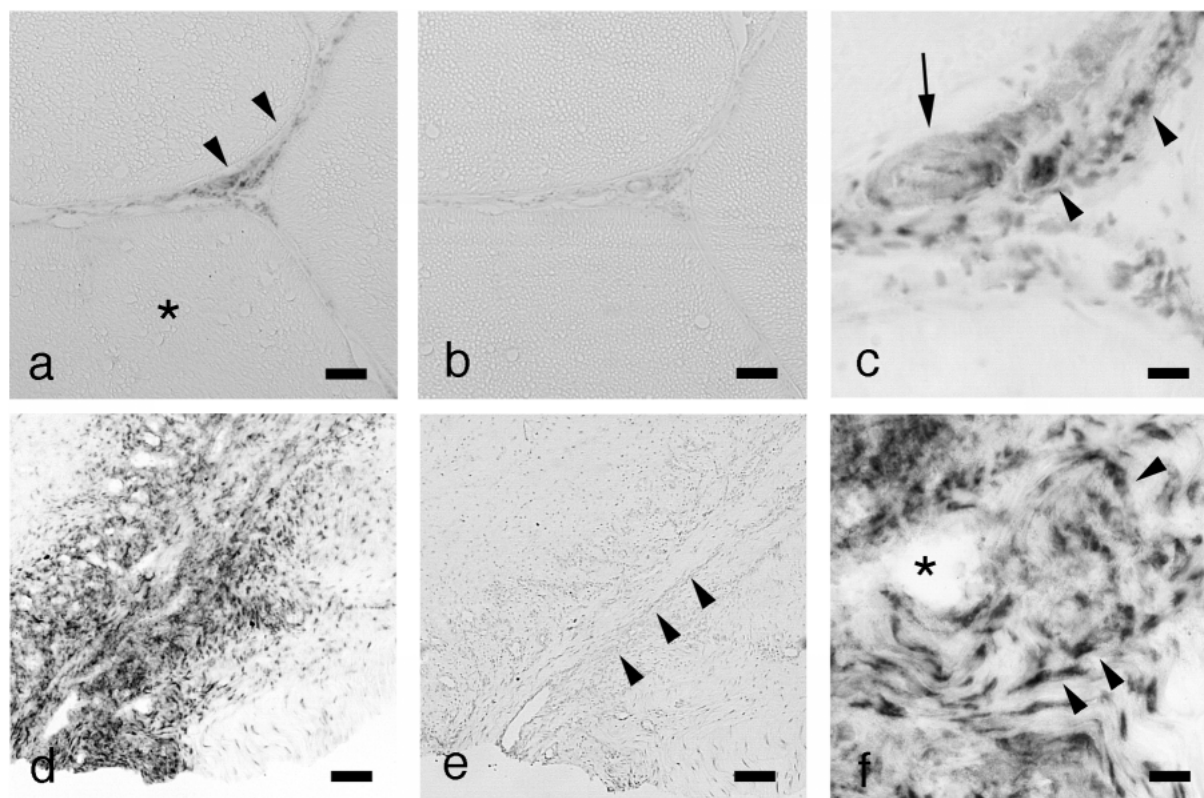


Fig. 2. Photomicrographs of *in situ* hybridization. a) Normal fetal tendon. Antisense probe positively hybridized with the endotenon (arrowheads), but no positive reaction was seen in tendon bundles (asterisk). Bar=100  $\mu$ m. b) Normal fetal tendon. No hybridization signal with sense probe was observed. Bar=100  $\mu$ m. c) Normal fetal tendon. High power view of a) indicated positive reaction with fibroblastic cells (arrowheads) in the endotenon. Vascular smooth muscle and endothelium were barely hybridized with antisense probe (arrow). Bar=20  $\mu$ m. d) SDF tendinitis. Positive reaction was observed in the granulation tissue neighboring the endotenon. Bar=100  $\mu$ m. e) SDF tendinitis. No hybridization signal with sense probe was observed. Arrowheads indicated the remnant of the original endotenon. Bar=100  $\mu$ m. f) SDF tendinitis. High power view of d) indicated active expression of MMP-13 mRNA in most fibroblastic cells proliferated in the granulation tissue (arrowheads). Some cellular components adjacent to the vessel (asterisk) were also hybridized with antisense probe. Bar=20  $\mu$ m.

were also slightly up-regulated in the tendinitis compared with normal adult SDFT, whereas no significant change in MMP-11 (stromelysin-3) and MMP-14 (MT1-MMP) was observed (Fig. 1). Based on the results of mRNA expression profiles, localization of MMP-13 mRNA was further examined by ISH. In normal fetal tendon, positive signals were restricted in the endotenon (Fig. 2a), and a few fibroblastic cells and vascular components showed positive signals for MMP-13 (Fig. 2c). In the tendinitis lesion, its mRNA signal concentrated in the granulation tissue generated along the endotenon (Fig. 2c) and most mRNA-expressing cells seemed to be fibroblastic cells proliferated in the granulation tissue (Fig. 2f). Sense probe did not hybridize any part of these tissues (Figs. 2b, 2e).

In addition to tumor invasion and metastasis [6], MMP-13 is known to function in some developing stage including bone formation [23], or several healing events after bone fracture [10] and tendon laceration [13]. In normal tendon, expression of MMP-13 was restricted in some fibroblastic cells and vascular components in the endotenon, suggesting the participation of this enzyme in maintaining normal homeostasis in matrix turnover of endotenon. We also showed apparent expression of MMP-13 by fibroblastic cells proliferated in the granulation tissue generated in the tendinitis. It was known that equine SDF tendinitis was experimentally induced by collagenase injection into the midmetacarpal region [3–5, 14] and MMP-13 participated in collagen degradation during flexor tendon healing of the rat [18]. Furthermore, its mRNA expression could be stimulated by inflammatory cytokines such as IL-1 and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) [2] and down-regulated by anti-TNF $\alpha$  antibody in human rheumatoid tenosynovitis [12]. These observation indicate that MMP-13 is thought to play an important role in tendon injuries including equine SDF tendinitis. Although the origin of these MMP-13-expressing cells in the tendinitis are not clear until now, this enzyme could significantly act in a certain process of SDF tendinitis. Further investigation on the kinetics of MMP-13-related collagenolytic activity in the tendinitis could clear a part of the molecular basis of the tendon injury.

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