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## Distribution of TNF receptors and TNF receptor-associated intracellular signaling factors on equine tendinocytes *in vitro*

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### Abstract

Although tumor necrosis factor (TNF) $\alpha$  is an important key factor in degeneration of equine superficial digital flexor tendon (SDFT), the dynamism of TNF receptors and associated factors on tendinocytes has not been elucidated. To reveal signaling events mediated by TNF-receptors (TNF-Rs) in tendinocytes, we focused on four signaling factors, TNF-R1, TNF-R2, TNF-R-associated factor 2 (TRAF2) and nuclear factor-kappa B (NF- $\kappa$ B), and investigated the distribution and production of these factors. Cultured tendinocytes were obtained from SDFTs of thoroughbred horses. The tendinocytes were treated with 10ng/ml equine TNF $\alpha$  medium for 6 hours and then the four factors on tendinocytes were visualized by using an immunohistochemical method, and the amounts of the four factors were determined by Western blot analysis. Although TNF-R1 and TNF-R2 co-localized on the same tendinocyte, in untreated control cells (normal condition), immunoreactivity against TNF-R1 was very weak but TNF-R2 showed a strong reaction. However, TNF-R1 showed the same high level of reaction as TNF-R2 in TNF $\alpha$ -treated cells (inflamed condition). Intense TRAF2 and NF- $\kappa$ B were detected at inflamed condition, however both factors were also detected at normal condition. The distinct distributions of the four factors under different conditions (normal and inflamed condition) *in vitro* not only reflect the dynamism of the cytokines but

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may also provide important clues for a means to prevent from occurrence of tendonitis and progress of tendon degeneration.

Key words : NF- $\kappa$ B, tendinocyte, TNF $\alpha$ , TNF-receptors, TRAF 2

### Introduction

Tumor necrosis factor (TNF) $\alpha$  is a cytokine that has various functions and plays a key role in the orchestration of complex events involved in inflammation, immunoreactions, cellular growth and differentiation<sup>8,12,14,28</sup>. Moreover this cytokine has the ability to collect inflammatory cells in a focal part and increase lymph flow and also have the ability to initiate apoptosis or proliferation of not only hematopoietic cells but also mesenchymal cells and nerve cells<sup>8,14,28</sup>. The effects of TNF $\alpha$  are mediated by at least two cognate TNF receptors, the 55-kDa type I receptor (TNF-R1) and 75-kDa type II receptor (TNF-R2)<sup>15,19,21</sup>, which belong to the TNF receptor superfamily<sup>5,11,22</sup>. The death domain is localized on the cytoplasmic tail of TNF-R1, and TNF-R1 initiates the process of cell death by activating the caspase cascade<sup>12</sup>. TNF-R1 is therefore called the "death receptor"<sup>24</sup>. TNF-R1 has another signal transduction pathway of apoptosis suppression<sup>16</sup>.

Many cells possess TNF-R1 and TNF-R2, and the two receptors being co-distributed on the same cell surface<sup>25</sup>; however, TNF-R2 has no intracellular death domain and does not induce apoptosis but mediates cell proliferation signals<sup>25,27</sup>. Signals through TNF-R2 are mediated *via* TNF-R-associated factor 2 (TRAF 2), which activates nuclear factor-kappa B (NF- $\kappa$ B), and NF- $\kappa$ B promotes transcription and prevention of apoptotic cell death<sup>3,4,6</sup>. TNF $\alpha$  in the signal transduction pathway activated by TRAF2 usually protects cells against apoptosis<sup>6,8</sup>. In normal conditions *in vivo*, the ability of TNF $\alpha$  to bind to

TNF-R2 is much greater than the ability of TNF $\alpha$  to bind to TNF-R1, and TNF $\alpha$  therefore dominantly binds to TNF-R2 in the presence of a low concentration of TNF $\alpha$ . In the presence of a high concentration of TNF $\alpha$ , TNF $\alpha$  tends to change the binding receptor from TNF-R2 to TNF-R1, in a process termed "ligand passing"<sup>26</sup>.

We have already revealed that production of many kinds of inflammatory cytokines such as TNF $\alpha$ , interleukin (IL) -1 $\alpha$ , IL-1 $\beta$  and interferon  $\gamma$  is greatly increased in an inflamed superficial digital flexor tendon (SDFT) compared to that in a normal SDFT in horses<sup>9</sup>. Moreover, we have found that many apoptotic tendinocytes are present in an inflamed equine SDFT and speculated that degeneration of an SDFT is caused by excessive apoptosis<sup>10</sup>. We have also found that two distinct signaling pathways, cell survival and apoptosis, are mediated by TNF-R1, which is distributed on equine SDFT tendinocytes *in vivo* (manuscript in preparation). Although an understanding of the cellular signaling pathway is important for preventing the occurrence of tendonitis in equine SDFT, it is very difficult to understand the cellular signaling pathways after being stimulated by inflammatory cytokines *in vivo*.

In this study, to determine the cellular signaling pathways stimulated by inflammatory cytokines, especially TNF $\alpha$ , we focused on four signaling factors, TNF-R1, TNF-R2, TRAF2 and NF $\kappa$ B, and investigated the distribution of these factors on tendinocytes *in vitro*.

## Materials and Methods

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

### *Isolation and culture of horse tendinocytes*

Clones of tendinocytes were isolated from SDFTs of thoroughbred horses (two males and three females, 1-4 years old) with no histological alteration of the tendons. The tendinocytes were isolated by the method of Arai *et al.*<sup>1)</sup> with some modifications. After sedation with an injection of medetomidine hydrochloride (16 mg/kg, administrated intermuscularly) and induction of anesthesia with thiopental sodium (6 mg/kg, administrated intravenously), the animals were killed with blood-letting and tissue samples were removed. Tendon specimens were cut into pieces of approximately 10 x 10 x 10 mm (tendon tissue blocks) and incubated in growth medium consisting of Dullbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; LifeTechnologies, Grand Island, NY, USA) and an antibiotic-antimycotic solution (100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; LifeTechnologies) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in T25 culture flasks for 7 to 10 days. During the harvested period, all tendon tissue blocks were mounted on cover glass slides in order to promote outgrowth of the tendinocytes. The growth medium was replaced every three days. Non-adhering cells were removed by changing the growth medium. To confirm that the cells were confluent, a small piece of the tendon tissue blocks were removed from the flask and, the attached cells were harvested with trypsin-EDTA (0.25% trypsin, 1mM EDTA; Sigma, USA) and replaced with cells 1:2 dilutions in the growth

medium. All experiments were performed during passage 3 or 4. At each passage, trypsin-released cells were seeded at a density of 5 x 10<sup>4</sup> cells/ml on a 12-well plastic culture plate seated with glass cover slips for immunohistochemistry.

### *TNF $\alpha$ treatment of tendinocytes*

TNF $\alpha$  is the most important key mediator of intracellular signal transduction of TNF-Rs. To evaluate the cellular reaction with TNF $\alpha$ , some cultured tendinocytes were treated with purified equine 10ng/ml TNF $\alpha$  (manuscript in preparation) for 6 h.

### *Immunofluorescence microscopy*

The control and TNF $\alpha$  treated (Tt) tendinocytes were rinsed with phosphate-buffered saline (PBS; 0.01M, pH7.4) and fixed with ethanol-acetone (1:1) for 5min at room temperature. After blocking unspecific binding sites with 10% normal donkey serum (Sigma, USA) diluted with PBS for 30 min, cells were incubated with primary antibodies for 1 h at room temperature with four primary antibodies: mouse anti-human TNF-R1 (monoclonal, diluted 1:50 in PBS), rabbit anti-human TNF-R2 (polyclonal, diluted 1:50), rabbit anti-human TRAF2 (polyclonal, diluted 1:50), and mouse anti-human NF- $\kappa$ Bp65 (monoclonal, diluted 1:50). All antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-human TNF-R1 antibody used in this study has already been confirmed to have no cross-reactivity with the anti-TNF-R2 antibody used in this study. Anti-NF- $\kappa$ Bp65 antibody is able to detect the activated domain. After being washed three times for 5 min each time with PBS, cells were incubated for 1 h at room temperature with rhodamine (Chemicon International, Temecula, CA, USA) -or FITC (Jackson Immuno Research, West Grove, PA, USA) -labeled secondary an-

tibodies diluted 1:50 in PBS. Coverslipped cells were observed under a confocal laser microscope (Fluoview ; Olympus, Tokyo, Japan).

### Western blot analysis

The tendinocytes were solubilized in ice-cold buffer consisting of 50 mM Tris-HCl (pH 7.6), 10mM EDTA, 1mM phenylmethylsulphonylfluoride and Triton X-100 1 % for 5 min. The solubilized proteins were recovered by centrifugation and subjected to sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS/ PAGE) (7.5 or 10% gels). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Cor-

poration, Billerica, MA, USA), and blots were blocked in Blockace (Yukijirushi, Tokyo, Japan) for 1 h. Each membrane was then washed four times with Tris-buffered saline (pH7.6) containing 0.2% Tween 20 (TBST) and incubated for 1h at room temperature with one of the following antibodies : mouse anti-human TNF-R1 (diluted 1:100 in TBST), rabbit anti-human TNF-R2 (diluted 1:100), rabbit anti-human TRAF2 (diluted 1:200), and mouse anti-human NF- $\kappa$ Bp65 (monoclonal, diluted 1:200). After incubation, the membranes were washed and incubated with the secondary antibodies peroxidase-conjugated goat anti-mouse IgG-horse radish

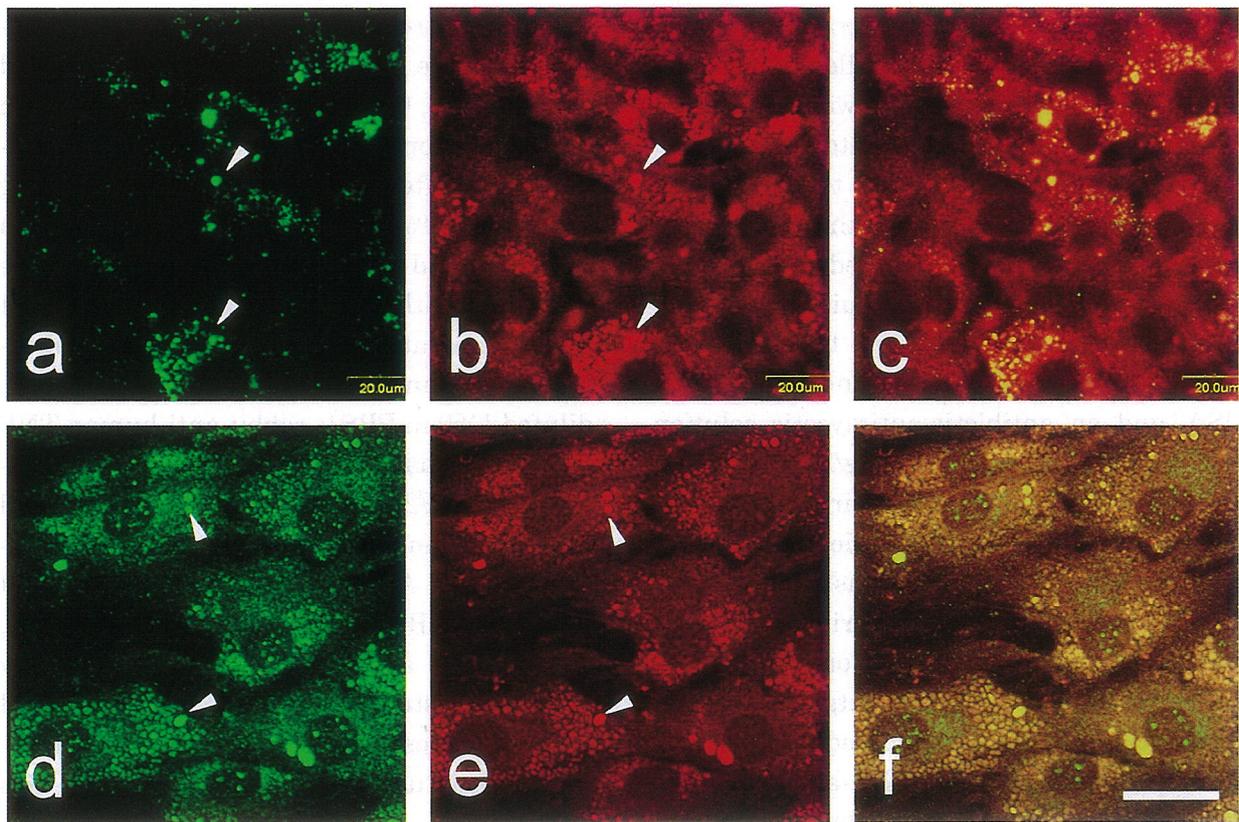


Figure 1 . Immunofluorescence staining with anti-TNF-R1 and anti-TNF-R2 antibodies. Upper panels indicate immunoreactivity in control tendinocytes (a-c) and lower panels indicate immunoreactivity in Tt tendinocytes (d-f) . Although the immunoreactions for TNF-R1 were weak in control tendinocytes (a), the immunoreactions for TNF-R2 were intense (b) . In Tt tendinocytes, both TNF-R1 and R2 showed intense reaction (d, e) . Immunoreactions were observed in the endosome (arrowheads) . Immunopositive reactions against the two receptors co-existed on the same tendinocyte in the merged image (c, f) . Bar : 20 $\mu$ m

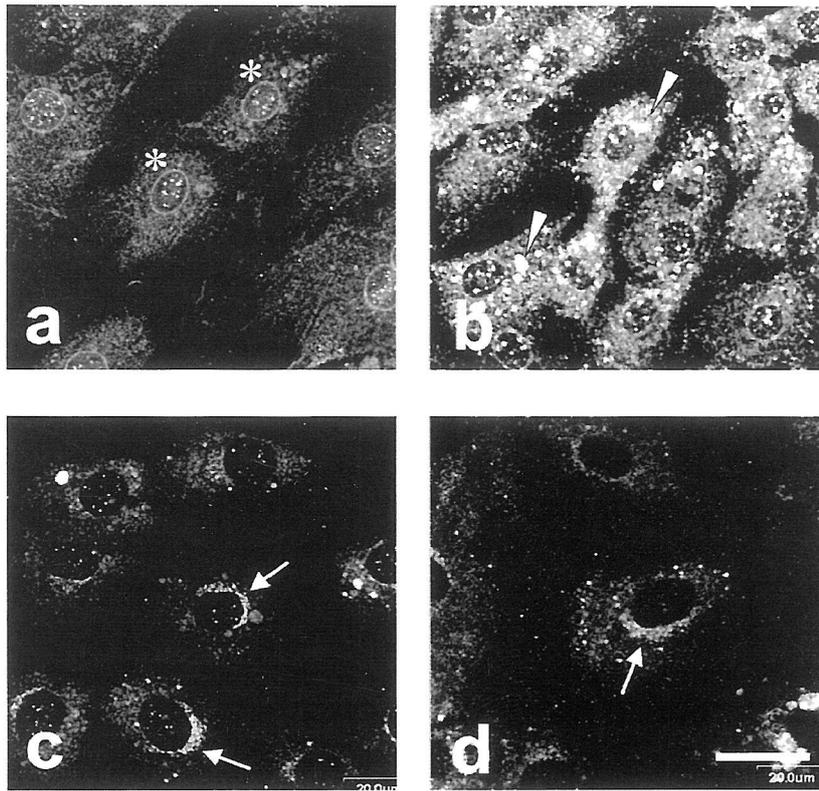


Figure 2 . Immunofluorescence staining with anti-TRAF 2 (a, b) and anti-NF- $\kappa$ Bp65 (c, d) antibodies. In normal conditions, immunopositive reactions were observed in nuclei with some spots (a : asterisks), while many endosome-like structures in Tt cells showed strong immunopositive reactions (b : arrowheads). NF- $\kappa$ Bp65-positive cells were observed in the vicinity of the cell nucleus in both control and Tt tendinocytes. In both normal (c) and Tt cells (d), the immunopositive reactions were localized in the perinuclear area, forming a crescent shape (arrows). Bar : 20 $\mu$ m

peroxidase (HRP) (diluted 1 : 10,000 in 0.05% Tween 20 in TBS) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and peroxidase-conjugated goat anti-rabbit IgG-HRP (diluted 1 : 50,000) for 1 h. Membranes were then washed and developed according to chemiluminescence (ECL) protocols (Amersham Pharmacia Biotech) and exposed to X-ray film (Kodak, Stuttgart, Germany) at room temperature. The density of the band on the developed film was analyzed by using NIH image software.

## Results

### Immunofluorescence staining

The localization of TNF-R1, TNF-R2,

TRAF2 and NF- $\kappa$ B in normal and Tt tendinocytes was examined by immunofluorescence staining. Tt cells showed strong immunopositive reactions (Fig. 1). The reactions of TNF-R1-positive cells were considerably less intense in control tendinocytes than in Tt ones (Fig. 1a and 1d). In contrast, both control and Tt tendinocytes showed intense immunoreactions against TNF-R2 antibody (Fig. 1b and 1e). The staining pattern of the two TNF receptors on control tendinocytes showed that TNF-R2 was more widely distributed than was TNF-R1 (Fig. 1a and 1b), while both receptors showed immunopositive-reactions on Tt cells (Fig. 1d and 1e). It was also found that these two receptors are co-

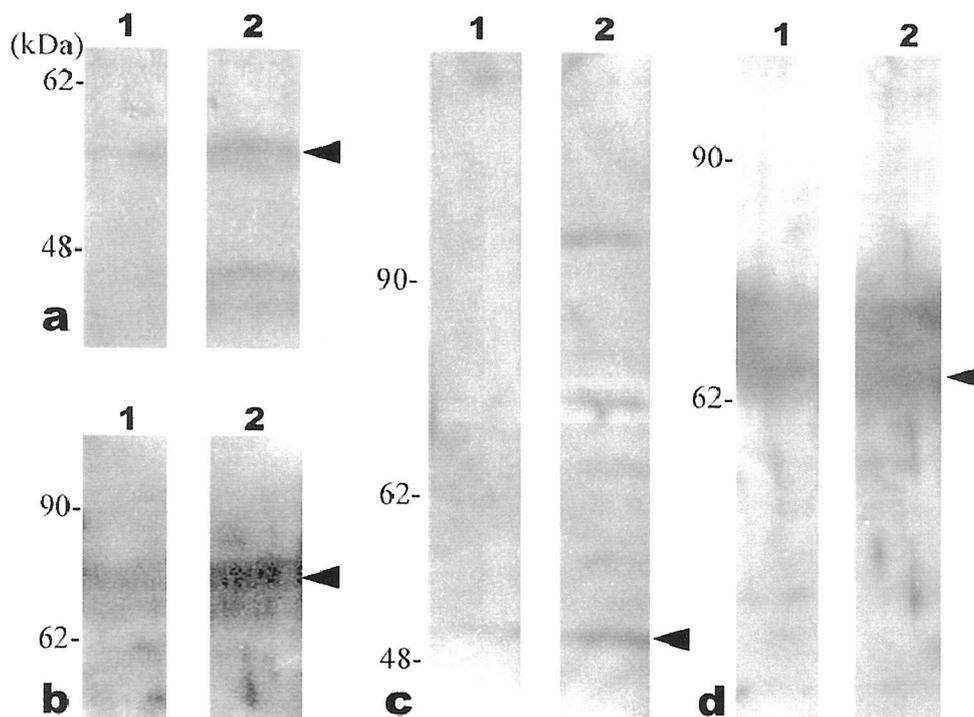


Figure 3. Western blot analysis revealed the presence of immunoreactive proteins, TNF-R1 (a), TNF-R2 (b), TRAF2 (c) and NF- $\kappa$ Bp65 (d). The activation levels of each of the four factors were up-regulated in inflamed tendon cells compared with the levels in control cells. The density of TNF-R1 in the inflamed tendon was twice that in the normal tendon, and the densities of TNF-R2, TRAF2 and NF- $\kappa$ Bp65 in inflamed tendons were about 1.4-, 3.8- and 1.7-times greater than the densities in the normal tendon, respectively.

distributed on the same cell (Fig. 1c and 1f). Interestingly, only small member of TNF-R1 and TNF-R2 were observed on the cell surface, but both TNF-Rs were distributed in endosomes-like structures.

Use of anti-TRAF2 antibody resulted in positive immunofluorescence staining for TRAF2 in both normal and Tt tendinocytes (Fig. 2a and 2b), but immunopositive reactions showed different patterns. In normal condition, the immunopositive reactions were observed in only nuclei with some spots, while many endosome-like structures in Tt cells showed strong immunopositive reactions. NF- $\kappa$ Bp65-positive cells were also observed in the vicinity of the cell nucleus of both control and Tt tendinocytes (Fig. 2c and 2d). In Tt cells assumed somewhat intense immunoreactivities

for NF- $\kappa$ Bp65 antibody. In both control and Tt cells, immunopositive reactions were localized in the perinuclear area, forming a crescent shape.

#### Western blot analysis

Western blot analysis revealed the presence of immunoreactive proteins, TNF-R1, TNF-R2, TRAF2 and NF- $\kappa$ Bp65 (Fig. 3). The activation levels of each four factors were all up-regulated in Tt cells (lane 2) compared with the level of in control cells (lane 1). The density of TNF-R 1 on Tt cells was twice that on control cells. All other factors studied also showed up-regulation of protein production; the densities of TNF-R2, TRAF2 and NF- $\kappa$ Bp 65 were about 1.4-, 3.8- and 1.7-times greater on Tt cells than on control cells, respectively.

## Discussion

In this study, we found that both TNF-R1 and TNF-R2 were distributed in the same tendinocyte and that both receptors were co-localized on the same organelle in the endosome-like structure. Interestingly, it has been reported that the distribution of TNF-R1 is different from that of TNF-R2 depending on the condition of the tendinocyte (normal or inflamed with TNF $\alpha$  treatment). The protein levels of TNF-R1 on Tt tendinocytes were much higher than those on control cells, whereas, both control and Tt tendinocytes showed intense reactions against TNF-R2. TNF-R1, a death receptor, was up-regulated after treatment with TNF $\alpha$ , its ligand, although mechanism by which up-regulation of TNF-R1 occurs has not been elucidated. By up-regulation of TNF-R1, some tendinocytes may be induced the apoptosis, however, TNF-R1 also mediates the signal transduction for cell survival as same as TNF-R2<sup>27)</sup>. This up-regulation may be a phenomenon for opposing acceleration of apoptosis in the tendinocytes.

Our previous study revealed that TNF $\alpha$  is expressed and distributed mRNA and protein levels even in a normal tendon, although the levels were very low (manuscript in preparation). TNF $\alpha$  at a low level has higher binding affinity for TNF-R2 than for TNF-R1 (under normal conditions)<sup>26)</sup>. Moreover, the lower level of TNF-R1 may indicate that the TNF-R2 signal pathway takes precedence over the TNF-R1 signal pathway in the TNF $\alpha$ -mediated signaling event under normal conditions. Continuous production of TRAF2 and NF- $\kappa$ B, may be deeply related to the TNF-R2 signaling pathway, has been confirmed in normal tendinocytes. By continuous production of these factors, tendinocytes would protect the cells against TNF $\alpha$ -induced apoptosis in normal condition.

We have also reported that amount of apoptotic tendinocytes and caspase-3-positive cells in inflamed tendons were significantly greater than the amounts in normal tendons *in vivo* and that excessive apoptosis in an inflamed tendon accelerates tendon degeneration<sup>10)</sup>. In another of our previous studies, TNF $\alpha$  mRNA expression level was found to be three-times higher under an inflamed condition than that in the normal state. The "ligand passing" phenomenon has not been clear in tendinocytes, however, these results of excessive apoptosis and marked expression of TNF $\alpha$  in an inflamed tendon and Tt tendinocytes support the speculation that tendinocytes possess distinct TNF receptors. And the inflamed condition might cause ligand passing from TNF-R2 to TNF-R1 and TNF $\alpha$  might activate both pathways, resulting in apoptosis or cell survival.

TNF-R1 and TNF-R2 were found to be mainly co-localized in endosome-like structures in tendinocytes. At first we estimated the possibility that the anti-TNF-R1 or TNF-R2 antibodies happened to detect some substances, which possess as similar epitopes as TNF-R1 or TNF-R2. A search of the GenBank database revealed no substances with a similar amino acid sequence alignment in with the regions of TNF-R1<sup>2)</sup> (accession ID : X59238, area: 30-301) and TNF-R2<sup>18)</sup> (accession ID : M88067, area: 260-461) used in this study. It has recently been reported that many cells respond by internalization of TNF-R1 and shedding of TNF-R2 to exposure to a variety of stimuli in the extracellular region of these receptors<sup>7,23)</sup> and that TNF $\alpha$  also possesses the ability to induce internalization or shedding of these two receptors<sup>16,20)</sup>. Results of these studies suggest that, immunopositive reactions in the tendinocytes might be a result of shedding of receptors and storage in organelles such as endosomes.

The results of different pattern of TRAF2 positive area between normal and Tt cells were also very characteristic. The immunopositive reactions in control cells were observed in nuclei with some spots, while many endosome-like structures in Tt cells showed strong immunopositive reactions. As stated in above, if ligand passing from TNF-R2 to TNF-R1 occurs at a high concentration of TNF $\alpha$  in tendinocytes, reinforcement of TRAF2 in Tt cells would be evidence of the presence of a TNF-R1-TRAF2 pathway in tendinocytes.

In conclusion, although further detailed study will be required, these distinct distributions of four factors under different conditions (normal and inflammation condition) *in vitro* not only reflect the dynamism of the cytokines but may also provide important clues for a means to prevent the occurrence of tendonitis and progress of tendon degeneration.

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