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Author(s)	KITAMURA, Hiroko; OKUMURA, Masahiro; SATO, Fumio; KIMOTO, Keita; KOHAMA, Morimasa; HASHIMOTO, Yoshiharu; TAGAMI, Masaaki; IWANAGA, Toshihiko
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## Increased concentrations of protein gene product 9.5 in the synovial fluid from horses with osteoarthritis

Hiroko Kitamura<sup>1</sup>, Masahiro Okumura<sup>2</sup>, Fumio Sato<sup>3</sup>, Keita Kimoto<sup>1</sup>,  
Morimasa Kohama<sup>1</sup>, Yoshiharu Hashimoto<sup>1</sup>, Masaaki Tagami<sup>4</sup> and Toshihiko Iwanaga<sup>1\*</sup>

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### Abstract

Our previous study established protein gene product 9.5 (PGP 9.5), a ubiquitin C-terminal hydrolase, as a specific cytochemical marker of synovial lining cells (type B synoviocytes) in the horse joint. The present study aimed to detect PGP 9.5 in the synovial fluid and shows that PGP 9.5 is a valuable marker of osteoarthritis in the horse. Immunohistochemical staining confirmed rich and consistent localization of PGP 9.5 immunoreactivity in the cytoplasm of synovial lining cells in the normal horse joint. Western blot analysis of synovial fluid from normal joints could detect a significant band corresponding to that contained in the brain and synovial membrane extracts.

When 60 synovial fluid samples from normal and abnormal joints were assayed with an enzyme-linked immunosorbent assay (ELISA) system, the concentration of PGP 9.5 tended to be elevated in osteochondrosis dissecance, inflammatory arthropathy and intra-articular fracture, among which a statistically significant elevation was recognizable between the intra-articular fracture and the control. Thus, this study demonstrated the possibility that PGP 9.5, derived from synovial lining cells, may be a new biochemical marker for arthritic disorders of the horse.

**Key words :** PGP 9.5, synovial fluid, ELISA, osteoarthritis, horse

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<sup>1</sup>Laboratory of Anatomy, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818

<sup>2</sup>Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818

<sup>3</sup>Laboratory of Molecular and Cellular Biology, Equine Research Institute, Japan Racing Association, Utsunomiya 320-0856

<sup>4</sup>Shadai Horse Clinic, Tomakomai 059-1361

\*Corresponding author

Fax : +81-11-706-5189

E-mail : tiwanaga@vetmed.hokudai.ac.jp

## Introduction

Osteoarthritis (OA) is a frequent disease of joints, characterized by variable degrees of pain, inflammation, and subsequent cartilage breakdown and regeneration. The pathogenesis of OA is not completely understood. Many investigators have considered that chondrocytes, synovial lining cells and inflammatory cells infiltrating in the synovial membrane contribute significantly to the progressive destruction of the joint by releasing cytokines, degradative enzymes, prostaglandins, reactive oxygen species, and nitric oxide<sup>25, 32, 33</sup>. Others have emphasized an important role of the local nervous system and its products, particularly substance P, in modulating articular inflammation<sup>13, 19</sup>.

For diagnosis of OA, radiographic examination of the affected joints, in addition to physical examination for lameness, is useful, but does not correlate with the clinical symptomatology and stage of the disease, and is not available for early stages of inflammation, since degradation of the cartilage matrix begins long before macroscopic changes occur in the joint. Clearly, for identifying abnormal animals at high risk for destructive OA and for monitoring the efficacy of therapies, there is a need for more convenient and reliable diagnostic methods than plain physical examination and radiography. In recent years, progress has been made in evaluating biochemical markers that might be valuable in identifying early OA and detectable in body fluids, such as synovial fluid, blood and urine<sup>2, 10, 20, 28</sup>. These contain proteoglycans, cytokines, eicosanoids (prostaglandin E<sub>2</sub>), nitric oxide, and neuropeptides. However, all of these candidate markers are derived mainly from chondrocytes, infiltrating immune cells and nerve elements; there have been no marker substances specific for synovial lining cells re-

flecting conditions of the synovial intima.

The joint capsule is underlined by a cell-rich layer, termed the synovial membrane, that is responsible for production of synovial fluid and homeostasis of the joint cavity. Morphologically, two types of synovial lining cells (intimal cells) constitute the cell layer<sup>11</sup>. Type A cells are macrophagic cells derived from the bone marrow, while type B cells are unique for the synovial membrane, secreting proteoglycans and collagens into the synovial matrix and joint cavity. Our previous study revealed protein gene product 9.5 (PGP 9.5), originally identified as a neuron-specific ubiquitin C-terminal hydrolase, to be a cytochemical marker of the type B cells in the horse joint, since essentially all type B cells contain the protein in the cytoplasm<sup>17</sup>.

The present immunochemical study reports the rich existence of PGP 9.5, a novel marker of type B synoviocytes, in the synovial fluid and a change of the concentration in arthritic disorders, suggesting that measurement of the PGP 9.5 concentration in synovial fluid is useful for diagnosis of OA.

## Materials and Methods

### *Synovial membranes and brain extract*

Three female thoroughbred horses (300-500kg in body weight) without any abnormalities were used for immunohistochemistry and preparation of brain extract. The animals were deeply anesthetized with pentobarbital and thiopental sodium, and were sacrificed by bloodletting from the cervical artery. All experiments were conducted in accordance with the Animal Care and Use Guidelines of the Graduate School of Veterinary Medicine, Hokkaido University.

Synovial membranes were obtained from the carpal joint, and fixed in 10% buffered formalin for 48 hr. After immersion in 30% sucrose solution (0.1 M phosphate buffer, pH7.4)

overnight, they were rapidly frozen in liquid nitrogen. Cryostat sections, 15  $\mu\text{m}$  in thickness, were processed for the avidin-biotin complex (ABC) method. After pretreatment with normal goat serum, the sections were incubated with a rabbit anti-human PGP 9.5 serum (RA 95101; Ultracclone, Isle of Wight, UK) diluted 1 : 10,000. The antigen-antibody reactions were detected by incubation with a Histofine kit (Nichirei, Tokyo, Japan) including goat anti-rabbit IgG and streptavidin-biotin complex, and visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.002%  $\text{H}_2\text{O}_2$ .

A brain sample (cerebral cortex) for deciding the standard unit in the ELISA and the synovial membrane for control in Western blotting were obtained from one of the horses, homogenized with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM sodium ethylenediamine-tetraacetic acid (EDTA), and solubilized in the presence of 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, and 1% Triton X-100 for 30 min on ice. The sample was centrifuged at 200 x g for 15 min and the supernatant was collected.

#### *Synovial fluid samples*

Synovial fluid samples were collected from 10 female thoroughbred horses with normal joints and 50 horses with joint diseases, including osteochondrosis dissecans ( $n=15$ ), intraarticular fracture ( $n=28$ ), and inflammatory arthropathy ( $n=7$ ) at arthroscopy for therapeutic reasons. Samples of clinical cases were collected from synovial fluids obtained for diagnosis at Shadai Horse Clinic.

#### *Western blotting*

Proteins in the brain and synovial membrane extracts described above, and the synovial fluids from normal joints were reduced with 0.7 M 2-mercaptoethanol and subjected to 12% polyacrylamide electrophoresis.

The proteins were electrophoretically transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% powdered milk in 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween 20, and incubated with peroxidase-labeled anti-rabbit immunoglobulins (Dako, Carpinteria, CA, USA). The antigen-antibody complex was visualized with an ECL kit (Amersham, Buckinghamshire, England) on X-ray film.

#### *Inhibition ELISA technique*

**Antigen plate:** The brain extract was reduced with 20 mM sodium carbonate-bicarbonate buffer containing 0.02% sodium azide (pH 9.2), and introduced into 96-well ELISA plates (200  $\mu\text{l}$  per well). The plates were sealed with parafilm and incubated for 2 hr at room temperature, kept at 4°C overnight, and then stored at 4°C until use.

**Inhibition step:** One hundred microliters of the samples or PGP 9.5-standard solution (brain extract) was mixed with an equal volume of the rabbit polyclonal antiserum against human PGP 9.5 diluted in proper concentration with PBS containing 0.05% Tween 20, 1% bovine serum albumin (BSA), and 0.05 M EDTA at pH 5.5. Plates were incubated for 1 hr at room temperature, then kept overnight at 4°C. On the next day, antigenic plates were washed 5 times with PBS containing 0.05% Tween 20 and 1% BSA (BSA-Tween-PBS) and incubated with 200  $\mu\text{l}$  of the inhibition mixture for 1 hr. After washing, 200  $\mu\text{l}$  of peroxidase-linked goat anti-rabbit immunoglobulin was added to each well and kept for 1 hr. The free second antibodies were removed by washing, and the plates were incubated with 200  $\mu\text{l}$  of color reagent which contained 0.012%  $\text{H}_2\text{O}_2$  and 2 mg/ml *o*-phenylenediamine in 0.05 M citric acid and 0.05 M sodium citrate (0.1 M citrate buffer, pH 4.5). The color reaction was stopped by adding 50  $\mu\text{l}$  of 2.5 M  $\text{H}_2\text{SO}_4$ , and the plates were read at 490 nm using an

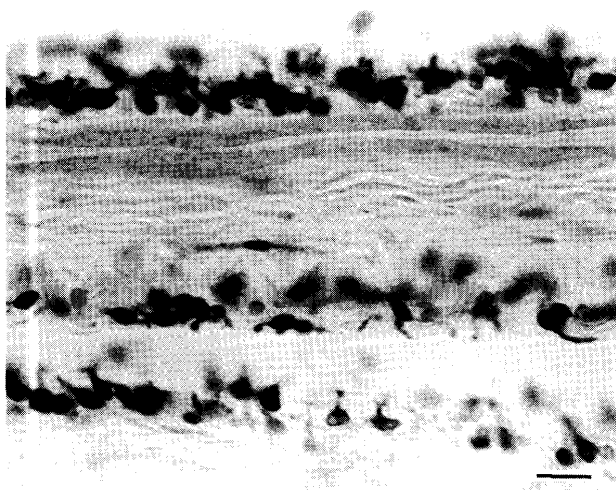


Fig. 1. Immunostaining for PGP 9.5 in the synovial membrane from a normal carpal joint. Numerous synoviocytes with dendritic processes in synovial villi are positively stained. Scale bar = 20  $\mu$ m

ELISA plate reader (model 550 microplate reader, Bio-Rad, CA, USA). The concentrations of PGP 9.5 in the samples were calculated by extrapolation from the standard curve of PGP9.5 standard solution.

## Results

### *Immunostaining and Western blot analysis*

Immunostaining of the synovial membrane for PGP 9.5 specifically detected synovial lining cells, which were densely distributed in the villous region and scattered in the fibrous part of the carpal joint. They were dendritic in shape and extended cytoplasmic processes towards the luminal surface (Fig. 1). Some immunoreactive nerve fibers were distributed around blood vessels, while only a few nerve fibers were scattered in the synovial membrane.

Western blotting yielded a predominant immunoreactive band, at approximately 23 kDa, in the extracts of brain and synovial membrane used for controls. Synovial fluid samples from normal horse displayed the most intensely immunoreactive band at the

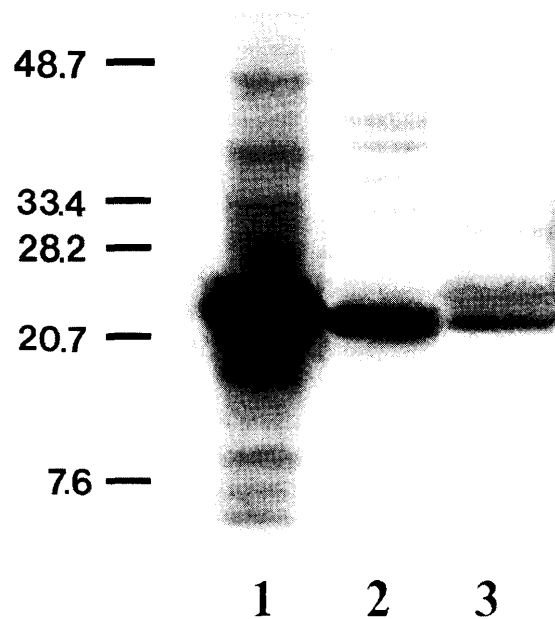


Fig. 2. Western blotting of PGP 9.5 immunoreactivity. An intensely immunoreactive band at about 23 kDa is seen in extracts of brain (lane 1) and synovial membrane (lane 2). A similar band is shared by a synovial fluid sample from a normal carpal joint (lane 3).

same position (Fig. 2).

### *Optimization of the conditions of ELISA*

The conditions of an inhibition ELISA for determination of the PGP 9.5 concentration from synovial fluid were optimized. In the assay, the brain extract in solutions was coated on wells of the ELISA microtitration plates for binding of a rabbit anti-human PGP 9.5 antibody (primary antibody), and the binding was detected by addition of peroxidase-linked goat anti-rabbit immunoglobulin (second antibody).

The sensitivity of the assay could be substantially improved by increasing the dilution of the primary antibody (1 : 10,000). The standard curve was displaced to a lower measuring range by decreasing the concentration of the primary antibody (Fig. 3). Further dilution resulted in too low an absorbance and decreased precision. The within- and between-assay coefficients of variation for this PGP

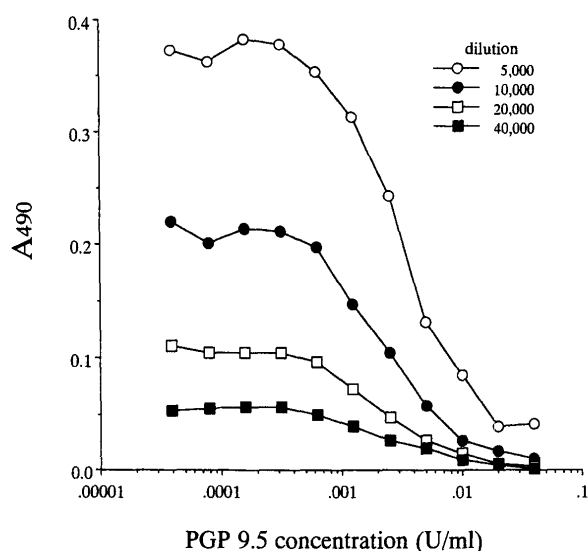


Fig. 3. Effect of dilution of the anti-PGP 9.5 serum used as the primary antiserum on a standard curve in inhibition ELISA

9.5 assay system were satisfactory (8.8 and 10.5%, respectively).

The optimal dilution of the second antibody also was determined (1 : 1,000). The standard curve was displaced to a lower measuring range by decreasing the concentration of the second antibody, while the peak value of absorbance of the color reaction was at 60 min after supplementation of the reactant (Fig. 4). Further dilution resulted in too low an absorbance and decreased precision.

The concentration of PGP 9.5 in the samples was calculated by comparing the absorbance value with values generated from known concentrations of brain extracts treated in a manner similar to the sample plates. Five milligrams of protein in the brain extracts was regarded to contain 100 units of PGP 9.5.

#### *PGP 9.5 concentration in synovial fluid from horses*

The results of the PGP 9.5 assay in synovial fluids are summarized in Fig. 5. The concentrations of synovial PGP 9.5 in horses with osteoarthritic joints were likely to be in-

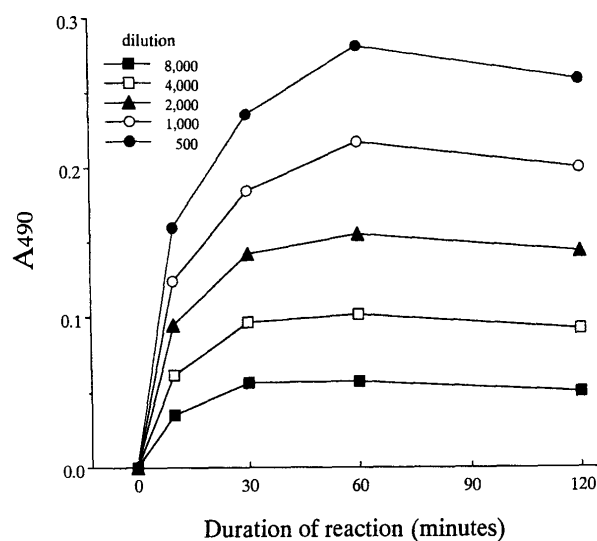


Fig. 4. Effect of dilution and incubation time of the peroxidase-labeled anti-rabbit IgG used as the second antiserum on a standard curve in inhibition ELISA

creased, as compared to normal horses. The concentration of PGP 9.5 in synovial fluids from horses with intra-articular fracture ( $0.871 \pm 0.462$ ) was significantly higher than that from normal horses ( $0.329 \pm 0.033$ ). Although there was no significant difference in PGP 9.5 concentrations between normal horses and those with osteochondrosis dissecans ( $0.701 \pm 0.436$ ) or inflammatory arthropathy ( $0.567 \pm 0.132$ ), synovial fluid samples from the latter two conditions tended to have increased PGP 9.5 concentrations.

#### Discussion

This is the first report on the concentration of a synoviocyte-specific protein in the synovial fluids of horses with and without arthritic disorders. Western blotting and ELISA clearly demonstrated the abundant existence of PGP 9.5 in the synovial fluid, and showed that the concentration changed depending on the condition of the joint. The present immunostaining by the anti-PGP 9.5 serum selectively stained the cytoplasm of type B synoviocytes in the horse joints, in accord

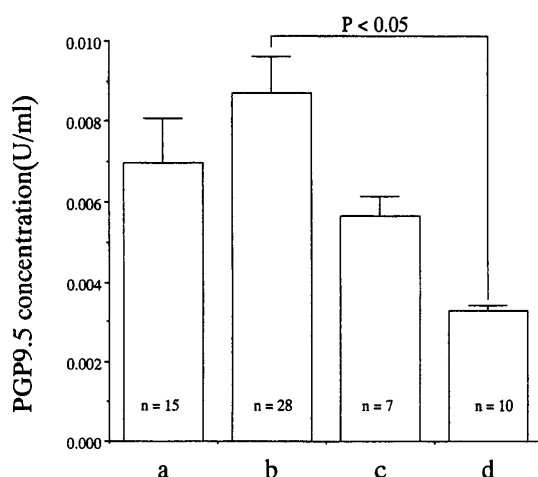


Fig. 5. PGP 9.5 concentrations in synovial fluids from horses with osteochondrosis dissecans (a), intra-articular fracture (b), and inflammatory arthropathy (c), and from normal control (d). The concentration of PGP 9.5 in synovial fluids from horses with intra-articular fracture is significantly higher than that from normal horses.

with our previous study<sup>17</sup>). Since there were very few PGP 9.5-immunoreactive nerve fibers in the horse synovial membrane, PGP 9.5 in the synovial fluid may be exclusively released from the synovial lining cells. PGP 9.5 is a cytosolic protein, but not a secretory protein contained in granular components, i. e. the secretory machinery. Although the releasing mechanism of PGP 9.5 remains unknown, there is a possibility that damaged synoviocytes release the cytoplasmic protein into the synovial fluid, as seen in the case of another neuron-specific cytoplasmic protein, neuron-specific enolase (NSE)<sup>23</sup>. The NSE concentration in cerebrospinal fluid has been established as a reliable marker of tissue damage in various neurologic disorders, including head injury, Creutzfeldt-Jacob disease and bacterial meningitis<sup>15,27,34</sup>. The PGP 9.5 concentrations tended to increase in all three types of arthritic disorders examined, especially in intra-articular fracture. The significant increase in the intra-articular fracture

may be explained by the fact that destruction of the synovial membrane and pain sensation are more severe in the intra-articular fracture than in the other two cases.

Synovial fluid in OA, and also in human rheumatoid arthritis, contains enhanced levels of neurogenic substances, cytokines, prostaglandins, and nitric oxide (NO), which are principally indicators of active synovial inflammation. Nerve fibers distributed in the horse synovium contain several bioactive peptides, including substance P, and release them into synovial fluid<sup>3,4</sup>. The increased substance P concentrations in the synovial fluid of the abnormal joint, and suppression of inflammation by neuropeptide depletion suggest the involvement of substance P in the etiopathogenesis of OA, especially neurogenic inflammation<sup>6</sup>. Elevated concentrations of substance P have been documented in synovial fluids of horses with joint disorders, including OA<sup>5,13,16</sup>. The release of substance P from stimulated nerve terminals may explain the pain sensation and neurogenic inflammation; however the number of nerve elements in the synovial membrane may be too small to suggest the leading roles of nerves in synovial inflammation<sup>13</sup>. Interleukin (IL) -1 and -6 and PGE<sub>2</sub> act as important mediators in articular inflammation, and increases in their concentrations (or activities) in the synovial fluid have been observed in clinical cases of inflammatory arthritis of horses<sup>1,2,16,21,24,30</sup>. The markers reported thus far are all substances derived from nerves, infiltrating inflammatory cells and/or damaged cartilage as well as synoviocytes; no reports are available about substances specifically derived from synovial intimal cells.

Cellular release of proteolytic enzymes and inflammatory mediators is responsible for destruction of articular cartilage by degradation of the cartilage matrix proteoglycans.

Considerable evidence suggests the involvement of degradative proteinases, matrix metalloproteinases (MMPs), in the progressive destruction of the cartilage matrix<sup>7)</sup>. Type II collagen is the predominant type of cartilage collagen, and is degraded by MMPs such as MMP-3 and MMP-13, which are secreted by chondrocytes and synoviocytes<sup>10)</sup>. In the horse, however, even a sensitive RT-PCR assay could detect only low levels of MMP-3 mRNA expression in some articular cartilage and synovial membrane samples, indicating low MMP production<sup>8)</sup>. Elevated production of NO and several cytokines such as tumor necrosis factor- $\alpha$ , IL-1 and IL-6 is believed to promote bone and cartilage destruction in affected joints<sup>12,26,29,31,32)</sup>. PGP 9.5 is not only useful as a marker of synoviocytes, but is also involved in the protein metabolism due to its ubiquitin C-terminal hydrolase activity. PGP 9.5 was originally identified as a ubiquitin C-terminal hydrolase showing brain-specific expression, but subsequent examinations revealed its wide distribution in non-neuronal cells. Ubiquitin is a normal component of most eukaryotic cells, and it is assumed that the covalent attachment of ubiquitin to proteins (ubiquitination) plays a role in metabolic processes of intracellular proteins<sup>9)</sup>. The ubiquitin C-terminal hydrolase is a key molecule that controls ubiquitination by the cleavage of amide bonds at the C-terminus of ubiquitin<sup>14)</sup>. Therefore, we can postulate its involvement in degradation or modulation of proteins in synovial fluid. The elevated concentrations of the proteolytic enzymes in synovial fluid may be functionally related to degradation of the increased protein and leukocytosis in acute articular inflammation of the horse.

Routinely applicable methods to monitor the condition of joint cartilage have been needed in the clinical field for many years. Cy-

tological and biochemical markers may also be valuable in monitoring disease progression and drug treatment. Markers of joint metabolism are released into synovial fluid and are mainly eliminated via lymphatic or blood circulation<sup>20)</sup>. Therefore, they might be detected in all body fluids as long as they are not captured and metabolized in regional lymph nodes. The existence of some brain-specific proteins in serum and their elevated concentrations in neurologic disorders<sup>18,22,27)</sup> encourages the measurement of PGP 9.5 in serum and urine for diagnosis of arthritis. A highly sensitive assay system for PGP 9.5 using antisera raised in two different animal species makes it possible to measure PGP 9.5 contents in blood and urine.

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