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**A study on keratan sulphate as a molecular marker
to monitor cartilage metabolism in horses**

(ウマにおける軟骨代謝を検索するための分子マーカーとしての
ケラタン硫酸に関する研究)

Masahiro OKUMURA

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1. Preface

1.1. Equine joint diseases

Lameness is the most important cause of wastage in racing horses as well as in horses that perform in other athletic events (Rossdale *et al.*, 1985; Todhunter *et al.*, 1993). Joint disease is a common and major cause of morbidity in horses. While a certain incidence of acute primary synovitis has not been determined, the development of an acutely inflamed joint in racing or training horses is a prevalent problem for owners, trainers and veterinarians (Palmer and Bertone, 1994). Joint destruction in inflammatory and degenerative joint diseases is characterized by an imbalance of enzyme catalyzed cartilage breakdown and cartilage matrix regeneration (Landoni *et al.*, 1996). Many medical and surgical treatments exist for various joint problems, but most of them are controversial, particularly with regards to its selection and definitive treatment results.

Much has been written on the management of different joint diseases, but little information exists documenting the clinical appearance of horses with different types of joint disease (Bramlage *et al.*, 1988; Raker, 1968). With the ready availability of new imaging modalities, the physical examination for lameness may often be overlooked. The radiographic features of joint disease, however, do not directly correlate with the clinical symptomatology and stage of the disease.

Careful observation and palpation can provide very useful additional information regarding the clinical significance of the disease beyond what can be seen on radiographs and therefore should be part of the evaluation of any horse with problems emanating from within or around a joint.

Obviously, clinicians should use a comprehensive physical examination to determine the extent of involvement of the clinically affected joint, but also to ensure that subtle problems in other joints are not overlooked. This indicates that only primary and essential devices are available to understand the pathology of joint diseases in equine practice. Definitive and quantitative assessment of the damage in joints must be given intense attention for more accurate diagnosis and precise treatment.

1.2. Aggrecans and keratan sulphate in articular cartilage

Articular cartilage is a specialized tissue composed of mesenchymal cells and chondrocytes, embedded in an extensive extracellular matrix. The cartilage matrix has a high content of proteoglycans and collagens, which are important for normal function of the tissue, and is synthesized and organized by the chondrocytes. Mature cartilage is characterized by the absence of blood vessels and nerve fibers. Homeostasis of chondrocyte function and responses to traumatic or inflammatory tissue injury can be induced by regulatory factors that are produced by cells in the synovial tissue or fluid. An alternative mechanism for the induction of chondrocyte response is the generation of regulatory factors within cartilage. Turnover of cartilage matrix molecules is normally controlled by chondrocytes that synthesize all the normal matrix components as well as proteinases, which can degrade them (Buttle *et al.*, 1995). The chondrocyte is itself subject to regulation by various cytokines and growth factors that can shift the homeostatic balance in an anabolic or catabolic direction (Trippel, 1995).

Most proteoglycans in the cartilage are high molecular weight macromolecules that enable cartilage to undergo reversible deformation (Fisher and Barclay, 1984). The

mechanisms and rate of cartilage proteoglycan turnover in normal and abnormal cartilage remain unclear (Heinegard and Paulsson, 1984; Maroudas, 1975; Revell and Muir, 1972; Thonar *et al.*, 1985). Lack of substantial amounts of partially degraded proteoglycans in the normal cartilage matrix suggests that proteoglycan molecules are usually rapidly degraded into smaller fragments, which are either engulfed by the chondrocytes or diffused into the body fluids that are in contact with cartilage (Thonar *et al.*, 1985). Consequently, proteoglycan fragments and glycosaminoglycans were found in the synovial fluid (Heinegard *et al.*, 1985; Saxone *et al.*, 1986), and several species of glycosaminoglycans including chondroitin sulphate and keratan sulphate have also been detected in the blood (Block *et al.*, 1989; Calatroni *et al.*, 1969; Heinegard *et al.*, 1984; Kliner *et al.*, 1987; Thonar *et al.*, 1985).

Keratan sulphate is a glycosaminoglycan that is distributed in the extracellular matrix of the joint cartilage. Keratan sulphate is mainly found in the aggrecans (approximately 99%), and to a lesser extent, in the cornea and other tissues (Axelsson and Heinegard, 1978). Therefore, to estimate the total catabolic activity of cartilage, keratan sulphate was measured as a metabolite of cartilage in the synovial fluid and blood (Block *et al.*, 1989; Kliner *et al.*, 1987; Poole *et al.*, 1990; Sweet *et al.*, 1988; Thonar *et al.*, 1985).

Recently, an enzyme-linked immunosorbent assay (ELISA) using an anti-human keratan sulphate antibody (1/20/5D4; Caterson *et al.*, 1983) has improved the measurement of low concentration of keratan sulphate (Thonar *et al.*, 1985). Measurement of serum keratan sulphate concentration has been done in several species, and its use as a diagnostic test also has been suggested (Alwan *et al.*, 1990; Leipold *et al.*, 1989; Mehraban *et al.*, 1991; Poole *et al.*, 1990; Okumura *et al.*, 1997; Okumura and Fujinaga, 1998; Todhunter *et al.*, 1993; Todhunter *et al.*, 1997).

1.3. Joint cartilage and biological markers in arthropathies

Measurement of the severity and extent of a joint damage is troublesome. In advanced inflammatory joint diseases, radiographs show cartilage loss and bone erosions at the joint. However, radiography is an insensitive technique that can provide only a historical record of the gross anatomical changes to a joint. Studies on a particular joint damage must be conducted over many years, during which the disease status may change radically. Much damage may be done early in the disease process, before the radiographic change, although it is uncertain which of the many aspects of synovitis are responsible for the joint damage. Sensitive ways of assessing the current status of cartilage and bone changes during the early stages of inflammatory joint disease would be of potential value to clinicians. As a result of considerable recent works on musculoskeletal tissues, particularly articular cartilage and bone, as well as the inflammatory process itself, better understanding could be given of the structure and metabolism of these tissues and how they are affected and changed by inflammation. Moreover, it has been possible to identify either specific molecules that are synthesized by only one connective tissue, such as cartilage or bone, or molecules that are predominantly produced and released from a single tissue. These molecules can be detected, mainly by immunoassay, in synovial fluid, serum and urine, in which their contents can be measured. Their presence is a product of the metabolism of the tissue from which they were derived: they may represent synthetic or degradative markers. Thus, it is now possible to examine body fluids for the presence of specific molecules and their degradation products. This information can be used to study and better understand the metabolism of the tissue from which they are

derived, and how the turnover of these tissue is influenced by the disease process and its therapy (Poole, 1994).

1.4. Aims of this study

The objective of this study was to establish definitive and quantitative assessment of the damage in joint by measurement of keratan sulphate, reacting to 2 types of monoclonal antibodies, in serum and synovial fluids for more accurate diagnosis and precise treatment in horses. The following experiments were therefore done in this study; (i) to establish a sensitive and specific monoclonal antibody to equine keratan sulphate and to develop an enzyme immunoassay for the measurement of its concentration in serum and synovial fluid from horses, (ii) to reproduce proteoglycan deposition and turnover of equine articular cartilage *in vitro* and to assess the use of keratan sulphate, reacting to 1/20/5D4 or newly established 1/14/16H9 antibody, as a cartilage catabolic or anabolic marker, and (iii) to determine keratan sulphate concentration in serum and synovial fluid from healthy horses, growing foals, and horses with joint problems and to evaluate clinically the role of keratan sulphate, reacting to 1/20/5D4 or newly established 1/14/16H9 antibody, as one of molecular markers to monitor cartilage metabolism in equine joint diseases.

2. Establishment of a Monoclonal Antibody (1/14/16H9) for Quantification of Equine Keratan Sulphate

2.1. Summary

To develop an enzyme immunoassay for measurement of the concentration of keratan sulphate in serum and synovial fluid from horses, a sensitive and specific monoclonal antibody to equine keratan sulphate, 1/14/16H9, was established.

BALB/c mice were immunized with chondroitinase-ABC-digested proteoglycan monomer from equine joint cartilage, and monoclonal antibodies were raised, using Sp2/0 cells as a fusion partner. A high-titer monoclonal antibody 1/14/16H9, which specifically recognizes the epitope on equine keratan sulphate, was identified. This antibody had no reactivity with chondroitin sulphate and core protein of proteoglycan monomers, hyaluronan, heparin, dermatan sulphate and heparan sulphate.

An inhibition enzyme-linked immunosorbent assay (ELISA) was optimized by using 1/14/16H9 antibody, and keratan sulphate concentration in 18 synovial fluids and 48 serum samples obtained from clinically normal horses and horses with arthritis was measured. Precision data were obtained over the range of 10 to 160 ng/ml. The within- and between-assay coefficients of variation of the assay were 10.0 and 12.7%, respectively.

A monoclonal antibody 1/14/16H9 that specifically recognized equine keratan sulphate was used for an enzyme immunoassay to measure the concentration of keratan sulphate in later chapter of this thesis.

2.2. Introduction

Articular cartilage has a high content of proteoglycans, which are important for normal function of the tissue. Proteoglycans consist of a protein core containing sulphated glycosaminoglycan side chains of chondroitin sulphate and keratan sulphate. These molecules, which are released as fragments into synovial fluid, blood and urine where they may be detected, could be used to diagnose osteoarthritis, monitor effects of treatment on cartilage turnover, and understand the catabolic and anabolic processes of articular cartilage metabolism (Alwan *et al.*, 1990; Caterson, 1991; Heinegard and Saxne, 1991; Okumura *et al.*, 1997; Palmer *et al.*, 1995; Thonar *et al.*, 1991).

Immunologic assays have been generally used to measure the concentration of fragments of the cartilage aggregating proteoglycan, aggrecan. Polyclonal aggrecan antibodies and monoclonal antibodies reacting with human keratan sulphate side chains have been used in assays for determination of aggrecan fragments in serum and synovial fluid (Heinegard *et al.*, 1985; Kongtawelert *et al.*, 1990; Thonar *et al.*, 1985), the most widely used being human keratan sulphate monoclonal antibody 1/20/5D4. Several investigators have reported measurement of the concentration of keratan sulphate in equine serum and synovial fluid, using this antibody (Alwan *et al.*, 1990; Okumura *et al.*, 1997; Todhunter *et al.*, 1997), however affinity of 1/20/5D4 antibody to keratan sulphate of horses is not well evaluated.

The objectives of this study were to establish a sensitive and specific monoclonal antibody to equine keratan sulphate, and to develop an enzyme immunoassay system for the measurement of the concentration of keratan sulphate in serum and synovial fluid from horses.

2.3. Materials and methods

2.3.1. Purification of equine cartilage proteoglycan monomers

Equine proteoglycan monomers were purified by use of a described method (Caterson *et al.*, 1983; Okumura *et al.*, 1997). Briefly, hyaline cartilage was collected from the synovial joints of a 1-year-old Thoroughbred foal which was euthanized because of a problem unrelated to the musculoskeletal system. The cartilage was cut into small pieces, and extracted with 4 mol/l (*M*) guanidine HCl-0.05*M* sodium acetate. The extracts were separated from the cartilage residue by centrifugation (15,000 × *g* for 30 minutes at 4 °C), and dialyzed against 0.05*M* sodium acetate, pH 6.5. Equine proteoglycan monomers were obtained after CsCl equilibrium density gradient centrifugation (194,000 × *g* at 10 °C) under 2 conditions at densities of 1.65 (40 hours) and 1.50 g/cm³ (48 hours), respectively, and dialyzed against 0.1*M* sodium chloride. Equine proteoglycan monomers were then frozen and dried.

The procedure of this experiment and handling of the animals were planned and done according to the guideline for animal experiments in Graduate School of Veterinary Medicine, Hokkaido University.

2.3.2. Enzymatically generated proteoglycan monomers

Equine proteoglycan monomer fragments were obtained by enzymatic digestion of proteoglycan monomers as follows. Chondroitinase-ABC-digested proteoglycan monomers were obtained by incubating proteoglycan monomers with chondroitinase ABC (Sigma Chemical Co., St Louis, Mo., U.S.A.; 0.4 mU/μg proteoglycan monomer)

in 50 mM Tris, 0.015 mM sodium chloride (pH 7.5) at 37 °C for 4 hours. Keratanase-digested proteoglycan monomers were obtained by incubating proteoglycan monomers with keratanase (Seikagaku-kogyo, Tokyo; 0.0057 U/ μ g proteoglycan monomer) in 50 mM Tris, 35 mM sodium acetate, 1.7 mM calcium acetate, 50 mM sodium chloride, 0.1 g of bovine serum albumin/l (pH 7.4) at 37 °C for 4 hours. Papain-digested proteoglycan monomers were obtained by incubating proteoglycan monomers with papain (Sigma Chemical Co.; 10 μ g/mg proteoglycan monomers) in a solution containing 0.2M sodium acetate, 5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM cysteine (pH 5.0) at 37 °C. After 4 hours, another 10 μ g of papain/mg of proteoglycan monomers was added and incubation continued for a total of 24 hours at 37 °C (Poole *et al.*, 1989).

2.3.3. Glycosaminoglycan chains and corneal keratan sulphate

Bovine kidney heparan sulphate (Seikagaku-kogyo), hog skin dermatan sulphate (chondroitin sulphate B; Seikagaku-kogyo), bovine corneal keratan sulphate (Seikagaku-kogyo), heparin (Upjohn Japan, Tokyo), and hyaluronan (Bayer AG, Leverkusen, Germany) were purchased.

2.3.4. Immunization and antibody production

Ten BALB/c mice were inoculated every 2 weeks for a total of 4 subcutaneous injections of chondroitinase-ABC-digested proteoglycan monomers (100 μ g/mouse /dose). The first injection was made with Freund's complete adjuvant; the 3 subsequent injections were done with incomplete adjuvant. One to 3 months after the last injection (3 days before fusion), 100 μ g of chondroitinase-ABC-digested proteoglycan monomers

was administered intraperitoneally as a booster injection. Fusion was carried out by use of a described method (Gefter *et al.*, 1977; Galfre and Milstein, 1981), with the myeloma cell line Sp2/O as a fusion partner. Using a direct enzyme-linked immunosorbent assay (ELISA) with chondroitinase ABC-digested proteoglycan monomer-coated wells, hybridoma supernatants were screened for proteoglycan monomers binding antibodies. The hybridoma cells were injected into primed BALB/c mice, where they grew as ascitic tumors. Ascitic fluids were harvested 1 to 3 weeks later, cells were then removed by centrifugation at $600 \times g$ for 10 minutes, and fluids were stored at $-20\text{ }^{\circ}\text{C}$ until used. Immunoglobulin class was determined by direct ELISA, using a mouse immunoglobulin typing kit (Mouse immunoglobulin typer kit, Bio-Rad, Hercules, CA, U.S.A.).

2.3.5. Quantification of synovial fluid and serum keratan sulphate concentration

An inhibition ELISA, established by Thonar *et al.* (1985), with some modifications (Okumura *et al.*, 1997), was used to measure keratan sulphate concentration in synovial fluid and serum from horses. One milligram of proteoglycan monomers was dissolved in $40\text{ }\mu\text{l}$ of buffer containing 0.2 U of chondroitinase-ABC, 0.1 M sodium acetate, and 0.1 M Tris, pH 7.3, followed by digestion for 2 hours at $37\text{ }^{\circ}\text{C}$. Just before coating the plates, the solution was diluted with 20 mM carbonate bicarbonate coating buffer, 0.02% sodium azide, pH 9.2, bringing the final concentration of proteoglycan monomers to $5\text{ }\mu\text{g/ml}$. Coating buffer containing chondroitinase-ABC-digested proteoglycan monomers ($200\text{ }\mu\text{l}$), prepared immediately prior to use, was added to each well of the ELISA plate (Nunc, Copenhagen, Denmark), which was then kept at $20\text{ to }22\text{ }^{\circ}\text{C}$ (room temperature) for 2 hours, then at $4\text{ }^{\circ}\text{C}$ until assayed.

Two hundred μl of monoclonal antibody 1/20/5D4 (Seikagaku-kogyo) or established monoclonal antibody 1/14/16H9 (ascites fluid) diluted to 1:5,000 and

1:25,000, respectively, in phosphate-buffered saline solution (PBS, pH 5.3) containing 0.05% Tween 20, 1% bovine serum albumin, and 0.05M EDTA was mixed with 200 μ l of test samples or the keratan sulphate standard diluted in PBS. These monoclonal antibody-keratan sulphate inhibition mixtures were incubated at room temperature for 1 hour, then were kept at 4 °C overnight. The mixtures were allowed to equilibrate to room temperature, and a standard ELISA was then performed.

Chondroitinase-ABC-digested proteoglycan monomer-coated wells were washed 3 times (5 minutes each) with PBS (pH 7.0) containing 0.05% Tween 20 to remove excess antigen, then were incubated for 60 minutes at room temperature with 200 μ l of the inhibition mixture (monoclonal antibody plus sample), prepared as described previously. After the incubation period, the wells were washed 3 times with PBS-0.05% Tween 20, after which 200 μ l of a peroxidase-conjugated rabbit anti-mouse IgG₁ (Zymed Laboratories, San Francisco, CA, U.S.A.) was added to the wells. This IgG₁ antibody was diluted 2,500-fold with PBS-0.05% Tween 20, 0.05M EDTA. After incubation for 30 to 60 minutes at room temperature, the unbound antibody was removed by washing the plates 5 times (5 minutes each) with PBS-0.05% Tween 20. Two hundred μ l of substrate, 0.015% H₂O₂ plus *o*-phenylenediamine (2 mg/ml), was added to each well, followed by incubation for 10 to 60 minutes. The reaction was stopped by addition of 50 μ l of 2M H₂SO₄. The plates were read at a wavelength of 490 nm, using an ELISA plate reader (Microplate reader MTP-120, Corona Electric, Katsuta, Ibaraki).

The concentration of keratan sulphate in the samples was calculated by comparing the absorbance value with values generated from known concentrations of chondroitinase-ABC-digested proteoglycan monomers treated in similar manner as sample plates. The keratan sulphate content in the chondroitinase-ABC-digested

proteoglycan monomers was measured, using a dimethylmethylen blue assay (Farndale *et al.*, 1982).

2.3.6. Preparation of sera and synovial fluid

Forty-eight serum and 18 synovial fluid samples were obtained from clinically normal horses and horses with arthritis at Hokkaido University and Shadai farm, Hokkaido.

2.3.7. Statistics

For analysis of correlation, Spearman's rank test was used.

2.4. Results

2.4.1. Characterization of monoclonal antibody 1/14/16H9

The BALB/c mice were inoculated with chondroitinase-ABC-digested proteoglycan monomers, and hybrids secreting proteoglycan monomer antibodies were identified by direct ELISA, using chondroitinase-ABC-digested proteoglycan monomer coated wells. Several antibodies were characterized for their epitope specificity, and most proved to be keratan sulphate antibodies. After 4 sequences of cloning, a monoclonal antibody clone 1/14/16H9 that was secreting high-titer antibodies to chondroitinase-ABC-digested proteoglycan monomers was obtained. The subclass of 1/14/16H9 antibody was IgG₁, with κ type light chains.

Reactivity of monoclonal antibody 1/14/16H9 with the enzyme-treated proteoglycan monomers was examined. Digestion of proteoglycan monomers with keratanase almost completely eliminated its reactivity (Figure 1). Removal of the chondroitin sulphate side chains by enzymatic treatment of proteoglycan monomers with chondroitinase-ABC resulted in almost identical inhibition to that of the native, not enzymatically digested proteoglycan monomers. Dissolution of the core protein with papain slightly decreased the inhibition.

Reactivity of monoclonal antibody 1/14/16H9 with glycosaminoglycans, including corneal keratan sulphate, dermatan sulphate, heparan sulphate, heparin, and hyaluronan, was also examined by use of an inhibition ELISA. Inhibition with bovine corneal keratan sulphate indicated that monoclonal antibody 1/14/16H9 obviously bound to the keratan sulphate from the cornea (Figure 2). Inhibition of binding of monoclonal antibody 1/14/16H9 was not found for sodium hyaluronan, sodium heparin, dermatan sulphate

and heparan sulphate (Figure 3).

2.4.2 Optimization of the conditions of ELISA

The conditions of an inhibition ELISA for determination of keratan sulphate concentration from cartilage was optimized. In the assay, proteoglycan monomers in solution (the analyte) and chondroitinase-ABC-digested proteoglycan monomers were coated on wells of the ELISA microtitration plate for binding of monoclonal antibody (primary antibody) and binding was detected by addition of a peroxidase-conjugated antibody (second antibody).

Sensitivity of the assay could be substantially improved by increasing the dilution of the primary antibody. The standard curve was displaced to a lower measuring range by decreasing the concentration of the primary antibody (Figure 4). Further dilution resulted in too low an absorbance and decreased precision.

Optimal dilution of the secondary antibody also was determined. The standard curve was displaced to a lower measuring range by decreasing the concentration of the secondary antibody (Figure 5). Further dilution resulted in too low an absorbance and decreased precision. The standard data was not affected by the properties of serum or synovial fluid.

Assay precision was estimated by testing a serum and a synovial fluid sample 10 times each in 1 assay and in 6 consecutive assays. The within- and between-assay coefficients of variation were 10.0 and 12.7%, respectively (Table 1). Precision data was obtained over the range of 10.0 to 160 ng/ml (Figure 6).

2.4.3. Comparison of keratan sulphate concentration in serum and synovial fluid, using monoclonal antibodies 1/14/16H9 and 1/20/5D4

Concentration of keratan sulphate was measured in 48 serum and 18 synovial fluid samples by use of an inhibition ELISA, monoclonal antibodies 1/14/16H9 and 1/20/5D4. In serum at the concentration of < 150 ng/ml, lower concentrations were detected by monoclonal antibody 1/14/16H9 than by monoclonal antibody 1/20/5D4 (Figure 7). Conversely, synovial fluid contained higher concentration of keratan sulphate that was immunoreactive with monoclonal antibody 1/14/16H9 than with monoclonal antibody 1/20/5D4 (Figure 8). For measurements of keratan sulphate concentration in serum and synovial fluid samples, correlation between the 2 monoclonal antibodies was obtained ($r = 0.857$ and 0.871 , respectively).

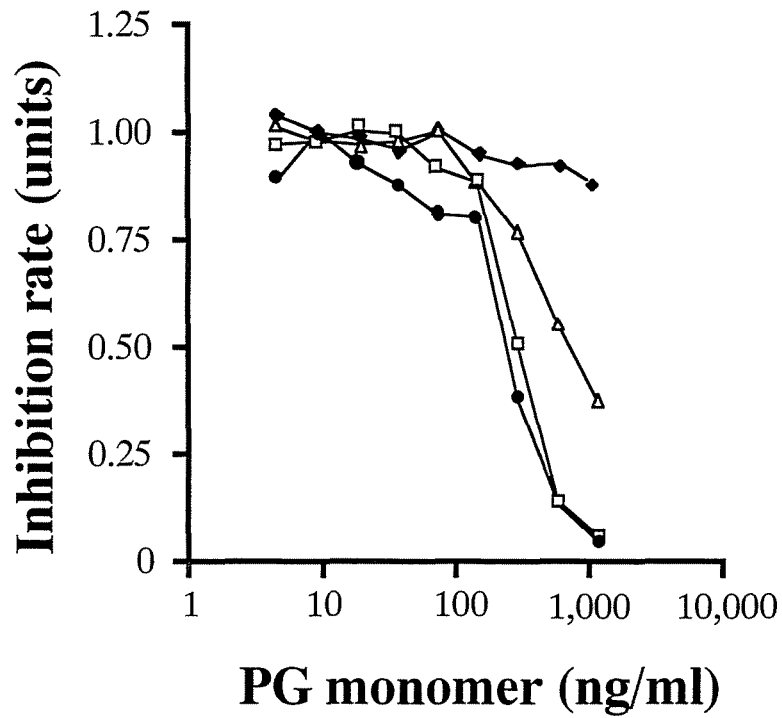


Figure 1-Reactivity of monoclonal antibody 1/14/16H9 with enzyme-treated proteoglycan (PG) monomer fragments. (Okumura and Fujinaga, 1998)

- △— Papain-digested PG monomer (keratan sulfate +)
- Non-treated PG monomer (keratan sulfate +)
- Chondroitinase ABC-digested PG monomer (keratan sulfate +)
- ◆— Keratanase-digested PG monomer (keratan sulfate -)

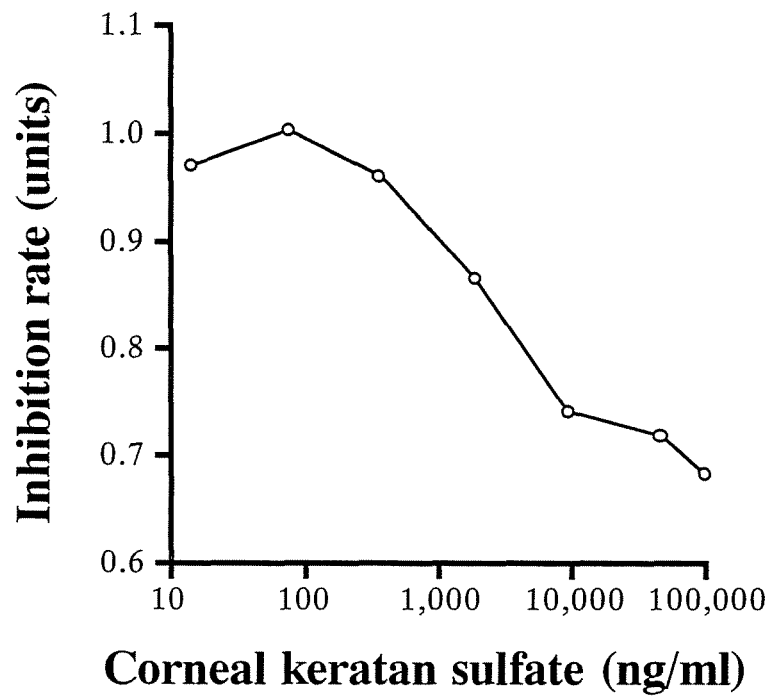


Figure 2-Reactivity of monoclonal antibody 1/14/16H9 with bovine corneal keratan sulfate.
(Okumura and Fujinaga, 1998)

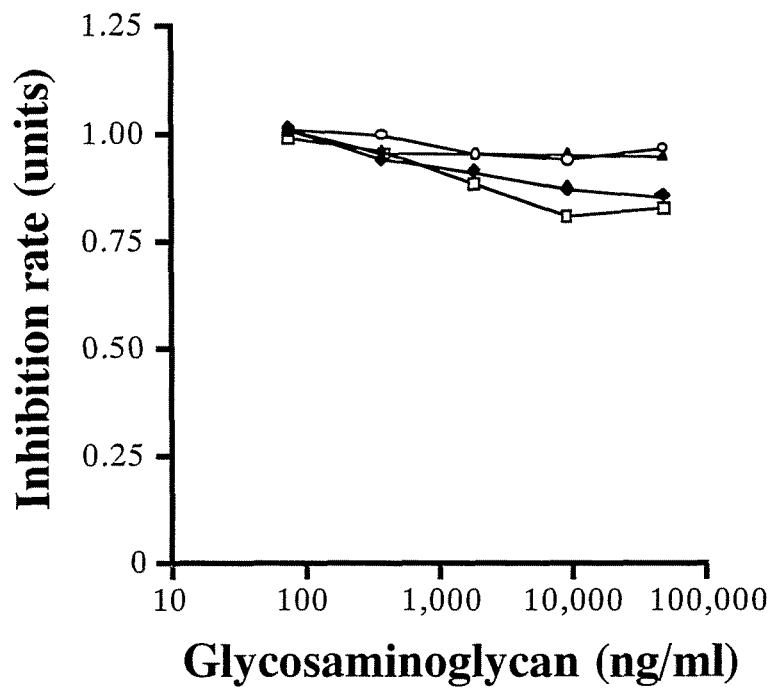


Figure 3-Reactivity of monoclonal antibody 1/14/16H9 with glycosaminoglycans. (Okumura and Fujinaga, 1998)

- Dermatan sulfate
- ◆— Heparan sulfate
- Hyaluronic acid
- ▲— Heparin

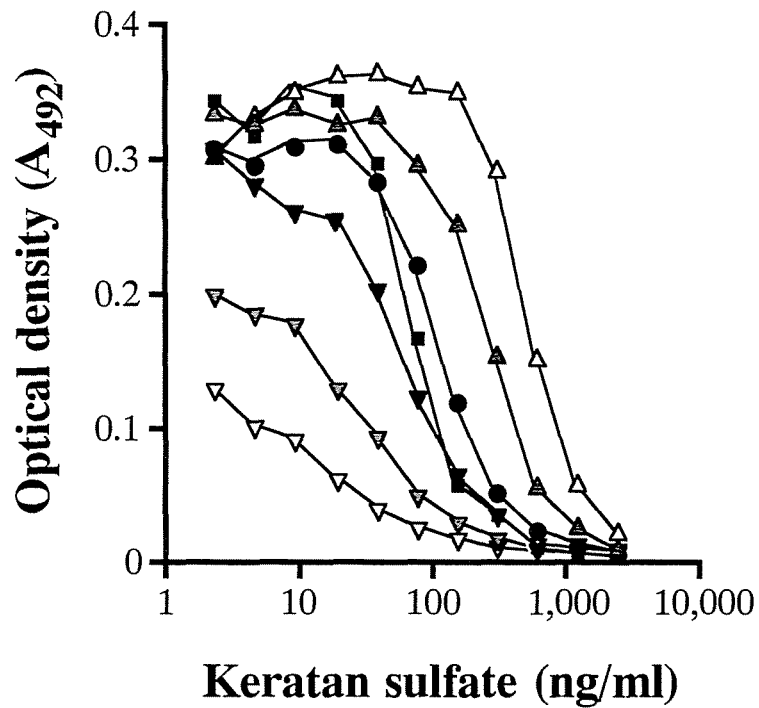


Figure 4-Effect of dilution of monoclonal antibody 1/14/16H9 as the primary antibody on a standard curve. (Okumura and Fujinaga, 1998)

- △— 1 : 6,400 (1/14/16H9)
- ▲— 1 : 12,800 (1/14/16H9)
- 1 : 25,600 (1/14/16H9)
- ▼— 1 : 51,200 (1/14/16H9)
- ▽— 1 : 102,400 (1/14/16H9)
- ▽— 1 : 204,800 (1/14/16H9)
- 1 : 5,000 (1/20/5D4)

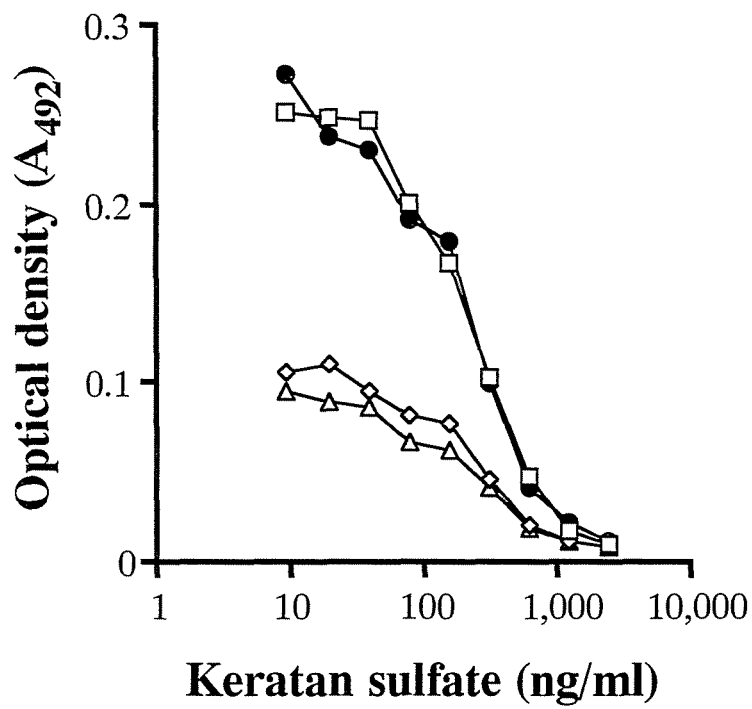


Figure 5-Effect of dilution of the secondary antibody on a standard curve.
 First antibody: 1/14/16H9.
 (Okumura and Fujinaga, 1998)

- 1 : 2,000
- 1 : 2,500
- ◇— 1 : 5,000
- △— 1 : 7,500

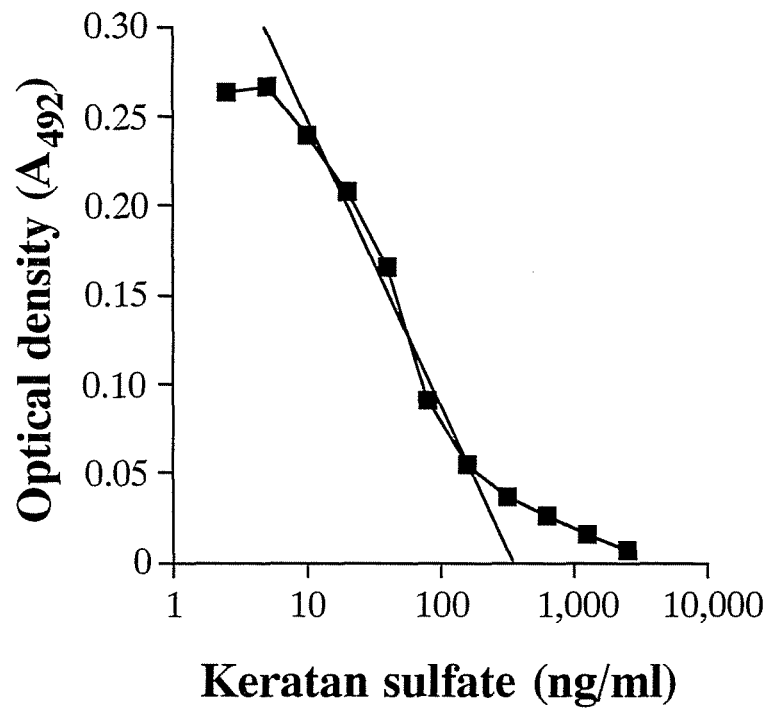


Figure 6-Standard curve for quantification of keratan sulfate.
First antibody: 1/14/16H9.
(Okumura and Fujinaga, 1998)

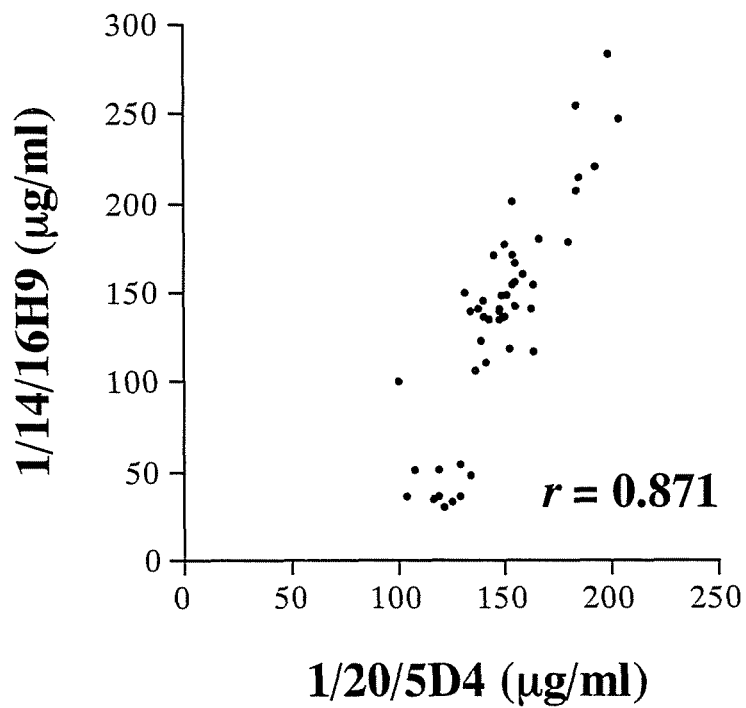


Figure 7-Comparison of keratan sulfate concentration in serum, using monoclonal antibodies 1/14/16H9 and 1/20/5D4. (Okumura and Fujinaga, 1998)

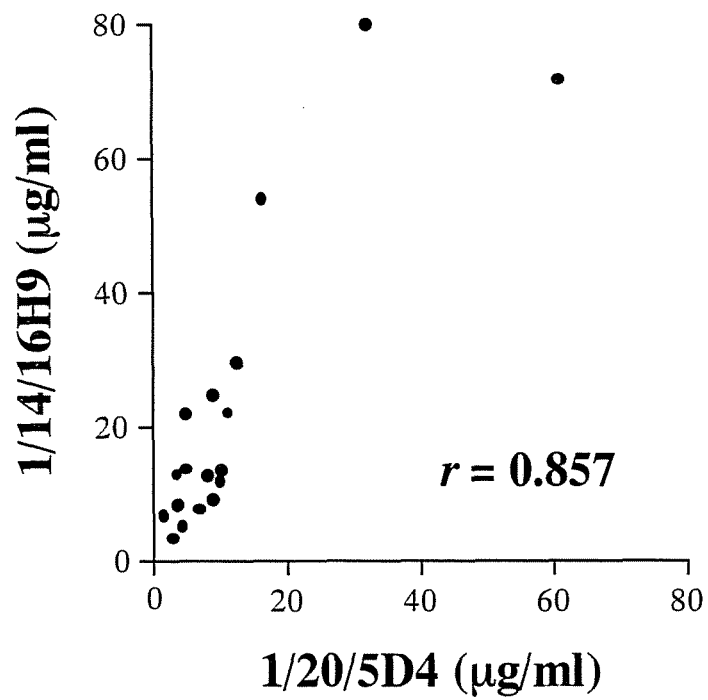


Figure 8-Comparison of keratan sulfate concentration in synovial fluid, using monoclonal antibody 1/14/16H9 and 1/20/5D4. (Okumura and Fujinaga, 1998)

Table 1. Precision of the keratan sulfate ELISA
using 1/14/16H9 antibody

Parameter	n	Mean (ng/ml)	SD (ng/ml)	CV (%)
Within-assay	12	199.8	19.6	10.0
Between-assay	12	199.3	18.6	12.7

n = number of examinations; S.D. = standard deviation
C.V. = coefficient of variation
(Okumura and Fujinaga, 1998)

2.5. Discussion

Determination of proteoglycan monomer fragments in serum and synovial fluids has been used to monitor normal and abnormal catabolism of cartilage (Campion *et al.*, 1991; Cruz *et al.*, 1989; Kliner *et al.*, 1987; Mehraban *et al.*, 1991; Okumura *et al.*, 1997; Saxne *et al.*, 1993; Sweet *et al.*, 1988; Todhunter *et al.*, 1997), as well as to evaluate the response to clinical intervention (Carroll *et al.*, 1992; Kongtawelert *et al.*, 1989; Saxne *et al.*, 1986). In horses with osteoarthritis, concentration of keratan sulphate in serum and synovial fluid is reported to be significantly higher than in clinically normal horses (Alwan *et al.*, 1990). In ponies with experimentally induced articular cartilage injury, keratan sulphate concentrations in serum and synovial fluids were found to increase (Todhunter *et al.*, 1993). The increases associated with acute generalized cartilage degradation (*eg*, intra-articular injection of chymopapain) were greater than those associated with osteochondral defects (Todhunter *et al.*, 1993). Other studies suggested that the concentrations of keratan sulphate in serum and synovial fluids from horses were affected by various joint diseases (Okumura *et al.*, 1997; Palmer *et al.*, 1995; Todhunter *et al.*, 1997). For this purpose keratan sulphate antibodies, notably the monoclonal antibody clone 1/20/5D4 that recognized human cartilage keratan sulphate, have been widely used (Caterson *et al.*, 1983).

The clone that secreted high-titer monoclonal antibody 1/14/16H9 was selected from some hybridomas that reacted with chondroitinase-ABC-digested equine proteoglycan monomers. Treatment of proteoglycan monomers with keratanase, chondroitinase-ABC and papain resulted in generation of glycosaminoglycans and a core protein. Native proteoglycan monomers have 2 types of glycosaminoglycan chains,

chondroitin sulphate and keratan sulphate, and a core protein. The epitope on equine proteoglycan monomers was susceptible to keratanase, but the antibody reacted strongly with chondroitinase-ABC-digested and nondigested equine proteoglycan monomers, and considerably with papain-digested equine proteoglycan monomers. This indicates that the antibody recognizes an epitope residing in the keratan sulphate region.

Reactivity of this antibody with corneal keratan sulphate or other glycosaminoglycans was examined. Inhibition of monoclonal antibody 1/14/16H9 by bovine corneal keratan sulphate was obvious, but inhibition was not detected for sodium hyaluronan, sodium heparin, dermatan sulphate and heparan sulphate. Corneal keratan sulphate has structure identical to that of keratan sulphate in cartilage (Caterson, 1991). Other glycosaminoglycan chains of proteoglycans tend to have the same linear disaccharide repeat sequences (Caterson, 1991). The antibody had appreciable reactivity with bovine corneal keratan sulphate, and less with dermatan sulphate, heparan sulphate, heparin and hyaluronan. It is, therefore, evident that the only glycosaminoglycan recognized by the antibody is keratan sulphate.

Effort was made to optimize an inhibition ELISA for determination of keratan sulphate of equine proteoglycan monomers, using the monoclonal antibody clone 1/14/16H9. This produced a stable, sensitive assay, which made detection of keratan sulphate in synovial fluid and serum possible, with an acceptable intra- and inter-assay precision. The sensitivity of the assay could be greatly improved by increasing dilution of the primary antibody.

Mean concentration of keratan sulphate measured by monoclonal antibody 1/14/16H9 in synovial fluid was approximately 170 times higher than the mean concentration in serum. Concentration measured by using monoclonal antibody 1/14/16H9 was not exactly the same value by monoclonal antibody 1/20/5D4 in synovial

fluid and serum. The concentration of the 1/14/16H9 epitopes was, however, directly proportional to that of 1/20/5D4 epitope in serum and synovial fluid, and correlation was found between the 2 antibody epitopes, 1/14/16H9 and 1/20/5D4, in serum and synovial fluid. It is also suggested that monoclonal antibody 1/14/16H9 recognizes an epitope on equine keratan sulphate.

Removal of the chondroitin sulphate side chains by enzymatic treatment of proteoglycan monomers with chondroitinase-ABC did not affect reactivity of the native proteoglycan monomers. This suggested that the affinity of monoclonal antibody 1/14/16H9 was not susceptible to degradation of the structure of proteoglycan monomers, and that the assay using monoclonal antibody 1/14/16H9 is relatively stable.

Furthermore, the within- and between-assay coefficient of variation for monoclonal antibody 1/14/16H9 were preferable (10.0 and 12.7%, respectively). These scores should be satisfactory not only for research purposes but also for clinical use (Kongtawelert *et al.*, 1989). Although it would be more useful that this antibody may be applied for more stable immunoassay, such as a sandwich ELISA, even the inhibition ELISA using this monoclonal antibody would be valuable for multiparameter clinicobiological testing.

In this chapter, a sensitive and specific monoclonal antibody, 1/14/16H9, that specifically detected equine keratan sulphate was established, and used for development of an enzyme immunoassay system for measurement of the concentration of keratan sulphate in serum and synovial fluids from horses. It is expected that this assay system will contribute to evaluation of cartilage metabolic activity in horses.

3. The Usefulness of the Measurement of Keratan Sulphate Reacting to 1/14/16H9 and 1/20/5D4 Antibodies to Monitor Cartilage Metabolism in *in vitro* Pellet-Cultured Equine Articular Chondrocytes

3.1. Summary

The usefulness of the measurement of keratan sulphate, reacting to newly established 1/14/16H9 and 1/20/5D4 antibodies, as a marker of cartilage metabolism was evaluated by using an *in vitro* model of equine articular cartilage.

Articular cartilage was harvested from 3 clinically healthy 6 month-old Thoroughbred foals. Articular chondrocytes were centrifuged and cultured as pellets in centrifuge tubes. Chondrocytes were incubated with insulin-like growth factor (IGF)-I α , transforming growth factor (TGF)- β 1 or interleukin (IL)-1 α for 2 weeks. Before incubation with IL-1 α , chondrocytes were pre-incubated for 1 week with 10% fetal bovine serum. The keratan sulphate concentrations and sulfated glycosaminoglycans (GAG) in pellets or media were measured by an inhibition ELISA and a 1,9-dimethyl-methylene blue colorimetric assay, respectively.

Synthesis of GAG was significantly increased by IGF-I α and TGF- β 1. Deposition of GAG in pellets was significantly increased by IGF-I α , but not by TGF- β 1.

The GAG and keratan sulphate concentrations in the culture media, which were stimulated by IGF-I α or IL-1 α , were also measured by an inhibition ELISA using 1/14/16H9 or 1/20/5D4 antibody. Keratan sulphate concentrations were significantly increased in those stimulated by IL-1 α , while no significant change was found in those

stimulated by IGF-I α . A high correlation between keratan sulphate concentrations and GAG was found in the media of pellets stimulated by IL-1 α ($r = 0.87$ and 0.84 ; GAG to 1/14/16H9 epitope and to 1/20/5D4 epitope, respectively), but lower in those stimulated by IGF-I α ($r = 0.43$ and 0.59 ; GAG to 1/14/16H9 epitope and to 1/20/5D4 epitope, respectively).

The results suggest that the *in vitro* concentration of keratan sulphate reacting to 1/14/16H9 mirrors the GAG concentration during the stage of cartilage catabolism but not during the cartilage anabolic stage. On the other hands, the results also suggest that the *in vitro* concentration of keratan sulphate reacting to 1/20/5D4 mirrors GAG concentration during the stage of cartilage catabolism and slightly during cartilage anabolism. These two differently reactive keratan sulphate concentrations in serum and synovial fluid could contribute as a marker to understand principally the catabolic process of articular cartilage metabolism and also represent in part some aspects of the cartilage anabolic process.

3.2. Introduction

The cartilage matrix has a high content of proteoglycans and collagens, which are important for normal function of cartilage, and is synthesized and organized by the chondrocytes. The chondrocyte is itself subject to regulation by various cytokines and growth factors that can shift the homeostatic balance in an anabolic or catabolic direction (Tippel, 1995). Insulin-like growth factor (IGF)-I and transforming growth factor (TGF)- β are thought to have a major influence on cartilage matrix synthesis (Trippel, 1995). Interleukin (IL)-1 induces increased synthesis of various matrix metalloproteinases by chondrocytes (Hembry *et al.*, 1994; Mort *et al.*, 1993).

A high-density pellet culture system was originally established for the study of growth plate chondrocyte hypertrophy (Kato *et al.*, 1988). Intimate interaction between chondrocytes and the extracellular matrix in a pellet mass is believed to stabilize the ability of chondrocytes to deposit cartilaginous matrix and induce mineralization (Kato *et al.*, 1988). This system has also been applied to cultures of chondrocytes from bovine nasal and articular cartilage (Xu *et al.*, 1996).

In Chapter 2 of this thesis, an anti-equine keratan sulphate antibody 1/14/16H9 was established, and serum and synovial fluid keratan sulphate concentration was measured by an ELISA using this antibody (Okumura and Fujinaga, 1998). It is however unclear whether or not keratan sulphate can be used as a marker for cartilage anabolism and/or catabolism.

The objectives of this chapter were to measure antigenic keratan sulphate concentration, using newly established 1/14/16H9 (Okumura and Fujinaga, 1998) or 1/20/5D4 (Caterson *et al.*, 1983) antibody, in the media of cultured equine articular chondrocytes in high-density pellets, the novel culture system of articular chondrocytes from horses, under anabolic or catabolic condition, and to evaluate whether keratan sulphate concentration can be used as an indicator to monitor cartilage metabolism in the biological fluid.

3.3. Materials and methods

3.3.1. Isolation and culture of chondrocytes

Articular cartilage was obtained from the femoropatellar joint of 6 month-old Thoroughbred foals (n=3) immediately after euthanasia, which was performed for a reason except musculoskeletal abnormalities. Full-thickness articular cartilage was aseptically harvested from the femoropatellar joint. The cartilage was then washed three times for 15 minutes in sterile PBS (pH7.2) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50 mg/ml gentamicin. Chondrocytes were released from freshly dissected articular cartilage specimens by sequential enzymatic digestion at 37 °C on a rotator with 2.5 mg/ml trypsin (Trypsin 1:250, Difco Lab., Detroit, MI, U.S.A.) for 30 minutes, and 2.5 mg/ml collagenase (Wako Pure Chemical Industry Ltd., Osaka) for 15 hours. The viability of the isolated cells was estimated with trypan blue exclusion test and was always above 92%. The isolated chondrocytes were resuspended at a density of 5×10^5 cells/ml in Dulbecco's Modified Eagle Medium (DMEM). In all experiments the medium contained 50 µg/ml ascorbic acid, 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin throughout the culture period. In some experiments, the DMEM contained 10% fetal bovine serum (FBS).

To prepare high-density pellet cultures, 1-ml aliquots of the cell suspension (5×10^5 cells) were transferred into 15-ml polypropylene centrifuge tubes. The cells were centrifuged at $200 \times g$ for 5 minutes. The resulting cell pellets were cultured in the same centrifuge tubes with loosened lids at 37 °C in a humidified atmosphere of 5 % CO₂/95% air. The medium was changed every 3 to 4 days and chondrocyte pellets were maintained for up to 2 weeks. Culture media were stored at the time of the medium replacement and at the end of culture, and stored at -20°C.

3.3.2. Supplements and culture procedures

In the study on extracellular matrix synthesis, recombinant human (rh) IGF-I α (Sigma Chemical Co.) or rhTGF- β 1 (Sigma Chemical Co.) was added in the medium for cultures grown for 2 weeks. In studies of extracellular matrix degradation, chondrocyte pellets were incubated in DMEM containing 10% FBS for 1 week, then supplemented with rhIL-1 α (Sigma Chemical Co.) for another 2 weeks. In all these experiments, IGF-I α or IL-1 α was added at each time the medium was changed.

3.3.3. Purification of equine cartilage proteoglycan monomers

Equine proteoglycan monomers were purified similarly as previously described in chapter 2, Section 2.3.1.

3.3.4. Measurement of sulfated glycosaminoglycan (GAG) concentration in the culture media

The proteoglycan released in the culture media was evaluated by assaying GAG concentration colorimetrically using dimethylmethylene blue (DMMB) (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) (Farndale *et al.*, 1982). The concentration of GAG in the samples were calculated by comparing the absorbance value with values generated from known concentrations of bovine corneal keratan sulphate (Seikagaku-kogyo) in similar manner as sample plates.

3.3.5. Measurement of keratan sulphate concentration in the culture media

An inhibition ELISA, established by Okumura and Fujinaga (1998), was used to measure keratan sulphate concentration in the culture medias described in Chapter 2, Section 2.3.5.

3.3.6. Statistical analysis

Comparisons between groups were made by the two-tailed Mann-Whitney U test with $P < 0.05$ taken as significant. The correlation between total GAG and keratan sulphate concentrations was evaluated by Spearman's correlation coefficient.

3.4. Results

3.4.1. Proteoglycan concentration in the extracellular matrix of articular chondrocyte pellets cultured with growth factors

Concentration of proteoglycans as extracellular matrix of articular chondrocytes could be detected in the chondrocyte pellets (Figure 9). Concentration of proteoglycans was significantly increased when the chondrocyte pellets were cultured with FBS (1,5 and 10%), or with IGF-I α (1.0 and 10.0 ng/ml). Transforming growth factor- β 1 did not significantly influence proteoglycan concentration in the matrix.

3.4.2. Proteoglycan concentration in the media of articular chondrocyte pellets cultured with growth factors

Changes in the concentration of proteoglycans from chondrocyte pellets in response to growth factors were detected by assaying the GAG in pellets and media (Table 2) and the proportion of GAG concentration in the tissue culture medium (Table 3) during the culture period. Both IGF-I α and TGF- β 1 significantly increased the concentration of GAG compared with that of control cultures, though the effect of IGF-I α to chondrocyte pellets was greater and more clearly dose-dependent than that of TGF- β 1 (Table 2). Only a small proportion of total GAG (both in-pellet and in-medium) was incorporated into the extracellular matrix during a 14-day period in control cultures; approximately 76% of total GAG was in the tissue culture medium (Table 3). This proportion was not affected significantly by IGF-I α , however TGF- β 1, at 1.0 and 10.0 ng/ml, significantly increased that of GAG in the medium (Table 3).

3.4.3. The change of GAG concentration in the extracellular matrix of articular chondrocyte pellets cultured with IL-1 α

Articular chondrocytes were cultured for 1 week with 10%FBS in DMEM, and 2 weeks further in the same medium with 1.0, 5.0, 10.0 and 20.0 ng/ml of IL-1 α . The cell pellets were then harvested for the assay of GAG. There was a significant decrease in the GAG concentration of articular chondrocyte pellets treated with 20.0 ng/ml of IL-1 α compared with that of the control pellets (Figure 10).

3.4.4. The change of keratan sulphate concentration, reacting to 1/14/16H9 and 1/20/5D4 antibodies, in the media of the pellets cultured with IGF-I α and IL-1 α

Changes in the concentration of keratan sulphate reacting to either 1/14/16H9 or 1/20/5D4 antibody in response to IGF-I α and IL-1 α were evaluated by measuring the keratan sulphate in the culture media using an inhibition ELISA. The concentration of keratan sulphate reacting to either 1/14/16H9 or 1/20/5D4 antibody in the media of pellets cultured with IL-1 α was significantly increased, compared with those of control cultures (Tables 4 & 5). Significant change of keratan sulphate concentration was not found in the media of pellets stimulated by IGF-I α , compared with those of control cultures (Tables 6 & 7).

3.4.5. Correlation between proteoglycans and keratan sulphate concentrations in the media of equine chondrocytes pellets

Changes in the proteoglycan concentration in response to IGF-I α and IL-1 α were examined by assaying both GAG and keratan sulphate concentrations in the media of chondrocyte pellets during the entire culture period, and then the comparison between

these two parameters was done. A high correlation between GAG and keratan sulphate concentrations was found in the media of pellets cultured with IL-1 α ($r = 0.87$ or 0.84 , 1/14/16H9 or 1/20/5D4, respectively; Figure 11), while a low value was detected in the group of IGF-I α ($r = 0.43$ or 0.59 , 1/14/16H9 or 1/20/5D4, respectively; Figure 12).

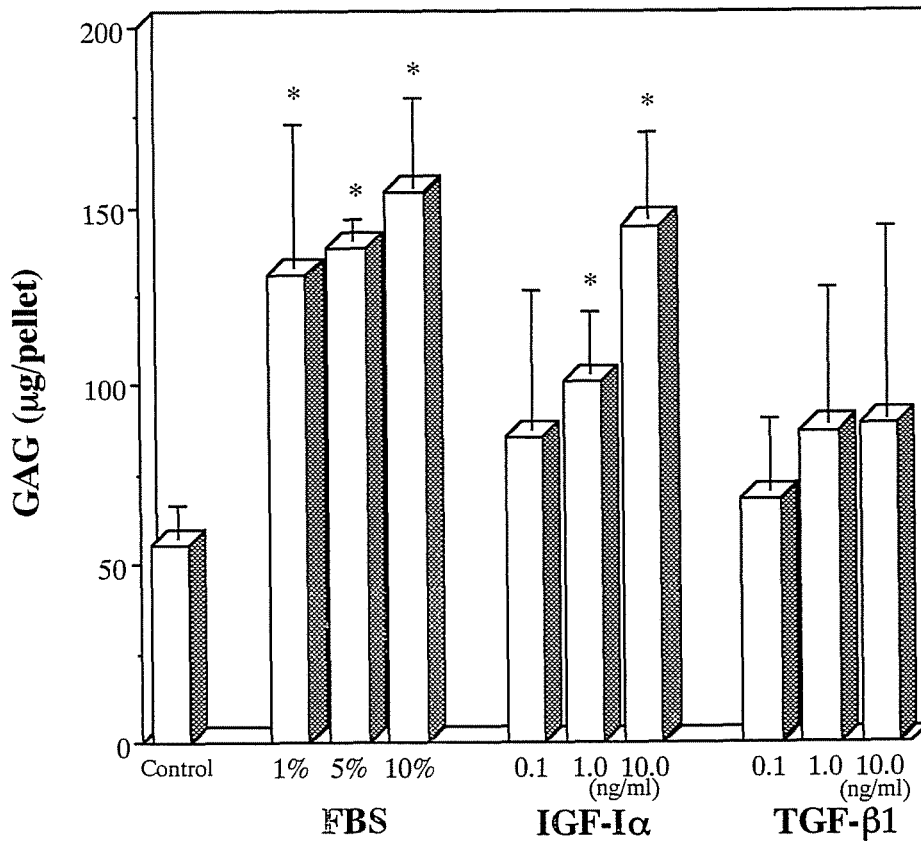


Figure 9- Contents of sulphated glycosaminoglycans (GAG) in the extracellular matrix of equine articular chondrocyte pellets. Pellets were incubated for 2 weeks in Dulbecco's modified Eagle medium (DMEM) without supplement (Control), with fetal bovine serum (FBS) or insulin-like growth factor (IGF)-I α or transforming growth factor (TGF)- β 1. Each bar is the mean \pm standard deviation (SD). n=12. *: P<0.05 compared with control cultures. (modified from Okumura *et al.*, 2000)

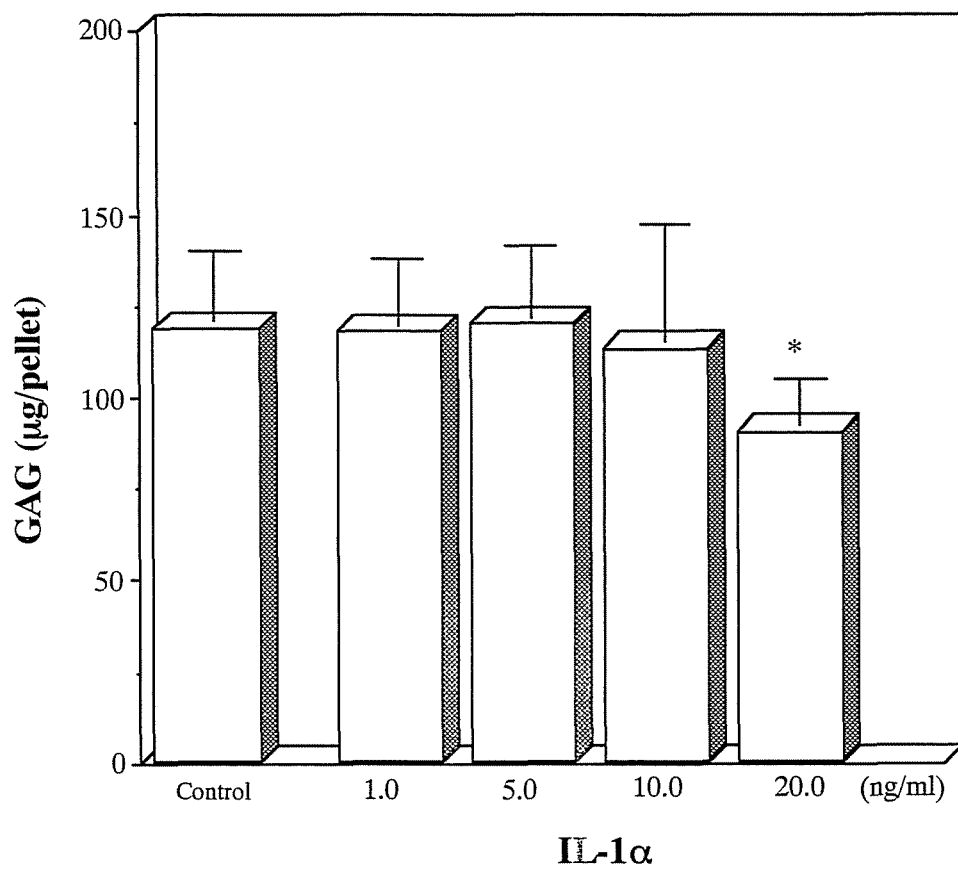


Figure 10- Changes of proteoglycan content in the extracellular matrix of equine articular chondrocyte pellets by interleukin (IL)-1 α supplement.
 Each bar is the mean \pm SD. n=12.
 *: P<0.05 compared with control cultures.
 (modified from Okumura *et al.*, 2000)

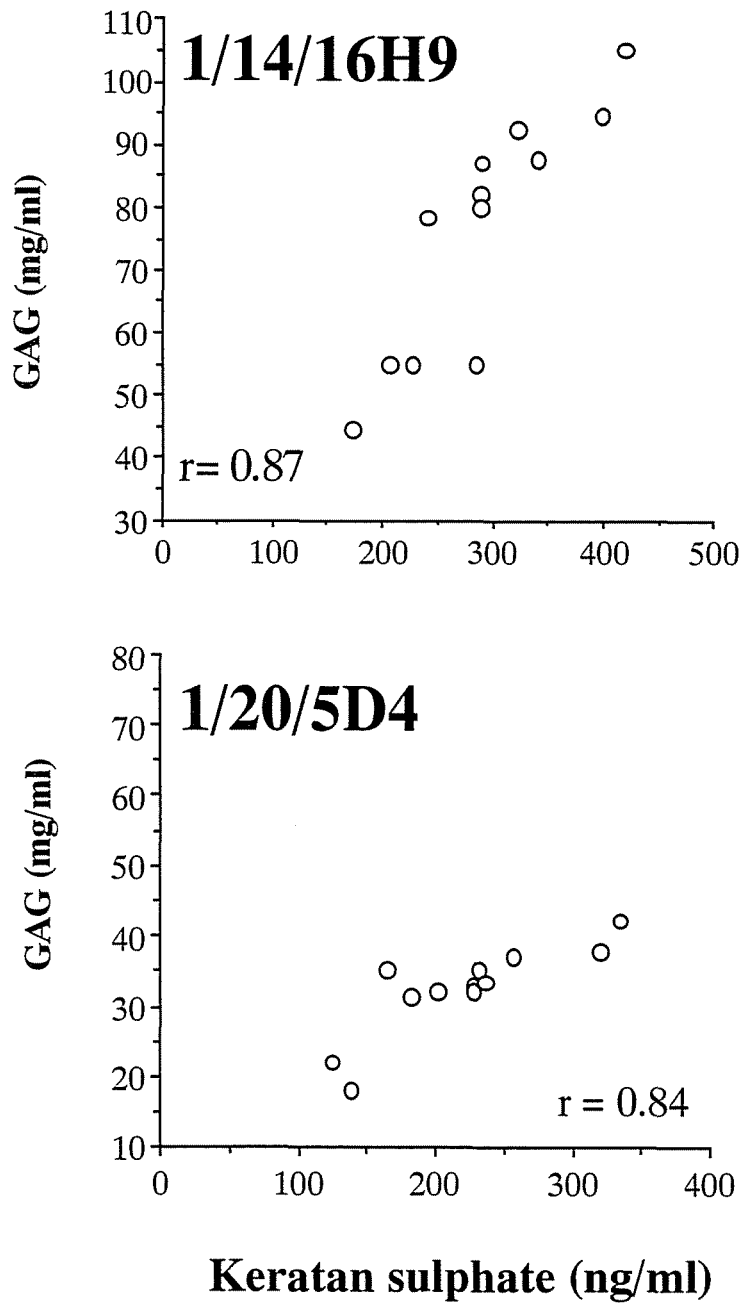


Figure 11- Comparison of keratan sulphate reacting to 1/14/16H9 or 1/20/5D4 antibody and GAG concentrations in the medium of equine articular chondrocyte pellets cultured with IL-1 α . (modified from Okumura *et al.*, 2000 & Okumura and Fujinaga, 2000)

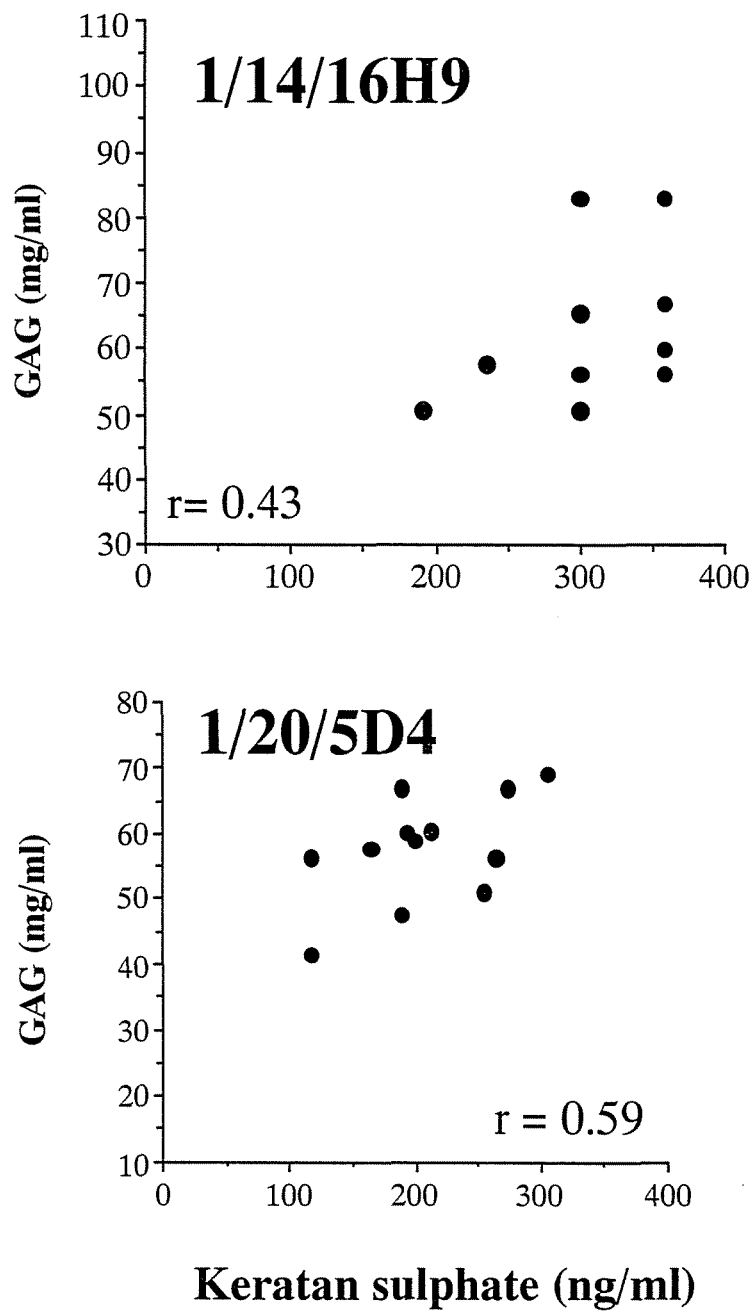


Figure 12- Comparison of keratan sulphate reacting to 1/14/16H9 or 1/20/5D4 and GAG concentrations in the medium of equine articular chondrocyte pellets cultured with IGF-I α .
(Okumura *et al.*, 2000 & Okumura and Fujinaga, 2000)

Table 2. GAG content in pellets and media cultured with growth factors

Growth factor concentration (ng/ml)	GAG content in 2 weeks ($\mu\text{g/pellet}$)			
	IGF-I α		TGF- β 1	
	Mean	SD	Mean	SD
0.0(Control)	241	59	241	59
0.1	377*	77	323	80
1.0	391*	27	386*	40
10.0	526*	64	443*	59

Each value was calculated with 12 samples.

*P<0.05 compared with Control

(modified from Okumura *et al.*, 2000)

Table 3. Percentage of GAG content in media of pellets

Growth factor concentration (ng/ml)	GAG content in media (% of total)			
	IGF-I α		TGF- β 1	
	Mean	SD	Mean	SD
0.0(Control)	76.2	5.9	76.2	5.9
0.1	78.3	6.8	81.3	4.7
1.0	74.3	2.9	85.3*	5.0
10.0	72.5	2.5	86.7*	4.4

Each value was calculated with 12 samples.

*P<0.05 compared with Control

(modified from Okumura et al., 2000)

Table 4. Concentrations of GAG and keratan sulphate (KS) reacting to 1/14/16H9 in the media of pellets cultured with IGF-I

IGF-I (ng/ml)	GAG ($\mu\text{g/ml}$)		KS (ng/ml)	
	Mean	SD	Mean	SD
0.0 (Control)	37.1	3.4	229	19
0.1	60.2*	8.1	240	18
1.0	58.5*	2.0	243	11
10.0	74.5*	9.7	214	9

Each value was calculated with 12 samples .

*P<0.05 compared with Control.

(modified from Okumura *et al.*, 2000)

Table 5. Concentrations of GAG and KS reacting to 1/20/5D4 in the media of pellets cultured with IGF-I

IGF-I (ng/ml)	GAG ($\mu\text{g/ml}$)		KS (ng/ml)	
	Mean	SD	Mean	SD
0.0 (Control)	38.0	10.5	182	77
0.1	51.4	10.8	228	73
1.0	58.5*	2.0	257	188
10.0	74.7*	9.7	249	85

Each value was calculated with 12 samples.

*P<0.05 compared with Control.

(modified from Okumura *et al.*, 2000)

Table 6. Concentrations of GAG and KS reacting to 1/14/16H9 in the media of pellets cultured with IL-1

IL-1 (ng/ml)	GAG ($\mu\text{g/ml}$)		KS (ng/ml)	
	Mean	SD	Mean	SD
0.0 (Control)	65.6	11.2	213	64
1.0	68.9	13.4	234	70
5.0	65.2	19.7	241	75
10.0	72.0	15.5	288*	60
20.0	85.1*	18.6	342*	58

Each value was calculated with 12 samples .

*P<0.05 compared with Control.

(modified from Okumura *et al.*, 2000)

Table 7. Concentrations of GAG and KS reacting to 1/20/5D4 in the media of pellets cultured with IL-1

IL-1 (ng/ml)	GAG ($\mu\text{g/ml}$)		KS (ng/ml)	
	Mean	SD	Mean	SD
0.0 (Control)	29.0	3.0	580	60
1.0	29.2	5.6	584	111
5.0	28.7	8.8	575*	177
10.0	33.7*	1.3	675*	27
20.0	39.4*	3.0	789*	61

Each value was calculated with 12 samples .

*P<0.05 compared with Control.

(modified from Okumura *et al.*, 2000)

3.5. Discussion

Equine articular chondrocytes from 6 month-old foals were cultured as pellets and synthesized a cartilage-like matrix that contained abundant proteoglycans. This *in vitro* deposition of extracellular matrix of articular cartilage was demonstrated by the quantitative assay of GAG. The matrix accumulated externally around the chondrocytes, unlike in monolayer cultures of chondrocytes where there is an intracellular accumulation of the matrix (Frazer et al., 1994). The pellet-cultured equine chondrocytes maintained good viability and showed no tendency to change phenotypic appearance towards dedifferentiation. This indicates that the pellet-cultured equine articular chondrocytes maintained their own biological properties *in vitro*.

Formation of the organized extracellular matrix occurred in the absence of growth factors, but the rate of matrix deposition was significantly increased by 1, 5 and 10% FBS and 1.0 and 10.0 ng/ml IGF-I α . The effect of IGF-I on cultured cartilage was reported to be a forcefull stimulator of proteoglycan synthesis by chondrocytes (Fosang et al., 1991; Luyten et al., 1988; McQuillan et al., 1986; Schalkwijk et al., 1989; Tyler, 1989), and it may also include a chondroprotective effect in IL-1-conditioned explants (Frisbie and Nixon, 1997), by possibly inhibiting the synthesis of proteolytic enzymes (Tyler et al., 1989; Fosang et al., 1991). The results in this study confirm previous studies that documented the ability of IGF-I on chondrocyte-mediated matrix metabolism in the pellet-cultures. Rogachefsky et al. (1993) demonstrated similar effects of IGF-I to chondrocytes *in vivo*, when combined with anti-inflammatory agents, and furthermore the role of IGF-I in cartilage repair. It was also reported that the *in vivo* anabolic effects of joint loading on the cartilage should be, at least partially, mediated by IGF-I in the synovial fluid of ponies (van den Hoogen et al., 1998).

TGF- β 1 mRNA expression was observed in the chondrocyte clusters immediately surrounding a lesion of dyschondroplasia in horses, and this alteration could be involved in the pathogenesis of this condition in horses (Henson *et al.*, 1997). TGF- β 1 (0.1-10.0 ng/ml) did not significantly increase or inhibit proteoglycan deposition of the matrix in the chondrocyte pellets, while it evidently increased synthesis of proteoglycans. TGF is well known to be a pleiotropic growth factor, which has complex effects on cartilage metabolism and is secreted by many cells, including chondrocytes (Fortier *et al.*, 1997; Massague, 1990; Morales *et al.*, 1991; Rosier *et al.*, 1989). Many studies on this growth factor have focused on proteoglycan and type II collagen synthesis in cartilage explant and chondrocyte monolayer cultures. It has been demonstrated that TGF- β has either stimulated (Frazer *et al.*, 1994; Hiraki *et al.*, 1988; Morales and Roberts, 1988) or inhibited (Horton *et al.*, 1989; Van der Kraan *et al.*, 1992) the cartilage matrix synthesis. The pellet culture system that was employed in this study contributes to the measurement of synthesis, deposition, release and degradation of matrix macromolecules in any one culture well, for which previous culture systems could not be applied (Xu *et al.*, 1996). These conflicting results can be explained by the increased release of GAG into the medium at 1.0 and 10.0 ng/ml of TGF- β 1. It was detected that the level of aggrecan concentration in medium was increased by TGF- β 1-stimulation on cultured bovine cartilage (Xu *et al.*, 1996). Possible interpretation of these results may include an increased degradation of the newly synthesized proteoglycans.

Changes in the concentration of proteoglycans in response to growth factors were examined by assaying the GAG synthesized and the proportion of GAG released into the tissue culture medium during the culture period. Both IGF-I α and TGF- β 1 significantly increased the synthesis of proteoglycans compared with that of control cultures, and the proportion of proteoglycan released was not significantly affected by IGF-I α , but TGF

- β 1 significantly increased this proportion at 1.0 and 10.0 ng/ml. It is suggested that TGF- β 1 may increase turnover of the chondrocyte extracellular matrix in contrast to IGF- $\text{I}\alpha$, which increases synthesis and deposition of the matrix proteins. In the present study the deposition of cartilage extracellular matrix was neither increased nor decreased by TGF- β stimuli, unlike in a previous study using pellet-cultured bovine nasal chondrocytes (Xu *et al.*, 1996). This minor contrast would be caused by the difference of the age or the maturation of the horse, from which the cartilage samples were harvested (Xu *et al.*, 1996). The results reported here would mean some degradation of the articular cartilage induced by TGF- β 1.

There was a significant decrease in GAG contents of equine articular chondrocyte pellets treated with 20 ng/ml of IL-1 α compared with that of control pellets. IL-1 derived from various cells, including synoviocytes and chondrocytes, is considered the predominant catabolic mediator responsible for cartilage degradation (Hasty *et al.*, 1990). IL-1 has been shown to stimulate chondrocyte production of metalloproteinases (Chandrasekhar and Phadke, 1988; MacDonald *et al.*, 1992; Morris and Treadwell, 1994; Tyler, 1989), and to inhibit the synthesis of collagen and proteoglycans by chondrocytes (Hasty *et al.*, 1990; Morris and Treadwell, 1994; Platt and Bayliss, 1994; Tyler, 1989). The results observed from the pellet-cultured chondrocytes in the present study support previous observations (Benya and Shaffer, 1982; Chandrasekhar and Phadke, 1988; Hasty *et al.*, 1990; Morris and Treadwell, 1994; Platt and Bayliss, 1994; Tyler, 1989). This pellet culture system established for equine articular chondrocytes was used for the following investigation of keratan sulphate as a metabolic marker of cartilage.

Equine articular chondrocytes from 6 month-old foals were cultured as pellets to allow them to synthesize or degrade their own extracellular matrix when supplemented with IGF- $\text{I}\alpha$ or IL-1 α as described above. This *in vitro* turnover of extracellular matrix

of the articular cartilage was evaluated by quantitative assays of GAG and keratan sulphate concentration reacting to 1/14/16H9 or 1/20/5D4 antibody.

The concentration of keratan sulphate reacting to the newly established anti-equine 1/14/16H9 and the anti-human keratan sulphate 1/20/5D4 antibodies in the media showed similar change as GAG did. The concentration of keratan sulphate in the serum was suggested to serve as a diagnostic test for generalized osteoarthritis (Thonar *et al.*, 1985). It was reported that levels of keratan sulphate in serum might provide information on the systemic changes in metabolism of cartilage proteoglycans (Block *et al.*, 1989; Roos *et al.*, 1995; Sweet *et al.*, 1988; Williams *et al.*, 1988). A higher correlation between GAG contents and keratan sulphate concentrations reacting to these antibodies was found in the media of the pellets cultured with IL-1 α , while a low value was in the media of the pellets cultured with IGF-I α . An *in vitro* study implied that keratan sulphate concentration, reacting to 1/20/5D4 antibody (Block *et al.*, 1989), may be used as a marker of cartilage degeneration and chondroitin sulphate as a marker of its synthesis (Poole, 1994; Poole *et al.*, 1990). The change of keratan sulphate concentration reacting to 1/14/16H9 or 1/20/5D4 antibody in the media of the pellets stimulated by IGF-I α did not show the same manner as proteoglycan concentration did, a better correlation value with GAG was found in keratan sulphate concentration measured by 1/20/5D4 ($r = 0.59$) antibody than by 1/14/16H9 ($r = 0.43$) antibody. This indicates that keratan sulphate concentration does not entirely represent the synthesis of cartilage matrix.

In this chapter, the pellet culture system has been successfully adapted for further understanding of cartilage matrix metabolism of equine articular chondrocytes. The results suggest that the *in vitro* concentration of keratan sulphate reacting to 1/14/16H9 mirrors the GAG concentration during the stage of cartilage catabolism but not during the cartilage anabolic stage. On the other hands, the results also suggest that the *in vitro* concentration of keratan sulphate reacting to 1/20/5D4 mirrors GAG concentration during

the stage of cartilage catabolism and slightly during cartilage anabolism. These two differently reactive keratan sulphate concentrations in serum and synovial fluid could contribute as a marker to understand principally the catabolic process of articular cartilage metabolism and also represent in part some aspects of the cartilage anabolic process.

4. Evaluation of Clinical Significance of Keratan Sulphate Reacting to 1/14/16H9 and 1/20/5D4 Antibodies as a Cartilage Metabolic Marker in Serum and Synovial Fluid from Horses

4.1 Summary

The clinical significance of keratan sulphate as a cartilage metabolic marker was evaluated by measurement of keratan sulphate concentration reacting to the newly established anti-equine keratan sulphate 1/14/16H9 and an anti-human keratan sulphate 1/20/5D4 antibodies in sera and synovial fluid from horses. Serum and synovial fluid samples were obtained from training and growing foals, resting horses, and horses with spontaneous or experimentally-induced osteoarthritis. Keratan sulphate concentration in serum and synovial fluid was measured by an inhibition ELISA using 1/14/16H9 and 1/20/5D4 antibodies. Serum alkaline phosphatase (ALP) activity, as well as radiographic and physical examinations, was measured in growing foals as a background data.

Serum keratan sulphate concentration, reacting to 1/14/16H9 and 1/20/5D4 antibodies, in growing foals (up to 3 months of age) was significantly higher than the value in foals over 4 months old. Serum keratan sulphate concentration in three foals, which had joint abnormalities, was higher than those in 12 normally growing foals during the first 3 months after birth.

In on-training horses, keratan sulphate concentrations in 2-year old horses was significantly higher than 3- or 4-year old horses while, in long-term resting group, that in 2-year old horses was not statistically higher than of the other older age groups. Significantly higher concentration of keratan sulphate was found in the on-training group

than in the long-term resting group in 2-year old horses. Keratan sulphate concentration significantly increased immediately after the training in healthy horses, while keratan sulphate concentrations at 1-, 5-, 9- and 24-hours after the training remained at similar levels to the pre-training conditions.

There was no significant difference in the levels of two different keratan sulphate concentrations reacting to 1/14/16H9 or 1/20/5D4 antibodies in synovial fluid between normal horses and horses with osteoarthritis.

The concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera peaked 3 days after the intra-articular injection of chymopapain, and were approximately 1.4- and 1.9-folds higher than the preinjected value, respectively. The concentrations of keratan sulphate in synovial fluid peaked 3 days after the intra-articular injection of chymopapain, and were approximately 4-folds higher than the value before the injection, in keratan sulphate reacting to both antibodies.

The results suggest that the measurement of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera and synovial fluid is valuable in understanding the cartilage metabolic activity related to various conditions of the joints. Both the newly established 1/14/16H9 antibody and the common antibody for the detection of keratan sulphate, 1/20/5D4, could contribute to search for the key of the catabolic activity of joint cartilage in the various conditions of the joints in horses.

4.2. Introduction

Much work has progressed out in the search for a marker of cartilage metabolism in human beings and animals with arthropathies and secondary osteoarthritis (Alwan *et al.*, 1990; Block *et al.*, 1989; Heinegard *et al.*, 1985; Kliner *et al.*, 1987; Okumura *et al.*, 1997; Okumura and Fujinaga, 1998; Saxone *et al.*, 1986; Thonar *et al.*, 1985; Todhunter *et al.*, 1993). Most proteoglycans in the cartilage are high molecular weight macromolecules that enable cartilage to undergo reversible deformation (Fisher and Barclay, 1984). The mechanisms and rate of cartilage proteoglycan turnover in normal and abnormal cartilage remain unclear (Heinegard and Paulsson, 1984; Maroudas, 1975; Revell and Muir, 1972; Thonar *et al.*, 1985). Lack of substantial amounts of partially degraded proteoglycans in the normal cartilage matrix suggests that proteoglycan molecules are usually rapidly degraded into smaller fragments, which are either engulfed by the chondrocytes or diffused into the body fluids that are in contact with cartilage (Thonar *et al.*, 1985). Consequently, proteoglycan fragments and GAG were found in synovial fluid (Heinegard *et al.*, 1985; Saxone *et al.*, 1986), and several species of GAG, including chondroitin sulphate and keratan sulphate, have also been detected in blood (Block *et al.*, 1989; Heinegard and Paulsson, 1984; Kliner *et al.*, 1987; Thonar *et al.*, 1985).

Keratan sulphate is a GAG that is distributed in the extracellular matrix of joint cartilage. Recently, an ELISA using an anti-human keratan sulphate antibody 1/20/5D4 (Caterson *et al.*, 1983) has led to improvements in measurement of low concentration of keratan sulphate (Thonar *et al.*, 1985).

The objectives of this chapter were to determine the amount of keratan sulphate,

reacting to the newly established anti-equine keratan sulphate 1/14/16H9 and an anti-human keratan sulphate 1/20/5D4 antibodies, in serum and synovial fluid of foals at the early stage of life, horses in resting or training period, and horses with spontaneous or experimental osteoarthritis, and to evaluate the clinical significance of keratan sulphate as a marker to understand cartilage metabolism of the joints.

4.3. Materials and methods

4.3.1. Serum and synovial fluid samples from horses

4.3.1.1. Serum samples from growing foals

Twenty neonatal foals were chosen at random from Shadai farm in Hokkaido, and 15 foals were further selected for this study. Monthly radiographic and physical examinations were done. Foal 1-12 had normal growth and Foal 13-15 had joint abnormalities (Table 8). Blood was collected from the foals 1 week after birth, then monthly for 6 months. Sera were stored at -20 °C until assayed.

For each time of blood sample collection, physical examination of the neck and limbs and radiography of the carpal joint (lateral and ventrodorsal[VD] views), hock (lateral and laterodorsal oblique views) and fetlocks (lateral and VD views) on the right side were done to investigate for any abnormalities of the joints. Additionally, serum ALP activity was also measured as a quantification of osteoblastic activity in foals.

4.3.1.2. Serum samples from long-term resting and on-training horses

Serum samples were obtained from long-term resting horses, which had not raced for more than 3 months for the reasons other than joint problems, and on-training horses, which had daily racetrack training for more than 3 months. Each group consisted of 3 generations including 2, 3 and 4-year old horses (each generation had 10 horses). Blood samples were collected from on-training horses after more than 24 hours from the last practice. Horses in either long-term resting or on-training had no clinical signs of joint diseases. Serum samples were stored at -20 °C until assayed.

4.3.1.3. Serum samples from on-training horses within 24 hours after training

Serum samples were obtained from five training horses (five males, 3 years of age)

before and immediately after 2-minutes-gallop, and 1, 5, 9 and 24 hours after training. The horses had no clinical signs of joint diseases. Serum samples were stored at -20 °C until assayed.

4.3.1.4. Serum and synovial fluid samples from horses with spontaneous and experimentally-induced osteoarthritis

Serum and synovial fluid samples were obtained from horses with no clinical signs of joint diseases and horses diagnosed clinically as having osteoarthritis in Shadai Horse Clinic, Hokkaido. The diagnosis of osteoarthritis was based on history, complaint, signalment, clinical examination and radiographic assessment.

Serum and synovial fluid samples were obtained from horses (four Thoroughbreds and one Arabian with ages ranging from 3 to 6 years old) with experimentally-induced osteoarthritis by an injection of 30 mg of chymopapain (Sigma Chem. Co.) into unilateral carpal joints. Blood was collected 0, 3, 7, 10, 14, 21, 28, 35, 42, 52 and 49 days after chymopapain injection. Synovial fluid was collected 0, 3, 10, 17, 24 and 35 days after the injection. Serum and synovial fluid samples were stored at -20 °C until assayed.

The procedure of this experiment and handling of the animals were planned and done according to the guideline for animal experiments in Graduate School of Veterinary Medicine, Hokkaido University.

4.3.2. Purification of equine cartilage proteoglycan monomers

Equine proteoglycan monomers were purified similarly as previously described in Chapter 2, Section 2.3.1.

4.3.3. Measurement of keratan sulphate concentration in sera and synovial fluid

An inhibition ELISA, established by Okumura and Fujinaga (1998), was used to measure keratan sulphate concentration in sera and synovial fluid as described in Chapter 2, Section 2.3.5.

4.3.4. Measurement of serum alkaline phosphatase (ALP) activity

Serum ALP activity was measured in foals by use of a serum analyzer (COBAS MIRA, F. Hoffmann-La Rosch Co. LTD, Basel, Switzerland).

4.3.5. Statistical analysis

For analysis of significance of data obtained for normally growing foals, repeated measures analysis of variance was used. Fisher's protected least significant difference was used as a post-hoc test. A P value < 0.05 was considered statistically significant.

Comparisons of serum or synovial fluid keratan sulphate concentration between normal and arthritic horses were made by the two-tailed Mann-Whitney U test with $P < 0.05$ taken as significant. The correlation between two different concentration of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies was evaluated by Spearman's correlation coefficient.

4.4. Results

4.4.1. Serum samples from growing foals

In all foals, serum keratan sulphate concentration was high from 1 week after birth to 3 months of age, and started to decrease rapidly from 4 to 5 months of age (Figure 15). In three foals (No. 13-15), which had joint problems, keratan sulphate concentration was higher than that in normally growing 12 foals at 1 week, and 1, 2, and 3 months of age. Both keratan sulphate epitopes, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed the same manner of changes in growing foals (Figure 13). Keratan sulphate concentration reacting to 1/20/5D4 antibody was always higher than that to 1/14/16H9 antibody, however prominent change of keratan sulphate concentration in three horses with joint diseases was likely to be more clearly represented in keratan sulphate concentration reacting to 1/14/16H9 antibody.

Radiographic findings for the hock, carpal joint, and fetlocks on the right side in normally growing 12 foals were not significant. The left hock of Foal No.13 had an osteochondrosis dissecans at the distal intermediate ridge of the tibia at 9 months of age, accompanied by swelling of the same joint (Table 8). In Foal No.14, swelling on both sides of the carpal joint and hock, and severe extroversion of both forelimbs were seen without significant radiographic findings. In Foal No.15, swelling but not a radiographic abnormality was found on both hocks (Table 8). Any other problems of the skeletal system were not detected during physical examination of Foal Nos.13-15 (Table 8).

The ALP value during the first 3 months was higher than the stable value after 5 months of age, despite no significant difference between male and female normally growing foals (Figure 14). The fluctuation in ALP activity in Foal Nos. 13-15 was

similar to that seen in other foals.

4.4.2. Serum keratan sulphate concentration in long-term resting and on-training horses

Both keratan sulphate concentrations, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed the same manner of changes in each group of horses.

In long-term resting horses, keratan sulphate concentration in 2-year old horses was higher than that of 3- or 4-year old horses, but a significant difference could not be detected among them (Figure 15). In on-training horses, keratan sulphate concentration in 2-year old horses was significantly higher than that of the 3- or 4-year old horses (Figure 15). In the 2-year old horses, significantly higher keratan sulphate concentration was found in the on-training group than in the long-term resting group (Figure 15). The 1/14/16H9 keratan sulphate likely showed more clearly the tendency (Figure 15).

4.4.3. Short-term change of keratan sulphate concentration in the blood from horses within 24 hours after training

Keratan sulphate concentration before and after the training of 2-minute-gallop was measured in five horses. Both keratan sulphate concentrations, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed the same manner of changes in these horses (Figure 16). Keratan sulphate concentration immediately after the training was significantly higher than that before the training, while keratan sulphate concentration 1-, 5-, 9- and 24-hours after training was the same level as before the training (Figure 16).

4.4.4. Serum and synovial fluid samples from horses with experimentally-induced osteoarthritis

The serum concentration of keratan sulphate peaked 3 days after the injection, and

was approximately 1.4- and 1.9-folds higher in horses that received chymopapain intra-articularly than the preinjected value, when estimated using 1/14/16H9 and 1/20/5D4 antibodies, respectively (Figure 17). The concentration of keratan sulphate reacting to 1/20/5D4 in sera was lowest 21 days after the injection and increased to a slightly higher value than the preinjected values, and then returned towards the preinjected value up to 49 days after the injection, while the concentration of keratan sulphate reacting to 1/14/16H9 antibody did not show the second increase after 21 days (Figure 17). Both keratan sulphate concentrations in sera, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed the same manner of changes (Figure 17). Although keratan sulphate level in 1/20/5D4 was always higher than that in 1/14/16H9, prominent change of keratan sulphate concentration at the first peak after the injection was apparently represented in the keratan sulphate reacting to 1/14/16H9 antibody (Figure 17).

The concentration of keratan sulphate in synovial fluid peaked 3 days after the injection of chymopapain, and was approximately 4-fold higher after the injection than that before the injection, and keratan sulphate concentration 10 days after the injection was still significantly higher than that before the injection (Figure 18). The concentration of keratan sulphate returned toward the preinjection values 24 days after the injection. Keratan sulphate concentrations, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed similar change and value in these horses (Figure 18).

4.4.5. Serum and synovial fluid samples from horses with spontaneous osteoarthritis

The results of the keratan sulphate assay in sera and synovial fluid are summarized in Table 9. The concentrations of serum keratan sulphate reacting to 1/14/16H9 (159.2 ± 41.9 ng/ml) and 1/20/5D4 (204.0 ± 82.4 ng/ml) antibodies in horses were both significantly increased, as compared to normal horses (98.6 ± 21.6 and 139.4 ± 43.7

ng/ml, respectively). There was no significant difference ($P>0.05$) in concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in synovial fluid between normal (36.7 ± 29.3 and 36.7 ± 29.3 $\mu\text{g/ml}$, respectively) and osteoarthritis (33.5 ± 18.5 and 28.3 ± 19.9 $\mu\text{g/ml}$, respectively).

In serum samples, the concentration of keratan sulphate reacting to 1/14/16H9 antibody (159.2 ± 41.9 ng/ml) was significantly lower than that to 1/20/5D4 antibody (204.0 ± 82.4 ng/ml), while no statistical difference was found between concentrations of keratan sulphate to 1/14/16H9 (36.7 ± 29.3 $\mu\text{g/ml}$) and 1/20/5D4 (37.9 ± 22.2 $\mu\text{g/ml}$) antibodies in synovial fluids.

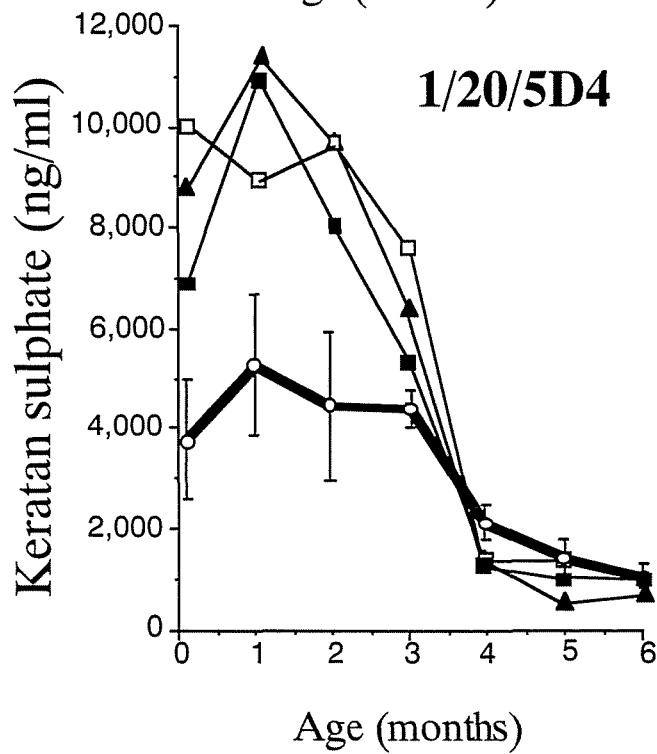
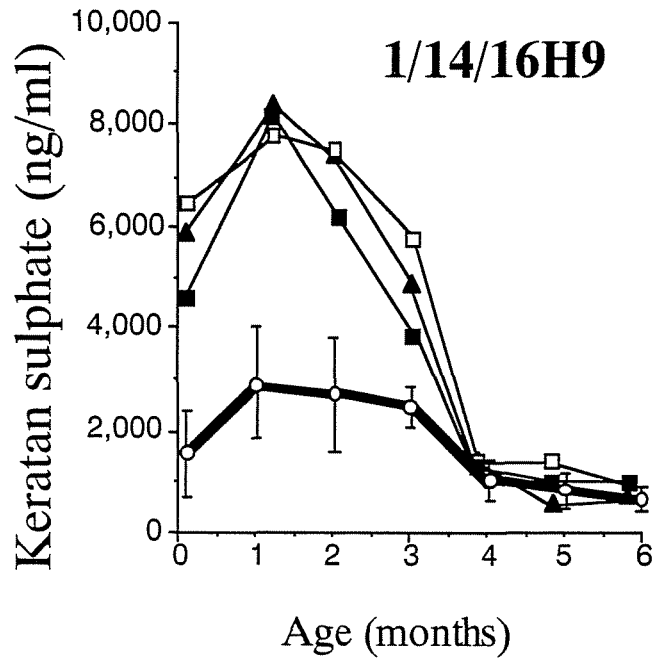


Figure 13-Changes in serum keratan sulfate reacting to 1/14/16H9 or 1/20/5D4 antibody concentrations with aging in foals.

Values are mean \pm standard error (SE).

○= Foal No. 1-12 (normally growing foals; n=12)

▲= Foal No. 13, □= Foal No. 14, ■= Foal No. 15 (for details, see Table 8).

(modified from Okumura *et al.*, 1997)

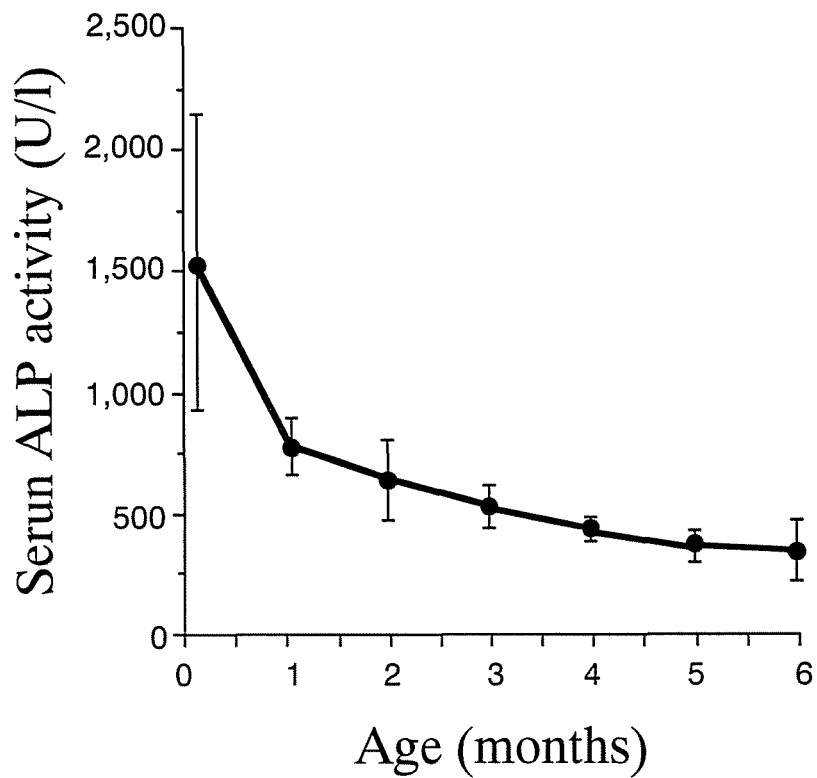


Figure 14-Changes of serum alkaline phosphatase (ALP) activity in foals.

Values are mean \pm SE.

● = Foal No. 1-15 (n=15).

(modified from Okumura *et al.*, 1997)

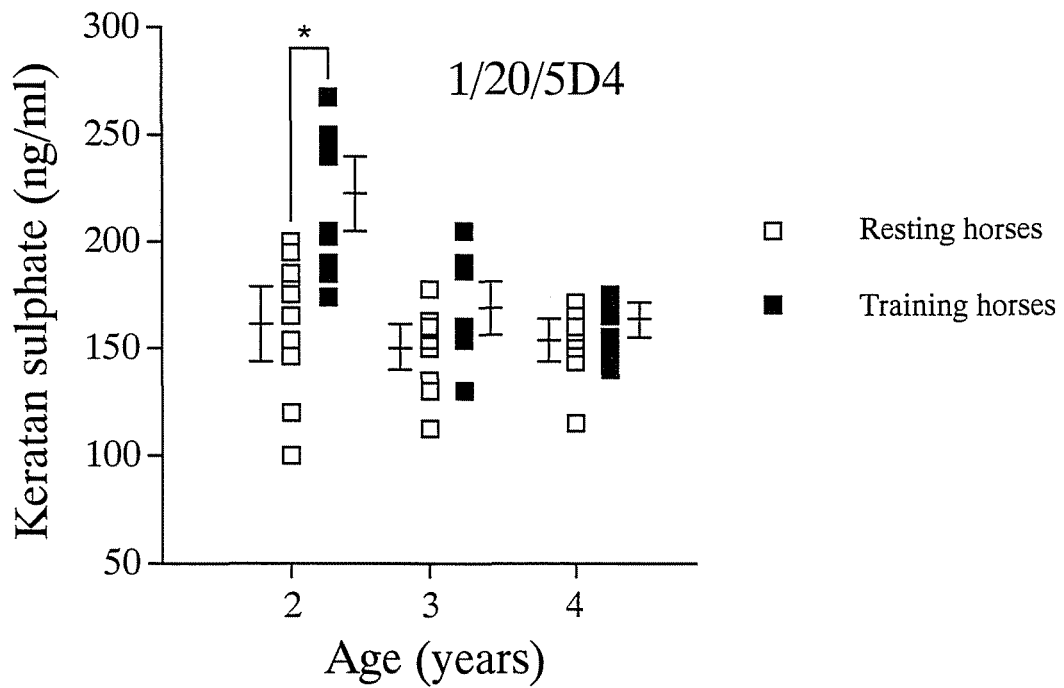
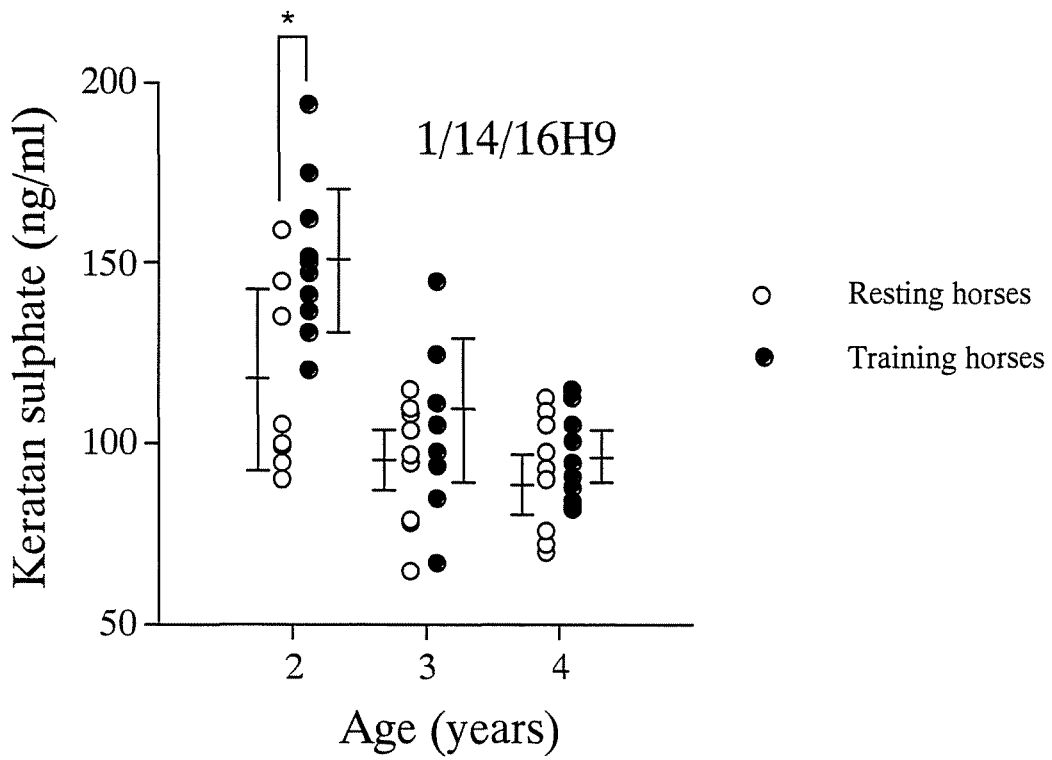


Figure 15-Serum keratan sulphate concentration reacting to 1/14/16H9 or 1/20/5D4 antibody in long-term resting and on-training horses.

Values are mean \pm standard error (SE).

* $P < 0.05$ compared between resting and training horses.

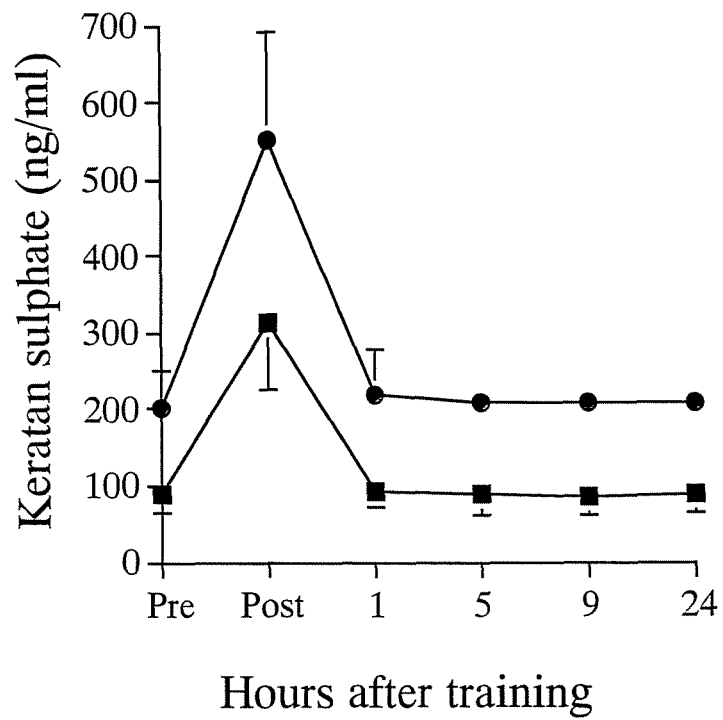


Figure 16-Keratan sulphate concentration before and after the training of 2-minutes-gallop

Values are mean \pm SD.

Pre: before training.

Post: within 5 minutes after training.

—■— 1/14/16H9 antibody

—●— 1/20/5D4 antibody

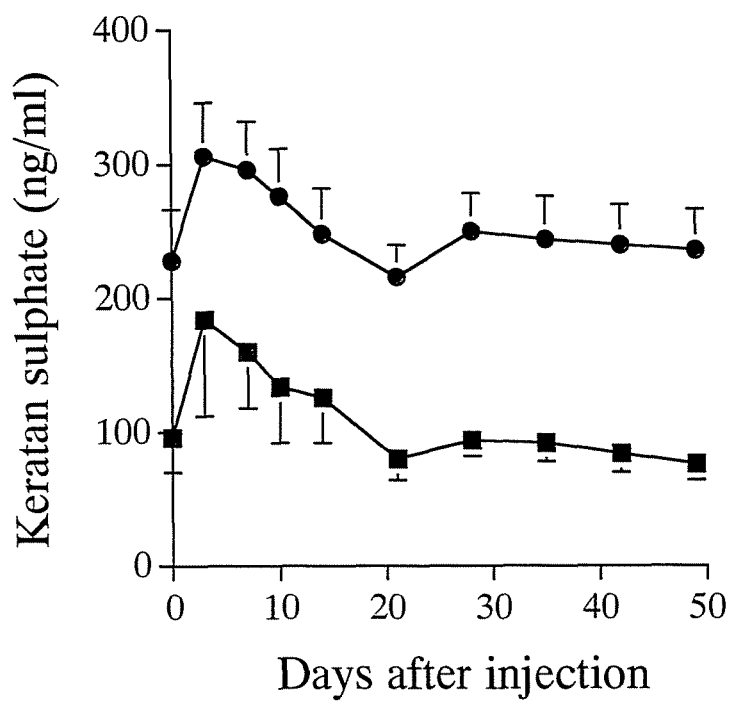


Figure 17-The concentration of keratan sulphate in sera from horses with experimentally-induced osteoarthritis.

Values are mean \pm SD.

- 1/14/16H9 antibody
- 1/20/5D4 antibody

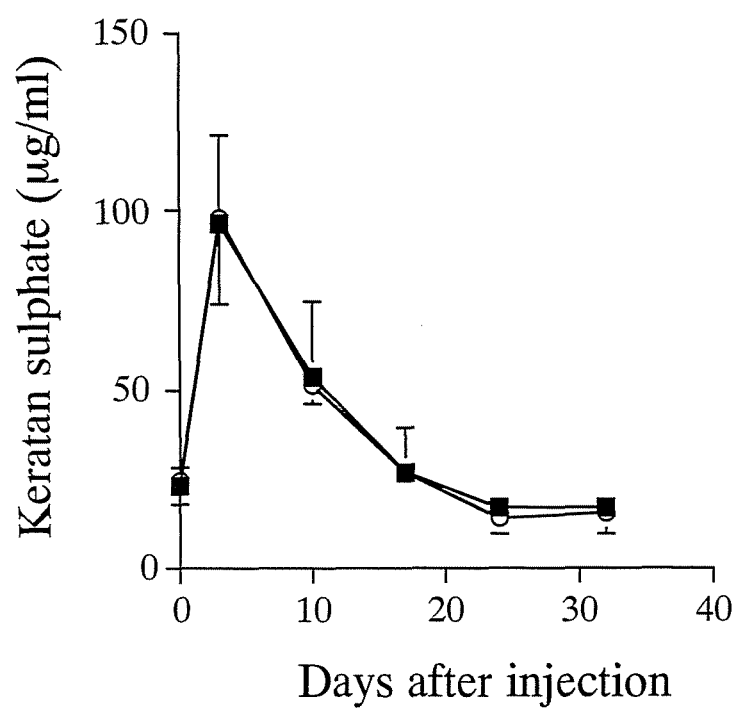


Figure 18-The concentration of keratan sulphate in synovial fluid from horses with experimentally-induced osteoarthritis.

Values are mean \pm SD.

—■— 1/14/16H9 antibody

—○— 1/20/5D4 antibody

Table 8. Clinical findings of Foals No.13-15

No.	Sex	Clinical signs	Radiologygraph	Other findings
13	Male	Swelling on the left at 9 months	OCD at 9 months	Arthroscopic surgery to remove osteochondral fragment from the left hock at 9 months.
14	Male	Joint effusion on the both hocks and severe extroversion of both forelimbs at 3 months	NS	Necropsy findings at 6 months. - Septic arthritis on both hocks - Degenerative lesion on both affected joints
15	Male	Joint effusion on both hocks at 3 months	NS	Recovered at 5months.

OCD = Osteochondrosis dissecans; NS = not significant.

(Okumura *et al.*, 1997)

Table 9. Keratan sulphate concentration reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera and synovial fluid (SF) from normal horses (Controls) and horses with osteoarthritis (OA)

Diagnostic groups	n	Age (years)	1/14/16H9			1/20/5D4			
			mean± SD	median	range	mean± SD	median	range	
Controls	Serum#	20	5.20	98.6± 21.6 ¹⁾	102.5 ¹⁾	60.1-135.9 ¹⁾	139.4± 43.7 ¹⁾	128.2 ¹⁾	60.3-263.1 ¹⁾
	SF	20	5.20	37.9± 22.2 ²⁾	33.5 ²⁾	8.0-80.1 ²⁾	36.7± 29.3 ²⁾	39.8 ²⁾	2.3-102.4 ²⁾
OA	Serum#	38	4.61	159.2± 41.9 ^{1)*}	150.0 ¹⁾	112.2-299.9 ¹⁾	204.0± 82.4 ^{1)*}	205.1 ¹⁾	80.5-400.4 ¹⁾
	SF	20	4.93	33.5± 18.5 ²⁾	28.5 ²⁾	8.0-73.0 ²⁾	28.3± 19.9 ²⁾	29.0 ²⁾	4.8-68.9 ²⁾

keratan sulphate: 1) ng/ml, 2) µg/ml.

*: P<0.05 as compared to Controls.

#: P<0.05 as compared between 1/14/16H9 and 1/20/5D4.
(modified from "Okumura *et al.*, 1998")

4.5. Discussion

According to the results of *in vitro* study in Chapter 3, keratan sulphate concentration in 1/14/16H9 could be a more accurate marker to evaluate cartilage catabolic activity in the culture media than that in 1/20/5D4.

Serum keratan sulphate concentration in growing foals (up to 3 months of age) was significantly higher than the value in foals more than 4 months of age. This suggests that the metabolic activity of cartilage, the rate of both catabolism and possibly anabolism are high at the initial growth stage in foals (Poole and Dieppe, 1994). Similarly, the keratan sulphate concentrations in children (5 to 12 years old) are higher than these in adult human beings (Thonar *et al.*, 1985). Proteoglycan concentration in puppy (2 weeks to 2 months old) was 3 times more than that of adult values (Lust and Summers, 1981).

Serum keratan sulphate concentrations in Foals Nos.13-15, which had joint abnormalities, was higher than in the normally growing foals 1-12 during the first 3 months after birth. Foal No.13 was diagnosed to have osteochondrosis dissecans after 6 months of age, although its serum keratan sulphate concentration decreased in normal manner, similarly in the other growing foals. Osteochondrosis dissecans is usually diagnosed by findings of mild abnormalities in the affected joints long time after its onset. Though it is not more than an inference because only one case of osteochondrosis dissecans was found during this study, higher cartilage catabolic activity might indicate a weakness of the cartilage matrix during the early growing stages in this foal. On the other hand, high keratan sulphate values in sera from all foals examined during the first 3 months suggest that the immature joint during the time could be easily affected by any factor of loading. In Foals Nos.14 and 15, serum keratan sulphate concentration was

higher than that in normally growing foals at the time they had joint problems. This might indicate that serum keratan sulphate concentration would be of value for monitoring the cartilage damage associated with joint diseases during the early stages of life.

Serum keratan sulphate concentration in foals decreased remarkably more than 3 months of age. In a previous report (Thonar *et al.*, 1985), serum keratan sulphate values were found to decrease in human beings from 13 to 15 years of age. This indicated that the catabolic activity of cartilage in foals was likely to be higher than that during any other phase of their life, similar to that in human beings (Heinegard *et al.*, 1985).

Both keratan sulphate epitopes, reacting to 1/14/16H9 and 1/20/5D4 antibodies, showed the same manner of changes in growing foals. Keratan sulphate concentration reacting to 1/20/5D4 was always higher than 1/14/16H9, and prominent change of keratan sulphate concentration in three horses with joint diseases was clearly represented in 1/14/16H9. This suggests that serum keratan sulphate concentration in three horses with joint abnormalities represented mainly the catabolism of the cartilage, and that the values in the healthy foals expresses not only the catabolism but also the anabolism of the cartilage.

Serum ALP activity peaked 1 week of age, then decreased rapidly for 1 month after birth. Each foal had almost the same fluctuation for 6 months. Alkaline phosphatase is one of important diagnostic makers in bone metabolism, or liver and bile duct function (Blackmore and Elton, 1975; Kramer and Sleight, 1968; Pickrell *et al.*, 1974). In kittens, increased serum ALP activity was suggested to reflect higher activity of osteoblasts (Kramer and Sleight, 1968). High serum ALP values were also found in the foals in this study, similar to that in kittens. However, serum ALP activity did not parallel serum keratan sulphate concentration, which reached the peak value at 1 month of age and was maintained for 3 months. It also suggests that higher serum keratan sulphate

concentration might reflect not only growth of cartilage but cartilage damage for the first 3 months of life.

In on-training horses, keratan sulphate concentration in 2-year old horses was significantly higher than that of 3- or 4-year old horses, while those in 2-year old horses was not significantly higher than other older age groups in long-term resting horses. Significantly higher concentration of keratan sulphate was found in the on-training group than in the long-term resting group in 2-year old horses. Serum keratan sulphate concentration was 3.5-folds higher in dogs younger than 2 months of age than in older dogs (Leipold *et al.*, 1989). This suggests that cartilage of younger animals is actively degrading itself and is mechanically immature. It was also reported that the concentration of keratan sulphate in serum was increased after exercise in 33 healthy athletes (Roos *et al.*, 1995). Because keratan sulphate concentration to 1/14/16H9 is likely to show more remarkable increase in younger horses than that to 1/20/5D4, the change after exercise may mainly represent the catabolic activity of mechanical stress to the joint cartilage. The increase of serum keratan sulphate reacting to both two antibodies is therefore suggested to reflect an effect of mechanical loading in combination with a possible high turnover rate of cartilage matrix in these individuals. The results in the present study suggest that young horses have low tolerance to striking mechanical stress during a high-speed run on a racetrack.

Short-term catabolism and discharge of keratan sulphate fragments in blood was examined by measurement of keratan sulphate concentration before and after the training of 2-minutes gallop in five horses. A 2-minutes-gallop is a remarkable stress for Thoroughbred horses, so that much mechanical stress will be bore by their bones and joints. This would cause a transient joint cartilage degradation. The time-course transient increase of degradation of joint cartilage was examined by measurement of serum keratan

sulphate for 24 hours. Immediately after the training in healthy horses, keratan sulphate was significantly higher than that before the training, while keratan sulphate concentration at 1-, 5-, 9- and 24-hours after training was at the same level as before the training. The immediate return of keratan sulphate concentration in the serum to pretraining values suggests the possible discharge of proteoglycan fragments from the blood to the urine, liver or other tissues for a short term, and the prolonged increase of keratan sulphate might demonstrate the active degradation of cartilage present in the joints or other cartilagenous tissues.

The serum concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 peaked 3 days after the intra-articular injection of chymopapain, and were approximately 1.4- and 1.9-folds higher than the preinjected value, respectively. The concentration of keratan sulphate reacting to both antibodies in synovial fluid peaked on the 3rd day after the injection of chymopapain, and was approximately 4-fold higher after the injection than that before the injection. Chymopapain is a serine proteinase which, when activated by cysteine, will cleave the protein core of aggrecan, releasing keratan sulphate into the synovial fluid and, hence, into the systemic circulation. Further degradation of aggrecan probably occurred because of synovial inflammation that accompanied chymopapain injection (Todhunter *et al.*, 1993).

The concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies showed almost the same change and level in the synovial fluid from the horse with experimentally-induced osteoarthritis. This suggests that keratan sulphate, up to 31 days after chymopapain injection, represented mainly the catabolic activity of joint cartilage. On the other hand, the concentration of keratan sulphate in sera peaked 3 days after the injection, and was approximately 1.4- and 1.9-folds higher in horses that received chymopapain intra-articularly than the preinjected value, in the epitopes of

1/14/16H9 and 1/20/5D4, respectively. This suggests that the base-line value of serum keratan sulphate may reflect systemic cartilage metabolism, so that the pre-injection value of 1/20/5D4 epitope is higher than that of 1/14/16H9 value.

The concentration of keratan sulphate reacting to 1/20/5D4 in sera was lowest 21 days after the injection and again slightly increased to higher value than the pre-injection values, then returned towards the preinjected value by 49 days after the injection, while the concentration of keratan sulphate reacting to 1/14/16H9 antibody did not show any remarkable change after the 21st day of a chymopapain injection. The second peak may represent the repairing response of damaged cartilage including both cartilage anabolic and catabolic activities, while the first peak represents the destruction of cartilage by chymopapain.

The concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera from horses with osteoarthritis were both significantly increased, as compared to normal horses. It was reported that the concentration of keratan sulphate in serum from horses with osteoarthritis (Alwan *et al.*, 1990) and inflammatory arthritis (Todhunter *et al.*, 1997) was significantly higher than in normal horses, although a finding contrary to this was also stated in osteoarthritis (Todhunter *et al.*, 1997). It is therefore suggested that this increase of keratan sulphate in sera may mirror cartilage catabolic activity in the joint.

There was no significant difference in the concentrations of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in the synovial fluid between the normal horses and horses with osteoarthritis. The keratan sulphate in synovial fluid should have a wide range of concentration (Todhunter *et al.*, 1997). Total GAG concentration in synovial fluid of human inflammatory arthritis was reported to be of a higher value in acute inflammatory conditions and a border range of lower value in chronic conditions

(Ractcliffe *et al.*, 1988). It is thought that the metabolism of cartilage and synovial function should be changed strikingly for a short time after the onset of joint diseases, and that plasma effusion into synovial fluid and inflammatory reaction afterwards might contribute for the concentration to compromise itself.

In this chapter, keratan sulphate concentration reacting to 1/14/16H9 and 1/20/5D4 antibodies were measured in growing foals, training and resting horses and horses with experimentally-induced and spontaneous osteoarthritis. The results demonstrate that the measurement of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera and synovial fluid would be of value to understand the cartilage catabolic activity related to various conditions of the joints. By comparing of the concentrations of keratan sulphate reacting to newly established 1/14/16H9 and the common antibody 1/20/5D4, it was shown that more accurate estimation of the catabolic or anabolic activity of cartilage could be made in the various conditions of the joints in horses.

5. General Conclusion

Joint damage is difficult to estimate its term of severity and extent objectively. Much damage may be done early in the disease process, before the detection of any radiographical changes, although it is uncertain which of the many aspects of synovitis are responsible for joint damage. Sensitive ways of assessing the current status of cartilage and bone changes during the early inflammatory joint disease would be of potential value to clinicians for more accurate diagnosis and precise treatment.

Articular cartilage is a specialized tissue composed of mesenchymal cells and chondrocytes, embedded in an extensive extracellular matrix. Proteoglycan molecules are usually rapidly degraded into smaller fragments, which are either engulfed by the chondrocytes or diffused into the body fluids that are in contact with cartilage. Consequently, proteoglycan fragments and glycosaminoglycans are found in the synovial fluid, and several species of glycosaminoglycans, including chondroitin sulphate and keratan sulphate, are also detected in the blood or urine. Keratan sulphate is a glycosaminoglycan that is distributed in the extracellular matrix of joint cartilage. Keratan sulphate is mainly found in aggrecans (approximately 99%), and to a lesser extent, in the cornea and other tissues. Therefore, to estimate the total catabolic activity of cartilage, keratan sulphate could be used as a representative metabolite of cartilage in the synovial fluid and blood.

In this study, firstly, a sensitive and specific monoclonal antibody to equine keratan sulphate, 1/14/16H9, was established to develop an enzyme immunoassay for measurement of the concentration of keratan sulphate in serum and synovial fluid from

horses. An inhibition enzyme linked immunosorbent assay (ELISA) was optimized by using the established monoclonal antibody, and keratan sulphate concentration in 18 synovial fluid and 48 serum samples obtained from clinically normal horses and horses with arthritis was measured. Precision data were obtained over the range of 10 to 160 ng/ml. The within- and between-assay coefficients of variation of the assay were 10.0 and 12.7 %, respectively.

Secondly, the significance of keratan sulphate reacting to the newly-established anti-equine keratan sulphate 1/14/16H9 antibody and an anti-human keratan sulphate 1/20/5D4 antibody as a marker of cartilage metabolism was evaluated by using an *in vitro* model of equine articular cartilage. Articular cartilage was harvested from clinically healthy 6 month-old Thoroughbred foals. Articular chondrocytes were centrifuged and cultured as pellets in centrifuge tubes. Chondrocytes were incubated with insulin-like growth factor (IGF)-I α , transforming growth factor (TGF)- β 1 or interleukin (IL)-1 α for 2 weeks. The sulfated glycosaminoglycans (GAG) and keratan sulfate concentrations in pellets or media were measured by a 1,9-dimethyl-methylene blue colorimetric assay and an inhibition ELISA, respectively. The concentration of GAG in the culture media was significantly increased by the supplement of IGF-I α and TGF- β 1. The concentration of GAG in pellets was significantly increased by the supplement of IGF-I α , but not by TGF- β 1.

The concentration of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in the culture media were also measured. A high correlation between GAG and keratan sulphate concentrations was found in the media of pellets stimulated by IL-1 α (r

= 0.87 and 0.84; GAG to 1/14/16H9 and to 1/14/16H9 keratan sulphate, respectively), but lower in those stimulated by IGF-I α ($r = 0.43$ and 0.59 ; GAG to 1/14/16H9 and to 1/14/16H9 keratan sulphate, respectively).

These two differently reactive keratan sulphate, 1/14/16H9 and 1/20/5D4, concentrations in serum and synovial fluid could contribute as a marker to understand principally the catabolic process of articular cartilage metabolism and also represent in part some aspects of the cartilage anabolic process.

Finally, the clinical significance of keratan sulphate as a cartilage metabolic marker was evaluated by measurement of keratan sulphate concentration reacting to 1/14/16H9 and 1/20/5D4 antibodies in serum and synovial fluid from horses.

Serum keratan sulphate concentration in growing foals (up to 3 months of age) was significantly higher than the value in foals over 4 months old. Serum keratan sulphate concentration in three foals, which had joint abnormalities, was higher than those in the normally growing 12 foals during the first 3 months after birth.

In on-training horses, keratan sulphate concentration in 2-year old horses was significantly higher than 3- or 4-year old horses. Significantly higher concentration of keratan sulphate was found in the on-training group than in the long-term resting group in 2-year old horses. Immediately after the training in healthy horses, keratan sulphate concentration was significantly higher than before the training.

The concentration of keratan sulphate in sera peaked 3 days after the experimental injection of chymopapain intra-articularly, and was approximately 1.9- and 1.4-folds higher than the preinjected value, in 1/14/16H9 and 1/20/5D4 keratan sulphate, respectively. The concentration of keratan sulphate in the synovial fluid peaked 3 days

after injection of chymopapain, and was approximately 4-folds higher than before the injection, in both keratan sulphates. There was no significant difference in the levels of keratan sulphate concentration reacting to 1/14/16H9 and 1/20/5D4 antibodies in synovial fluid between normal horses and horses with osteoarthritis.

Both established 1/14/16H9 antibody and the common antibody(1/20/5D4) for the detection of keratan sulphate could contribute to understand the catabolic activity of joint cartilage in the various conditions of the joints in horses.

In conclusion, my study showed that the measurement of keratan sulphate reacting to 1/14/16H9 and 1/20/5D4 antibodies in sera and synovial fluid would be of value to understand the cartilage metabolic activity related to various conditions of the joints. Comparison of the concentrations of keratan sulphate reacting to both newly established 1/14/16H9 and the common antibody 1/20/5D4 would contribute to understand more accurately the catabolic or anabolic activity of joint cartilage in the various conditions of the joints in horses. The measurement of keratan sulphate in sera and synovial fluid would be of value to understand the catabolic activity of joint cartilage, so that keratan sulphate could be an noninvasive and possibly direct informative marker to detect cartilage pathology in horses with joint diseases.

6. References

1. Alwan WH, Carter SD, Bennett D, May SA, Edwards GB. Cartilage breakdown in equine osteoarthritis: measurement of keratan sulphate by an ELISA system. Res Vet Sci 1990;49:56-60.
2. Axelsson I, Heinegard D. Characterization of the keratan sulphate proteoglycans from bovine corneal stroma. Biochem J 1978;169:517-530.
3. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215-224.
4. Blackmore DJ, Elton D. Enzyme activity in the serum of thoroughbred horses in the United Kingdom. Equine Vet J 1975;7:34-39.
5. Block JA, Schnitzer TJ, Andersson GB, Lenz ME, Jeffery R, McNeill TW, Thonar EJ. The effect of chemonucleolysis on serum keratan sulfate levels in humans. Arthritis Rheum 1989;32:100-104.
6. Bramlage L, Schneider RK, Gabel AA. A clinical perspective on lameness originating in the carpus. Equine Vet J 1988;Suppl.6:12-618.
7. Buttle DJ, Bramwell H, Hollander AP. Proteolytic mechanisms of cartilage breakdown: a target for arthritis therapy. Arthritis Rheum 1995;32: 894-900.
8. Calatroni A, Donnelly PV, Di Ferrante N. The glycosaminoglycans of human plasma. J Clin Invest 1969;48:332-343.
9. Champion GV, McCrae F, Schnitzer TJ, Lenz ME, Dieppe PA, Thonar EJ. Levels of keratan sulfate in the serum and synovial fluid of patients with osteoarthritis of the knee. Arthritis Rheum 1991;34:1254-1259.
10. Carroll GJ, Bell MC, Laing BA, McCappin S, Blumer C, Leslie A. Reduction of the concentration and total amount of keratan sulphate in synovial fluid from patients with osteoarthritis during treatment with piroxicam. Ann Rheum Dis 1992;51:850-854.
11. Caterson B, Christner JE, Baker JR. Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. Monoclonal antibodies to cartilage proteoglycan. J Biol Chem 1983;258:8848-8854.
12. Caterson B. Immunological aspects of markers of joint disease. J Rheumatol Suppl 1991;27:19-23.
13. Chandrasekhar S, Phadke K. Interleukin-1-induced alterations in proteoglycan metabolism and matrix assembly. Arch Biochem Biophys 1988;265:294-301.

14. Cruz TF, Malcolm AJ, Adams ME. The effect of maturation and anterior cruciate ligament transection on the level of keratan sulfate in the serum of dogs. J Rheumatol 1989;16:1345-1350.
15. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 1982;9:247-248.
16. Fisher AT, Barclay WP. Osteochondrosis dissecans in the horse. Comp Cont Educ 1984;6:123-131.
17. Fortier LA, Nixon AJ, Mohammed HO, Lust G. Altered biological activity of equine chondrocytes cultured in a three-dimensional fibrin matrix and supplemented with transforming growth factor beta-1. Am J Vet Res 1997;58:66-70.
18. Fosang AJ, Tyler JA, Hardingham TE. Effect of interleukin-1 and insulin like growth factor-1 on the release of proteoglycan components and hyaluronan from pig articular cartilage in explant culture. Matrix 1991;11:17-24.
19. Frazer A, Bunning RAD, Thavarajah M, Seid JM, Russell RGG. Studies on type II collagen and aggrecan production in human articular chondrocytes in vitro and effects transforming growth factor-b and interleukin-1b. Osteoarthritis Cartilage 1994;2:235-245.
20. Frisbie DD, Nixon AJ. Insulin-like growth factor 1 and corticosteroid modulation of chondrocyte metabolic and mitogenic activities in interleukin 1-conditioned equine cartilage. Am J Vet Res 1997;58:524-530.
21. Galfre G, Milstein C. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol 1981;73B:3-46.
22. Gefter ML, Margulies DH, Scharff MD. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genet 1977;3:231-236.
23. Hastly KA, Reife RA, Kang AH, Stuart JM. The role of stromelysin in the cartilage destruction that accompanies inflammatory arthritis. Arthritis Rheum 1990;33:388-397.
24. Heinegard D, Paulsson M. Structure and metabolism of proteoglycans, extracellular matrix biochemistry. In: Structure and Metabolism of Proteoglycans, Extracellular Matrix Biochemistry. Piez KA, Reddi AH eds. Elsevier, New York, USA 1984;277-328.
25. Heinegard D, Inerot S, Wieslander J, Lindblad G. A method for the quantification of cartilage proteoglycan structures liberated to the synovial fluid during developing degenerative joint disease. Scand J Clin Lab Invest 1985;45:421-427.
26. Heinegard D, Saxne T. Macromolecular markers in joint disease. J Rheumatol Suppl 1991;27:27-29.

27. Hembry RM, Bagga MR, Dingle JT, Thomas PP, Reynolds JJ. Metalloproteinase production by rabbit articular cartilage: comparison of the effects of interleukin-1 alpha in vitro and in vivo. Virchows Arch 1994;425:413-424.
28. Henson FM, Schofield PN, Jeffcott LB. Expression of transforming growth factor-beta 1 in normal and dyschondroplastic articular growth cartilage of the young horse. Equine Vet J 1997;29:434-439.
29. Hiraki Y, Inoue H, Hirai R, Kato Y, Suzuki F. Effect of transforming growth factor beta on cell proliferation and glycosaminoglycan synthesis by rabbit growth-plate chondrocytes in culture. Biochim Biophys Acta 1988;969:91-99.
30. Horton WE Jr, Higginbotham JD, Chandrasekhar S. Transforming growth factor-beta and fibroblast growth factor act synergistically to inhibit collagen II synthesis through a mechanism involving regulatory DNA sequences. J Cell Physiol 1989;141:8-15.
31. Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci USA 1988;85:9552-9556.
32. Klinner DJ, Gorski JP, Thonar EJ. Keratan sulfate levels in sera of patients bearing cartilage tumors. Cancer 1987;59:1931-1935.
33. Kongtawelert P, Francis DJ, Brooks PM, Ghosh P. Application of an enzyme-linked immunosorbent-inhibition assay to quantitate the release of KS peptides into fluids of the rat subcutaneous air-pouch model and the effects of chondroprotective drugs on the release process. Rheumatol Int 1989;9:77-83.
34. Kongtawelert P, Ghosh P. A new sandwich-ELISA method for the determination of keratan sulphate peptides in biological fluids employing a monoclonal antibody and labelled avidin biotin technique. Clin Chim Acta 1990;195:17-26.
35. Kramer JW, Sleight SD. The isoenzymes of serum alkaline phosphatase in cats. Am J Vet Clin Path 1968;2: 87-91.
36. Landoni MF, Foot R, Frean S, Lees P. Effects of flunixin, tolfenamic acid, R(-) and S(+) ketoprofen on the response of equine synoviocytes to lipopolysaccharide stimulation. Equine Vet J 1996;28:468-475.
37. Leipold H, Goldberg RL, Lust G. Canine serum keratan sulfate and hyaluronate concentrations. Relationship to age and osteoarthritis. Arthritis Rheum 1989; 32:312-321.
38. Lust G, Summers BA. Early, asymptomatic stage of degenerative joint disease in canine hip joints. Am J Vet Res 1981;42:1849-1855.

39. Luyten F, Hascall VC, Nissley SP, Morales TI, Reddi AH. Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. Arch Biochem Biophys 1988;267:416-425.
40. MacDonald M, Stover SM, Willits NH, Benton HP. Regulation of matrix metabolism in equine cartilage explant cultures by interleukin 1. Am J Vet Res 1992;53:2278-2285.
41. Maroudas A. Biophysical chemistry of cartilaginous tissues with special reference to solute and fluid transport. Biorheology 1975;12:233-248.
42. Massague J. Transforming growth factor-alpha. A model for membrane-anchored growth factors. J Biol Chem 1990;265:21393-1396.
43. McQuillan DJ, Handley CJ, Campbell MA, Bolis S, Milway VE, Herington AC. Stimulation of proteoglycan biosynthesis by serum and insulin-like growth factor-I in cultured bovine articular cartilage. Biochem J 1986;240:423-430.
44. Mehraban F, Finegan CK, Moskowitz RW. Serum keratan sulfate. Quantitative and qualitative comparisons in inflammatory versus noninflammatory arthritides. Arthritis Rheum 1991;34:383-392.
45. Morales TI, Roberts AB. Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem 1988;263:12828-12831.
46. Morales TI, Joyce ME, Sobel ME, Danielpour D, Roberts AB. Transforming growth factor-beta in calf articular cartilage organ cultures: synthesis and distribution. Arch Biochem Biophys 1991;288:397-405.
47. Morris EA, Treadwell BV. Effect of interleukin 1 on articular cartilage from young and aged horses and comparison with metabolism of osteoarthritic cartilage. Am J Vet Res 1994;55:138-146.
48. Mort J, Dodge GR, Roughley PJ, Liu J, Finch SJ, DiPasquale G, Poole AR. Direct evidence for active metalloproteinases mediating matrix degradation in interleukin 1-stimulated human articular cartilage. Matrix 1993;13:95-102.
49. Okumura M, Fujinaga T. Evaluation of the role of keratan sulphate as a molecular marker to monitor cartilage metabolism in horses. Vet Med A 2000;47:99-105.
50. Okumura M, Tagami M, Fujinaga T. Consideration of the role of antigenic keratan sulphate reacting to a 1/14/16H9 antibody as a molecular marker to monitor cartilage metabolism in horses. J Vet Med Sci 2000;62:281-285.
51. Okumura M, Fujinaga T. Establishment of a monoclonal antibody (1/14/16H9) for detection of equine keratan sulfate. Am J Vet Res 1998;59:1203-1208.

52. Okumura M, Tagami M, Fujinaga T. Measurement of serum and synovial fluid keratan sulphate and antibody to collagen type II in equine osteoarthritis. Vet Med A 1998;45:513-516.
53. Okumura M, Fujinaga T, Urakawa E, Tagami M, Tsukiyama K. Evaluation of the catabolic activity of cartilage by measurement of serum keratan sulfate concentration in foals. Am J Vet Res 1997;58:925-929.
54. Palmer JL, Bertone AL. Joint structure, biochemistry and biochemical disequilibrium in synovitis and equine joint disease. Equine Vet J 1994;26:263-277.
55. Palmer JL, Bertone AL, McClain H. Assessment of glycosaminoglycan concentration in equine synovial fluid as a marker of joint disease. Can J Vet Res 1995;59:205-212.
56. Pickrell JA, Schluter SJ, Belasich JJ, Stewart EV, Meyer J, Hobbs CH, Jones RK. Relationship of age of normal dogs to blood serum constituents and reliability of measured single values. Am J Vet Res 1974;35:897-903.
57. Platt D, Bayliss MT. An investigation of the proteoglycan metabolism of mature equine articular cartilage and its regulation by interleukin-1. Equine Vet J 1994;26:297-303.
58. Poole AR, Witter J, Roberts N, Piccolo F, Brandt R, Paquin J, Baron M. Inflammation and cartilage metabolism in rheumatoid arthritis. Studies of the blood markers hyaluronic acid, orosomucoid, and keratan sulfate. Arthritis Rheum 1990;33:790-799.
59. Poole AR. Immunochemical markers of joint inflammation, skeletal damage and repair: where are we now? Ann Rheum Dis 1994;53:3-5.
60. Poole AR, Dieppe P. Biological markers in rheumatoid arthritis. Semin Arthritis Rheum 1994;23(Suppl 2):17-31.
61. Raker C. Clinical observations of bone and joint diseases in horses. Cornell Vet 1968;58:15-28.
62. Ratcliffe A, Doherty M, Maini RN, Hardingham TE. Increased concentrations of proteoglycan components in the synovial fluids of patients with acute but not chronic joint disease. Ann Rheum Dis 1988;47:826-832.
63. Revell PA, Muir H. The excretion and degradation of chondroitin 4-sulphate administered to guinea pigs as free chondroitin sulphate and as proteoglycan. Biochem J 1972;130:597-606.
64. Rogachefsky RA, Dean DD, Howell DS, Altman RD. Treatment of canine osteoarthritis with insulin-like growth factor-1 (IGF-1) and sodium pentosan polysulfate. Osteoarthritis Cartilage 1993;1:105-114.

65. Roos H, Dahlberg L, Hoerner LA, Lark MW, Thonar EJ, Shinmei M, Lindqvist U, Lohmander LS. Markers of cartilage matrix metabolism in human joint fluid and serum: the effect of exercise. Osteoarthritis Cartilage 1995;3:7-14.
66. Rosier RN, O'Keefe RJ, Crabb ID, Puzas JE. Transforming growth factor beta: an autocrine regulator of chondrocytes. Connect Tissue Res 1989;20:295-301.
67. Rossdale P, Hopes R, Digby NJ, Offord K,. Epidemiological study of wastage among racehorses 1982 and 1983. Vet Rec 1985;116:66-69.
68. Saxne T, Heinegard D, Wollheim FA. Therapeutic effects on cartilage metabolism in arthritis as measured by release of proteoglycan structures into the synovial fluid. Ann Rheum Dis 1986;45:491-497.
69. Saxne T, Glennas A, Kvien TK, Melby K, Heinegard D. Release of cartilage macromolecules into the synovial fluid in patients with acute and prolonged phases of reactive arthritis. Arthritis Rheum 1993;36:20-25.
70. Schalkwijk J, Joosten LA, van den Berg WB, van de Putte LB. Chondrocyte nonresponsiveness to insulin-like growth factor 1 in experimental arthritis. Arthritis Rheum 1989;32:894-900.
71. Sweet MB, Coelho A, Schnitzler CM, Schnitzer TJ, Lenz ME, Jakim I, Kuettner KE, Thonar EJ. Serum keratan sulfate levels in osteoarthritis patients. Arthritis Rheum 1988;31:648-652.
72. Thonar EJ, Lenz ME, Klintworth GK, Caterson B, Pachman LM, Glickman P, Katz R, Huff J, Kuettner KE. Quantification of keratan sulfate in blood as a marker of cartilage catabolism. Arthritis Rheum 1985;28:1367-1376.
73. Thonar EJ, Manicourt DM, Williams J, Lenz ME, Sweet MB, Schnitzer TJ, Otten L, Glant T, Kuettner KE. Circulating keratan sulfate: a marker of cartilage proteoglycan catabolism in osteoarthritis. J Rheumatol Suppl 1991;27:24-26.
74. Todhunter RJ, Yeager AE, Freeman KP, Parente EJ, Lust G. Keratan sulfate as a marker of articular cartilage catabolism and joint treatment in ponies. Am J Vet Res 1993;54:1007-1016.
75. Todhunter RJ, Fubini SL, Freeman KP, Lust G. Concentrations of keratan sulfate in plasma and synovial fluid from clinically normal horses and horses with joint disease. J Am Vet Med Assoc 1997;210:369-374.
76. Trippel SB. Growth factor actions on articular cartilage. J Rheumatol Suppl. 1995;43:129-32.
77. Tyler JA, Benton HP. Synthesis of type II collagen is decreased in cartilage cultured with interleukin 1 while the rate of intracellular degradation remains unchanged. Coll Relat Res 1988;8:393-405.

78. Tyler JA. Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. Biochem J 1989; 260:543-548.
79. Van den Hoogen BM, van de Lest CH, van Weeren PR, Lafeber FP, Lopes-Cardozo M, van Golde LM, Barneveld A. Loading-induced changes in synovial fluid affect cartilage metabolism. Br J Rheumatol 1998;37:671-676.
80. Van der Kraan PM, Vitters EL, van den Berg WB. Inhibition of proteoglycan synthesis by transforming growth factor beta in anatomically intact articular cartilage of murine patellae. Ann Rheum Dis 1992;51:643-647.
81. Williams JM, Downey C, Thonar EJ. Increase in levels of serum keratan sulfate following cartilage proteoglycan degradation in the rabbit knee joint. Arthritis Rheum 1988;31:557-560.
82. Xu C, Oyajobi BO, Frazer A, Kozaci LD, Russell RG, Hollander AP. Effects of growth factors and interleukin-1 alpha on proteoglycan and type II collagen turnover in bovine nasal and articular chondrocyte pellet cultures. Endocrinology 1996;137:3557-3565.

7. 和文要約

関節障害の程度およびその病態を具体的に把握することは、通常非常に困難である。関節障害発症機序のいずれの場面において滑膜炎が最も重要な役割を演じているかは明らかではないが、関節疾患の初期、つまりX線検査で明らかな病変が発見される前に関節内では軟骨の大きな傷害が発生する。初期の炎症性関節疾患における軟骨および骨病変の状態を評価するための感度の高い検査手法は、臨床獣医師が関節疾患のより正確な診断と適切な治療法を選択する上で有用である。

関節軟骨は、非常に多くの細胞外基質の中にその実質細胞である軟骨細胞が散在する特殊な組織である。通常の代謝過程において、プロテオグリカン分子は一定の速度で小断片に分解され、軟骨に隣接する体液中に拡散する。体液中に拡散したそれらプロテオグリカン断片は、コンドロイチン硫酸あるいはケラタン硫酸等のグリコサミノグリカンとして血液や尿中で確認される。ケラタン硫酸は、軟骨の細胞外基質に多く存在するグリコサミノグリカンであり（軟骨中に体内含量の99%が存在）、軟骨以外では角膜などの組織にわずかに存在するのみである。そのため、血中あるいは関節液中における代謝産物としてのケラタン硫酸を測定することは軟骨固有の基質代謝をほぼ反映しているものと考えられ、ケラタン硫酸は非侵襲的な軟骨代謝マーカーとして期待されるようになった。

本研究は、ウマにおいて関節軟骨代謝の変動から関節疾患の病態を把握することを目標に、1) ウマケラタン硫酸を特異的に認識するモノクローナル抗体を開発し、高感度のケラタン硫酸測定法を開発すること、2) ペレット培養法を応用した*in vitro*培養軟骨細胞を、インスリン様成長因子 (IGF)-1 α 、形質転換

成長因子 (TGF)- β 1 およびインターロイキン (IL)-1 α で刺激し、関節軟骨代謝を変動させて培養液中およびペレット中のケラタン硫酸濃度を、本研究で新たに開発した抗ウマケラタン硫酸抗体および抗ヒトケラタン硫酸抗体の2種類を用いて測定し、ケラタン硫酸測定の意義を評価すること、および3) ウマの血清および関節液を用いて、成長、機械的刺激による短期の代謝変動、実験的および自然発症の関節炎におけるケラタン硫酸濃度の変動を評価し、軟骨代謝におけるケラタン硫酸濃度測定の意義を検討することを目的とした。

まず、ウマの血清および関節液中ケラタン硫酸濃度を測定する酵素免疫測定法を開発するために、ウマのケラタン硫酸を特異的に認識する高感度のモノクローナル抗体 (1/14/16H9) を作成した。開発したモノクローナル抗体を用いて競合酵素免疫測定法を最適化し、臨床的に正常なウマおよび関節炎発症馬から得られた血清48材料および関節液18材料のケラタン硫酸濃度を測定した。最適化した測定法では、ケラタン硫酸濃度が10から160 ng/mlの範囲で測定でき、同一測定内誤差および時差測定間誤差がそれぞれ10.0%および12.7%であった。

次に、今回開発した抗ウマケラタン硫酸抗体1/14/16H9と抗ヒトケラタン硫酸抗体1/20/5D4に反応するケラタン硫酸の軟骨代謝マーカーとしての有用性を、*in vitro*培養軟骨モデルを用いて評価した。6か月齢のサラブレッド種ウマ3頭から関節軟骨を採取し、軟骨細胞に分離後、遠心管内でペレットとして培養した。軟骨細胞をIGF-I α 、TGF- β 1あるいはIL-1 α を添加した培養液で2週間培養し、ペレット中あるいはその培養液中のポリ硫酸化グリコサミノグリカン (GAG) およびケラタン硫酸 (1/14/16H9 および 1/20/5D4) 濃度を、それぞれ1,9-ジメチルメチレンブルー比色定量法および競合酵素免疫測定法によって定量した。培養液中のGAG濃度は、IGF-I α および TGF- β 1 添加によって

有意に増加した。ペレット中のGAG濃度は、IGF-I α の添加によって有意に増加したが、TGF- β 1の添加では有意な変化はみられなかった。一方、GAG濃度と抗ウマケラタン硫酸抗体1/14/16H9および抗ヒトケラタン硫酸抗体1/20/5D4に反応するケラタン硫酸濃度を比較したところ、IL-1 α 添加時にGAG濃度とケラタン硫酸は高い相関性（GAGと1/14/16H9抗体：0.87；GAGと1/20/5D4抗体：0.84）を示したが、IGF-I α 添加時には、その相関性は低かった（GAGと1/14/16H9抗体：0.43；GAGと1/20/5D4抗体：0.59）。

以上の結果から、これら2つの抗体（1/14/16H9および1/20/5D4）によって測定されるケラタン硫酸濃度は、主に軟骨異化を示すマーカーとして有用であると考えられ、場合によっては軟骨同化をも反映する可能性があると考えられた。

最後に、*in vivo*における軟骨代謝マーカーとしてのケラタン硫酸の臨床的な有用性を評価するため、今回作成した抗ウマケラタン硫酸抗体1/14/16H9および抗ヒトケラタン硫酸抗体1/20/5D4を用いて、ウマの成長過程、機械的刺激による短期の代謝変動、実験的および自然発症の関節炎における血清および関節液中ケラタン硫酸濃度を測定した。

出生直後から3か月齢までの血清ケラタン硫酸濃度（1/14/16H9および1/20/5D4）は4か月以降のそれと比較して有意に高値を示した。また、関節疾患を発症した3頭のウマの血清ケラタン硫酸濃度（1/14/16H9および1/20/5D4）は正常に発育した12頭のウマのそれに比較して著しく高値を示した。

トレーニング中のウマでは、3および4歳馬に比較して2歳馬で血清ケラタン硫酸濃度（1/14/16H9および1/20/5D4）が有意に高値を示した。常時運動を行っている2歳馬では6か月以上休養している2歳馬に比較して血清ケラ

タン硫酸濃度が有意に高値を示した。また、運動直後の血清ケラタン硫酸濃度（1/14/16H9および1/20/5D4）は、運動直前のそれに比較して有意に増加し、運動終了後1時間以内に運動直前のレベルに低下した。

キモパイン関節内投与によって実験的に変形性関節症を作出したウマにおける血清および関節液中のケラタン硫酸濃度を測定した。キモパイン投与3日目の1/14/16H9および1/20/5D4抗体で測定した血清ケラタン硫酸濃度は、投与前のそれぞれ1.9および1.4倍に増加した。同様に関節液中ケラタン硫酸濃度（1/14/16H9および1/20/5D4）はキモパイン投与3日目に、投与前の約4倍に増加した。また、変形性関節症を自然発症したウマにおける関節液中ケラタン硫酸濃度は、正常のウマのそれと比較して両抗体とも有意な変動はみられなかったが、血清中ケラタン硫酸濃度は、正常のウマのそれと比較して両抗体とも高い傾向が認められた。

今回作出した抗ウマケラタン硫酸抗体1/14/16H9および抗ヒトケラタン硫酸抗体1/20/5D4によって測定されたケラタン硫酸濃度は、生体のウマの関節における関節異化を理解する上で有用な指標になりうるものが、*in vitro*培養軟骨モデルを用いた試験で示された。また、生体の馬の血清および関節液を用いた試験においても、1/14/16H9抗体と1/20/5D4抗体それぞれに反応するケラタン硫酸濃度の比較によって、軟骨基質が異化あるいは同化作用をうけている状況を把握するのに有用であることが示された。ウマの関節疾患の病態を把握する上で、ケラタン硫酸の測定は特に軟骨の損傷を表現する、非侵襲的かつ直接的な情報を提供するマーカーであることが示唆された。

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