CHAPTER II

LITERATURE REVIEW

The review was divided into seven parts as follows:

I) Effect of orthodontic treatment on periodontium

II) The periodontal tissue (periodontium)

III) Extracellular matrix (ECM) of the periodontal tissue

IV) Glycosaminoglycans and chondroitin sulfate in periodontal tissue

V) Gingival crevicular fluid (GCF)

VI) Glycosaminoglycans (GAGs) in GCF associated with orthodontic tooth movement

VII) Methods for detecting GAGs in GCF

   Electrophoresis

   Enzyme-linked immunosorbent assay (ELISA)

VIII) Monoclonal antibodies (mAb) and mAb WF6

I) Effect of orthodontic treatment on periodontium

During orthodontic tooth movement, the forces transmitted to the tooth induce changes in the periodontium. Classically, the movement of the teeth has been explained via the pressure:a tension theory, in which bone is resorbed in areas perceived to be subjected to pressure and deposited at sites under tension. In this theory, an alteration in blood flow within the periodontal ligament is produced by the sustained pressure that causes the tooth to shift position within the periodontal ligament space, compressing the ligament in some areas while stretching it in the other. Blood flow is decreased where the periodontal ligament is compressed, while it is usually maintained or increased where the periodontal ligament is under tension. Alterations in blood flow quickly create changes in the chemical environment. These chemical changes, acting either directly

or by stimulating the release of other biologically active agents, would stimulate cellular differentiation and activity (Proffit, 2000). Therefore, the molecular mechanisms that induce bone remodeling still remain largely unknown. The current evidence suggests that periodontal ligament cells have a mechanism to respond directly to mechanical forces by activating a wide variety of mechanosensory signaling molecules. For example, the stretching of the periodontal ligament abruptly activates ion channels on cell membrane and changes in cytoskeletal organization. These alterations quickly induce the generation of intracellular secondary messengers, such as calcium ion, inositol triphosphate (IP3), and cyclic adenosine monophosphate (cAMP). Subsequently, an intermediate response to applied force includes the generation of arachidonic acid metabolites, such as prostaglandin and interleukin –1 (McCulloch *et al.*, 2000). The findings from one study have shown that prostaglandin and interleukin-1 levels increase within a short time after the application of pressure (Grieve *et al.*, 1994). Prostaglandin is an important mediator of the cellular response because it has the ability to stimulate both osteoclastic and osteoblastic activities (Proffit, 2000).

At the sites of alveolar bone compression, osteoclasts proliferate and an initial resorption of the superficial bone takes place. Osteoblasts are also involved in the initial response by producing collageneolytic enzymes to remove a portion of the unmineralized ECM of alveolar bone, thereby facilitating access for osteoclast precursors to the bone surface. In addition, osteoblasts produce chemokines and cytokines, which can attract monocyte precursors and promote osteoclast differentiation and activity. In contrast, at the site of tension, osteoblasts are activated to produce osteoid layers that are subsequently mineralized to form new bone (Sodek and McKee, 2000). The monitoring of acid and alkaline phosphatase activity in tissue and serum has been performed assess bone turn over rate. It was found that bone resorption induced the elevations in acid phosphatase levels, whereas bone formation was associated with higher alkaline phosphatase levels (Insoft *et al.*, 1996). However, a study examining the uptake and release of ^{3}H –thymidine and ^{3}H -proline by the periodontal ligament *in vivo* indicated that metabolic activity at both the pressure and tension sides of teeth

subjected to an orthodontic force was not different as mentioned above (Baumrind, 1969).

With regard to the molecular mechanisms, the applied mechanical forces produce a distortion of the ECM of the periodontium, resulting in alterations in the cellular shape. This creates short-lived configuration piezoelectric spikes that can lead to cellular activation by changing membrane potential and ion channel activity. This distortion also induces a release of neuropeptides from afferent nerve endings that cause vasodilatation and migration of leukocytes into the extravascular space. Thus, a wide variety of cytokines and growth factors are synthesized and secreted. In addition, as the capillaries are stretched or compressed excessively, tissue damage may occur. Such events lead to the secretion of ECM components, tissue degradation enzymes, and local factors. These local factors induce cellular proliferation and differentiation which promote wound healing and tissue remodeling. The degradation of ECM by enzyme is considered important in influencing cellular function via altering cell – matrix interaction, thus activating cell signaling pathways (Streuli, 1999). Moreover, some degraded components of ECM from periodontal tissue, such as GAGs, can diffuse into gingival crevicular fluid (GCF). Therefore, the changes in the deeper periodontal tissue, such as alveolar bone and periodontal ligament, will modify both the GCF flow rate and its components (Davidovitch, 1995). So, we may be able to take this advantage to predict the outcomes of orthodontic treatment by monitoring these degraded products released into GCF over a period of treatment.

II) The periodontal tissue (periodontium)

The periodontium is defined as supporting and investing tissue of the tooth. It consists of cementum, periodontal ligament, alveolar bone, and gingiva facing the tooth (Figure 2.1). In relation to orthodontic tooth movement, the primary function tissues are periodontal ligament and alveolar bone.
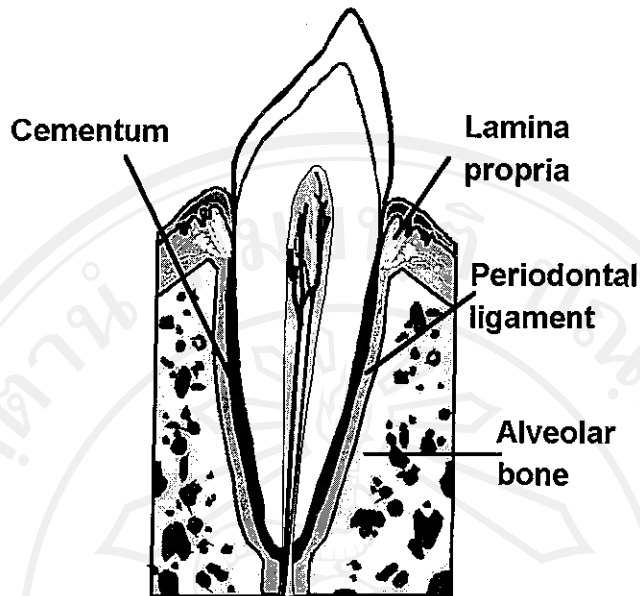
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Figure 2.1 Structure of periodontium in supporting the tooth (Shore, 2002. [Online].
available at http://www.dentistry.leeds.ac.uk/OROFACE/PAGES/chapt3.html)

The periodontal ligament is a complex, vascular, and highly cellular connective tissue about 0.2 mm in width. It situates between the root of the tooth and alveolar bone. It consists of two main parts, cellular and extracellular components. The cellular components include fibroblasts, osteoblasts, osteoclasts, epithelial cells rest of Malassez, macrophages, undifferentiated mesenchymal cells, and cementoblasts. The extracellular matrix (ECM) components of periodontal ligament consist of well – defined collagen fiber bundles and ground substance. The ground substance consists largely of proteoglycans, glycoproteins, and glycolipids (Ten Cate, 1998). The principal functions of periodontal ligament are to support the teeth and to withstand the considerable forces of mastication. The periodontal ligament also has another important function by acting as a sensory receptor. This function is necessary for the proper positioning of the jaw during normal function and in response to the orthodontic force.

The alveolar bone is a mineralized connective tissue that forms the primary supporting structure for teeth. It is composed of bundle bone, which is formed in layers in a parallel orientation to the coronal-apical direction of the tooth (Schroeder, 1992). A

thick outer layer of alveolar bone is cortical bone that extends from the jawbone and forms the lingual and labial surfaces of the alveolar process. Alveolar bone is largely made up of spongy cancellous bone. Within the cancelluos bone are numerous marrow spaces. However, there are smaller endosteal spaces in the cortical bone. Some of these small endosteal spaces extend into, and are contiguous with the periodontal ligament (Sodek and McKee, 2000). Similar to the periodontal ligament, the alveolar bone consists of two components, cellular and extracellular matrix (ECM) components. The cellular components include osteoblasts, osteocytes, bone-lining cells, and osteoclasts. The ECM components of alveolar bone consist of organic and inorganic matrix. The organic matrix is composed of collagen fiber and non-collagen proteins. The non-collagen proteins include osteonectin, osteocalcin, bone morphogenetic proteins, proteoglycans, and bone sialoprotein. The inorganic matrix is permeated by hydroxyapatites, which make up approximately 67% of total volume/weight (Ten Cate, 1998).

III) Extracellular matrix (ECM) of the periodontal tissue

The ECM provides important functions within the connective tissue of the periodontium in maintaining structural integrity and regulating cellular activity and function. The principal elements are collagenous fibrous networks providing structural support. The collagen fibers are embedded in and interact with non-collagenous matrix consisting of proteoglycans and various glycoproteins. Within connective tissue of periodontal tissue, proteoglycans represent an important and diverse family of ECM components (Waddington, 2001).

Proteoglycans are macromolecules being composed of a core protein to which one or more highly anionic glycosaminoglycan (GAG) chains are attached by covalent bonds. They look like bottle-brushes, and all attach to a central chain, made of hyaluronic acid. To a long strand of hyaluronic acid, several proteins, called core proteins, attach non-covalently. From these core proteins, which come out of the hyaluronic acid strand, several other glycosaminoglycans are linked covalently forming

glycosidic bonds, but instead of being linked to the oxygen of asparagine residues, they are N-linked to the amide nitrogen. (Figure 2.2)
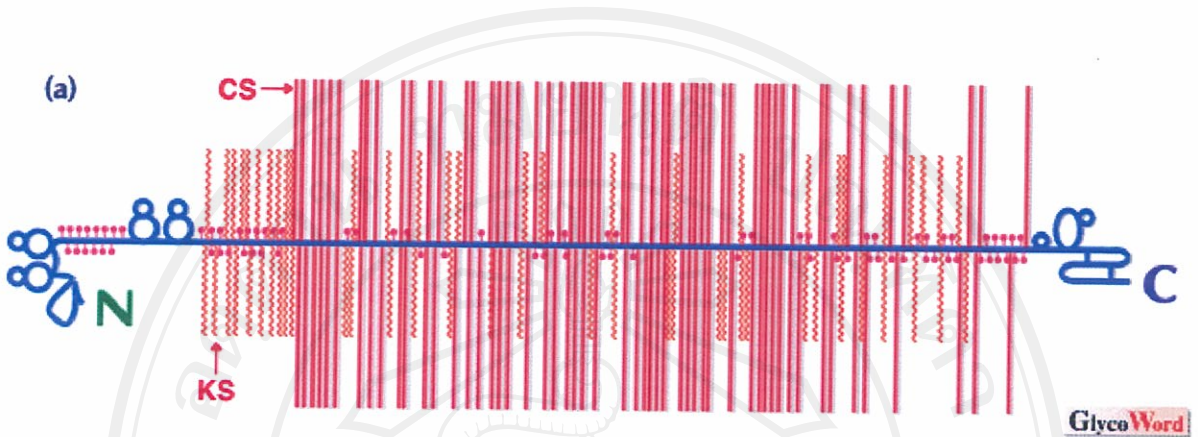

Figure 2.2   Models of typical proteoglycans. NH2 and COOH termini of core proteins are   indicated by N and C, respectively. Glycosaminoglycans are depicted by red lines (i.e., solid line, chondroitin sulfate / dermatan sulfate CS / DS; broken line, heparan sulfate HS; wavy line, keratan sulfate KS). (Hardingham, 2004. [Online]. Available at http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA00E.html)

The physical characteristics and biological functions of proteoglycans are partly determined by the physicochemical characteristic of the glycosaminoglycan chain, partly by the structure of core proteins, and interactions between proteoglycans and other ECM molecules (Embery et al., 2000). Proteoglycans have a variety of functions, including tissue hydration, regulation of collagen fibril formation, growth factor binding, and cell adhesion and growth (Ruoslahti and Yamaguchi, 1991).

IV) Glycosaminoglycans and chondroitin sulfate in periodontal tissue

In recent years, interests have been directed to a particular group of glycosaminoglycans (GAGs). GAGs are defined as linear polysaccharide units consisting of repeating disaccharide units, of which one is a hexosamine (either D-glucosamine or D-galactosamine) and the other is a hexuronic acid (D-glucuronic acid, L-galacturonic acid or iduronic acid). Seven species of GAGs exist: hyaluronic acid

(HA), chondroitin-4-sulfate (C-4-S), chondroitin-6-sulfate (C-6-S), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS). All except HA are invariably sulfated (Figure 2.3). (Waddington, 2001)
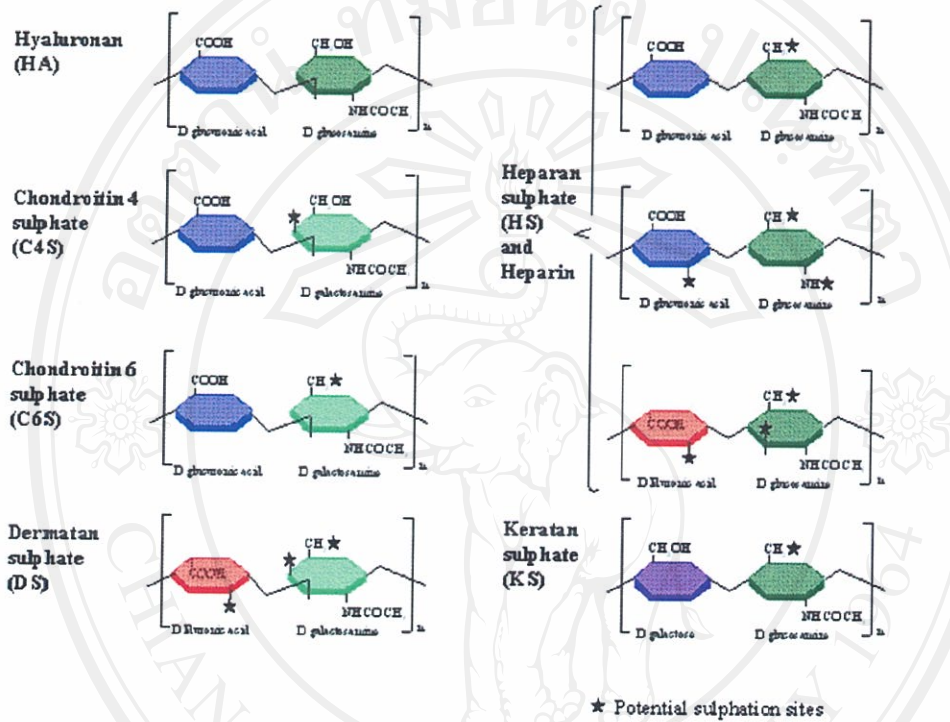

★ Potential sulphation sites

Figure 2.3 Classification and repeating disaccharide units of glycosaminoglycans
(Waddington, 2001)

DS and HS are present in the soft tissues of the periodontium, but not consistently found in alveolar bone. The non-sulfated GAG or HA is distributed throughout the periodontium, but present in particularly high amounts in gingiva (Kavadia-Tsatala et al., 2002). The chondroitin sulfate, like the HA, is widely present throughout the connective tissue of mammals (Bartold, 1987).

Chondroitin sulfate is the predominant glycosaminoglycan in alveolar bone proteoglycans. It consists of repetitive disaccharides formed by D-glucuronic acid and N-acetylgalactosamine residues, with a mean of one sulfate ester per disaccharide, which is bound to carbon 4 or 6 in the N-acetyl hexosamine residue. Chondroitin 4-sulfate and 6-sulfate can be present in the same proteoglycan molecule. Variations in

chain size, degree of sulfation, and chondroitin 4-sulfate/6-sulfate ratio are associated with tissue physiology, age of the individual, and the pathological state .Chondroitin sulfate is mostly composed of   C-6-S, C-4-S, and a minority of unsulfate (C-0-S). The ratio of C-4-S to C-6-S is greater in the calcified than that in the uncalcified tissue (Okazaki *et al.*, 1993). Chondroitin sulfate proteoglycans have been shown to aggregate non-covalently with hyaluronic acid, forming a much larger structure. They appear to exist *in vivo* in this aggregated form in the ground substance of cartilage, and have also been isolated from tendons, ligament, and aorta (Schwartz, 1986).

In periodontium, chondroitin sulfate has been identified as the predominant GAG in human alveolar bone, and C-4-S is a major component (Waddington *et al.*, 1989). However, C-6-S isomer is also present in a low amount but increases with age (Bartold, 1987). In the present study, we were interested in the changes of C-6-S levels in GCF which can be used to monitor the remodeling process of deep periodontal tissues during orthodontic tooth movement.

V) Gingival crevicular fluid (GCF)

GCF is an exudate of varying compositions found in the sulcus or periodontal pocket between the tooth and gingiva. It is a complex mixture of substance derived from the serum, leukocytes, structural cells of the periodontium, and oral bacteria (Uitto, 2003), as shown in Figure 2.4.

B = bacteria
D = degradation products
E = epithelium
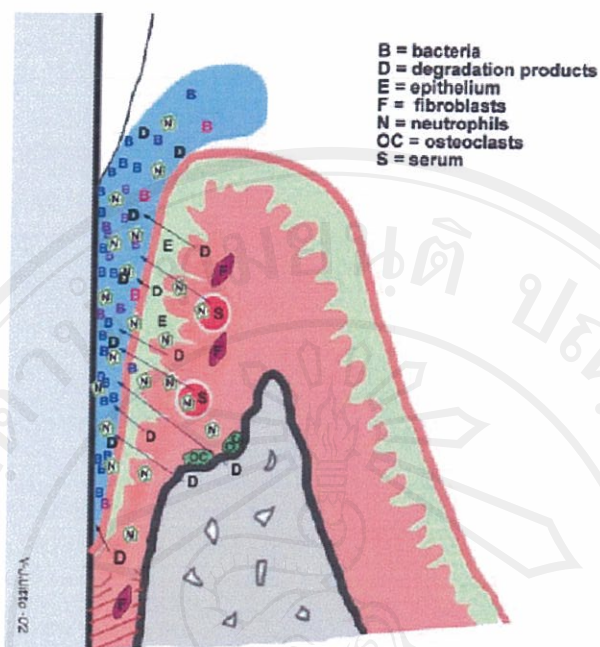F = fibroblasts
N = neutrophils
OC = osteoclasts
S = serum

Figure 2.4   Compositions of gingival crevicular fluid (Uitto, 2003)

In healthy gingiva, GCF represents the transudation of interstitial fluid in gingival tissue. The production of GCF is primarily a result of an increase in the permeability of the vessels underlying junctional and sulcular epithelium (Griffiths, 2003). In the course of gingivitis and periodontitis, GCF is transformed into true inflammatory exudates, which are induced by a chemical or mechanical means. GCF plays a special role in maintaining the structure of junctional epithelium and defending against microbial organisms in periodontal tissue. The major cellular components of the GCF include bacteria, epithelial cells, leukocytes, and erythrocytes (Delima and Van Dyke, 2003). The host–derived substances in GCF include antibodies, cytokines, enzymes, and tissue degradation products (Uitto, 2003). Many degradation products of the ECM from the periodontium can be used for analysis of the periodontal tissue condition. These molecules include, collagen degradation products (pyrimidine cross-linked carboxy-terminal telopeptide, and hydroxyproline peptide), osteocalcin, fibronectin, and proteoglycans (Giannobile et al., 2003).

VI) Glycosaminoglycans (GAGs) in gingival crevicular fluid associated with orthodontic tooth movement

The detection of GAGs in GCF was first reported by Embery *et al.* (1982). GAGs have been detected in GCF samples in sites around teeth affected by chronic gingivitis, chronic periodontitis, and juvenile periodontitis (Embery *et al.*, 1982; Last *et al.*, 1985). The presence of C-4-S and C-6-S in GCF samples has been associated with those clinical situations in which degradative changes occur in deep periodontal tissues. However, the C-4-S and C-6-S were not detected at sites affected by chronic gingivitis (Last *et al.*, 1985). Smith *et al.* (1995) found that the level of C-4-S was significantly higher at periodontitis sites compared to healthy periodontal tissue and correlated with increased alveolar bone destruction.

The detection of GAGs in GCF associated with orthodontic moved teeth was first reported by Last *et al.* (1988). They carried out a cross-sectional quantitative investigation by cellulose acetate electrophoresis of samples collected simultaneously from the mesial and distal surfaces of teeth in three groups of young persons (no treatment, active treatment with fixed appliances, or in an early retention phase). They detected the increase in C-4-S levels on the side of a tooth towards which pressure was being directed by an orthodontic appliance, but no significant increase in the levels of HA was detected at the mesial and distal surface of either the active or the retention group. Later, in a study conducted by Samuels *et al.* (1993), GCF was collected with microcapillary tubes alternately from the mesial and distal sides of a canine before and during orthodontic treatment in two groups of children, one undergoing therapy with fixed appliances and the other with functional appliances. Electrophoretic identification and densitometric quantification of GAG components were followed. Furthermore, they measured the vertical and horizontal movement of the tooth. They reported that the chondroitin sulfate (CS) levels were significantly increased from both vertically and horizontally moved teeth. This increase was unlikely to be due to the effects of plaque, gingival inflammation or pocket probing depth because their tendency to increase during treatment was not statistically significant. In addition, the movement of the canine

into the extraction site did not have a significant effect on the GAG levels in GCF, and the researchers concluded that the vertical movement might be important in producing the change in CS levels in GCF. Similarly, Baldwin *et al.* (1999) also found that the increase in CS levels was related to the amount of tooth movement and the presence of vertical movement.

In contrast, Last *et al.* (1988) reported that there were no significant differences in the CS levels between the mesial and distal sides of teeth undergoing active movement, although the CS levels at the distal side tended to be higher. Pender *et al.* (1994) carried out a study over a two-year period of orthodontic tooth movement and investigated the changes in the GAG components in GCF in 3 stages of orthodontic treatment (before orthodontic treatment, during retraction, and in retention stage). They found that the HA and CS levels were not significantly increased during active canine retraction compared with before orthodontic treatment. However, they observed a decrease in the CS levels in the retention stage.

In conclusion, the findings from these studies show the conflicting results about the association between changes in C-4-S levels in GCF and degradative changes in the deep periodontal tissues. Therefore in the present study, we were interested in the changes in CS levels particularly chondroitin-6-sulfate, present in human GCF in association with a potential diagnostic marker of periodontal tissue condition during orthodontic tooth movement.

VII) Methods for detecting GAGs in GCF

Electrophoresis

When a charged protein is placed in an electric field, it will migrate toward the oppositely charged region, and this is the basis of electrophoresis. In most electrophoresis methods, the molecules being analyzed are placed on a solid support and then allowed to migrate. For proteins, a polyacrylamide gel support is commonly used. The proteins are applied to the gel, and the gel is contained in an electrophoresis cell, which in turn is connected to a power supply that creates a positive and a negative

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electrode in the cell. Buffer is used to complete the circuit in the cell between the gel
and the electrode wires. The buffer in the cell and contained in the gel is important,
since its pH determines the charge on the protein molecules.

Usually, the determining factor in the separation of the molecules is their
charges. The more highly charged the molecule, the faster and farther it will move
during electrophoresis. With proteins, however, the second effect is seen, namely the
size of the protein. As a protein moves through the gel, it must overcome frictional
forces, which oppose its movement. The larger the protein, the greater the frictional
force. Thus, in most gels, the exact rate of movement of a particular protein depends on
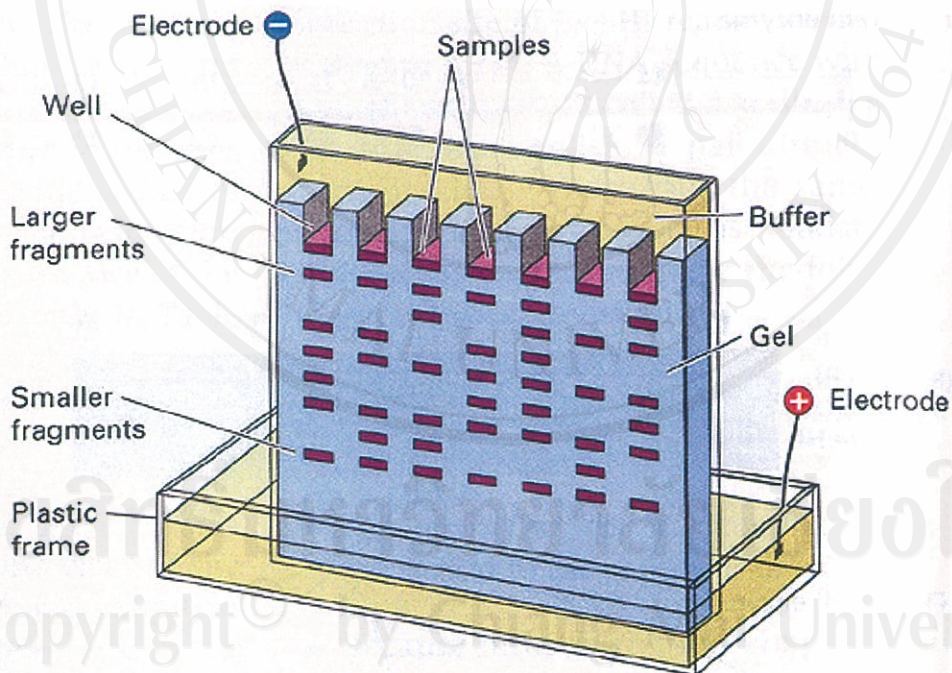both its charge and size. (Figure 2.5)

Figure 2.5 Separation of molecule after moving through an electric field (Krempels,
2005. [Online]. available at http://www.bio.miami.edu/dana/250/
25003_10.html)

One type of electrophoresis methods is SDS-gel electrophoresis. In this method, the proteins to be separated are denatured (usually in urea) and then mixed with the detergent sodium dodecyl sulfate (SDS). SDS binds along the length of the protein, obscuring its own charges and giving all proteins the same negative charge per unit length. Thus, charge is essentially removed as a factor in the separation and size alone becomes important. All proteins will move toward the positive electrode, but large proteins will move more slowly than small proteins. The distance moved is inversely proportional to the log of the molecular weight. It is therefore possible to run several proteins of known molecular weight in an SDS-gel electrophoresis procedure, measure their migration distances, and construct a calibration curve. The distance moved by a protein of unknown molecular weight can be compared to the standards and its size is determined. Some proteins are colored and can be seen directly on a gel, but most are colorless. To visualize most proteins, a staining procedure is needed.

Electrophoresis has been used to identify proteoglycan metabolites in GCF for a long time. This method is a lengthy procedure and requires manipulations of the sample. Therefore, it is not unsuitable for a quickly chair-side method for GAG quantification.

Enzyme-linked immunosorbent assay (ELISA)

ELISA is a widely-used method for measuring the concentration of a particular molecule (e.g., a hormone or drug) in a fluid, such as serum or urine. The molecule is detected by antibodies that have been made against it; that is, for which it is the antigen. This method is one of the immunoassays which use two different antibodies. The antibodies react with the antigen whose concentration is to be measured. A fixed quantity of one antibody is attached to a series of replicate solid supports, such as plastic microtiter wells. Test solution containing antigen at an unknown concentration or a series of standard solutions with known concentrations of antigen are added to the wells and allowed to bind. Unbound antigen is removed by washing, and the second antibody, which is enzyme linked or radiolabeled, is allowed to bind. The antigen serves as a bridge, so the more antigens in the test or standard solutions, the more enzyme-linked or radiolabeled second antibody will bind. The results from the standards are

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used to construct a binding curve for the second antibody as a function of antigen concentration, from which quantities of antigen in the test solutions may be inferred. When this test is performed with two monoclonal antibodies, it is essential that these antibodies see non-overlapping determinants on the antigen; otherwise, the second antibody cannot bind (Abbas *et al.*, 2000) (Figure 2.6).

The ELISA method can be used to detect GAGs in trace amounts of GCF; therefore, the GCF left after the first ELISA method can be used to detect other biomolecules. It can analyze many samples at a time, so it is appropriate in the epidemiological study. Furthermore, GAGs may be analyzed more quickly and readily by ELISA than by electrophoresis (Nishino *et al.*, 1990). In addition, the ELISA has the advantage of comparing GAGs to the historical observation using the same antibodies under the same experiment setup.
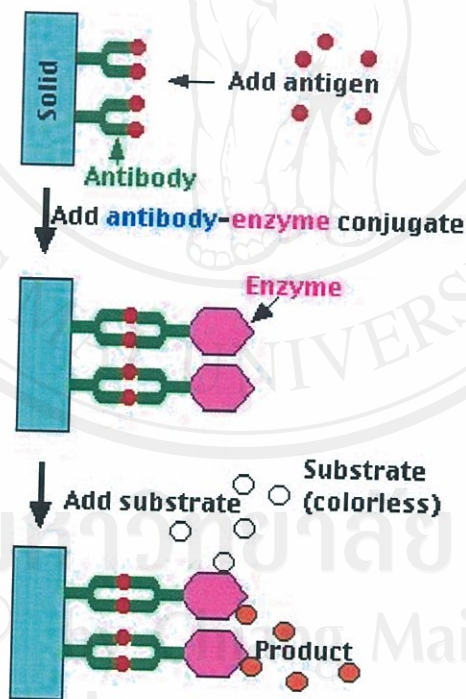

Figure 2.6 ELISA and monoclonal antibody in detecting antigen (Kimball, 2004. [Online] available at http://users.rcn.com/jkimball.ma.ultranet/Biology Pages /E/Elisa.html)

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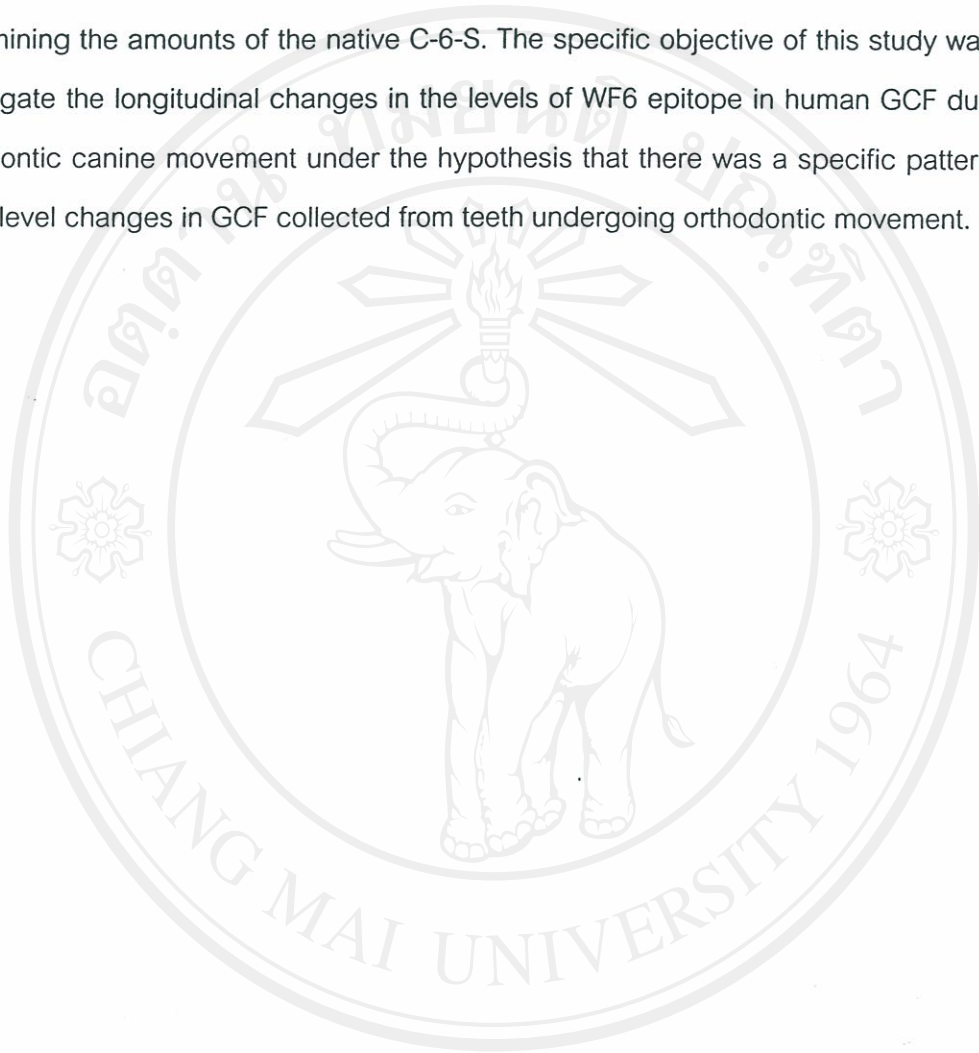
VIII) Monoclonal antibodies (mAbs) and mAb WF6

mAbs are the antibody molecules which direct against a specific determinant, or epitope, on an antigen molecule (Lodish *et al.*, 2000). The method for producing monoclonal antibodies is based on the fact that B-lymphocytes can produce antibodies which can react specifically with antigen. However, each B-lymphocyte cannot grow indefinitely; therefore, it is necessary to immortalize B cells that produce a specific antibody. This is achieved by cell fusion, or somatic cell hybridization, between a normal antibody-producing B cell and a myeloma cell, followed by selection of fused cells that secrete antibody of the desired specificity derived from the normal B cell (Abbas *et al.*, 2000).

mAb WF6 was transduced and characterized at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University. It recognizes an epitope on a native chondroitin-6 sulfate and a competitive ELISA method was then developed to detect the WF6 epitopes in human serum using aggrecan as standard (A1D1 fraction) (Pothacharoen, 2000). The synthesis of the mAb WF6 was described in a previous study (Tiengburanatam, 1996). Pothachareon (2000) reported that the levels of this epitope were higher in osteoarthritic patient's serum than in normal serum, and were also significantly higher in rheumatoid arthritis patient's serum. Therefore, it was suggested that the levels of WF6 epitope reflect the degradation of the cartilage similar to increased levels of 3B3 epitope in degenerative joint disease. However, the detection by using the mAb WF6 does not need the step of chondoitinase digestion like the detection by the mAb 3B3 because the mAb WF6 detects a native state of C-6-S.

The pattern of C-6-S level changes in GCF during orthodontic tooth movement has never been studied before. Consequently, we would like to determine changes of the C-6-S levels in the present study. This study would provide some useful data regarding the longitudinal changes of C-6-S levels in GCF during orthodontic canine movement, which will be beneficial for developing a quick chair-side diagnosis tool to assess the deep periodontal tissue condition in the future.

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In this study, mAb WF6 was used to detect the WF6 epitope of chondroitin-6-sulfate in GCF collected from the teeth undergoing orthodontic tooth movement by determining the amounts of the native C-6-S. The specific objective of this study was to investigate the longitudinal changes in the levels of WF6 epitope in human GCF during orthodontic canine movement under the hypothesis that there was a specific pattern of C-6-S level changes in GCF collected from teeth undergoing orthodontic movement.