CHAPTER III

MATERIALS AND METHODS

Materials

The following materials were used in this study

1. Orthodontic appliance
   - Preadjusted bracket ,Roth prescription 0.018" slot (3M,Unitek,USA)
   - Bonding agent (System 1+, Omco, USA)
   - Closed coil spring
2. Sample collection instrument
   - 1.5 ml Eppendorf tubes
   - Scissors
   - 2.0X10.0 mm filter paper strip (Whatman® No.1)
3. Chemical reagents and materials for an ELISA technique
   - Microtiter plates (Maxisorp®, Nunc)
   - Blue and yellow tips
   - 1.5 ml Eppendorf tubes
   - Multichannel pipette
   - Auto pipette
   - Tray
   - Shaker, vortex
   - IgM-specific peroxidase conjugated anti-mouse immunoglobulin
   - mAb WF6
   - PBS-tween
   - 1% w/v BSA
   - Peroxidase substrate

- 4M H_2SO_4

Methods

Informed consent

Prior to the collection of GCF from volunteers, an informed consent was obtained.

Experimental subjects

Eleven teeth from four orthodontic patients were selected in this study. Seven canines were used as experimental teeth and four incisors were used as control teeth. The inclusion criteria were:

1) Good general health.
2) Lack of antibiotic therapy during the past six months.
3) Absence of anti-inflammatory drug administration in the month before the study.
4) Healthy periodontal tissue and no radiographic evidence of periodontal bone loss.
5) Requirement of first premolar extraction and canine distal tooth movement as part of their orthodontic treatment plan.

Experimental design

Seven canines undergoing distal movement were induced in this study as the experimental teeth; four incisors were the control teeth. Orthodontic brackets or bands were placed on all teeth. For the orthodontic edgewise technique, 0.018x0.025 – inch preadjusted slot brackets (3M, Unitek Inc.) with transpalatal arch (0.9 mm stainless steel wire) were used. After appliance insertion, all teeth were leveled until the main archwire reached a 0.016x0.016 inch rectangular wire (stainless steel, Omco®). This treatment period was called a leveling phase (L phase). Subsequently, a canine was retracted with a closed coil spring on a 0.016x0.016-inch rectangular wire. The coil spring was approximately changed every 4-5 weeks to maintain force magnitude. A canine was moved distally until it contacted a second premolar. This treatment period

was called a movement phase. Thereafter, a canine was stabilized in this position with a ligature wire for 8 weeks. This treatment period was called a stop phase or S phase.

GCF collection

GCF samples were collected from all canines and incisors at base line (prior to orthodontic appliance insertion) and called as T0. After appliance insertion, the samples were collected every 4 weeks and recorded as an L phase (L0, L4, L8....). During the distal movement of canines, GCF samples were collected every week and called an M phase (M0, M1, M2, M3.....). After complete canine movement, GCF samples were collected every week for 8 weeks and called an S phase (S0, S1, S2 .....S8). The GCF collecting technique was performed by using the method of Offenbacher *et al.* (1986). Briefly, after tooth isolation from saliva and gentle air drying, Whatman® No.1 filter paper strips (2x10 mm) were placed into the gingival sulcus at mesial and distal of canines (Figure 3.1). Care was taken to avoid mechanical injury to the periodontal tissue. The filter paper strip containing the sample was cut (size 2.0x2.0 mm) and placed in a 1.5 ml Eppendorf tube. Samples collected from the mesial and distal side of the tooth were pooled and kept in the same Eppendorf tube. Then, the tube was labeled and stored at -80^{0} C until further processing.

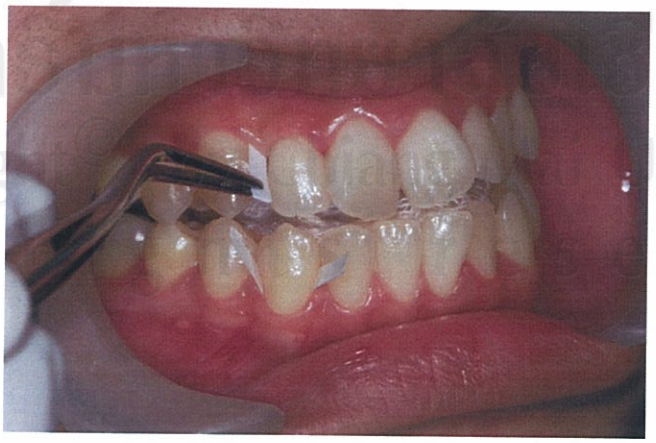

Figure 3.1 Paper strips used to collect GCF.

A Competitive Inhibition ELISA for mAb WF6

An ELISA method was performed as follows.

1. Microtiter plates (Maxisorp®, Nunc) were coated overnight at room temperature with 10 µg/ml shark PG-A1 fraction (100 µl/well) in the coating buffer.

2. In the following morning, plates were washed with PBS-tween (150 µl/well) three times and left air-dried.

3. Plate was then blocked with 150 µl/well of 1% (w/v) BSA in the incubating buffer for 60 min at 37 °C. After washing, 100 µl of the mixture, containing samples or a standard competitor (Shark PG-A1D1 fraction: range 39.06-10,000 ng/ml) in mAb WF6 (1:100), was added.

4. After incubation for 60 min at 37 °C, plates were washed and then the IgM-specific anti-mouse immunoglobulin conjugated with peroxidase (100 µl/well; 1:2,000) was added and incubated for 60 min at 37 °C.

5. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37 °C for 20 min to allow the color to develop.

6. The reaction was stopped by addition of 50 µl of 4M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

Protein assay

Total Protein concentration was determined using the Bio-Rad protein assay, based on the Bradford dye-binding procedure (Bradford,1976). It is a simple colorimetric assay for measuring total protein concentration. The Bio-Rad protein assay is based on the color change of Coomassie Brilliant Blue G-250 dye in response to various concentrations of protein. The dye primarily binds to basic (especially arginine) and aromatic amino acid residues. Bovine serum albumin (BSA) standards (0-1,000 µg/µl) and samples were added to the

microtitre plates (10 µl/well) in triplicate. A concentrated dye reagent and deionized distilled water were mixed together (1:4) and then added to each well (200 µl/well). The plates were incubated at room temperature for 5 min and the absorbance was measured at 620 nm. Protein concentrations were determined from a standard curve.