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Gingival crevicular fluid (GCF) levels of interleukin-6 (IL-6), soluble glycoprotein 130 (sgp130), and soluble interleukin-6 receptor (sIL-6R) during orthodontic tooth movement

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GINGIVAL CREVICULAR FLUID (GCF) LEVELS OF INTERLEUKIN-6 (IL-6), SOLUBLE GLYCOPROTEIN 130 (SGP130), AND SOLUBLE INTERLEUKIN-6 RECEPTOR (SIL-6R) DURING ORTHODONTIC TOOTH MOVEMENT

By

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Abstract

Introduction: Orthodontic tooth movement requires the precise coordination of a multitude of biological factors in order for proper bone remodeling to occur. Cytokines, such as Interleukin-6 (IL-6), play a critical role in the communication necessary for both bone resorption and bone apposition. IL-6 reacts with receptor proteins, such as soluble IL-6 receptor (sIL-6R), on target cells in order to transmit signals important to both osteoclast and osteoblast activities. Soluble glycoprotein 130 (sgp130), on the other hand, acts as a natural inhibitor to IL-6 activity. The purpose of this investigation was to evaluate the levels of IL-6, sIL-6R, and sgp130 in the gingival crevicular fluid (GCF) of human teeth undergoing orthodontic forces on both the tension and compression sides in the initial stages of orthodontic tooth movement. **Methods:** GCF samples were obtained from 9 healthy orthodontic patients [5 males, 4 females, age range 11 to 31 years (mean 17.445 years)] just prior to initial wire placement (0.012 or 0.014 Nickel Titanium archwire) with fixed orthodontic appliances. GCF samples were then collected 1, 6, and 24 hours after orthodontic force application. Patients returned in 5-7 weeks for a 2nd visit and orthodontic re-activation. GCF samples were again collected immediately before orthodontic activation, as well as 1, 6, and 24 hours after force application. Sampling sites included the mesiobuccal and distolingual of an experimental tooth, as well as the mesiobuccal of a control. GCF volumes were assessed with a Periotron 6000 (OraFlow, Smithtown, New York), while a Bradford assay was performed to obtain protein levels. Processing was carried out with a multiplex bead-based Luminex assay to detect IL-6, sgp130, and sIL-6R levels in the GCF samples. **Results:** GCF volumes were significantly higher in the experimental samples at both the initial (p=0.009) and recall

visits (p=0.055). The greatest difference between GCF volumes for the experimental and control sites was after 6 hours of orthodontic activation (p=0.02). Relative to the control site, GCF IL-6 levels were significantly elevated for the mesiobuccal experimental site at 6 (p=0.001) and 24 hours (p=0.004) post-activation, whereas the IL-6 levels for the distobuccal experimental site were only elevated at 24 hours post-activation (p=0.034). GCF sgp130 levels were elevated for the mesiobuccal experimental site at 1, 6, and 24 hours post-activation relative to both the distobuccal experimental site (p=0.005, p=0.012, p=0.001) and the control site (p=0.005, p=0.001, p=0.000). Similarly, GCF sIL-6R levels were also elevated for the mesiobuccal experimental site at 1, 6, and 24 hours post-activation relative to both the distobuccal experimental site (p=0.035, p=0.029, p=0.001) and the control site (p=0.037, p=0.006, p=0.006). **Conclusions:** GCF volumes increase in a time-dependent fashion after orthodontic forces are applied to a tooth. Meanwhile, IL-6 levels in the GCF of orthodontically treated teeth increase earlier than previously reported. In the present study, IL-6 levels peaked at approximately 6 hours post-activation. Additionally, sgp130 and sIL-6R levels in the GCF are elevated throughout the first 24-hour post-activation period in orthodontic tooth movement. While there was a definitive difference in protein levels between opposing sides of the experimental teeth, further research is needed to differentiate the levels of IL-6, sgp130, and sIL-6R on the compression and tension sides during orthodontic tooth movement.

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Introduction

The study of bone remodeling was introduced in 1892 when German surgeon, Julius Wolff, proposed that a transformation of both the internal and external bone architecture occurs in response to prolonged stresses acting upon it (Frost, 2004). Since that time, Wolff's Law has been updated, modified, and expounded upon as researchers continue to explore the complex field of bone biology. An understanding of bone remodeling is of particular interest to the profession of orthodontics.

Unperturbed, the native pattern of alveolar bone remodeling maintains a homeostatic state. The introduction of orthodontic treatment, however, disrupts the equilibrium between bone apposition and bone resorption (Wise and King, 2008). When orthodontic forces are applied to a tooth, they are transmitted to the periodontal ligament (PDL) and adjacent alveolar bone. These forces, in turn, initiate a complex cascade of events that result in the remodeling of the surrounding bone and eventual movement of the tooth (Masella and Meister, 2006). While the sequence of events at the tissue and cellular levels of bone remodeling is well described, there remains a lack of comprehensive understanding in the coordination of biochemical events at the molecular level (Krishnan and Davidovitch, 2006). As such, there have been numerous studies that have sought to further elucidate the molecular biology of bone remodeling during orthodontic tooth movement.

In order to examine the molecular biology involved, a cellular understanding of tooth movement is prerequisite. Osteoclasts are the primary bone resorbing cells in the body, whereas osteoblasts are the primary bone forming cells. A large number of osteoclasts are recruited to the resorptive front during tooth movement (Rygh, 1974;

Rody *et al.*, 2001). As the tooth begins to move into the resorbed area, osteoblasts proliferate and begin to secrete the precursors of new bone in the vacated area (King *et al.*, 1991; Roberts and Chase, 1981). Cycling between osteoclast and osteoblast activity, the body must maintain a type of equilibrium in order to obtain efficient bone remodeling during tooth movement.

While there are numerous interconnected systems in place that control the levels of mature osteoclasts and osteoblasts active during bone remodeling, one predominate pathway uses the cytokine family interleukin-6 (IL-6) as a signal transducer (Liu *et al.,* 2006). Most importantly, IL-6 plays a critical role in the differentiation of both osteoclasts and osteoblasts. Osteoclasts are derived from hematopoietic precursors in the bone marrow, whereas osteoblasts are mesenchymal in origin (Manolagas, 1998). Regardless of the cell's origin, however, IL-6 type cytokines stimulate the differentiation of the respective precursors into osteoblasts and osteoclasts (Blanchard *et al.,* 2009). IL-6 also interacts with other factors that are critically involved with bone homeostasis, including the receptor activator of nuclear factor kappa-β (RANK)/receptor activator of nuclear factor kappa-β ligand (RANKL)/osteoprotegerin (OPG) system, sex steroids, prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF)-α, parathyroid hormone (PTH), IL-11, and IL-1 (Liu *et al.*, 2006). The natural inhibitor of these IL-6 signaling responses is soluble glycoprotein 130 (sgp130) (Jostock *et al.*, 2001).

While IL-6 has been extensively studied in other models of bone metabolism, the investigation of the role of IL-6 during orthodontic tooth movement is relatively limited. A convenient and non-invasive method to study molecules involved with tooth movement is gingival crevicular fluid (GCF) analysis. Using this approach, the presence

of many inflammatory factors has been identified, and the role these factors play in orthodontic tooth movement has become more clearly understood (Ren and Vissink, 2008; Kavadia-Tsatala *et al.*, 2002). While the increased levels of IL-6 during orthodontic tooth movement have been confirmed by GCF analysis (Uematsu *et al.*, 1996), a definitive temporal pattern has yet to be established. Furthermore, a comparison between levels of IL-6 and the natural inhibitor, sgp130, has not been examined during orthodontic tooth movement. The goal of this research, therefore, is to gain a better understanding of IL-6, sgp130, and sIL-6R by determining whether a clear relationship exists between their respective levels in bone remodeling, and by establishing a temporal pattern of their production relative to initial orthodontic force application. Understanding the relationship between orthodontic mechanics and the levels of IL-6 and sgp130 could assist the clinician with providing optimal treatment. Additionally, since tooth movement is a reliable model of overall bone metabolism, the results of this study will aid in more clearly understanding somatic bone remodeling and repair. Furthermore, orthodontic tooth movement and periodontal disease involve similar factors, and knowledge of the cytokines involved in both processes may lead to an increased comprehension of the complications involved with tooth movement such as loss of attachment and external apical root resorption. Lastly, future research may provide the clinician with novel techniques that make use of the complex role of IL-6 in orthodontic tooth movement.

Literature Review

Orthodontic Tooth Movement

At first glance, it may appear that orthodontic tooth movement can be explained by simple physics: a force is applied to a tooth until friction is overcome and the tooth begins to move. Whereas this may explain the interface between the tooth and the orthodontic appliance, it does not address the biological mechanisms involved. Orthodontic tooth movement is a unique phenomenon where a solid object moves through a solid medium. Therefore the biological intricacies involved with this type of movement are novel and not entirely understood. As orthodontic forces are applied, the mechanical loading within the system increases, and a complex cascade of biological events lead to the remodeling of the surrounding tissues and eventual movement of the tooth (Masella and Meister, 2006).

Theories of Orthodontic Tooth Movement

Historically, there have been several major theories of orthodontic tooth movement, essentially revolving around two themes; namely, the periodontal ligament and the alveolar bone. Presently, the most widely accepted theory explaining orthodontic tooth movement is referred o as the pressure tension theory.

Pressure Tension Theory. According to the pressure tension theory, whenever a tooth is subjected to an orthodontic force, areas of pressure and tension are created within the periodontal ligament space. The periodontal ligament is compressed on the side opposite of the force causing an increase in pressure, whereas the periodontal ligament on the same side of the force increases in tension (Midgett *et al.*, 1981). The changes in pressure and tension within the periodontal ligament space initiate cellular changes

produced by chemical messengers. On the pressure side, there is a decrease in cellular replication as a result of vascular constriction, whereas on the tension side, there is an increase in cellular replication because of the stimulation afforded by the stretching of the fiber bundles of the periodontal ligament (Midgett *et al.*, 1981).

Contemporary Pressure Tension Theory. In 2008, Henneman proposed a theoretical model that modified and updated the pressure-tension theory. This model divides tissue and cellular responses to force application into four stages: (1) matrix strain and fluid flow, (2) cell strain, (3) cell activation and differentiation, and (4) tissue remodeling (Henneman, 2008).

Stage 1. The first stage occurs when tooth movement is initiated by the increased mechanical loading of an orthodontic force. The initial movement of the tooth within the periodontal ligament space results in a positive strain on the bone apposition side of the periodontal ligament (Reitan, 1951). The side of bone apposition is often referred to as the tension side in the pressure-tension theory but may be more accurately described as the appositional side. This positive strain creates a stretching of the collagen fibers connecting the tooth to the bone (Rygh *et al.*, 1986; Melsen, 1999), which in turn, can activate residing periodontal cells such as osteoblasts and fibroblasts. The side opposite of the appositional side, on the other hand, undergoes a negative strain within the periodontal ligament (Reitan, 1951), and is commonly referred to as the pressure side. This term, however, can be somewhat misleading as the collagen fibers on this side actually relax upon force application due to the negative strain (Melsen, 1999; Binderman *et al.*, 2002). Therefore, it may be more accurately referred to as the resorptive side. As negative strain occurs on the resorptive side, the fluid within the periodontal ligament

space undergoes an almost immediate fluid flow (Henneman, 2008). The fluid flow on both the appositional and resorptive sides may activate entrapped osteocytes within the bone through canaliculi (Weinbaum *et al.*, 1994; Cowin *et al.*, 1995; Mak *et al.*, 1997). This concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data (Klein-Nulend *et al.*, 1995c; Ajubi *et al.,* 1996; Owan *et al.*, 1997; Westbroek *et al.*, 2000; Tan *et al.*, 2006) and *in vivo* data (Pitsillides *et al.*, 1995; Turner *et al*., 1995, Knothe Tate *et al.*, 2000).

Stage 2. Cell strain is the next step in the response to orthodontic tooth movement. Collagen fiber tension and fluid flow cause deformation of periodontal ligament cells through a network of cell-matrix attachments (Beertsen *et al*., 1997; Davidovitch, 1991). These cells include both fibroblasts and osteoblasts. This strain is then transferred within the cell by the focal adhesion complex to the cytoskeleton and protein kinases that initiate intracellular signaling pathways (Hynes, 1992; Wang and Thampatty, 2006). These pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins (Davidovitch *et al.*, 1988; Alhashimi *et al.*, 2001; Bakker *et al.*, 2003; Mullender *et al.*, 2004; Tang *et al.*, 2006; Klein-Nulend *et al*., 1995b; Westbroek *et al*., 2000) that activate several types of cells.

Stages 3 & 4. The last two stages occur simultaneously and in conjunction with one another. The mediators released by the activated periodontal cells stimulate osteocytes and a variety of periodontal ligament cells including osteoblasts, fibroblasts, and osteoclast-precursors (Henneman, 2008). Osteocytes play a key role in both the resorptive and apposition sides. On the appositional side, activated osteocytes release mediators, which stimulate precursors in the periodontal ligament to differentiate into

active osteoblasts (Dereka *et al.*, 2006). Meanwhile, osteocytes release cytokines, which stimulate the differentiation of precursors into osteoclasts on the resorptive side (Yoo *et al.*, 2004). These osteoclast associated regulating cytokines have been shown *in vitro* and *in vivo* to include colony-stimulating factor, receptor activator of nuclear factor kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins (Nomura and Takano-Yamamoto, 2000; Zhao *et al.*, 2002; Kurata *et al.*, 2006; Shiotani *et al*., 2001).

After osteoclast differentiation has begun, the osteoclasts are stimulated by osteoblast and osteocyte released osteopontin to begin attaching to the bone surface by specific integrins (Gay and Weber, 2000). These attached osteoclasts undergo a morphological change and differentiate internally into functional zones. The clear zone isolates the bone surface; the body of the osteoclast contains an extensive lysosomal system; and the ruffled border is the site of actual bone resorption (Hill, 1998). It is at this site that hydrogen ions are released to dissolve the bone, and enzymes resorb residual organic matrix (Teitelbaum *et al.,* 1997; Phan *et al.,* 2004). As the bone undergoes resorption, space is created to allow movement in the direction of force application.

The appositional side, meanwhile, is undergoing bone formation that is mediated by the production of alkaline phosphatase, osteocalcin, and other non-collagenous proteins (Matsuda *et al.*, 1998; Ozaki *et al.*, 2005; Yang *et al.*, 2006). These cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including

prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β (Nahm *et al.,* 2004; Apajalahti *et al.,* 2003; Takahashi *et al.,* 2003, 2006; Ingman *et al.,* 2005; Owan *et al.*, 1997; Howard *et al.,* 1998; You *et al.,* 2000; Yamaguchi *et al.,* 2004).

Molecular Level of Tooth Movement

As discussed, orthodontic tooth movement is a complex process involving many types of cells, mediators, and a complex system of cytokine cascades. It is important to understand tooth movement on a cellular and molecular level in order to better control and modify it clinically.

Cell types. Bone remodeling is primarily a function of three cell types: osteoclasts, osteoblasts, and osteocytes. Osteoclasts are multinucleated giant cells of hematopoietic origin that have the capacity to resorb mineralized tissue. These descendants of the monocyte-macrophage lineage can be distinguished by their polarized morphology and ruffled border. Osteoblasts, on the other hand, are derived from bone marrow mesenchymal stem cells and are the principal cells of bone matrix apposition. These bone-forming cells are progressively transformed into osteocytes as their own secretion products trap the cells. Bone remodeling is the result of the coordinate and interactive effects of these three osteogenic cell types. Through cell-cell contacts and soluble factors, a complex balance between bone resorption and bone apposition is maintained.

Cytokines. As in most biological functions, cytokines are the soluble factors primarily involved in the coordinate activities of osteogenic cells. While typically only present in nanomolar-to-picomolar concentrations (Heinrich *et al.*, 1998), cytokines regulate the proliferative, differentiative, and maturation events in most cells throughout

the body. Unlike hormones, which are stored for later secretion, cytokines are usually rapidly synthesized locally and then immediately secreted when stimulated (Heinrich *et al.*, 1998). When the two communicating cells are in close proximity, the rapidity of synthesis and secretion can make cytokine detection difficult.

Cytokines are often described as pleiotropic due to the variety of effects they can stimulate depending on the nature of the target cell (Simpson *et al.*, 1997). Some cytokines may elicit agonistic or antagonistic effects on the same target cell depending on the surrounding factors. In addition to the pleiotropism that they may exhibit, cytokines often display overlapping biological activities and may even share the same receptors (Simpson *et al.*, 1997). A plethora of cytokines are involved in bone metabolism including RANK, RANKL, OPG, bone morphogenetic proteins (BMPs), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 11 (IL-11), and TNF-α amongst many others (Krishnan & Davidovitch, 2009).

Osteogenic Cytokines. A system of cytokines of particular significance in understanding the relationship between osteoclastogenesis and osteoblastogenesis in tooth movement is the OPG/RANKL/RANK system. The discovery of osteoprotegerin was the first step in understanding this complex interaction. The molecule was discovered independently by two groups conducting contrasting experiments. While studying therapeutic utilities of TNF receptor-related molecules, the Amgen, Inc. group stumbled upon a relatively innocuous protein whose overexpression caused marked osteopetrosis in rats (Simonet *et al.*, 1997). It was found that this was due to a decrease in osteoclasts, indicating that OPG played a clear role in regulating osteoclastogenesis. The Snow Brand Milk Group, meanwhile, was systematically searching for a theoretical

molecule fulfilling the role that OPG occupies now when they discovered the same protein as the Amgen, Inc. group (Yasuda *et al.*, 1998). OPG has since undergone significant research to further characterize the protein (Khosla, 2001).

RANK/RANKL/OPG System. The discovery of OPG warranted the search for an osteoclast differentiation factor expressed on osteoblastic cells that was essential for osteoclast development. The identification of RANKL as this protein was quickly realized by the two previously mentioned groups (Lacey *et al.*, 1998; Yasuda *et al.*, 1998). RANKL was shown to play a key role in osteoclast differentiation stimulation, osteoclast activity, and the inhibition of osteoclast apoptosis (Malyankar *et al.*, 2000; Lacey *et al.*, 1998; Fuller *et al.*, 1998). RANK had previously been identified as the receptor for RANKL, so its involvement in osteoclastogenesis regulation became readily apparent (Anderson *et al.*, 1997).

The interaction between the three proteins is actually quite simple. Osteoblasts produce both osteoclastogenesis-stimulating RANKL and osteoclastogenesis-inhibiting OPG (Kearns *et al.*, 2008; Khosla, 2001). If both RANKL and OPG are released by the osteoblastic cell, then OPG binds to RANKL that effectively inhibits the osteoclastogenic activity of RANKL (Schneeweis *et al.*, 2005). If OPG is not present, however, then RANKL is free to bind to its natural receptor, RANK (Nakagawa *et al.*, 1998; Kearns *et al.*, 2008). The RANK receptor is located on osteoclast precursor cells, and when bound by RANKL, it promotes the differentiation of the cell into a mature osteoclast (Khosla, 2001; Kearns *et al.*, 2008). Through this system, bone remodeling is regulated by a balance between RANK-RANKL binding and OPG production (Theoleyre *et al.*, 2004).

Interleukin-6 and Orthodontic Tooth Movement

As explained, bone tissue remodeling results from the coordinate activities of osteoblasts, osteoclasts, and the RANK-OPG system. An important integrating factor in all of these elements is interleukin-6 (IL-6), a protein that functions as a cytokine in a myriad of biological functions including bone metabolism. Derived primarily in the bone marrow from osteoblastic cells, IL-6 is unique in that it may function in contradictory roles depending on the target cell (Kwan Tat *et al.*, 2004; Liu *et al*., 2006). In fact, with a role in both bone apposition and bone resorption, IL-6 is a critical factor in skeletal homeostasis as well as bone remodeling (Manolagas and Jilka, 1995; Martin *et al.*, 1998; Suda *et al.*, 1999).

Signaling through the IL-6 Receptor. IL-6 signal transduction begins with the binding of IL-6 to IL-6Rα, a glycoprotein that lacks intrinsic signaling properties (Franchimont *et al.*, 2005). The binding of IL-6 to IL-6Rα, in turn, stimulates the association of the IL-6/IL-6Rα complex with the signal-transducing receptor, glycoprotein 130 (gp130) (Kamimura *et al.*, 2003). Since bonding is between a pair of IL-6/IL-6Rα complexes and two gp130 molecules, this newly formed complex is hexameric in structure (Franchimont *et al.*, 2005). While IL-6Rα is often a membrane bound protein, the soluble form (sIL-6Rα) may initiate the activation of the signal transduction pathway as well (Franchimont *et al.*, 2005). This agonistic process, referred to as *transsignaling*, can been seen on cells expressing only gp130, and allows IL-6 to function in most parts of the body (Franchimont *et al.*, 2005; Jones *et al.*, 2001; Kamimura *et al.*, 2003). In contrast to sIL-6Rα, soluble gp130 (sgp130) acts in an antagonistic manner and may take part in turning off gp130 signaling (Kamimura *et al.*,

2003; Franchimont *et al.*, 2005). According to Franchimont (2005), the levels of sgp130 present in the bone microenvironment in physiological conditions or in inflammation states are currently unknown.

After the activation of the gp130 complex is initiated, signal transduction continues via the activation of Janus kinases (JAK), which leads to the activation of one of the two major signaling pathways: the mitogen-activated protein kinase (MAPK) cascade, or the signal transducers and activators of transcription (STAT) cascade (Kwan Tat *et al.*, 2004). These two major pathways are dependent on one another both qualitatively and quantitatively (Franchimont *et al.*, 2005). With the use of knock-in experimentation, it has also been shown that silencing of one pathway will lead to an exaggerated activation of the remaining signaling pathway (Ernst & Jenkins, 2004; Heinrich *et al.*, 2003).

IL-6 Type Cytokines. Since gp130 has been identified as the IL-6 signal transducer, other cytokines have been found to use gp130 as a receptor subunit as well (Kamimura *et al.*, 2003). Cytokines using gp130 as a signal transducer are commonly referred to as IL-6 Type Cytokines and include IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), leukemia-inhibitory factor (LIF), cardiotrophin-like cytokine (CLC), neuropoietin (NP), and oncostatin M (OSM) (Blanchard *et al.*, 2009). Similar to the complex formed by IL-6 and IL-6Rα, most IL-6 Type Cytokines bind to a ligand-specific receptor prior to interacting with the gp130 receptor subunit (Kamimura *et al.*, 2003).

Since all IL-6 Type Cytokines use gp130 as a signal transducer, there is a large amount of redundancy in biological activities among the included cytokines (Kamimura

et al., 2003). For example, all known IL-6 Type Cytokines can initiate the production of acute-phase proteins (APPs) (Kamimura *et al.*, 2003). Several *in vivo* studies have indicated, however, that some effects are specific to individual IL-6 Type Cytokines and that each of these cytokines could have a predominant role in a particular pathophysiological situation (Blanchard *et al.*, 2009).

IL-6 Signal Orchestration Model. As has been discussed, IL-6 acts upon a multitude of different cells throughout the body, and may even initiate different signal pathways in the same cell type. In addition to this, there may be similar IL-6 Type Cytokines acting in coordination with the IL-6 induced signaling pathways. Using both *in vivo* and *in vitro* experimentation, Kamimura *et al.* (2003) found that gp130 can even simultaneously activate contradictory signals in a given target cell. It is theorized that the overall balance of these distinct signals could determine the final biological outputs by a given ligand in a target cell (Kamimura *et al.*, 2003). This theory is termed the *signal orchestration model* (Hirano *et al.*, 1997; Hirano, 1999; Hirano and Fukada, 2001). This theory may also aid in the explanation of how a pleotropic cytokine like IL-6 can exert multiple functions in a variety of cells. The target cells may be exposed to a variety of cytokine signaling factors. The complex interaction among different signaling networks can influence the activation state of a certain pathway, which will then modulate the balance of the signaling, and effectually determine the biological outputs (Kamimura *et al.*, 2003). With regards to orthodontic tooth movement, the *signal orchestration model* provides an explanation for the involvement of IL-6 in both bone resorption and bone apposition.

Effects of Interleukin-6 on Bone Resorption. Derived from hematopoietic precursors in the bone marrow (Roodman, 1996), osteoclasts are the primary cells involved in bone resorption. These highly specialized, multinucleated cells dissolve crystalline hydroxyapatite and degrade organic bone matrix rich in collagen fibers by use of specialized machinery associated with the osteoclasts unique cell structure (Vaananen *et al*., 2000). Osteoclast activity may either be modulated directly or indirectly via a host of regulatory factors including growth factors, hormones, and cytokines such as IL-6 (Liu *et al.*, 2006).

IL-6 can indirectly stimulate osteoclastic activity by increasing interactions between osteoblasts and osteoclasts via the RANK/RANKL/OPG system (Blanchard *et al.*, 2009). Interestingly, the protein will stimulate osteoclast activity when co-cultured with osteoblasts, but has no osteoclastic activity in an osteoclast culture devoid of osteoblasts (Kwan Tat *et al.*, 2004). In fact, some studies have shown that IL-6 may have an inhibitory role on osteoclast differentiation and bone resorption when obtained from purified osteoclast precursors (Blanchard *et al.*, 2009). Other studies have shown that along with IL-11 and LIF, IL-6 can stimulate the osteoblastic secretion of several downstream effectors such as RANKL, which then activate osteoclast activity and differentiation (Blanchard *et al.*, 2009). In order for RANKL secretion to be stimulated, however, sIL-6R must be present due to the low levels of IL-6R expressed on osteoblasts (Liu *et al.*, 2006; Blanchard *et al.*, 2009). Furthermore, TNF-α, IL-1, and PGE2 may stimulate the osteoblastic production of IL-11, LIF, and IL-6, which will further amplify the osteoclastic signal (Blanchard *et al.*, 2009). In addition to its effects on RANKL secretion, IL-6 has been shown *in vitro* to both increase RANK expression on osteoclasts

and decrease OPG secretion by osteoblasts when combined with sIL-6R (Liu *et al.*, 2006). The net result of this stimulation on the RANK/RANKL/OPG system is increased osteoclastic activity. O'Brien *et al.* (2000) and Kwan Tat *et al.* (2004), however, have reported a *decrease* in RANK expression and an *increase* in OPG secretion when IL-6 and sIL-6R are introduced in combination. Recently, IL-6 has been shown to induce osteoclast formation by a RANKL-independent mechanism, but the method by which it does so is still unclear (Kudo *et al.*, 2003).

Effects of Interleukin-6 on Bone Apposition. Osteoblasts, the principal bone apposition cells, originate from multipotent mesenchymal stem cells of the bone marrow, which also give rise to fibroblasts, chondrocytes, adipocytes, and muscle cells (Manolagas, 1998; Liu *et al.*, 2006; Aubin, 1998). IL-6 promotes osteogenic lineage commitment of the mesenchymal marrow cells by stimulating their differentiation into osteoblastic precursors (Liu *et al.*, 2006; Blanchard *et al.*, 2009). Due to the low levels of IL-6R on the osteoblast cell surface, sIL-6R or LIF must be present in combination with IL-6 in order for the stimulation of mesenchymal cells to occur (Blanchard *et al.*, 2009; Liu *et al.*, 2006). IL-6 then stimulates the further differentiation and development of the osteoblastic precursor cells into functional osteoblasts by activation of the JAK/STAT pathway (Liu *et al.*, 2006; Iwasaki *et al.*, 2008; Blanchard *et al.*, 2009; Manolagas, 1998; Itoh *et al.*, 2006). Lastly, IL-6 will initiate apoptosis in mature osteoblasts (Li *et al.*, 2008).

In addition to its role in the stimulation of osteoblast differentiation, IL-6 has recently been shown to directly suppress osteoclastogenesis by inhibition of the RANK-

signaling pathway (Yoshitake *et al.*, 2008; Duplomb *et al.*, 2008). IL-6 accomplishes this by diverting precursor cells into the macrophage lineage (Duplomb *et al.*, 2008).

Gingival Crevicular Fluid

In recent years, gingival crevicular fluid (GCF) has been used as a medium to measure a variety of molecules and bacteria present in both the oral cavity and the periodontal ligament space. GCF is an osmotically mediated exudate found in the gingival sulcus that tends to increase in volume with inflammation and greater capillary permeability (Yamaguchi, 2009). While GCF is primarily composed of serum, the composition can be modified by the surrounding gingival tissue and any bacteria present in the area (Cimasoni, 1983). As such, GCF can be found to contain a variety of substances including immunoglobulins, microorganisms, toxins, cells, and lysosomal enzymes (Yamaguchi, 2009). Recently, it has been observed that markers in bone remodeling and destruction can be found with the GCF as well (Lamster and Novak, 1992). It has been shown to accurately reflect the immune and inflammatory reactions arising from both periodontitis and orthodontic force application (Lamster, 1992; McCulloch, 1994; Ren *et al.*, 2002; Ren *et al.*, 2007).

GCF Levels of IL-6, sgp130, and sIL-6R. While many studies have used GCF levels to examine bone remodeling during active periodontal disease, more research is needed to examine the same markers during orthodontic force application. Several cytokines and growth factors have, however, been shown to have a strong correlation with orthodontic force application (Uematsu *et al.*, 1996; Grieve *et al.*, 1994; Lowney *et al.*, 1995). To this point, the GCF levels of IL-6 during orthodontic tooth movement have been examined in several previous studies. Uematsu *et al.* (1996) found a peak increase

in IL-6 after 24 hours of force application in canine retraction cases. Since that time, several studies have replicated the result of human IL-6 peak activation after 24 hours (Ren *et al.*, 2007; Ren *et al.*, 2002; Zhang and Ren, 2001). None of these reports, however, examined time points between 1 and 24 hours post-orthodontic activation. Yao *et al.* (2003), on the other hand, found that IL-6 levels were elevated in the GCF for at least 72 hours after orthodontic force application was induced. Two studies showed that IL-6 levels were not significantly elevated in adult orthodontic patients (Ren *et al.*, 2002; Zhang and Ren, 2001). At this time, neither the natural inhibitor of IL-6, spg130, nor the soluble receptor for IL-6, sIL-6R, have been examined in GCF levels from an orthodontic or periodontal perspective.

Summary

Orthodontic tooth movement is not a simple process and involves a plethora of molecules both known and unknown at this time. This process is most easily introduced by examining the four stages of tooth movement as introduced by Henneman (2008). It is during the initial stages of this process that IL-6 plays a critical role in both bone apposition and bone resorption. Sgp130, on the other hand, is an important antagonist to the signaling of the cytokine IL-6. The current study examined the roles that these two proteins play in orthodontic tooth movement. The null hypothesis was that the levels of IL-6 in GCF peak at a shorter interval than previously examined in orthodontic tooth movement. The ratio of IL-6 to sgp130 levels in the GCF was also expected to exhibit a temporal pattern. This knowledge will allow orthodontists to be more effective in their clinical decisions during force application and may allow for future research to examine possibilities in orthodontic tooth movement acceleration.

Methods and Materials

Study Population

GCF samples were collected from nine orthodontic patients [5 males, 4 females, age range 11 to 31 years (mean 17.445 years)] treated at the University of Nevada, Las Vegas School of Dental Medicine. All patients were approached during the screening appointment, and proper consent was obtained prior to beginning orthodontic treatment. Research protocol and consent forms were approved by the Office of Research Integrity – Human Subjects.

All patients were treatment planned to receive orthodontic treatment with fixed appliances. The study inclusion criteria were: no self-reported use of anti-inflammatory medications, good periodontal health (generalized probing depths ≤ 3mm and no radiographic evidence of periodontal bone loss), and the presence of one or more teeth that did not require immediate orthodontic movement. Patients were excluded from the study based on the following criteria: presence of periodontal disease and/or moderate to severe gingivitis, only minimal required tooth movement, diabetes, self-reported use of anti-inflammatory medication within the last 30 days, history of bleeding problems, use of tobacco, and/or poor oral hygiene.

Data Collection

Pre-treatment records including photographs, panoramic and cephalometric radiographs, and study models were obtained for each patient. Prior to treatment, a detailed periodontal examination was also performed. Probing depths, bleeding on probing (BOP), and a plaque index (PI) were recorded at both initial and recall appointments. One test tooth per patient was selected based on the amount of predicted

movement while also taking into consideration the ease of accurate sample collection. Of the nine selected experimental teeth, five were maxillary lateral incisors, three were mandibular lateral incisors, and one was a maxillary central incisor. Tension and compression sites for each test tooth were predicted based on preliminary study models. One control tooth per patient was selected based on the individual treatment plans by determining teeth that did not require immediate orthodontic bonding. Of the nine selected control teeth, one was a maxillary lateral incisor, one was a maxillary canine, one was a mandibular canine, two were mandibular incisors, two were mandibular premolars, and two were maxillary premolars. Control teeth were not bonded with orthodontic appliances either before or during the duration of the study.

Initial GCF samples were obtained prior to the bonding of any orthodontic appliances at three separate sites for each patient. Samples were taken on the mesiobuccal and distolingual of the experimental teeth as well as the mesiobuccal of the control teeth. Prior to sampling, the teeth were isolated with cotton rolls and gently dried with air. Thereafter, Periopaper (Oraflow, Smithtown, New York) was carefully inserted into the sulci to collect the GCF from each site for approximately 90 seconds. After removal from the sulci, each Periopaper was placed in a Periotron 6000 (Oraflow, Smithtown, New York) to measure the amount of GCF fluid obtained. This procedure was carried out three times per sampling site, and the combined Periopapers were immediately placed in a single vial of 200 μL of 1X phosphate buffered saline (PBS). At each time point, samples were collected from three sites: the mesiobuccal of the test teeth, the distolingual of the test teeth, and the mesiobuccal of the control teeth. Samples were then placed in a standard freezer for interim storage until they could be moved on

the same day to a -20°C freezer for final storage until assayed. The number of freeze thaw cycles of the samples was kept to a minimum.

After an initial sampling procedure, orthodontic appliances were bonded on each patient. Orthodontic forces were then applied by use of a 0.012 or 0.014 Nickel Titanium archwire. One hour after initial orthodontic force application, a second set of GCF samples was collected in the same manner as the initial samples. This procedure was repeated again after 6 and 24 hours of orthodontic force application.

Patients returned for recall appointments after 5-7 weeks. A periodontal examination was performed to record probing depths, BOP, and PI. Exclusion criteria were reevaluated for each patient, and no patients were eliminated from the study based on the aforementioned criteria. Another sampling procedure was carried out prior to any changes in orthodontic treatment. New orthodontic forces were then applied by use of a new 0.016 or 0.018 Nickel Titanium archwire for seven of the nine patients. The initial archwire was removed and retied for the remaining two patients. Samples were again collected 1, 6, and 24 hours after the orthodontic forces were reactivated.

Calibration of Periotron 6000

The Periotron 6000 was calibrated by adding 14 sequentially increasing known fluid volumes (0.1-1.4 μL) of fetal bovine serum (FBS) to Periopaper strips that were then placed in the Periotron. The numerical readings given by the Periotron per volume were recorded. Each volume was tested three times to minimize error. The mean value per volume was calculated and a calibration curve was plotted using MasterPlex 2010 (Hitachi Solutions) analysis software. A fourth polynomial regression equation was formulated from the mean values, and was used to calculate the GCF volume absorbed to

the periopaper. The three volumes obtained per sample site were added together to calculate total GCF volume per sample.

GCF Protein Concentration

GCF protein concentration was determined by a commercially available protein assay reagent based upon the Bradford method of protein concentration determination (Bradford, 1976). In brief, 20 μl of sample was combined with 275 μl of Protein Dye Reagent (Bio-Rad, Hercules, CA) following the microtiter plate assay directions as suggested by the manufacturer. After a 10-minute room temperature incubation with shaking, the absorbance at 595 nm was determined using a SpectaMax Plus (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer. Bovine serum albumin was used as the protein standards for generation of the standard curve and unknown concentrations were calculated. All standards and samples were assayed in duplicate.

IL-6, sIL-6R, and sgp130 Levels in GCF

IL-6, sIL-6R, and sgp130 levels in GCF were determined by MILLIPLEX MAP assays (Millipore, Billercia, MA) according to manufacturer's instructions. In preparation for the multiplex assay, the GCF samples were thawed on ice and centrifuged at 1000x*g* for 10 minutes. Two hundred microliters of assay buffer were added to each well of the microtiter filter plate. The plate was then placed on a plate shaker for 10 minutes at room temperature. The assay buffer was then removed from each well by vacuum. Standards and control were added to the appropriate wells (25 μL). 25 μL of assay buffer was then added to the sample wells only, followed by the addition of 25 μL of matrix solution to the background, standards, and control wells. Samples (25 μL) were then added to the appropriate wells. Finally, 25 μL of mixed beads were added to each well. The

microtiter filter plate was then incubated overnight on a plate shaker at 4°C. After incubation, all liquid was removed from the plate by gentle vacuuming. The plate was then washed 3 times with 200 μL/well of wash buffer with vacuuming between each wash. Detection antibodies (50 μL) were added and the plate was sealed and incubated on a plate shaker for 1 hour at room temperature. At that point, Streptavidin-Phycoerythrin (50 μL) was added to each well and the plate was incubated for an additional 30 minutes at room temperature. After the incubations, the liquid was removed by vacuum filtration and plate was washed 3 times with 200 μL/well of wash buffer with vacuum filtration between each wash. This was followed by the addition of 100 μL of sheath fluid to each well. The plate was then placed on the plate shaker for 5 minutes in order to re-suspend the labeled beads. The plate was then analyzed on a Luminex 200 (Luminex Corp. Austin, TX). Raw data was exported from the Luminex instrument and standard curves and unknowns were analyzed using MasterPlex 2010 (Hitachi Solutions) analysis software.

Statistical Analysis

The means for the GCF volume, total protein levels, IL-6 levels, sgp130 levels, and the sIL-6R levels for both experimental sites and the control site were compared using Student's *t* test using Microsoft Excel and SPSS 16. Significance was accepted for a $p < 0.05$.

Results

Bone remodeling associated with orthodontic tooth movement is a complex process involving a myriad of cytokines. During the initial stages of tooth movement, IL-6 plays a critical role in both osteoclast and osteoblast activity. In order to initiate the signaling cascade, IL-6 binds to the target cells via a membrane bound receptor complex that may include sIL-6R. Meanwhile, sgp130 acts as a natural inhibitor for the signaling of the cytokine IL-6. The present study examined the levels of IL-6, sgp130, and sIL-6R in the GCF throughout the initial stages of orthodontic tooth movement. In coordination with these efforts, the volume and protein concentration of GCF secreted during orthodontic tooth movement was also monitored.

Patient and Sample Characteristics

Five males and four females ranging from 11 to 31 years of age volunteered for participation in the study (Table 1). Initial GCF samples were obtained prior to the bonding of any orthodontic appliances at three separate sites for each patient. Samples were taken on the mesiobuccal and distolingual of the experimental teeth as well as the mesiobuccal of the control teeth. Periopaper (Oraflow, Smithtown, New York) was carefully inserted into the sulci to collect the GCF from each site for approximately 90 seconds. After removal from the sulci, each Periopaper was placed in a Periotron 6000 (Oraflow, Smithtown, New York) to measure the amount of GCF fluid obtained. This procedure was carried out three times per sampling site, and the Periopaper samples were immediately combined in a single vial of phosphate buffered saline. At each time point, samples were collected from three sites: the mesiobuccal of the test teeth, the

distolingual of the test teeth, and the mesiobuccal of the control teeth. Two sets of samples were not collected due to patient scheduling (Table 2).

Table 1. Patient Characteristics

Sex	n	Age	n
Male	5	Mean	17.445
Female	4	Min	11.167
		Max	31.250

Table 2. Summary of GCF Samples Collected

Visit	Post-activation (hours)	Test Site MB (n)	Test Site DL (n)	Control Site MB (n)
Initial	0	9	9	9
	1	9	9	9
	6	9	9	9
	24	9	9	9
Recall (5-7 weeks)	0	9	9	9
	1	8	8	8
	6	9	9	9
	24	8	8	8
Total		70	70	70 = 210

Gingival Crevicular Fluid Volume

The Periotron readings for each Periopaper were converted into GCF volume (μL) by use of a standard curve. The 4-parameter standard curve was created by placing sequentially known volumes of fetal bovine serum onto pieces of Periopaper, which were then placed into the Periotron to produce a reading (Figure 1). Recorded Periotron readings for each Periopaper were then converted into GCF volume for analysis and total GCF for each sample was calculated by adding together the individual readings for each of the 3 Periopapers that constituent a single sample.

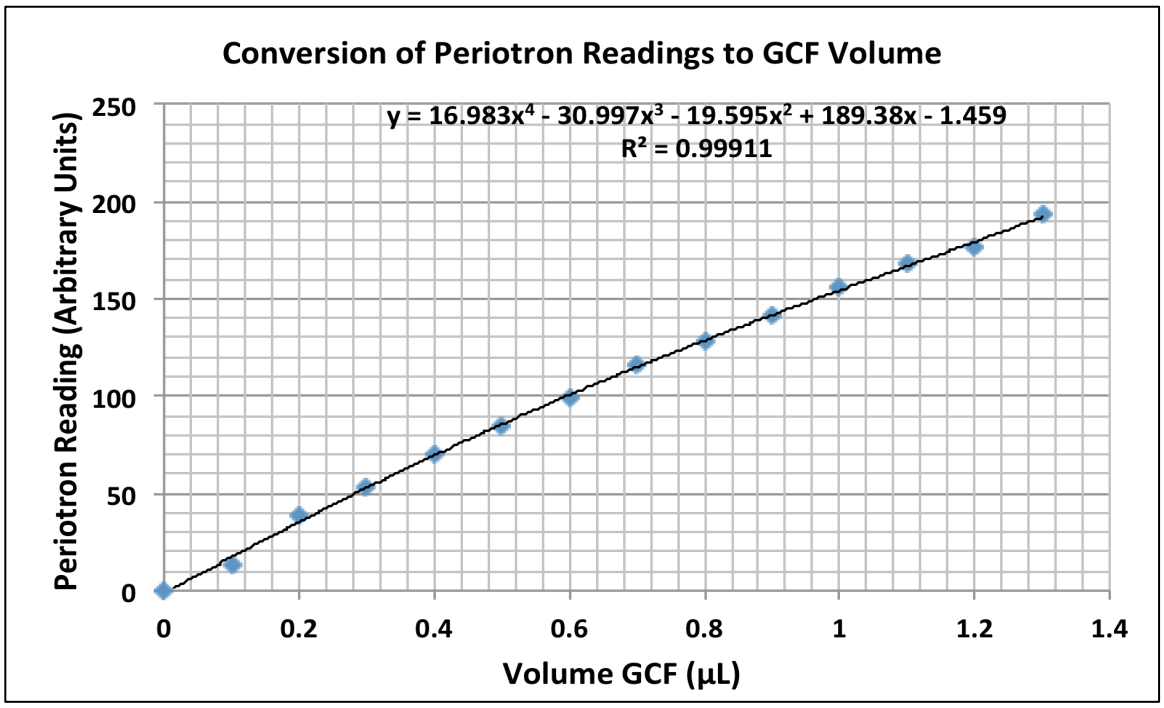

Figure 1. Conversion of Periotron Readings to GCF Volume (µL)

The GCF volumes were analyzed to determine any temporal patterns during orthodontic tooth movement. The distribution of measured GCF volumes for control and experimental sites (MB and DL) at both the initial (first) and recall (second) appointment are depicted in Figure 2. Control GCF volume ranged from 0.1 µL to 1.9 µL, while experimental GCF volumes ranged from 0.1 µL to 4.3 µL. Mean GCF values were calculated for each. The difference between the mean GCF volumes of the experimental samples from the initial visit (1.13±0.50 µL) and the mean GCF volumes of the experimental samples from the recall visit (1.05±0.50 µL) was not statistically significant ($p=0.25$). Additionally, there was not a statistically significant difference between the control sample mean GCF volumes from the initial (0.80±0.22 µL) and recall appointments (0.85±0.32 µL; $p=0.26$). When comparing the mean GCF volumes of the experimental samples to the mean GCF volumes of the control samples, however, there

was a statistically significant difference for both the initial visit (p=0.009) and the recall visit (p=0.05) (Figure 2).

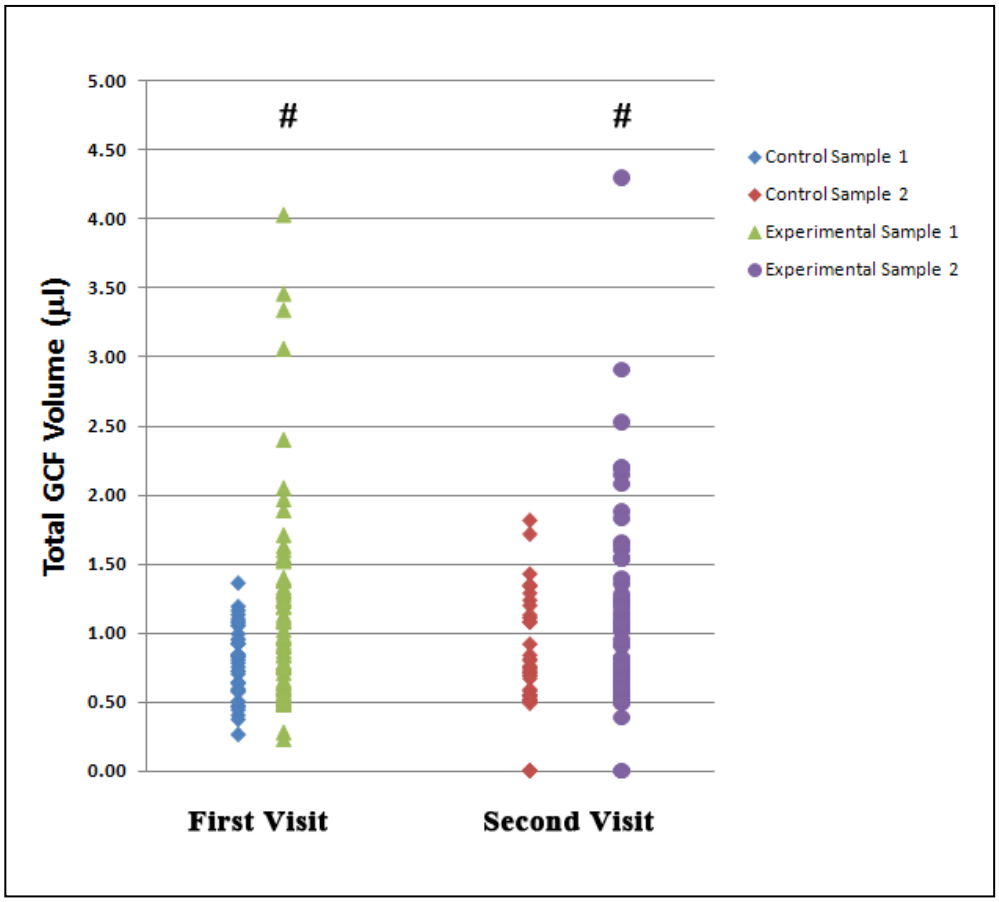

Figure 2. Total GCF Volume Collected During First and Second Visit
There is no statistical difference between the amount of GCF collected between the first and second visit for both the control and experimental samples. There is, however, a significant increase in the amount of GCF collected from experimental teeth versus control teeth at both the first and the second visit (# $p < 0.05$).

When the mean GCF volumes for experimental samples were compared according to the time since activation regardless of visit, there was a statistically significant increase in volume at 6 hours relative to all other time points ($p<0.05$). At both the 6 hour ($p=0.02$) and 24 hour ($p=0.03$) time points, there was also a statistically significant difference between the experimental and control mean GCF volumes. There

was no statistically significant difference among any of the control mean GCF volumes regardless of sample time (Figure 3).

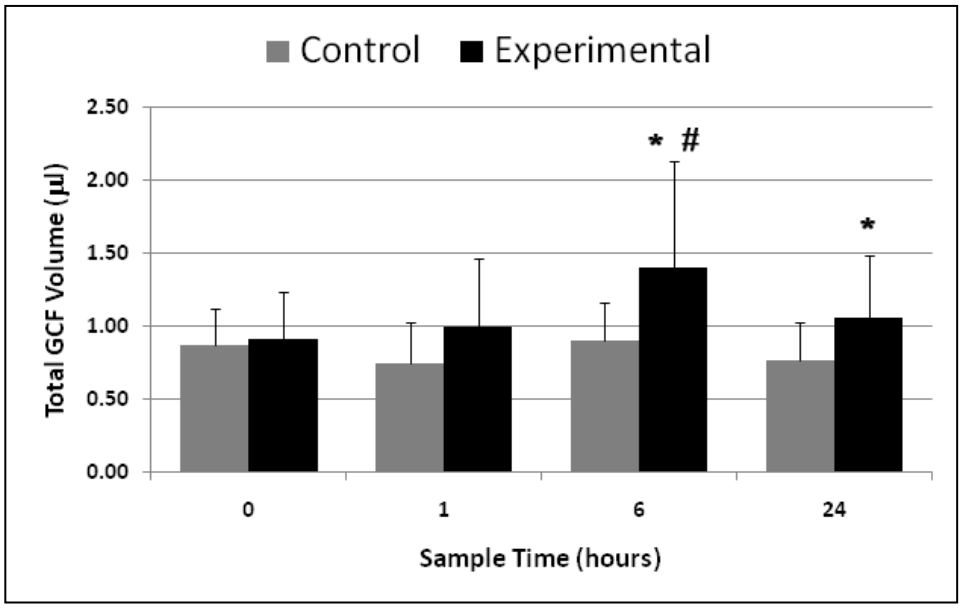

Figure 3. GCF Volumes Reported in Hours Since Activation
For experimental mean GCF volumes, there was a statistically significant increase at the 6-hour time point versus all other experimental time points (# $p < 0.05$). There was a statistical significant difference in mean GCF volumes for experimental and control samples at both 6 hours (* $p=0.02$) and 24 hours (* $p=0.03$). Note that at 1 hour, $p=0.08$, and although increased was not considered statistically significant as significance was set at $p < 0.05$.

Gingival Crevicular Fluid Protein Levels

The protein levels in each GCF sample were determined by the assay method first developed by Bradford (1976). Known quantities of bovine serum albumin were used as proteins standards and the protein concentrations were plotted against the observed absorbance at 595 nm (Figure 4). Protein concentrations in the samples were extrapolated from the standard curve. Total protein was calculated by multiplying the determined sample concentration (mg/ml) by the 0.2 ml sample volume. Figure 5 illustrates the distribution of protein levels detected in the collected samples. Control

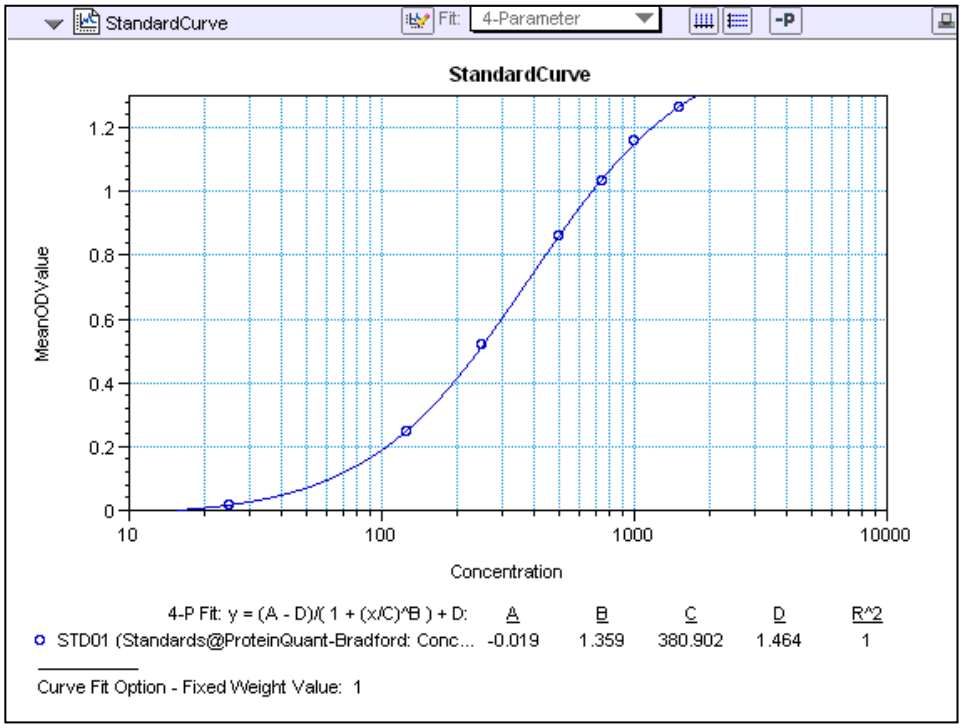

Figure 4. Standard Curve Obtained for Bradford Assay
Known quantities of BSA were measured at absorbance of 595 nm on a spectrophotometer (Molecular Devices SpectraMax) in order to create a standard curve. The Bradford analysis was performed in duplicate with 20 µL of sample in order to determine the protein concentration (µg/mL) in the previously eluted samples.

total protein levels ranged from 3 µg to 85 µg, while experimental GCF total protein levels ranged from 4 µg to 175 µg. Mean total protein values were calculated for each. When comparing the mean total protein amounts for the experimental samples (MB and DL), there was not a significant difference between total protein levels from the first (36.55±27.59 µg) and second visits (35.24±20.31 µg). In contrast, there was a statistically significant difference between the control mean total protein levels from the first (23.66±12.79 µg) and second visits (35.36±19.40 µg; p=0.005). Likewise, when comparing mean total protein levels between the control and experimental samples, there

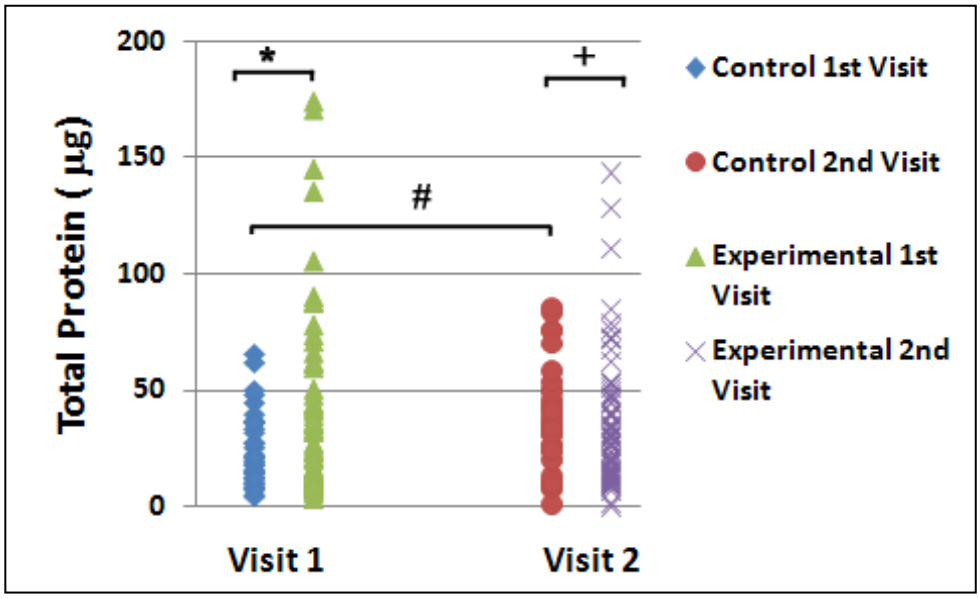

Figure 5. Total Protein Levels By Visit
Statistically significant differences were found between the experimental and control samples from the first visit (* $p < .03$), as well as between the control samples of the first and second visit (# $p < .01$). It was noted that there was not a significant difference between the control and experimental values from the second visit (+ $p > 0.05$).

was a statistically significant difference from samples obtained during the first visit ($p = 0.028$). On the other hand, there was not a significant difference between the experimental and control mean total protein levels from the samples obtained during the second visit (Figure 5). Interestingly, when the mean total protein levels are combined by visit, but separated by hours since activation, there is no significant difference between the three sample sites at any time point (Figure 6).

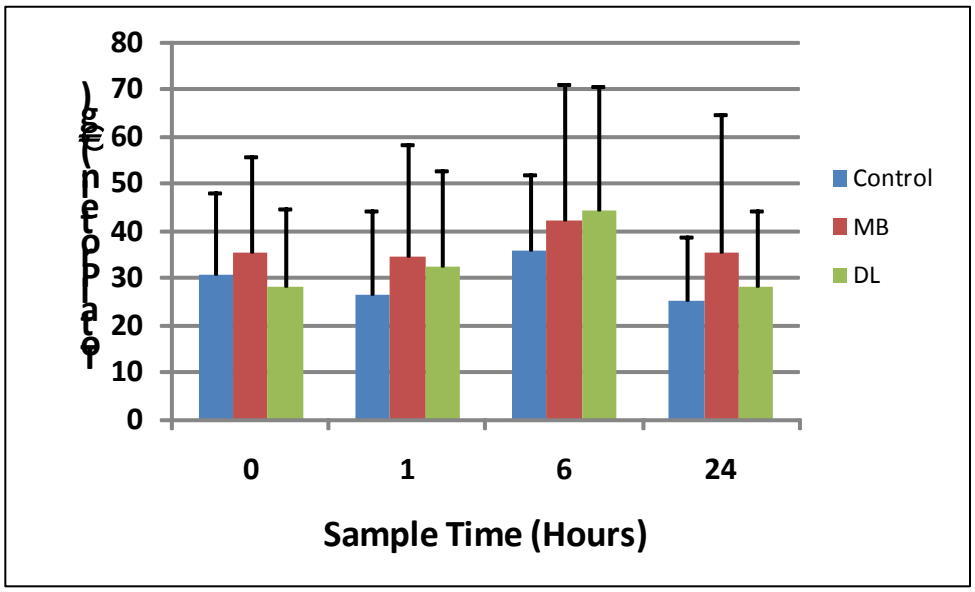

Figure 6. Total Protein Levels in Hours Since Activation
No statistically significant difference with time for either control or experimental

In addition to examining GCF volume and total protein levels, the protein concentration in the GCF was calculated (total protein/ GCF volume) to ascertain if any differences existed between the samples. Control GCF protein concentrations ranged from 6 µg/µL to 102 µg/µL, while experimental GCF protein concentrations ranged from 7 µg/µL to 135 µg/µL. Mean protein concentration values were calculated for each. When comparing the control to the experimental samples at the first or second visit, no significant differences were detected. For both control ($p=0.002$) and experimental ($p=0.006$) GCF protein concentrations, however, there was a significant increase at the second visit (40.41±15.60 µg/µL, 37.46±15.38 µg/µL) compared to the first visit (28.76±11.00 µg/µL, 29.39±13.80 µg/µL; Figure 7). When control and experimental protein concentrations were examined by time since activation regardless of visit, no statistically significant differences were found (Figure 8).

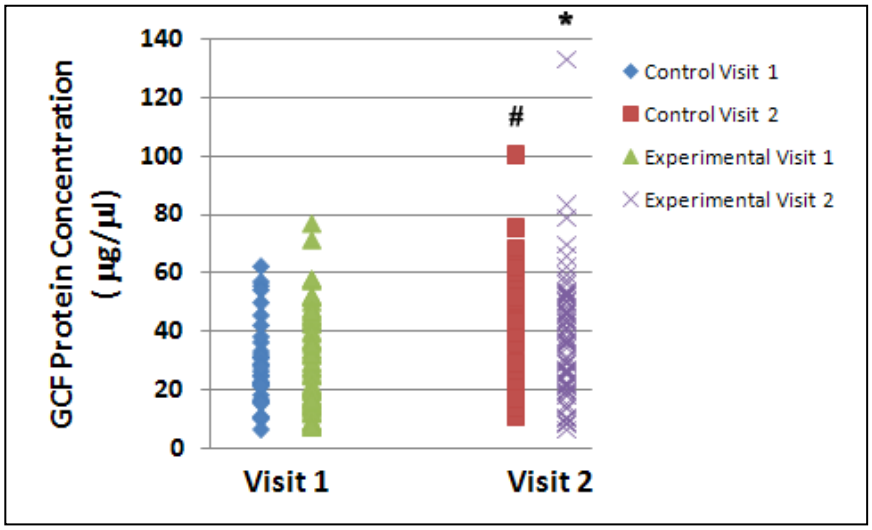

Figure 7. Protein Concentration By Visit
Statistically significant differences were found between the first and second visits for the control samples (# $p < 0.003$) and the experimental samples (* $p < 0.006$). There was no statistically significant difference between the control and experimental samples at either visit.

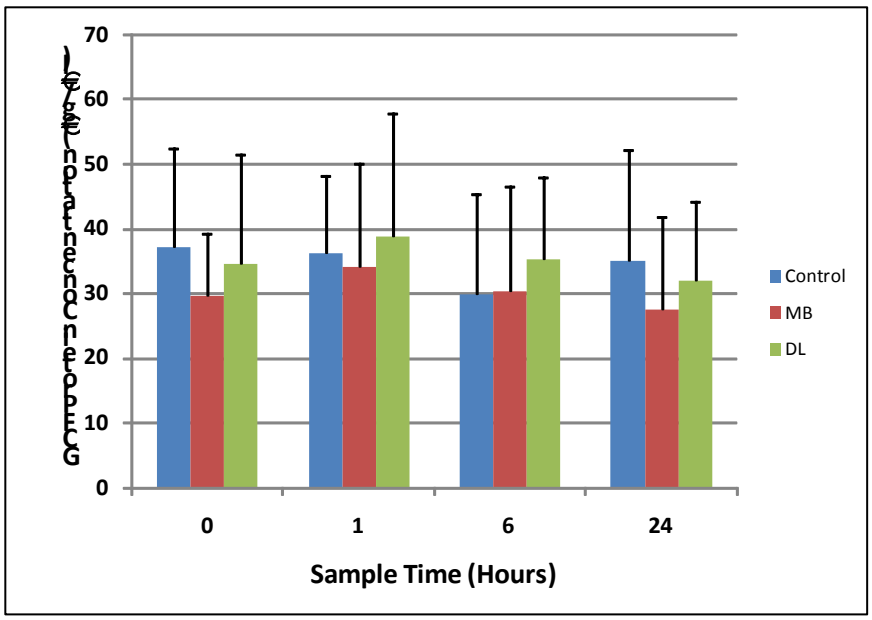

Figure 8. Protein Concentration in Hours Since Activation
No statistically significant difference with time for either control or experimental

IL-6, sgp130, and sIL-6R Levels

The purpose of the present study was to investigate and establish temporal patterns of the levels of IL-6, sgp130, and sIL-6R in the GCF during orthodontic tooth movement. Since the alveolar bone surrounding teeth undergoing orthodontic tooth movement contains both sites of bone resorption and bone apposition, IL-6 levels (fg/μg protein) were investigated on opposing sides (MB and DL) of the experimental teeth in order to examine any differences in cytokine activity between the two sites. Both sample sites were also compared to a control site in order to establish baseline activity for IL-6 for teeth not undergoing orthodontic tooth movement. Control GCF IL-6 concentrations ranged from 8 fg/μg to 480 fg/μg, while experimental GCF IL-6 concentrations ranged from 10 fg/μg to 795 fg/μg. Mean IL-6 protein concentration values were calculated for each. The mean GCF IL-6 protein concentration for the MB of the experimental teeth (83.85±74.73 fg/μg) was significantly higher than the mean GCF IL-6 protein concentration for both the DL of the experimental teeth (49.10±45.55 fg/μg; $p=0.025$) and the control site (50.67±50.07 fg/μg; $p=0.032$; Figure 9a). In order to investigate the temporal pattern of the mean IL-6 levels in the GCF, the samples were sorted by sample site according to hours since activation. Relative to the control site, the mean IL-6 values on the MB were significantly higher after both 6 hours ($p=0.001$) and 24 hours ($p=0.004$), whereas there was only a significant difference at 24 hours on the DL ($p=0.034$; Figure 9b). Despite the elevated appearance of figure 9b at 1 hour for the MB site, there was no statistical significance due to a high SEM. Figure 9c depicts individual mean IL-6 values at each post-activation time point, and highlights the reason for an elevated SEM at the MB site after 1 hour of activation. Mean IL-6 protein concentration levels appear to peak

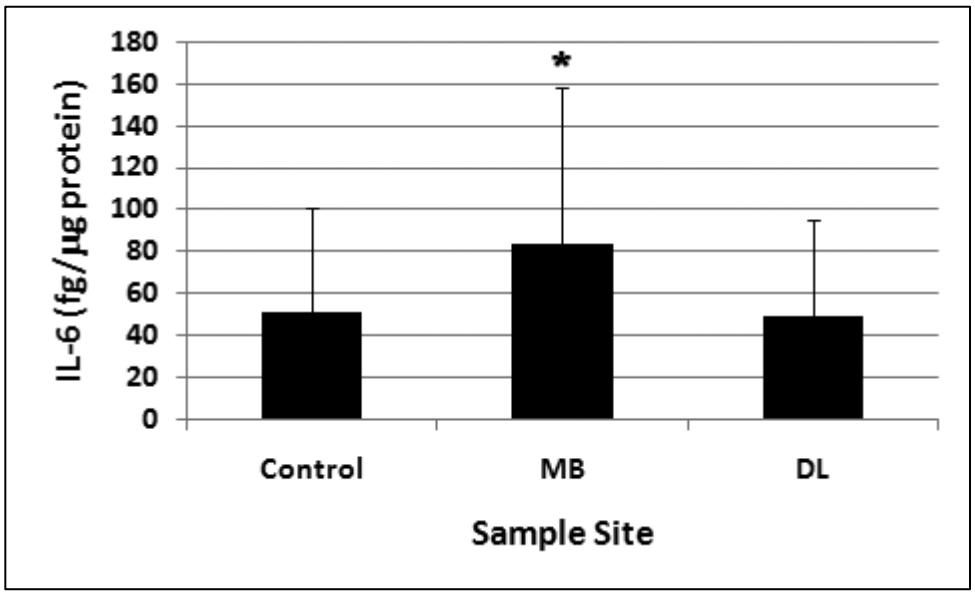

Figure 9a. Mean IL-6 Levels by Sample Site
While there was not a statistically significant difference between the control and DL experimental site for mean GCF IL-6 levels, the MB experimental site GCF IL-6 levels were significantly elevated relative to both the DL experimental site and the control site (* $p<0.04$).

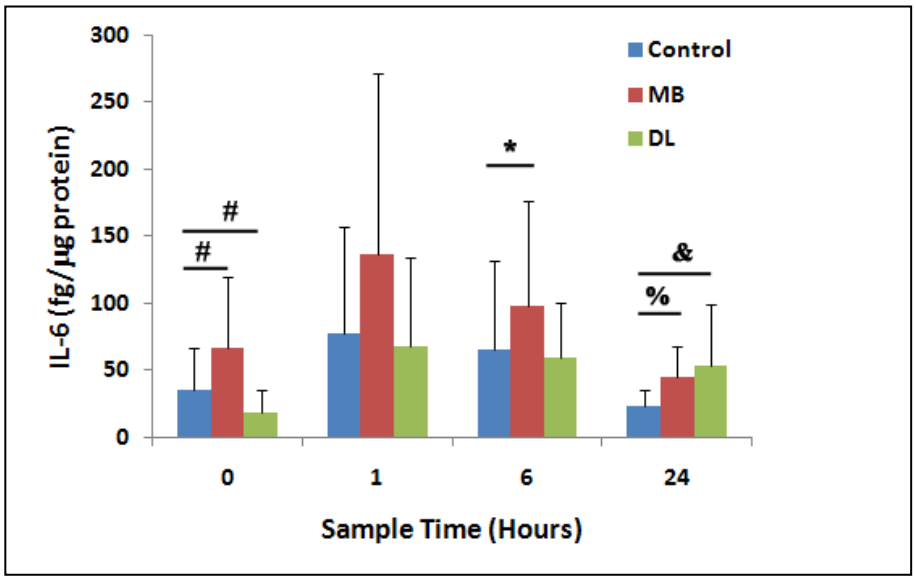

Figure 9b. Mean IL-6 Levels Reported in Hours Since Activation
There was a statistically significant increase in IL-6 levels at 6 hours (*$p < 0.002$) and 24 hours (% $p < 0.005$) on the MB of the experimental teeth relative to the control. There was also a statistically significant increase in IL-6 levels on the DL of the experimental teeth at 24 hours (& $p < 0.04$). Lastly, there was a statistically significant difference in overall IL-6 levels at 0 hours for both of the experimental sites relative to the control (# $p < 0.05$). It is noted, however, that these measurements include the second visit IL-6 protein levels which may influence the baseline.

after approximately 6 hours of orthodontic activation. It must also be noted that there was a statistically significant difference between both the MB and DL experimental sites (p=0.035, p=0.046) relative to the control sites at 0 hours (Figure 9a).

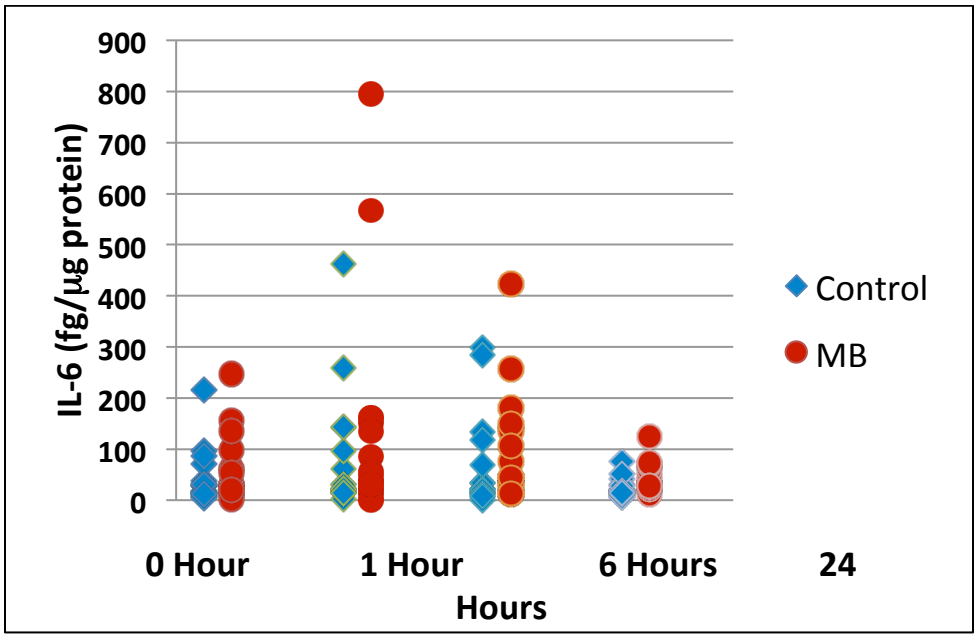

Figure 9c. Individual IL-6 Levels in Hours Since Activation
While the mean IL-6 levels appeared to be highest for the mesiobuccal at 1 hour, the results were not statistically significant due to a large standard deviation. The results of this figure indicate that IL-6 levels peaked after 6 hours.

Since sgp130 has been shown to act as a natural inhibitor to IL-6 activity (Franchimont *et al.*, 2005; Jostock *et al.*, 2001; Kamimura *et al.*, 2003), mean sgp130 GCF levels (pg/µg protein) were also measured in order to establish a temporal pattern relative to IL-6 levels. Control GCF sgp130 concentrations ranged from 0.2 pg/µg to 5.8 pg/µg, while experimental GCF sgp130 concentrations ranged from 2.0 pg/µg to 16.5 pg/µg. Mean sgp130 protein concentration values were calculated for each. The mean GCF sgp130 protein concentration for the MB of the experimental teeth (6.16±2.55

pg/μg) was significantly higher than the mean GCF sgp130 protein concentration for both the DL of the experimental teeth (3.58±1.03 pg/μg; p=0.000) and the control site (3.21±0.94 pg/μg; p=0.000; Figure 10a). The difference in mean GCF sgp130 levels between the DL site of the experimental teeth and the control site was not statistically significant (p=0.07; Figure 10a). In order to investigate the temporal pattern of the mean sgp130 levels in the GCF, the samples were sorted by sample site according to hours since activation. Mean sgp130 levels were significantly elevated at the MB experimental site at 1 (p=0.005), 6 (p=0.001), and 24 hours (p=0.000) post-activation relative to the control (Figure 10b). Similarly, Figure 10b shows that the mean sgp130 levels on the MB experimental site were elevated at 1 (p=0.005), 6 (p=0.012), and 24 hours (p=0.001) post-activation relative to the experimental DL site as well. There was no significant difference for sgp130 levels between the control and DL samples.

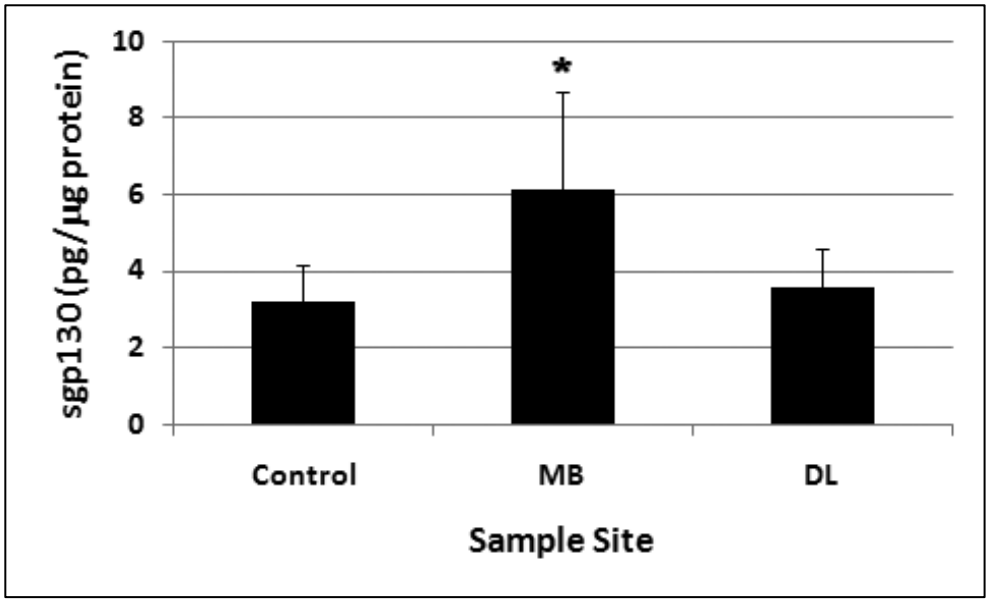

Figure 10a. Mean sgp130 Levels by Sample Site
While there was not a statistically significant difference between the control and DL experimental site for mean GCF sgp130 levels, the MB experimental site GCF sgp130 levels were significantly elevated relative to both the DL experimental site and the control site (* p<0.01).

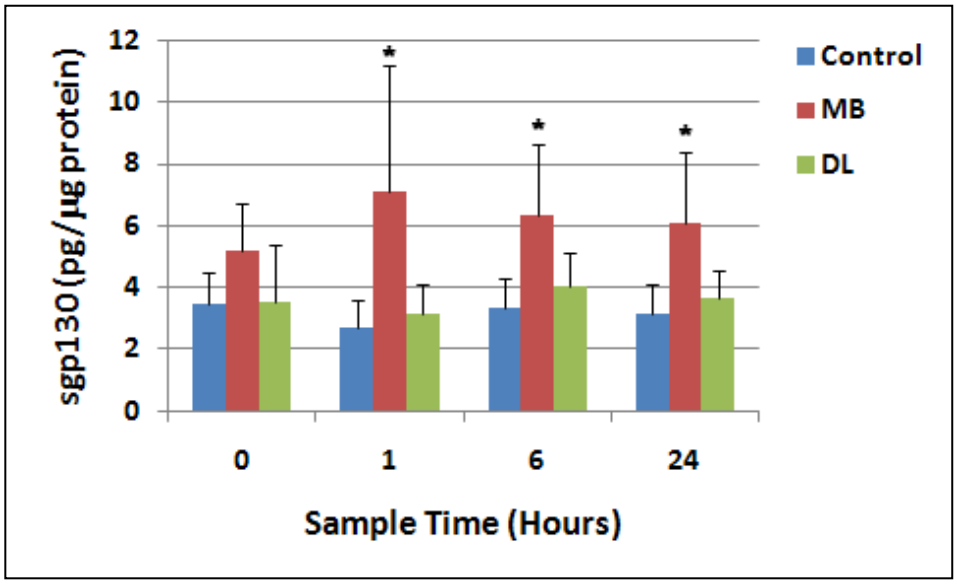

Figure 10b. Mean Sgp130 Levels Reported in Hours Since Activation Sgp130 levels were elevated at the mesiobuccal experimental site at 1 hour, 6 hours, and 24 hours relative to both the control and distolingual experimental sites (* $p < 0.02$).

Since sIL-6R often forms part of the receptor complex for IL-6 on the target cell, it would be expected that sIL-6R may increase as IL-6 levels increase. In order to investigate the relationship between IL-6 and sIL-6R, the levels of GCF sIL-6R (pg/µg protein) were examined during orthodontic tooth movement as well. Control GCF sIL-6R concentrations ranged from 0.3 pg/µg to 1.2 pg/µg, while experimental GCF sIL-6R concentrations ranged from 0.4 pg/µg to 2.3 pg/µg. Mean sIL-6R protein concentration values were calculated for each. The mean GCF sIL-6R protein concentration for the MB of the experimental teeth (1.22±0.55 pg/µg) was significantly higher than the mean GCF sIL-6R protein concentration for both the DL of the experimental teeth (0.65±0.27 pg/µg; p=0.000) and the control site (0.67±0.29 pg/µg; p=0.000; Figure 11a). In order to investigate the temporal pattern of the mean IL-6 levels in the GCF, the samples were sorted by sample site according to hours since activation. Similar to mean sgp130 levels,

the mean GCF sIL-6R levels were significantly elevated on the MB of the experimental site at 1 hour, 6 hours, and 24 hours post-activation relative to both the control (p=0.037, p=0.006, p=0.006) and DL experimental sites (p=0.035, p=0.029, p=0.001). Additionally, mean GCF sIL-6R levels from the experimental MB site were significantly higher than the control and experimental DL sites at 0 hours post-activation (p=0.001, p=0.003; Figure 11b).

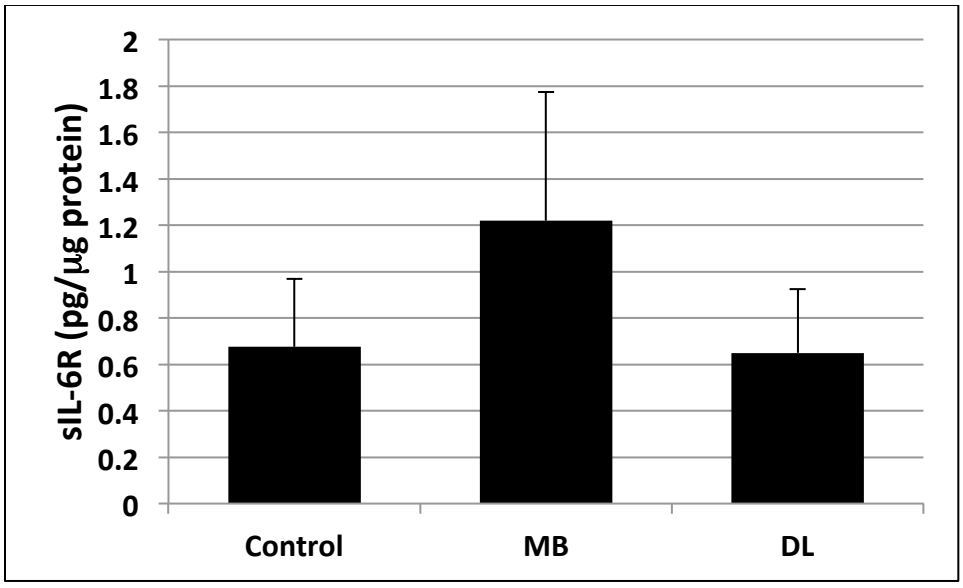

Figure 11a. Mean sIL-6R Levels by Sample Site
While there was not a statistically significant difference between the control and DL experimental site for mean GCF sIL-6R levels, the MB experimental site GCF sIL-6R levels were significantly elevated relative to both the DL experimental site and the control site (* $p<0.01$).

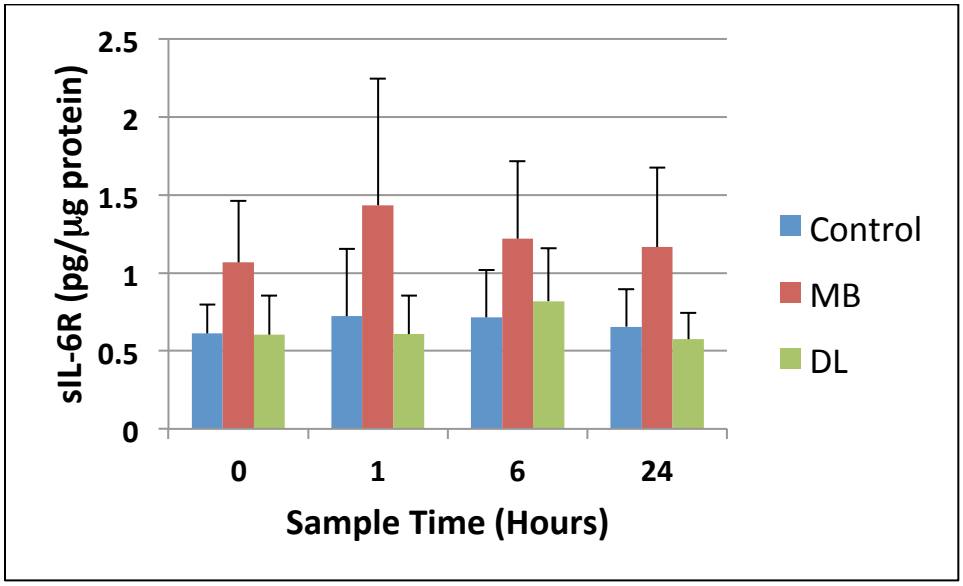

Figure 11b. Mean SIL-6R Levels Reported in Hours Since Activation
SIL-6R levels were elevated at the mesiobuccal experimental site at 0 hours, 1 hour, 6 hours, and 24 hours relative to both the control and distolingual experimental sites ($p < 0.04$).

Discussion

Gingival Crevicular Fluid Volume

The present study evaluated the changes in IL-6, sgp130, and sIL-6R in gingival crevicular fluid (GCF) after the application of orthodontic force. GCF samples were collected at eight different time points over 5-7 weeks. At both the initial bonding appointment and at the first recall appointment, baseline samples were collected. After orthodontic force application, samples were then collected at three additional time points (1, 6, and 24 hours) for each appointment.

Analysis of GCF volume results did not show a statistically significant difference in GCF volumes between the first and second visit for either the control or experimental groups. Comparison of the experimental GCF volumes to the control volumes, however, clearly demonstrated increased GCF volumes associated with the experimental teeth. While these results indicate that increased GCF volume may be used as suitable biomarker for tissue remodeling during orthodontic treatment, previous reports in the literature regarding orthodontic tooth movement and GCF volume have been conflicting. Studies by both Tersin (1978) and Baldwin *et al.* (1999) reported an increase in GCF volume during orthodontic tooth movement, whereas Miyajima (1991), Uematsu *et al.* (1996), and Drummond *et al.* (2011) reported no statistically significant difference in the GCF volumes of orthodontically treated teeth. The conflicting results may be due to a difference in study design as well as the time points at which GCF volumes were measured. The present study is the only study in which GCF volume was measured at the 6-hour time point.

Since there was not a statistically significant difference between the first and second visit for GCF volumes within the same sample groups, further analysis of GCF volume combined the two appointments and examined GCF volumes by hours since activation (0, 1, 6, and 24 hours). With the exception of the 0-hour time point, all experimental samples showed a higher GCF volume than the corresponding control samples at every time point. In addition, the 6-hour time point showed a statistically significant increase in GCF volume for experiment samples relative to all other experimental time points. Previous studies on GCF volume during tooth movement have not examined volumes after 6 hours of orthodontic force application. The increase in GCF volume may be an early indicator of the inflammation associated with orthodontic tooth movement, and an examination of GCF volumes at 6 hours after force application may provide the optimal time for assaying the degree of inflammation associated with tooth movement.

Total Protein Concentration

Since GCF volumes vary between samples, it is possible that total protein levels may vary accordingly. Thus, examining GCF protein concentration gives a more accurate view of the protein levels in each sample. The present study reported a statistically significant increase in protein concentration between the first and second visits for both the control group and the experimental group. There was, however, no significant difference in protein concentration between the control and experimental samples at either visit. When examining the protein concentration relative to the time since force activation, there was no statistically significant differences at any time point for experimental or control groups. These findings suggest that there may be a

generalized increase in GCF protein concentration levels as orthodontic forces are applied.

Interleukin-6, sgp130, and sIL-6R Levels

Orthodontic tooth movement is a complex process, which involves a delicate balance of bone apposition and bone resorption. IL-6 has been shown to influence both osteoblastic and osteoclastic activity, and thus, plays a critical role in orthodontic bone remodeling (Liu *et al.*, 2006). In fact, IL-6 can simultaneously generate both osteoclastic and osteoblastic signals in a target cell through its receptor complex, IL-6Rα and gp130 (Kamimura *et al.*, 2003). While the soluble form of IL-6Rα, sIL-6R, may act synergistically with IL-6 (Blanchard *et al.*, 2009), the soluble form of gp130 , sgp130, has been shown to act as a natural inhibitor to IL-6 activity (Jostock *et al.*, 2001; Kamimura *et al.*, 2003; Franchimont *et al.*, 2005). The present study examined the GCF levels of IL-6, sgp130, and sIL-6R after orthodontic force application.

While previous studies have shown a peak in IL-6 levels during orthodontic tooth movement after 24 hours (Uematsu *et al.*, 1996; Ren *et al.*, 2007; Ren *et al.*, 2002; Zhang and Ren, 2001), none of the studies examined IL-6 GCF levels between 1 and 24 hours after orthodontic activation. The present study investigated an additional time point at 6 hours post-activation, as well as opposing sites (mesiobuccal and distolingual) on the same experimental tooth. Opposing sites were chosen in order to measure possible differences between the compression and tension sides of tooth movement. For most of the experimental teeth, however, rotation was the primary predicted movement after initial force activation, and as such, it was not possible to accurately designate either site as the compression or tension side. Based off preliminary models, however, it was

predicted that bone resorption would be more likely to occur on the mesiobuccal side of the experimental teeth in seven of the nine patients. In the remaining two patients, it was difficult to predict which site may undergo bone resorption. It must also be noted that the possibility of contamination between the two opposing sites in the gingival crevice could not be excluded.

The data from this study showed a statistically significant increase of approximately two-fold for the mean IL-6 GCF levels at 6 and 24 hours on the mesiobuccal of the experimental teeth relative to controls at the corresponding time points. In fact, IL-6 levels appeared to peak after 6 hours of orthodontic activation on the mesiobuccal of the experimental teeth. Meanwhile, the IL-6 GCF levels on the distolingual of the experimental teeth were significantly higher than controls at 24 hours. There also appears to be a clear increase in IL-6 GCF levels on the distolingual of the experimental teeth after orthodontic activation.

It must also be noted that while the mesiobuccal experimental IL-6 GCF levels were significantly higher than the control samples at 0 hours, and the distolingual experimental IL-6 GCF levels were significantly lower than the control samples at 0 hours, the measured protein levels were a combination of both the first and second visits. Due to the nature of the Nickel Titanium orthodontic wires used in the study, that some residual activation was still present at 0 hours of the second visit in some cases.

The results of this study indicate that the peak IL-6 levels may be earlier than previously reported. While it was not possible to differentiate the compression and tension sides of tooth movement, it would be expected that IL-6 levels would increase regardless due to the involvement of IL-6 in both osteoclastic and osteoblastic activities.

Since sgp130 has been found to be a natural inhibitor to IL-6 (Jostock *et al.*, 2001; Kamimura *et al.*, 2003; Franchimont *et al.*, 2005), it may be expected that GCF levels of sgp130 would decrease as IL-6 levels increase. The present study, however, showed an increase is sgp130 levels on the mesiobuccal of experimental teeth 1, 6, and 24 hours. It may be that as IL-6 is produced, sgp130 levels gradually increase until an inhibitory effect is produced on IL-6 activity. It may be predicted, in such a scenario, that sgp130 levels would continue to increase past the time constraints of the present study. At present, there are no other published studies that have examined GCF levels of sgp130 during orthodontic tooth movement. Future studies may be able to solidify a stronger relationship between the GCF levels of IL-6 and sgp130 during orthodontic tooth movement by expanding the time line.

The mean concentration levels of sIL-6R were significantly elevated in the mesiobuccal site of experimental teeth at all time points relative to the control and distolingual sites. IL-6 signal transduction typically occurs through a gp130/IL-6R receptor complex (Kamimura *et al.*, 2003). While the soluble form of gp130, sgp130, acts as an antagonist to IL-6 activity (Jostock *et al.*, 2001; Kamimura *et al.*, 2003; Franchimont *et al.*, 2005), the soluble form of IL-6R, sIL-6R, may replace its membrane-bound counterpart in the receptor complex (Kamimura *et al.*, 2003). As such, levels of sIL-6R may be expected to increase as IL-6 activity increases as was seen in the present study. If enough membrane bound IL-6R is present, however, sIL-6R would not be necessary for IL-6 signal transduction. As with mean IL-6 concentration levels, it should be noted that sIL-6R GCF levels were elevated in the mesiobuccal site at 0 hours as well. It must be taken into consideration that since first and second visit samples were

combined for the sake of analysis, continued activation of the Nickel Titanium orthodontic wires may have elevated sIL-6R levels at the 0 hour time point of the second visit.

For IL-6, sgp130, and sIL-6R, there was a statistically significant difference between the mesiobuccal and distolingual experimental sites for most time points. While it is difficult to accurately predict which site was undergoing bone resorption versus bone apposition, it is clear that orthodontic forces affect protein levels on the compression and tension sides to a different extent.

Conclusions

1. Gingival crevicular fluid volume increases after orthodontic forces are applied. In the present study, GCF fluid volume peaked at 6 hours post-activation but remained elevated at 24 hours post-activation.
2. The IL-6 concentration levels in the GCF increase during orthodontic tooth movement, with a peak at approximately 6 hours post-activation. While earlier studies reported a peak at 24 hours, previous studies did not measure levels between 1 and 24 hours.
3. Sgp130 and sIL-6R are detectable in the GCF during initial tooth movement. While the concentration levels of both proteins increase after orthodontic forces are applied, further research must be completed to establish a strong temporal pattern relative to IL-6 levels.
4. Orthodontic forces affect IL-6, sgp130, and sIL-6R protein levels on the compression and tension sides to a different extent. Further research must be completed in order to establish a strong relationship between the proteins and the type of bone remodeling occurring.

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