Immunohistochemistry study on expression of Tumor Necrosis Factor Like Weak Inducer of Apoptosis (TWEAK) and its receptor FN14 in normal and periodontitis tissues

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

Nupur Grover Kataria BDS (Adel)

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Declared by: Witnessed by:

_____ _____

N. Kataria

Date:_____ Date:_____

Acknowledgements

It is with great respect and regards that I take this opportunity to express my profound sense of gratitude to my supervisor, Professor P Mark Bartold, for his constant support, encouragement and help throughout this research project. His invaluable advice, constructive criticism, consummate skill to guide and positive energy has been a tremendous source of inspiration during these last three years.

I extend my sincere thanks to Dr David Haynes, Department of Pathology, University of Adelaide. He has played an integral role in the conceptualization of this project and his continuous flow of ideas was indispensable for the project.

I am much indebted to Dr AASK Dharmapatni, Department of Pathology, University of Adelaide, who has been extremely kind and considerate to me. Her incredible patience and expertise provided crucial guidance in the actual execution of this project. I would also like to thank Ms Ruth Wyszynski for her assistance with the immunohistochemistry. I am thankful to Mr. Christopher Holding for his readiness to assist with any queries in the laboratory.

I am very grateful to Mr. Hans Schoppe and the staff of Department of Histology, University of Adelaide, for their hard work and help with the preparation of the histologic sections.

My special thanks to Mr. Dale Caville, Department of photography, University of Adelaide. His painstaking supervision and meticulous approach has resulted in the clear and sharp pictures displayed in the research report.

This project was generously supported by a grant from the Australian Dental Research Foundation Inc (ADRF). My sincere thanks to them for the same.

At this juncture I would like to express my gratefulness to my wonderful colleagues Dr Teck Tang, Dr Andre Bendyk, Dr Raymond Chan, Dr Christopher Bates, Dr Emma Megson and Dr Tina Choo for their unwavering support and friendship.

This project would not have been possible without the love and understanding of my husband Gopal and my beautiful girls Navya and Nishka. Above all I would like to thank all my family for their constant moral support.

Contents

1.1 Abstract

Periodontitis is a chronic inflammatory disease wherein microbial factors induce complex inflammatory and immune responses in a susceptible host. In periodontitis host-derived enzymes, cytokines and other proinflammatory mediators play an integral role in the destruction of tooth supporting structures and alveolar bone. TWEAK (TNF-like weak inducer of apoptosis), one of the members of the TNF superfamily, has recently been identified as an important inflammatory mediator. Fn14 (fibroblast growth factor-inducible 14) protein/TWEAKR has been identified as the cell surface receptor for TWEAK. TWEAK/Fn14 signaling results in multiple biologic effects including induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis. TWEAK has also been shown to promote osteoclastic differentiation of cells from the monocyte/macrophage lineage. Expression of TWEAK and its receptor Fn14 is elevated in tissues and cells cultured from a number of chronic inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases. This review considers the biology of TWEAK and its receptor Fn14 in periodontitis.

2.1 Abstract

Background: Periodontitis is a chronic inflammatory disease wherein microbial factors induce complex inflammatory and immune responses in a susceptible host. In periodontitis host derived enzymes, cytokines and other proinflammatory mediators play an integral role in the destruction of tooth supporting structures and alveolar bone. TWEAK (TNF-like weak inducer of apoptosis) is one of the newest members of the TNF superfamily to be identified. Fibroblast growth factor-inducible 14 (Fn14) protein/TWEAKR has been identified as the cell surface receptor for TWEAK. TWEAK/Fn14 signaling results in multiple biologic effects including induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis. Recently, TWEAK has also been shown to promote osteoclastic differentiation of cells from the monocyte/macrophage lineage. Expression of TWEAK and its receptor Fn14, is elevated in tissues and cells cultured from a number of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases. Accordingly, we hypothesised that the expression of TWEAK and Fn14/TWEAKR will be increased in tissue samples from periodontitis patients.

Aim: The aim of this study was to investigate the expression of TWEAK and its receptor Fn14, in gingival biopsies from periodontitis patients and clinically normal patients using immunohistochemistry techniques.

Materials and Methods: The study included 27 patients (18 females and 9 males, aged 30-77 years, mean age = 55.2 years) with generalised chronic and aggressive periodontitis. The gingival biopsy sites in the chronic and aggressive periodontitis group had clinical probing pocket depths and clinical attachment loss greater than 5 mm with radiographic evidence of bone loss ranging from 50-90% of the root length. The non-periodontitis tissue samples consisted of gingival tissue resected from seven patients (4 males and 3 females, aged 23-70 years at the time of surgery; mean age = 45.5 years) undergoing crown lengthening surgery at sites not affected by periodontitis. Using monoclonal antibodies, the expression of TWEAK and its receptor Fn14 was investigated by immunohistochemistry in formalin-fixed paraffin embedded tissues. The specimens were evaluated by a semiquantitiative analysis (SQA). The Mann Whitney U-test was used to compare mean rank of SQA between two groups and Kendall’s tau_b test was used to determine correlations between different parameters.

Results: Semiquantiative analyses demonstrated that the expression of TWEAK protein was significantly higher in periodontitis tissue compared to healthy tissue (Mann Whitney U-test, p value 0.002). Similarly, in comparison with healthy tissue, periodontitis-affected tissues expressed significantly higher Fn14 protein (Mann Whitney U-test, p value 0.013). A strong positive correlation was found between TWEAK and Fn14 expression (Kendall's tau_b test; p value 0.007 and r value 0.395). In periodontitis-affected tissue specimens, TWEAK and Fn14 protein was mainly expressed by mononuclear leukocytes (morphologically resembling lymphocytes and plasma cells), cells lining blood vessels, spindle shaped cells resembling fibroblasts and multinucleated cells.

Conclusion: This study demonstrates that there is a higher expression of TWEAK and Fn14 protein in periodontitis tissues as compared to clinically normal controls. This suggests that TWEAK /Fn14 signaling could be an additional player in the pathogenesis of periodontitis and adds to the increasing number of cytokine networks involved in periodontal inflammation.

Chapter 1

A REVIEW OF TUMOR NECROSIS FACTOR LIKE WEAK INDUCER OF APOPTOSIS (TWEAK) AND ITS RECEPTOR FN14

NG KATARIA[1], PM BARTOLD[2]

[1]Post Graduate Student (Periodontology), School of Dentistry, University of Adelaide

[2]Professor of Periodontology, Colgate Australian Clinical Dental Research Centre, School of Dentistry, University of Adelaide

1.1 Abstract

Periodontitis is a chronic inflammatory disease wherein microbial factors induce complex inflammatory and immune responses in a susceptible host. In periodontitis host-derived enzymes, cytokines and other proinflammatory mediators play an integral role in the destruction of tooth supporting structures and alveolar bone. TWEAK (TNF-like weak inducer of apoptosis), one of the members of the TNF superfamily, has recently been identified as an important inflammatory mediator. Fn14 (fibroblast growth factor-inducible 14) protein/TWEAKR has been identified as the cell surface receptor for TWEAK. TWEAK/Fn14 signaling results in multiple biologic effects including induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis. TWEAK has also been shown to promote osteoclastic differentiation of cells from the monocyte/macrophage lineage. Expression of TWEAK and its receptor Fn14 is elevated in tissues and cells cultured from a number of chronic inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases. This review considers the biology of TWEAK and its receptor Fn14 in periodontitis.

1.2 Introduction

Periodontitis is an inflammatory disease characterised by destruction of the supporting tissues of the teeth and involves an interplay between microbial, host, genetic and environmental factors (Page and Kornman, 1997). The microbial factors are the primary etiological factors which induce complex inflammatory and immune responses in a susceptible host (Baker 2000, Haffajee and Socransky 1994). Thus, bacteria are considered to be necessary factors for periodontal disease initiation but they are not necessarily sufficient to cause disease progression. The inflammatory process which ensues is an attempt by the host to eliminate the microbial assault and repair the injury caused to periodontal tissues. It is a carefully orchestrated process in which many cells and molecular mediators play interactive roles at various stages. Specifically, it is characterised by the accumulation of B and T lymphocytes, monocytes and neutrophils (Page and Schroeder, 1976). Periodontitis can vary in the rate of progression, magnitude of inflammatory response and its potential response to treatment due to differences in microbial composition, host response and factors predisposing certain individuals and sites to disease progression.

As part of the host response, various cell types release inflammatory mediators. These include cytokines, low molecular weight lipids derived from arachidonic acid, chemokines, gases including nitric oxide, carbon monoxide and reactive oxygen species (Van Dyke and Serhan, 2003). All of these play critical roles in the initiation and progression of inflammation. The so called proinflammatory cytokines (e.g. tumor necrosis factor α, Interleukin-1, Interleukin-8) are responsible for driving the inflammatory response. Cytokines which suppress the activity of proinflammatory cytokines and promote healing are called anti-inflammatory cytokines (e.g. IL-4, IL-10 and IL-13). The destruction of gingival connective tissue, periodontal ligament and bone in periodontitis is largely brought about by the proinflammatory mediators released during this process (Gemmell *et al* 1997, Graves and Cochran 2003, Irwin and Myrillas 1998). Proinflammatory cytokines modulate immune function and affect tissue destruction by either acting directly on other cells or through the induction of other cytokines. The inflammatory process is thus a double edged sword. Although primarily a protective response, inflammation can also turn into a destructive process.

Once the host is able to neutralize the microbial insult, the inflammatory process is resolved by catabolism of proinflammatory mediators and the process of repair is initiated. This is a well coordinated event in which secreted “stop” signals result in shut down and clearance of various participating inflammatory cells. Thus, the body tries to limit the destruction once the initiating causes have been eliminated. Periodontal diseases are chronic inflammatory diseases wherein induction and resolution of inflammation go on side by side and this is largely driven by cytokines (Kantarci et al., 2006).

Understanding the role of cytokines in the pathology of periodontal disease would help in the development of host-modulation therapies to be used along with conventional periodontal therapy.

1.3 Role of cytokines in periodontal disease

The role of various cytokines such as Interleukin-1 (IL-1), Interleukin-8 (IL-8), Interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α), Transforming growth factor-β (TGF-β) and Interferon-γ (IFN-γ) in chronic inflammatory diseases such as periodontitis is well established. In particular IL-1 and TNF-α have been considered to have pivotal roles

in the pathogenesis of periodontitis. IL-1 and TNF-α can upregulate adhesion molecules on leucocytes and endothelial cells and stimulate the production of chemokines to recruit circulating leucocytes (Graves and Cochran, 2003). They can stimulate the production of other inflammatory mediators such as prostaglandins and can enhance bacterial killing and phagocytic activity (Pfizenmaier et al., 1996). They can also cause connective tissue loss by stimulating the production of lytic enzymes such as matrix metalloproteinases and enhance bone resorption by promoting osteoclast formation and activity (Pfizenmaier *et al* 1996, Stashenko *et al* 1987). IL-1 and tumor necrosis factor antagonists have been shown to significantly reduce the loss of connective tissue attachment and the loss of alveolar bone height in a Macaca fascicularis primate model of experimental periodontitis (Delima et al., 2001). Another study in non human primates demonstrated that the inhibition of IL-1/TNF activity resulted in significant reduction in inflammatory cell recruitment and alveolar bone loss (Assuma et al., 1998).

1.4 Apoptosis and periodontitis

Apoptosis, or programmed cell death, is a normal physiologic process which contributes to maintainance of tissue homeostasis (Cohen, 1991). However, it also has a crucial role in the regulation of inflammation and host immune responses. Various factors such as hormones, cytokines, growth factors, bacterial or viral infections and immune responses mediate the apoptotic process (Thompson, 1995). Apoptosis is driven by a family of cysteine proteases called caspases (Thornberry, 1985).

In chronic inflammatory diseases such as periodontitis tissue loss occurs as a result of pathologic tissue breakdown and inadequate tissue repair. Induction of apoptosis of host cells such as fibroblasts by periodontal pathogens or their products might add to periodontal tissue destruction. A reduction in the number of fibroblasts has been reported during inflammation of the gingiva (Zappa et al., 1992). Apoptosis of periodontal fibroblasts and osteoblasts has been shown to contribute to pathogenesis of experimental periodontitis in rats (Liu et al., 2003). Apoptosis of fibroblasts has been reported in the areas of gingival inflammation (Koulouri et al., 1999). Various *in vitro* studies have shown that bacterial products induce apoptosis of fibroblast and osteoblast cell lines (Gadhavi *et al* 2000, Wang *et al* 1999, Yamamoto *et al* 1999). The role of cytokines such as TNF in inducing apoptosis of fibroblasts has been investigated (Graves *et al* 2001). Graves and coworkers in an *in vivo* study inoculated *P. gingivalis* below periosteum of mice calvaria

(Graves *et al* 2001). They observed that *P. gingivalis*-induced fibroblast apoptosis was greatly reduced in mice lacking TNF receptors and proposed that TNF caused most of the programmed cell death of fibroblasts. They further concluded that cytokines such as TNF might play a more important role in apoptosis of fibroblasts than the direct effects of bacterial products (Graves *et al* 2001).

Apoptosis of other resident host cells such as lymphocytes might also shift the balance in favor of tissue destruction. *In vitro* studies have shown direct cytotoxic effect of bacteria on lymphocytes and human gingival keratinocytes (Geatch *et al* 1999, Sorkin and Niederman 1998). Induction of apoptosis of peripheral blood mononuclear cells and B lymphocytes in periodontal tissue by bacterial toxins and lipopolysaccharides has also been displayed (Kurita-Ochiai *et al* 1999, Ohguchi *et al* 1998).

Apoptosis also has a major role to play in the resolution of inflammation by bringing about programmed cell death of inflammatory cells. Thus, another possible mechanism contributing to periodontal pathogenesis might be the delayed apoptosis of neutrophils. Incubation of neutrophils with lipopolysaccharides from *P. gingivalis* delays the apoptosis of polymorphonuclear leucocytes (PMNL) (Hiroi *et al* 1998, Ichinose *et al* 1990, Preshaw *et al* 1999). Granulocyte-monocyte colony stimulating factor (GM-CSF) is secreted during the inflammatory response in adult periodontitis and reduces apoptosis of neutrophils (Gamonal *et al* 2001). Van Dyke and Serhan proposed that periodontitis resulted from the lack of resolution of inflammation at the site of chronic inflammation (Van Dyke and Serhan, 2003). This could be due to failure of endogenous anti-inflammatory mediators to overcome proinflammatory mechanisms (e.g. neutrophil mediated tissue injury in localised aggressive periodontitis) (Van Dyke and Serhan, 2003). A possible mechanism of delayed apoptosis has been proposed, whereby neutrophils accumulate in periodontitis lesions resulting in exacerbation of neutrophil-mediated tissue damage (Berker et al., 2005). Neutrophil apoptosis changes the phenotype of the monocyte resulting in the production of anti-inflammatory cytokines (IL-10) and suppression of proinflammatory cytokines (IL-1β) in response to Porphyromonas gingivalis lipopolysaccharide (Berker et al., 2005). All this evidence suggests an important role for apoptotic mechanisms in the pathogenesis of periodontal diseases.

1.5 TNF (Tumor Necrosis Factor) superfamily

To date there have been 17 TNF ligands identified within the TNF superfamily and 21 members within the TNF Receptor (TNFR) superfamily. Prominent among these are TNF-α (Tumor necrosis factor-α), Fas ligand, CD40L/CD 154, lymphotoxins, Receptor activator NF κβ ligand (RANKL), TNF related apoptosis inducing ligand (TRAIL) and osteoprotegerin (OPG) (Chicheportiche et al., 1997). Interactions between these ligands and their receptors take part in a number of biological processes including inflammation, immune responses, cell proliferation and differentiation, apoptosis, bone remodeling and anti-tumor activity (Aggarwal 2003, Locksley *et al* 2001). Any disturbance in the delicate balance of various biological mediators might result in progressive chronic inflammatory diseases.

Following binding with their ligand, TNFRs transmit signals through recruiting various adaptor molecules that bind to their cytoplasmic domains. The adaptor molecules include TNFR-associated factors (TRAFs), which generally activate the transcription factor nuclear factor-κβ and kinases of the mitogen-activated protein kinase (MAPK) family. Others such as TNFR-associated death domain, Fas-associated death domain and receptor interactive protein 1 lead to the recruitment of caspases which could cause apoptotic cell death (Baud and Karin 2001, Darnay and Aggarwal 1999).

Tumor Necrosis Factor-α (TNF-α) is one of the major proinflammatory cytokines involved in the tissue destruction seen in periodontitis. It is a multifunctional cytokine involved in inflammation, acute-phase response, cell proliferation, differentiation and apoptosis (Tracey and Cerami, 1993). It is produced by many cell types, including macrophages, monocytes, lymphocytes, keratinocytes and fibroblasts, in response to inflammation, infection, injury and other environmental challenges (Baud and Karin, 2001). The binding of TNF-α to its receptors TNFR1 and TNFR2 causes activation of caspases and two major transcription factors, NF-κβ and AP-1 which in turn regulate genes involved in chronic and acute inflammatory responses (Barnes and Karin, 1997). It recruits neutrophils and stimulates their degranulation, stimulates IL-1 synthesised by fibroblasts and monocytes, triggers the release of various vasoactive amines and prostaglandins and stimulates matrix metalloproteinase production by various cells. Although, TNF-α stimulates bone resorption, it is not as potent as IL-1 (Gemmell et al., 1997). A schematic representation of these processes is shown in Figure 1.1.

-

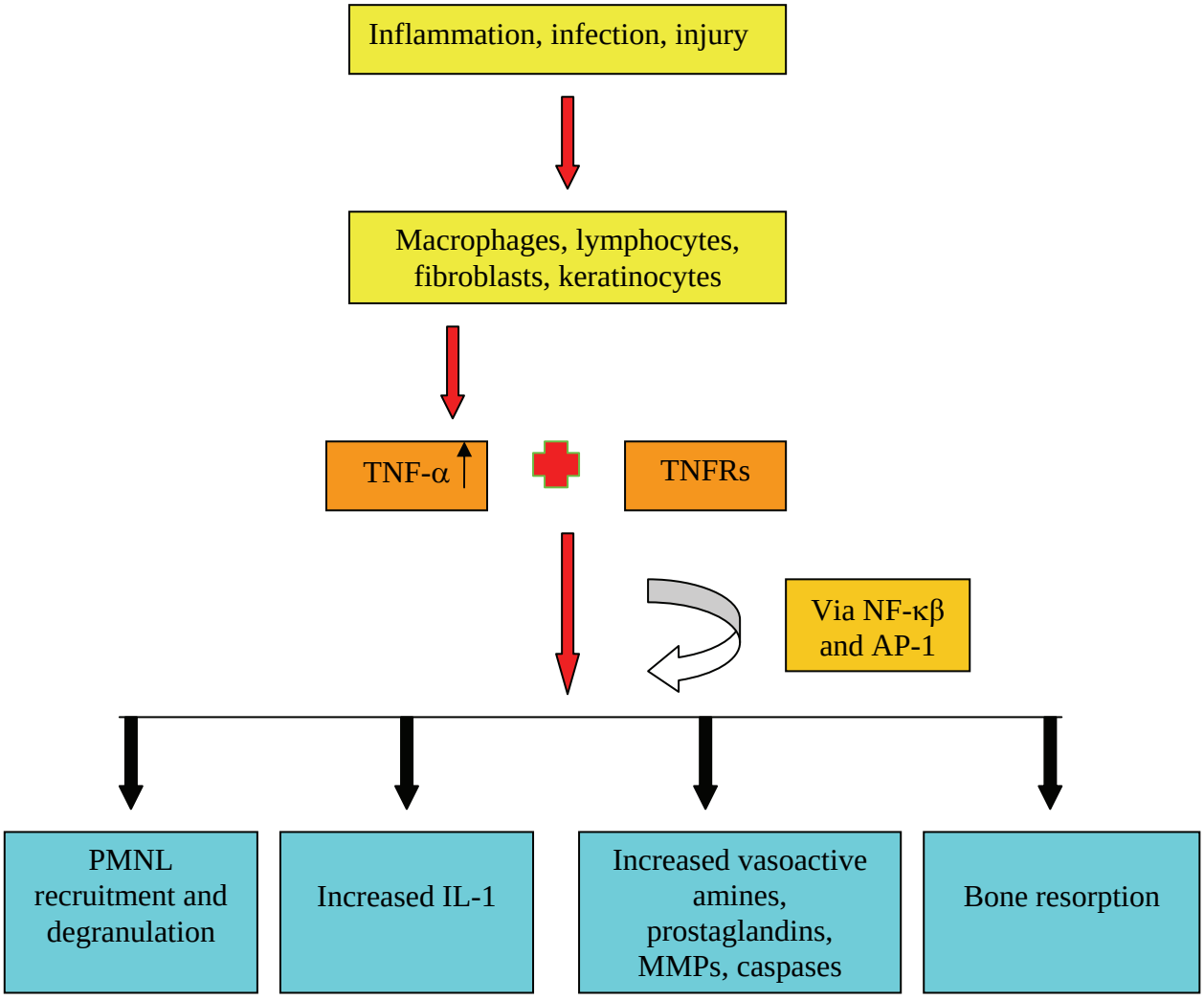


Figure 1.1 Role of TNF-α in inflammation, infection and injury

1.6 Role of RANKL and OPG in periodontitis

One of the members of the TNF superfamily is Receptor Activator NF κβ ligand (RANKL). Human RANKL is a type II transmembrane protein with an approximate mass of 45kD and is regarded as a major regulator of pathological bone resorption. RANKL is expressed by osteoblasts and stromal cells (Yasuda *et al* 1998), fibroblasts (Quinn *et al* 2000, Takayanagi *et al* 2000) and activated T cells (Horwood *et al* 1999, Kotake *et al* 2001, Teng *et al* 2000, Vernal *et al* 2006) and B-cells (Yun et al., 1998). The receptor for RANKL is RANK (receptor activator of NFκβ). RANK is a member of Tumor Necrosis Factor-Receptor (TNF-R) superfamily. RANK is expressed as a transmembrane heterotrimer on the surface of haematopoietic osteoclasts progenitors, mature osteoclasts, chondrocytes, mammary gland epithelial cells (Fata *et al* 2000, Nakagawa *et al* 1998).

RANK is also expressed by cells of the monocyte/macrophage lineage, B and T cells, dendritic cells and fibroblasts (Khosla, 2001).

Binding of RANKL to RANK expressed on osteoclast progenitors and osteoclasts, results in osteoclastogenesis by stimulating differentiation of osteoclast progenitors and activation of mature osteoclasts (Burgess *et al* 1999, Hsu *et al* 1999, Lacey *et al* 1998, Nakagawa *et al* 1998). It also inhibits osteoclast apoptosis (Fuller et al., 1998). *In vitro* studies report that RANKL can both activate mature osteoclasts and mediate osteoclastogenesis in the presence of macrophage colony stimulating factor-CSF1 (Lacey *et al* 1998, Yasuda *et al* 1998). After RANKL binding, RANK communicates with the cytoplasm of cells through tumor necrosis factor receptor associated factors (TRAFs) 1, 2, 3, 5 and 6 and activate downstream pathways such as NF-κβ, JNK/SAPK, p38 and Akt/PKB (Arron and Choi, 2000).

Osteoprotegerin (OPG) is another soluble TNF "receptor-like" molecule which is a natural inhibitor of RANKL. It binds to RANKL with high affinity and prevents its ligation to RANK (Akatsu *et al* 1998, Lacey *et al* 1998, Yasuda *et al* 1998). It has been shown to be expressed by human periodontal ligament cells, gingival fibroblasts and epithelial cells (Kanzaki *et al* 2002, Sakata *et al* 1999) as well as endothelial cells (Crotti *et al* 2003).

OPG and RANKL are modulated by various inflammatory cytokines present in periodontitis such as IL-1, IL-6, IL-7 and IL-11 (Brandstrom *et al* 1998, Hofbauer *et al* 1999, Liu *et al* 2003, Mogi *et al* 2004, Nakashima *et al* 2000, Teng *et al* 2000, Toraldo *et al* 2003). Activated T-cells and B-cells play a key role in pathogenesis of periodontal disease (Takeichi et al., 2000). Activated T-cells can act directly via promoting RANKL expression and indirectly via production of cytokines such as TNFα, IL-11 and Il-17 that in turn induce RANKL expression by osteoblasts and bone marrow cells. Furthermore, Interferon-γ from T-cells and lipopolysaccharides from bacteria can induce the production of IL-1, TNF-α or IL-6 by macrophages which in turn increases RANKL expression in osteoblasts and bone marrow cells (Theill et al., 2002). Other factors produced by activated T cells such as IL-3 or TNF related apoptosis inducing ligand (TRAIL) inhibit RANKL-mediated proliferation and inhibition of osteoclasts (Zauli *et al* 2004). Depending on the presence of Th1 or Th2 cytokines, activated B-cells can affect osteoclastogenesis in a positive or negative fashion (Choi *et al* 2001). B-cells can up-regulate RANKL and

contribute to bone resorption (Han et al., 2006). A schematic representation of these interactions is shown in Figure 1.2.

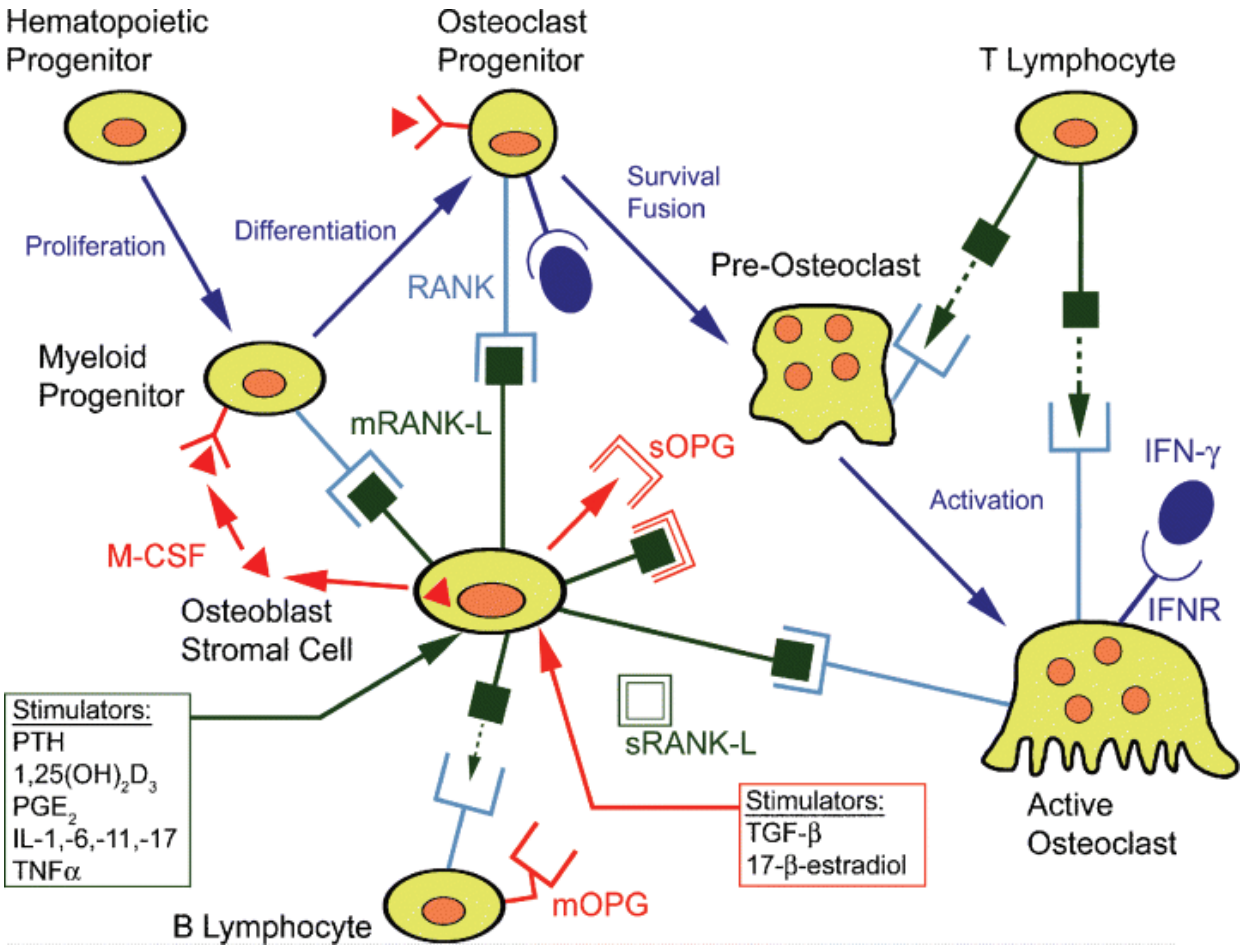


Figure 1.2 Schematic illustration of the RANK-RANKL-osteoprotegerin system. Adapted from Nanci and Bosshardt (2006).

The pathological role of RANKL and OPG in periodontitis has been studied. Significantly higher levels of RANKL and lower levels of OPG in the periodontitis tissues have been reported (Crotti *et al* 2003). Furthermore, RANKL protein is associated with lymphocytes and macrophages and many leukocytes expressed RANK mRNA in periodontitis tissue (Crotti *et al* 2003). A semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) study showed that the level of RANKL mRNA is highest in advanced periodontitis (Liu et al., 2003). In contrast, the level of OPG mRNA in both advanced and moderate periodontitis is lower than that in the healthy tissues (Liu et al., 2003).

Lu and coworkers observed high levels of RANKL and IL-6, but not OPG, in gingival crevicular fluid (GCF) of patients with chronic periodontitis. RANKL-positive cells were found in significant numbers in the inflammatory connective tissue zone of diseased

gingiva compared to healthy gingiva. These results indicate that expression of RANKL is positively correlated with IL-6 in the GCF (Lu *et al* 2006).

RANKL and OPG expression are differentially regulated in various forms of periodontitis and the relative RANKL/OPG ratio appears to be indicative of disease occurrence (Bostanci et al., 2007b). Stronger RANKL expression and high relative RANKL/OPG ratio is seen in chronic and aggressive periodontitis patients compared to patients with gingivitis and healthy individuals (Bostanci et al., 2007b, Wara-aswapati et al., 2007). Although, the relative RANKL/OPG ratio is similar in both generalised aggressive and chronic periodontitis, stronger RANKL expression is seen in generalised aggressive periodontitis, whereas chronic periodontitis patients have weaker OPG expression (Bostanci et al., 2007b). High levels of RANKL and low levels of OPG, resulting in an enhanced RANKL/OPG ratio, has also been found in GCF of patients with chronic and generalised aggressive periodontitis (Wara-aswapati et al., 2007, Bostanci et al., 2007a, Mogi et al., 2004).

From these studies it is evident that up-regulation of the RANKL and down-regulation of OPG might be one of the major mechanisms modulating local bone destruction in periodontitis. The bone resorption seen in periodontitis may be driven by proinflammatory cytokines that regulate the RANKL expression on mesenchymal cells, activated T-cells and B-cells (Nanci and Bosshardt, 2006).

1.7 TNF-like weak inducer of apoptosis (TWEAK)

TNF-like weak inducer of apoptosis (TWEAK) is one of the members of the TNF superfamily. The cDNA of TWEAK was discovered during cloning of an erythropoietin related gene in murine peritoneal macrophages (Chicheportiche et al., 1997). The gene was assigned to the TNF superfamily based on characteristic sequence motifs. It was named TWEAK due to its relationship to TNF and weak apoptotic properties. It was also known as Apo3 ligand as it was shown to bind to the TNFR superfamily member called Apoptosis antigen-3/Death Receptor-3 (Apo3/DR3) (Marsters et al., 1998). However, other investigators could not demonstrate its binding to DR3 *in vitro* and reported that TWEAK could act on DR3- negative cells (Kaptein *et al* 2000, Schneider *et al* 1999). Thus, TWEAK is the name generally used and accepted for this protein (Kaptein et al., 2000).

1.7.1 Structure

TWEAK is a 249 amino acid type II transmembrane protein and its active form has a compact trimer structure similar to other members of TNF superfamily (Chicheportiche et al., 1997). TWEAK has a C-terminal extracellular region (206 amino acids, comprised of a stalk region and a prototypical TNF-homology domain containing one potential N-glycosylation site); a transmembrane domain (25 amino acids); and an N-terminal intracellular domain (18 amino acids, containing a potential protein kinase C phosphorylation site) (Chicheportiche *et al* 1997, Marsters *et al* 1998).

1.7.2 Expression

TWEAK mRNA is abundant in the human heart, pancreas, colon, small intestine, lung, ovary and prostate, while its levels are lowest in kidney, testis and liver (Chicheportiche et al., 1997). The secondary immune system has been suggested to express TWEAK and lymphoid organs including spleen, lymph node, appendix and peripheral blood lymphocytes contain abundant TWEAK mRNA (Chicheportiche *et al* 1997, Marsters *et al* 1998). However, even though TWEAK mRNA is present in many tissues, this may not translate to actual expression of TWEAK protein. Other than in peripheral blood lymphocytes (Chicheportiche *et al* 1997, Marsters *et al* 1998, Pradet-Balade *et al* 2002), TWEAK mRNA has been found in mouse peritoneal macrophages (Chicheportiche et al., 2002) and human fibroblasts (Semov et al., 2002).

TWEAK expression in macrophages has been shown to be upregulated by Interferon-γ (Nakayama *et al* 2000). Thus, TWEAK is expressed by many inflammatory leukocytes such as monocytes/macrophages, dendritic cells, natural killer cells, activated T cells and plasma cells (Dharmapatni *et al* 2008, Kaplan *et al* 2002, Kawakita *et al* 2004, Zheng and Burkly 2008).

TWEAK is initially synthesised as a full-length, plasma-membrane-bound protein which can undergo intracellular proteolysis to form a soluble active form of TWEAK which is released extracellularly (Chicheportiche et al., 1997). This secreted form of TWEAK makes it available as a long-range biological signal (Chicheportiche et al., 1997). As all of the membrane bound TWEAK does not undergo proteolysis, it can be detected in different cell lines (Winkles, 2008).

1.8 Fibroblast growth factor-inducible 14 (Fn14)/TWEAK receptor

1.8.1 Structure

Although initially Death Receptor-3 (DR3) was thought to be the receptor of TWEAK (Marsters et al., 1998), later studies reported that TWEAK could act on DR3 negative cells and did not always promote apoptosis (Kaptein *et al* 2000, Lynch *et al* 1999). Since then, human fibroblast growth factor-inducible 14 protein (Fn14)/TWEAKR has been identified as the cell surface receptor for TWEAK (Wiley et al., 2001). It has been cloned as a fibroblast growth factor-inducible gene (Meighan-Mantha *et al* 1999). It belongs to the TNF-receptor superfamily and is expressed on endothelial cells and smooth muscle cells. Fn14 encodes a 102 amino acids type I transmembrane protein, having 6 cysteine residues in its extracellular regions. Its short cytoplasmic domain is composed of 28 amino acids which contains a TNF receptor-associated factor (TRAF)-binding motif (Wiley et al., 2001).

Polek and coworkers found that TWEAK ligand induced differentiation into osteoclasts by a murine monocyte/macrophage cell line which did not display Fn14 (Polek et al., 2003). They suggested this may occur via a second TWEAK receptor (Polek et al., 2003). It was later noted that the clone of Fn14 antibody used for the above mentioned study was not optimal for detection of mouse Fn14 (Campbell et al., 2004). In general, most studies suggest that Fn14 is the major receptor for TWEAK and Fn14 solely mediates the known biologic effects of TWEAK (Bossen et al., 2006, Harada et al., 2002, Nakayama et al., 2003). However, other unidentified cell receptors of TWEAK might be present (Winkles, 2008).

1.8.2 Expression

Fn14 is widely expressed in a variety of tissues, cells and cell lines. Fn14 mRNA is present at relatively high levels in brain, heart, aorta, pituitary, adrenal, mammary gland, kidney, lymph node, lung, liver, spleen, thymus, bladder and uterus (Wiley and Winkles, 2003). Fn14 protein is expressed by many cell types including epithelial cells (Michaelson et al., 2005), mesenchymal cells (Girgenrath *et al* 2006) and endothelial cells (Donohue *et al* 2003, Harada *et al* 2002, Perper *et al* 2006). Fn14 is also expressed by all tissue progenitor cells of mesenchymal lineage (Girgenrath *et al* 2006) and embryonic stem cells (Ramalho-Santos et al., 2002). Other cells expressing Fn14 are fibroblasts (Hosokawa *et al* 2006,

Perper *et al* 2006), monocytes/macrophages (Perper et al., 2006), osteoblasts and chondrocytes like cells (Perper et al., 2006). An elevated amount of Fn14 protein has been detected on certain but not all neoplastic tissues, including breast, liver and brain tumors (Brown *et al* 2003, Michaelson *et al* 2005, Tran *et al* 2003). It is upregulated in several experimental models of tissue regeneration and repair (Feng *et al* 2000, Wiley *et al* 2001).

The expression of Fn14 is regulated by many growth factors, cytokines, hormones and compounds including IL-1 (Hosokawa *et al* 2006), TNF-α (Tran *et al* 2006), INF-γ (Maecker *et al* 2005), bacterial lipopolysaccharides (Chacon *et al* 2006), bone morphogenetic protein-6 (Ren et al., 2007), epidermal growth factor, platelet derived growth factor (Meighan-Mantha *et al* 1999), transforming growth factor-β (Hosokawa *et al* 2006), vascular endothelial growth factor-A (Donohue *et al* 2003) and fibroblast growth factor 1 (FGF1) or fibroblast growth factor 2 (FGF2) (Feng et al., 2000). TWEAK has also been demonstrated to induce Fn14 expression in an *in vitro* study involving glioma cell lines (Tran *et al* 2006). Thus, these mechanisms might amplify TWEAK-stimulated cellular responses under certain conditions *in vivo* (Winkles, 2008).

1.9 Mechanism of TWEAK/Fn14 interaction

Fn14/TWEAKR serves as a receptor for TWEAK, bringing about its proinflammtory and cell death effects by stimulating several different signaling cascades (Winkles, 2008). In particular, prolonged NF-κB pathway activation (via biphasic activation of both canonical and noncanonical pathways) following binding of TWEAK to Fn14 has been demonstrated (Brown *et al* 2003, Harada *et al* 2002, Nakayama *et al* 2003, Saitoh *et al* 2003).

The extracellular domain of human TWEAK binds to murine Fn14 protein and stimulates the NF-Kappa B transcription factor signaling pathway (Brown *et al* 2003). Fn14 is the functional TWEAK receptor associated with four distinct TRAF (tumor necrosis factor-receptor associated factors 1, 2, 3, 5) (Brown *et al* 2003). The TRAF family of signal transducers plays an important role in TWEAKR/Fn14 mediated NF-κβ activation (Han et al., 2003). This has been explained by the potential interaction between the cytoplasmic tail of TWEAKR and the TRAF molecule through a TRAF-binding motif present in TWEAKR (Han et al., 2003). A schematic representation of this interaction is shown in Figure 1.3A.

There is evidence to indicate that multiple intercellular signaling cascades including ERK1/2, JNK 1/2 and p38 might be involved in the biological effect of TWEAK (Donohue *et al* 2003, Saas *et al* 2000). In another study, phosphatidylinositol 3-kinase (PI3K) and nuclear factor-kappa B (NF-kappa B) inhibitor was shown to inhibit both intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression induced by TWEAK (Hosokawa *et al* 2006). Most of the proinflammatory molecules such as matrix metalloproteinases (MMPs), interleukin-8 (IL-8), granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES), ICAM-1, VCAM-1 which are induced by TWEAK/Fn14 interaction are mediated by the NF-κβ signaling pathway (Winkles, 2008).

From the current literature it is evident that the soluble form of TWEAK can bind to its receptor Fn14 and induce cellular responses, but it is not known whether membrane bound TWEAK can also act in a cell contact-dependent, juxtacrine manner to activate Fn14-positive cells (Winkles, 2008).

Some studies have suggested Fn14 signaling events might be ligand-independent (Tanabe et al., 2003) or TWEAK-independent (Dogra et al., 2007). Fn14 overexpression on the cell surface induces monomer trimerization and trimer multimerization, which then triggers TRAF association and the subsequent molecular and cellular events (Winkles, 2008). This is similar to ligand-independent signaling displayed in the TNF receptor family under conditions of high expression (Burkly *et al* 2007, Xu and Shu 2002). TWEAK activity independent of Fn14 has also been suggested whereby soluble TWEAK internalizes into many cell lines in an Fn14 independent manner and translocate directly into nucleus resulting in nuclear translocation of glycogen sunthase kinase-3β (GSK-3β) and p65 leading to induction of NF-κβ-driven gene expression (De Ketelaere *et al* 2004). Recently, TWEAK was reported to interact with CD163, a scavenger receptor present exclusively on monocyte/macrophage line (Bover *et al* 2007). The varied mechanisms of TWEAK and Fn14 signaling suggest a heterogenic role in biologic processes. Nonetheless, TWEAK independent/Fn14 independent pathways might indicate a separate as yet unidentified receptor or ligand (Winkles, 2008). A schematic representation of TWEAK independent signaling is shown in Figure 1.3B.

Figure 1.3 Mechanism of TWEAK/Fn14 interaction

A. TWEAK and Fn14 binding

B. TWEAK independent signaling

Adapted from Burkly *et al* (2007).

1.10 Biological effects of TWEAK

TWEAK has been demonstrated to have multiple roles in various biological processes (Figure 1.4). These include induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis (Campbell *et al* 2006, Chicheportiche *et al* 1997, Kaplan *et al* 2000, Lynch *et al* 1999, Maecker *et al* 2005). Recent studies have also shown that TWEAK has a role to play in tissue repair and regeneration following acute tissue injury (Girgenrath *et al* 2006). It has been proposed that TWEAK/Fn14 signaling might be a universal mechanism which plays a role in various inflammatory and autoimmune disorders mediating pathologic tissue remodeling (Zheng and Burkly, 2008).

Figure 1.4 TWEAK is a multifunctional cytokine. Adapted from Burkly *et al* (2007).

1.10.1 Proinflammatory effects

Many authors have reported the proinflammatory effects of TWEAK including stimulation of production of cytokines and extracellular matrix degrading enzymes. The proinflammatory role of TWEAK has been investigated in detail in chronic immunoinflammatory diseases such as rheumatoid arthritis. Most of these studies indicate that TWEAK might be considered as an additional player in the pathogenesis of chronic immunoinflammatory diseases leading to tissue destruction.

TWEAK induces secretion of chemokines such as IL-8 from fibroblasts and cell lines of colon carcinoma and melanoma (Chicheportiche et al., 1997). Later, Saas and coworkers reported the proinflammatory effects of TWEAK including increased IL-6 secretion and intercellular adhesion molecule-1 (ICAM-1) expression when added to astrocyte cell cultures suggesting that TWEAK could play a significant role in brain inflammation (Saas et al., 2000). It was found that TWEAK upregulates surface expression of adhesion molecules ICAM-1 and E-selectin in human umbilical vein endothelial cells and also enhances secretion of MCP-1 (Monocyte Chemotactic protein-1) and IL-8 by these cells (Harada *et al* 2002). TWEAK induces production of matrix metalloproteinase-1 (MMP-1) and other proinflammatory molecules such as PGE2, IL-8, IL-6, RANTES and IP-10 (interferon-inducible protein 10) in human dermal fibroblasts and human synoviocytes

from patients with rheumatoid arthritis and advance osteoarthritis in a dose-dependent fashion (Chicheportiche et al., 2002). Although, the proinflammatory properties of TWEAK are much less pronounced than those of IL-1β and TNF, TWEAK has been shown to result in a four-fold increase in the production of PGE2, MMP-1 and IL-8 in the presence of TNF or IL-1β (Chicheportiche et al., 2002). TWEAK also significantly upregulates RNA message for macrophage inflammatory protein (MIP)-1 alpha in rheumatoid synoviocytes but not dermal fibroblasts (Chicheportiche et al., 2002) . Induction by TWEAK of most of the inflammatory mediators requires a longer incubation time due to induction of one or more intermediate mediators, which are as yet unknown (Chicheportiche et al., 2002). TWEAK might also contribute to the persistence of the inflammatory response by recruiting neutrophils and macrophages (Chicheportiche et al., 2002). Chicheportiche *et al* (2002) reported that the effects of TWEAK, although not affected by antibodies to TNF, were completely abrogated by a blocking antibody to TWEAK. Referring to the non-response of patients to current host-modulation therapies, Chicheportiche *et al* (2002) proposed that TWEAK might have a synergistic destructive role in the pathogenesis of chronic immunoinflammatory diseases. In a recent *in vitro* study, a major role of TWEAK/Fn14 interaction was noted in the synovitis associated with rheumatoid arthritis by directly inducing the proliferation of synovial fibroblasts and by up-regulating the production of inflammatory cytokines/chemokines as well as the expression of ICAM-1 (Kamijo et al., 2008). High serum levels of TWEAK, TNF alpha and IL-6 are seen in rheumatoid arthritis patients compared to normal controls (Park et al., 2008). Moreover, serum TWEAK levels correlate with disease activity of rheumatoid arthritis (Park et al., 2008) and high TWEAK/Fn14 expression is seen in these patients (Dharmapatni et al., 2008).

In a murine model of collagen-induced arthritis (CIA), dramatically elevated serum levels of TWEAK have been found and it has been suggested that TWEAK might be a new therapeutic target for human rheumatoid arthritis (Perper et al., 2006). Furthermore, the clinical severity of CIA was reduced significantly when TWEAK was blocked by a neutralizing monoclonal antibody (mAb) (Perper et al., 2006). In the same study, the anti-TWEAK treatment proved efficacious when administered just before the disease onset but not during the priming phase of CIA (Perper et al., 2006). TWEAK inhibition significantly reduced serum levels of a panel of arthritogenic mediators, including chemokines such as macrophage inflammatory protein 1β (MIP-1β/CCL-4), lymphotactin (XCL-1), IFN-γ-inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1) and RANTES, as

well as the matrix metalloprotease-9 (Perper et al., 2006). Thus, there is strong evidence for TWEAK contributing to joint tissue inflammation, angiogenesis and damage, as well as inhibition of endogenous repair in rheumatoid arthritis (Perper et al., 2006). The inhibitory effect of anti-TWEAK antibodies has also been confirmed in another study in a murine model of collagen-induced arthritis (Kamata et al., 2006).

On the basis of *in vitro* studies, multiple mechanisms have been proposed by which TWEAK could contribute to the pathology of rheumatoid arthritis (Perper et al., 2006). Firstly, TWEAK, produced by infiltrating or synovium-resident macrophages, may promote joint inflammation by stimulating synovial fibroblasts to replace inflammatory cytokines and chemokines, including IL-1, IL-6, IL-8, IP-10, RANTES, IL-15 and IL-17. Secondly, TWEAK could directly trigger cartilage and bone damage as observed by the production of a number of matrixmetalloproteases in chondrocytes in response to TWEAK and by promoting osteoclastogenesis. Thirdly, TWEAK may contribute to joint pathology through directly promoting synovial angiogenesis. Finally, it was reported that TWEAK may promote an immature osteoblast phenotype by blocking the differentiation of precursor cells of the osteoblast and chrondrocyte lineage (Perper et al., 2006).

A possible role for TWEAK in the pathophysiology of skin disorders has been reported whereby TWEAK induced RANTES via its interaction with Fn14 on primary cultured normal human keratinocytes. This production of RANTES could be augmented by transfroming growth factor-β1 (Jin et al., 2004). Xu and co-workers (2004) demonstrated that TWEAK/Fn14 interaction might be involved in airway inflammatory responses as it stimulated the production of IL-8 and GM-CSF from human bronchial epithelial cells. It has been suggested that TWEAK might be involved in the pathogenesis of artheroscelrosis (Kim *et al* 2004). TWEAK induces various proinflammatory cytokines such as IL-6, MCP-1, IL-8 and MMP-9 from macrophages in atherosclerotic plaques, thus reducing the plaque stability (Kim *et al* 2004). An unpublished study by Putterman and coworkers has demonstrated that mesangial cells from lupus-prone mice released MCP-1, RANTES and IP-10 in response to Fn14 engagement by TWEAK suggesting that TWEAK/Fn14 interaction might play a crucial role in the inflammatory cascade leading to lupus nephritis (Campbell *et al* 2004). Another *in vivo* study confirmed the key role of TWEAK/Fn14 interaction in the pathogenesis of nephritis in the chronic graft-versus-host model of systemic lupus erythematosus by promoting local inflammatory events (Zhao *et al*

2007).TWEAK has NF-κβ-dependent proinflammatory effects on kidney tubular epithelial cells *in vitro* and *in vivo* (Sanz et al., 2008).

The proinflammatory effect of TWEAK/Fn14 interaction contributes to the pathogenesis of experimental autoimmune encephalitis (Mueller *et al* 2005). TWEAK has also been shown to increase the secretion of MCP-1 by CNS- endothelial cells and its production could be prevented by blocking Fn14 signaling (Mueller *et al* 2005). Campbell and coworkers (2006) found that murine mesangial cells express cell surface TWEAK receptors. TWEAK had a substantial direct proinflammatory effect on these cells which was time and dose-dependent. The effects were mediated via Fn14 receptor and the NF-κβ signaling pathway. The chemokine induction was abolished by blocking TWEAK with soluble Fn14 and by murine IgG2a anti-TWEAK antibodies.

In a mouse model, TWEAK has been reported to regulate IFN-γ production by decreasing NK cell numbers and by decreasing the amount of IFN-γ from activated NK cells (Maecker *et al* 2005). TWEAK also suppresses IL-12 production by macrophages (Maecker *et al* 2005). IL-12 and INF-γ in turn are known to play a key role in transition of innate to adaptive immune response by directing the development of T helper 1 subtype (Chehimi and Trinchieri, 1994). Moreover, the balance of IL-12 production versus IL-10 and IL-4 production during immune response might play an important role in determining Th-1 type versus Th-2 type immune responses (Trinchieri et al., 1992). Thus, TWEAK/Fn14 pathway might also have a possible role in modulating immune responses (Maecker *et al* 2005).

Hence, TWEAK has shown to have proinflammatory role in various chronic inflammatory diseases such as rheumatoid arthritis (Perper et al., 2006), inflammatory renal disease seen in systemic lupus erythematosus (Schwartz *et al* 2006), neuroinflammation (Desplat-Jego *et al* 2002, Desplat-Jego *et al* 2005, Iocca *et al* 2008) and autoimmune diseases such as multiple sclerosis (unpublished study by D. Gveric, University College London, cited in Burkly *et al* 2007).

1.10.2 TWEAK - the cell growth factor

TWEAK has been shown to act as a growth factor for multiple cell types, including synoviocytes, astrocytes and vascular cells (Desplat-Jego *et al* 2002, Donohue *et al* 2003,

Kamata *et al* 2006). In a recent three-dimensional model culture system study, the murine Eph4 mammary epithelial cell line exhibited enhanced proliferation at lower TWEAK concentrations (2–10 ng/ml) and was associated with the appearance of a branching phenotype and reduced differentiation, while relatively high doses of TWEAK (100 ng/ml) induced Eph4 cell death (Michaelson et al., 2005). Thus, variation of the relative levels of TWEAK and Fn14 under different physiological conditions may be one means of regulating context-dependent, cellular responses induced by TWEAK (Burkly et al., 2007). The ability of TWEAK to induce cell growth might be suggestive of TWEAK's role in tissue repair after acute injury and in pathological hyperplasia seen in arthritis (pannus formation), colitis (crypt epithelial deformity), neurodegenerative diseases (gliosis) and cancer (Burkly et al., 2007).

1.10.3 TWEAK and angiogenesis

Angiogenesis is an important process which plays an integral role in repair and regeneration. TWEAK has been shown to induce angiogenesis and result in proliferation of endothelial cells (Jakubowski *et al* 2002, Lynch *et al* 1999). TWEAK may either act alone or in combination with other growth factors like FGF-2 and VEGF-A to regulate pathological angiogenesis (Donohue et al., 2003). Alone TWEAK is able to promote endothelial cell survival and resistance to apoptosis (Jakubowski *et al* 2002). In the presence of bFGF (fibroblast growth factor), TWEAK can contribute to endothelial cell proliferation and migration whereas it antagonizes the morphogenic response of endothelial cells to VEGF (Jakubowski *et al* 2002). Moreover, TWEAK appears to be a growth and migration factor but not a survival factor for endothelial cells (Donohue et al., 2003). It has been noted that TWEAK contributes to the pathogenesis of rheumatoid arthritis by promoting synovial angiogenesis (Perper et al., 2006).

1.10.4 TWEAK's role in tissue repair and regeneration

Our understanding of the role of TWEAK in tissue repair and regeneration is still evolving. Following acute tissue injury, inflammation promotes the removal of cellular debris and directly influences the behavior of progenitor cells (Duffield, 2003, Arnett et al., 2001, Mutsaers et al., 2002). Fn14 expression is upregulated at sites of injury (Winkles, 2008). In *in vitro* models, a serum fraction of coagulated blood has been shown to induce Fn14 expression (Meighan-Mantha et al., 1999, Feng et al., 2000). In *in vivo* conditions this

would be possible only at sites of tissue injury and remodeling (Winkles, 2008). Other animal studies support these findings wherein Fn14 expression has been found to be increased at sites of injury (Feng et al., 2000, Wiley et al., 2001).

Girgenrath and coworkers found that TWEAK/Fn14 interaction is a novel regulator of skeletal muscle precursor cells (Girgenrath *et al* 2006). TWEAK and Fn14 are minimally expressed in the undamaged skeletal muscle of normal adult mice and markedly increased in damaged areas after injury. Following cardiotoxin-induced injury, mice deficient in Fn14 showed reduced inflammatory response, including delayed infiltration of macrophages and neutrophils and chemokine expression and delayed muscle fiber regeneration. Interestingly, in Fn14 deficient mice, other pathways such as TNF/TNFR showed increased expression to compensate for lack of TWEAK/Fn14 interaction (Girgenrath *et al* 2006). Fn14 is expressed on all mesenchymal lineage progenitor cells indicating a broad involvement of TWEAK/Fn14 pathway in various tissue injuries and disease settings (Girgenrath *et al* 2006).

TWEAK is a mitogen for liver progenitor cells and following chemical injury promotes liver progenitor expansion (Jakubowski et al., 2002). On the basis of TWEAK's proinflammatory and progenitor cell expanding activities (Girgenrath et al., 2006, Jakubowski et al., 2002, Perper et al., 2006), it has been suggested that TWEAK has a physiological role in tissue regeneration after acute injury (Burkly et al., 2007). It has been proposed that following tissue injury, Fn14 on stromal and/or tissue progenitor cells is upregulated through a growth factor-mediated mechanism (Burkly et al., 2007). Initially TWEAK may be provided at a low level by tissue-resident cells such as fibroblasts; however, later on the infiltrating inflammatory cells become a major source of TWEAK (Burkly et al., 2007). Once engaged, the TWEAK/Fn14 pathway drives chemokine production by tissue-resident cells, including progenitors (Girgenrath et al., 2006), resulting in additional inflammatory cell infiltration, as well as the expansion of tissue progenitor cells. Thus, the TWEAK/Fn14 complex provides an important positive feedback loop to facilitate tissue repair. As successful repair proceeds, the resolution of inflammation and reduced expression of TWEAK/Fn14 then allow expanded progenitor cells to differentiate and regenerate the tissue (Burkly et al., 2007).

Thus, there is increasing evidence that TWEAK plays a distinct and opposing role in acute inflammation following acute tissue injury compared to chronic inflammation. In the case

of acute injury TWEAK promotes tissue repair. When there is persistent injury or disease, TWEAK has an inherently different role. Here it promotes production of cytokines, chemokines and matrix metalloproteinases, pathological hyperplasia angiogenesis and endothelial cell activation (Perper et al., 2006, Chicheportiche et al., 2002, Campbell et al., 2006, Hosokawa et al., 2006). This leads to chronic inflammation and tissue degeneration. TWEAK may also hinder tissue repair by inhibiting progenitor cell differentiation (Perper et al., 2006, Ando et al., 2006). The roles of TWEAK/Fn14 interaction in acute and chronic inflammation are shown in Figure 1.5.

Figure 1.5 TWEAK in acute and chronic inflammation. Adapted from Burkly *et al* (2007).

1.10.5 TWEAK and apoptosis

TWEAK has been described to have a weak apoptotic effect on a number of cell types (Chicheportiche et al., 1997, Nakayama et al., 2002, Marsters et al., 1998). Multiple pathways of TWEAK induced cell death have been described in literature. Two cell-type specific mechanisms of cell death induced by TWEAK have been described, including caspase-dependent apoptosis and cathepsin B-dependent necrosis (Nakayama et al., 2002). However, as Fn14 lacks the canonical death domain, binding of TWEAK to Fn14 might not directly trigger the extrinsic apoptotic pathway characterised by formation of the death-inducing signaling complex and caspase 8 (or caspase 10) activation (Peter and Krammer, 2003). It has been proposed that TWEAK induces apoptosis indirectly via endogenously produced TNF-alpha interacting with the TNF receptor (Schneider et al., 1999). Wilson and coworkers showed that the cell death of HT29 adenocarcinoma cells induced by TWEAK is caspase-independent, encompassing features of both necrosis and apoptosis

(Wilson and Browning, 2002, Winkles, 2008). It has also been suggested that TWEAK might have indirect effects that promote killing of tumor cells (Campbell et al., 2004). This was attributed to its ability to induce some of the pro-inflammatory chemokines which could promote the migration and retention of inflammatory cells within the tumor environment, facilitating a cytotoxic anti-tumor immune response (Campbell et al., 2004). A study reported that TWEAK/Fn14 interaction induced cell death in cultured murine tubular epithelial kidney cells costimulated with TNF-α/interferon-gamma, wherein apoptosis was associated with activation of caspase-8, caspase-9 and caspase-3 (Justo et al., 2006) .

In *in vitro* studies TWEAK has also been shown to induce death of non-tumor cell types such as human peripheral blood monocytes and human natural killer cells (Kaplan et al., 2002, Maecker et al., 2005). It still remains to be seen whether the ability to kill monocytes indicates role of TWEAK in resolution of inflammation (Zheng and Burkly, 2008).

Overall, TWEAK has a relatively weak ability to induce cell death and more studies are needed to elucidate its significance in chronic inflammatory diseases.

1.10.6 TWEAK and its effect on bone cells

TWEAK has also been described to cause differentiation of monocytes/macrophages cells into osteoclasts which might indicate a role in pathologic bone destruction (Winkles, 2008).

Polek and coworkers reported that TWEAK can induce RAW 264.7 (monocytes/macrophages) cells to differentiate into tartrate-resistant acid phosphatase (TRAP) positive multinucleated, functional osteoclasts (Polek et al., 2003). However, the RAW cells did not display Fn14 receptors. As the Fn14/TWEAKR neutralizing antibodies did not block TWEAK-induced osteoclast cell differentiation of RAW cells, it was concluded that this effect was not brought about by the interaction of Fn14 and TWEAK (Polek et al., 2003). They suggested that a second TWEAK receptor or TWEAK R2 may be present on the RAW cells. The effect of TWEAK was direct and not mediated by RANKL, as shown by the use of TWEAK- or RANKL neutralizing antibody and by osteoprotegerin (OPG). Thus, it was proposed that TWEAK might be a potentially novel osteoclastic factor acting independently of RANKL (Polek et al., 2003). Both TWEAK and RANKL were observed to have an osteoclastic differentiation effect on RAW cells.

However, TWEAK was unable to induce primary human monocyte precursors into osteoclasts (Polek et al., 2003). It has been speculated that RAW cells, which are more mature, could be more easily differentiated into osteoclasts by TWEAK than the more immature primary human monocyte precursor cells which require additional stimulation from macrophage-colony stimulating factor (M-CSF) (Polek et al., 2003). Factors other than M-CSF might be required for the induction of differentiation of primary human monocyte precursors by TWEAK (Polek et al., 2003). An additive (but not a synergistic) osteoclastic effect of TWEAK on RANKL has been described which has been attributed to the TWEAK activated downstream signaling pathways including mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and NF-κβ, similar to those noted for RANKL (Polek et al., 2003).

It has also been suggested that TWEAK might be a novel regulator of bone homeostasis and play a role in regulation of osteoblast function and differentiation (Ando et al., 2006). This suggestion was based on a study of the biological effects of TWEAK on mouse MC3T3-E1 cells (Ando et al., 2006). MC3T3-E1 is a clonal osteogenic cell line which maintains characteristics of primary osteoblast progenitors and is used for studying osteoblast differentiation and function *in vitro*. It was observed that TWEAK/Fn14 interaction induced RANTES production through the PI3K-Akt pathway, inhibited BMP-2-induced differentiation through MAPK ErK pathway and upregulated RANKL expression through the MAPK ErK pathway in osteoblastic MC3T3-E1 cells (Ando et al., 2006). Moreover, it was also demonstrated that cultured human osteoblasts responded to TWEAK by inducing RANTES production (which was blocked by Fn14-Fc chimera) and expression of the RANKL protein (Ando et al., 2006). RANTES has been shown to be an important chemokine for the migration of osteoclasts (Yu et al., 2004). Also, the role of RANKL in osteoclast differentiation is well understood (Yasuda et al., 1998).

However, unpublished studies by T. Zheng and D. Findlay (cited in Burkly *et al* 2007) reported that TWEAK did not induce osteoclast differentiation in a human monocytic cell line (THP-1) or human peripheral blood monocytes. Thus, the direct effect of TWEAK on osteoclast differentiation is debatable, even though it has been shown to indirectly result in osteoclastic differentiation by upregulating RANKL in osteoblasts (Ando et al., 2006).

1.11 Role of TWEAK/Fn14 signalling in periodontitis

TWEAK has also been studied in periodontal tissues (Hosokawa *et al* 2006). Reverse transcription–polymerase chain reaction (RT-PCR) analysis and immunohistochemistry revealed that TWEAK and TWEAK receptor, Fn14, mRNA and protein were expressed in periodontally diseased tissues (Hosokawa *et al* 2006). TWEAK and Fn14 were mainly expressed by mononuclear cells. When stimulated by TWEAK, human gingival fibroblasts produced IL-8 and vascular endothelial growth factor (VEGF) and enhanced intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression on HGF in a dose-dependent fashion (Hosokawa *et al* 2006). The IL-8 and VEGF production induced by TWEAK was augmented synergistically by simultaneous stimulation with transforming growth factor (TGF)-β1 or IL-1β. This synergistic effect was attributed to the ability of IL-1β and TGF-β1 to enhance the expression of Fn14 in HGF in a dose-dependent manner (Hosokawa *et al* 2006). Furthermore, in the same study, it was reported that TGF-β 1 augments TWEAK induced ICAM-1 expression and inhibits TWEAK-induced VCAM-1 expression. The authors concluded that as IL-8, VEGF, ICAM-1 and VCAM-1 are involved in the inflammatory response in periodontal diseased tissues; TWEAK might be involved in the pathophysiology of periodontal disease (Hosokawa *et al* 2006). Thus, along with other cytokines such as IL-1β and TGF-β1, TWEAK might exacerbate periodontal disease.

From the existing literature it is evident that TWEAK/Fn14 signaling contributes to chronic inflammatory conditions via its proinflammatory actions, modulation of immune response, angiogenesis, repair, regeneration and stimulation of apoptosis. The majority of destruction in periodontitis is due to the production of host derived enzymes, cytokines and other proinflammatory mediators resulting in the activation of immune cells and enzymes such as matrix metalloproteinases (Page and Kornman 1997, Boch *et al* 2001, Van Dyke and Serhan 2003, Kornman 2008). Moreover, inflammatory process in periodontitis is marked with disruption of repair and regeneration (Bartold and Narayanan 2006). Any impediment in the crucial process of angiogenesis might contribute to pathogenesis of periodontitis by interfering with repair and regeneration. Imbalance of Th-1 and Th-2 type immune response resulting in the relative dominance of B cell and plasma cells in periodontitis is another paradigm which is associated with periodontal disease (Berglundh and Donati 2005). The general consensus seems to be that Th1 is associated with periodontal health and Th2 is associated with periodontal disease (Dennison and Van Dyke

1997, Seymour *et al* 1993). TWEAK /Fn14 pathway might also modulate immune responses by suppressing IL-12 and INF-γ which might in turn decrease Th-1 type immune response leading to imbalance of Th-1 type and Th-2 type immune responses (Maecker *et al* 2005, Trinchieri *et al* 1992, Chehimi and Trinchieri 1994). There is evidence that apoptosis might play a role in the pathogenesis of periodontal diseases. This could be due to apoptosis of fibroblasts and resident lymphocytes in periodontal tissue brought about by cytokines such as TNF and bacterial lipopolysaccharides which might result in decreased repair (Graves *et al* 2001, Kurita-Ochiai *et al* 1999, Ohguchi *et al* 1998, Liu *et al* 2003). TWAEK/Fn14 can also contribute to pathologic bone destruction in periodontitis by promoting osteoclast differentiation and migration, inhibiting osteoblast differentiation and upregulating RANKL on osteoblasts (Polek *et al* 2003, Ando *et al* 2006). Hence, there are number of possible ways in which TWEAK/Fn14 signalling might play a role in the pathogenesis of periodontitis.

1.12 Future perspectives

One of the important underlying mechanisms of destruction in periodontal disease is an imbalance in proinflammatory and anti-inflammatory mediators. Modulation of the host response offers a novel approach to the treatment of periodontal disease. It involves the manipulation of immune response to suppress undesired processes and stimulate protective processes (Kantarci et al., 2006). However, it must be noted that removal of the biofilm will still be fundamental to the treatment of periodontitis.

Human clinical trials have already confirmed the usefulness of IL-1 and TNF antagonists in treating rheumatoid arthritis and their use is being investigated in a number of other inflammatory conditions such as septic shock, glomerulonephritis and arthritis in animal models (Dayer et al., 2001, Dinarello, 2000, Feghali and Wright, 1997, Henderson, 1994). Infliximab, a chimeric anti-TNFα monoclonal antibody and Etanercept, a soluble dimeric fusion protein containing the ligand-binding domain of TNFR2, are now licensed for use in rheumatoid arthritis and Crohn's disease (Jones and Moreland, 1999, Bell and Kamm, 2000).

In vitro studies have demonstrated TWEAK's ability to regulate numerous cellular responses including cell proliferation, migration, survival, differentiation and apoptosis. TWEAK is also a proangiogenic and proinflammatory factor *in vivo*. In recent studies

TWEAK inhibition by anti-TWEAK antibodies have proved efficacious in treating collagen induced arthritis in animal models (Kamata et al., 2006, Perper et al., 2006). Recently, rheumatoid arthritis patients responding favorably to Etanercept displayed a significant decrease in serum TWEAK levels (Park et al., 2008). Other than rheumatoid arthritis, pharmacological inhibition of TWEAK activity might have therapeutic efficacy in other inflammatory and degenerative diseases such as ischemic stroke, cerebral edema, multiple sclerosis and periodontitis (Potrovita et al., 2004, Desplat-Jego et al., 2005, Yepes and Winkles, 2006, Williams et al., 1985).

Inhibitors of prostaglandins, MMPs, IL-1 and TNF have shown to suppress inflammatory processes and bone resorption in experimental periodontitis in animal models (Nyman et al., 1979, Delima et al., 2002, Di Paola et al., 2007, Williams et al., 1985, Assuma et al., 1998). By embarking on the identification and understanding the role of new cytokines like TWEAK in pathological destruction in periodontitis, we can get new insights into the pathogenesis of periodontal disease. In the future, this might pave the way for the development of new host modulation therapies involving anti-cytokine therapy to be used as an adjunct to conventional periodontal therapy.

1.13 Conclusion

Various studies have demonstrated increased expression of TWEAK and its receptor Fn14 in tissues and cells cultured from a number of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases. The studies indicate that TWEAK might play a role in the pathogenesis of these chronic inflammatory diseases. In addition, TWEAK has been shown to be involved in bone homeostasis and apoptosis of tumor cells. Some of these studies have indicated that TWEAK might serve as an additional therapeutic target in the treatment of rheumatoid arthritis, autoimmune diseases such as systemic lupus erythematosus and cancer.

Current literature has reported varied biologic effects of TWEAK. Most of the effects are mediated via Fn14 receptor and NF-κβ signaling pathway. However, TWEAK independent Fn14 signaling has also been demonstrated. On the other hand, TWEAK has also displayed Fn14 independent activity. TWEAK has also been shown to act by signaling pathways other than NF-κβ. The proinflammatory activities of TWEAK include induction of cytokines, chemokines and MMPs. It has a significant role in angiogenesis and activation of endothelial cells by upregulating surface expression of adhesion molecules i.e. ICAM-1

and VACM-1. TWEAK has also displayed inhibitory effects on differentiation of osteoblasts and promotes osteoclastic differentiation of cells from the monocyte/macrophage lineage. Furthermore, TWEAK /Fn14 pathway might also modulate immune responses by suppressing IL-12 and INF-γ which might in turn decrease Th-1 type immune response leading to imbalance of Th-1 type and Th-2 type immune responses.

1.14 Hypothesis

On the basis of the existing literature on TWEAK and its cognate receptor Fn14, it is hypothesised that the expression of TWEAK and Fn14/TWEAKR will be increased in tissue samples from periodontitis patients.

1.15 Aim of the study

The aim of the present study was to investigate the expression of TWEAK and TWEAK's receptor Fn14 through immunohistochemistry techniques using TWEAK and Fn14 antibodies.

1.16 References

Aggarwal BB (2003). Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3: 745-56.

Akatsu T, Murakami T, Nishikawa M, Ono K, Shinomiya N, Tsuda E, *et al* (1998). Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. *Biochem Biophys Res Commun* 250: 229-34.

Ando T, Ichikawa J, Wako M, Hatsushika K, Watanabe Y, Sakuma M, *et al* (2006). TWEAK/Fn14 interaction regulates RANTES production, BMP-2-induced differentiation and RANKL expression in mouse osteoblastic MC3T3-E1 cells. *Arthritis Res Ther* 8: R146.

Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP (2001). TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci* 4: 1116-22.

Arron JR, Choi Y (2000). Bone versus immune system. *Nature* 408: 535-6.

Assuma R, Oates T, Cochran D, Amar S, Graves DT (1998). IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. *J Immunol* 160: 403-9.

Baker PJ (2000). The role of immune responses in bone loss during periodontal disease. *Microbes Infect* 2: 1181-92.

Barnes PJ, Karin M (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 336: 1066-71.

Bartold PM, Marshall RI, Haynes DR (2005). Periodontitis and rheumatoid arthritis: a review. *J Periodontol* 76(11 Suppl): 2066-74.

Bartold PM, Narayanan AS. (2006). Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 40: 29-49.

Baud V, Karin M (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11: 372-7.

Bell S, Kamm MA (2000). Antibodies to tumour necrosis factor alpha as treatment for Crohn's disease. *Lancet* 355: 858-60.

Berglundh T, Donati M. (2005). Aspects of adaptive host response in periodontitis. *J Clin Periodontol* 32 Suppl 6: 87-107.

Berker E, Kantarci A, Hasturk H, Van Dyke TE (2005). Effect of neutrophil apoptosis on monocytic cytokine response to Porphyromonas gingivalis lipopolysaccharide. *J Periodontol* 76: 964-71.

Boch JA, Wara-aswapati N, Auron PE. (2001). Interleukin 1 signal transduction--current concepts and relevance to periodontitis. *J Dent Res* 80: 400-407.

Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, *et al* (2006). Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem* 281: 13964-71.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Toz H, *et al* (2007a). Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. *J Clin Periodontol* 34: 370-6.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Toz H, *et al* (2007b). Differential expression of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin mRNA in periodontal diseases. *J Periodontal Res* 42: 287-93.

Bover LC, Cardo-Vila M, Kuniyasu A, Sun J, Rangel R, Takeya M, *et al* (2007). A previously unrecognised protein-protein interaction between TWEAK and CD163: potential biological implications. *J Immunol* 178: 8183-94.

Brandstrom H, Jonsson KB, Ohlsson C, Vidal O, Ljunghall S, Ljunggren O (1998). Regulation of osteoprotegerin mRNA levels by prostaglandin E2 in human bone marrow stroma cells. *Biochem Biophys Res Commun* 247: 338-41.

Brown SA, Richards CM, Hanscom HN, Feng SL, Winkles JA (2003). The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation. *Biochem J* 371(Pt 2): 395-403.

Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, *et al* (1999). The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 145: 527-38.

Burkly LC, Michaelson JS, Hahm K, Jakubowski A, Zheng TS (2007). TWEAKing tissue remodeling by a multifunctional cytokine: role of TWEAK/Fn14 pathway in health and disease. *Cytokine* 40: 1-16.

Campbell S, Burkly LC, Gao HX, Berman JW, Su L, Browning B, *et al* (2006). Proinflammatory effects of TWEAK/Fn14 interactions in glomerular mesangial cells. *J Immunol* 176: 1889-98.

Campbell S, Michaelson J, Burkly L, Putterman C (2004). The role of TWEAK/Fn14 in the pathogenesis of inflammation and systemic autoimmunity. *Front Biosci* 9: 2273-84.

Chacon MR, Richart C, Gomez JM, Megia A, Vilarrasa N, Fernandez-Real JM, *et al* (2006). Expression of TWEAK and its receptor Fn14 in human subcutaneous adipose tissue. Relationship with other inflammatory cytokines in obesity. *Cytokine* 33: 129-37.

Chehimi J, Trinchieri G (1994). Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *J Clin Immunol* 14: 149-61.

Chicheportiche Y, Bourdon PR, Xu H, Hsu YM, Scott H, Hession C, *et al* (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem* 272: 32401-10.

Chicheportiche Y, Chicheportiche R, Sizing I, Thompson J, Benjamin CB, Ambrose C, *et al* (2002). Proinflammatory activity of TWEAK on human dermal fibroblasts and synoviocytes: blocking and enhancing effects of anti-TWEAK monoclonal antibodies. *Arthritis Res* 4: 126-33.

Choi Y, Woo KM, Ko SH, Lee YJ, Park SJ, Kim HM, *et al* (2001). Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8(+) T cells. *Eur J Immunol* 31: 2179-88.

Cohen JJ (1991). Programmed cell death in the immune system. *Adv Immunol* 50: 55-85.

Crotti T, Smith MD, Hirsch R, Soukoulis S, Weedon H, Capone M, *et al* (2003). Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J Periodontal Res* 38: 380-7.

Darnay BG, Aggarwal BB (1999). Signal transduction by tumour necrosis factor and tumour necrosis factor related ligands and their receptors. *Ann Rheum Dis* 58(Suppl 1): (I2-I13.

Dayer JM, Feige U, Edwards CK, 3rd, Burger D (2001). Anti-interleukin-1 therapy in rheumatic diseases. *Curr Opin Rheumatol* 13: 170-6.

De Ketelaere A, Vermeulen L, Vialard J, Van De Weyer I, Van Wauwe J, Haegeman G, *et al* (2004). Involvement of GSK-3β in TWEAK-mediated NF-kappaB activation. *FEBS Lett* 566(1-3):60-4.

Delima AJ, Karatzas S, Amar S, Graves DT (2002). Inflammation and tissue loss caused by periodontal pathogens is reduced by interleukin-1 antagonists. *J Infect Dis* 186: 511-6.

Delima AJ, Oates T, Assuma R, Schwartz Z, Cochran D, Amar S, *et al* (2001). Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis. *J Clin Periodontol* 28: 233-40.

Dennison DK, Van Dyke TE. (1997). The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol 2000* 14: 54-78.

Desplat-Jego S, Creidy R, Varriale S, Allaire N, Luo Y, Bernard D, *et al* (2005). Anti-TWEAK monoclonal antibodies reduce immune cell infiltration in the central nervous

system and severity of experimental autoimmune encephalomyelitis. *Clin Immunol* 117: 15-23.

Desplat-Jego S, Varriale S, Creidy R, Terra R, Bernard D, Khrestchatisky M, *et al* (2002). TWEAK is expressed by glial cells, induces astrocyte proliferation and increases EAE severity. *J Neuroimmunol* 133: 116-23.

Dharmapatni AASSK, Smith MD, Weedon H, Holding C, Haynes DR (2008). The expression of TNF-like weak inducer of apoptosis (TWEAK) and TWEAK receptor (Fn14) in synovial tissues from patients with various types of arthritis. Poster presented in Australian *Rheumatology* Association 50th Annual Scientific Conference 2008, Adelaide, Australia.

Di Paola R, Mazzon E, Muia C, Crisafulli C, Terrana D, Greco S, *et al* (2007). Effects of etanercept, a tumour necrosis factor-alpha antagonist, in an experimental model of periodontitis in rats. *Br J Pharmacol* 150: 286-97.

Dinarello CA (2000). Proinflammatory cytokines. *Chest* 118: 503-8.

Dogra C, Hall SL, Wedhas N, Linkhart TA, Kumar A (2007). Fibroblast growth factor inducible 14 (Fn14) is required for the expression of myogenic regulatory factors and differentiation of myoblasts into myotubes. Evidence for TWEAK-independent functions of Fn14 during myogenesis. *J Biol Chem* 282: 15000-10.

Donohue PJ, Richards CM, Brown SA, Hanscom HN, Buschman J, Thangada S, *et al* (2003). TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. *Arterioscler Thromb Vasc Biol* 23: 594-600.

Duffield JS (2003). The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci* 104: 27-38.

Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, *et al* (2000). The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 103: 41-50.

Feghali CA, Wright TM (1997). Cytokines in acute and chronic inflammation. *Front Biosci* 2: 12-26.

Feng SL, Guo Y, Factor VM, Thorgeirsson SS, Bell DW, Testa JR, *et al* (2000). The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas. *Am J Pathol* 156: 1253-61.

Fuller K, Wong B, Fox S, Choi Y, Chambers TJ (1998). TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* 188: 997-1001.

Gadhavi A, Wilson M, Tabona P, Newman HN, Henderson B, Bennett JH (2000). Inhibition of mitosis and induction of apoptosis in MG63 human osteosarcoma-derived cells *in vitro* by surface proteins from Actinobacillus actinomycetemcomintans. *Arch Oral Biol* 45: 707-11.

Gamonal J, Bascones A, Acevedo A, Blanco E, Silva A (2001). Apoptosis in chronic adult periodontitis analysed by in situ DNA breaks, electron microscopy and immunohistochemistry. *J Periodontol* 72: 517-25.

Geatch DR, Harris JI, Heasman PA, Taylor JJ (1999). *In vitro* studies of lymphocyte apoptosis induced by the periodontal pathogen Porphyromonas gingivalis. *J Periodontal Res* 34: 70-8.

Gemmell E, Marshall RI, Seymour GJ (1997). Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontol 2000* 14: 112-43.

Girgenrath M, Weng S, Kostek CA, Browning B, Wang M, Brown SA, *et al* (2006). TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. *Embo J* 25: 5826-39.

Graves DT, Cochran D (2003). The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol* 74: 391-401.

Graves DT, Oskoui M, Volejnikova S, Naguib G, Cai S, Desta T, *et al* (2001). Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment and osteoclast formation in response to *P. gingivalis* infection. *J Dent Res* 80: 1875-9.

Haffajee AD, Socransky SS (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 5: 78-111.

Hamill CA, Michaelson JS, Hahm K, Burkly LC, Kessler JA (2007). Age-dependent effects of TWEAK/Fn14 receptor activation on neural progenitor cells. *J Neurosci Res* 85: 3535-44.

Han S, Yoon K, Lee K, Kim K, Jang H, Lee NK, *et al* (2003). TNF-related weak inducer of apoptosis receptor, a TNF receptor superfamily member, activates NF-kappa B through TNF receptor-associated factors. *Biochem Biophys Res Commun* 305: 789-96.

Han X, Kawai T, Eastcott JW, Taubman MA (2006). Bacterial-responsive B lymphocytes induce periodontal bone resorption. *J Immunol* 176: 625-31.

Harada N, Nakayama M, Nakano H, Fukuchi Y, Yagita H, Okumura K (2002). Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 299: 488-93.

Haynes DR, Crotti TN, Loric M, Bain GI, Atkins GJ, Findlay DM (2001). Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. *Rheumatology* 40: 623-30.

Henderson B (1994). Cytokine and proteinases inhibitors in the modulation of connective tissue destruction in rheumatoid arthritis. *Clin Immunother* 2: 167-174.

Hiroi M, Shimojima T, Kashimata M, Miyata T, Takano H, Takahama M, *et al* (1998). Inhibition by Porphyromonas gingivalis LPS of apoptosis induction in human peripheral blood polymorphonuclear leukocytes. *Anti Cancer Res* 18: 3475-9.

Ho DH, Vu H, Brown SA, Donohue PJ, Hanscom HN, Winkles JA (2004). Soluble tumor necrosis factor-like weak inducer of apoptosis overexpression in HEK293 cells promotes tumor growth and angiogenesis in athymic nude mice. *Cancer Res* 64: 8968-72.

Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S (1999). Interleukin-1β and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone* 25: 255-9.

Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT (1999). Activated T lymphocytes support osteoclast formation *in vitro*. *Biochem Biophys Res Commun* 265: 144-50.

Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T (2006). Proinflammatory effects of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) on human gingival fibroblasts. *Clin Exp Immunol* 146: 540-9.

Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, *et al* (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 96: 3540-5.

Ichinose Y, Hara N, Ohta M, Aso H, Chikama H, Kawasaki M, *et al* (1990). Recombinant granulocyte colony-stimulating factor and lipopolysaccharide maintain the phenotype of and superoxide anion generation by neutrophils. *Infect Immun* 58: 1647-52.

Iocca HA, Plant SR, Wang Y, Runkel L, O'Connor BP, Lundsmith ET, *et al* (2008). TNF superfamily member TWEAK exacerbates inflammation and demyelination in the cuprizone-induced model. *J Neuroimmunol* 194: 97-106.

Irwin CR, Myrillas TT (1998). The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis* 4: 43-7.

Jakubowski A, Browning B, Lukashev M, Sizing I, Thompson JS, Benjamin CD, *et al* (2002). Dual role for TWEAK in angiogenic regulation. *J Cell Sci* 115(Pt 2): 267-74.

Jin L, Nakao A, Nakayama M, Yamaguchi N, Kojima Y, Nakano N, *et al* (2004). Induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes. *J Invest Dermatol* 122: 1175-9.

Jones RE, Moreland LW (1999). Tumor necrosis factor inhibitors for rheumatoid arthritis. *Bull Rheum Dis* 48: 1-4.

Justo P, Sanz AB, Sanchez-Nino MD, Winkles JA, Lorz C, Egido J, *et al* (2006). Cytokine cooperation in renal tubular cell injury: the role of TWEAK. *Kidney Int* 70: 1750-8.

Kamata K, Kamijo S, Nakajima A, Koyanagi A, Kurosawa H, Yagita H, *et al* (2006). Involvement of TNF-like weak inducer of apoptosis in the pathogenesis of collagen-induced arthritis. *J Immunol* 177: 6433-9.

Kamijo S, Nakajima A, Kamata K, Kurosawa H, Yagita H, Okumura K (2008). Involvement of TWEAK/Fn14 interaction in the synovial inflammation of RA. *Rheumatology* (Oxford) 47: 442-50.

Kantarci A, Hasturk H, Van Dyke TE (2006). Host-mediated resolution of inflammation in periodontal diseases. *Periodontol 2000* 40: 144-63.

Kanzaki H, Chiba M, Shimizu Y, Mitani H (2002). Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. *J Bone Miner Res* 17: 210-20.

Kaplan MJ, Lewis EE, Shelden EA, Somers E, Pavlic R, McCune WJ, *et al* (2002). The apoptotic ligands TRAIL, TWEAK and Fas ligand mediate monocyte death induced by autologous lupus T cells. *J Immunol* 169: 6020-9.

Kaplan MJ, Ray D, Mo RR, Yung RL, Richardson BC (2000). TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages. *J Immunol* 164: 2897-904.

Kaptein A, Jansen M, Dilaver G, Kitson J, Dash L, Wang E, *et al* (2000). Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3). *FEBS Lett* 485: 135-41.

Kawakita T, Shiraki K, Yamanaka Y, Yamaguchi Y, Saitou Y, Enokimura N, *et al* (2004). Functional expression of TWEAK in human hepatocellular carcinoma: possible implication in cell proliferation and tumor angiogenesis. *Biochem Biophys Res Commun* 318: 726-33.

Kawakita T, Shiraki K, Yamanaka Y, Yamaguchi Y, Saitou Y, Enokimura N, *et al* (2005). Functional expression of TWEAK in human colonic adenocarcinoma cells. *Int J Oncol* 26: 87-93.

Khosla S (2001). Minireview: the OPG/RANKL/RANK system. *Endocrinology* 142: 5050-5.

Kim SH, Kang YJ, Kim WJ, Woo DK, Lee Y, Kim DI, *et al* (2004). TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. *Circ J* 68: 396-9.

Kornman KS. (2008). Mapping the Pathogenesis of Periodontitis: A New Look. *J Periodont* 79: 1560-1568.

Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, *et al* (2001). Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 44: 1003-12.

Koulouri O, Lappin DF, Radvar M, Kinane DF (1999). Cell division, synthetic capacity and apoptosis in periodontal lesions analysed by in situ hybridisation and immunohistochemistry. *J Clin Periodontol* 26: 552-9.

Kurita-Ochiai T, Fukushima K, Ochiai K (1999). Lipopolysaccharide stimulates butyric acid-induced apoptosis in human peripheral blood mononuclear cells. *Infect Immun* 67: 22-9.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, *et al* (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 165-76.

Liu D, Xu JK, Figliomeni L, Huang L, Pavlos NJ, Rogers M, *et al* (2003). Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction. *Int J Mol Med* 11: 17-21.

Locksley RM, Killeen N, Lenardo MJ (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104: 487-501.

Lu HK, Chen YL, Chang HC, Li CL, Kuo MY (2006). Identification of the osteoprotegerin/receptor activator of nuclear factor-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis. *J Periodontal Res* 41: 354-60.

Lynch CN, Wang YC, Lund JK, Chen *YW*, Leal JA, Wiley SR (1999). TWEAK induces angiogenesis and proliferation of endothelial cells. *J Biol Chem* 274: 8455-9.

Maecker H, Varfolomeev E, Kischkel F, Lawrence D, LeBlanc H, Lee W, *et al* (2005). TWEAK attenuates the transition from innate to adaptive immunity. Cell 123: 931-44.

Marsters SA, Sheridan JP, Pitti RM, Brush J, Goddard A, Ashkenazi A (1998). Identification of a ligand for the death-domain-containing receptor Apo3. *Curr Biol* 8: 525-8.

Meighan-Mantha RL, Hsu DK, Guo Y, Brown SA, Feng SL, Peifley KA, *et al* (1999). The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration. *J Biol Chem* 274: 33166-76.

Michaelson JS, Cho S, Browning B, Zheng TS, Lincecum JM, Wang MZ, *et al* (2005). TWEAK induces mammary epithelial branching morphogenesis. *Oncogene* 24: 2613-24.

Mogi M, Otogoto J, Ota N, Togari A (2004). Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *J Dent Res* 83: 166-9.

Mueller AM, Pedre X, Kleiter I, Hornberg M, Steinbrecher A, Giegerich G (2005). Targeting fibroblast growth factor-inducible-14 signaling protects from chronic relapsing experimental autoimmune encephalomyelitis. *J Neuroimmunol* 159: 55-65.

Mutsaers SE, Whitaker D, Papadimitriou JM (2002). Stimulation of mesothelial cell proliferation by exudate macrophages enhances serosal wound healing in a murine model. *Am J Pathol* 160: 681-92.

Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, *et al* (1998). RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun* 253: 395-400.

Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, *et al* (2000). Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: modulation of the expression by osteotropic factors and cytokines. *Biochem Biophys Res Commun* 275: 768-75.

Nakayama M, Ishidoh K, Kayagaki N, Kojima Y, Yamaguchi N, Nakano H, *et al* (2002). Multiple pathways of TWEAK-induced cell death. *J Immunol* 168: 734-43.

Nakayama M, Ishidoh K, Kojima Y, Harada N, Kominami E, Okumura K, *et al* (2003). Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death. *J Immunol* 170: 341-8.

Nakayama M, Kayagaki N, Yamaguchi N, Okumura K, Yagita H (2000). Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. *J Exp Med* 192: 1373-80.

Nanci A, Bosshardt DD (2006). Structure of periodontal tissues in health and disease. *Periodontol 2000* 40: 11-28.

Nyman S, Schroeder HE, Lindhe J(1979). Suppression of inflammation and bone resorption by indomethacin during experimental periodontitis in dogs. *J Periodontol* 50: 450-61.

Offenbacher S (1996). Periodontal diseases: pathogenesis. *Ann Periodontol* 1: 821-78.

Ohguchi M, Ishisaki A, Okahashi N, Koide M, Koseki T, Yamato K, *et al* (1998). Actinobacillus actinomycetemcomitans toxin induces both cell cycle arrest in the G2/M phase and apoptosis. *Infect Immun* 66: 5980-7.

Page RC, Kornman KS (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 14: 9-11.

Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS (1997). Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol 2000* 14: 216-48.

Page RC, Schroeder HE (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 34: 235-49.

Park MC, Jung SJ, Park YB, Lee SK (2008). Relationship of serum TWEAK level to cytokine level, disease activity and response to anti-TNF treatment in patients with rheumatoid arthritis. *Scand J Rheumatol* 37: 173-8.

Perper SJ, Browning B, Burkly LC, Weng S, Gao C, Giza K, *et al* (2006). TWEAK is a novel arthritogenic mediator. *J Immunol* 177: 2610-20.

Peter ME, Krammer PH (2003). The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* 10: 26-35.

Pfizenmaier K, Wajant H, Grell M (1996). Tumor necrosis factors in 1996. *Cytokine Growth Factor Rev* 7: 271-7.

Polek TC, Talpaz M, Darnay BG, Spivak-Kroizman T (2003). TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TWEAKR. Evidence for a second TWEAK receptor. *J Biol Chem* 278: 32317-23.

Potrovita I, Zhang W, Burkly L, Hahm K, Lincecum J, Wang MZ, *et al* (2004). Tumor necrosis factor-like weak inducer of apoptosis-induced neurodegeneration. *J Neurosci* 24: 8237-44.

Pradet-Balade B, Medema JP, Lopez-Fraga M, Lozano JC, Kolfschoten GM, Picard A, *et al* (2002). An endogenous hybrid mRNA encodes TWE-PRIL, a functional cell surface TWEAK-APRIL fusion protein. *Embo J* 21: 5711-20.

Preshaw PM, Schifferle RE, Walters JD (1999). Porphyromonas gingivalis lipopolysaccharide delays human polymorphonuclear leukocyte apoptosis *in vitro*. *J Periodontal Res* 34: 197-202.

Quinn J, Horwood N, Elliott J, Gillespie M, Martin T (2000). Fibroblastic stromal cells express receptor activator of NF-kappa B ligand and support osteoclast differentiation. *J Bone Miner Res* 15: 1459-1466.

Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* 298: 597-600.

Ren R, Charles PC, Zhang C, Wu Y, Wang H, Patterson C (2007). Gene expression profiles identify a role for cyclooxygenase 2-dependent prostanoid generation in BMP6-induced angiogenic responses. *Blood* 109: 2847-53.

Saas P, Boucraut J, Walker PR, Quiquerez AL, Billot M, Desplat-Jego S, *et al* (2000). TWEAK stimulation of astrocytes and the proinflammatory consequences. *Glia* 32: 102-7.

Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S (2003). TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. *J Biol Chem* 278: 36005-12.

Sakata M, Shiba H, Komatsuzawa H, Fujita T, Ohta K, Sugai M, *et al* (1999). Expression of osteoprotegerin (osteoclastogenesis inhibitory factor) in cultures of human dental mesenchymal cells and epithelial cells. *J Bone Miner Res* 14: 1486-92.

Sanz AB, Justo P, Sanchez-Nino MD, Blanco-Colio LM, Winkles JA, Kreztler M, *et al* (2008). The cytokine TWEAK modulates renal tubulointerstitial inflammation. *J Am Soc Nephrol* 19: 695-703.

Schneider P, Schwenzer R, Haas E, Muhlenbeck F, Schubert G, Scheurich P, *et al* (1999). TWEAK can induce cell death via endogenous TNF and TNF receptor 1. *Eur J Immunol* 29: 1785-92.

Schwartz N, Michaelson JS, Putterman C (2007). Lipocalin-2, TWEAK and other cytokines as urinary biomarkers for lupus nephritis. *Ann NY Acad Sci* 1109: 265-74.

Schwartz N, Su L, Burkly LC, Mackay M, Aranow C, Kollaros M, *et al* (2006). Urinary TWEAK and the activity of lupus nephritis. *J Autoimmun* 27: 242-50.

Semov A, Semova N, Lacelle C, Marcotte R, Petroulakis E, Proestou G, *et al* (2002). Alterations in TNF- and IL-related gene expression in space-flown WI38 human fibroblasts. *Faseb J* 16: 899-901.

Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. (1993). Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *J Periodontal Res* 28: 478-486.

Sorkin BC, Niederman R (1998). Short chain carboxylic acids decrease human gingival keratinocyte proliferation and increase apoptosis and necrosis. *J Clin Periodontol* 25: 311-5.

Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM (1987). Synergistic interactions between interleukin 1, tumor necrosis factor and lymphotoxin in bone resorption. *J Immunol* 138: 1464-8.

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, *et al* (2000). T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* 408(6812):600-5.

Takeichi O, Haber J, Kawai T, Smith DJ, Moro I, Taubman MA (2000). Cytokine profiles of T-lymphocytes from gingival tissues with pathological pocketing. *J Dent Res* 79: 1548-55.

Tanabe K, Bonilla I, Winkles JA, Strittmatter SM (2003). Fibroblast growth factor-inducible-14 is induced in axotomised neurons and promotes neurite outgrowth. *J Neurosci* 23: 9675-86.

Teng YT, Nguyen H, Gao X, Kong YY, Gorczynski RM, Singh B, *et al* (2000). Functional human T-cell immunity and osteoprotegerin ligand control alveolar bone destruction in periodontal infection. *J Clin Invest* 106: R59-67.

Theill LE, Boyle WJ, Penninger JM (2002). RANK-L and RANK: T cells, bone loss and mammalian evolution. *Annu Rev Immunol* 20: 795-823.

Thompson CB (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456-62.

Thornberry N (1985). Caspases: Key mediators of apoptosis. *Chem Biol* 5: 50-85.

Toraldo G, Roggia C, Qian WP, Pacifici R, Weitzmann MN (2003). IL-7 induces bone loss *in vivo* by induction of receptor activator of nuclear factor kappa B ligand and tumor necrosis factor alpha from T cells. *Proc Natl Acad Sci USA* 100: 125-30.

Tracey KJ, Cerami A (1993). Tumor necrosis factor, other cytokines and disease. *Annu Rev Cell Biol* 9: 317-43.

Tran NL, McDonough WS, Donohue PJ, Winkles JA, Berens TJ, Ross KR, *et al* (2003). The human Fn14 receptor gene is up-regulated in migrating glioma cells *in vitro* and overexpressed in advanced glial tumors. *Am J Pathol* 162: 1313-21.

Tran NL, McDonough WS, Savitch BA, Fortin SP, Winkles JA, Symons M, *et al* (2006). Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-kappaB and correlate with poor patient outcome. *Cancer Res* 66: 9535-42.

Tran NL, McDonough WS, Savitch BA, Sawyer TF, Winkles JA, Berens ME (2005). The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-fibroblast growth factor-inducible 14 (Fn14) signaling system regulates glioma cell survival via NFkappaB pathway activation and BCL-XL/BCL-W expression. *J Biol Chem* 280: 3483-92.

Trinchieri G, Wysocka M, D'Andrea A, Rengaraju M, Aste-Amezaga M, Kubin M, *et al* (1992). Natural killer cell stimulatory factor (NKSF) or interleukin-12 is a key regulator of immune response and inflammation. *Prog Growth Factor Res* 4: 355-68.

Valverde P, Kawai T, Taubman MA (2005). Potassium channel-blockers as therapeutic agents to interfere with bone resorption of periodontal disease. *J Dent Res* 84: 488-99.

Van Dyke TE, Serhan CN (2003). Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. *J Dent Res* 82: 82-90.

Vernal R, Dutzan N, Hernandez M, Chandia S, Puente J, Leon R, *et al* (2006). High expression levels of receptor activator of nuclear factor-kappa B ligand associated with human chronic periodontitis are mainly secreted by CD4+ T lymphocytes. *J Periodontol* 77: 1772-80.

Wang PL, Shirasu S, Shinohara M, Daito M, Oido M, Kowashi Y, *et al* (1999). Induction of apoptosis in human gingival fibroblasts by a Porphyromonas gingivalis protease preparation. *Arch Oral Biol* 44: 337-42.

Wara-aswapati N, Surarit R, Chayasadom A, Boch JA, Pitiphat W (2007). RANKL upregulation associated with periodontitis and Porphyromonas gingivalis. *J Periodontol* 78: 1062-9.

Watts GS, Tran NL, Berens ME, Bhattacharyya AK, Nelson MA, Montgomery EA, *et al* (2007). Identification of Fn14/TWEAK receptor as a potential therapeutic target in esophageal adenocarcinoma. *Int J Cancer* 121: 2132-9.

Wiley SR, Cassiano L, Lofton T, Davis-Smith T, Winkles JA, Lindner V, *et al* (2001). A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 15: 837-46.

Wiley SR, Winkles JA (2003). TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TWEAKR/Fn14 receptor. *Cytokine Growth Factor Rev* 14: 241-9.

Williams RC, Jeffcoat MK, Kaplan ML, Goldhaber P, Johnson HG, Wechter WJ (1985). Flurbiprofen: a potent inhibitor of alveolar bone resorption in beagles. *Science* 227: 640-2.

Wilson CA, Browning JL (2002). Death of HT29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis. *Cell Death Differ* 9: 1321-33.

Winkles JA (2008). The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov* 7: 411-25.

Xu H, Okamoto A, Ichikawa J, Ando T, Tasaka K, Masuyama K, *et al* (2004). TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF. *Biochem Biophys Res Commun* 318: 422-7.

Xu LG, Shu HB (2002). TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production. *J Immunol* 169: 6883-9.

Yamamoto S, Mogi M, Kinpara K, Ishihara Y, Ueda N, Amano K, *et al* (1999). Anti-proliferative capsular-like polysaccharide antigen from *Actinobacillus actinomycetemcomitans* induces apoptotic cell death in mouse osteoblastic MC3T3-E1 cells. *J Dent Res* 78: 1230-7.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, *et al* (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 95: 3597-602.

Yepes M (2007a). TWEAK and the central nervous system. *Mol Neurobiol* 35: 255-65.

Yepes M (2007b). TWEAK and Fn14 in central nervous system health and disease. *Front Biosci* 12: 2772-81.

Yepes M, Winkles JA (2006). Inhibition of TWEAK activity as a new treatment for inflammatory and degenerative diseases. *Drug News Perspect* 19: 589-95.

Yu X, Huang Y, Collin-Osdoby P, Osdoby P (2004). CCR1 chemokines promote the chemotactic recruitment, RANKL development and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *J Bone Miner Res* 19: 2065-77.

Yun TJ, Chaudhary PM, Shu GL, Frazer JK, Ewings MK, Schwartz SM, *et al* (1998). OPG/FDCR-1, a TNF receptor family member, is expressed in lymphoid cells and is up-regulated by ligating CD40. *J Immunol* 161: 6113-21.

Zappa U, Reinking-Zappa M, Graf H, Case D (1992). Cell populations associated with active probing attachment loss. *J Periodont* 63: 748-52.

Zauli G, Rimondi E, Nicolin V, Melloni E, Celeghini C, Secchiero P (2004). TNF-related apoptosis-inducing ligand (TRAIL) blocks osteoclastic differentiation induced by RANKL plus M-CSF. *Blood* 104: 2044-50.

Zhao Z, Burkly LC, Campbell S, Schwartz N, Molano A, Choudhury A, *et al* (2007). TWEAK/Fn14 interactions are instrumental in the pathogenesis of nephritis in the chronic graft-versus-host model of systemic lupus erythematosus. *J Immunol* 179: 7949-58.

Zheng TS, Burkly LC (2008). No end in site: TWEAK/Fn14 activation and autoimmunity associated- end-organ pathologies. *J Leukoc Biol*.

Chapter 2

IMMUNOHISTOCHEMISTRY STUDY ON EXPRESSION OF TUMOR NECROSIS FACTOR LIKE WEAK INDUCER OF APOPTOSIS (TWEAK) AND ITS RECEPTOR FN14 IN NORMAL AND PERIODONTITIS TISSUES

NG KATARIA[1], PM BARTOLD[2], D HAYNES[3], AASK DHARMAPATNI[4]

[1]Post Graduate Student (Periodontology), School of Dentistry, University of Adelaide

[2]Professor of Periodontology, Colgate Australian Clinical Dental Research Centre, School of Dentistry, University of Adelaide

[3]Discipline of Pathology, School of Medical Sciences, University of Adelaide.

[4]Discipline of Pathology, School of Medical Sciences, University of Adelaide.

2.1 Abstract

Background: Periodontitis is a chronic inflammatory disease wherein microbial factors induce complex inflammatory and immune responses in a susceptible host. In periodontitis host derived enzymes, cytokines and other proinflammatory mediators play an integral role in the destruction of tooth supporting structures and alveolar bone. TWEAK (TNF-like weak inducer of apoptosis) is one of the newest members of the TNF superfamily to be identified. Fibroblast growth factor-inducible 14 (Fn14) protein/TWEAKR has been identified as the cell surface receptor for TWEAK. TWEAK/Fn14 signaling results in multiple biologic effects including induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis. Recently, TWEAK has also been shown to promote osteoclastic differentiation of cells from the monocyte/macrophage lineage. Expression of TWEAK and its receptor Fn14, is elevated in tissues and cells cultured from a number of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases. Accordingly, we hypothesised that the expression of TWEAK and Fn14/TWEAKR will be increased in tissue samples from periodontitis patients.

Aim: The aim of this study was to investigate the expression of TWEAK and its receptor Fn14, in gingival biopsies from periodontitis patients and clinically normal patients using immunohistochemistry techniques.

Materials and Methods: The study included 27 patients (18 females and 9 males, aged 30-77 years, mean age = 55.2 years) with generalised chronic and aggressive periodontitis. The gingival biopsy sites in the chronic and aggressive periodontitis group had clinical probing pocket depths and clinical attachment loss greater than 5 mm with radiographic evidence of bone loss ranging from 50-90% of the root length. The non-periodontitis tissue samples consisted of gingival tissue resected from seven patients (4 males and 3 females, aged 23-70 years at the time of surgery; mean age = 45.5 years) undergoing crown lengthening surgery at sites not affected by periodontitis. Using monoclonal antibodies, the expression of TWEAK and its receptor Fn14 was investigated by immunohistochemistry in formalin-fixed paraffin embedded tissues. The specimens were evaluated by a semiquantitiative analysis (SQA). The Mann Whitney U-test was used to compare mean rank of SQA between two groups and Kendall’s tau_b test was used to determine correlations between different parameters.

Results: Semiquantiative analyses demonstrated that the expression of TWEAK protein was significantly higher in periodontitis tissue compared to healthy tissue (Mann Whitney U-test, p value 0.002). Similarly, in comparison with healthy tissue, periodontitis-affected tissues expressed significantly higher Fn14 protein (Mann Whitney U-test, p value 0.013). A strong positive correlation was found between TWEAK and Fn14 expression (Kendall's tau_b test; p value 0.007 and r value 0.395). In periodontitis-affected tissue specimens, TWEAK and Fn14 protein was mainly expressed by mononuclear leukocytes (morphologically resembling lymphocytes and plasma cells), cells lining blood vessels, spindle shaped cells resembling fibroblasts and multinucleated cells.

Conclusion: This study demonstrates that there is a higher expression of TWEAK and Fn14 protein in periodontitis tissues as compared to clinically normal controls. This suggests that TWEAK /Fn14 signaling could be an additional player in the pathogenesis of periodontitis and adds to the increasing number of cytokine networks involved in periodontal inflammation.

2.2 Introduction

Periodontitis is a chronic inflammatory disease wherein microbial factors induce complex inflammatory and immune responses in a susceptible host (Baker 2000, Haffajee and Socransky 1994). In periodontitis host derived enzymes, cytokines and other proinflammatory mediators play an integral role in the destruction of tooth supporting structures (Page and Kornman 1997, Boch *et al* 2001, Van Dyke and Serhan 2003). Thus, bacteria are considered to be necessary factors for periodontal disease initiation but they are not sufficient to cause disease progression. Cytokines, the major regulators of the immunoinflammatory response seen in periodontitis, affect tissue destruction by either acting directly on cells or through induction of production of other cytokines. Thus, the balance between proinflammatory and anti-inflammatory cytokines determines the outcome. The role of proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), IL-1, IL-8 and IL-6 in periodontitis is well recognised (Gemmell *et al* 1997, Graves and Cochran 2003, Irwin and Myrillas 1998). IL-1 and TNF-α can promote leukocyte recruitment, production of other inflammatory mediators such as prostaglandins, enhanced bacterial killing and phagocytic activity, production of lytic enzymes such as matrix

metalloproteinases and enhance bone resorption by promoting osteoclast formation and activity (Graves and Cochran, 2003, Pfizenmaier *et al* 1996, Stashenko *et al* 1987).

Receptor Activator NF κβ Ligand (RANKL), a member of the TNF superfamily, binds to its receptor RANK and mediates bone loss by stimulating differentiation of osteoclast progenitors, activation of mature osteoclasts and inhibiting osteoclast apoptosis (Burgess *et al* 1999, Fuller *et al* 1998, Hsu *et al* 1999, Lacey *et al* 1998, Nakagawa *et al* 1998). Osteoprotegerin (OPG) is a natural inhibitor of RANKL and binds to RANKL with high affinity and prevents its ligation to RANK (Lacey *et al* 1998, Akatsu *et al* 1998, Yasuda *et al* 1998). Recognition of the RANKL-RANK-OPG axis has enhanced our understanding of the mechanisms behind the pathologic bone loss seen in periodontitis. High levels of RANKL and low levels of OPG have been found in the tissue and gingival crevicular fluid of periodontitis patients compared to tissues from clinically normal patients (Crotti *et al* 2003, Liu *et al* 2003, Lu *et al* 2006, Bostanci *et al* 2007a, Bostanci *et al* 2007b, Mogi *et al* 2004, Wara-aswapati *et al* 2007). It has been suggested that bone resorption in periodontitis may be driven by proinflammatory cytokines that regulate RANKL expression on mesenchymal cells, activated T-cells and B-cells (Nanci and Bosshardt 2006).

TNF-like weak inducer of apoptosis (TWEAK) is one of the members of the TNF superfamily (Chicheportiche *et al* 1997). It is expressed by various inflammatory cells including macrophages (Chicheportiche *et al* 1997, Nakayama *et al* 2000), monocytes (Kaplan *et al* 2002), activated T-cells (Kawakita *et al* 2004) and plasma cells (Dharmapatni *et al* 2008). In addition it is expressed by fibroblasts (Semov *et al* 2002). Fn14 (fibroblast growth factor-inducible 14) protein/TWEAKR has been identified as the cell surface receptor for TWEAK (Wiley *et al* 2001). Fn14 is expressed by many cell types including epithelial cells (Michaelson *et al* 2005), mesenchymal cells (Girgenrath *et al* 2006) and endothelial cells (Harada *et al* 2002, Donohue *et al* 2003, Perper *et al* 2006). Moreover, many tissue progenitor cells of mesenchymal lineage (Girgenrath *et al* 2006), embryonic stem cells (Ramalho-Santos *et al* 2002) and osteoblasts (Perper *et al* 2006) also express Fn14. Binding of TWEAK to Fn14 results in the stimulation of NF-B and several other signaling cascades (Saitoh *et al* 2003, Winkles 2008). However, TWEAK independent Fn14 signaling has been demonstrated (Dogra *et al* 2007). On the other hand, TWEAK has also displayed Fn14 independent activity (De Ketelaere *et al* 2004).

TWEAK/Fn14 signaling results in multiple biologic effects including induction of inflammatory cytokines, modulating immune response angiogenesis and stimulation of apoptosis (Chicheportiche *et al* 1997, Campbell *et al* 2006, Kaplan *et al* 2000, Lynch *et al* 1999, Maecker *et al* 2005). The proinflammatory activities of TWEAK include induction of cytokines, chemokines and matrix metalloproteinases (Chicheportiche *et al* 1997, Chicheportiche *et al* 2002, Harada *et al* 2002, Perper *et al* 2006, Kamijo *et al* 2008). This protein plays a significant role in angiogenesis and activation of endothelial cells by upregulating surface expression of adhesion molecules such as ICAM-1 and VACM-1 (Kamijo *et al* 2008, Chicheportiche *et al* 2002, Hosokawa *et al* 2006). Recent studies have also shown that TWEAK has a role to play in tissue repair and regeneration following acute tissue injury (Girgenrath *et al* 2006).

TWEAK also has an inhibitory effect on the differentiation of osteoblasts and promotes osteoclastic differentiation of cells from the monocyte/macrophage lineage (Polek *et al* 2003 ando *et al* 2006). Furthermore, the TWEAK/Fn14 pathway might modulate immune responses by suppressing IL-12 and INF-γ which might in turn decrease Th-1 type immune response leading to imbalance of Th-1 type and Th-2 type immune responses (Maecker *et al* 2005).

Many studies have demonstrated increased expression of TWEAK and its receptor Fn14 in tissues and cells from a number of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis, inflammatory skin, kidney and airway diseases (Chicheportiche *et al* 2002, Perper *et al* 2006, Jin *et al* 2004, Xu *et al* 2004, Kim *et al* 2004, Putterman *et al* 2004). These studies indicate that TWEAK might play a role in the pathogenesis of these chronic inflammatory diseases. It has been proposed that TWEAK/Fn14 signaling might be a universal mechanism which plays a role in various inflammatory and autoimmune disorders mediating pathologic tissue remodeling (Zheng and Burkly 2008). Accordingly TWEAK might serve as an additional therapeutic target in the treatment of chronic inflammatory diseases (Potrovita *et al* 2004, Desplat-Jego *et al* 2005, Yepes and Winkles 2006).

TWEAK/Fn14 signalling has also been investigated in periodontally diseased tissues utilizing reverse transcription–polymerase chain reaction (RT-PCR) analysis and immunohistochemistry (Hosokawa *et al* 2006). TWEAK and Fn14 were shown to be expressed in both mRNA and protein forms in periodontitis tissues (Hosokawa *et al* 2006).

TWEAK and Fn14 were mainly expressed by mononuclear cells. When stimulated by TWEAK, human gingival fibroblasts (HGF) produced IL-8 and vascular endothelial growth factor (VEGF) and enhanced intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression on HGF in a dose-dependent fashion (Hosokawa *et al* 2006). The IL-8 and VEGF production induced by TWEAK was augmented synergistically by simultaneous stimulation with transforming growth factor (TGF)-β1 or IL-1β. The authors concluded that as IL-8, VEGF, ICAM-1 and VCAM-1 are involved in the inflammatory response in periodontal diseased tissues; TWEAK might be involved in the pathophysiology of periodontal disease (Hosokawa *et al* 2006). Thus, along with other cytokines such as IL-1β and TGF-β1, TWEAK might exacerbate periodontal disease.

In light of the above, it is hypothesised that the expression of TWEAK and Fn14/TWEAKR will be increased in tissue samples from periodontitis patients. The aim of the present study was to investigate the expression of TWEAK and TWEAK's receptor Fn14 through immunohistochemistry techniques using TWEAK and Fn14 antibodies.

2.3 Materials and methods

2.3.1 Patient demographics

The study included gingival samples taken from 27 patients (18 females and 9 males, aged 30-77 years, mean age = 55.2 years) with generalised chronic and aggressive (moderate to severe) periodontitis. The study population included 23 patients with generalised chronic periodontitis and 4 patients with generalised aggressive periodontitis. The patients were classified on the basis of American Academy of Periodontology 1999 classification (Armitage 1999). These patients were undergoing active periodontal treatment which included a non-surgical initial phase therapy (involving oral hygiene instructions and scaling and root debridement). The gingival biopsy sites in the chronic and aggressive periodontitis group had clinical probing pocket depths and clinical attachment loss greater than 5 mm with radiographic evidence of bone loss ranging from 50-90% of the root length.

The soft tissue biopsy was obtained by the postgraduate students during the flap surgery carried out for the treatment of the patients. The test samples included mostly interdental col tissue and granulation tissue removed next to periodontal bony defects. The healthy

tissue samples consisted of gingival tissue resected from seven patients (4 males and 3 females, aged 23-70 years at the time of surgery; mean age: 45.5 years) undergoing crown lengthening surgery not affected by periodontitis. 24 of the periodontitis tissue samples and three of the healthy samples were obtained from patients attending Periodontal Postgraduate Clinic at the Dental School, University of Adelaide between the time period of 2006-2007, while the remaining tissue samples of periodontitis and non-periodontitis patients were obtained from archival tissue samples stored at the Department of Pathology, University of Adelaide. Informed consent was obtained from the patients attending the Periodontics postgraduate clinic. This study was approved by the University of Adelaide Ethics committee in accordance with the guidelines of the National Health and Medical Research Council of Australia.

2.3.2 Antibodies

The following monoclonal antibodies (mAbs) were used for this study: mouse anti-human TWEAK antibody (P2D10 , subclass IgG2a, gifted by Timothy S Zheng, Biogen Idec Inc, Cambridge, USA) (Ho *et al* 2004) and Purified mouse antihuman Fn14 (subclass IgG1, clone ITEM 1, Biolegend, catalogue number 314002) (Hosokawa *et al* 2006). For control sections, the primary antibodies were omitted or irrelevant isotype-matched mouse mAbs (ID4.5 for IgG2a and IB.5 for IgG1) were applied.

2.3.3 Preparation of tissue for immunohistochemical detection

Following collection of the periodontal tissues from patients, the samples were immediately immersed in 10% normal buffered formalin overnight and then processed for embedding in paraffin. Sections were cut using a microtome at 5 μm and stored at room temperature. The paraffin embedded sections were dewaxed in two changes of xylene and two changes of alcohol. The sections were rehydrated in Milli Q water twice for 5 minutes. Both TWEAK and Fn14 expression was detected using a three step immunohistochemical detection method (Kraan *et al* 1999).

2.3.4 Routine histology staining (Hematoxylin-Eosin staining)

Routine histological assessment of the tissue samples was examined following Hematoxylin and Eosin staining to ensure that the tissues included were appropriate for

immunohistochemical detection and analysis. Following deparaffinisation, the sections were immersed in Hematoxylin solution for five minutes and then washed, followed by dipping in acid alcohol 5-7 times. Afterwards, the sections were washed in water and then dipped in lithium carbonate solution for 2 minute, washed in water and dipped in eosin for 3 minutes. After a final wash in water, the sections were immersed in two changes of alcohol and two changes of xylene and finally mounted with coverslips using DPX (dibutyl phthalate) as a mounting medium.

Each sample was given an inflammatory score on the basis of a microscopic analysis. Since no known reference was available for microscopic grading of inflammation of periodontal tissue, at the time of the study, a grading system was devised. The grading system was based on the percentage of chronic inflammatory cells in the connective tissue layer of periodontal tissue (both unhealthy and healthy) samples. A similar scale for grading of inflammation in soft tissues has been utilized in a previous study for assessing immune response to demineralized freeze-dried bone graft (Garraway *et al*, 1998).

Score 0 (minimal) - Chronic inflammatory cells from 0 to 10%
Score 1 (mild) - Chronic inflammatory cells from 11 to 25%
Score 2 (mild to moderate) - Chronic inflammatory cells from 26 to 50%
Score 3 (moderate to severe) - Chronic inflammatory cells from 51 to 75%
Score 4 (severe) - Chronic inflammatory cells from 76 to 100%

The grading system was performed by two observers (one of whom was blinded) to enhance the objectivity of the grading system.

2.3.5 Immunohistochemical staining for TWEAK expression

For detecting of TWEAK, tissue sections were pretreated to unmask antigen epitopes using Proteinase K (200 µg/ml) for 37ºC for 30 minutes. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 in 0.1% Sodium Azide and Phosphate buffered saline (PBS). A working concentration of 14.5 µg/ml of the primary antibody (P2D10; subclass IgG2a mouse anti-human TWEAK antibody, was determined in a preliminary experiment. The primary antibody was diluted in PBS with 1% Bovine Serum Albumin (BSA). The primary antibody was added and incubated in a wet chamber overnight at room temperature. On the second day, the sections were incubated with a secondary antibody, horseradish peroxidase

(HRP) conjugated goat anti-mouse antibody for 30 minutes at room temperature. HRP conjugated swine anti-goat IgG was then added for another 30 minutes at room temperature. Secondary and tertiary antibodies were diluted in PBS/1% BSA in the presence of 10% normal human serum (NHS). The sections were washed in PBS between each step. Colour reaction was developed using hydrogen peroxide as substrate and 3,9 Aminoethylcarbazole (AEC) as the dye giving a red hue to positive sections.

2.3.6 Immunohistochemical staining for Fn14 expression

In order to detect Fn14 expression, sections underwent HER (Heat Epitope Retrieval) in Sodium Citrate buffer (pH 6) for 20 minutes at 95ºC in a water bath. Following this, the sections were bench cooled for 20 minutes before undergoing the next step of the immunohistochemical process. Endogenous peroxidase activity was inhibited with 1% H_2O_2 in methanol. Blocking serum (normal horse serum provided in the Vectastain Universal Elite ABC kit) was added to the sections to reduce non-specific staining. A working concentration of 20µg/ml of the primary antibody against Fn14 was determined in a preliminary experiment. The primary antibody was diluted in PBS with 1% BSA to obtain the working concentration and added to the tissue sections. The slides were incubated in a wet chamber overnight at room temperature. The next day sections were incubated with a secondary antibody (biotinylated universal antibody raised in horse provided in the Vectastain Universal Elite ABC kit) at the concentration recommended by manufacturers for 1 hour at room temperature. Following this enhancement of primary and secondary antibody was carried out using ABC Reagent (Avidin and Biotinylated horse radish peroxidase macromolecular Complex) provided in the ABC kit. The sections were washed in PBS between each step except the slides were left unwashed after the addition of the blocking serum. Colour reaction was developed using H_2O_2 as the substrate and DAB (Diaminobenzidine tetrahydrochloride) dye giving a brown hue to positive sections.

2.3.7 Counterstaining of tissue samples (for both TWEAK and Fn14 detection)

Counterstaining was performed using Harris Hematoxylin for 10 seconds. The sections were then washed in water and immediately immersed in saturated lithium carbonate solution for 30 seconds. After a final wash in water, the slides were mounted in Gurr Aquamount (BDH, Poole, UK). Staining was performed at the same time in all samples for TWEAK detection to reduce day to day staining variability. For Fn14 the staining was

repeated twice to confirm the reproducibility of the results. Omission of primary antibodies and isotype matched antibodies (ID4.5 for IgG2a and IB.5 for IgG1) were used as the negative controls. Isotype matched antibodies were used at an equal concentration to that of the primary antibodies tested. Positive controls were performed on rheumatoid arthritis tissue samples known to express TWEAK and Fn14 (Dharmapatni *et al* 2008) personal communication).

2.3.8 Microscopic analysis

After immunohistochemical staining, the sections stained with mouse antihuman TWEAK antibody and purified mouse antihuman Fn14 were scored using a semiquantitative method. One sample was analysed from each patient. Due to the small size of the tissue biopsies the total area of the section was assessed for staining using a 5 point scale (0–4), by two independent observers in a random order, as described previously (Kraan *et al* 2000, Tak *et al* 1995).

Scores from 0 to 4 were given depending on the number of chronic inflammatory cells staining positive for tested antibodies.

Score 0: 0 to 10% of the chronic inflammatory cells stain positive
Score 1: 11 to 25% of the chronic inflammatory cells stain positive
Score 2: 26 to 50% of the chronic inflammatory cells stain positive
Score 3: 51 to 75% of the chronic inflammatory cells stain positive
Score 4: 76 to 100% of the chronic inflammatory cells stain positive

2.3.9 Statistical analysis

Statistical analysis for the SQA results in the two groups (periodontitis and clinically normal patients) studied was performed using SPSS version 11.5 (SPSS Inc, Chicago, IL, USA). Mann-Whitney U test for non-parametric data was used to analyze the mean ranks of semi-quantitative scores for TWEAK and Fn14. Kendall's tau_b test was used to detect correlation between TWEAK and Fn14 SQA. Same test was used to determine the correlation between inflammatory score and TWEAK/Fn14 expression. For both Mann-Whitney U test and Kendall's tau_b test, a p value of <0.05 was considered as being

statistically significant. Correlation was considered to be significant at r value (correlation coefficient) of 0.01.

2.4 Results

The gingival biopsy sites in the chronic and aggressive periodontitis group had clinical probing pocket depths and clinical attachment loss greater than 5 mm with radiographic evidence of bone loss ranging from 50-90% of the root length. Patient details including age, gender, current medication and smoking history at the time of surgery are shown in Tables 2.1 and 2.3. Tables 2.2 and 2.4 depict in detail probing pocket depth, recession, radiographic bone loss and level of inflammation at the sites of tissue biopsy.

Sl #	Biopsy #	G	Dg	Age	Smoking	Medical History /Medications
1	CP2/06	F	CP	62	N	Nil
2	CP4/06	M	CP	52	X	RA, hypertension, high cholesterol, lower back problem, Plaquenil, Celebrex, Enalpril, Lipitor, Aspirin
3	CP1/07	F	CP	69	N	Nil
4	CP2B/07	M	CP	58	N	Type 2 DM, hypertension, Barrett's disease, heart bypass; Plavax, Ritalin, Ezetrol, Nexium, Nifedipine
5	CP3/07	F	CP	47	N	Nil
6	CP4/07	M	AP	42	Y, 3-4/day	Anxiety disorder
7	CP5/07	F	CP	45	Y, marijuana	Nil
8	CP6/07	F	CP	57	N	Nil, HRT
9	CP7/07	M	CP	42	X	Nil, Losec
10	CP8/07	M	CP	77	N	Type 2 DM (controlled), hypertension, high cholesterol, arthritis, Atacand, Volatran, Celebrex
11	CP9/07	F	CP	71	N	Osteoporosis, Caltrate, Fosamax
12	CP10/07	F	CP	40	Y, 10-15/day	Nil
13	CP11/07	F	CP	75	N	Asthma, glaucoma, Seretide, Ventolin, Xalatan, Alphagan
14	CP14/07	F	CP	48	N	Asthma, Type 2 DM (diet controlled), Seretide, Ventolin
15	CP15/07	M	CP	45	X	Schizophrenic disorder, depression, Sodium valproate, Seroquel, Edronx, Epilim
16	CP19/07	F	AP	36	N	Nil
17	CP20/07	F	CP	72	X	Type 2 DM, hypertension, hyperlipidemia, Avandia, Melezide, Diaformin, Tritace, Asprin, Zimstat
18	CP23/07	M	CP	74	X	Arthritis, Diclofenac
19	CP26/07	F	CP	54	N	Hypertension, arthritis, Karvea, Glucosamine
20	CP27/07	F	AP	30	N	Nil
21	CP28/07	M	CP	66	X	Osteoarthritis, Lipitor, Felodurer, Panadol osteo, Anapox
22	CP29/07	F	CP	41	S	Asthma, thyroid gland removed due to cancer, Pulmicort, Ventolin, Thyroxine
23	CP33/07	F	CP	77	X	Hypertension, Felodipine
24	CP34/07	F	AP	46	X	Nil
25	CP14/00	F	CP	45	N	Psoriasis
26	CP13/06	M	CP	48	X	Hypertension, Karvea
27	CP3/05	F	CP	49	N	Nil

Dg: Diagnosis
CP: Generalised chronic periodontitis
AP: Generalised aggressive periodontitis

N: Never smoker
Y: Smoker
X: Ex-smoker

Table 2.1 Patient demographics (periodontitis group)

Sl no	Biopsy no	Dg	Site of sample	Inflam score	PD at site (mm)	Recession (mm)	Bone loss (%)
1	CP2/06	CP	17	2	5-7	0	70-80
2	CP4/06	CP	23-27	4	5-7	0-1	50-55
3	CP1/07	CP	14-17	3	5-7	0-4	50-70
4	CP2B/07	CP	33-43	3	5-8	0-2	30-50
5	CP3/07	CP	42,43	3	5	2	55-60
6	CP4/07	AP	25-27	3	6-7	1	50-60
7	CP5/07	CP	24-27	1	5-6	0-1	50
8	CP6/07	CP	14,15,16	1	5-8	2-3	50-60
9	CP7/07	CP	17	2	5-8	2-4	50-60
10	CP8/07	CP	43	3	9-10	2-4	70
11	CP9/07	CP	34-38	4	5-9	0-2	50-60
12	CP10/07	CP	11,12-15	3	6-10	0-5	50-70
13	CP11/07	CP	13-16	3	5-8	0-3	50-70
14	CP14/07	CP	24-27	3	5-7	0-1	50-70
15	CP15/07	CP	22-27	4	5-7	0-2	50-60
16	CP19/07	AP	46	4	9	2	90
17	CP20/07	CP	12-1 4	4	7	2	80-85
18	CP23/07	CP	34-37	3	5-8	0-3	60-70
19	CP26/07	CP	47-48	4	9	0-1	60
20	CP27/07	AP	23-27	4	5-9	0-1	50-70
21	CP28/07	CP	23	3	9-11	2-4	80-90
22	CP29/07	CP	46-47	3	8-9	0	50-60
23	CP33/07	CP	23-26	3	5-7	0-3	60-65
24	CP34/07	AP	14-15	4	5-6	0-1	50
25	CP14/00	CP	16	1	6-8	0-2	70-80
26	CP13/06	CP	27,28	1	5-7	0	50
27	CP3/05	CP	34	2	4	0	20-30

Inflammatory score
Score 0 (minimal): chronic inflammatory cells from 0 to 10%
Score 1 (mild): chronic inflammatory cells from 11to 25%
Score 2 (mild to moderate): chronic inflammatory cells from 26 to 50%
Score 3 (moderate to severe): chronic inflammatory cells from 51 to 75%
Score 4 (severe): chronic inflammatory cells from 76 to 100%
Dg: Diagnosis PD: Probing pocket depth
CP: Generalised chronic periodontitis AP: Generalised aggressive periodontitis

Table 2.2 Clinical findings of sampling sites (peridontitis group)

Sl no	Biopsy no	Age (yrs)	G	Smoking	Medical History/medications
1	NG1/07	58	M	N	Nil
2	NG2/07	37	F	N	Nil
3	NG3/07	52	M	Y, 10/day	Migraine
4	NG03/04	49	F	N	Nil
5	NG17/01	64	F	N	Nil
6	NG04/04	42	M	N	Nil
7	NG13/01	17	M	N	supraventricular tachycardia

Table 2.3 Patient demographics (normal group)

Sl no	Biopsy no	Site	Inflammatory score	Bone Loss
1	NG1/07	11	0	Nil
2	NG2/07	26	2	Nil
3	NG3/07	36	0	Nil
4	NG03/04	13-23	2	Nil
5	NG17/01	13,14	1	20%
6	NG04/04	36	1	Nil
7	NG13/01	42	2	Nil

Inflammatory score
Score 0 (minimal): chronic inflammatory cells from 0 to 10%
Score 1 (mild): chronic inflammatory cells from 11to 25%
Score 2 (mild to moderate): chronic inflammatory cells from 26 to 50%
Score 3 (moderate to severe): chronic inflammatory cells from 51 to 75%
Score 4 (severe): chronic inflammatory cells from 76 to 100%

Table 2.4 Clinical findings of sampling sites (normal group)

2.4.1 TWEAK expression in periodontitis tissue and normal control

The tissue sections were considered appropriate for immunohistochemical study if they had a significant proportion of gingival epithelium along with underlying connective tissue. In this study the clinically normal tissues samples showed mild to moderate levels of inflammatory response (Table 2.4). The periodontitis tissue samples showed moderate to severe levels of inflammation (Table 2.2) and included areas of granulation tissue

characterised by the presence of blood vessels, loose connective tissue, spindle shaped cells and leucocytic infiltration.

Positive staining for TWEAK protein was seen in leukocytes that formed large mononuclear cell infiltrates in tissue samples from periodontitis lesions (Figure 2.1A). Most of the leucocytes staining positively for TWEAK antibody had an eccentrically placed round nucleus with a rim of cytoplasm around it. These mononuclear cells appeared to be morphologically similar to lymphocytes or plasma cells (Wheater *et al* 1987) (Figures 2.1A, 2.1C). Some of the sections showed positive staining for TWEAK protein in multinucleated cells (Figures 2.2A, 2.2B)

Semiquantitative assessment (SQA) for TWEAK reflected the proportion of positive cells in the periodontitis tissue. TWEAK SQA scoring showed a mean value of 2.27 (standard error: 0.245; Table 2.9. Range: 0 to 4; Table 2.5). 16 of the 27 periodontitis samples showed a high SQA of >3.

Mononuclear cells in 17/27 periodontitis sections, showed very bright cell staining with the TWEAK antibody (Figures 2.2E, 2.2F). This percentage ranged from no expression to 50% (Table 2.5).

In 20 of the 27 periodontitis sections, cells lining the blood vessels had positive staining for TWEAK protein (Figure 2.2C, Table 2.5). Positive TWEAK staining of spindle shaped cells was observed in 12/27 tissue samples (Figures 2.2A, 2.2B, Table 2.5). These spindle shaped cells had morphology similar to fibroblasts.

TWEAK expression was less obvious in the non-periodontitis tissues. (Figures 2.1B, 2.1D). The mean TWEAK SQA was 0.43 (Standard error: 0.297; Table 2.9. Range: 0 to 2; Table 2.6) with five out of seven clinically normal samples having TWEAK SQA <1. TWEAK was associated with cells lining blood vessels and spindle shaped cells in five out of seven clinically normal samples. However, staining was lot weaker in non-periodontitis tissues (Figure 2.2D, Table 2.6). Figure 2.5 shows the mean TWEAK SQA in periodontitis and normal patients. Bars depict mean SQA +/- standard error.

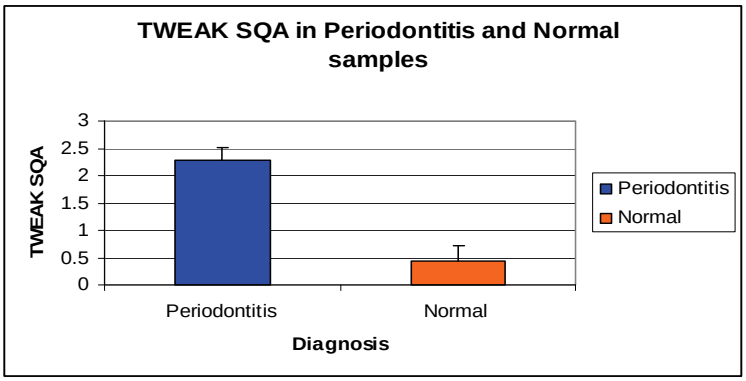


Figure 2.5 TWEAK SQA in periodontitis and normal patients. Bars depict mean SQA +/- standard error

Sl no	Biopsy no	TWEAK SQA	BS (%)	BV	Sp
1	CP2/06	3	10-20	P	P
2	CP4/06	1	5-10	SP	P
3	CP1/07	3	nil	P	P
4	CP2B/07	2	10-20	P	N
5	CP3/07	3	10-20	N	N
6	CP4/07	2	10	P	N
7	CP5/07	3	10-25	SP	P
8	CP6/07	1	Nil	SP	N
9	CP7/07	3	Nil	N	N
10	CP8/07	0	Nil	P	N
11	CP9/07	3	10	N	N
12	CP10/07	3	10-20	P	N
13	CP11/07	3	10-15	N	N
14	CP14/07	3	10-20	P	P
15	CP15/07	3	10	P	N
16	CP19/07	3	10	SP	P
17	CP20/07	4	10	P	N
18	CP23/07	4	50	N	N
19	CP26/07	3	50	N	N
20	CP27/07	3	10	P	N
21	CP28/07	0	nil	P	P
22	CP29/07	1	nil	P	P
23	CP33/07	3	50	P	P
24	CP34/07	not incl	not incl	P	P
25	CP14/00	0	nil	P	P
26	CP13/06	2	nil	N	N
27	CP3/05	0	nil	P	P
	Mean	2.27			
	Range	0-4			
	SD	1.25			

P: positive
SP: strongly positive
WP: weakly positive
N: no staining noted

BS: bright staining
BV: blood vessel
Sp: spindle shaped cells

Table 2.5 TWEAK expression in periodontitis samples - 16 samples with TWEAK SQA >3

Sl no	Biopsy no	TWEAK SQA	BS (%)	BV	Sp
1	NG1/07	0	N	N	P
2	NG2/07	2	10	WP	P
3	NG3/07	0	N	N	WP
4	NG03/04	0	N	WP	WP
5	NG17/01	0	N	WP	WP
6	NG04/04	0	N	WP	N
7	NG13/01	1	10	WP	N
	Mean	0.4286			
	Range	0-2			
	SD	0.7868			

P: positive BS: bright staining
WP: weakly positive BV: blood vessel
N: no staining noted Sp: spindle shaped cells

Table 2.6 TWEAK expression in normal patients - 6 samples with SQA $\leq$1

Sl no	Biopsy no	Fn14 SQA	BV	Sp
1	CP2/06	3	P	P
2	CP4/06	4	P	P
3	CP1/07	2	P	P
4	CP2B/07	3	P	P
5	CP3/07	4	P	P
6	CP4/07	2	P	N
7	CP5/07	1	WP	N
8	CP6/07	0	N	N
9	CP7/07	3	SP	P
10	CP8/07	1	P	P
11	CP9/07	3	P	N
12	CP10/07	2	WP	N
13	CP11/07	1	P	N
14	CP14/07	1	P	P
15	CP15/07	4	P	N
16	CP19/07	3	SP	P
17	CP20/07	2	P	N
18	CP23/07	3	SP	SP
19	CP26/07	4	N	P
20	CP27/07	4	P	P
21	CP28/07	4	P	P
22	CP29/07	1	WP	N
23	CP33/07	4	P	SP
24	CP34/07	3	P	N
25	CP14/00	0	P	N
26	CP13/06	3	P	P
27	CP3/05	2	N	P
	Mean	2.48		
	range	0-4		
	SD	1.28		

P: positive
SP: Strongly positive
WP: weakly positive
N: no staining noted
SD: standard deviation

SQA: semiquantitative assessment
BS: bright staining
BV: blood vessel
Sp: spindle shaped cells

Table 2.7 Fn14 in periodontitis patients - 14 samples with Fn14 SQA >3

Sl no	Biopsy no	Fn14 SQA	BV	Sp cells
1	NG1/07	0	N	N
2	NG2/07	2	WP	N
3	NG3/07	1	WP	N
4	NG03/04	0	N	N
5	NG17/01	1	P	N
6	NG04/04	0	N	N
7	NG13/01	3	P	P
Mean		1		
Range		0 to 3		
SD		1.1547		

P: positive
SP: Strongly positive
WP: weakly positive
N: no staining noted
SD: standard deviation
SQA: semiquantitative assessment
BS: bright staining
BV: blood vessel
Sp: spindle shaped cells

Table 2.8 Fn14 in normal samples - 5 samples with SQA <1

2.4.2 Fn14 expression in periodontitis tissue and normal control

In tissue samples from periodontitis patients, high Fn14 protein expression was observed in leucocytes forming mononuclear infiltrates (Figure 2.3A). These cells were found to be morphologically similar to plasma cells and lymphocytes (Figure 2.3C). In periodontitis tissue samples mean Fn14 SQA was 2.48 (standard error: 0.247, Table 2.9). However, a range of 0 to 4 was seen (Table 2.7). 14 out of 27 patients had high Fn14 SQA of 3 or more. Mean Fn14 SQA of periodontitis sample was found to be significantly greater than that seen in tissue samples collected from non-periodontitis patients (Figures 2.3B, 2.3D). In normal patients Fn14 SQA was 1.00 (Standard Error: 0.436; Table 2.9. Range: 0 to 2; Table 2.8). Five out of seven samples from clinically normal patients had Fn14 SQA <1 (Table 2.8). Cells lining blood vessels showed positive staining for Fn14 protein in all but three of the periodontitis tissue samples (Figure 4A, Table 2.7). In periodontitis patients, Fn14 protein expression was also noted in spindle shaped cells resembling fibroblasts (Figure 2.4A, Table 2.7).

In contrast, blood vessels were positive to Fn14 protein in four out of seven tissue samples from non-periodontitis patients. Out of these two samples showed very weak staining for Fn14 protein (Figure 2.4B, Table 2.8). All but one of the samples from non-periodontitis patients showed no evidence of Fn14 expression in spindle shaped cells (Table 2.8). Figure 2.6 shows the mean Fn14 SQA in periodontitis and normal patients. Bars depict mean SQA +/- standard error.

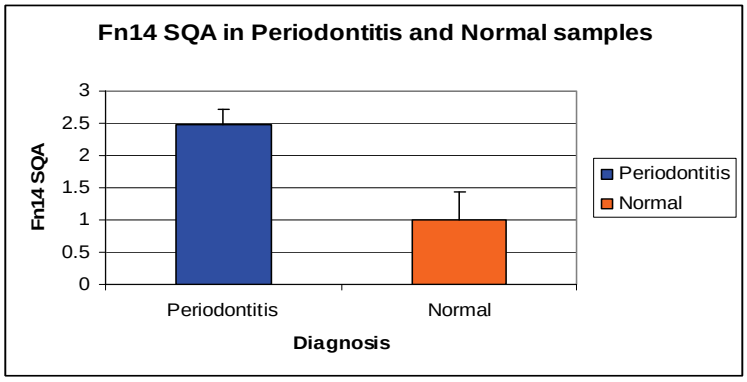


Figure 2.6 Fn14 SQA in periodontitis and normal patients. Bars depict mean SQA +/- standard error

Sections which were used as negative controls showed absence of staining (Figures 2.1E, 2.3E). Rheumatoid arthritis tissue samples were used as positive control. The sections from active RA lesions showed high expression for both TWEAK and Fn14 protein (Figures 2.1F, 2.3F).

2.4.3 TWEAK/Fn14 expression in periodontitis patients with medical history of other chronic inflammatory diseases

It is interesting to observe that all the five patients suffering from either rheumatoid arthritis or osteoarthritis and periodontitis had high inflammatory score (Table 2.9). Whilst the TWEAK SQA $\geq$ 3 was seen in only two of these samples, Fn14 SQA $\geq$ 3 was observed in four of them (Table 2.9).

Sl no	Biopsy no	Med H/o	Medications	Inflam score	TWEAK SQA	Fn14 SQA
1	CP 4/06	RA	Aspirin, celebrex, plaquenil	4	1	4
2	CP8/07	A	celebrex	3	0	1
3	CP23/07	A	diclofenac	3	4	3
4	CP26/07	A	glucosamine	4	3	4
5	CP28/07	A	Panadol osteo	3	0	4

Med H/o: Medical history RA: Rheumatoid arthritis
Inflam score: inflammatory score A: Arthritis

Table 2.9 TWEAK/Fn14 expression in periodontitis patients with concomitant relevant medical history of other chronic inflammatory diseases.

2.4.4 Statistical analysis

The expression of TWEAK protein was significantly higher in periodontitis tissue as compared to normal tissue (Mann Whitney U-test, p value 0.002). Similarly, in comparison with normal tissue, periodontitis tissue expressed significantly higher Fn14 protein (Mann Whitney U-test, p value 0.013). A strong positive correlation was found between TWEAK and Fn14 expression (Kendall's tau_b test; p value 0.007 and r value 0.395). In addition, a strong correlation was found between inflammatory score and TWEAK (Kendall's tau_b test; p value 0.001 and r value 0.494) and Fn14 expression (Kendall's tau_b test; p value 0.0001 and r value 0.530).

	TWEAK SQA Mean	TWEAK SQA Std error	Fn14 SQA Mean	Fn14 SQA Std Error
Periodontitis	2.27	0.245	2.48	0.247
Normal	0.43	0.297	1	0.436

Table 2.10 TWEAK and Fn 14 SQA in periodontitis tissues and normal sample

2.5 Discussion

This immunohistochemical study demonstrates that the expression of TWEAK and its receptor Fn14, is increased in periodontitis tissue. As reflected by high SQA scores and

statistical analysis, there was a significantly higher TWEAK protein expression in the periodontitis group than the healthy group. In periodontitis affected tissue, TWEAK protein was expressed by the mononuclear leukocytes forming large infiltrates. These mononuclear cells had an eccentrically placed nucleus and were morphologically similar to lymphocytes or plasma cells (Wheater *et al* 1987). TWEAK protein was displayed in fibroblast-like spindle shaped cells too. In the past many studies have shown expression of TWEAK by various inflammatory cells, including macrophages (Chicheportiche *et al* 1997, Nakayama *et al* 2000), monocytes (Kaplan *et al* 2002), activated T-cells (Kawakita *et al* 2005) and plasma cells (Dharmapatni *et al* 2008). The findings of this study confirm the results from a previous immunohistochemical study which reported presence of TWEAK protein in mononuclear cells and human gingival fibroblasts in tissue samples from eight periodontitis patients (Hosokawa *et al* 2006). High TWEAK expression has also been noted in tissues from patients with active rheumatoid arthritis (Dharmapatni *et al* 2008).

This study additionally reports the expression of TWEAK protein by multinucleated cells and cells lining blood vessels in periodontitis samples. In healthy gingival samples TWEAK protein was weakly expressed by cells lining blood vessels and spindle shaped cells. An earlier study has also demonstrated that TWEAK is expressed by endothelial cells (Donohue *et al* 2003). TWEAK has been shown to induce monocytes/macrophages to differentiate into Tartrate-resistant acid phosphatase (TRAP) positive multinucleated, functional osteoclasts (Polek *et al* 2003). However, there is no existing report in the current literature describing the expression of TWEAK by osteoclasts.

In the present study, Fn14 expression was found to be present in significantly higher amounts in periodontitis patients than in clinically healthy patients. The cells expressing Fn14 were observed to be mononuclear cells with morphology similar to lymphocytes and plasma cells (Wheater *et al* 1987). The majority of the periodontitis samples investigated expressed Fn14 in the cells lining blood vessels. Fibroblasts and some multinucleated cells were also positive for Fn14 in periodontitis samples. In comparison Fn14 was very weakly expressed in cells lining blood vessels in normal patients. Only one healthy sample showed fibroblasts staining with Fn14 antibody.

Fn14 is expressed by many cell types including epithelial cells (Michaelson *et al* 2005), mesenchymal cells (Girgenrath *et al* 2006) and endothelial cells (Harada *et al* 2002,

Donohue *et al* 2003, Perper *et al* 2006). Fn14 is also expressed by many progenitor cells of mesenchymal lineage (Girgenrath *et al* 2006) and embryonic stem cells (Ramalho-Santos *et al* 2002). This study supports the findings of a previous study which reported expression of Fn14 by mononuclear cells and fibroblasts in periodontitis tissue (Hosokawa *et al* 2006). Another study reported higher expression of Fn14 in active rheumatoid arthritis tissue samples as compared to normal synovial tissue (Dharmapatni *et al* 2008).

The present study demonstrates samples with high inflammatory score had high TWEAK and Fn14 expression. This strong correlation between TWEAK and Fn14 expression and inflammatory score in the periodontitis samples might indicate that TWEAK and Fn14 are expressed by infiltrating inflammatory cells rather than resident cell populations. This was in stark contrast to the findings in normal samples wherein a low inflammatory score coorelated with low TWEAK and Fn14 SQA score. These observations might elicit a possible role of TWEAK/Fn14 signalling in destruction seen in periodontitis. Some of the periodontitis samples did show high TWEAK but low Fn14 expression and vice versa. However, TWEAK independent Fn14 signaling does exist (Dogra *et al* 2007). TWEAK has also displayed Fn14 independent activity (De Ketelaere *et al* 2004). Moreover, there is a possibility that another receptor for TWEAK might mediate its action (Polek *et al* 2003, Bover *et al* 2007). This might explain the intra-sample variability seen in expression of TWEAK and Fn14 in periodontitis tissue in the present study.

Samples with both rheumatoid arthritis or osteoarthritis and periodontitis were marked with high inflammatory score and high expression of TWEAK and or Fn14. There have been number of studies which have indiacted a likely interrelationship between Rheumatoid arthritis and periodontitis (Mercado *et al* 2000, Ramamurthy *et al* 2005, de Pablo *et al* 2007). TWEAK / Fn14 signalling might be a likely pathway which is common to both the chronic immunoinflammatory diseases in question. However, this study is not designed to investigate the influence of other chronic inflammatory conditions such as rheumatoid arthritis and osteoarthritis on the expression of TWEAK and Fn14 in periodontally diseased tissues. In the present study the number of samples having both rheumatoid arthritis or osteoarthritis and periodontitis were few. Hence a conclusive inference could not be made and merits further research in the future.

Periodontitis is a chronic immunoinflammatory disease in response to microbial factors resulting in the destruction of tooth supporting tissues and pathologic bone loss. The

majority of destruction in periodontitis is not due to the direct action of enzymes or other agents from the involved microorganisms but due to the production of host derived enzymes, cytokines and other proinflammatory mediators resulting in the activation of immune cells and enzymes such as matrix metalloproteinases (Page and Kornman 1997, Boch *et al* 2001, Van Dyke and Serhan 2003, Kornman 2008). Page and Schroeder described four stages of the periodontal lesion and reported that the advanced lesion was characterised by loss of the alveolar bone and periodontal ligament which was predominated by plasma cells and displayed both tissue destruction and fibrosis (Page and Schroeder 1976). Although dominated by B cells and plasma cells, the periodontal inflammatory infiltrate also contains T lymphocytes, macrophages and neutrophils (Page *et al* 1997). In this study high expression of TWEAK and Fn14 by mononuclear cells resembling plasma cells and lymphocytes in periodontitis tissue might suggest the possible contribution of this pathway to the pathogenesis of the disease.

TWEAK is known for its multiple biologic effects which are mediated via the Fn14 receptor and NF-κβ signaling pathway (Saitoh *et al* 2003, Brown *et al* 2003, Harada *et al* 2002, Nakayama *et al* 2003). Signaling pathways other than NF-κβ such as MAPK, ERK1/2, JNK 1/2 and p38 also mediate TWEAK/Fn14 interaction (Donohue *et al* 2003, Saas *et al* 2000, Hosokawa *et al* 2006). The proinflammatory role of TWEAK has been investigated in chronic immunoinflammatroy and autoimmune diseases such as rheumatoid arthritis, artherosclerosis, systemic lupus erythematosus and multiple sclerosis (Chicheportiche *et al* 2002, Kim *et al* 2004, Kamijo *et al* 2008, Park *et al* 2008, Campbell *et al* 2004, Zhao *et al* 2007). The activities of TWEAK include induction of proinflammatory molecules such as cytokines (IL-6, IP-10) chemokines (IL-8, MCP-1), PGE2 and MMPs (Chicheportiche *et al* 2002, Chicheportiche *et al* 1997, Harada *et al* 2002, Perper *et al* 2006, Kamijo *et al* 2008). It has a significant role in angiogenesis and activation of endothelial cells by upregulating surface expression of adhesion molecules i.e. ICAM-1 and VCAM-1 (Chicheportiche *et al* 2002, Kamijo *et al* 2008, Hosokawa *et al* 2006). IL-1β and TNF are known to potentiate the proinflammatory actions of TWEAK/Fn14 pathway by upregualting the expression of Fn14 (Chicheportiche *et al* 2002, Hosokawa *et al* 2006).

The role of proinflammatory cytokines in the initiation and progression of periodontitis is well documented (Gemmell *et al* 1997, Graves and Cochran 2003, Irvin and Myrillas 1998,

Kantarci *et al* 2006). The proinflammatory effect of TWEAK/ Fn14 signalling might be an additional factor contributing to the tissue destruction in periodontitis.

TWEAK/Fn14 is known to effect endothelial cells in multiple ways. These include endothelial cell proliferation, migration, capillary formation, promoting cell survival, increasing the expression of ICAM-1 and E-selectin and inducing the secretion of proinflammatory cytokines (Lynch *et al* 1999, Wiley *et al* 2001, Harada *et al* 2002, Donohue *et al* 2003). TWEAK can either act alone or in sync with other growth factors to bring about differential tissue response on endothelial cells. Alone it is able to promote endothelial cell survival and resistance to apoptosis (Jakubowski *et al* 2002). In the presence of fibroblast growth factor (bFGF), TWEAK can contribute to endothelial cell proliferation and migration whereas it antagonizes the morphogenic response of endothelial cells to vascular endothelial growth factor (VEGF) (Jakubowski *et al* 2002). Angiogenesis is an important process which plays an integral role in repair and regeneration. Periodontal diseases are chronic inflammatory diseases wherein there is disruption in repair and regeneration (Bartold and Narayanan 2006). In this study endothelial cells were shown to express TWEAK and Fn14 in periodontitis tissue. Any impediment in the crucial process of angiogenesis might be a possible pathway by which TWEAK/Fn14 signalling can contribute to pathogenesis of periodontitis by interfering with repair and regeneration.

TWEAK also has an inhibitory effect on the differentiation of osteoblasts and promotes osteoclastic differentiation of cells from the monocyte/macrophage lineage (Ando *et al* 2006, Polek *et al* 2003). TWEAK induces osteoclast differentiation independent of RANKL (Polek *et al* 2003). Along with RANKL, an additive (but not a synergistic) osteoclastic effect of TWEAK has been described (Polek *et al* 2003). TWEAK has also been shown to upregulate RANKL expression on osteoblasts and induces secretion of chemokines from osteoblasts such as RANTES thereby promoting osteoclast differentiation and migration (Ando *et al* 2006). There are few reports which debate the osteoclastic effect of TWEAK (unpublished studies by T. Zheng and D. Findlay cited in Burkly *et al* 2007). Overall current evidence supports the notion that TWEAK can contribute to pathologic bone loss by interfering with osteoblast and osteoclast differentiation. Thus, TWEAK/Fn14 complex might be a potential player in contributing to pathologic bone destruction in periodontitis.

TWEAK /Fn14 pathway might also modulate immune responses by suppressing IL-12 and INF-γ which might in turn decrease Th-1 type immune response leading to imbalance of Th-1 type and Th-2 type immune responses (Maecker *et al* 2005, Trinchieri *et al* 1992, Chehimi and Trinchieri 1994). Three hypotheses have been proposed as to which T cell subsets are associated with periodontitis (Seymour *et al* 1993, Ebersole and Taubman 1994, Dennison and Van Dyke 1997). Several studies have shown that there are more abundant Th2 than Th1 cells in diseased periodontal tissues (Yamazaki *et al* 1997, Lappin *et al* 2001). These findings are consistent with early observations that B cells are predominant in "established" periodontal lesion while T cells are more dominant in "early" stages of disease (Page and Schroeder 1976). The general consensus seems to be that Th1 is associated with periodontal health and Th2 is associated with periodontal disease (Dennison and Van Dyke 1997, Seymour *et al* 1993). However, it has been suggested that the relative dominance of B cell and plasma cells in periodontitis may be due to an imbalance in Th1 and Th2 cells (Berglundh and Donati 2005). Hence, TWEAK can play a role in periodontitis by shifting the balance more towards Th2 response.

TWEAK also has weak apoptotic effects directly by caspase dependent and independent pathways or indirectly by stimulating TNF-alpha (Chicheportiche *et al* 1997, Nakayama *et al* 2002, Marsters *et al* 1998, Schneider *et al* 1999, Wilson and Browning 2002). There is evidence that apoptosis might play a role in the pathogenesis of periodontal diseases. This could be due to apoptosis of fibroblasts and resident lymphocytes in periodontal tissue brought about by cytokines such as TNF and bacterial lipopolysaccharides which might result in decreased repair (Graves *et al* 2001, Kurita-Ochiai *et al* 1999, Ohguchi *et al* 1998, Liu *et al* 2003). Although TWEAK and Fn14 was expressed more in periodontitis tissue in this study it still remains to be evaluated whether TWEAK has any apoptotic effect on cells of the periodontium. At least one study has shown that human gingival fibroblast produced IL-8 and VEGF upon TWEAK stimulation (Hosokawa *et al* 2006).

2.6 Future directions

In this study many mononuclear cells, which are most likely to be lymphocytes, exhibited increased TWEAK and Fn14 expression. However, there were subsets of these cells which expressed little or negative staining. Hence it will be necessary to identify the specific cell types expressing TWEAK and Fn14, especially within lymphocyte subsets, by carrying out dual immunohistochemistry studies. It would also be interesting to investigate whether the

multinucleated cells positive for TWEAK expression are TRAP positive cells/osteoclasts. The expression of TWEAK by leuckocytes also needs to be further studied to understand its relevance, if any, to the pathologic process. *In vitro* and *in vivo* functional studies are called for to understand the physiologic and pathologic roles of TWEAK/Fn14 signaling in both diseased and normal periodontal tissue. Although, this study was not designed to stratify the study population according to pocket depth, clinical attachment loss and type of periodontitis (chronic versus aggressive), the majority of patients had generalised chronic periodontitis and only four had generalised aggressive periodontitis. The samples were taken from moderate to advanced cases and the pocket depths ranged from 5 to 7mm. It would be interesting to evaluate whether there is any difference in TWEAK and Fn14 expression based on pocket depth, clinical attachment loss and type of periodontitis.

2.7 Conclusion

There is sufficient evidence that TWEAK/Fn14 signaling contributes to chronic inflammatory conditions via its proinflammatory actions, modulation of immune response, angiogenesis and stimulation of apoptosis. Furthermore, TWAEK/Fn14 has a role to play in tissue repair and regeneration and can contribute to pathologic bone destruction by promoting osteoclast differentiation and migration, inhibiting osteoblast differentiation and upregulating RANKL on osteoblasts. This study demonstrated a higher expression of TWEAK and Fn14 in mononuclear leucocytes, spindle shaped cells resembling fibroblasts, cells lining blood vessels and multinucleated cells in tissue samples from periodontitis patients compared to clinically normal periodontal tissues. This suggests that TWEAK/Fn14 signaling might be an additional player in the pathogenesis of periodontitis.

2.8 References

Akatsu T, Murakami T, Nishikawa M, Ono K, Shinomiya N, Tsuda, E, Mochizuki S, Yamaguchi K, Kinosaki M, Higashio K, Yamamoto M, Motoyoshi K, Nagata N. (1998). Osteoclastogenesis inhibitory factor suppresses osteoclast survival by interfering in the interaction of stromal cells with osteoclast. *Biochem Biophys Res Commun* 250: 229-234.

Ando T, Ichikawa J, Wako M, Hatsushika K, Watanabe Y, Sakuma M, Tasaka K, Ogawa H, Hamada Y, Yagita H, Nakao A. (2006). TWEAK/Fn14 interaction regulates RANTES production, BMP-2-induced differentiation and RANKL expression in mouse osteoblastic MC3T3-E1 cells. *Arthritis Res Ther* 8: R146.

Armitage GC. (1999). Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* 4: 1-6.

Baker PJ. (2000). The role of immune responses in bone loss during periodontal disease. Microbes Infect 2: 1181-1192.

Bartold PM, Narayanan AS. (2006). Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 40: 29-49.

Berglundh T, Donati M. (2005). Aspects of adaptive host response in periodontitis. *J Clin Periodontol* 32 Suppl 6: 87-107.

Boch JA, Wara-aswapati N, Auron PE. (2001). Interleukin 1 signal transduction--current concepts and relevance to periodontitis. *J Dent Res* 80: 400-407.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Toz H, Atilla G, Hughes, FJ, Belibasakis GN. (2007a). Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. *J Clin Periodontol* 34: 370-376.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Toz H, Berdeli A, Atilla G, McKay IJ, Hughes, FJ, Belibasakis GN. (2007b). Differential expression of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin mRNA in periodontal diseases. *J Periodontal Res* 42: 287-293.

Bover LC, Cardo-Vila M, Kuniyasu A, Sun J, Rangel R, Takeya M, Aggarwal BB, Arap W, Pasqualini R. (2007). A previously unrecognised protein-protein interaction between TWEAK and CD163: potential biological implications. *J Immunol* 178: 8183-8194.

Brown SA, Richards, CM, Hanscom HN, Feng SL, Winkles JA. (2003). The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation. *Biochem J* 371: 395-403.

Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli, C, Kelley M, Hsu H, Boyle WJ, Dunstan, CR, Hu S, Lacey DL. (1999). The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 145: 527-538.

Campbell S, Burkly LC, Gao HX, Berman JW, Su L, Browning B, Zheng T, Schiffer L, Michaelson JS, Putterman, C. (2006). Proinflammatory effects of TWEAK/Fn14 interactions in glomerular mesangial cells. *J Immunol* 176: 1889-1898.

Campbell S, Michaelson J, Burkly L, Putterman C (2004). The role of TWEAK/Fn14 in the pathogenesis of inflammation and systemic autoimmunity. *Front Biosci* 9: 2273-84.

Chehimi J, Trinchieri G. (1994). Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *J Clin Immunol* 14: 149-161.

Chicheportiche Y, Bourdon PR, Xu H, Hsu YM, Scott H, Hession, C, Garcia I, Browning JL. (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem* 272: 32401-32410.

Chicheportiche Y, Chicheportiche R, Sizing I, Thompson J, Benjamin, CB, Ambrose, C, Dayer JM. (2002). Proinflammatory activity of TWEAK on human dermal fibroblasts and synoviocytes: blocking and enhancing effects of anti-TWEAK monoclonal antibodies. *Arthritis Res* 4: 126-133.

Crotti T, Smith MD, Hirsch R, Soukoulis S, Weedon H, Capone M, Ahern MJ, Haynes D. (2003). Receptor activator NF kappaB ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis. *J Periodontal Res* 38: 380-387.

De Ketelaere A, Vermeulen L, Vialard J, Van De Weyer I, Van Wauwe J, Haegeman G, Moelans I. (2004). Involvement of GSK-3β in TWEAK-mediated NF-kappaB activation. *FEBS Lett* 566: 60-64.

Dennison DK, Van Dyke TE. (1997). The acute inflammatory response and the role of phagocytic cells in periodontal health and disease. *Periodontol 2000* 14: 54-78.

De Pablo P, Dietrich T, McAlindon TE. (2007). Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. *J Rheumatol* 15:70-76.

Desplat-Jego S, Creidy R, Varriale S, Allaire N, Luo Y, Bernard D, Hahm K, Burkly L, Boucraut J. (2005). Anti-TWEAK monoclonal antibodies reduce immune cell infiltration in the central nervous system and severity of experimental autoimmune encephalomyelitis. *Clin Immunol* 117: 15-23.

Dharmapatni AASSK, Smith MD, Weedon H, Holding, C, Haynes DR. (2008) The expression of TNF-like weak inducer of apoptosis (TWEAK) and TWEAK receptor (Fn14) in synovial tissues from patients with various types of arthritis. Poster presented at

Australian Rheumatology Association 50th Annual Scientific Conference 2008. Adelaide, Australia.

Dogra, C, Hall SL, Wedhas N, Linkhart TA, and Kumar A. (2007). Fibroblast growth factor inducible 14 (Fn14) is required for the expression of myogenic regulatory factors and differentiation of myoblasts into myotubes. Evidence for TWEAK-independent functions of Fn14 during myogenesis. *J Biol Chem* 282: 15000-15010.

Donohue PJ, Richards, CM, Brown SA, Hanscom HN, Buschman J, Thangada S, Hla T, Williams MS, Winkles JA. (2003). TWEAK is an endothelial cell growth and chemotactic factor that also potentiates FGF-2 and VEGF-A mitogenic activity. *Arterioscler Thromb Vasc Biol* 23: 594-600.

Ebersole JL, Taubman MA. (1994). The protective nature of host responses in periodontal diseases. *Periodontol 2000* 5: 112-141.

Fuller K, Wong B, Fox S, Choi Y, Chambers TJ. (1998). TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med* 188: 997-1001.

Garraway R, Young WG, Daley T, Harbrow D, Bartold PM. (1998). An assessment of the osteoinductive potential of commercial demineralized freeze-dried bone in the murine thigh muscle implantation model. *J Periodontol* 69: 1325-1336.

Gemmell E, Marshall RI, Seymour GJ. (1997). Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. *Periodontol 2000* 14: 112-143.

Girgenrath M, Weng S, Kostek, CA, Browning B, Wang M, Brown, SA, Winkles JA, Michaelson JS, Allaire N, Schneider P, Scott ML, Hsu YM, Yagita H, Flavell RA, Miller JB, Burkly LC, Zheng TS. (2006). TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration. *Embo J* 25: 5826-5839.

Graves DT, Cochran D. (2003). The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol* 74: 391-401.

Graves DT, Oskoui M, Volejnikova S, Naguib G, Cai S, Desta T, Kakouras A, Jiang Y. (2001). Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment and osteoclast formation in response to *P. gingivalis* infection. *J Dent Res* 80: 1875-1879.

Haffajee AD, Socransky SS. (1994). Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* 5: 78-111.

Harada N, Nakayama M, Nakano H, Fukuchi Y, Yagita H, Okumura K. (2002). Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 299: 488-493.

Ho DH, Vu H, Brown SA, Donohue PJ, Hanscom HN, Winkles JA. (2004). Soluble tumor necrosis factor-like weak inducer of apoptosis overexpression in HEK293 cells promotes tumor growth and angiogenesis in athymic nude mice. *Cancer Res* 64: 8968-8972.

Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. (2006). Proinflammatory effects of tumour necrosis factor-like weak inducer of apoptosis (TWEAK) on human gingival fibroblasts. *Clin Exp Immunol* 146: 540-549.

Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli, C, Morony S, Shimamoto G, Bass MB, Boyle WJ. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA* 96: 3540-3545.

Irwin, CR, Myrillas TT. (1998). The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis* 4: 43-47.

Jakubowski A, Browning B, Lukashev M, Sizing I, Thompson JS, Benjamin, CD, Hsu YM, Ambrose, C, Zheng TS, Burkly LC. (2002). Dual role for TWEAK in angiogenic regulation. *J Cell Sci* 115: 267-274.

Jin L, Nakao A, Nakayama M, Yamaguchi N, Kojima Y, Nakano N, Tsuboi R, Okumura K, Yagita H, Ogawa H. (2004). Induction of RANTES by TWEAK/Fn14 interaction in human keratinocytes. *J Invest Dermatol* 122: 1175-1179.

Kamijo S, Nakajima A, Kamata K, Kurosawa H, Yagita H, Okumura K. (2008). Involvement of TWEAK/Fn14 interaction in the synovial inflammation of RA. *Rheumatology* 47: 442-450.

Kantarci A, Hasturk H, Van Dyke TE (2006). Host-mediated resolution of inflammation in periodontal diseases. *Periodontol 2000* 40: 144-63.

Kaplan MJ, Lewis EE, Shelden EA, Somers E, Pavlic R, McCune WJ, Richardson BC. (2002). The apoptotic ligands TRAIL, TWEAK and Fas ligand mediate monocyte death induced by autologous lupus T cells. *J Immunol* 169: 6020-6029.

Kaplan MJ, Ray D, Mo RR, Yung RL, Richardson BC. (2000). TRAIL (Apo2 ligand) and TWEAK (Apo3 ligand) mediate CD4+ T cell killing of antigen-presenting macrophages. *J Immunol* 164: 2897-2904.

Kawakita T, Shiraki K, Yamanaka Y, Yamaguchi Y, Saitou Y, Enokimura N, Yamamoto N, Okano H, Sugimoto K, Murata K, Nakano T. (2004). Functional expression of TWEAK in human hepatocellular carcinoma: possible implication in cell proliferation and tumor angiogenesis. *Biochem Biophys Res Commun* 318: 726-733.

Kawakita T, Shiraki K, Yamanaka Y, Yamaguchi Y, Saitou Y, Enokimura N, Yamamoto N, Okano H, Sugimoto K, Murata K, Nakano T. (2005). Functional expression of TWEAK in human colonic adenocarcinoma cells. *Int J Oncol* 26: 87-93.

Kim SH, Kang YJ, Kim WJ, Woo DK, Lee Y, Kim DI, Park YB, Kwon BS, Park JE, Lee WH. (2004). TWEAK can induce pro-inflammatory cytokines and matrix metalloproteinase-9 in macrophages. *Circ J* 68: 396-399.

Kornman KS. (2008). Mapping the Pathogenesis of Periodontitis: A New Look. *J Periodont* 79: 1560-1568.

Kraan MC, Haringman JJ, Ahern MJ, Breedveld, FC, Smith MD, Tak PP. (2000). Quantification of the cell infiltrate in synovial tissue by digital image analysis. *Rheumatology* 39: 43-49.

Kraan MC, Haringman JJ, Post WJ, Versendaal J, Breedveld, FC, Tak PP. (1999). Immunohistological analysis of synovial tissue for differential diagnosis in early arthritis. *Rheumatology* (Oxford) 38: 1074-1080.

Kurita-Ochiai T, Fukushima K, Ochiai K. (1999). Lipopolysaccharide stimulates butyric acid-induced apoptosis in human peripheral blood mononuclear cells. *Infect Immun* 67: 22-29.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan, CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli, C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 165-176.

Lappin DF, MacLeod, CP, Kerr A, Mitchell T, Kinane DF. (2001). Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clin Exp Immunol* 123: 294-300.

Liu D, Xu JK, Figliomeni L, Huang L, Pavlos NJ, Rogers M, Tan A, Price P, Zheng MH. (2003). Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction. *Int J Mol Med* 11: 17-21.

Lu HK, Chen YL, Chang HC, Li, CL, Kuo MY. (2006). Identification of the osteoprotegerin/receptor activator of nuclear factor-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis. *J Periodontal Res* 41: 354-360.

Lynch, CN, Wang YC, Lund JK, Chen YW, Leal JA, and Wiley SR. (1999). TWEAK induces angiogenesis and proliferation of endothelial cells. *J Biol Chem* 274: 8455-8459.

Maecker H, Varfolomeev E, Kischkel, F, Lawrence D, LeBlanc H, Lee W, Hurst S, Danilenko D, Li J, Filvaroff E, Yang B, Daniel D, Ashkenazi A. (2005). TWEAK attenuates the transition from innate to adaptive immunity. *Cell* 123: 931-944.

Marsters SA, Sheridan JP, Pitti RM, Brush J, Goddard A, and Ashkenazi A. (1998). Identification of a ligand for the death-domain-containing receptor Apo3. *Curr Biol* 8: 525-528.

Mercado F, Marshall RI, Klestov AC, Bartold PM (2000). Is there a relationship between rheumatoid arthritis and periodontal disease? *J Clin Periodontol* 27:267-272.

Michaelson JS, Cho S, Browning B, Zheng TS, Lincecum JM, Wang MZ, Hsu YM Burkly LC. (2005). TWEAK induces mammary epithelial branching morphogenesis. *Oncogene* 24: 2613-2624.

Mogi M, Otogoto J, Ota N, Togari A. (2004). Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. *J Dent Res* 83: 166-169.

Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Yano K, Morinaga T, Higashio K. (1998). RANK is the essential signaling receptor for osteoclast differentiation factor in osteoclastogenesis. *Biochem Biophys Res Commun* 253: 395-400.

Nakayama M, Ishidoh K, Kayagaki N, Kojima Y, Yamaguchi N, Nakano H, Kominami E, Okumura K, Yagita H. (2002). Multiple pathways of TWEAK-induced cell death. *J Immunol* 168: 734-743.

Nakayama M, Ishidoh K, Kojima Y, Harada N, Kominami E, Okumura K, Yagita H. (2003). Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death. *J Immunol* 170: 341-348.

Nakayama M, Kayagaki N, Yamaguchi N, Okumura K, Yagita H. (2000). Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. *J Exp Med* 192: 1373-1380.

Nanci A, and Bosshardt DD. (2006). Structure of periodontal tissues in health and disease. *Periodontol 2000* 40: 11-28.

Ohguchi M, Ishisaki A, Okahashi N, Koide M, Koseki T, Yamato K, Noguchi T, Nishihara T. (1998). Actinobacillus actinomycetemcomitans toxin induces both cell cycle arrest in the G2/M phase and apoptosis. *Infect Immun* 66: 5980-5987.

Page RC, Kornman KS. (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 14: 9-11.

Page RC, Schroeder HE. (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest* 34: 235-249.

Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. (1997). Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol 2000* 14: 216-248.

Park MC, Jung SJ, Park YB, Lee SK. (2008). Relationship of serum TWEAK level to cytokine level, disease activity and response to anti-TNF treatment in patients with rheumatoid arthritis. *Scand J Rheumatol* 37: 173-178.

Perper SJ, Browning B, Burkly LC, Weng S, Gao, C, Giza K, Su L, Tarilonte L, Crowell T, Rajman L, Runkel L, Scott M, Atkins GJ, Findlay DM, Zheng TS, Hess H. (2006). TWEAK is a novel arthritogenic mediator. *J Immunol* 177: 2610-2620.

Pfizenmaier K, Wajant H, Grell M. (1996). Tumor necrosis factors in 1996. *Cytokine Growth Factor Rev* 7: 271-277.

Polek TC, Talpaz M, Darnay BG, Spivak-Kroizman T. (2003). TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TWEAKR. Evidence for a second TWEAK receptor. *J Biol Chem* 278: 32317-32323.

Potrovita I, Zhang W, Burkly L, Hahm K, Lincecum J, Wang MZ, Maurer MH, Rossner M, Schneider A, and Schwaninger M. (2004). Tumor necrosis factor-like weak inducer of apoptosis-induced neurodegeneration. *J Neurosci* 24: 8237-8244.

Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* 298: 597-600.

Ramamurthy NS, Greenwald RA, Celiker MY, Shi EY (2005). Experimental arthritis in rats induces biomarkers of periodontitis which are ameliorated by gene therapy with tissue inhibitor of metalloproteinases. *J Periodontol* 76:229-233.

Saas P, Boucraut J, Walker PR, Quiquerez AL, Billot M, Desplat-Jego S, Chicheportiche Y, Dietrich PY. (2000). TWEAK stimulation of astrocytes and the proinflammatory consequences. *Glia* 32: 102-107.

Saitoh T, Nakayama M, Nakano H, Yagita H, Yamamoto N, Yamaoka S. (2003). TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation. *J Biol Chem* 278: 36005-36012.

Schneider P, Schwenzer R, Haas E, Muhlenbeck, F, Schubert G, Scheurich P, Tschopp J, Wajant H. (1999). TWEAK can induce cell death via endogenous TNF and TNF receptor 1. *Eur J Immunol* 29: 1785-1792.

Semov A, Semova N, Lacelle, C, Marcotte R, Petroulakis E, Proestou G, Wang E. (2002). Alterations in TNF- and IL-related gene expression in space-flown WI38 human fibroblasts. *Faseb J* 16: 899-901.

Seymour GJ, Gemmell E, Reinhardt RA, Eastcott J, Taubman MA. (1993). Immunopathogenesis of chronic inflammatory periodontal disease: cellular and molecular mechanisms. *J Periodontal Res* 28: 478-486.

Stashenko P, Dewhirst, FE, Peros WJ, Kent RL, Ago JM. (1987). Synergistic interactions between interleukin 1, tumor necrosis factor and lymphotoxin in bone resorption. *J Immunol* 138: 1464-1468.

Tak PP, van der Lubbe PA, Cauli A, Daha MR, Smeets TJ, Kluin PM, Meinders AE, Yanni G, Panayi GS, Breedveld, FC. (1995). Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis. *Arthritis Rheum* 38: 1457-1465.

Trinchieri G, Wysocka M, D'Andrea A, Rengaraju M, Aste-Amezaga M, Kubin M, Valiante NM, Chehimi J. (1992). Natural killer cell stimulatory factor (NKSF) or interleukin-12 is a key regulator of immune response and inflammation. *Prog Growth Factor Res* 4: 355-368.

Van Dyke TE, Serhan, CN. (2003). Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. *J Dent Res* 82: 82-90.

Wara-aswapati N, Surarit R, Chayasadom A, Boch JA and Pitiphat W. (2007). RANKL upregulation associated with periodontitis and Porphyromonas gingivalis. *J Periodontol* 78: 1062-1069.

Wheater, PR, Burkitt HG, Daniels VG. (1987) Functional histology. A text and colour atlas. Churchill Livingston

Wiley SR, Cassiano L, Lofton T, Davis-Smith T, Winkles JA, Lindner V, Liu H, Daniel TO, Smith, CA, and Fanslow WC. (2001). A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 15: 837-846.

Wilson, CA, and Browning JL. (2002). Death of HT29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis. *Cell Death Differ* 9: 1321-1333.

Winkles JA, (2008). The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov* 7: 411-425.

Xu H, Okamoto A, Ichikawa J, Ando T, Tasaka K, Masuyama K, Ogawa H, Yagita H, Okumura K, Nakao A, (2004). TWEAK/Fn14 interaction stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF. *Biochem Biophys Res Commun* 318: 422-427.

Yamazaki K, Nakajima T, Kubota Y, Gemmell E, Seymour GJ. and Hara K. (1997). Cytokine messenger RNA expression in chronic inflammatory periodontal disease. *Oral Microbiol Immunol* 12: 281-287.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 95: 3597-3602.

Yepes M, Winkles JA, (2006). Inhibition of TWEAK activity as a new treatment for inflammatory and degenerative diseases. *Drug News Perspect* 19: 589-595.

Zhao, Z, Burkly LC, Campbell S, Schwartz N, Molano A, Choudhury A, Eisenberg RA, Michaelson JS, Putterman, C. (2007). TWEAK/Fn14 interactions are instrumental in the pathogenesis of nephritis in the chronic graft-versus-host model of systemic lupus erythematosus. *J Immunol* 179: 7949-7958.

Zheng TS, Burkly LC. (2008). No end in site: TWEAK/Fn14 activation and autoimmunity associated- end-organ pathologies. *J Leukoc Biol*, May 15 (Epub ahead of print)

2.9 Appendix 1 - Tables

NPar Tests
Mann-Whitney Test

Ranks

	diagnosis	N	Mean Rank	Sum of Ranks
SQA TWEAK	periodontitis	26	19.58	509.00
	normal control	7	7.43	52.00
	Total	33		

Test Statistics[b]

	SQA TWEAK
Mann-Whitney U	24.000
Wilcoxon W	52.000
Z	-3.109
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.002[a]

a. Not corrected for ties.

b. Grouping Variable: diagnosis

Explore
diagnosis

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	diagnosis	N	Percent	N	Percent	N	Percent
SQA TWEAK	periodontitis	26	96.3%	1	3.7%	27	100.0%
	normal control	7	100.0%	0	.0%	7	100.0%

Descriptives

	diagnosis			Statistic	Std. Error
SQA TWEAK	periodontitis	Mean		2.27	.245
		95% Confidence Interval for Mean	Lower Bound	1.76	
			Upper Bound	2.77	
		5% Trimmed Mean		2.30	
		Median		3.00	
		Variance		1.565	
		Std. Deviation		1.251	
		Minimum		0	
		Maximum		4	
		Range		4	
		Interquartile Range		2.00	
		Skewness		-.821	.456
		Kurtosis		-.575	.887
	normal control	Mean		.43	.297
		95% Confidence Interval for Mean	Lower Bound	-.30	
			Upper Bound	1.16	
		5% Trimmed Mean		.37	
		Median		.00	
		Variance		.619	
		Std. Deviation		.787	
		Minimum		0	
		Maximum		2	
		Range		2	
		Interquartile Range		1.00	
		Skewness		1.760	.794
		Kurtosis		2.361	1.587

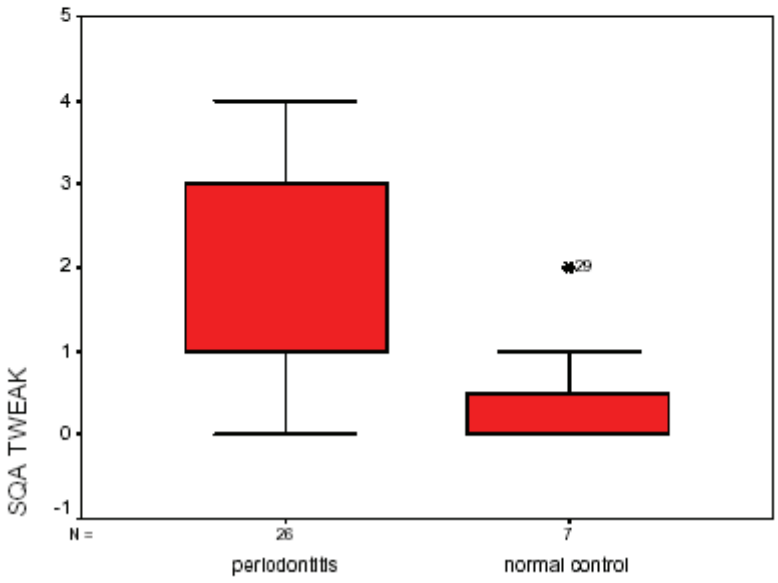
SQA TWEAK

Stem-and-Leaf Plots

SQA TWEAK Stem-and-Leaf Plot for
DIAGNOSI= periodontitis

```
 Frequency     Stem &  Leaf

     4.00        0 .  0000
      .00        0 .
     3.00        1 .  000
      .00        1 .
     3.00        2 .  000
      .00        2 .
    14.00        3 .  00000000000000
      .00        3 .
     2.00        4 .  00

 Stem width:          1
 Each leaf:       1 case(s)
```

```
SQA TWEAK Stem-and-Leaf Plot for
DIAGNOSI= normal control

 Frequency    Stem &  Leaf

     5.00        0 .  00000
      .00        0 .
     1.00        1 .  0
     1.00 Extremes    (>=2.0)

 Stem width:         1
 Each leaf:       1 case(s)
```



diagnosis

Explore
diagnosis

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	diagnosis	N	Percent	N	Percent	N	Percent
FN14SQA	periodontitis	27	100.0%	0	.0%	27	100.0%
	normal control	7	100.0%	0	.0%	7	100.0%

Descriptives

	diagnosis			Statistic	Std. Error
FN14SQA	periodontitis	Mean		2.48	.247
		95% Confidence Interval for Mean	Lower Bound	1.97	
			Upper Bound	2.99	
		5% Trimmed Mean		2.53	
		Median		3.00	
		Variance		1.644	
		Std. Deviation		1.282	
		Minimum		0	
		Maximum		4	
		Range		4	
		Interquartile Range		3.00	
		Skewness		-.429	.448
		Kurtosis		-.905	.872
	normal control	Mean		1.00	.436
		95% Confidence Interval for Mean	Lower Bound	-.07	
			Upper Bound	2.07	
		5% Trimmed Mean		.94	
		Median		1.00	
		Variance		1.333	
		Std. Deviation		1.155	
		Minimum		0	
		Maximum		3	
		Range		3	
		Interquartile Range		2.00	
		Skewness		.909	.794
		Kurtosis		-.150	1.587

FN14SQA

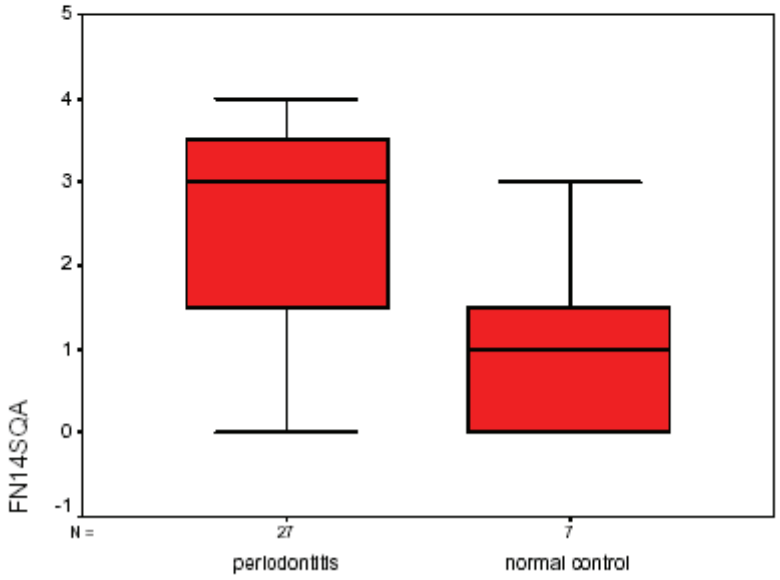
Stem-and-Leaf Plots

```
FN14SQA Stem-and-Leaf Plot for
DIAGNOSI= periodontitis

 Frequency    Stem &  Leaf

     2.00        0 .  00
      .00        0 .
     5.00        1 .  00000
      .00        1 .
     5.00        2 .  00000
      .00        2 .
     8.00        3 .  00000000
      .00        3 .
     7.00        4 .  0000000

 Stem width:         1
 Each leaf:       1 case(s)
```

FN14SQA Stem-and-Leaf Plot for
DIAGNOSI= normal control

Frequency	Stem &	Leaf
3.00	0 .	000
2.00	1 .	00
1.00	2 .	0
1.00	3 .	0

Stem width: 1
Each leaf: 1 case(s)



diagnosis

NPar Tests

Mann-Whitney Test

Ranks

	diagnosis	N	Mean Rank	Sum of Ranks
FN14SQA	periodontitis	27	19.61	529.50
	normal control	7	9.36	65.50
	Total	34		

Test Statistics[b]

	FN14SQA
Mann-Whitney U	37.500
Wilcoxon W	65.500
Z	-2.483
Asymp. Sig. (2-tailed)	.013
Exact Sig. [2*(1-tailed Sig.)]	.013[a]

a. Not corrected for ties.

b. Grouping Variable: diagnosis

Correlations

Correlations

		FN14SQA	SQA TWEAK
FN14SQA	Pearson Correlation	1	.511**
	Sig. (2-tailed)	.	.002
	N	34	33
SQA TWEAK	Pearson Correlation	.511**	1
	Sig. (2-tailed)	.002	.
	N	33	33

**. Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Correlations

Correlations

			FN14SQA	SQA TWEAK
Kendall's tau_b	FN14SQA	Correlation Coefficient	1.000	.395**
		Sig. (2-tailed)	.	.007
		N	34	33
	SQA TWEAK	Correlation Coefficient	.395**	1.000
		Sig. (2-tailed)	.007	.
		N	33	33

**. Correlation is significant at the .01 level (2-tailed).

Correlations

Correlations

		inflammatory score	FN14SQA
inflammatory score	Pearson Correlation	1	.546**
	Sig. (2-tailed)	.	.003
	N	27	27
FN14SQA	Pearson Correlation	.546**	1
	Sig. (2-tailed)	.003	.
	N	27	34

**. Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Correlations

Correlations

		FN14SQA	inflammatory score
FN14SQA	Pearson Correlation	1	.649**
	Sig. (2-tailed)	.	.000
	N	34	34
inflammatory score	Pearson Correlation	.649**	1
	Sig. (2-tailed)	.000	.
	N	34	35

**. Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Correlations

Correlations

			FN14SQA	inflammatory score
Kendall's tau_b	FN14SQA	Correlation Coefficient	1.000	.530**
		Sig. (2-tailed)	.	.000
		N	34	34
	inflammatory score	Correlation Coefficient	.530**	1.000
		Sig. (2-tailed)	.000	.
		N	34	35

**. Correlation is significant at the .01 level (2-tailed).

Correlations

Correlations

		inflammatory score	SQA TWEAK
inflammatory score	Pearson Correlation	1	.580**
	Sig. (2-tailed)	.	.000
	N	35	33
SQA TWEAK	Pearson Correlation	.580**	1
	Sig. (2-tailed)	.000	.
	N	33	33

**. Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Correlations

Correlations

			inflammatory score	SQA TWEAK
Kendall's tau_b	inflammatory score	Correlation Coefficient	1.000	.494**
		Sig. (2-tailed)	.	.001
		N	35	33
	SQA TWEAK	Correlation Coefficient	.494**	1.000
		Sig. (2-tailed)	.001	.
		N	33	33

**. Correlation is significant at the .01 level (2-tailed).

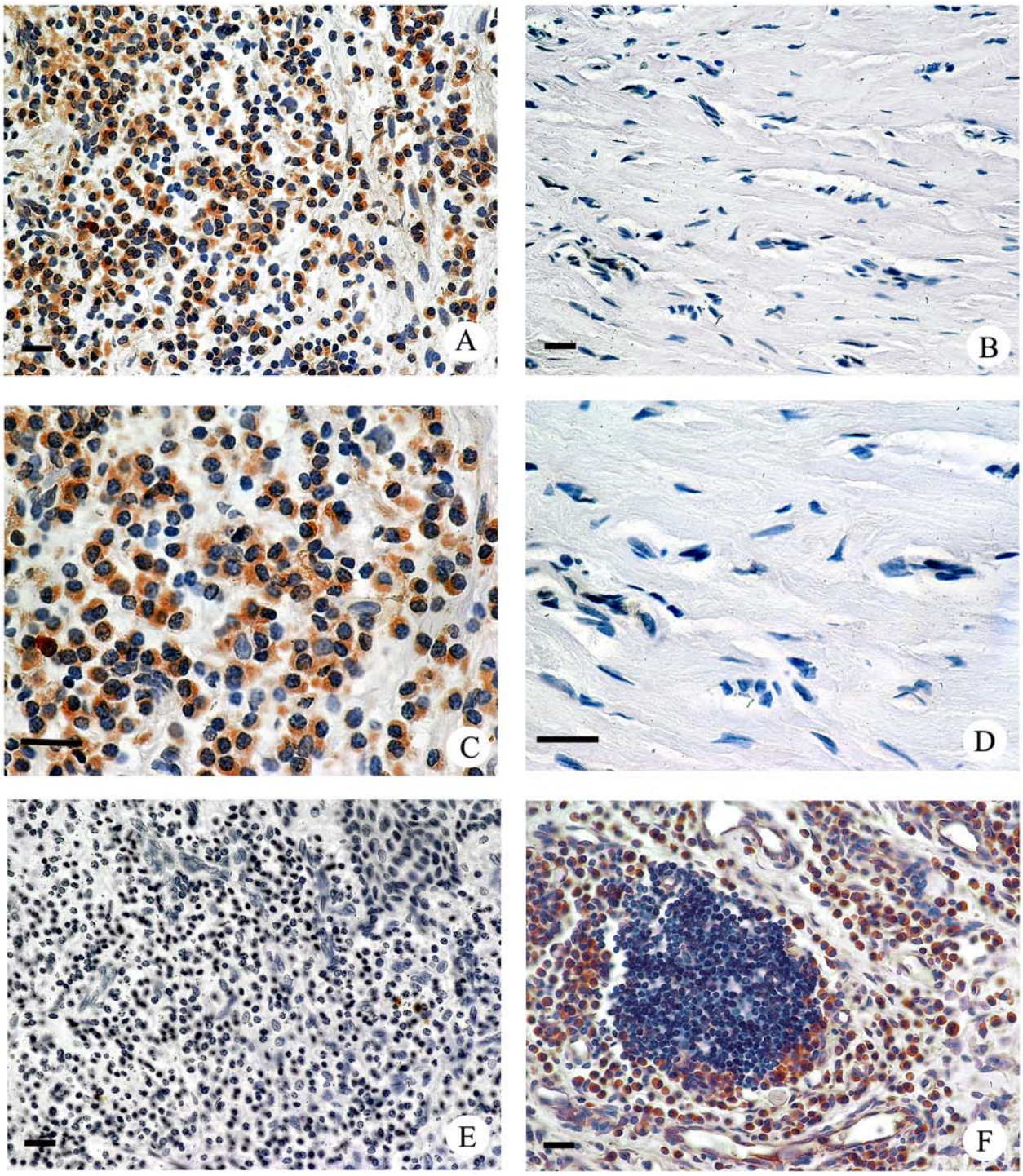


Fig 2.1: A- TWEAK in periodontitis tissue (200 times magnification), B- TWEAK in normal samples (200 times magnification), C-TWEAK in periodontitis tissue (400 times magnification), D- TWEAK in in normal samples (400 times magnification), E- negative control in periodontitis tissue (200 times magnification), F- positive control, TWEAK in Rheumatoid Arthritis tissue (200 times magnification)
Bar = 25 microns

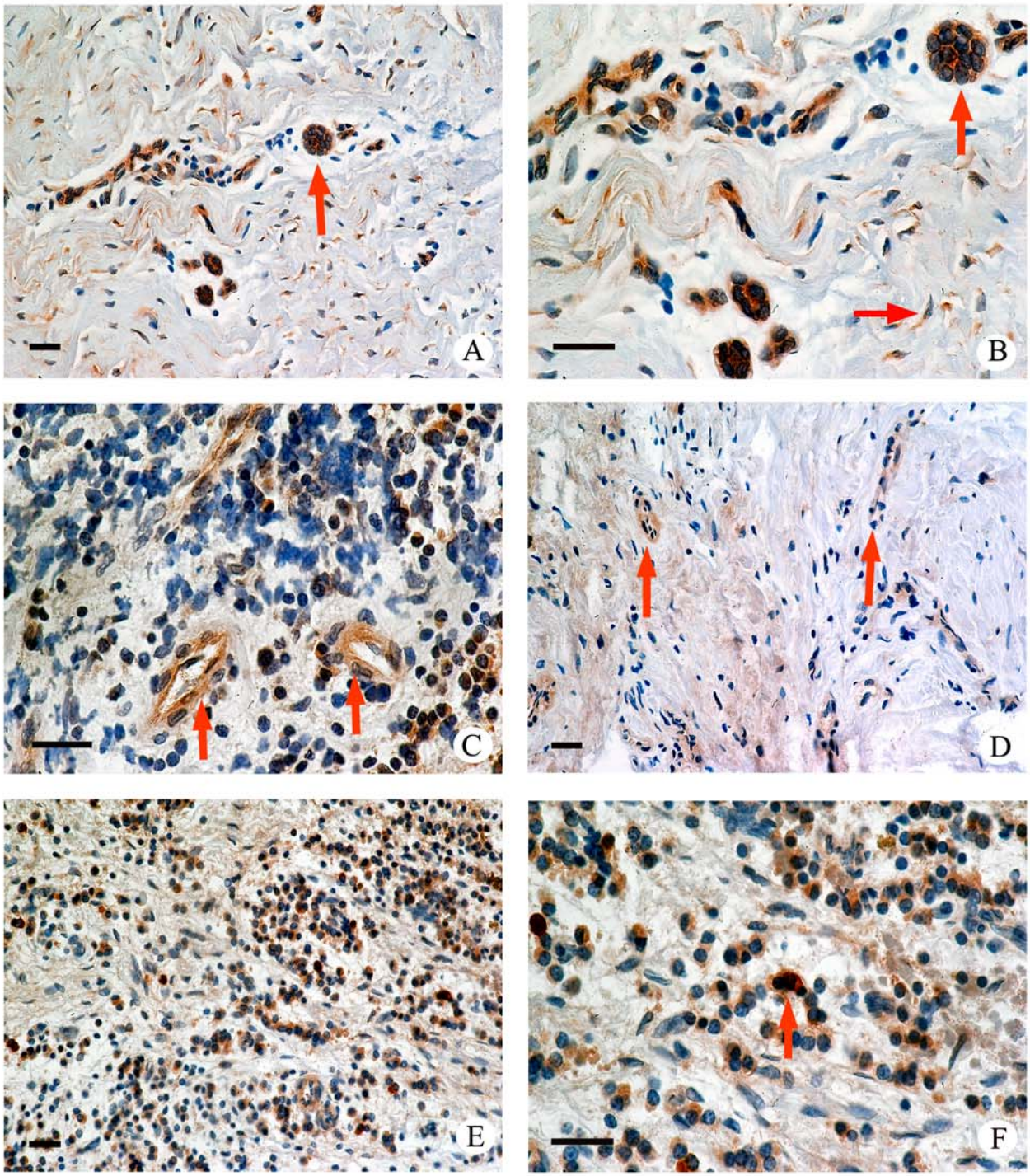


Fig 2.2: A - TWEAK expression in multinucleated cells (MNC), spindle shaped cells in periodontitis tissue (200 times magnification), arrowhead indicates positively stained MNC, B- TWEAK expression in MNC, spindle shaped cells in periodontitis tissue (400 X magnification) indicated by arrowheads, C- TWEAK expression in blood vessels in periodontitis tissue (400 times magnification) indicated by arrowheads, D - TWEAK in blood vessels of normal samples (200 times magnification) indicated by arrowheads, E- bright cell staining with TWEAK antibody in periodontitis tissue (200 times magnifiation), F- bright cell staining with TWEAK antibody in periodontitis tissue (400 times magnifiaction) indicated by arrowhead; Bar = 25 microns.

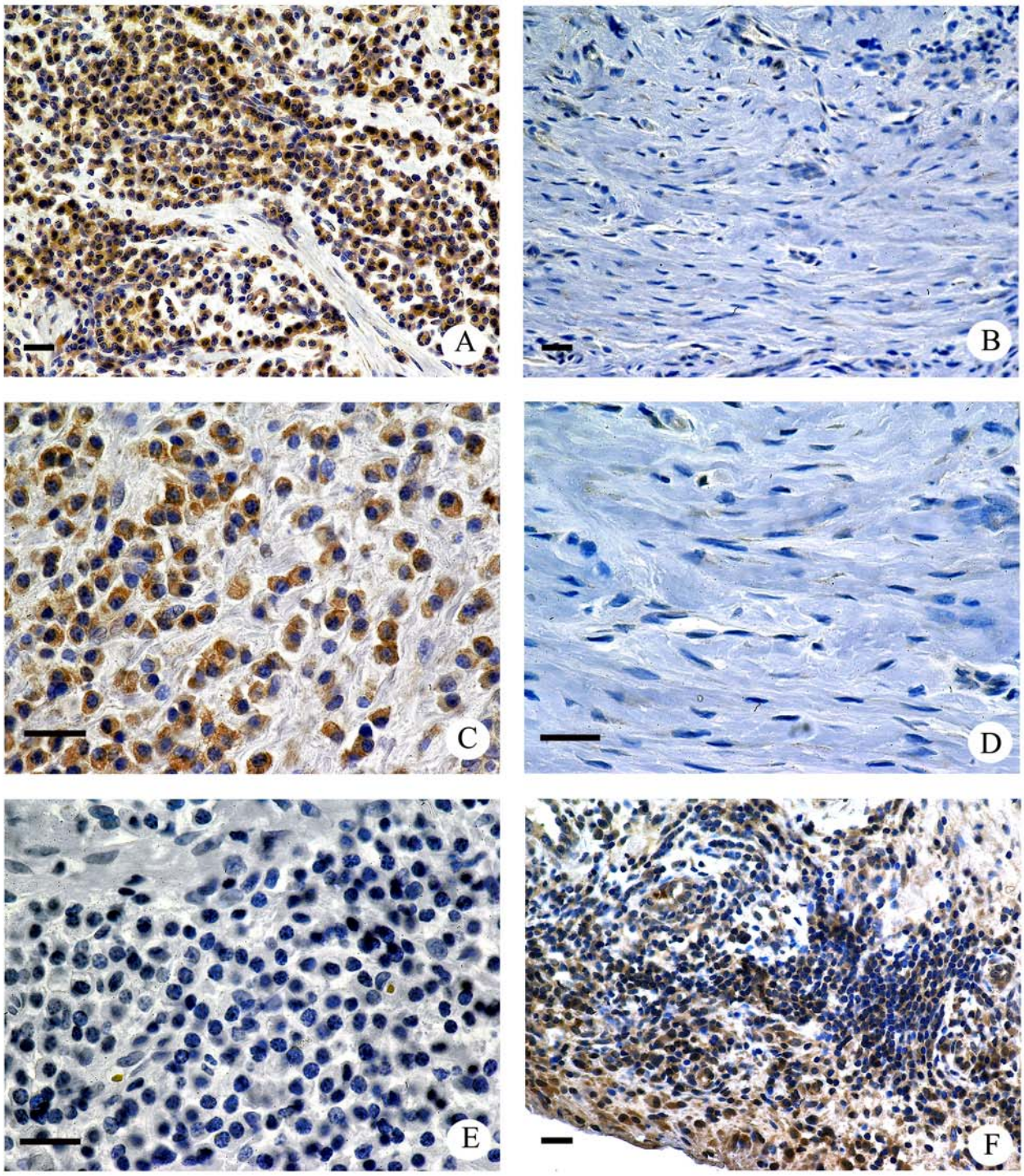


Fig 2.3: A- Fn14 in periodontitis tissue (200 times magnification), B- Fn14 in normal samples (200 times magnification), C- Fn14 in periodontitis tissue (400 times magnification), D- Fn14 in normal samples (400 times magnification), E- negative control in periodontitis tissue (400 times magnification), F- positive control, Fn14 in rheumatoid arthritis tissue (200 times magnification)
Bar = 25 microns

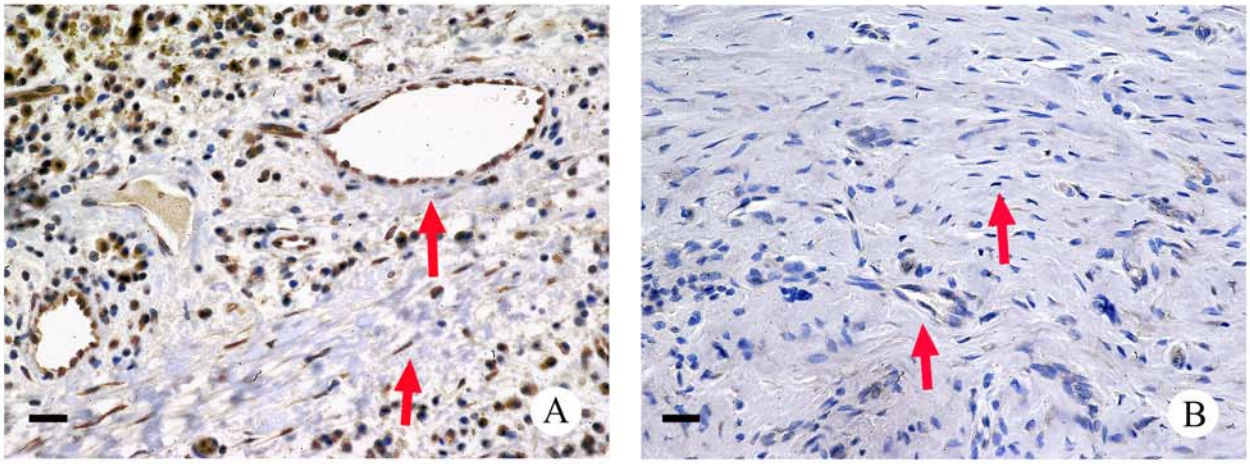


Fig 2.4: A- Fn14 expression in cells lining blood vessels and spindle shaped cells in periodontitis tissue (200 times magnification) as indicated by arrownheads, B- Fn14 in cells lining blood vessels and spindle shaped cells of normal samples (200 times magnification) as indicated by arrowheads; Bar = 25 microns