CELLULAR REGULATION OF INFLAMMATORY PROCESSES IN PERIODONTAL DISEASE CONTROLLED BY THE αvβ6 INTEGRIN, A JUNCTIONAL EPITHELIUM CELL RECEPTOR

By

DANIELA NICA

BSc, The University of British Columbia

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ABSTRACT

Periodontitis is an inflammatory disease caused by a combination of specific bacteria, host susceptibility and environmental factors. The sulcular and junctional epithelia act as the barrier for the plaque biofilm. Increased anaerobic pathogens are potent virulent factors that interact with the gingival epithelium inducing inflammatory cytokines including TGFβ. As an immunosuppressive cytokine, TGFβ acts as a growth inhibitor of the junctional epithelium. The latent form of TGFβ can be activated by αvβ6 integrin, a keratinocyte cell receptor found in the junctional epithelium (JE). In this study we have used a rat model of periodontitis induced by LPS and blocking antibodies for αvβ6 integrin to manipulate the function of this integrin in order to observe its role in inflammation. Pocket epithelium formation was also investigated in a knock out (KO) mouse models for β6, TSP-1 and for both β6 and TSP-1.

In the rat model, 24 animals were divided in four groups that were treated for 8 weeks. The experimental groups (n=6) were treated with blocking (6.3G9) or non-blocking (7.8B3) anti-β6 antibody and LPS. The control group was treated with pyrogen free water and one group was treated with LPS alone. Measurements of the junctional epithelium migration for area and length below the cementum enamel junction (CEJ) were quantified and compared statistically. Our findings showed that there was significant differences in JE migration between all rat treatment groups (ANOVA and Tukey test, $p<0.05$). Also inflammatory cellular infiltration was evaluated and was statistically different between the 6.3G9+LPS group and 7.8B3+LPS group and between

the 6.3G9+LPS group and LPS group alone($p<0.05$ ANOVA) and not statistically different between LPS and 7.8B3+LPS group ($p>0.05$ unpaired t-Test).

In the second model histological analysis was carried out for the knock out mice. In the murine KO model JE area and length of migration bellow the CEJ was found statistically different between the three different KO strains of mouse (ANOVA, $p<0.05$). The Tukey test found that the difference between the double KO and β6 KO was not significant for area only.

We concluded that αvβ6 integrin plays an important role in inflammation possibly through activation of TGFβ.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

$\alpha v\beta 6$	Alpha v beta 6
$\beta 6$-/-	Beta 6 Knock Out
CAV 9	Coxsackievirus A9
CEJ	Cementum Enamel Junction
CT	Connective Tissue
DAT	Directly Attached to the Tooth
ECM	Extra Cellular Matrix
EBL	External Basal Lamina
FMDV	Foot-and-Mouth Disease Virus
fMLP(fMetLeuPhe)	Formyl peptide
ICAM	Intercellular Adhesion Molecule
IBL	Internal Basal Lamina
IFN-γ	Interferon gamma

ABBREVIATIONS

IL	Interleukin
JE	Junctional Epithelium

LAP	Latency-Associated Protein
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharide
LTBP	Latent TGFβ-Binding Protein
MMP	Matrix Metalloproteinases
PD	Periodontal disease
PDL	Periodontal Ligament
PE	Pocket Epithelium
PKC	Proteine Kinase C
PMN	Polymorphonuclear Leukocyte
RGD	Arg-Gly-Asp
ROI	Reactive Oxygen Intermediates
SCC	Squamous Cell Carcinoma
TGFβ	Transforming Growth Factor Beta
Th1/2	T helper cell 1/2
TNF	Tumor Necrosis Factor
TSF	Transseptal Fibers
TSP	Thrombospondin
TSP1 KO	Thrombospondin 1 Knock Out
VCAM	Vascular Cell Adhesion Molecule

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DEDICATION

This work is dedicated to my children Emilia Ana, Timothy Jonathan Nica and Julia Maria. Their understanding and support was essential for the completion of my graduate studies. I hope this will inspire them to achieve higher education and be successful in their careers. I am also dedicating this work to my mother Maria Costea who has helped me in my work and to my grandmother who suffered from rheumatism and periodontal disease and wished that someone in the family will have an impact in the medical world. I love you all and I am especially grateful to have you in my life.

INTRODUCTION

Integrin αvβ6 is present in moderate levels in healthy junctional epithelium and in pocket epithelium (Johnson et al., personal communication) as well as throughout the epithelium of the interdental papilla (Csiszar et al., 2007). Multiple functions have been attributed to an increased expression of αvβ6 integrin including promotion of cell migration, control of cell proliferation, inflammation, activation of TGFβ, suppression of apoptosis, modulation of protease activity and mediation of invasion of carcinoma cells (Thomas et al., 2006). An αvβ6 knockout mice study found that the absence of the integrin caused development of exaggerated inflammation and protected the mice from experimental pulmonary fibrosis, due to impaired TGF-β activation (Munger et al., 1999). Also β6-overexpressing transgenic mice had wounds that presented different types of chronic scars that were associated with increased levels of TGF-β (Häkkinen et al., 2004).

As a cell membrane receptor, integrin αvβ6 recognizes the RGD sequence of latency-associated protein LAP and after a conformational change the activation of TGFβ occurs (Keski-Oja et al., 2004). Fibronectin, an abundant ECM glycoprotein, and a ligand for integrin αvβ6 is required to mediate the activation of latent TGFβ complexes contained in the latent TGFβ-binding protein LTBP-1 that is concentrated in the ECM (Fontana et al., 2005). Activation of TGFβ can also be

modulated by other molecules and one of them is thrombospondin-1 (TSP-1), that could also regulate inflammation. TSP1-KO mice present epithelial hyperplasia, leukocyte infiltration, and acute and chronic inflammation in the lungs (Lawler, et al., 1998), as well as milder inflammatory changes in the pancreas (Crawford et al., 1998). Transforming growth factor β (TGFβ), an immunosuppressive cytokine, was shown to act as a growth inhibitor of the junctional epithelium (presenting less sensitivity to this cytokine than oral gingival epithelium) (Lu H et al., 1997). Therefore the absence of the cytokine would results in epithelium growth and possibly increasing inflammation. Chronic inflammation, as seen in periodontitis leads to connective tissue degradation and bone loss. Therefore manipulation of αvβ6 integrin could be an important therapeutic tool in order to control the inflammatory processes in periodontitis.

CHAPTER I: Review of the Literature

1.1 Periodontal disease (PD)

1.1.1 Periodontal Disease classification

Periodontal disease (PD) is an inflammatory disease comprising a group of inflammatory conditions in the supporting tissue around the teeth, caused by a combination of specific bacteria, host susceptibility and environmental factors. There are over forty different gingival diseases classified as periodontal disease(s) or conditions. However, periodontal disease has been

traditionally been subdivided into two clinical conditions; gingivitis and periodontitis. Gingivitis, inflammation of the gingiva, can be plaque or non-plaque induced and fails to generate any significant connective tissue loss. Periodontitis, however, involves gingival inflammation at areas of apical migration of epithelial attachment at root surfaces with concurrent loss of connective tissue and alveolar bone. Periodontitis is classified in four major types: *Chronic Periodontitis* with either a localized or generalized form, *Aggressive Periodontitis* that can also be localized or generalized, *Necrotizing Ulcerative Gingivitis* and *Periodontitis as a manifestation of systemic diseases* with a provenance of hematologic or genetic disorders (Carranza et al., 2002).

1.1.2 Etiology of Periodontal Disease(s)

It has been demonstrated that there are at least 500 different bacteria taxa found in sub-gingival plaque (either by cultivation or by 16S ribotyping) with a possible 300 unknown species. Periodontopathogens were identified early by using a comparative technique; comparing bacterial composition of subgingival plaque from healthy and diseased sites. It is now generally agreed that periodontitis is not associated with a single bacterium and that the disease can be considered as a polyspecies outgrowth leading to a pathogenic subgingival biofilm. Such an outgrowth occurs as a result of a change in the microenvironment of bacterial plaque providing a more hospitable ecological niche for the colonization of periodontopathogens after horizontal

transfer of these bacteria. As periodonto-pathogens increase in the plaque, the microenvironment of the community changes from facultative to obligate anaerobic (Haffajee & Socransky, 1994).

The consensus report of the World Workshop on Clinical Periodontics concluded that three bacterial species, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Tannerella forsythia should be considered as the major periodontal pathogens (Anonymis, 1996). However, other gram negative species have been described to be present in advanced forms of chronic periodontitis such as Treponema denticola (Socransky et al., 1998). These bacteria are associated agents for periodontitis due to their capacity to increase the virulence of the plaque through structural and/or secreted virulence factors (endotoxin, exotoxins, and tissue destroying enzymes).

1.1.3 Immunological Involvement in Periodontal Disease

The sulcular and junctional epithelia act as the barrier for the plaque biofilm. With a change in the microflora (towards anaerobes) virulence factors interact with the gingival epithelium inducing proinflammatory cytokines and chemokines. With time these virulence factors ultimately cause cellular damage. Once the junctional epithelium is breached, blood vessels underlying the epithelium become more permeable and express adhesion molecules, thus

allowing adherence of leukocytes in the areas of tissue damage (Pöllänen et al., 2003). A gradient of chemoattractant signals derived from both the endothelium and/or bacterial products (fMLP) allows leukocyte extravasation and provides a navigational system for the leukocytes in the gingival tissues. In this way neutrophils migrate through the junctional epithelium and into the gingival sulcus. The action of these phagocytic cells constitutes the first line of defense against bacterial infection.

Neutrophils, primary effector cells in the innate immune response, have been described as playing a major role in periodontal disease that is both protective and destructive (Dennison and Van Dyke, 1997). Their protective function was observed in individuals who have neutrophil disorders such as cyclic neutropenia (Cohen and Morris, 1961), Chédiak-Higashi syndrome (Hamilton and Giansanti, 1974), and leukocyte adhesion deficiency syndrome (Springer et al., 1984; Waldrop et al., 1987) which have an increased susceptibility to periodontal destruction. Neutrophils form a barrier between the epithelium and plaque limiting bacterial invasion of the epithelium and underlying connective tissue (Hemmerle & Frank 1991). The neutrophils minimize the destructive effects of plaque bacteria through opsonization and production of reactive oxygen intermediates (ROI) and release of compounds found within their lysosomal

granules during their response to bacterial challenge (Altman et al., 1992). Unfortunately, the above products of these activated neutrophils can also cause tissue damage.

Neutrophils activated by phagocytosis of bacteria generate a number of ROIs, O_2-, HOCl, O., hydrogen peroxide (H_2O_2), and NO, with the potential to destroy bacteria and gingival cells (Pöllänen et al., 2003). They also have two main types of secretory granules that contain agents with bactericidal activity: the azurophilic (primary) granules containing myeloperoxidase, lysosome, elastase, cathepsin G, urokinase, acid hydrolases, and defensins, while the specific (secondary) granules contain lactoferrin, elastase and lysozyme (Dennison and Van Dyke, 1997).

If the early inflammation is not controlled by the neutrophil response and bacterial products persist, a secondary inflammatory infiltrate in the connective tissue will accumulate and predominate. This infiltrate of lymphocytes (T-cells) and macrophages accumulates in the proximity of the junctional epithelium. Macrophages are important mediators of inflammation in the connective tissue infiltrate, where they produce several inflammatory cytokines and as well as present antigens to T-cells to initiate the adaptive immune response (Dennison and Van Dyke, 1997). Activated macrophages are essential for innate resistance to intracellular infection in that they produce pro-inflammatory cytokines that enhance phagocytosis and elimination of the

pathogen (Trinchieri, 1997). Macrophages are also involved in the activation of the monocyte/lymphocyte axis triggering the adaptive immune response and generating primed T and B effector cells.

Thus, the effector mechanisms of innate and adaptive immune responses are inter-dependent (Trinchieri, 1997). Cells of the innate response influence T-cell differentiation by controlling the cytokine milieu in the tissues or draining lymph nodes where antigen-specific T-cells expand in response to antigen presentation. A high level of IL-12 during T-cell expansion will influence Th1 differentiation, while IL-4 is necessary for Th2 cells (Mosmann and Coffman, 1989). In susceptible individuals this adaptive response does not contain enough microbial challenge and the need of bacterial clearance is consequently leading to a change in the nature of the inflammatory response relying on B-cells and plasma cells. This step results in either protective antibody production and subsequent control of the infection (Th2 response) or non-protective antibodies and connective tissue destruction and bone loss (Th1 response). The ability of the innate immune system (neutrophils and macrophages) to regulate the adaptive immune response via the production of cytokines such as the IL-1 receptor antagonist, IL-4 and IL-12 is being recognized. A strong innate immune response may, on the one hand, clear the periodontopathic

bacteria and, on the other, determine the nature of the lymphocytic response, which may subsequently result in a stable or progressive form of the disease (Gemmell et al., 2002).

1.1.4 Periodontal disease in rodents

Studies utilizing various animal species have been used to obtain valuable information about periodontal disease. These studies of periodontal disease involved diverse subjects such as non-human primates (Holt et al., 1988), sheep (Ismaiel et al., 1989), dogs (Renvert et al., 1996), hamsters (Makris and Saffer, 1986), mice (Shapiro et al., 1960) and rats (Alencar et. al., 2002). Normal oral structure and physiology and the pathogenesis of periodontal diseases have been studied more extensively in the rat than in any other rodent. Since the rat was widely used as an animal model in periodontal research, its anatomical and histological features for the normal periodontium, especially the dentogingival area and the physiological age-dependent changes in the molar regions are well documented. Although particular dental and periodontal tissue differs in size among various rat strains the tissue structure and growth are identical in all strains (Page and Hubert, 1982).

The structure of dentogingival area in rat, including the junctional epithelium and its attachment to the tooth surface, the configuration and topography of the epithelial tissue at the gingival

margin is very similar to that of man (Skillen, 1930; Listgarten, 1975; Eggert et al., 1980). There are two exceptions; first, the crevicular epithelium in rats is keratinized (Thilander, 1961) and second, in the area between oral gingival and junctional epithelium in rats, the gingival epithelium bends apically and joins the coronal portion of the junctional epithelium at the buccal or lingual aspects of the molars. This structural detail is important with respect to pocket formation in the rat gingival as it becomes displaced by an overgrowth of bacteria (König and Mühlemann, 1959). Moreover, the junctional epithelium is a site of entry for the pathogens and also for the pathway for inflammatory exudation in rat as in human.

Lipopolysaccharide (LPS) from E. coli has been previously used as an important factor for studying the initiation and progression of human periodontal diseases in vitro (Haffajee and Socransky, 1994). In vivo experiments have also used rat models in which topical application of LPS resulted in apical migration of junctional epithelium along the root surface (Suzumara et al., 1989, Iwamoto et al., 1998). Apical migration of junctional epithelium along the root surface is an important factor in periodontal pocket formation.

LPS treatment in conjunction with bacterial protease caused intraepithelial cleavage and increased B cell density in the tissues (Ekuni et al., 2003). Bacterial protease also has an effect

on junctional epithelial elongation by promoting apical proliferation of the cells (Suzumura et al., 1989). Following application of LPS and proteases to rat gingival sulci, the apical migration of the junctional epithelium appears to occur simultaneously with the apoptosis of the periodontal ligament (PDL) fibroblasts, which in turn follows proliferation of the basal cells and collagen destruction (Ekuni, et al., 2005). With respect to the etiology of periodontal disease, LPS from a number of different gram negative periodontal pathogens also increased epithelial cell growth indicating that these bacteria can also stimulate junctional epithelial proliferation, an initial step in periodontal disease.

1.2. Gingival Attachment Apparatus

1.2.1. Structure and function of gingival attachment apparatus

In the oral cavity, the attachment between the gingival epithelium and root surface is mediated by a unique epithelium called the junctional epithelium. The junctional epithelium is a stratified squamous non-keratinized epithelium, composed coronally of up to 15-30 cells (thick) with about 1-3 cell layers apically. Also associated with the junctional epithelium are infiltrated neutrophils and monocytes (Schroeder and Listgarten, 1971).

Cells of the junctional epithelium are specialized in attachment formation as well as being capable of movement and of positional change. This latter feature provides the junctional epithelium with the ability to advance and retract (Stern, 1981). Junctional epithelium develops when tooth erupts through the oral epithelium and fuses with the reduced enamel epithelium (Bartold et al., 2000).

Attachment of the junctional epithelium to the tooth is termed the epithelial attachment apparatus (Schroeder and Listgarten, 1971). The attachment is effected by both the hemidesmosomes found in the plasma membrane of the cells directly attached to the tooth (DAT) cells and the internal basal lamina (Pöllanen et al., 2003).

1.2.2. JE integrity and function of gingival attachment apparatus in disease

There are two strata that form the junctional epithelium, the basal layer facing the connective tissue and the suprabasal layer attaching to the tooth. Both layers have cells that exhibit rapid turnover. This feature is both essential for repair caused by mechanical damage and as well as forming a barrier against plaque bacteria (the rapid shedding as an effective removal of bacterial products (Pöllanen et al., 2003)).

Compared to sulcular epithelium, junctional epithelium has wider intercellular spaces. When mild inflammation occurs, in gingivitis for example, the junctional epithelium is the major path for the inflammatory exudates from adjacent blood vessels. Neutrophils and mononuclear cells (mainly lymphocytes) are found in the connective tissue in the proximity of the junctional epithelium. These cells can be passively carried into the sulcus as a result of junctional epithelium turnover, as well as in response to chemoattractant gradients provided by bacterial products. When more than 30% of neutrophils occupy the intercellular spaces, the junctional epithelium becomes more permeable with outward flow of gingival fluid and enhanced transmigration of leukocytes (Schroeder and Listgarten, 1971; Schroeder and Listgarten, 1997; and Bosshardt and Lang, 2005). Conversely, the above mentioned property can also be considered detrimental because it allows bacterial products to enter in the junctional epithelium and connective tissue (Swartz et al., 1972; Ijuhin, 1988).

1.2.2.1 Cell adhesion molecules

Transendothelial migration of leukocytes along the endothelium involves multiple steps. In a sequential order these include initiating rolling of leukocytes along the endothelium (mediated by the selectin family of adhesion molecules), firm adhesion of the leukocytes which results from

activation of leukocyte integrins, and finally the extravasation of the leukocytes through the endothelial intercellular junctions (Butcher, 1991).

Intercellular adhesion molecule-1 (ICAM-1) is a membrane bound glycoprotein which appears to be weakly expressed on resting lymphocytes, but strongly expressed on activated lymphocytes (Clark et al., 1986). ICAM-1 or CD54 is one of the ligands for lymphocyte function-associated Ag -1 (LFA-1) and CR3 that are leukocyte integrins. ICAM-1 and ICAM-2 are members of the Ig superfamily (Simmons et al., 1988) whose functions are two-fold. They mediate cell-to-cell interactions in inflammatory reactions as well as allow for the passage of leukocytes from the capillaries into the surrounding tissue (Boyd et al., 1988). As an example of these functions monoclonal antibodies for ICAM-1 block completely or partially the homotypic aggregation of lymphoblasts (Boyd et al., 1988), monocytes (Patarroyo et al., 1988), and cell lines of lymphoid and myeloid origin (Schultz et al., 1988; Boyd et al., 1989). The monoclonal antibody also blocks lymphocyte or neutrophil adhesion to culture endothelial cells (Dustin et al., 1988a), adhesion of T lymphoblast to epidermal keratinocytes (Dustinet al., 1988b) or fibroblasts (Dustin et al., 1996c), T cell- mediated cytotoxicity (Makgoba et al., 1988), and monocyte-dependent T cell stimulation (Dougherty et al., 1988; Neumayer et al., 1990). Interferon gamma (IFN-γ) and

tumor necrosis factor alpha (TNF-α) are shown to enhance ICAM-1 expression on endothelial cells, keratinocytes and fibroblasts (Dustin et al., 1998c).

1.2.2.2 Cytokines

LPS from gram-negative bacteria induces the synthesis of cytokines such as IL-1 and TNF-α (Lindemann et al. 1988; McFarllane et al. 1990) from macrophages, fibroblast and endothelial cells as well as IL-6 from neutrophils. These cytokines, as cell regulators play a central role in the pathogenesis of inflammatory periodontal diseases by further activating leukocytes and osteoclasts. In the junctional epithelium it has been shown that TNF-α, IL-1α and IL-1 β are expressed by cells of the superficial layers of the coronal half of the epithelium (Miyauchi et al., 2001). Transforming growth factor β (TGFβ) was shown to act as a growth inhibitor of the junctional epithelium (presenting less sensitivity to this cytokine than oral gingival epithelium) (Lu H et al., 1997).

TGF-β messenger RNA is synthesized in a variety of normal and transformed cells including: degranulated platelets, endothelial cells, fibroblasts, keratinocytes, tumor cells and T cells responding to antigen (Derynck et al., 1985). Activated macrophages can secrete TGF-β when

exposed to LPS (Assoian et al., 1987). Also TGF-β as an immunoregulator cytokine is a potent chemoattractant for human peripheral blood monocytes and induces monocyte migration in vitro thus providing an important signal for monocyte recruitment (Wahl et al., 1987).

TGF-β is found to be both an immunosuppressive and a potent pro-inflammatory cytokine. These opposing effects are controlled by the selective production, latency, and receptor modulation as well as the susceptibility of target cells at various stages of development, maturation and activation (Wahl, 1992). In rheumatoid arthritis, activated lymphocytes, macrophages, neutrophils and synovial fibroblasts are capable of producing TGF-β (Wahl et al., 1990). Also the cytokine production can be amplified via autocrine and paracrine circuits (Kim et al., 1990). In addition it can trigger a pro-inflammatory cascade by increasing mRNA levels for IL-1, TNF and IL-6 which in turn stimulate T cells to produce more TGF- β creating a positive feed back loop (Bristol et al., 1990, Wahl, 1992). In general immature cells are turned on by TGF- β to be involved in inflammation, whereas mature cells are suppressed and deactivated to favor resolution of inflammation (McCartney and Wahl, 1994). However locally delivered or endogenous TGF- β initiates and exacerbates an inflammatory response by promoting adherence, monocyte recruitment and activation of leukocytes (Allen et al.,1990). In

contrast when administered systemically, TGF- β acts as an immunosuppressant by interrupting cell adhesion and inhibiting cell proliferation (Brandes et al., 1991).

1.2.2.3. Proteases

In response to bacteria and inflammatory cytokines, junctional epithelial cells, macrophages and PMNs release proteinases that are involved in the defense against microbes (Pöllanen et al., 2003). Proteinases of host origin are involved in the degradation of extracellular components of connective tissue and epithelium including components of both the external basal lamina (basement membrane at the connective tissue–junctional epithelium interface) and the internal basal lamina at the epithelium–tooth interface. Therefore, these enzymes seem to have the potential to contribute to the lateral and apical proliferation of the junctional epithelium into the connective tissue (Pöllanen et al., 2003). Plasminogen activators (PA) are proteases that play a major role in ECM proteolysis by degrading matrix proteins and by activating matrix metalloproteinases (MMPs) (Skrzydlewska et al., 2005). MMPs (MMP1, MMP2, MMP7, MMP9 and MMP13) are expressed in situ by inflammatory cells i.e. monocytes, macrophages, lymphocytes, polymorphonuclear cells and residential cells i.e. fibroblasts, epithelial cells, and endothelial cells (Reynolds et al., 1994). One study showed that MMP2 and MMP9 are present

in health but increased levels of MMP9 and pro-MMP9 were expressed in mild inflammation while MMP2 was significantly much lower (Ejiel et al., 2003).

1.3. Integrins

1.3.1. Structure, function, types

Integrins are a large family of transmembrane glycoproteins consisting of noncovalent heterodimers that interact with a wide variety of ligands including extracellular matrix glycoproteins, complement and other cells, while their intracellular domains interact with the cell cytoskeleton (Hynes, 1987). 18 α and 8 β subunits have been identified that form 24 different heterodimer integrins (Fig 1; Hynes, 2002). Each subunit has a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain or tail (Fernandez et al., 1998). Integrins have binding sites for divalent cations Mg^{++} and Ca^{++} that are involved in ligand binding facilitating the process of cell adhesion and migration (Plow et al., 2000). Integrin binding to their ligands results in the formation of multi-protein assemblies composed of cytoskeletal and signaling molecules. These assemblies are regulated by the nature of integrin-ligand interactions, as well as by intracellular regulators that include tyrosine kinases and phosphatases, PKC, and small GTPases. Therefore integrin-mediated cellular physiological

responses include the activation of signal transduction, cytoskeletal rearrangements, and co-regulation of growth factor activities (Katz & Yamada, 1997).

Integrins bind to matrix proteins; fibronectin, vitronectin, collagen, laminin and fibrinogen (Häkkinen et al., 2000). Some integrins also recognize integral membrane proteins of the immunoglobulin superfamily (ICAM-1, ICAM-2, VCAM-1) and mediate direct cell-cell adhesion (Hynes, 1992). Integrins bind to the extracellular matrix glycoproteins by recognition of the Arg-Gly-Asp (RGD) motif (i.e. tenascin binding to $\alpha v\beta 6$ integrin (Häkkinen et al., 2002)). The RGD tripeptide sequence is found in such molecules as fibronectin, vitronectin and laminin (Figure 1; Hynes, 2002). In addition, in vertebrates, integrins have a set of receptors for collagens and other related molecules (Fig 1) as well a receptor for Ig-superfamily receptors like VCAM-1 (Hynes, 2002).

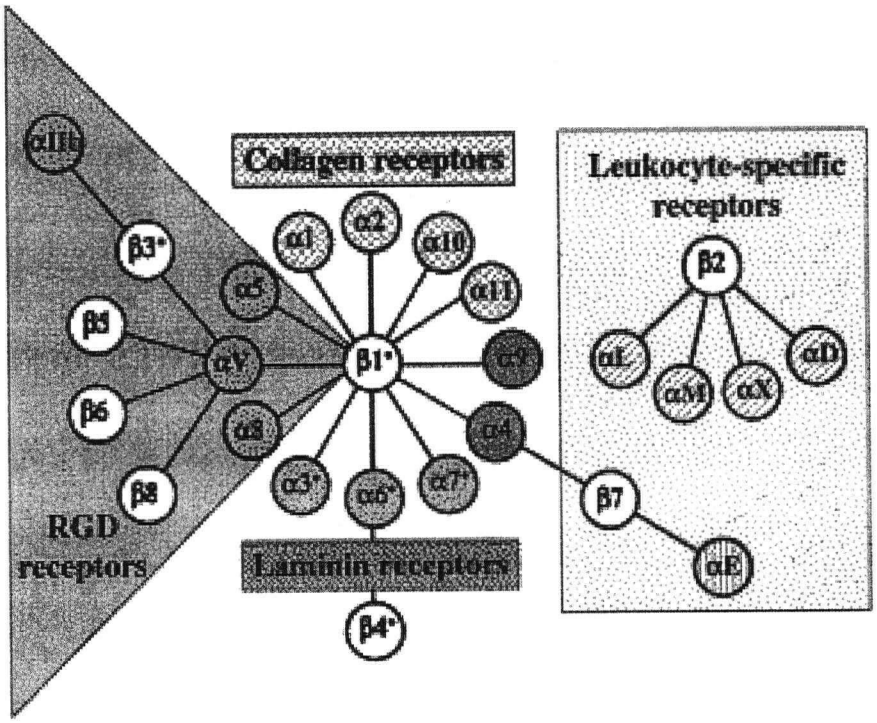


Figure 1. The Integrin Receptor Family: αβ heterodimers; each subunit crosses the membrane once, with most of each polypeptide (>1600 amino acids in total) in the extracellular space and two short cytoplasmic domains (20–50 amino acids). The figure depicts the mammalian subunits and their αβ associations; 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationships (coloring of α subunits), ligand specificity and, in the case of β2 and β7 integrins, restricted expression on white blood cells. α subunits with gray hatching or stippling have inserted I/A domains (see text). Such α subunits are restricted to chordates, as are α4 and α9 (green) and subunits β2-β8. In contrast, α subunits with specificity for laminins (purple) or RGD (blue) are found throughout the metazoa and are clearly ancient (see text). Asterisks denote alternatively spliced cytoplasmic domains. A few extracellular domains are also alternatively spliced (not shown).

Figure used with permission from publisher. Richard O. Hynes, review 2002. Integrins Bidirectional, Allosteric Signaling Machines.Cell: 110(6); 673-87

All 24 integrins have specific functions as determined by their receptor diversity as well as experiments using integrin knock out mice. Knocking out the genes for β subunits and all except four α subunits gave different phenotypes, reflecting the functional specificity of each integrin. The phenotypes range from a complete block in preimplantation development (β1), through major developmental defects (α4, α5, αv, β8), to perinatal lethality (α3, α6, α8, αv, β4, β8) and defects in leukocyte function (αL, αM, αE, β2, β7), inflammation (β6), hemostasis (αIIb, β3. α2), bone remodeling (β3), and angiogenesis (α1, β3) (Hynes 1996, 2002; DeArcangelis and George-Labouesse, 2000; Sheppard, 2000; Bouvard et al., 2001).

The common function of the integrin is to mediate cell-to-cell or cell-to-extracellular matrix (ECM) interactions of which the most important processes include: embryological development, hemostasis, thrombosis, wound healing, immune and nonimmune defense mechanisms, and oncogenic transformation (Hynes, 1987). Integrins provide adhesion for stationary cells, traction during cell movement and, the promotion of signaling pathways that regulate various processes including proliferation, migration, cell survival, differentiation, tumor invasion and metastasis (Hynes, 1992).

In gingiva, junctional epithelial cells express α6β4 that is a major constituent of the hemidesmosomes attaching the basal epithelial cells to the basement membrane (Stepp et al., 1990; Jones et al., 1991; Hormia et al., 1999). Hopkinson et al.(1995) demonstrated that α6β4 interacts with type XVII collagen that stabilizes the integrin complex of the hemidesmosome. Basal keratinocytes in the healthy junctional epithelium express a family of β1 integrins that have a role in cell-cell contact in the basal keratinocytes (Larjava et al., 1990).

The α2β1, α3β1 integrins are present in the stationary epithelium and are found in the basal epithelial cells in healthy oral mucosa and gingiva. The α2β1 binds collagen, laminin and tenascin and α3β1 binds collagen, laminin and fibronectin (Larjava et al., 1996). In normal JE α5β1, a classical fibronectin receptor is absent while α8β1, also a fibronectin receptor, is present in the basal keratinocytes together with α9β1 that binds to tenacin (Bossy et al., 1991).

In pocket epithelium integrin expression is variable, where focal losses of α2β1, α3β1 were found in most areas. In other areas the entire pocket epithelium was found to be strongly positive for β1 integrins, and α6β4 was expressed weak in epithelium associated with chronic inflammation (Haapasalmi et al., 1995). Also Del Castillo et al. (1996) investigated healthy gingiva (n=5) and gingival inflammation at different stages of periodontitis (n=18). In healthy

human gingiva α 2, α 3 and α 6 integrin chains were expressed and distributed characteristically on the basal cell layers and in some cells of the stratum spinosum, in the junctional and sulcular epithelium sites. Inflammatory stages of periodontitis revealed further upregulation of α 2, α 3 and α 6 integrins in the junctional and sulcular epithelial cells, which correlated with the stage of the periodontitis and the extent of the cellular infiltration. The α 4 and α 6 integrins were also found to be the predominant beta 1 integrin receptors on inflammatory cells. The amount of α 4 and α 6 positive cells correlated with the number of the inflammatory cells (Del Castillo et al.. 1996).

Johnson et al. (personal communication) investigated the expression of β1, β4, β6 and α5 integrins in periodontally "healthy" and periodontitis patients. In her immunohistochemical study, β1 and β4 are expressed in both health and periodontitis, in which intense staining is present in the keratinocytes facing the IBL and moderate staining in the basal epithelial cells facing the EBL as well as the suprabasal cells of the sulcular and oral epithelium. The apical portion of the sulcular and junctional epithelium was the most stained. Expression of β6 localized to the JE cells where the staining was more intense coronal to the most apical portion of the sulcular epithelium. The presence of α5 is observed in both healthy and periodontitis specimens in the basal epithelial cells of the JE facing the tooth (Johnson et al., unpublished). No

members of the alpha v integrin family were found in any pocket epithelia studied (Haapasalmi et al., 1995).

1.3.2. Integrin αvβ6

1.3.2.1. Localization and function

Complete amino acid sequence of a novel integrin beta subunit (beta 6) was identified using the polymerase chain reaction in primary cultures of airway epithelium (Shepard et al., 1990). In healthy adult primate tissues, β6 mRNA and protein are rarely detected in most organs with the exception of the endometrium and macula densa (Breuss et al., 1993).

Integrin αvβ6 is expressed in malignant colonic and oral epithelium (Jones et al., 1997), in wound healing (Busk et al., 1992, Breuss et al., 1995; Haapasalmi et al. 1996), and in serous epithelial ovarian cancer (Ahmed et al., 2002). In gingival, it was localized throughout the junctional epithelium in health (Garcia et al personal communication). Its strongest expression observed on the basal epithelial cells and granulation tissue formation when the edges of the wound have closed (Haapasalmi et al. 1996, Häkkinen et al., 2000), in the papilla (Csiszar et al., 2007) and through the long epithelial lining of erupting teeth (Häkkinen, personal communication). Previous human cutaneous and mucosal wound healing studies found that αvβ6

expression was up-regulated at day 3 in the migrating epithelium and remains up-regulated at least until day 14 when re-epithelialization is complete (Breuss et al., 1995; Clark et al., 1996; Häkkinen et al., 2000; Larjava et al., 2002). Similar to these studies, Johnson et al. (personal communication), found that up-regulation of αvβ6 expressed by the migrating keratinocytes in day 3-day-old wounds decreased but never disappeared after wound closure. The authors postulated that the role of integrin is to participate in the regulation of gingival inflammation by activating TGFβ-1.

Multiple functions have been attributed to an increased expression of αvβ6 including promotion of apical cell migration, cell proliferation, inflammation, activation of TGFβ, suppression of apoptosis, modulation of protease activity and mediation of invasion of carcinoma cells. As a promoter of migration and invasion it binds to the RGD motif in its ligands including fibronectin (Busk et al., 1992), tenascin-C (Prieto et al., 1993), and vitronectin (Huang et al., 1998).

As a mediator for inflammation it binds to the latency-associated peptide (LAP) of TGFβ-1 (Munger et al., 1999; Annes et al., 2004) and TGFβ-3 (Annes et al., 2002). Migration of human oral keratinocytes on fibronectin occurred when αvβ6 was bound to this ligand, and resulted in up-regulated secretion of the pro-enzyme form of type IV collagenase, MMP-9 (Thomas et al.,

2001). The integrin αvβ6 was highly expressed throughout the whole lesion of 90% of squamous cell carcinomas (SCC). Moreover, it was expressed in 41% of leukoplakia specimens, and 85% of lichen planus samples. However, it could not be found but in tissues with inflammatory hyperplasia or chronic inflammation (Hamidi et al 2000). It also has a role in malignant transformation of the oral epithelium in SCC by facilitating adhesion and migration (Koivisto et al., 2000) and a role in cell invasion demonstrated *in vivo* where expression in the SCC is often strongest at the invasive front of the tumor (Hamidi et al., 2000).

Integrin αvβ6 is a recognized receptor for foot-and-mouth disease virus (FMDV). Its expression on epithelial cells correlates with the tissue tropism of the virus that has been shown to use the RGD-dependent integrin sequence for binding (Jackson et al., 2000). As well coxsackievirus A9 (CAV9), another virus containing the functional RGD motif in one of their capsid proteins, binds to αvβ6 (Williams et al., 2004).

Mice homozygous for a null mutation (knock-out) in the gene encoding the beta 6 subunit (beta 6-/- mice) had juvenile baldness associated with infiltration of macrophages into the skin, and accumulated activated lymphocytes around conducting airways in the lungs (Huang et al., 1996). Another study using αvβ6 knockout mice found that the absence of the integrin caused

development of exaggerated inflammation and protected the mice from experimental pulmonary fibrosis, due to impaired TGF-β activation (Munger et al., 1999). Beta 6-/- mice also demonstrated airway hyper responsiveness to acetylcholine, a hallmark feature of asthma. These results suggest that αvβ6 participates in the modulation of epithelial inflammation (Huang et al 1996). Transgenic mouse lines that constitutively expressed human β6-integrin were used to study whether αvβ6 integrin plays an active role in abnormal wound healing (Häkkinen et al., 2004). The β6-overexpressing transgenic animals did not show any developmental abnormalities but they developed spontaneous wounds that presented different types of chronic scars. Since different expression levels of αvβ6 integrin used in wound healing experiments, i.e. β6 -/- (Huang et al., 1996) and β6-overexpressing (Häkkinen et al., 2004) developed chronic progressing scars and ulcerations, it suggests that αvβ6 integrin expressed by the epithelium contributes to the chronic fibrotic response. This result tells that it is possible that αvβ6 integrin may play a role when wound healing is compromised by infection or immunosuppression. We hypothesize that in periodontal disease it plays an important role by activating TGF-β.

1.3.2.2. αvβ6-integrin has a role in TGFβ *activation*

The transforming growth factor TGFβ family of cytokines consists of three closely related isoforms TGFβ -1, TGFβ -2 and TGFβ -3 that regulate cell growth and differentiation,

inflammatory responses and ECM production. Two latent forms have been described: the small complex (TGFβ -LAP) consisting of a mature TGFβ dimer noncovalently associated with latency-associated protein (LAP) and the large latent complex containing in addition to TGFβ–LAP, a latent TGFβ-binding protein (LTBP) that binds covalently to LAP (Munger et al., 1998). Experiments conducted by Annes et al. (2004), found that LTBP-1 enables αvβ6 -integrin-mediated activation by fixing and concentrating the latent complex in the ECM. As αvβ6 recognizes the RGD sequence of LAP and the integrin presenting cell retracts (Figure 2), conformational change and activation of TGFβ occurs through mechanical traction (Keski-Oja et al., 2004). Fibronectin is required for integrin αvβ6 -mediated activation of latent TGFβ complexes containing LTBP-1 (Fontana et al., 2005).

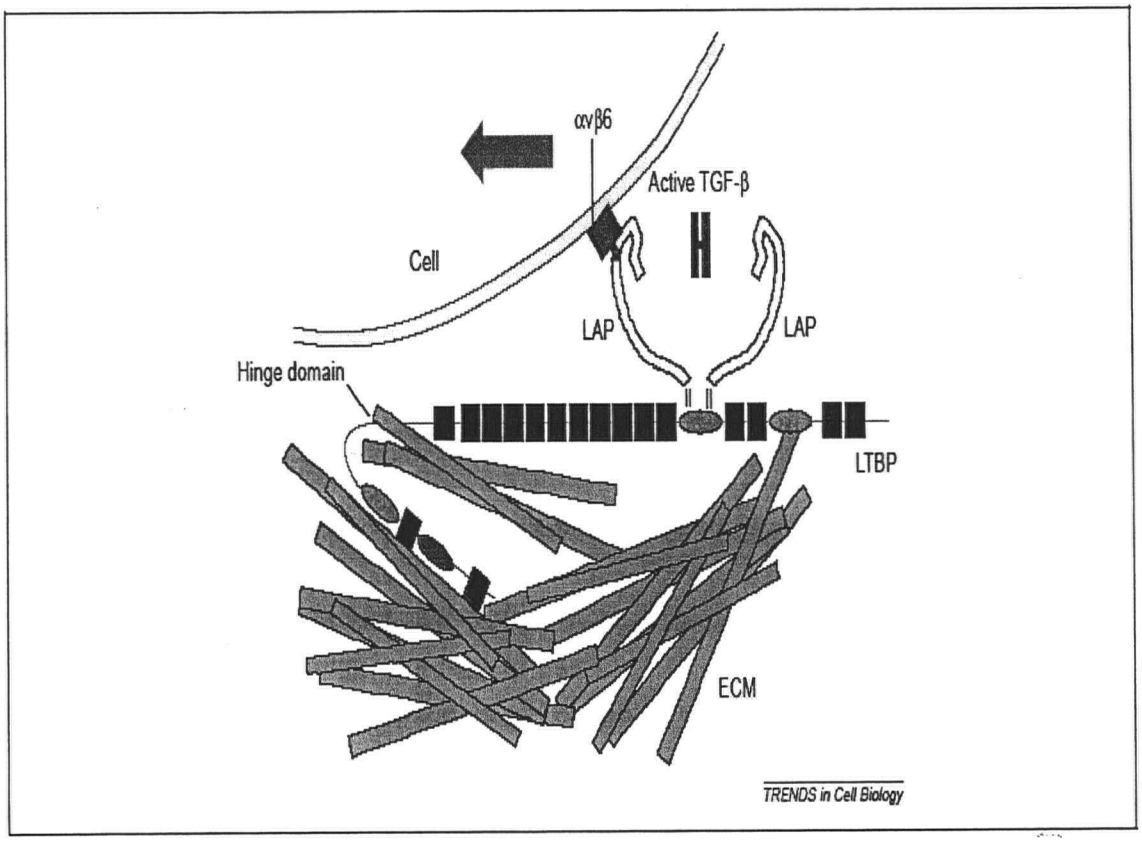


Figure 2. Alpha v beta 6 mediated activation of latent transforming growth factor (TGF-ß) by mechanical traction. The RGD domain is recognized by an αvβ6-integrin expressing cell in Latency-associated protein (LAP). The hinge region of the large latent complex associates with the ECM by unknown interactions. Both interactions are essential for αvß6-integrin mediated TGF- β activation. Cell movement (grey arrow) changes conformation of the structure of the latent complex. Cytoskeleton movement caused by a reduction in cell size by for example, retracts membrane protrusion and pulls LAP from the mature TGF-β to release the active cytokine from the protein complex. Black squares denote calcium binding epidermal growth factor (EGF) –like domain; green squares denote non-calcium binding EGF-like repeats; brown oval denotes EGF-like-8-Cys repeat hybrid domain. Figure used by permission from publisher. (Keski-Oja et al., 2004.)

1.3.2.3. Thrombospondin TSP-1 activates TGFβ

Thrombospondins (TSPs) are a small family of secreted, modular glycoproteins consisting of two homo trimers, TSP-1 and TSP-2, that form one subgroup, and the three smaller pentameric TSPs (TSP-3, TSP-4 and TSP-5) that form the second subgroup (Bornstein and Sage, 1994). TSP-1 is expressed by a variety of cell types including platelets, vascular smooth muscle cells and mesangial cells (MCs). It is also frequently expressed at sites of inflammation and wound healing (Bornstein, 1995) In vitro studies using various cell types have demonstrated multiple functions for TSP-1 in modulating platelet aggregation, angiogenesis, cell proliferation, cell adhesion and migration, and also TGF- β activity (Lawler et al., 1998).

Multiple receptors such as heparin sulphate, calreticulin, CD36, αvβ3-integrin and CD47 as well as specific peptide sequences such as the TGFβ-activating sequence RFK trigger TSP-1 functions (Lawler et al., 2000). The most prominent features of the TSP1-KO mouse are epithelial hyperplasia, leukocyte infiltration, and acute and chronic inflammatory changes involving the lungs (Lawler, et al., 1998), as well as milder inflammatory changes in the pancreas (Crawford et al., 1998). However, the inflammatory changes seen inTSP1-KO mice are not as severe as those seen in TGFβ 1-knockout mice (Crawford et al., 1998) suggesting that several overlapping mechanisms are simultaneously responsible for TGFβ activation.

Specific sequences in TSP-1 and in the LAP have been determined to be essential for activation of latent TGFβ by TSP-1 (Murphy and Poczatek, 2000). The physiological relevance of the ability of TSP-1 to activate latent TGFβ-1 has been the subject of much attention and some controversy, since there are other means of activation, including limited proteolysis by plasmin and interaction with αvβ6 and αvβ8 integrin. TSP-1 is a ligand for several integrins, including αvβ3, αIIβ3, α3β1, α4β1, and α5β1, and TSP-1-integrin interactions appear to mediate numerous TSP-1 functions. TSP-1 is transiently expressed at high concentrations in damaged and inflamed tissues, where TSP-1-integrin interactions function in the host inflammatory response (Chen et al., 2000).

Murphy-Ullrich et al. (2000) observed for the first time that growth inhibitory effects of platelet TSP-1 were partially TGF-β dependent. Subsequently, it was demonstrated that for TGF-β activation, TSP1 binds directly to the LAP and TGF- β protein forming an active ternary LAP–TSP1–TGF- β complex (Figure 3). While LTBP does not play a role, the failure of protease inhibitors to influence this process and the persistence of TGF- β activity within this LAP–TSP1–TGF- β complex suggests that binding of TSP1 to LAP–TGF- β mediates a conformational

change within the LAP–TGF- β pro-cytokine complex that abolishes a specific interaction required for latency (Schultz-Cherry and Murphy-Ullrich, 1993).

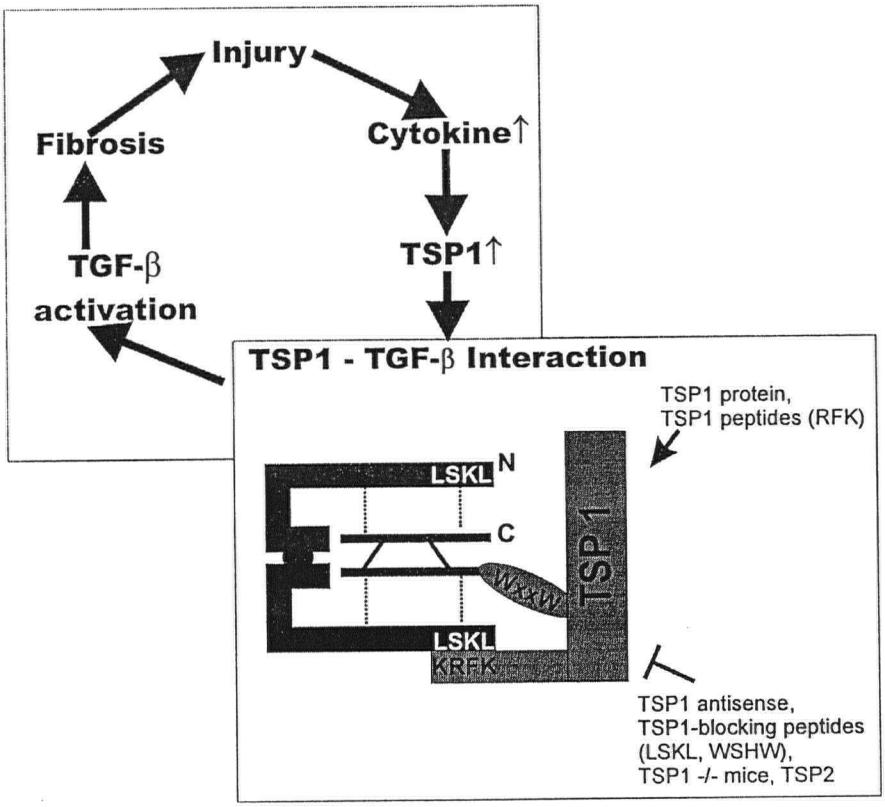


Figure 3. Activation of TGF- β mediated by TSP1. More detailed studies identified two important sites within the TSP1 molecule that are responsible for this complex interaction. That is the WxxW (WSHW, WSPW or WGPW) motif from the type I repeats of the TSP1 molecule that is used as the binding site to the active TGF- β domain (Schultz-Cherry etal., 1999). Binding of this site is essential to correctly orient the second TSP1 motif, the KRFK-sequence, which interacts with the corresponding complementary site on the latent TGF- molecule, the N-terminal LSKL-sequence of the LAP. Once the complex forms as a result of these interactions that leads to a conformational change in LAP that allows the mature TGF- β protein to bind to its receptors still being incorporated in the ternary complex (Ribeiro et al., 1999).

(Figure used by permission from publisher; courtesy of Hugo et al., 2003).

Murphy-Ullrich and Poczatek (2000) suggest that TSP-2 could also compete with TSP-1 for binding to latent TGFβ-1 thus inhibiting its activation. Some of the changes observed in wound healing in TSP-2-null mice are indeed compatible with the presence of higher levels of active TGFβ-1 in the wounds. Higher levels of active TGFβ-1 were found in cultured dermal TSP-2-null fibroblasts similar to observed changes in wound healing in TSP-2-null mice, where higher levels of active TGFβ-1 were found in the wounds (Bornstein, 2001).

1.4. Thesis Objectives and Hypotheses

Hypothesis: αvβ6 integrin plays a major role in activating TGFβ-1 in the JE. In periodontal disease there is decrease in TGFβ-1 activation. This results in a decrease in immunological suppression leading to increased inflammation of the gingiva. This, decrease in TGFβ-1 also results in a concomitant increase in epithelial cell proliferation.

Goals: To investigate functional role of ***αvβ6 integrin*** in JE in a rat and mouse model of periodontal disease.

CHAPTER II: Material and Methods

2.1. Animals

2.1.1 Mice

Three eight months old αvβ6 KO mice (2 females and one male) and four eight months old TSP-1 KO mice (all males) were received and prepared for histological analysis (a gift from Dr. Jack Lawler, Division of Cancer Biology and Angiogenesis, Harvard University, Boston USA). At a later date seven double KO eight months old (6 females and one male) and two wild type nine months old (female and male) were received as a gift from Dr. Jack Lawler as well.

2.1.2 Rats

Twenty four, 8 weeks old male Sprague Dawley rats (Animal Care Centre, University of British Columbia, Vancouver), were divided in four groups of six rats that were treated individually for eight weeks. All animal studies were conducted in compliance with the Canadian Council on Animal Care guidelines and approved by the University of British Columbia Animal Care Committee.

2.2 Antibodies

The following are a list of the antibodies used in the rat functional experiment.

Antigen	Primary Antibody	Host animal	Mono or Polyclonal	Dilution used	Company or source	Secondary Antibody used
ICAM	Ab 1A29	Mouse	Monoclonal	1:50	Abcam Ltd (Cambridge, MA)	Vectastain biotinylated
CD68	ED1	Mouse	Monoclonal	1:50	Serotec Ltd. Oxford, UK)	Vectastain biotinylated
CD 4	W3/25	Mouse	Monoclonal	1:50	Serotec Ltd. Oxford, UK)	Vectastain biotinylated
Pan-B Cells	Ki-B1R	Mouse	Monoclonal	1:50	Fitzgerald Ind. Int. Inc Concord, MA	Vectastain biotinylated
Pan-G Granulocyte	RK-4	Mouse	Monoclonal	1:50	Fitzgerald Ind. Int. Inc Concord, MA	Vectastain biotinylated
CD3 (T cell)	Clone not specified	Rabbit	Polyclonal	1:50	Zymed Laboratories Inc, San Francisco, CA	Vectastain biotinylated
Ki67	Ab-3	Rabbit	Polyclonal	1:50	Lab Vision Corporation, Fremont CA	Vectastain biotinylated
Anti-mouse Collagen I	Clone not specified	Rabbit	Polyclonal	1:300	Chemicon Int. Inc, Temecula CA	Vectastain biotinylated
Anti-Human Tenascin	BC-24	Mouse	Monoclonal	1:4,000	Sigma Biosciences, St Louis MI	Vectastain biotinylated
Anti-Human Fibronectin	Clone not specified	Rabbit	Polyclonal	1:400	Sigma Biosciences, St Louis MI	Vectastain biotinylated
TGFβ1 (V)	sc-146	Goat	Polyclonal	1:50	Santa Cruz Biotechnology INC, CA	Alexa Fluor 546 Donkey anti-goat IgG Red Eugene, Oregon, USA
Anti-β6	β6-4B5	Rabbit	Polyclonal	1:10	Biogen Ab	Alexa Fluor 488 Chicken anti Rabbit IgG green Eugene Oregon
Anti-αvβ6	6.3G9				Biogen Ab	Alexa Fluor 488 goat anti-mouse IgG Invitrogen, Eugene Oregon,
Anti-αvβ6	7.8B3				Biogen Ab	Alexa Fluor 488 goat anti-mouse IgG Invitrogen, Eugene Oregon,

Table 1. List of the antibodies used in the rat functional experiment

2.3. Study Protocols

2.3.1 Murine Model

All mice KOs were produced on a C57Bl/6 background (129Sv). The heads from all mice, previously fixed in formaldehyde (FA) were dissected so that the maxilla was used for buccal-lingual sectioning while the mandible was cut sagitaly and used for mesial-distal sectioning.

2.3.2 Rat Model

The rats were divided randomly into 4 groups (n=6) prior to treatment. Each group was treated at the same time of the day. All six rats were anesthetized with AErrane isoflurane (Baxter Corporation, Mississauga, ON, Canada). They were placed on their backs and kept under anesthesia, while their mouth was kept open with an orthodontic stainless steal wire piece. The antibodies and LPS were administered with a micropipette directly in the sulcus of the two molar teeth on both sides of the maxilla. The rats were then placed back in their cage to recover. After 8 weeks all animals were euthanized and dissected. The rat maxilla was dissected and preserved for experimentation according to Figure 4.

The first group was treated with 3 x 0.3 μL pyrogen free water. The second group was treated with 3 x 0.3 μL 6.3G9 monoclonal antibody (5.9 ng/ μL) + LPS E.coli 0127:B8 (25 μg/ μL; Sigma-Aldrich, St. Louis, MI). The third group was treated with 3 x 0.3 μL 7.8B3 monoclonal antibody (1.46 ng/ μL) + LPS LPS E.coli 0127:B8; (25 μg/ μL; Sigma-Aldrich, St. Louis, MI). The fourth group was treated with LPS E.coli 0127:B8 (25 μg/ μL; Sigma-Aldrich, St. Louis, MI) alone.

The function monoclonal antibodies 6.3G9 and 7.8B3 are not commercially available (a gift and collaboration with Dr.Weinreib, Biogen Idec, Cambridge MA and Lung Biology Center, Cardioascular Research Institute, and Department of Medicine, University of California, San Francisco, CA). The 6.3G9 anti αvβ6 antibody is described as being a non-ligand-mimetic blocking Ab that is cation independent. This Ab is a potent inhibitor of αvβ6-mediated TGFβ activation by blocking binding to fibronectin, tenacin and vitronectin. In turn this antibody is not blocked by RGD-containing peptides and not internalized by αvβ6 expressing cells and does not contain RGD or related sequences. Also it blocks both human and murine αvβ6 function. Meanwhile 7.8B3 Ab has very low blocking potency in the cell adhesion format on LAP (Dr.Weinreib et. al., 2004).

2.4. Tissue processing

2.4.1 Murine Model

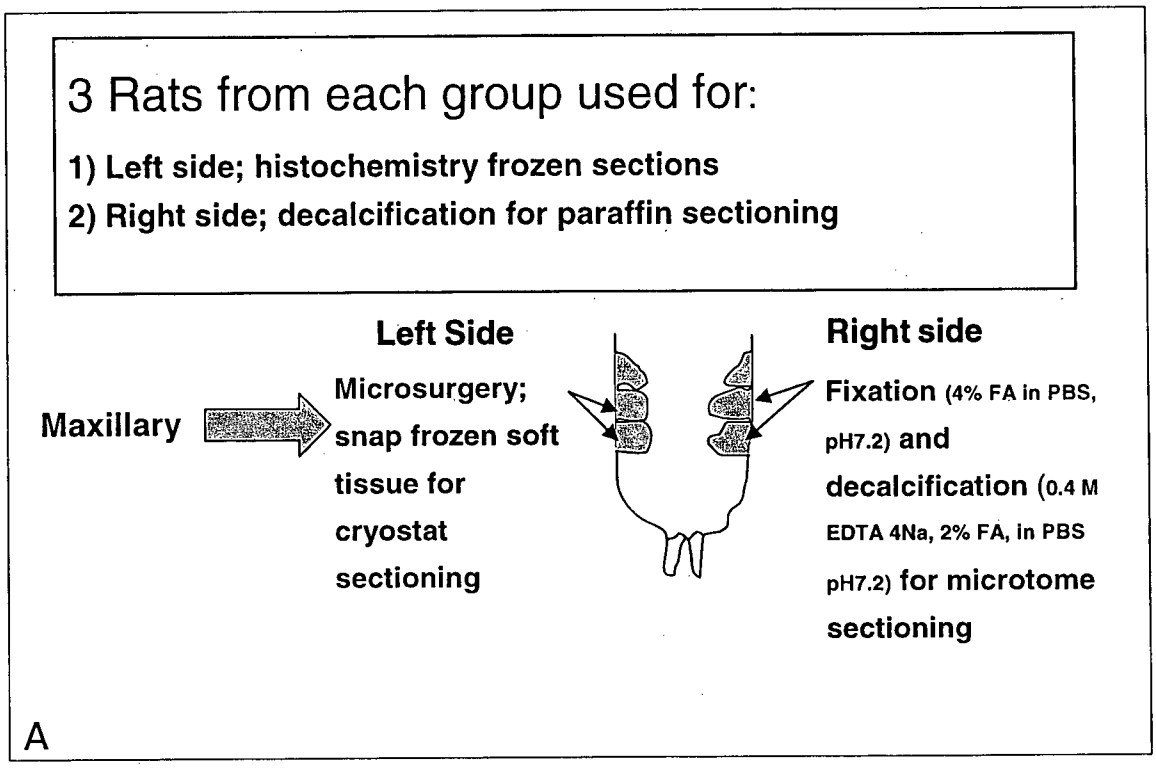
Fixed maxilla and mandible of the mice were placed in decalcification solution (0.4 M EDTA and 2% formaldehyde in PBS (pH= 7.2). The solution was changed daily after microwaving. The tissue was placed in ice filled container in the Pelco 3470 Hornet Microwave System (Redding, CA) for 30 minutes at the time at power 3 for up to six times a day. The number of days was not the same for all specimens. The ideal bone density for sectioning was decided by x-ray examination at which point the decalcification process was terminated. The length of the decalcification process varied from 4 to 6 days depending on the size of the tissue. The tissue was processed for paraffin embedding using a routine protocol. Paraffin sections (5-6 μm thick) were cut with the AO Spencer "820" microtome (American Optical Co., Instrument Division, Buffalo 15, NY, USA), left to dry overnight and then stored at room temperature.

2.4.2 Rat Model

From each of the four groups the JE tissue from three rats was surgically removed under a dissecting microscope Leica Mz6 (Leica, Heerbrugg, Switzerland) and snap frozen in liquid nitrogen (see Fig 4 for details). The tissue was then stored in the - 80°C freezer for further analysis.

Similarly, JE from the remaining three rats in each group, was removed under microscope from the left side of the maxilla around the three molars. This JE was immersed in Tissue TeK™ (OCT compound, Sakura Finetek USA Inc., Torrance, CA, USA), placed on dry ice and then submerged in liquid nitrogen. The tissue was oriented for buccal lingual sectioning. Once frozen it was stored in the - 80°C freezer until sectioning could be performed. The specimens were sectioned with 2800 Eclipse TS 100 Cryostat at 6 μm thickness settings.

The heads of the same three rats were dissected and only the right hand side of the maxilla was fixed in 4 % formaldehyde in PBS formalin for one day. After one day it was placed in decalcification solution and was decalcified as described above for the mice tissue. The ideal bone density for sectioning was decided by x-ray examination at which point the decalcification process was terminated. The length of the decalcification process varied from 5 days to 8 depending on the size of the tissue. The tissue was processed for paraffin embedding using a routine protocol. Paraffin sections (5-6 μm thick) were cut with the AO Spencer "820" microtome (American Optical Co., Instrument Division, Buffalo 15, NY, USA), left to dry at 40°C on Fisher slide warmer overnight and then stored in boxes at room temperature.



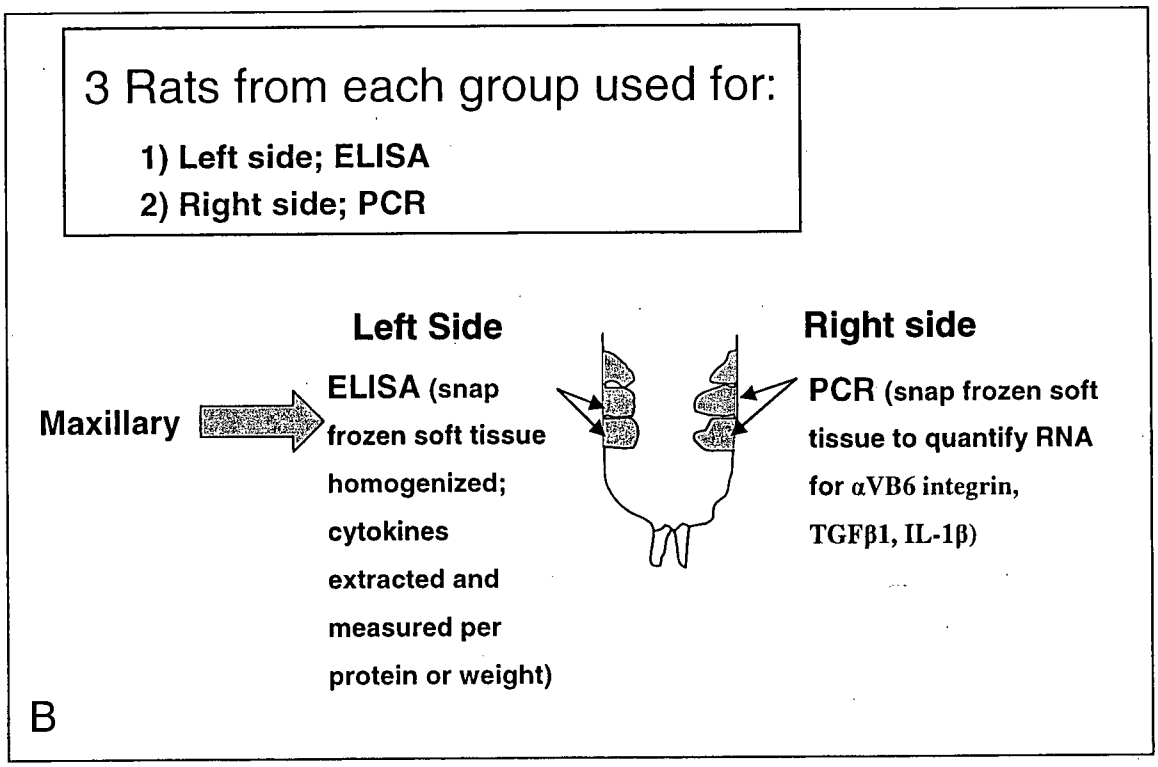


Figure 4. Experimental design: Figure A describes the histological sample and figure B describes the molecular and cytokine study samples

2.5. Staining Procedure and Evaluation of Sections

2.5.1 Murine model

Every tenth slide was used for basic H&E staining and evaluated under the light microscope (Axiolab E, Zeiss, North York, ON, Canada) equipped with a 4x, 10x, 20x and 40x objective. Sectioned tissue was photographed using a digital camera (Nikon Coolpix, Torance, CA, USA) attached to a Nikon Eclipse TS 100 microscope (Torance, CA, USA) equipped with a 4x, 10x, 20x and 40x objective. Panels of buccal lingual paraffin sections were prepared with Photo Shop. Measurements for area of the junctional epithelium migration were taken from the CEJ down towards the root of the tooth using the Image J program. Similarly the length of the pocket epithelium was measured and compared between groups for statistical significance. A total of 45 sections were taken for each mouse for the area of maximum migration.

2.5.2 Rat model

2.5.2.1 H & E Staining

Following sectioning of each sample one in every ten slides was stained with Hematoxilin and Eosin (H&E) then evaluated under the light microscope (Axiolab E, Zeiss, North York ON) equipped with a 4x, 10x, 20x and 40x objective. Sectioned tissue was photographed using a

digital camera (Nikon Coolpix, Torance, CA) attached to a Nikon Eclipse TS 100 microscope (Torance, CA) equipped with a 4x, 10x, 20x and 40x objective. (Torance, CA). Panels with the buccal lingual paraffin sections were prepared with Photo Shop. Measurements for the area of migration were taken with ImageJ from the CEJ down towards the root of the tooth. Similarly the length of the pocket epithelium was measured. A total of 45 measurements for tissue samples with the best morphology found in the proximity to the area of maximum migration were used for statistical analyses. Sections of the frozen tissue were also stained with H&E and observed under microscope.

2.5.2.2 Immunohistological Staining of Cells

The sections demonstrating areas where the JE had migrated were used to identify cells and markers involved in the inflammatory response. This was accomplished using immunohistochemistry to localize lymphocytes (anti-CD4 Ab) macrophages (anti-ED1 Ab) anti-ICAM-1 Ab. As well proliferating nuclei were localized with Ki67 Ab (See Table 1).

Immunohistochemical staining was performed using the ABC Vectastain Kit (Vector Laboratories Inc., Burlingame, CA). For localizing macrophages (ED1), sections were fixed for 2 hours in 4% formaldehyde (Fisher Scientific, Nepean, ON) / 0.2 % glutaraldehyde (Sigma-

Aldrich, St. Louis, MI) in PBS. Sections were circled with PAP pen (ImmEdge Pen, Vector Laboratories Inc.), and washed in 1mg/ml phosphate buffer saline (PBS) and bovine serum albumin (BSA,) and 0.01 Triton X-100. This was followed by incubation in normal blocking serum (Vectastain, Vector Laboratories Inc., Burlinghame, CA) in a humidified chamber for 30 minutes at room temperature, then incubation with the primary anti-ED1 antibody diluted (1:50) in PBS and BSA overnight. After rinsing with PBS/BSA 0.01 Triton X-100, the samples were incubated with biotinylated secondary antibody for one hour then rinsed again and incubated for 30 min with Vectastain ABC reagent (Vectastain, Vector Laboratories Inc., Burlinghame, CA. The last wash was followed by the color reaction with Vector VIP substrate kit for peroxidase. The negative control was a section stained with non-specific mouse IgG (Sigma Immuno Chemicals).

Localization of T-cells was done by using both anti-CD4 antibody and ICAM-1 antibody (Table 1). The protocol described above was similar for both CD4 and ICAM-1 staining, except that for fixation 70% methanol was used for 10 minutes, the wash solution did not contain Triton X-100 and the tissue was treated for 30 minutes with 0.5 ml of 30% H_2O_2 in 50ml methanol for blocking the endogenous peroxidase activity.

For Ki67 (Table 1) staining the frozen sections were fixed in acetone (- 20°C) for 5 minutes. The rest of the protocol is similar to the one used for ED1 (Table 1). Each antibody was developed for the color reaction on an individual bases after the ideal time was determined. Then a series of slides from each group were stained and developed at the same time to maintain similar conditions for the best comparison of the four experimental groups. The reaction was stopped with distilled water and slides were air dried and then mounted with Vectamount permanent mounting medium (Vector Laboratories Inc., Burlinghame, CA). Selected images were recorded using Nikon Coolpix digital camera (Torance, CA).

2.5.2.3 Double Immunohistological Staining of αvβ6 and active TGF-β1

Two antibodies from different animals were used to localize αvβ6 integrin and active TGF-β1 cytokine. Sections from all four groups were left to melt at room temperature and then fixed in acetone (- 20°C) for 5 minutes. After air drying the tissue was circled with a PAP pen (ImmEdge Pen, Vector Laboratories Inc) and then placed in a humidified dark chamber using a microscope slide storage box with damp paper towels placed at the bottom. The sections were washed with PBS/Triton X-100 0.01% for 5 minutes. For blocking the background a solution of PBS/BSA 10mg/ml Triton X-100 0.01% was used for 30 minutes at RT in the humid chamber. The blocking solution was removed and the first antibody anti-β6 (see list) was incubated overnight

at +4°C in the humid chamber. On the following day sections were washed twice for 5 minutes with PBS/BSA 1mg/ml Triton X-100 0.01%. The second primary antibody against active TGF-β1 was added to the sections which were then incubated overnight at +4°C in the humid chamber. Following this incubation, the sections were washed twice for 5 minutes with PBS/BSA 1mg/ml Triton X-100 0.01%. A solution containing 1ml of Alexa Fluor 546 Donkey anti-goat IgG and 1ml of Alexa Fluor 488 chicken anti rabbit (Table 1) in 100 ml of PBS/BSA 1mg/ml Triton X-100 0.01% was added after incubation for one hour in the dark in the humid chamber then the slides were washed twice for 5 minutes with PBS/BSA 1mg/ml Triton X-100 0.01%. Slides were mounted with (Immu-mount, Pittsburgh, PA) and pictures were taken with camera Q Imaging at 40X magnification with the filter for fluorescein isothiocyanate (FITC) for detection of Alexa Fluor 448 and Rodamine (Rodh) filter for detection of Alexa Fluor 546. Pictures were taken under both filters using Northern Eclipse softwear. Using the same program the pictures for αvβ6 integrin and the ones for active TGF-β1 were then merged to observe co-localization of the integrin and cytokine.

2.6. Quantification of Leukocyte Infiltrate

The inflammatory infiltrate was measured in the rat model using H&E stained tissue for each group. Fifteen tissue sections for three individual rats in a group were analyzed using a light

microscope with a microscopic grid 100 squares 1mm² (Axiolab E, Zeiss, North York Ontario) and 20X magnification. Two independent measurements were recorded. The first measurement was the inflammatory index. This was determined by counting the squares in the grid containing the inflammatory infiltrate around the JE migration. An index of 1 to 5 was assigned for this measurement based on the percentage of inflammatory infiltrate located in the observation grid. The second measurement was the density of infiltrate as determined by counting the number of leukocytes found in 10 randomly selected squares. Again each measurement was scaled from 1 to 5. For example for the density measurement an index of 1 was assigned when any number of cells from 0 to 9 cells was obtained, from 10 to 19 cells an index of 2 was assigned, from 20 to 29 an index of 3, 30 to 39 an index of 4 and anything above 40 cells received an index of 5. In order to determine the final histological score the two measurements were added together (Table 2).

The sum measurement 1(cells/10 squares) and 2 (total # squares around JE)	Histological score
0-9	1
10-19	2
20-29	3
30-39	4
40>	5

Table 2. Inflammatory infiltrate quantification

2.7. Zymography

Frozen tissue stored at -80°C was ground with a mortar and pestle under liquid nitrogen in a fume hood. The ground samples were dissolved in 1mL buffer obtained from one tablet Roche Complete Protease Inhibitor cocktail tabs (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany) in PBS. The buffered homogenized tissue was then stored at -80°C. From each sample, that is 3 samples for Pyrogen-free H20 group, LPS+6.3G9 Group and LPS and 2 samples from LPS+7.8B3 alone group, 25µl was used for zymographic analysis of gelatinases MMP-2 and MMP-9. Gelatinolytic activity was identified by zymography using sodium dodecylsulphate-polyacrylomide gel electrophoresis (SDS-PAGE) 10% containing 10mg/ml 2-Methoxy-2,4-diphenyl-3(2H)-furanone (MDPF) gelatin in H2O. The gels were run at constant current 25mA for 90m in running buffer. After this time the gels were washed by shaking for 30 minutes at room temperature in 50mM Tris, 2.5% Triton X-100, 0.02% NaN3, pH 7.5 then in 50mM Tris, 5mM CaCl2, 1µM ZnCl2, 2.5% Triton X-100, 0.02%NaN3 pH 7.5. The gels were then incubated and developed overnight in 50mM Tris, 5mM CaCl2, 1µM ZnCl2, 0.02% NaN3, pH 7.5 at 37°C. The zymograms were stained in 20% Methanol, 7% Acetic acid +0.2 % Coomasie blue R250. Pictures were taken under UV light using Electrophoresis Systems (Fisher Scientific, Pittsburgh, PA) and the Glow Box (Cheltenham, PA).

2.8. Statistical Analysis

ANOVA test was used to find out if there was a significant difference between all four groups, i.e. the difference between the area of tissue migration between double null mice, the αVβ6 KO, TSP-1 KO mice and the wild type mice and the difference between the epithelium length migrations for the same groups. The differences between the epithelium area migration and the JE length migration for the fours groups in the rat model were analyzed similarly with ANOVA.

In order to compare group means, the post hoc Tukey Test was used. The Tukey Test is designed to perform a pair wise comparison of the means to see where the significant difference is. The statistical analyses for the inflammatory infiltrate used a one way ANOVA analysis with Prism program as well as an unpaired t-test.

CHAPTER III: Results

3.1. Rat Model of Periodontal Disease

In order to investigate the role of TGF-β and αvβ6 integrin in the progression of periodontal disease the rat model described previously was used. The rats were divided in four groups of six rats that were treated individually for eight weeks. The treatments were as follows: Group 1:

pyrogen free water; Group 2: LPS +6.3G9 monoclonal anti-αvβ6 blocking antibody; Group 3: LPS+7.8B3 monoclonal non-blocking αvβ6 antibody; Group 4: LPS alone. At the end of the experiment the animals were sacrificed and the JE removed.

3.1.1. Epithelial Migration: Area and Length.

Junctional epithelium morphology was preserved better in the paraffin embedded sections compared to the frozen sections and therefore they were used for measurements of JE migration. The elongation of the JE down the root and inside the CT was observed in buccal-lingual sections and measured after picture images were taken and presented in panels (Figure 5). The epithelial cells in pocket epithelium are larger and elongated compared to the cells in the junctional epithelium. Large intercellular spaces can be seen in the migrating epithelium in which mononuclear cells are observed. Pocket epithelium forms along the tooth root or migrating in the interdental area into the connective tissue. Inflammatory infiltrate is present in the connective tissue along the pocket epithelium and accumulated mononuclear cells are found inside the epithelial gaps (Figure 6).

Significantly more pocket epithelium was found in both the LPS plus blocking antibody treated rats compared to the LPS and pyrogen-free water treated rats (Figure 7). Pocket epithelium area formed below CEJ was measured for total area migration as was JE length migration along the tooth root (Figure 8).

Buccal-Lingual Sections 10X for Rat Model

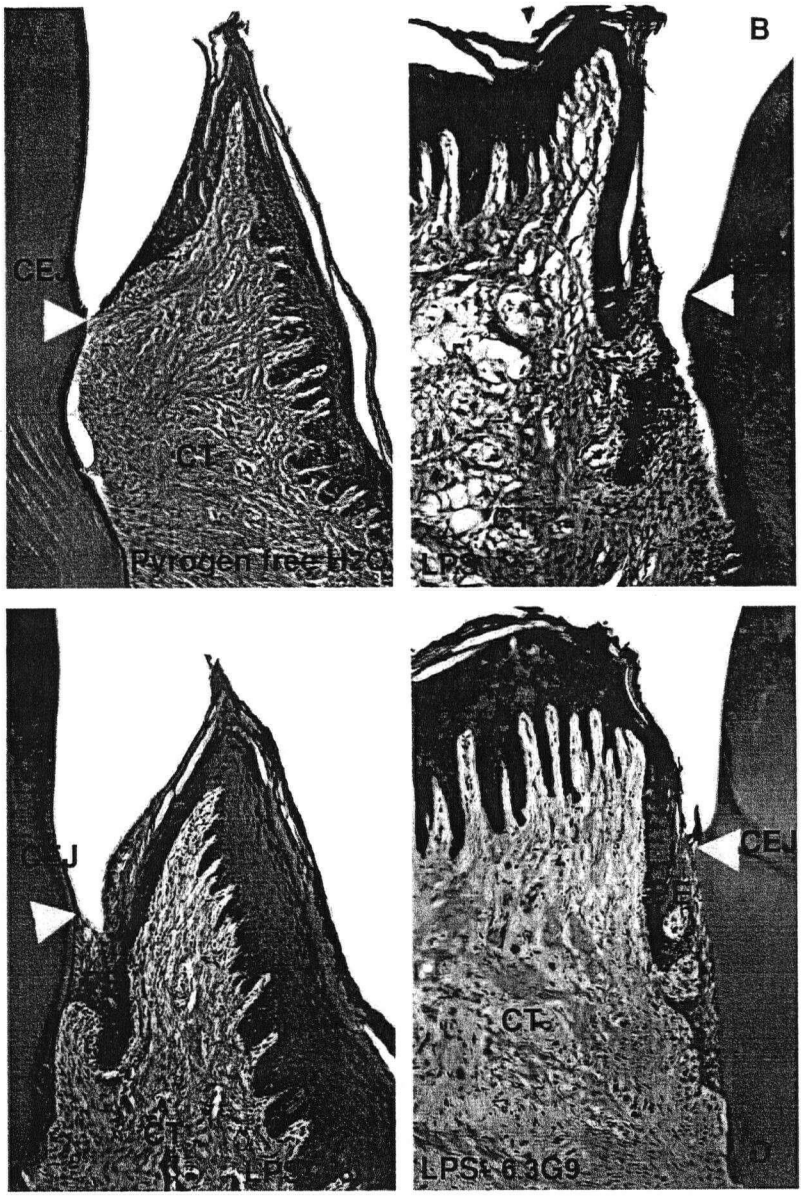


Figure 5. H&E staining of rat tissue 10X. White arrows pointing to Cementum Enamel Junction (CEJ), Pocket Epithelium (PE) formed in pictures B, C and D: Panels are representative of 3 animals for each group

Buccal-Lingual Sections 40X for Rat Model

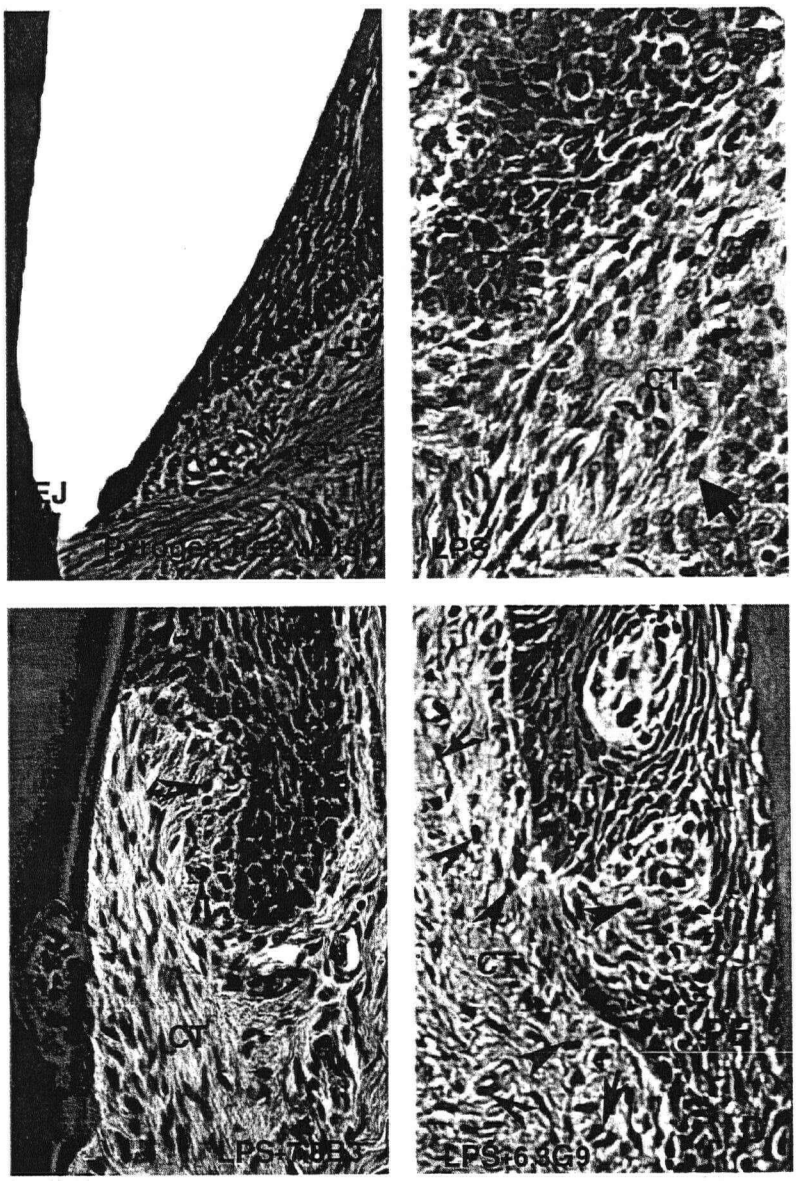


Figure 6. H&E staining for rat tissue 40X: pocket epithelium (PE) formed in images B, C and D. In panel B thick black arrow shows PMN in the LPS group. In panels C and D an increased number of mononuclear cells are present around the pocket epithelium, black arrow heads showing inflammatory infiltrate. Panels are representative of 3 animals in each group:

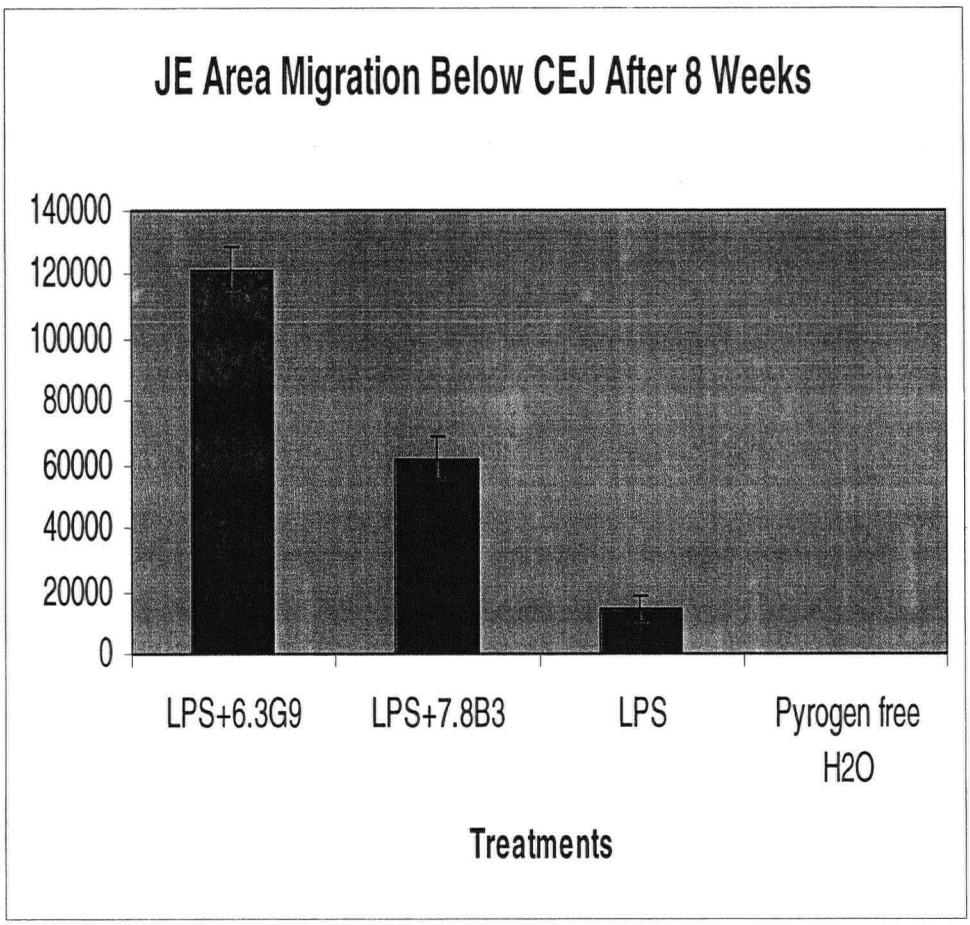


Figure7. JE area of migration in the connective tissue below the CEJ. ANOVA analysis found significant difference between all groups ($p<0.05$) and Tukey test found significant difference exist between groups when compared individually except the LPS and pyrogen free water that had no migration (all measurements were zero). Panels are representative of 12 animals: The units on y axis are relative (Image J units)

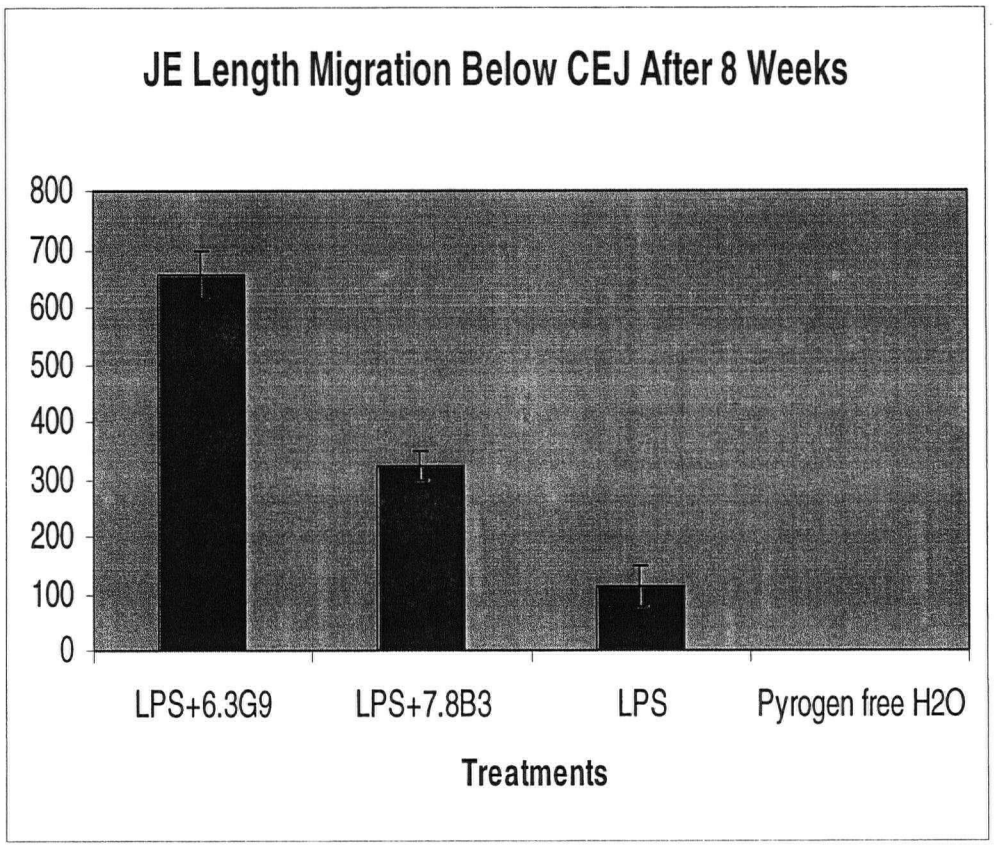


Figure 8: Length of JE migration along the tooth root. ANOVA analysis found significant difference between all groups ($p<0.05$) and with Tukey test all groups were significantly different. Panels are representative of 12 animals. The units on y axis are relative (Image J units).

3.1.2 Leukocyte Infiltrate

The leukocyte infiltrate was quantified in the four treatment groups as discussed in the materials and methods. It can be seen from Figure 9 that the blocking antibody elicited a significantly higher infiltrate than any of the other three treatment protocols.

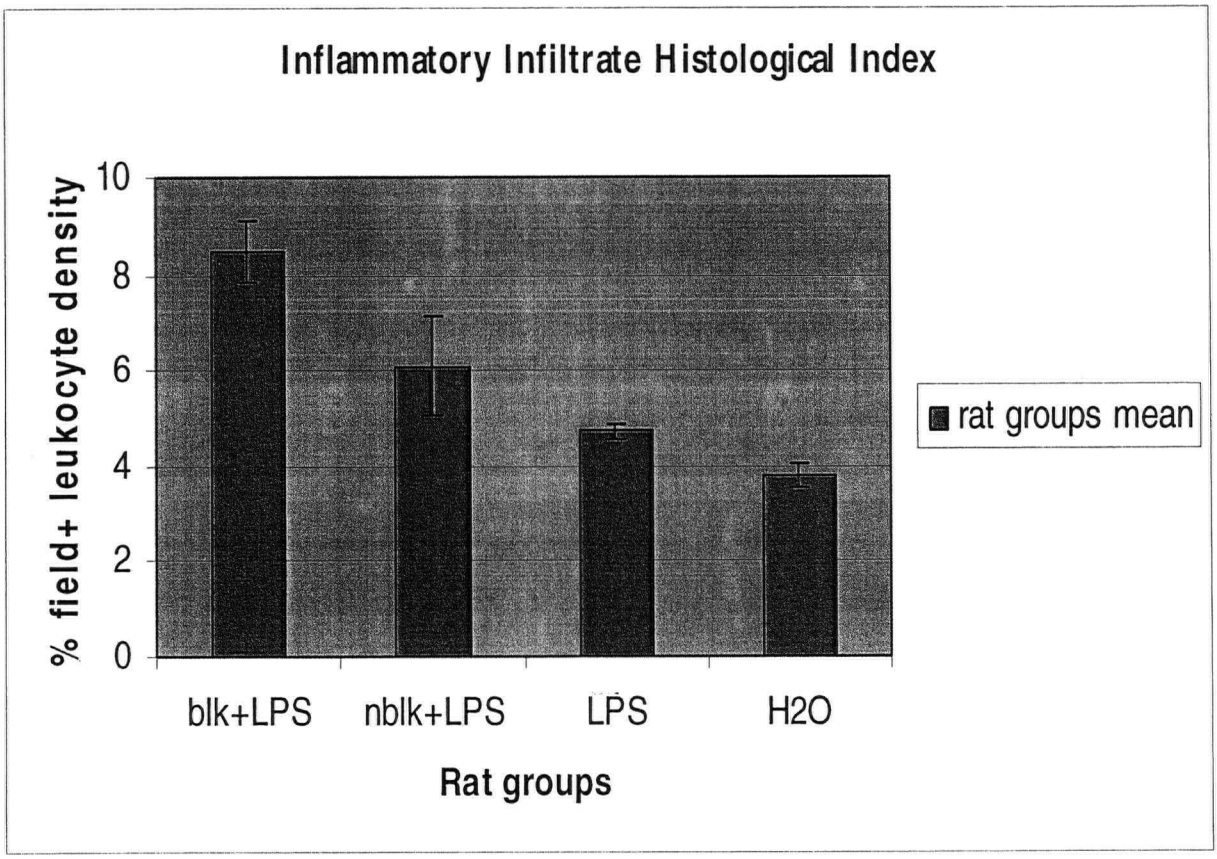


Figure 9. Inflammatory infiltrate: One-way ANOVA is significant between groups ($p < 0.05$). Unpaired t-Test found that between all groups there is significant difference with the exception of LPS group and non-blocking and LPS group. These values present data on 12 rats.

3.1.3 Gelatin Zymography

Gelatin zymography was carried out to determine whether MMP activity varied between the four treatment groups. In our study the gels were far from perfect. However, in the UV exposed image (green background Fig 10 A&B), the thick bands of 72 kDa (pro-gelatinase A, pro-MMP2) and the thin bands of 66 kDa (active gelatinase A, MMP-2) are observed below very thin bands of 92 kDa (pro-gelatinase B, pro-MMP-9) and thin bands of 88 kDa (active gelatinase B, MMP-9) that are slightly visible.

In figure 10 A taken under UV light (green background) can be seen bands of same intensity and width for pro-MMP2 (72 kDa) and activated MMP2 (66 kDA) in the LPS (first three bands from the left) and blocking antibody 6.3G9+LPS (three bands to the right in figure 10 A) groups. The Pyrogen free water group in figure 10 B is represented by the three bands at the left. The bands appear slightly thinner and more intense. The middle band and the third band in the water group are significantly different, where the middle is thinner than the one at the right. This difference could be accounted for biological variation within the group. The non-blocking antibody 7.8B3+LPS has two bands that are visible because there are only 2 specimens in that group. The thin band close to the water group could be the result of experimental error. Insufficient sample does not allow for running another gel.

In the Coomasie blue R250 (Figure 10) stained gels C and D the bands appear fainter due to the intense blue staining. Image C from left to right contains three bands for the LPS group and three bands for blocking antibody group (6.3G9+LPS). In figure 10 D from left to right contains two bands of non-blocking (7.8B3+LPS) antibody, third one is probably due to contamination and last three ones are the pyrogen free water group. Insufficient sample did not allow us to repeat SDS page zymography, therefore the results cannot be conclusive.

SDS Page Gels for MMP2, pro-MMP2, MMP 9 and pro-MMP9

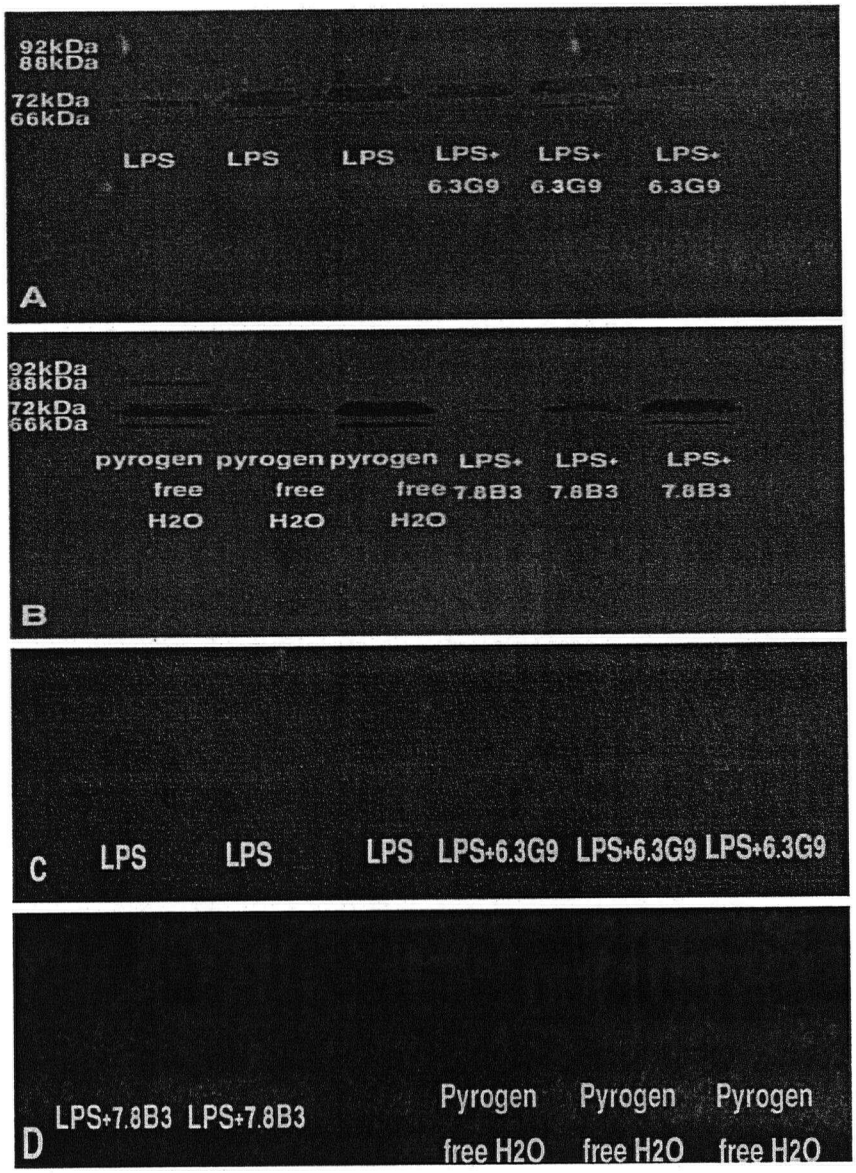


Figure 10. SDS Page Zymography for MMP2, pro-MMP2, MMP 9 and pro-MMP9: Image A and B represents a SDS page gelatin zymograpy imaged under UV Light. 92 kDa band is for pro-MMP-9, 88 kDA band is for active MMP-9, 72 kDA band for pro MMP-2 and 66 kDA band for active MMP-2. Image C and D represents SDS page gelatin zymograpy stained with Coomasic blue R250.

3.1.4 Double Immunohistological Staining of αvβ6 and active TGF-β1

As has been discussed, αvβ6 is important in the activation of TGF-β1, consequently we hypothesized that these molecules should be co-localized in the gingiva of animals undergoing experimental periodontitis. We evaluated this in the four treatment groups of our rat model. In the first column of Figure 11 can be seen the images for anti β6 (FITC) staining. The middle column contains the images for the anti-active TGF-β1 staining (Rodh filter image), while the right column contain the images obtained by merging the first two images (FITC filter image merged with the Rodh filter image) to observe co-localization of αvβ6 integrin and active TGF-β1. First row images are for the pyrogen free water group, the second row consist of images for the LPS group, third is the non-blocking antibody group (7.8B3+LPS) and last is blocking antibody group (6.3G9+LPS) TGF -β1 staining can be clearly seen in the LPS group and in the non blocking antibody 7.8B3 plus LPS treated group. Active TGF -β1 is not seen in the pocket epithelium of the blocking antibody 6.3G9 plus LPS treated group. Active TGF -β1 is only slightly present in the water treated group. It was noted that the staining for αvβ6 integrin was very weak and it was difficult to determine whether it truly reflected integrin staining or was a "bleed through" from the red channel. Therefore determination of co-localization was not possible.

Immunoflorescent double staining for Beta 6 and active TGF-beta 1

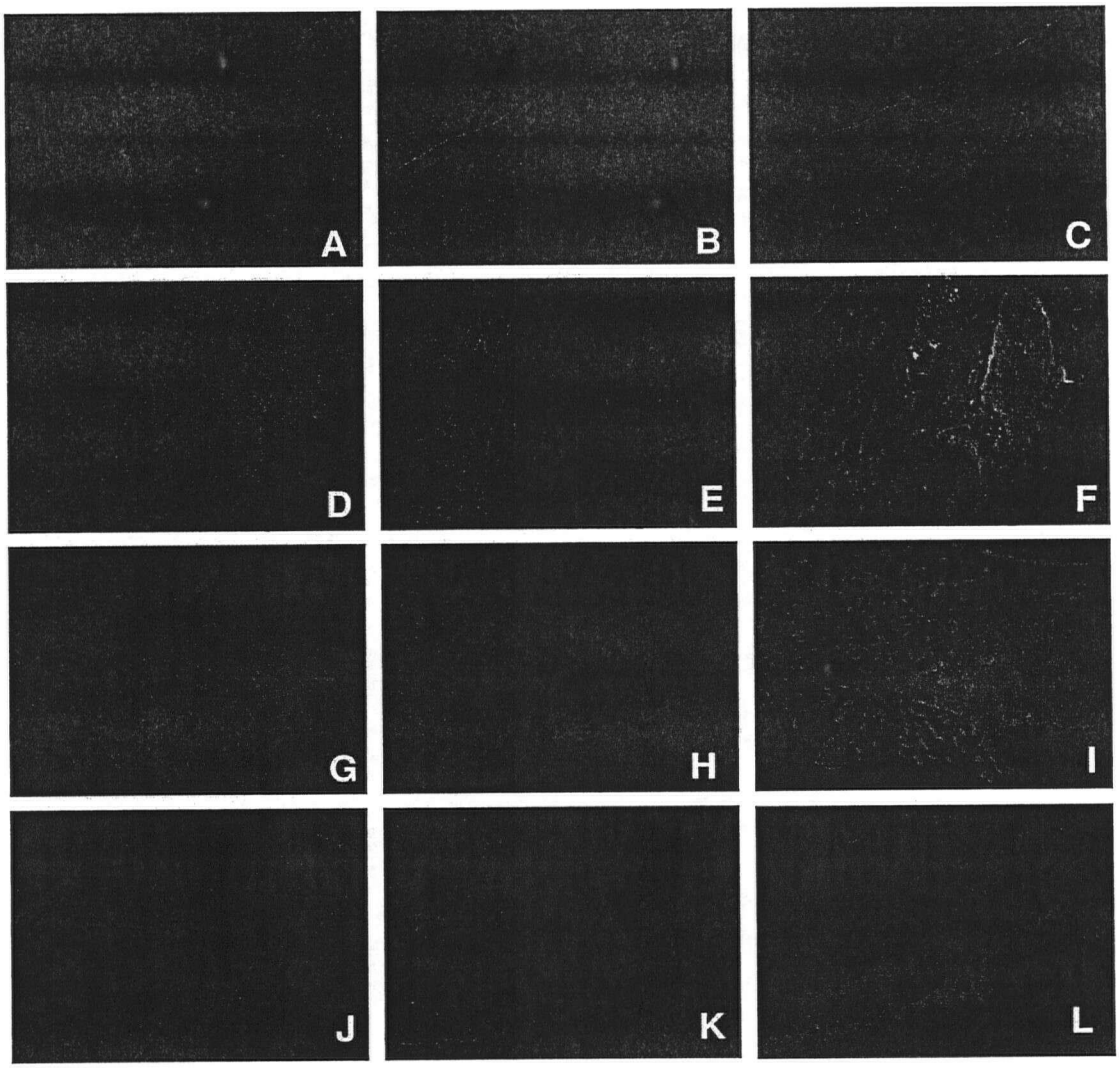


Figure 11: Immunoflorescent double staining for Beta 6 and active TGF-beta 1

A,D,G and J pictures are staining for β 6 taken using a FITC filter for blue light, where A is pyrogen free water group, D is the LPS group, G is the non-blocking Ab group and J is the blocking Ab group; pictures B,E,H and K are the staining for active TGF-β where B is the pyrogen free water, E is the LPS group, H is the non-blocking Ab group and K is the blocking Ab group. These latter pictures are taken with a Rodh filter. The C, F, I and L pictures are merged images of the FITC and Rodh pictures.

3.1.5 Immunostaining for inflammatory cells.

Immunostaining for inflammatory cells (pan B, pan G, CD4, CD3, ED1), proliferating marker Ki67 and ICAM-1 could not delineate any specific cells over and above background staining, This may or may not reflect treatment of the specimens prior to staining. Therefore there are no representative results presented. Extracellular matrix molecules markers were identified by staining for Collagen I, tenascin and fibronectin. No staining difference between groups was observed.

3.2. Mice Model of Periodontal Disease

3.2.1 Morphological analysis of mesial-distal sections

B6 KO mice, TSP-1 KO mice, and double KO mice were used to assess the possible role of two TGF-β1 activating agents (αvβ6 and TSP-1) in changes seen in junctional epithelium during periodontal disease. The wild type (mouse strain=129Sv) was used as control.

In the wild type control mice the epithelium does not migrate and the alveolar bone is close to the CEJ. In the TSP-1 KO mice the junctional epithelium is in general thicker than in the wild type and presents gaps within the JE. This unorganized and gap filled JE is even more evident in

the β6 KO and all through the pocket epithelium in the double KO. Alveolar bone degradation is present in the double KO as can be seen in Figure 12.

At higher magnification ((20X) Figure 13) it is more obvious that the distance between the CEJ and the alveolar bone increases from the wild type to TSP-1 KO, to β6 KO with the largest increase being seen in the double KO (where the alveolar bone is absent at this magnification). The sponge-like epithelium can be observed better at this magnification as well. Just below the CEJ and above the alveolar bone the transseptal fiber bundles (TSF) can be observed in panels A and B. In panel C due to the elongation of the JE and ECM reorganization the TSF seems to move along with the pocket epithelium formation to confer some surrogate support and to substitute the alveolar bone that sustains the tooth in health. In panel D the parallel arrangement of the TSF is visible throughout the entire surface between the teeth. The epithelium migrates along the tooth root for a longer distance in the double KO mice compared to the β6 KO. In both β6 KO and double KO intra-bone pocket formation cause the transseptal fibers to be oblique and the bone destruction pattern is vertical or angular.

Inflammatory leukocytes infiltrate can be observed under 40X magnification (Figure 14). In the wild type fewer mononuclear cells are observed comparing to the TSP-1 KO, β6 KO and double

KO mice. PMNs comprise the majority of the inflammatory infiltrate in the wild type mice. The epithelial cells in the TSP-1 KO are elongated and have large interstitial spaces in-between. Large gaps are seen all through the epithelium in TSP-1 KO, and in the pocket epithelium in β6 KO and double KO mice sections.

Mesial-Distal Sections 4X Murine Model

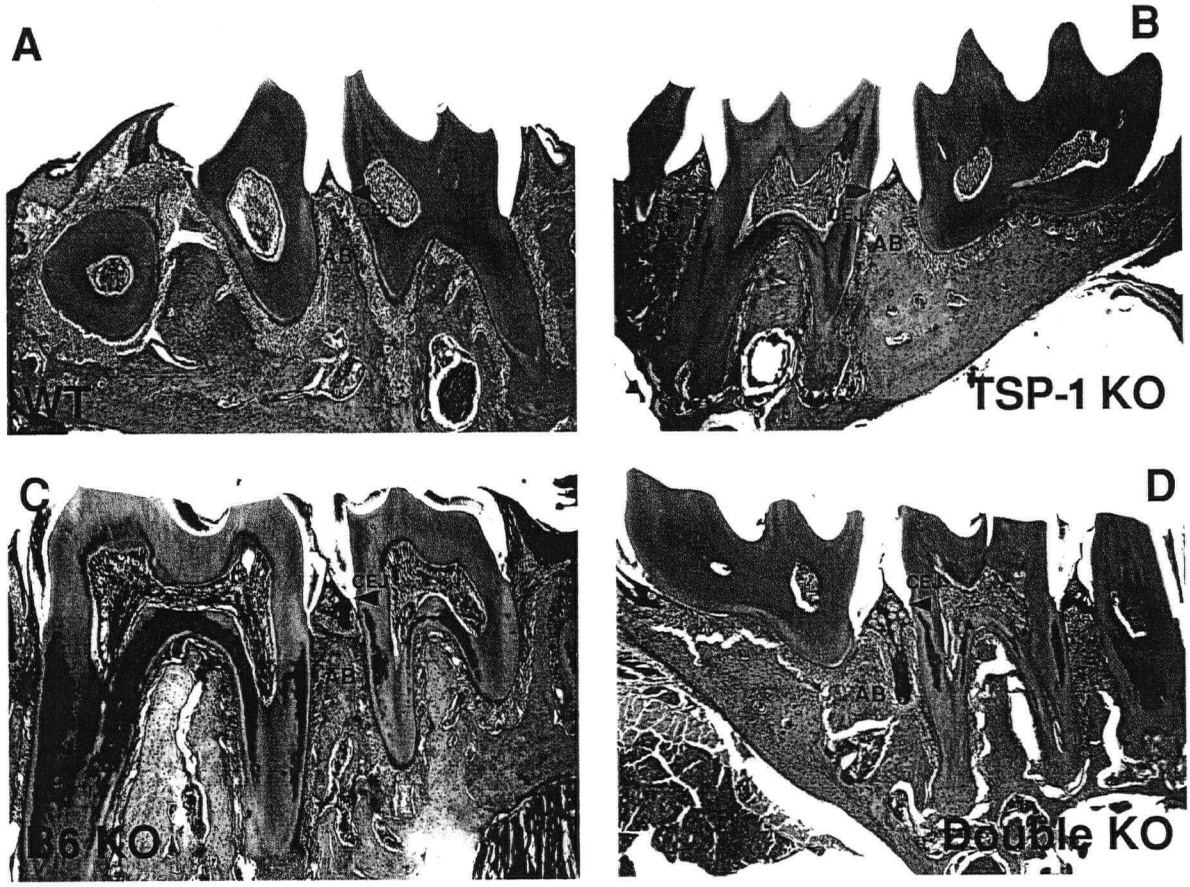


Figure 12: Mesial-Distal Sections 4X Murine Model in a Pentachrome Staining; nuclei stained dark purple to black, muscle, epithelia stained red, bone and dentin stained green to blue. The alveolar bone (AB) is intact in A, B and C while in picture D there is some bone loss accompanying the elongation of the junctional epithelium below the cementun enamel junction (CEJ)

Mesial-Distal Sections 20X for Murine Model

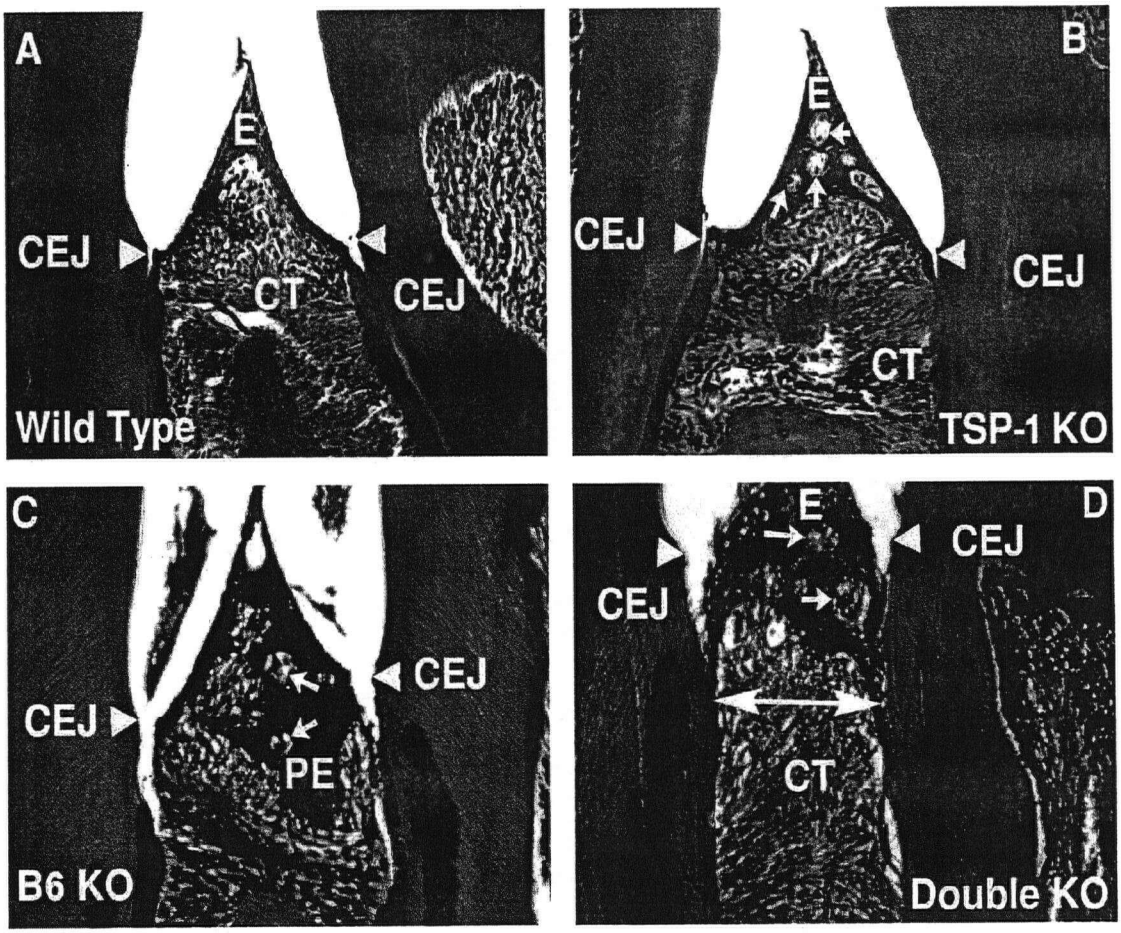


Figure 13. Mesial-Distal Sections 20X for Murine Model Pentachrome Staining: figure A and B present normal epithelium compared to images C and D where the epithelium migrated along the root of the tooth (double head arrow); in figures B,C and D epithelium present hollow spaces with inflammatory cells inside (small white arrows).

Mesial-Distal Sections 40X for Murine Model

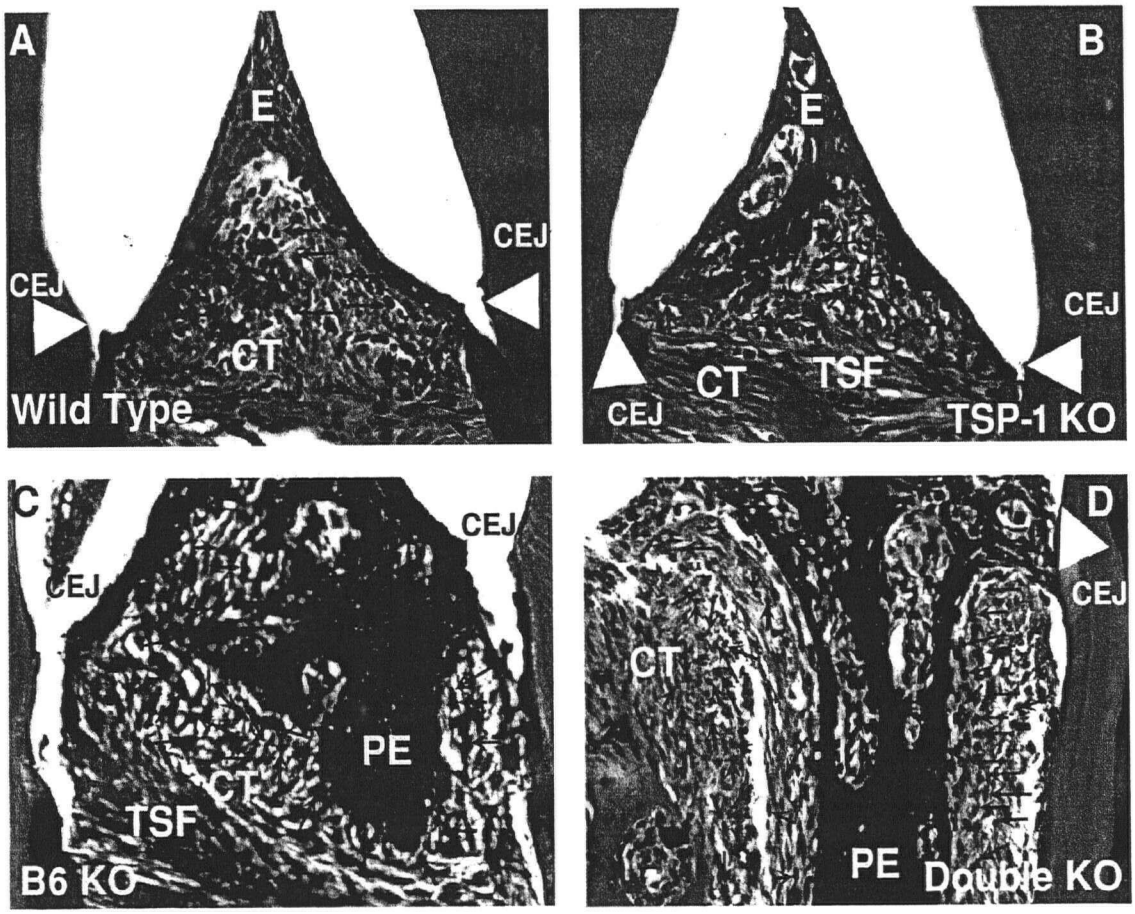


Figure 14: Mesial-Distal Sections 40X for Murine Model 40X magnification: PMNs are present in picture A (blue small arrow heads), few monocytes are present around the oral epithelium (red arrows). The oral epithelium appears to be thinner and denser (A) than in the other groups (B, C and D). In B large inflammatory infiltrate present around the oral epithelium (red arrows) and intact transseptal fiber bundles (TSF); In C a large inflammatory infiltrate (red arrows) shows around the pocket epithelium that formed below the cementum enamel junction (CEJ) and also can be seen displacement of the transseptal fiber bundles (TSF), that has an oblique orientation; All epithelia in B, C and D have hollow spaces in which inflammatory cells reside.

3.2.2. Morphological analysis of buccal-lingual sections

In buccal- lingual sections the morphology is also represented by an epithelium with a sponge like appearance in the TSP-1KO, β6 KO and double KO mice (Figure 15). Junctional epithelium migrates below the cementum enamel junction in both β6 KO and double KO mice. Inflammatory infiltrate is increased in all knock out groups (Figure 16). In the area between oral gingival and junctional epithelium, where the gingival epithelium bends apically and joins the coronal portion of the junctional epithelium it can be observed that junctional epithelium in all knock out groups is thicker compared to the wild type group and the intercellular spaces are larger. Epithelial cells are elongated in sections taken from both β6 KO and double KO mice. All area and length average measurements are presented in Figures 17 and 18. The areas and length of gingival migration was significantly different between all groups with the double KO and the B6 KO demonstrating the most effect. It was interesting that there was no migration in TSP-1 KO similarly to the wild type groups.

Buccal-Lingual Sections 20X for Murine Model

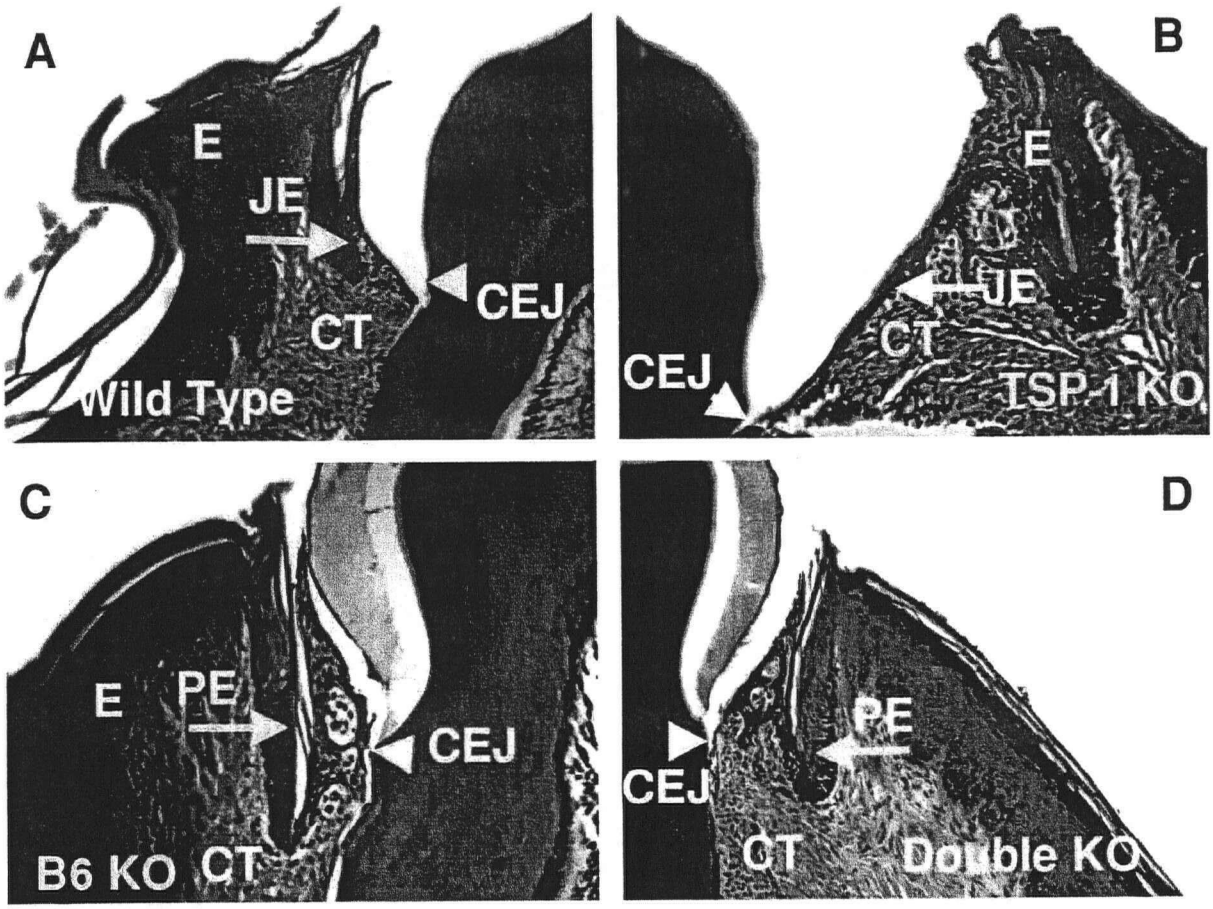


Figure 15: Buccal-Lingual Sections 20X for Murine Model 20X magnification: In (A) the gingival oral epithelium (OE) bends apically and joins the coronal portion of the junctional epithelium (JE) which is a characteristic morphological feature for rodents; in (B) compared with the wild type mice the epithelium is thicker with wider intercellular spaces and presents hollow gaps containing inflammatory infiltrate.

Buccal-Lingual Sections 40X for Murine Model

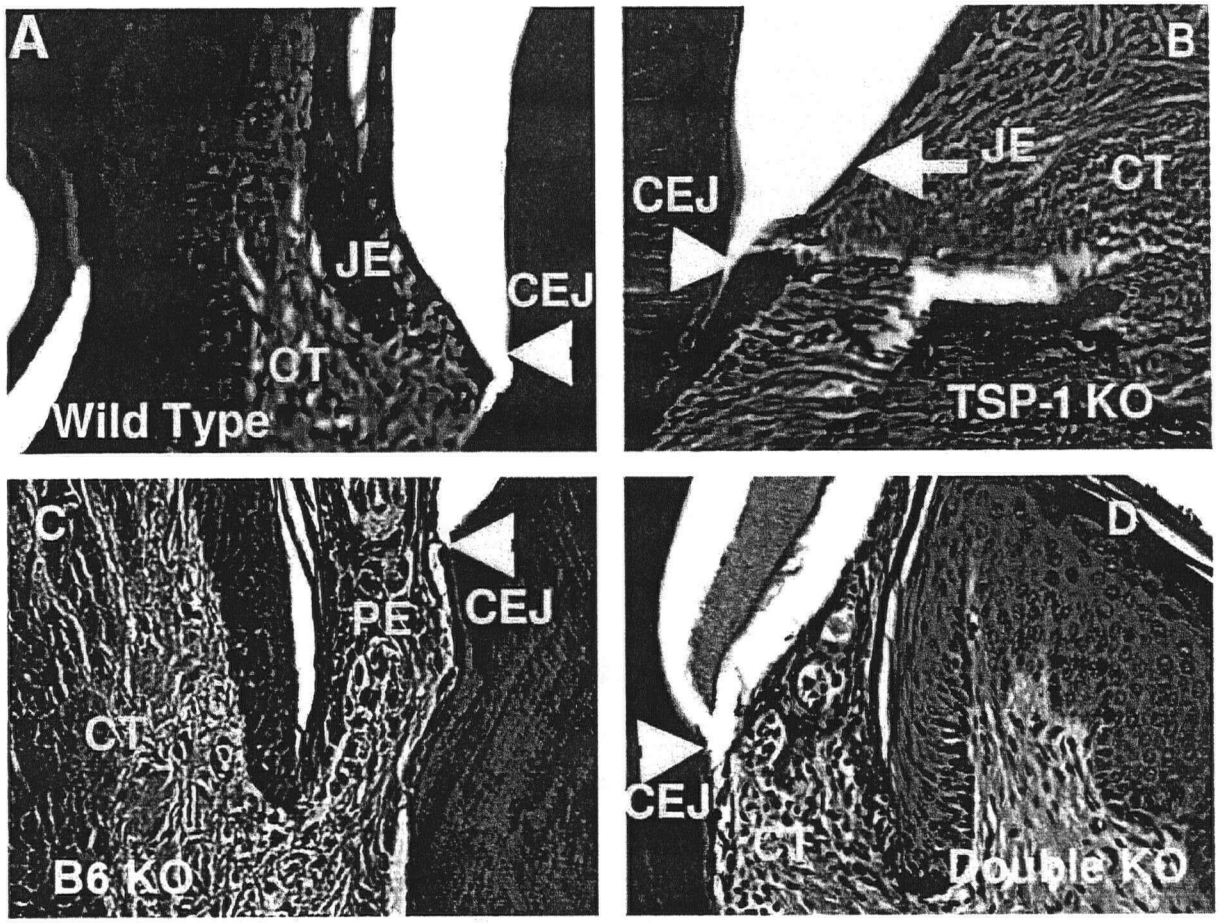


Figure 16: Buccal-Lingual Sections 40X for Murine Model: Inflammatory infiltrate preset around the junctional epithelium in C and D; Junctional epithelium has migrated down the root (C,D) and the loop formed by the oral epithelium and JE is longer than in the Wild Type or TSP-1 KO; the junctional epithelium presents large hollow spaces containing inflammatory cells (D).

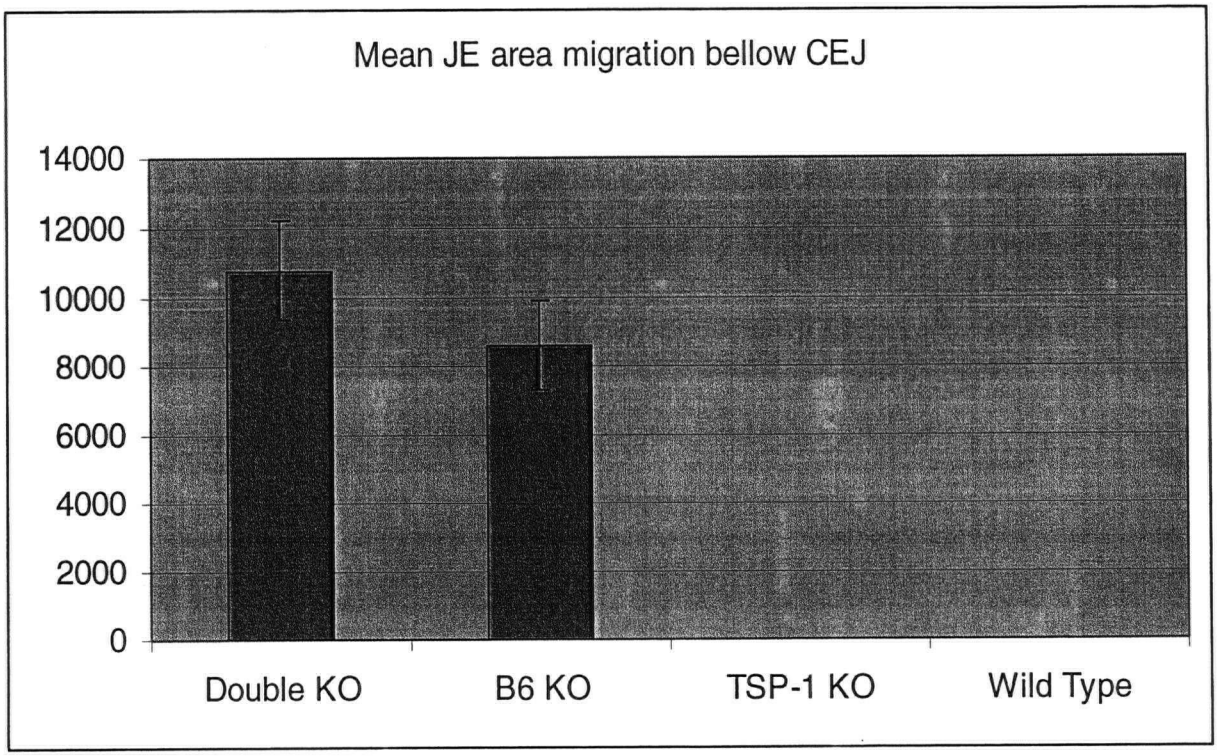


Figure 17. JE Area Below CEJ in the Murine KO Model. JE area migration below cementum enamel junction into the connective tissue was found statistically significant amongst the groups with ANOVA ($p<0.05$). Tukey test found that the difference between the double KO and β6 KO is not significant; the difference between these two and the wild type and TSP-1 KO is significant. The units on the y axis are arbitrary. These values present data on 14 mice.

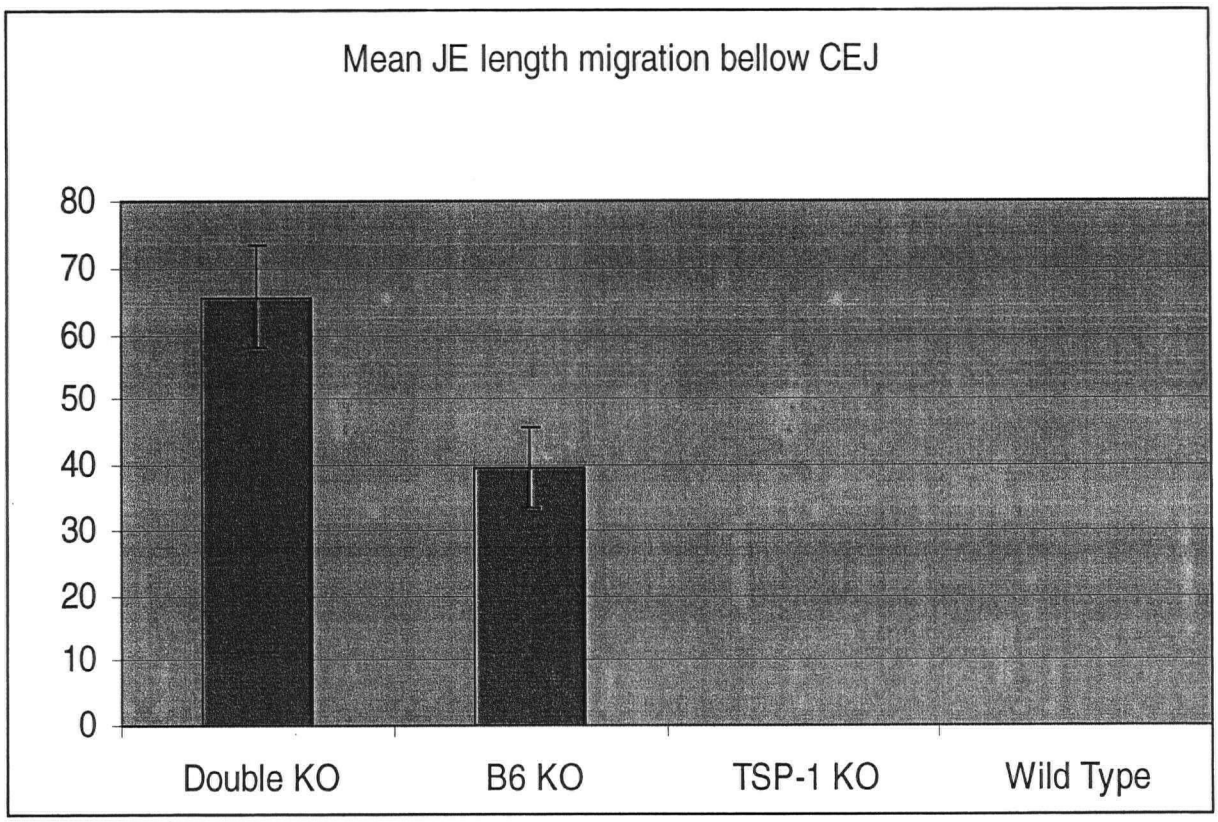


Figure 18. JE length Migration Below CEJ in the Murine KO Model: JE length migration was found statistically significant between all groups with ANOVA ($p<0.05$). Tukey test (95% confidence interval) found that there is significant difference between all groups ($p<0.05$): the measurements for the TSP-1 KO and the wild type group are zero. The y axis measurement units are arbitrary units. These values present data on 14 mice.

CHAPTER IV: Discussion

Periodontal disease is a clinical disease comprising a group of inflammatory conditions in the supporting tissue around the teeth, caused by a combination of specific bacteria, host susceptibility and environmental factors. Periodontitis presents as gingival inflammation at areas of apical migration of epithelial attachment at root surfaces with concurrent loss of connective tissue and alveolar bone.

In the oral cavity, the attachment between the gingival epithelium and the root surface is mediated by a unique epithelium called the junctional epithelium. The junctional epithelium provides a barrier against bacterial penetration. Cells of the junctional epithelium are specialized in attachment formation as well as being capable of movement and of positional change. This latter feature provides the junctional epithelium with the ability to advance and retract (Stern, 1981).

Pathologically, when mild inflammation occurs the junctional epithelium is the major path for the inflammatory exudates from adjacent blood vessels. Neutrophils and mononuclear cells (mainly lymphocytes) are found in the connective tissue in the proximity of the junctional epithelium. These cells can be passively carried into the sulcus as a result of junctional

epithelium turnover, as well as in response to chemoattractant gradients provided by bacterial products (Schroeder and Listgarten, 1971; Schroeder and Listgarten, 1997; Bosshardt and Lang, 2005). In the case where inflammation is prolonged the connective tissue breaks down and the junctional epithelium proliferates apically and migrates down the tooth root surface, extending long pseudo-rete ridges into the inflamed connective tissue thus forming the pocket epithelium (Larjava et al., 1996).

In this thesis we investigated the lack of αvβ6 integrin and its role in periodontal disease in two animal models.

4.1. Rat Model of Periodontal Disease

Active TGFβ-1 is an immunosuppressive cytokine suppressing LPS-induced TNFα responses in macrophages (Chen et al., 2001) as well as acting as a growth inhibitor of the junctional epithelium (Lu H et al., 1997). Recent evidence has indicated that apoptotic cells are possible sources of inflammatory regulators by releasing the anti-inflammatory cytokines, IL-10 (Gao et al., 1998 and Voller al., 1997) and TGF-β (Fadok et al., 1998).

In this study we show that αvβ6 is an epithelial regulator of inflammation with additional functions in inhibition of junctional epithelium growth and migration. Inhibiting this integrin with antibodies caused the formation of a long epithelial mass attached to the root surface hence providing protection against bacterial LPS. Morphological analysis consisting of quantification of the area and length migration of the JE below CEJ, demonstrated that in the experimental group where the integrin αvβ6 was blocked with a functional blocking antibody 6.3G9 there was a significant increase in junctional epithelium growth and migration in comparison to the control group treated with LPS and LPS plus the nonblocking antibody (7.8B3). Similarly, the inflammatory infiltrate when quantified appeared to be significantly increased in the blocking antibody group (6.3G9) in comparison to the other groups.

In our rat functional experiment, the presence of LPS and of the blocking antibody 6.3G9 also caused the formation of long epithelium formation or pocket epithelium. This was measured as area of migration and length migration below CEJ. This effect was significantly enhanced in the experimental group (6.3G9) when compared to the other treatment groups where a non-blocking Ab (7.8B3+LPS) or when just LPS was used suggesting that inhibition of the αvβ6 integrin directly affects epithelial cell growth. This could be a result of inhibition of activation of TGFβ-1 that functions as an epithelial growth inhibitor. In the rat group that was treated with the non-

blocking Ab (LPS+7.8B3 Ab) it was found that the antibody did partially blocked the $\alpha v\beta 6$ integrin and therefore the presence of pocket epithelium in that group was significantly larger ($p<0.05$) than the pocket formation in the LPS treated group when the area below CEJ was measured. The non-blocking antibody 7.8B3 was described as having very low blocking potency in the cell adhesion format (Weinreb et al., 2004).

Comparison of LPS and pyrogen free H_2O group was not significant for area of migration. The length of the long epithelium that migrated along the tooth root was significantly different between all groups indicating that epithelial migration occurs before epithelial growth and possibly plays a more important role in the defense against pathogens in the periodontal disease. Other researchers using LPS from E-coli reported similar results where the JE formed a long epithelium along the tooth root surface (Suzumara et al., 1989, Iwamoto et al., 1998, Ekuni et al., 2003). This can indicate that proliferation and growth occur and migration is not necessarily dependent on the two processes.

The results for the inflammatory infiltrate showed that the treatment with a blocking Ab for the $\alpha v\beta 6$ integrin (6.3G9+LPS group) increased the mononuclear cell infiltrate in comparison to the non-blocking Ab (7.8B3+LPS group) plus LPS group and the pyrogen free water treated group,

thus reflecting the indirect immunosuppressive role of this integrin through the possible activation of TGF-β. There is a high biological variability in the treatment groups as indicated by the high standard deviation within the groups.

As TGF-β is a ligand for αvβ6 integrin (Munger et al., 1999, Sheppard et al., 2001) we looked at the co-localization of these two molecules in sections from our treated groups. Active anti-TGF-β fluorescent staining for the rat sections is less visible in the junctional epithelium where the blocking antibody was used as a treatment. In contrast strong staining was present in the pocket epithelium of the LPS group sections- indicating that in the 6.3G9+LPS group TGF-β was not activated. There was a difference between the blocking and non-blocking treatment groups in activation of TGF-β. It was noted that the staining for αvβ6 integrin was very weak and it was difficult to determine whether it truly reflected integrin staining or was a "bleed through" from the red channel. Therefore determination of co-localization was not possible.

Proteinases of host origin are involved in the degradation of extracellular components of connective tissue and epithelium and seem to have the potential to contribute to the lateral and apical proliferation of the junctional epithelium into the connective tissue (Pöllanen et al., 2003). For example matrix metalloproteinases (MMPs) that are expressed by inflammatory cells i.e.

monocytes, macrophages, lymphocytes, polymorphonuclear cells and residential cells i.e. fibroblasts, epithelial cells, and endothelial cells are representative for connective tissue degradation (Reynolds et al., 1994). Specifically MMP2 and MMP9 are found in mild to advanced inflammation, in which high MMP9 levels are prevalent (Ejiel., et al. 2003). In our study the gels were far from perfect. However it appeared that active MMP9 and pro-MMP9 levels were low. In contrast high levels of pro-MMP2 and active MMP2 were observed. This suggests that after 8 weeks of treatment there is not much connective tissue degradation in spite of elongation of the JE. In comparison to a study in which the migration of human oral keratinocytes on fibronectin occurred when αvβ6 was bound to this ligand, and resulted in unregulated secretion of the pro-enzyme form of type IV collagenase, matrix metalloproteinase-9 MMP-9 (Thomas et al., 2001).

4.2 Mouse Model of Periodontal Disease

αvβ6 integrin was previously identified as a modulator of epithelial inflammation in a mouse knock out model for beta 6 subunit (beta 6-/-) . It was found that these mice presented with juvenile baldness associated with inflammatory infiltrate in the skin and accumulation of lymphocytes around conducting airways in the lungs (Huang et al., 1996). Both β6 KO and β6

over-expressing mouse models (Häkkinen et al., 2004) do affect normal wound healing. Therefore, it is possible that the importance of αvβ6 integrin dependent TGF- β activation could be reflected in immunosuppressive situations. To observe which the major activator of TGF- β is, knock out mice for TSP-1 have been studied. It was observed that the most prominent features of the TSP1-null mouse were epithelial hyperplasia, leukocyte infiltration, and acute and chronic inflammatory changes involving the lungs (Lawler, et al., 1998), as well as milder inflammatory changes in the pancreas (Crawford et al., 1998). The inflammatory changes seen inTSP1-knockout mice are not as severe as those seen in TGF-β1-knockout mice (Crawford et al., 1998).

Our study, comprising mice homozygous for a null mutation in the gene encoding the β6 subunit; TSP-1 KO mice; and mice having both a mutation for β6 subunit and TSP-1 (double KO). The control for the experiment was a wild type group of mice sharing the same genetic background as the KO's. It was found that αvβ6 integrin plays a role in both junctional epithelial (JE) growth and migration. It is possible that this effect could have occurred by activation of TGF- β. The difference between the double KO and β6 KO (JE area migration below the cementum enamel junction into the connective tissue) was not significant. The difference between these two and the wild type and TSP-1 KO was, however, significant. Therefore it can be concluded that TSP-1 has a slight effect on TGF- β activation that is synergistic in nature and

a difference can be noticed only when accompanied by the β6 KO gene as it is in the double KO group.

4.3 Conclusion

In conclusion we have shown that junctional epithelial cells expressing $\alpha v\beta 6$ have a protective role in periodontal disease. They form a barrier to bacterial insult by providing junctional epithelium integrity when epithelial cells migrate. In our studies we found that $\alpha v\beta 6$ expressing cells maintained normal tissue homeostasis. Disruption of $\alpha v\beta 6$ led to increased migration of JE migration and growth of epithelial tissue. Moreover $\alpha v\beta 6$ itself may have an indirect role in immunoregulation probably through activation of TGF- β. As above blocking $\alpha v\beta 6$ integrin increased inflammatory infiltration. Our findings suggest that over expression of $\alpha v\beta 6$ could lessen inflammation and periodontal destruction and thereby providing a proof of concept for $\alpha v\beta 6$ as a therapeutic modality in the treatment of periodontal disease.

4.4 Recommendation for future studies

Future studies will be needed to compare the anti-inflammatory role of TGF- β activated by $\alpha v\beta 6$ and the presence of IL10 that is also an anti-inflammatory cytokine. A functional experiment could use an anti-IL10 antibody in addition to the anti $\alpha v\beta 6$ antibody. Moreover it will be interesting to look at the JE in IL10 KO in comparison to β6 KO and quantify the inflammatory infiltrate in these two specimens.

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