EXPRESSION OF ALPHA v BETA 6 INTEGRIN IN PERIODONTAL DISEASE

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Dental Science)

THE UNIVERSITY OF BRITISH COLUMBIA

August 2007

Abstract

Integrins are the principal cell surface receptors that mediate cell-to-cell or cell-to-extracellular matrix (ECM) binding, providing adhesion for stationary cells, traction during cell movement and, importantly, translating extracellular matrix cues into intracellular signal transduction pathways. The αvβ6 integrin, an exclusively epithelial integrin, exhibits limited distribution in the body. In adult tissue, αvβ6 integrin is expressed during inflammation, carcinogenesis, and in wound healing. It is not expressed in oral gingival epithelium but it is constitutively expressed in junctional epithelium (JE). Its capability to bind and activate transforming growth factor-β (TGFβ) suggests immune regulation and it could therefore play a protective role against periodontal disease. When comparing hematoxylin and eosin stained paraffin sections of wild-type (FVB) and β6 integrin-knockout mice (β6 -/-) under the light microscope, apical migration of junctional epithelium beyond the cemento-enamel junction (CEJ) resulting in formation of pocket epithelium (PE) was clearly demonstrated only in specimens of β6 integrin-knockout animals. In addition, analysis of defleshed mandibles revealed a significant increase in alveolar bone loss and therefore enhanced exposed root surface area and furcation involvement for knockout mice in comparison to their age matched wild-type animals (FVB). The findings of this study suggest that αvβ6 integrin, exclusively expressed in JE, might play an important role in the pathogenesis of periodontal disease in mice. One possible mechanism could be through its regulatory function in the activation of TGFβ.

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LIST OF ABBREVIATIONS

αvβ6	Alpha-v- beta-6
AP	Alveolar process
BP	Bullous pemphegoid antigen
β6 -/-	Beta-6 integrin-Knockout
CEJ	Cementoenamel junction
CEM	Cell Adhesion Molecule
DAT	Directly Attached to Tooth
EBL	External Basal Lamina
ECM	Extracellular Matrix
EnaC	Apical sodium channel
EM	Electron microscopy
FoxP3	Forkhead transcription factor P3
FVB	Wild-Type
GCF	Gingival crevicular fluid
H&E	Hematoxylin and Eosin
HD	Hemidesmosome
IBL	Internal Basal Lamina
ICAM	Intercellular Adhesion Molecule
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin

IL-1Ra	Interleukin-1 receptor antagonist
INF-γ	Interferon-γ
iTreg	Induced T regulatory cells
JE	Junctional Epithelium
LAP	Latency-associated peptide
LFA	Lymphocyte function antigen
LLC	Large latent complex
LTBP	Latent TGFβ-binding protein
MHC	Major histocompatibility complex
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NK	Natural killer cell
nTreg	Natural T regulatory cells
OSCC	Oral squamous cell carcinoma
PE	Pocket epithelium
PGE2	Prostaglandin E2
PMN	Polymorphonuclear
RGD	Arginin-glycine-aspartate
SCC	Squamous cell carcinoma
SLC	Small latent complex
Teff	Effector T cells
TGFβ	Transforming Growth Factor-beta
TNF	Tumor Necrosis factor

TNF-R1	Tumor Necrosis factor receptor 1
TNF-R2	Tumor Necrosis factor receptor 2
Treg	Regulatory T cells
TSP	Thrombospondin

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Hannu Larjava, for his continuing support, guidance and patience during my studies. I would also like to thank Dr. Lari Häkkinen who has been very supportive and helpful and always willing to provide assistance.

I am very grateful to Dr. Tassos Irinakis, a great mentor who always went the extra mile to support and guide me throughout the last three years. Thank you for believing in me.

I would also like to thank Mr. Cristian Sperantia for his technical support.

I want to thank Dr. Leeni Koivisto, Ms. Daniela Nica, Dr. Ameneh Eslami, Mr. Heidar Alimohamad, Dr. Yangshuang Xie, Dr. Guoqiao Jiang, and Mr. Andre Wong for all their help and instruction during the laboratory work.

A special thanks to my classmate Dr. Tarek Ali, a great friend, for sharing the long hours of studying with me. Thank you Tarek for making this journey more pleasant.

Thank you to Mrs.Vicky Koulouris who was always there when the ride was rough and unbearable.

Thank you to Mrs. Ingrid Ellis who generously provided editorial assistance.

I am very grateful to all the professors and clinical instructors for sharing their knowledge and expertise with me. Thank you for teaching me.

I would also like to thank all the staff at UBC especially Mrs. Miriam Dexter, Mrs. Nancy Perl, and Mrs. Cherry Ong for their daily support and patience.

Special thanks goes to my brother, Farzan and my brother-in-law Arash for providing their assistance and expertise from a far distance in writing my thesis.

I would like to thank both families for supporting us tremendously throughout the last three years. Without your help this wouldn't be possible.

Most of all, I want to thank my loving wife, Azadeh and my little daughter, Rose. Thank you Azadeh for all your sacrifice, your love, your patience and most of all for being such a wonderful mom and dad for our precious Rose. You put your career on hold to support me in this journey. I will always be grateful. I love you very much and cannot wait to give you the same love and support when you go after your dreams. To you my little Rose, even though I knew you were in very good hands, I am still sorry that I wasn't there for you. I promise there won't be any days anymore that daddy closes the door. Soon will be your turn to lock out me. I love you very much.

DEDICATION

This work is respectfully dedicated to my parents Abdoljavad and Roohangiz. Both of you have sacrificed the most beautiful moments of life (seeing your children grow), given up your happiness and joy to provide me and my brother with a better life and a brighter future. I am who I am because of what you have taught me. You taught me to love, to respect, to share and most of all never to give up going after my dreams. Even being far away, you were and always will be by me. You left giant footprints in my world and in my heart. I love you very much and I am very proud of who you are.

CHAPTER I

1. αvβ6 integrin

1.1 Structure

Integrins are the principal cell surface receptors that enable normal as well as transformed cells to attach to and respond to their extra-cellular environment. They mediate cell-to-cell or cell-to-extracellular matrix (ECM) adhesion, providing adhesion for stationary cells, traction during cell movement and, importantly, the promotion of many signaling pathways that regulate diverse processes such as proliferation, cell survival, migration, differentiation, tumour invasion and metastasis (Watt, 2002). In addition to ligand binding, integrins aggregate together, resulting in side-by-side placement of many signaling and structural molecules that are associated with their cytoplasmic tails, allowing them to interact (Yamada and Even-Ram, 2002). In this way they serve as the major mechanism for translating extra-cellular matrix cues into intracellular signal transduction pathways (Liu et al., 2000).

Structurally, integrins are heterodimers composed of two different, non-covalently associated α and β subunits. Each subunit is a type I transmembrane glycoprotein that has relatively large extracellular domains and, with the exception of the β4 subunit, a short cytoplasmic tail (Hynes, 2002). The extracellular domain is composed of a membrane-distal, globular head (that contains the ligand binding) on two long stalks (Hynes, 2002). The carboxy (C) termini of the α and β subunits traverse the cell membrane and extend a short distance (usually < 60 amino-acid residues) into the cytoplasm (Hynes, 2002). Presently, 18 α and eight β subunits have been identified that form 24 different

heterodimers, each of which can bind to a specific repertoire of cell-surface, ECM or soluble protein ligands.

β6 integrin is composed of 788 amino acids. The cytoplasmic tail of β6 integrin differs from other related β-subunits by having an extra sequence of eleven amino acids at its carboxy terminal, suggesting different interactions with cytoplasmic components (Sheppard et al., 1990). β6 subunits can only form heterodimers with αv, whereas αv subunits can pair with multiple β subunits such as β1, β3, β5, β6, and β8 (Hynes, 2002).

The αvβ6 integrin is an exclusively epithelial integrin that is highly expressed during fetal development and is downregulated in differentiated adult epithelia (Breuss et al., 1995). Its distribution in the body is limited. αvβ6 integrin is expressed in epithelial cells of the kidney tubule (more specifically, the macula densa), endometrium in a uterus in the secretory phase, salivary gland ducts, gall bladder and epididymis (Breuss et al., 1993). Monaghan et al. (2005) recently published evidence of integrin-αvβ6 expression by cells within the stratified squamous epithelium of the tongue, ventral soft palate, interdigital skin, and coronary band of cattle. αvβ6 integrin is absent from normal healthy epidermis with the exception of root sheath cells of the hair follicles (Häkkinen et al., 2004) and oral mucosa with the exception of junctional epithelium and interdental papilla epithelium (Garcia, 2005; Csiszar et al., 2007). In adult tissue, αvβ6 integrin is expressed during inflammation, carcinogenesis, and in wound healing (Breuss et al., 1995).

1.2 Function

Integrin αvβ6 belongs to a subfamily of integrins which recognize amino acid sequences that share a common arginine-glycine-aspartate or RGD motif (Ruoslahti and Peirschbacher, 1987; Hynes, 2002). In addition to mediating cellular adhesion to extracellular matrix proteins such as fibronectin (Busk et al., 1992), tenascin-C (Prieto et al., 1993), and vitronectin (Huang et al., 1998), integerin-αvβ6 binds TGF-β1 latency-associated peptide (LAP) through an RGD-dependent mechanism and participates in the conversion of TGF-β1-LAP into active TGF-β1 (Munger et al., 1999; Annes et al., 2004). Over the last decade an increased expression of αvβ6 integrin has been associated with many different functions such as promotion of cell migration, control of cell proliferation, activation of TGFβs, suppression of apoptosis, modulation of protease activity and mediating invasion of carcinoma cells (Thomas et al., 2006). It has been demonstrated that αvβ6 integrin promotes migration of human primary oral keratinocytes on fibronectin, and the binding of αvβ6 integrin to this ligand upregulates secretion of the pro-enzyme form of type IV collagenase, matrix metalloproteinase-9 (MMP-9) (Thomas et al., 2001). Although αvβ6 integrin is not expressed constitutively in healthy epithelia, however it is upregulated during tissue remodelling, including wound healing and carcinogenesis (Breuss et al., 1995).

1.3 Expression of αvβ6 integrin in normal and wound keratinocytes

Integrins including αvβ6 play a significant role in the regulation of keratinocyte function and in wound healing (Häkkinen et al., 2004). The upregulation of type IV collagenase MMP-9 by αvβ6 would facilitate cell movement by allowing detachment from the

basement membrane (Thomas et al., 2001). Although αvβ6 integrin is not expressed in normal basal keratinocytes, its expression is upregulated on wounding (Larjava et al., 1993; Breuss et al., 1995; Haapasalmi et al., 1996; Häkkinen et al., 2000; Larjava et al., 2002). In wounds, at least two integrins, α5β1 and αvβ6, are expressed by keratinocytes. Both of these integrins are co-expressed, resulting in cellular interaction with fibronectin (Larjava et al., 1993; Clark et al., 1996). Several studies on human and animal wounds have shown that β6 is detectable in keratinocytes at the wound edge (Breuss et al., 1995; Clark et al., 1996; Haapasalmi et al., 1996). Despite the fact that αvβ6 integrin was expressed by migrating keratinocytes in early wounds, the maximal expression of this integrin was seen at a relatively late stage of mucosal and dermal wound healing during granulation tissue formation and basement membrane reorganization, when migrating edges of the wound epithelium have joined (Haapasalmi et al., 1996; Häkkinen et al., 2000; Larjava et al., 2002).

In vitro studies have repeatedly demonstrated that αvβ6 integrin facilitates keratinocyte adhesion and migration on fibronectin (Weinacker et al., 1994), tenascin (Prieto et al., 1993) and vitronectin (Huang et al., 1998), all of which are components of the early wound matrix (Häkkinen et al., 2000). This suggests that αvβ6 integrin may also regulate this process in vivo (Breuss et al., 1995; Clark et al., 1996; Häkkinen et al., 2000; Larjava et al., 2002). However, β6 integrin-deficient mice did not show any change in wound closure rates (Huang et al., 1996). Recently, Häkkinen et al. (2004) showed that expression of αvβ6 in murine skin was strong and relatively uniform on most basal keratinocytes close to the wound edge 3 days post injury which remained strongly

expressed, although less uniform, at initial wound closure. Although the de novo, but transient, expression of αvβ6 integrin by wound keratinocytes is well documented, the molecular mechanisms leading to its expression and eventual disappearance are still unclear (Clark et al., 1996).

1.4 Expression of αvβ6 integrin in carcinomas

Wound healing and carcinogenesis have many biological processes in common, to the point that carcinogenesis has been described as a mis-regulated form of wound healing (Dvorak, 1986). Many of the ECM ligands for αvβ6 integrin are usually modulated and often upregulated during both tissue remodelling and cancer (Chiquet-Ehrismann and Chiquet, 2003). When looking at oral squamous cell carcinoma (OSCC), most cells express high levels of αvβ6 integrin (Hamidi et al., 2000). However, unlike wound healing, carcinoma αvβ6 appears to be permanently expressed and may be responsible for promoting tumour progression (Thomas et al., 2006). Although expression of αvβ6 integrin is restricted to carcinomas, it is not limited to oral and skin SCC. αvβ6 integrin expression has been reported in carcinomas of the lung (Smythe et al., 1995), breast (Arihiro et al., 2000), pancreas (Sipos et al., 2004), stomach (Kawashima et al., 2003), colon (Bates et al., 2005), ovary (Ahmed et al., 2002), salivary gland (Breuss et al., 1995), malignant transformation of oral leukoplakia (Larjava et al., 1993; Hamidi et al., 2000), oral squamous cell carcinoma (Hamidi et al., 2000) as well as skin squamous cell carcinoma (Breuss et al., 1995; Bates et al., 2005). In addition, epithelial cells in samples of lichen planus have also been shown to express αvβ6 integrin (Hamidi et al., 2000). These data suggest that αvβ6 integrin expression may play an active role in these

processes and therefore may be useful in predicting malignant transformation (Impola et al., 2004).

1.5 TGFβ activation

The transforming growth factor βs (TGFβ) are powerful cytokines that affect a variety of cellular processes, including cell proliferation, integrin expression, immune function and development (Blobe et al., 2000). In addition, they also stimulate the expression of ECM proteins, such as tenascin, thormbospondin, fibronectin, vitronectin, and several proteoglycans (Taipale et al., 1998), and play a major role in the regulation of ECM degradation and remodeling (Koli et al., 2001). Defects in TGFβ function lead to a number of pathological conditions such as autoimmune disease and tumour cell growth (Prime et al., 2004). Furthermore, many fibrotic conditions show an increase in TGFβ expression, which appear to be modulated, in part, through the TGFβ-driven trans-differentiation of fibroblasts into myofibroblasts (Sharma and Ziyadeh, 1994). In the mammalian family, so far three different isoforms of TGFβ have been identified, namely TGF-β1, TGFβ-2 and TGFβ-3. They are secreted as heterotrimeric complexes derived from two genes. Each gene encodes a protein of 390-414 amino acids which is processed into two polypeptide chains. While the 249-282-amino acid N-terminal subunit forms the active TGFβ cytokine, the C-terminal subunit with the 112-amino-acid is known as the latency-associated protein (LAP). Each LAP dimer forms a non-covalent complex with a TGFβ dimer known as the small latent complex (SLC), which retains the TGFβ cytokine in an inactive conformation. The SLC is usually complexed with a protein called the latent TGFβ-binding protein (LTBP), forming a large latent complex (LLC). It is

predominantly found in the extracellular matrix (Massagué et al., 2000). LTBP is required for the proper folding and secretion of TGFβ and deposition to the extracellular matrix (Koli et al., 2001). Since TGFβs are secreted into the extracellular matrix as inactive (latent) precursors, activation from this latent state is required for their normal function.

When defining the mechanism by which the various TGFβ isoforms are activated, so far most work has focused on TGFβ1. Both proteolytic and non-protyeolytic mechanisms for activating latent TGFβ1 have been described. In the proteolytic process, proteases involved in TGFβ1 activation include plasmin (Lyons et al., 1990; Sato et al., 1990), urokinase-type and tissue-type plasminogen activators (Nunes et al., 1995; Chu and Kawinski, 1998), matrix metalloproteases-2 and -9 (MMP-2 and MMP-9) (Yu and Stamenkovic, 2000), and cathepsin (Lyons et al., 1988). Their effect is elicited by proteolytic degradation of TGFβ1-LAP. There are three ways in which these proteases might facilitate the activation of latent TGFβ. First they could target the protease-sensitive hinge region in LTBP, leading to the liberation of a still-latent remnant of the LLC, which would have to be further processed for activation (Taipale et al., 1994). Second, proteases could enable the conversion of pro-LLC to LLC in the extracellular environment and thereby render the latent complex activation competent. Third, proteolytic cleavage of LAP, which results in destabilization of LAP-TGFβ interactions, might release active TGFβ from its latent complex (Lyons et al., 1988). However, the non-proteolytic activation mechanism, which involves interactions with TGFβ1-LAP, induces a conformational change and exposes the receptor-binding site in TGF-β1.

Both thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1995; Ribeiro et al., 1999) and the integrins αvβ6 (Munger et al., 1999), αvβ1 (Munger et al., 1998), αvβ8 and, weakly, αvβ5 (Munger et al., 1998) could bind to TGFβ1-LAP. In vitro studies have demonstrated that the binding of thrombospondin-1, αvβ6, or αvβ8 to TGFβ1-LAP results in TGF-β1 activation. The thrombospondin and αvβ6 mechanisms have been further validated in vivo by analysis of thrombospondin-1 (Crawford et al., 1998) and β6 knockout mice (Huang et al., 1996; 1998), which show features that may be attributable to a loss of TGF-β1 activity. The mechanism for TGF-β1 activation by thrombospondin-1 (TSP-1) involves a direct interaction between TSP-1 and LAP (Murphy-Ullrich and Poczatek, 2000). A short amino acid sequence (RFK) is believed to be responsible for this activation. Furthermore, in vitro and in vivo studies have identified a tetrapeptide (KRFK) as an additional TGFβ activator which probably elicits effects by disrupting the non-covalent interactions between LAP and TGFβ. The fact that TSP-1 null mice demonstrate a partial phenotypic overlap with TGFβ1-null animals supports the contention that TSP-1 is an in vivo activator of latent TGFβ (Crawford et al., 1998). TSP-1 is also expressed throughout development in a number of tissues, where it may function as a TGFβ activator (Iruela-Arispe et al., 1993; Majack et al., 1987).

αvβ6 was the first integrin to be identified as a TGFβ activator (Munger et al., 1999). The activation results from a conformational change in the latent TGFβ molecule rather than via cleavage of the peptide and is dependent on the ability of αvβ6 to connect with the actin cytoskeleton of the cell. The mechanism of activation depends upon a direct

interaction between αvβ6 and the RGD amino acid sequence present in TGFβ1-LAP (Breuss et al., 1993). Cells expressing mutated β6 subunits, which were unable to interact with actin, could still bind LAP but not activate TGFβ. The LAP of TGF-β3 (LAP-3) also contains an RGD sequence and is similarly activated (Annes et al., 2002). However, TGF-β2 does not contain an RGD sequence (Ludbrook et al., 2003) and therefore can not be activated by αvβ6. Recently, Mu et al. (2002) reported that integrin αvβ8 can activate latent TGF-β1. It is interesting that activation by αvβ8 requires protease (MT1-MMP) activity in addition to the integrin. However, the exact roles of MT1-MMP and αvβ8 in this activation mechanism remain to be elucidated.

Recently, Annes et al. (2004) have identified LTBP-1 as one of the missing links in αvβ6-integrin-mediated TGFβ activation. Their results showed that the hinge region is necessary but not solely sufficient for this activation. Since this region also targets latent TGFβ to the ECM, they were able to demonstrate that without matrix fixation, there is no TGFβ activation. Overall, the results suggest that LTBP-1 enables αvβ6-integrin-mediated activation by both fixing and concentrating the latent complex in the ECM. Based on these observations, they concluded that αvβ6 integrin activates TGFβ by mechanical traction.

1.6 Roles of αvβ6-mediated TGFβ activation in pulmonary disease

Of the five integrins that contain the αv subunit, αvβ6 integrin plays an important and specific role in regulating tissue inflammation and fibrosis, and in models of several

common lung diseases such as acute lung injury and pulmonary emphysema (Sheppard, 2001).

αvβ6 integrin has been shown to regulate the immune system in the lung by activating TGFβ (Munger et al., 1999). In the lungs of healthy adults, TGF-β1 is present in a latent form (Sheppard, 2004). Activation of this latent cytokine through αvβ6 integrin will result in pulmonary fibrosis, even in the absence of any increase in TGFβ protein expression (Munger et al., 1999).

In vivo studies using mice homozygous for a null mutation of the integrin β6 subunit showed an enhanced inflammatory response in the lungs and skin, similar to the exaggerated inflammation seen in mice homozygous for a null mutation of TGFβ1 (Huang et al., 1996). Contrary to the expected progressive tissue fibrosis following inflammation, β6 knockout mice did not develop fibrotic lesions. In fact, they were protected from the pulmonary fibrosis (Munger et al., 1999). This suggests that αvβ6-mediated TGFβ activation possibly plays an important role in fibrosis of epithelial organs such as the lung (Sheppard, 2004).

When analyzing the mechanism by which the mice lacking the β6 subunit develop pulmonary disease, a single gene, the macrophage-restricted metaloprotease MMP-12, was identified as the most highly-induced gene in the lungs of these animals (Morris et al., 2003; Kaminski et al., 2000). MMP-12 is an extracellular matrix-degrading metalloproteinase expressed only by tissue macrophages and placental throphoblasts

(Belaaouaj et al., 1995). In vivo studies comparing β6 knockout to wild-type mice have demonstrated a marked increase in MMP-12 expression in β6 knockout mice (Morris et al., 2003; Kaminski et al., 2000). This finding, as well as the implication of MMP-12 in the development of cigarette-associated pulmonary emphysema in mice (Hautamaki et al., 1997), suggests that the lack of αvβ6-mediated TGFβ activation might have an effect on macrophage function resulting in emphysema (Sheppard, 2004). In fact, further investigation using β6 knockout mice revealed significant abnormalities associated with macrophage morphology and induction of surface markers (integrin αM and MHC II) which are involved in macrophage activation (Huang et al., 1998). Furthermore, aging β6 knockout mice developed a spontaneous, progressive alveolar enlargement over time (Morris et al., 2003). The fact that emphysema was absent in double knockout mice lacking both αvβ6 and MMP-12 indicates the central role of MMP-12 in the process of pulmonary emphysema (Morris et al., 2003).

Further investigations using αvβ6-deficient mice have demonstrated an additional role for αvβ6-mediated TGFβ activation in pulmonary disease. In a well-characterized model of pulmonary fibrosis induced by bleomycin, an anti-cancer drug, β6 knockout mice were protected from pulmonary edema (Pittet et al., 2001). Pulmonary edema occurs through imbalance between the rates of fluid movement into the alveolar spaces compared to the rate of re-absorption across the epithelium (Sheppard, 2004). In vitro studies revealed an increase in the permeability across endothelial monolayers (Hurst et al., 1999) and alveolar epithelial monolayers (Pittet et al., 2001) associated with αvβ6-dependant TGFβ activation. In addition, TGFβ also reduces epithelial sodium re-absorption by decreasing

the expression of the apical sodium channel, EnaC, on the apical surface of these cells (Frank et al., 2003).

CHAPTER II

2. Junctional epithelium

The junctional epithelium as the epithelial component of the dento-gingival unit forms gradually from reduced enamel epithelium, beginning orally and ending at the cemento-enamel junction 3-4 years after the crown breaches the oral mucosa (Ten Cate, 1998).

As part of the marginal free gingiva, JE is surrounded by connective tissue apically and laterally and sulcular epithelium coronally. This stratified squamous epithelium is not keratinized, comprising of the stratum basale adjacent to the gingival connective tissue and the stratum suprabasale facing the tooth surface (Bosshardt and Lang, 2005). Its attachment to the non-renewable tooth surface and the connective tissue occurs through transmembrane cell matrix junctional complexes known as hemidesmosomes (HD) and a basal lamina-like extracellular matrix.

While the external basal lamina (EBL) refers to the basement membrane located at the interface between the basal cells of the JE and the gingival connective tissue, the basal lamina on the tooth side is termed the internal basal lamina (IBL) (Bosshardt and Lang, 2005).

Despite many morphological similarities, IBL exhibits different structural and molecular characteristics when compared to a basement membrane. This is characterized by lacking the typical matrix constituents such as types IV and VII collagen, most laminin isoforms

(e.g. laminin-10/11), and basement membrane proteoglycan perlecan, but expressing laminin-5, an epithelium-specific variant (Hormia et al., 2001).

Hemidesmosomes at the IBL are intracellularly connected to the cytokeratin filaments of the epithelial innermost suprabasal cells, the so-called DAT cells (Directly Attached to the Tooth) (Salonen, 1994). This connection is mediated through the interaction of intracellular proteins bullous pemphegoid antigen (BP-230) and plectin with two transmembrane components of the HD known as bullous pemphegoid antigen (BP-180) and α6β4 integrins (Pöllänen et al., 2003).

The interaction between cell surface molecules involved with the intracellular cytoskeleton and components of the extracellular matrix are essential for cell adhesion, tissue stability, regeneration, and reaction to external signals (Uitto and Larjava, 1991). Therefore, α6β4 integrins mediating the interaction between the intracelluar plectin and IBL through their ligand, laminin-5, are considered to be one of the important components participating in the firm attachment of the epithelial cells to IBL (Ryan et al., 1999). The consensus that binding between integrin α6β4 and laminin-5 supports epithelial cell adhesion is further elucidated by the fact that epithelial downgrowth was noted in the presence of MMP-7 (matrilysin), which is capable of cleaving this binding (von Bredow et al., 1997). Due to its strategically-important location, remarkable cell and extracellular dynamics, as well as its high cellular turnover (Skougaard, 1965; 1970; Demetriou and Ramfjord, 1972), JE regulates tissue homeostasis and enables defense against microorganisms and their products (Schroeder and Listgarten, 1997).

The increased permeability of JE as compared to oral or sulcular epithelium is based on the wide intercellular spaces as a result of the small number of desmosomes connecting cells mechanically (Schroeder and Münzel-Pedrazzoli, 1970). These interstitial spaces provide a pathway for fluid movement to the bottom of the gingival sulcus. This transport of tissue fluid containing a variety of cell types such as PMN, macrophages, and lymphocytes, enables the JE to control and regulate the constant microbiological challenge. While PMNs are located more in the centre of the junctional epithelium and close to the tooth surface (Schroeder and Listgarten, 1997), lymphocytes and macrophages are found mainly in and near the basal cell layer (Schroeder, 1973). Additional cell types found in JE were antigen-presenting cells, Langerhans and other dendritic cells located primarily in the coronal one-fourth of JE and at the border zone to sulcular epithelium (Juhl et al., 1988).

Regulation and maintenance of tissue integrity such as the integrity of epithelial attachment to the tooth surface or epithelial-connective tissue interface seems to be important in initiation of periodontal disease (Bosshardt and Lang, 2005). Junctional epithelium, expressing numerous cell surface or cell membrane molecules involved in cell-to-cell or cell-to-matrix adhesion, is considered particularly important structurally in the regulation and maintenance of normal tissue architecture and function. While cell adhesion molecules (CAM) such as cadherins and integrins are associated with structural integrity, other CAMs like intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function antigen-3 (LFA-3), both members of the immunoglobulin superfamily of recognition molecules, participate in counteracting the bacterial challenge, demonstrating

the active involvement of JE in the host defense process (Tonetti, 1997; Tonetti et al., 1998).

In addition, epithelial cells of JE express various cytokines such as IL-1α, IL-1β, IL-8, and TNF-α. While IL-8 as a chemotactic cytokine is involved in routing PMN's into the bottom of the sulcus, the pro-inflammatory cytokines, IL-1α, IL-1β, TNF-α activate the release of additional cytokines such as interleukin-6 (IL-6) and inflammatory mediators such as prostaglandin-E2 (PGE2), contributing to periodontal tissue destruction (Gemmell et al., 1997). Following contact with lipopolysaccharide, almost all cells in the junctional epithelium demonstrate a strong expression of these cytokines (Miyauchi et al., 2001).

The fact that a clinically healthy gingiva contains an inflammatory infiltrate to a very limited extent resulting in microscopic signs of slight inflammation (Brecx et al., 1987) demonstrates the important role of JE in participating in the first line of host defense by providing a pathway for fluid and transmigrating leukocytes, especially PMNs (Schroeder and Listgarten, 1997). Overcoming this peripheral defense will lead to detachment of JE from the tooth surface and therefore formation of pocket epithelium (Schroeder and Listgarten, 1997). Since this is considered a hallmark in the development of periodontitis, research has been focused on understanding the mechanism involved in conversion of the junctional epithelium to pocket epithelium. Observations of pocket epithelium in humans (Takata and Donath, 1988) and animals (Hillmann et al., 1990) have demonstrated the detachment of DAT cells from the tooth surface resulting in an

intra-epithelial split. Due to the significant correlation between the degrees of gingival inflammation and GCF volume flowing through the intercellular space of the JE (Klinkhamer and Zimmerman, 1969; Attström and Egelberg, 1970; Kowashi et al., 1980), it is believed that the disintegration of the junctional epithelium is partly due to the increased number of transmigrating leukocytes such as monocytes/macrophages, PMNs, T- and B-lymphocytes (Schroeder and Listgarten, 1997).

The junctional epithelium as an 'open system' not only provides passage for cells and substances from the gingival connective tissue into the sulcus, but also allows bacteria and their products to enter the junctional epithelium and the underlying structure (Bosshardt and Lang, 2005). Many studies have demonstrated the ability of the two major pathogens in periodontal disease, Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis, to adhere to and invade epithelial cells (Deshpande et al., 1998; Huard-Delcourt et al., 1998; Lamont and Jenkinson, 1998; Fives-Taylor et al., 1999; Forng et al., 2000; Quirynen et al., 2001). Therefore, it seems reasonable to assume that pocket formation may also be due to subgingival microbial spreading in a susceptible host (Schroeder and Attström, 1980). Recent evidence has shown that the virulence factors produced by P. gingivalis, known as gingipains, are capable of degrading the components of the epithelial cell-to-cell junctional complexes, reducing the adhesion of the cells to extracellular matrices, changing cell morphology, impairing cell motility, and promoting apoptosis (Wang et al., 1999; Katz et al., 2000; Chen et al., 2001; Hintermann et al., 2002; Katz et al., 2002). Furthermore, in an in vitro study by Tada et al. (2003), gingipains reduced the expression of ICAM-1 on human oral epithelial cell lines and

degraded the ICAM-1 in the cell membranes, suggesting that gingipains may also disrupt the ICAM-1-dependent adhesion of PMNs to oral epithelial cells.

Despite this new evidence, the exact mechanisms that lead to formation of pocket epithelium still remain unresolved.

CHAPTER III

3. Pathogenesis of Periodontal Disease

3.1 Introduction

The pathogenesis of periodontitis was categorically explained by Page and Schroeder in 1976. Although the amount of information provided at that time was limited, the general principles and the overall conclusions are still largely accepted today. Periodontal disease is not a single homogenous disease. It is a multifactorial process that represents a group of related, usually chronic, and sometimes aggressive bacterial infectious diseases which result in destruction of periodontal support including alveolar bone and connective tissue (Ranney, 1992; Offenbacher, 1996). Although the bacteria are the initiating force in periodontal disease, the host immune response to the pathogenic infection is critical for disease progression (Genco, 1992; Socransky and Haffajee, 1992). This can be explained by the fact that the presence of specific periodontal pathogens is not sufficient to cause disease in the non-susceptible host (Page, 1999).

While tissue destruction is limited to epithelial cells and collagen fibers from the connective tissue during the initial stage of the disease, progression of periodontal disease is characterized by loss of periodontal ligament and disruption of its attachment to the cemental root surface, as well as resorption of alveolar bone (Offenbacher, 1996). Pocket epithelium is formed when junctional epithelium proliferates and migrates apically on the root surface. It extends long pseude rete ridges deep into the inflamed connective tissue, exposing the epithelial cells to the new matrix components of the chronic granulation tissue (Larjava et al., 1996).

3.2 Microbial challenge

Pellicle formation consisting of proteins and glycoproteins from saliva and crevicular fluid is rapidly observed on the freshly-cleaned tooth surface (Marsh and Bradshaw, 1995). This provides a surface for bacterial attachment and enhances the initial bacterial colonization (Skopek and Liljemark, 1994). As a result of autoaggregation (attraction between same species) and coaggregation (attraction between different species), the dental plaque biofilm matures, demonstrating an increase in numbers and species of bacteria (Lamont et al., 1993; Riviere et al., 1996). The clustering of bacteria within the biofilms shows specific associations among bacteria present (Socransky and Haffajee, 2002).

Based on the position on the tooth surface, there are two different types of dental plaque biofilms: supragingival plaque forming above, and subgingival plaque forming below the marginal gingiva. The first bacteria colonizing the supragingival tooth surface are mostly gram positive facultative microorganisms, followed by gram negative cocci as well as gram positive and gram negative rods (Listgarten, 1976).

Extending apically, a subgingival plaque biofilm is formed containing gram negative anaerobic and also motile bacteria. More than 600 bacterial species have been isolated from subgingival plaque. However, only a small number are strongly associated with the pathogenesis of periodontal disease in the susceptible host (Socransky and Haffajee, 2002).

When examining subgingival plaque samples, six closely associated groups of bacterial species were identified. These included the Actinomyces, a yellow complex consisting of members of the genus Streptococcus, a green complex consisting of Capnocytophaga species, Actinobacillus actinomycetemcomitans serotype a, Eikenella corrodens and Campylobacter concisus, a purple complex consisting of Veillonella parvula and Actinomyces odontolyticus, an orange complex consisting of Campylobacter species such as C. rectus, C. gracilis and C. showae, and a red complex consisting of P. gingivalis, T. forsythensis and T. denticola (Socransky et al., 1998). Except for the orange and red complexes, the other groups are the early colonizers of the tooth surface, and their growth usually precedes the multiplication of the predominantly gram negative orange and red complexes (Socransky et al., 1998). In subgingival plaque, certain complexes are present together more frequently than others. For example, it is unlikely to find the red complex species without the presence of the orange complex. However, members of the Actinomyces, yellow, green and purple complexes are often found without members of the red complex or even the red and orange complexes (Socransky and Haffajee, 2002).

3.3 Host response

In infectious diseases, bacteria or their products invade the host tissue, often resulting in a wide variety of inflammatory and immunopathological reactions (Takada et al., 1991). It is generally accepted that much of the periodontal tissue destruction observed in periodontal disease is host-mediated through release of pro-inflammatory cytokines by local tissues in response to the bacterial biofilm (Page, 1991).

Host response in periodontal disease can be divided into non-specific congenital immunity (innate immunity) and specific acquired immunity (adaptive immunity). In most cases, there is a cross-talk between the innate and adaptive immune responses (Graves and Cochran, 2003).

Non-specific immunity containing phagocytic cells such as polymorphonuclear leucocytes (PMN), monocytes/macrophages, natural killer cells (NK), as well as soluble effector molecules such as complement (C) and acute phase protein (C-reactive protein) presents the first line of defense which is activated following the initial contact of bacterial lipopolysaccharide with the cells of junctional epithelium (Madianos et al., 1997). At an early stage, monocytes and activated macrophages respond to endotoxin by releasing pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 which play a key role in the initiation, regulation, and perpetuation of the non-specific immune responses in the periodontium (Darveau, 2000; Gemmell et al., 1997). The release of these primary mediators stimulates the production of secondary mediators and chemokines, resulting in amplification of the inflammatory response, induction of connective tissue degrading enzymes, and osteoclastic bone resorption (Graves and Cochran, 2003). To counterbalance this inflammatory response and to regulate homeostatic stability of immune system, endogenous anti-inflammatory cytokines such as IL-4, IL-10, and TGFβ are released (Kantarci et al., 2006).

While the non-specific immunity is critical in the early control of bacterial replication and successful eradication of the infection, the adaptive immune response indicated by

antibody production is important in establishing specific immunity with a memory component. It is activated directly or indirectly through cytokine such as IL-1, IL-2, and IL-7, as well as antigen processing and presenting cells such as macrophages, neutrophils, and dendritic cells (Hornef et al., 2002; Teng, 2003).

3.3.1 Cytokine

Cytokines regulated at the level of synthesis, secretion and biological activity are important for pathogenesis of an increasing number of diseases such as diabetes mellitus, autoimmune thyroid disease (Gianoukakis and Smith, 2004), ulcerative colitis, and Crohn's disease (Papadakis and Targan, 2000), as well as periodontal disease (Seymour and Taylor, 2004). They are produced transiently, often in picomolar concentrations, and act locally in the tissue where they are produced (Page et al., 1997). Cytokines as intercellular messengers bind to specific receptors on their target cells, initiating intracellular second messengers. They do not act in isolation but rather function as a complex network, combining the elements of both innate and adaptive immunity in the defense against infection and disease (Banyer et al., 2000). Many cytokines demonstrate overlapping functions so that the absence of one but the presence of another may result in the same biological outcome (Seymour and Taylor, 2004). Depending on the nature of the cytokine response, their release could result in a protective immunity or lead to a destructive outcome (Gemmell and Seymour, 2004).

The central role of cytokines in focal immunopathologies such as periodontal disease is demonstrated by the fact that once the critical level of pro-inflammatory cytokine

production is achieved, the physiological response and the protective immunity becomes a pathologic and destructive process to the surrounding tissues (Graves and Cochran, 2003).

3.3.1.1 IL-1

The three cytokines originally described as the members of the IL-1 family were IL-1α and IL-1β with similar activities acting agonistic, and IL-1 receptor antagonist, IL-1Ra, which functions as a competitive inhibitor (Dinarello, 1997). The IL-1 cytokines produced by a variety of cells, in particular stimulated monocytes, macrophages and epithelial cells (Feghali and Wright, 1997), are of fundamental importance in health and disease as evidenced by their large array of biological activities and direct regulation of expression of several genes during inflammation (Dinarello, 1996). While elevated levels of IL-1β can be detrimental, resulting in tissue destruction and enhanced bone resorption (Kornman et al., 1997; Tatakis, 1993), low levels are beneficial in host responses to infection (Taylor et al., 2004). The importance of the IL-1 protective ability against infection was demonstrated in an animal study where spread of infection was prevented by IL-1β activity (Graves et al., 2000).

The wide-ranging roles of the IL-1β biological effect in innate and adaptive immune responses have been implicated in the pathogenesis of several disease processes such as asthma (Okada et al., 1995), preterm labour (Dudley, 1997), and periodontal disease (Jandinski et al., 1991; Kinane et al., 1992). The role of IL-1β in the initiation and progression of periodontal disease has been demonstrated in many animal and human

studies. In a rat model, increased inflammation and bone resoption was noted when IL-1β was applied to the gingiva (Koide et al., 1995). In experimental periodontitis of non-human primates, significant reduction in inflammation, connective tissue attachment loss, and bone resorption occurred when using IL-1β receptor inhibitor (Delima et al., 2002). Human studies revealed an increase of tissue IL-1β levels in the gingiva of patients with periodontal disease as compared to the healthy controls (Hönig et al., 1989). Higher levels were noted in active versus stable periodontal sites (Stashenko et al., 1991). In addition, IL-1β levels found in gingival crevicular fluid (GCF) of patients with periodontal disease were increased when compared to the levels of healthy subjects (Masada et al., 1990; Preiss and Meyle, 1994).

3.3.1.2 TNF-α

Tumor necrosis factor refers to two related proteins, TNF-α and lymphotoxin-α (also known as TNF-β), with a high degree of structural homology (Beyaert and Fiers, 1994). TNF interacts with two structurally similar cell surface receptors, TNF-R1 and TNF-R2 (Rothe et al., 1992; Pfizenmaiener et al., 1993). Most of the destructive effects of TNF have been attributed to TNF-R1 (Rothe et al., 1993; Amar et al., 1995). While macrophages are likely to be the most important source of TNF production in the early stages of gingival inflammation, it seems that in advanced periodontal disease other leukocytes such as the B cell may be the primarily source (Seymour et al., 2001). TNF-α, primarily produced by activated macrophages, shares many biological activities with IL-1β. Its implication in periodontal disease is due to its contribution to several events that are essential for the initiation of an inflammatory response and its ability to stimulate

bone resorption (Tatakis and Kumar, 2005). In an animal study comparing the wild type and TNF receptor-null mice, fibroblast apoptosis was greatly reduced in the knockout animals, suggesting the stimulating effect of TNF in the programmed cell death of fibroblasts (Graves et al., 2001). Therefore, it was concluded that the presence of TNF not only contributes to breakdown of gingival connective tissue, but also limits the ability to repair the destroyed tissue (Graves et al., 2001). In experimental periodontits in a Macaca fascicularis primate model, treatment with IL-1/TNF antagonists resulted in a significant decrease of connective tissue attachment loss and reduction in the number of osteoclasts, indicating the involvement of these cytokines as major contributors to periodontal bone destruction and connective tissue matrix degradation (Delima et al., 2001).

Based upon these findings, a potential effective periodontal therapy should aim in dampening the overreaction of the host response to bacteria by inhibiting the inappropriate cytokines such as IL-1β and TNF-α. Presently, IL-1 or TNF antagonists are used in human clinical trials in the treatment of other inflammatory conditions such as arthritis (Dayer et al., 2001).

3.3.1.3 TGFβ

Transforming growth factor-β1 as a multifunctional cytokine is secreted from many different cells including lymphocytes, monocytes, platelets and neutrophils (Wahl et al., 1993; Steinsvoll et al., 1999). In regulation of the host's response to bacterial and immunological challenge, TGF-β1 has both pro-inflammatory and anti-inflammatory

features. While the anti-inflammatory properties of TGF-β are achieved through suppression of innate and humoral immune responses (Prime et al., 2004; Gürkan et al., 2006), its pro-inflammatory properties are demonstrated by its capability to function as a chemoattractant for neutrophils, monocytes, mast cells and lymphocytes as well as its ability to mediate the release of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α by these cells (Kiritsy et al., 1993; Marek et al., 2002).

The nature of immune responses (inflammation or tolerance) is determined by the differentiation of naive T cells, which is influenced by the type of cytokines and co-stimulatory receptors engaged. While pathogen recognition promotes differentiation of effector T cells (Teffs) as a result of inflammatory cytokine production and T cell co-stimulation, recognition of self antigens results in induction of anergic T cells and the gradual accumulation of regulatory T cells (Tregs), promoting tolerance (Sundrud and Rao, 2007).

TGFβ, among other factors, affects the immune response through its regulatory effect on naive T cells, influencing their proliferation, differentiation, and survival (Gorelik and Flavell, 2002.). It blocks T cell proliferation by inhibiting IL-2 production, inhibits differentiation of Th1 and Th2 cells, prevents T cell activation-induced cell death, and induces the generation of forkhead transcription factor FoxP3 expressing Tregs (Li et al., 2006). FoxP3 expressing Tregs could either arise through differentiation in the thymus, known as natural Treg (nTreg), or through the differentiation of naive T cells in

peripheral lymphoid oranges, the so-called induced Tregs (iTreg) (Zheng et al., 2002; Chen et al., 2003).

The pivotal function of TGFβ in inflammation and tolerance is explained by its capability to promote differentiation of two opposing T cell lineages, iTreg, a subset of regulatory T cells that possesses immune suppression activities, and Th17, a specific effector T cells subset that secrets inflammatory cytokines interleukin-17 (IL-17) (Sundrud and Rao, 2007). It has been postulated that the presence or absence of co-stimulatory inputs and the cytokines IL-2, IL-6 and IL-23 play a key factor in the opposite cellular responses seen after activation of the same cell surface receptors by the same stimuli such as TGFβ.

Among the TGFβ superfamily, TGF-β1 is the predominant isoform expressed in the immune system. The potent immunosuppressive role of TGF-β1 has been demonstrated in many animal studies (Li et al., 2006). Knockout mice lacking TGF-β1 would either die in uterus as a result of defective yolk sac vasculogenesis and hematopoiesis (Dickson et al., 1995), or develop to term and die within 3-4 weeks due to a severe multi-organ inflammatory disorder, exhibiting development of mixed inflammatory cell infiltration into heart, stomach, liver, diaphragm, lung, salivary gland, and pancreas (Prud'homme and Piccirillo, 2000). Lack of functional TGF-β1 is also associated with increased mRNA expression of several inflammatory cytokines (Shull et al., 1992) such as IFN-γ, TNF-α, and IL-1β. In addition, TGF-β1 downregulates the antigen expression of the MHC class I and II of a variety of cell types including B cells and macrophages (Czarniecki et al., 1988). Its immunosuppressive effect is further elicited through its

inhibitory effect on IgG and IgM secretion by B lymphocytes (Letterio and Roberts, 1998; Kehrl et al., 1991). TGF-β1 can also interfere with the adhesion of neutrophils and lymphocytes to the vascular endothelial cells through modifying the expression of various adhesion molecules such as E-selectin (Prud'homme and Piccirillo, 2000).

TGFβ is also involved in angiogenesis, extracellular matrix synthesis, apoptosis and cell growth inhibition (Prime et al., 2004). As the major activator of extracellular matrix synthesis, it plays an important role in wound healing, tissue remodeling and regeneration (Sporn and Roberts, 1993). The increased expression of collagen and other extracellular matrix components mediated by TGFβ (Tabibzadeh, 2002) is due to its ability to suppress matrix degrading proteinases such as matrix metalloproteinases, and to induce formative fibroblast phenotype, which could synthesize connective tissue matrix (Overall et al., 1991).

The central role of TGF-β in regulation of collagen metabolism in physiologic as well as pathologic conditions (van der Zee et al., 1997) make it an interesting cytokine to monitor in the pathogenesis of periodontal disease. However, due to the complex interaction between and among different cytokines at the site of inflammation and the multi-factorial nature of periodontal disease, the involvement of TGFβ in the pathogenesis of periodontal disease is not completely understood (de Souza et al., 2003; Ejeil et al., 2003). Recent analysis of gingival crevicular fluid have shown increased TGFβ levels in patients with gingivitis, chronic periodontitis and generalized aggressive periodontitis as compared to healthy individuals (Gürkan et al., 2006). The total amount of GCF TGFβ

was significantly correlated to clinical periodontal parameters such as probing depth, clinical attachment loss and bleeding on probing (Gürkan et al., 2006). In another study, measuring the GCF TGFβ of the sites following a regenerative procedure or surgical periodontal therapy, an increase in TGFβ levels was demonstrated after both treatment modalities (Kuru et al., 2004). These findings suggest that TGFβ might contribute to both inflammatory regulation as well as remodelling processes during periodontal disease. It seems that the changes in the levels of TGFβ in GCF could be an effective tool in monitoring the progress of periodontal repair and regeneration (Kuru et al., 2004). Until more studies are undertaken, the precise role of TGFβ in the pathogenesis of periodontal disease remains unclear.

CHAPTER IV

Aim of the study

The αvβ6 integrin an exclusively epithelial integrin, is absent from oral gingival epithelium yet found in junctional epithelium. Although it is expressed in low levels in healthy tissue, its expression is rapidly upregulated by injury and inflammation. Considering its existence in the JE and its ability to activate TGF-β1, a cytokine with an anti-inflammatory effect, it was hypothesized that αvβ6 integrin plays a protective role in the development of periodontal disease. Therefore, this study was conducted to investigate our hypothesis that lack of αvβ6 integrin in mice has a negative impact on periodontal status.

For this purpose the project was divided in two parts:

1) Quantification and comparison of alveolar bone loss around the mandibular 1st and 2nd molars between β6 -/- and wild-type mice within different age groups.
2) Comparison of histological changes in JE in regards to migration and degree of inflammation between the two groups using paraffin sections of the maxilla.

CHAPTER V

Material & Methods

26 integrin-knockout mice (β6 -/-, Huang et al., 1996; generous gift from Dr. Dean Sheppard, University of California, San Francisco, USA) and 30 wild-type mice (FVB) were included in this experiment. Animals were labeled based on their age, sex and group. The integrin-knockout group consisted of six 3-month old (3 female & 3 male), six 6-month old (3 female & 3 male), and fourteen 12-month old (9 female & 5 male) animals as compared to the wild-type group which included six 3-month old (3 female & 3 male), six 6-month old (3 female & 3 male), and eighteen 12-month old (17 female & 1 male) animals. All data collected were based on assessment of 4 teeth per animal unless indicated otherwise. All mice were allowed free access to standard mouse chow (Purina 5001) and water. Animals were sacrificed by carbon dioxide inhalation. Upon decapitation, maxilla and mandible were separated and processed for different analyses.

5.1 Mandible

Mandibles cut in half were partially defleshed mechanically and exposed to 2% KOH (EM Science, Merck, Darmstadt, Germany) until completely defleshed. In order to further delineate the CEJ, dry mandibles were stained using Van Gieson's solution for 30 seconds followed by water diluted Ponceau-S (1:10) for 5 minutes. The stained jaws were then used for quantification of alveolar bone loss.

5.1.1 Alveolar bone loss measurement of mandible

Dried stained jaws were placed under a dissecting microscope (Leica MZ6, Switzerland) and aligned using dental impression putty so that the lingual and buccal cusps were superimposed. Using a digital camera (Nikon, Coolpix 995, Tokyo, Japan), images including the lingual view of first and second molars with a standardized scale (Thin Williams Periodontal probe, Hu Friedy, Chicago, Illinois, USA) were taken at X 40 magnification (Objective 4, Eyepiece 10) from all the jaws. Photoshop CS Me (Adobe, San Jose, California, USA) was utilized to standardize the image size before any measurements. Quantification of the area between the CEJ and the crest of alveolar bone was achieved using ImageJ software (http://rsb.info.nih.gov/ij/). The area of exposed root was documented for each tooth (1st & 2nd molar) in pixel units as well as in mm^2 (Figure 1). Repeated measurements on randomly selected teeth revealed intraexaminer agreement of > 98% (38 repeated measurements out of 221).

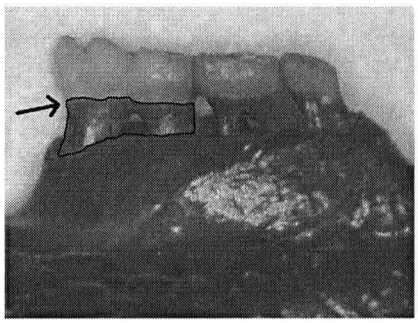


Figure 1.
Example of alveolar bone loss quantification. Surface area between CEJ (black arrow) and alveolar crest of the first molar is marked and measured using ImageJ software.

In order to evaluate possible eruption of the teeth, the most apical part of the alveolar process anteriorly and posteriorly were determined (blue X in Figure 2) and connected via a straight line. The vertical distance from this line to the CEJ was determined for each root of each tooth (mesial root of 1st molar, distal root of 1st molar, mesial root of 2st

molar, distal root of 2st molar). This distance was measured mid-lingually for each root (Figure 2).

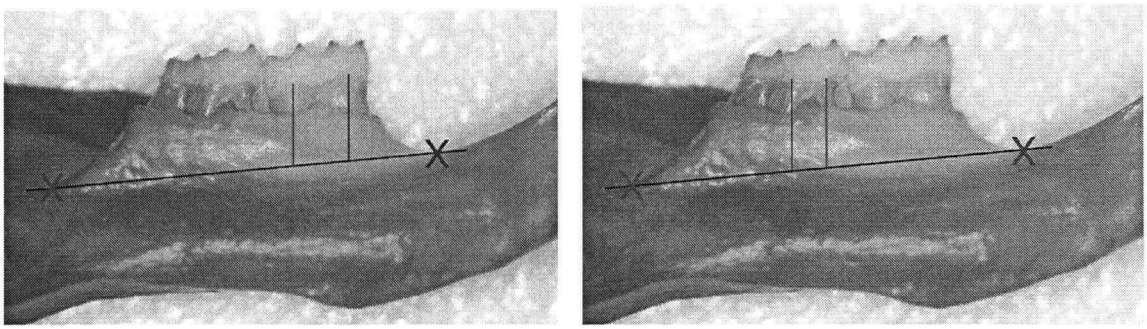


Figure 2. Example of measurement technique for the distance between alveolar process and CEJ. The most apical parts of the alveolar process anteriorly and posteriorly are indicated with blue X.

The alveolar bone loss assessment was finalized with evaluation of furcation involvement based on the classification described by Wiebe et al. (2001) (Table 1).

Table 1. Alveolar bone loss quantification scale by Wiebe et al. (2001)

Class	Description
0	No furcation involvement
I	Exposure of the furcation and horizontal bone loss extending into furcation
II	Exposure of the furcation with a through-and-through (tunnel) defect from the buccal to the oral surface of the toot
III	Through-and-through furcation defect with horizontal bone loss extending into the apical third of the root

To further determine the extent of bone loss, hemimandibles of twenty-two 12-month old animals (11 FVB & 11 β6 -/-) were selected for radiographic analysis using Faxitron x-rays. Qualitative assessment using an index between 0-3 was performed separately for the mesial, mid, and distal of the 1st molar as well as mesial and mid of the 2nd molar. The index utilized is explained in Table 2.

Table 2. Qualitative assessment of alveolar bone loss (Bone Loss Index)

Index	Description
0	No bone loss
1	Horizontal or vertical bone loss < 1/3
2	Horizontal or vertical bone loss between 1/3 – 2/3
3	Horizontal or vertical bone loss > 2/3

For better visual illustration of bone resorption, scanning electron microscopy (SEM) images of selected mandibles from each group (FVB & β6 -/-) were prepared after the clinical crowns had been removed using a fine bur. For this purpose defleshed mandibles were washed in PBS 0.1 M, dried for 3 days in 60° C oven, coated with gold, and observed with scanning electron microscope (Cambridge 260, StereoSEM, Cambridge, England). The images were purely for illustrative purposes and were not used for any measurements.

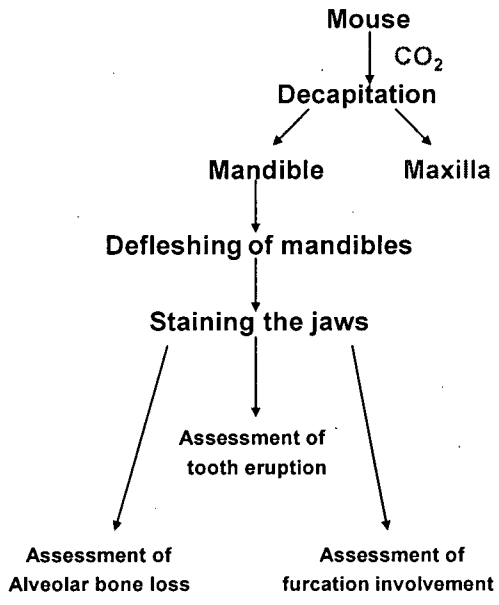
5.1.2 Assessment of attrition

The attrition of the mandibular posterior teeth was qualitatively determined using a scale between 0 and 1. While score 0 was used for teeth with attrition < 50% of cusp height, teeth demonstrating occlusal wear ≥ 50% of cusp height were given score 1.

5.1.3 Weight of the mandible

In order to determine whether there were any differences in body or jaw size between the two groups, thirty-five 12-month old animals (15 FVB, 20 β6 -/-) were compared for their body weights (Mettler PE160, Zürick, Switzerland) and defleshed mandibular jaw weights (Mettler AE260, Zürick, Switzerland) using calibrated scales.

Schematic illustration of the processes performed on the mandible



5.2 Maxilla

The maxillary jaws were decalcified in 0.4 M EDTA and 2% formaldehyde in PBS (pH=7.2) with the solution being changed every other day for a period of 6 weeks (EDTA: Sigma – Aldrich, St. Louis, MO, USA; Formaldehyde: Fisher Scientific, Fairlawn, NJ, USA; PBS: Invitrogen – GIBCO, Carlsbad, CA, USA). The specimens (36 blocks each containing one maxilla) were then processed for embedding in paraffin using a routine protocol and stored in the refrigerator until used.

5.2.1 Histological assessment of the JE

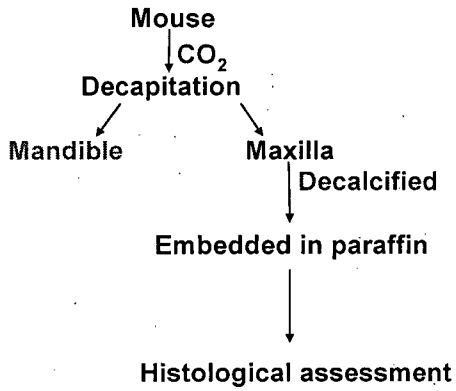
From the 36 paraffin specimens, 6 were included in the study (3 FVB & 3 β6 -/-). The specimens were then cut (7 μm) in the bucco-lingual direction, giving approximately 1400-1500 sections (350-375 slides) for each group of animals. Every tenth slide from each block of sections was stained with H&E, providing 39 stained slides for the FVB mice and 38 stained slides for the β6 -/- mice. Stained sections were used to assess the migration of JE as well as the degree of inflammation. Using an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan) at X 40 magnification (Objective 4, Eyepiece 10) and a digital camera (Canon Powershot A540, Tokyo, Japan) with a standard setting, digital images were captured from all stained sections with a standard scale and processed using Photoshop CS Me software. Utilizing ImageJ software, migration of JE was measured in pixels as well as in mm from standardized pictures. The images were further used for qualitative assessment of inflammation immediately under junctional epithelium (JE) or pocket epithelium (PE) on each side of each root. The scoring was done by a single examiner according to Table 3.

Table 3. Qualitative assessment of inflammation

Score	Description
0	No inflammatory infiltrate
1	Mild inflammation, scattered inflammatory infiltrate around JE or PE
2	Moderate inflammation, moderate inflammatory infiltrate around JE or PE
3	Severe inflammation, heavy inflammatory infiltrate around JE or PE

In addition to H&E staining, 10 slides were used for Movat Pentachrome staining (Schmidt and Wirtala, 1996) to better distinguish between different structures such as connective tissue (CT), junctional epithelium (JE), and cementum (C). This allowed for better visualization and assessment of any changes seen in the specimens.

Schematic illustration of the processes performed on the maxilla



5.3 Statistical analysis

Statistical analyses were performed using Student t-test and Mann-Whitney U test. Comparison of parametric data between FVB and β6 -/- mice including differences in alveolar bone loss, tooth eruption, animal weight, weights of defleshed mandibles, and JE apical migration were achieved utilizing Student t-test. For the nonparametric data including severity of inflammation from maxillary paraffin sections and degree of bone loss from mandibular radiographic images, Mann-Whitney U test was used.

CHAPTER VI

Results

The assessment of body size comparing the weight between 12 FVB (8 males & 4 females) and 12 β6 -/- (8 males & 4 females) at twelve months age revealed that β6 integrin-knockout mice, with an average weight of 32 g ± 3.80, were significantly lighter than their age-matched wild-types, which presented with a mean weight of 45 g ± 3.81 (P-value < 0.0005). To ensure that the size of mandibles between the two groups was comparable, the weight of defleshed, non-stained mandibular jaws of the same animals were recorded. With average values of 0.079 g ± 0.005 (FVB) and 0.080 g ± 0.006 (β6 -/-), no statistically significant difference was found (P-value > 0.3).

6.1 Mandible

Defleshed and stained mandibles were used for assessment of alveolar bone loss through quantification of exposed surface area between CEJ and alveolar bone crest using ImageJ software. The comparison of alveolar bone loss between the two groups (FVB & β6 -/-) was then performed for the same age combining both genders, among the first molars (Figure 3), second molars (Figure 4), and both molars combined (Figure 5) using Student t-test.

Figure 3 presents the data for the 1st molars. Mean bone loss was significantly higher in knockout animals in all three age groups. Among the 3-month old animals using 24 teeth (12 in each group), these values were 0.38 mm^2 ± 0.055 and 0.34 mm^2 ± 0.036 for β6 -/- and FVB, respectively. The difference was statistically significant (P-value < 0.05). For

6-month old animals, mean bone loss based on 24 teeth (12 in each group) was measured to be 0.44 mm^2 ± 0.063 for β6 -/- versus 0.34 mm^2 ± 0.043 for FVB. With a P-value < 0.0005, the difference was statistically very significant. Similar findings were observed for 12-month old animals when comparing 63 teeth (36 FVB & 27 β6 -/-). With a mean bone loss of 0.50 mm^2 ± 0.114 and 0.40 mm^2 ± 0.055 for β6 -/- and FVB, respectively, the difference was determined to be statistically very significant (P-value < 0.0005). It is noteworthy to mention that among the knockout mice in this group, one 1st molar was lost due to severe bone resorption and could not be included in the assessment.

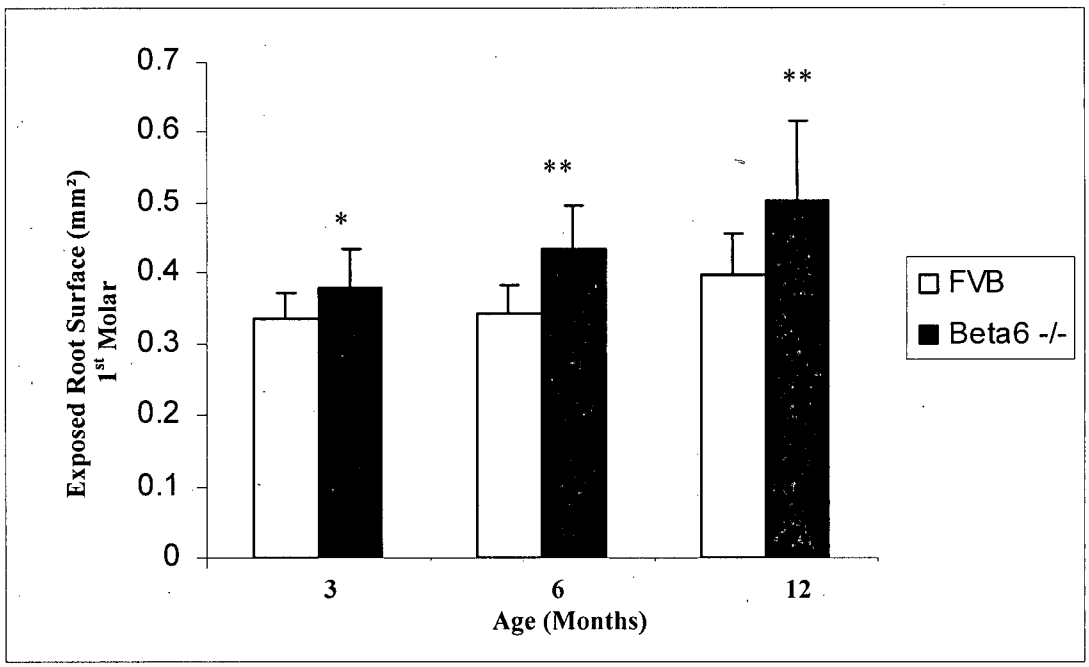


Figure 3. Quantification of the surface area between the CEJ and alveolar crest of the 1st mandibular molar using ImageJ software. Mean values of 24 teeth from 3- and 6-month and 63 teeth from 12-month old animals ± SD are presented. Statistical analysis was performed using Student t-test. (*): Significant difference, P-value < 0.05, (**): Very significant difference, P-value < 0.0005.

Figure 4 presents the results of the duplicate analysis for 2nd molars based on the same number of teeth per group. Compared to FVB, mean bone loss is significantly greater among knockout animals in the 3- and 6-month old age groups (P-value < 0.0005). However, for 12-month old animals, despite a higher mean value for bone loss in β6 -/- compared to FVB mice, the difference was found not to be statistically significant (P-value > 0.05). In the latter group, two teeth among the knockout animals were lost as a result of excessive bone loss and were therefore excluded from the analysis. For 3-month old animals, the measurement revealed a mean bone loss of 0.24 $mm^2 \pm 0.046$ and 0.18 $mm^2 \pm 0.018$ for β6 -/- and FVB, respectively. These values were 0.29 $mm^2 \pm 0.073$ versus 0.16 $mm^2 \pm 0.035$ and 0.33 $mm^2 \pm 0.096$ versus 0.30 $mm^2 \pm 0.079$ for 6- and 12-month old animals, respectively.

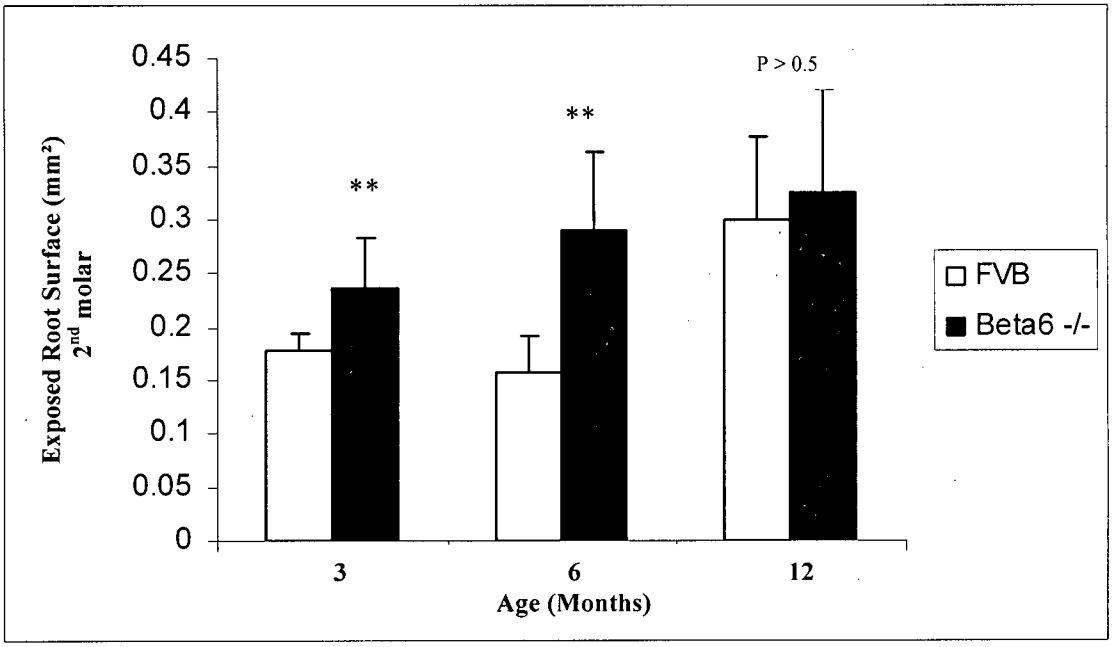


Figure 4. Quantification of the surface area between the CEJ and alveolar crest of the 2nd mandibular molar using ImageJ software. Mean values of 24 teeth from 3- and 6-month and 62 teeth from 12-month old animals ± SD are presented. Statistical analysis performed using Student t-test. (**): Very significant difference, P-value < 0.0005; No significant difference, P-value > 0.5.

Using the same criteria, data for both molars combined were used for the next statistical analysis (Figure 5). With a mean bone loss of 0.31 mm^2 ± 0.087 versus 0.26 mm^2 ± 0.085 in 3-month old, 0.36 mm^2 ± 0.099 versus 0.25 mm^2 ± 0.10 in 6-month old, and 0.42 mm^2 ± 0.14 versus 0.35 mm^2 ± 0.084 in 12-month old animals, β6 -/- mice demonstrated significantly more bone loss compared to their age-matched FVB counterparts.

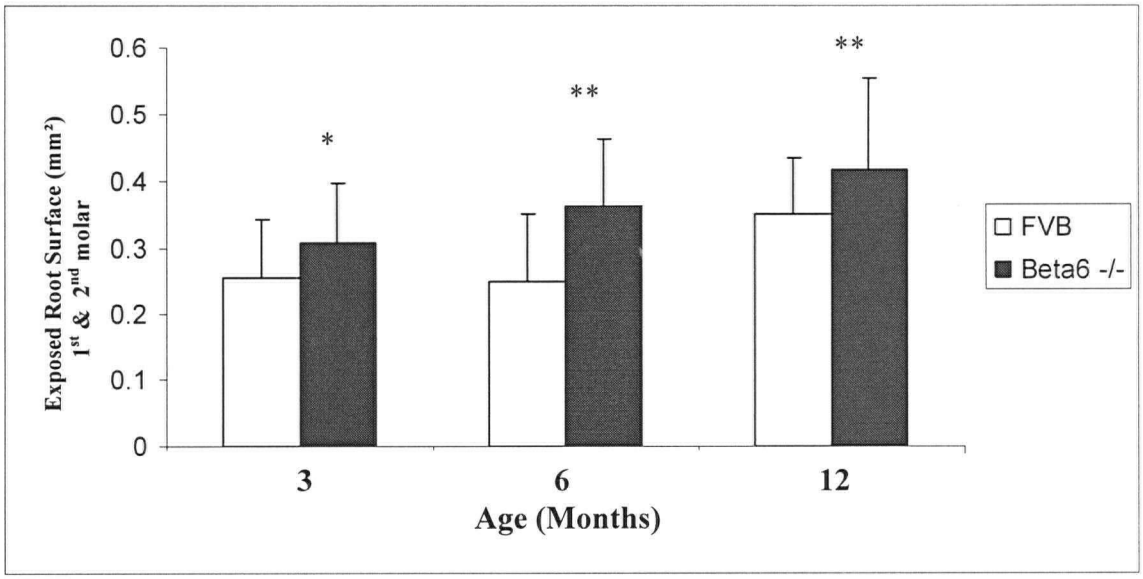


Figure 5. Quantification of the surface area between the CEJ and alveolar crest of the 1st and 2nd mandibular molars using ImageJ software. Mean values of 48 teeth from 3- and 6-month and 125 teeth from 12-month old animals ± SD are presented. Statistical analysis performed using Student t-test. (*): Significant difference, P-value < 0.05; (**): Very significant difference, P-value < 0.0005.

In order to determine whether gender played a role in the severity of alveolar bone loss, males and females were compared separately combining 1st and 2nd molars. The results showed no statistical difference in bone loss measurements between the sexes. Based on these findings, it was clearly demonstrated that, independent of age or the number of teeth involved, integrin-knockout mice (β6 -/-) exhibit significantly more bone loss as compared to age-matched wild-type (FVB) mice in almost all categories except for the 2nd molar in the 12-month old age group (Fig. 6 and Table 4).

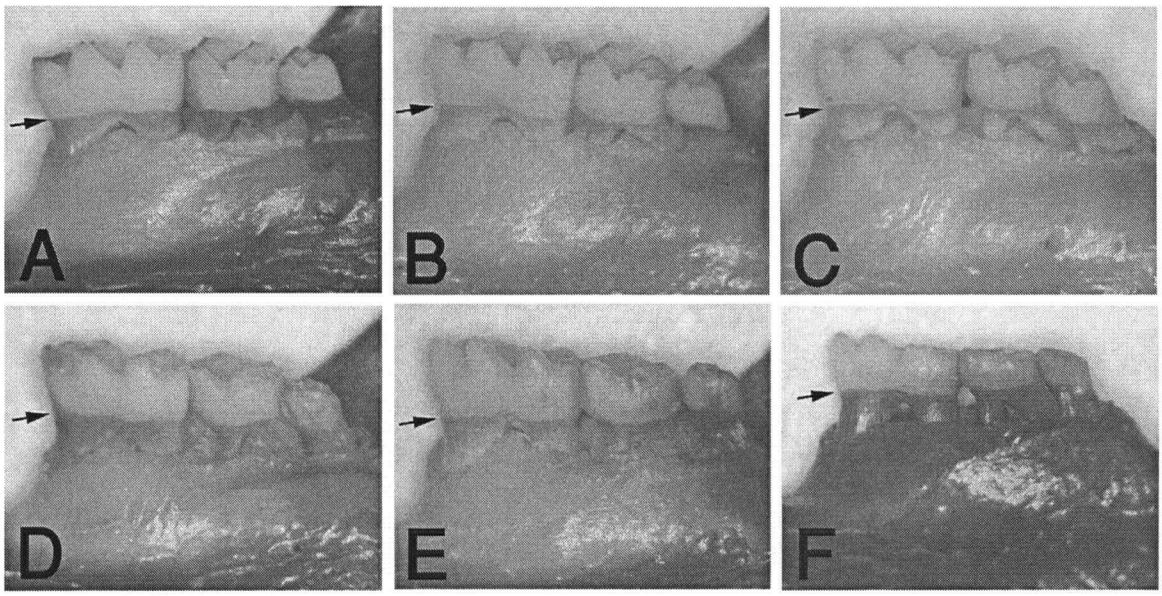


Figure 6. Example of degrees of alveolar bone loss (25 X magnification). Black arrow indicates CEJ. **A)** FVB, 3 months old; **B)** FVB, 6 months old; **C)** FVB, 12 months old; **D)** β6 -/-, 3 months old; **E)** β6 -/-, 6 months old; **F)** β6 -/-, 12 months old.

Table 4. Student t-test demonstrating statistical differences in degree of exposed root surface area (alveolar bone loss) in mandibular jaw between wild-type (FVB) and integrin-knockout (β6 -/-) mice. (*): Significant difference; (**): Very significant difference.

P values			
	1st Molar	**2nd Molar**	**1st & 2nd Molars**
3 months old	$P < 0.05$ *	$P < 0.0005$ **	$P < 0.05$ *
6 months old	$P < 0.0005$ **	$P < 0.0005$ **	$P < 0.0005$ **
12 months old	$P < 0.0005$ **	$P > 0.5$	$P < 0.005$ **

The enhanced bone loss in the β6 -/- mice was further confirmed when comparing the prevalence and the severity of furcation involvement between the two groups using the classification described by Wiebe et al. (2001) (Table 1). An example of different degrees of furcation involvement is demonstrated in Figure 7.

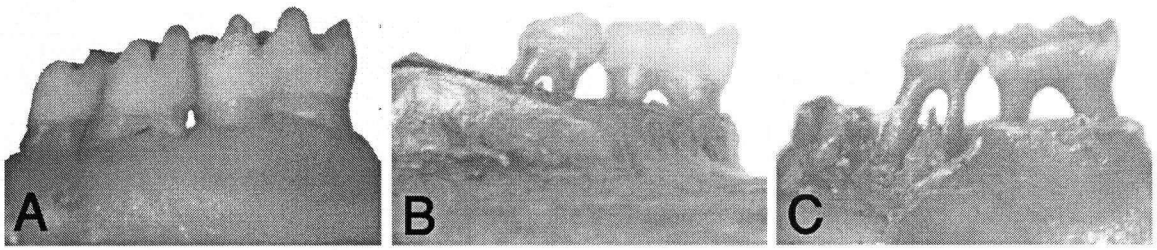


Figure 7. Examples of furcation involvement (40 X magnification). **A)** No furcation involvement on 1st molar, Class I furcation on 2nd molar; **B)** Class II furcation on 1st & 2nd molars; **C)** Class II on 1st molar, Class III on 2nd molar.

An analysis of 224 teeth (120 FVB and 104 β6 -/-) revealed severe furcation involvement (Class II and III) to be more prevalent in the knockout group (51% versus 35.8% for class II, and 4.8% versus 0% for Class III). On the contrary, wild-type animals (FVB) presented with more teeth with 0 or Class I involvement (17.5% versus 3.8% for class 0, and 46.7% versus 40.4% for Class I) (Table 5).

Table 5. Prevalence and severity of furcation involvement of mandibular teeth based on classification by Wiebe et al. (2001) (See Table 1 for classification criteria)

Furcation involvement of mandibular teeth		
	FVB 30 animals →120 teeth	**β6 -/-** 26 animals → 104 teeth
0	17.5%	3.8%
Class I	46.7%	40.4%
Class II	35.8%	51.0%
Class III	0%	4.8%

The bone loss was further assessed by comparing radiographic images of 22 hemimandibles (11 FVB & 11 β6 -/-). The scoring criteria, using a scale between 0 and 3 is explained in Table 2. The data collected from 110 sites (55 sites per group) were subject to statistical analysis utilizing Mann-Whitney U test. With P-value < 0.0005, significantly more bone loss was noted among the knockout animals. Data are presented in Figure 8 and Figure 9 illustrates an example of the radiographic as well as the scanning electron microscopy images (SEM).

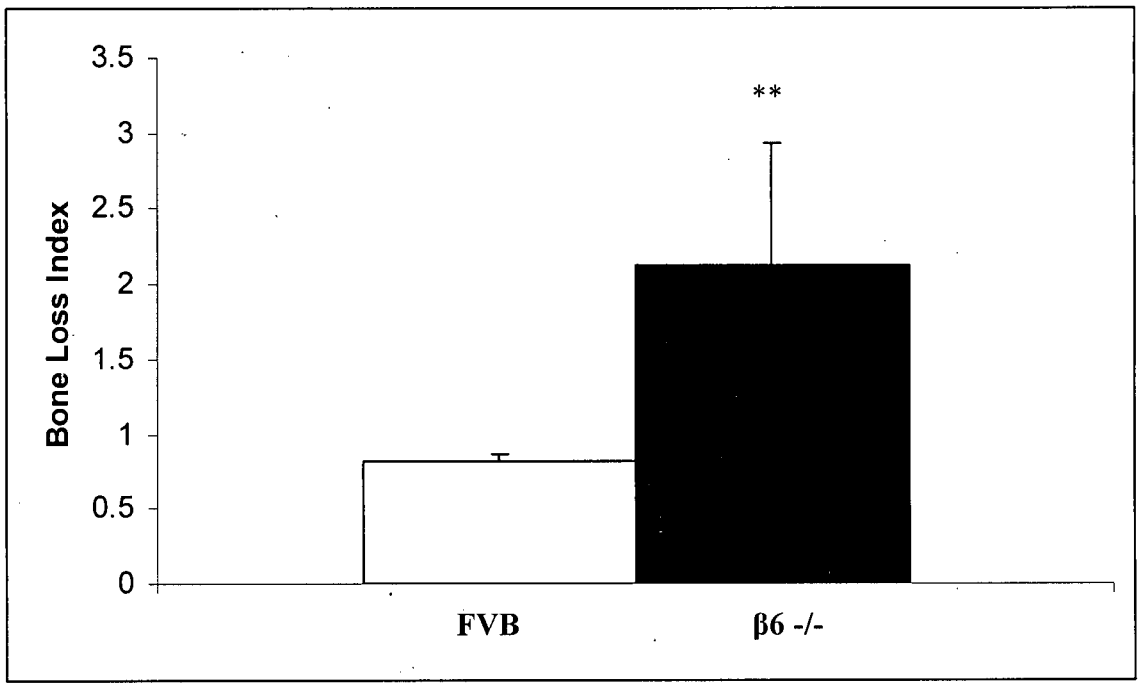


Figure 8. Qualitative assessment of the degree of bone loss using Faxitron x-ray images of the hemimandibles. Data presented are mean values ± SD from 22 hemimandibles (11 FVB & 11 β6 -/-) providing 110 sites. Statistical analysis performed using Mann-Whitney U test. (**): Very significant difference, P-value < 0.0005.

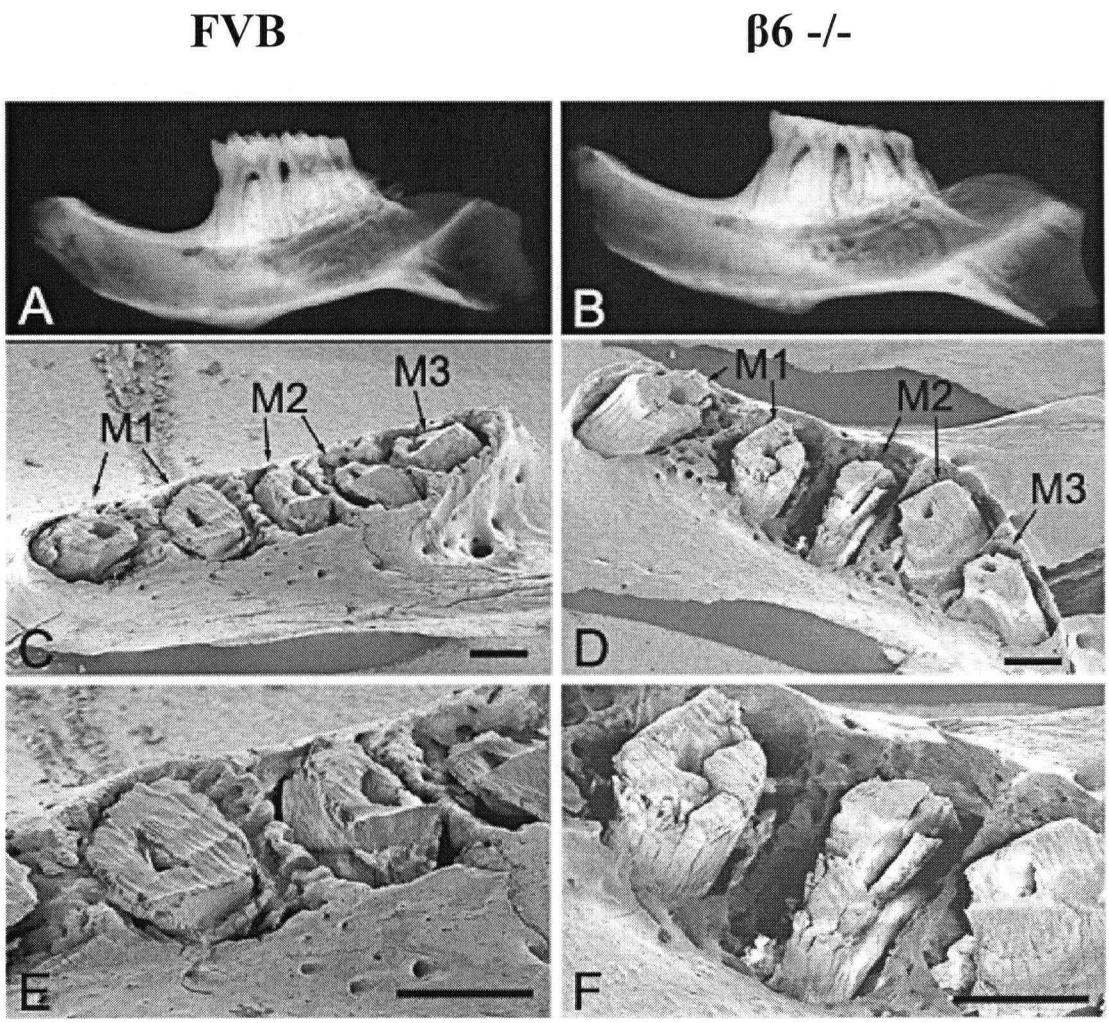


Figure 9. Radiographic and SEM images of hemimandibles. **A)** FVB, Radiographic image; **B)** β6 -/-, Radiographic image. Black arrows point at areas of decreased bone density, indicating bone loss; **C)** FVB, SEM image after removal of clinical crowns with high speed; **D)** β6 -/-, SEM image after removal of clinical crowns with high speed; **E)** & **F)** illustrate the enlarged views of **C)** & **D)**. Teeth are indicated with M1 for 1st Molar, M2 for 2nd Molar, and M3 for 3rd Molar. Arrowhead is pointing at the level of the interproximal bone. Interproximal crater (black triangle) is noted between M1 and M2 for β6 -/-. Bar = 500μm.

To elucidate the degree of periodontal disease, a classification of attachment loss based on the severity of furcation and the number of teeth involved was developed. Left and right sides of the mandibular jaw were assessed separately (Table 6).

Table 6. Classification of attachment loss (AL) of mandibular teeth based on degree of furcation involvement.

Classification of attachment loss	
No attachment loss (AL)	None of the molars show furcation involvement (0-0)
Mild AL	Only one molar has Class I furcation (0-I)
Mild-Moderate AL	Both molars have Class I furcation (I-I)
Moderate AL	One molar has Class I and one has Class II furcation (I-II)
Moderate-Severe AL	Both molars have Class II furcation (II-II)
Severe AL	One molar has Class II and one has Class III furcation (II-III)

An evaluation of 112 mandibular jaws (60 FVB and 52 β6 -/-) revealed that, while moderate-severe attachment loss or severe attachment loss was more prevalent within knockout animals (38.5% versus 21.7% for moderate-severe AL, 5.8% versus 0% for Severe AL), wild-type mice presented with more jaws demonstrating no attachment loss (5% versus 0%), or mild attachment loss (25% versus 7.7%) (Table 7).

Table 7. Severity of attachment loss (AL) based on classification described in Table 6 for wild-type (FVB) and integrin-knockout (β6 -/-) mice.

Severity of attachment loss		
	FVB (30 animals → 60 half jaws)	**β6-/-** (26 animals → 52 half jaws)
No attachment loss (AL)	**5%**	**0%**
Mild AL	**25%**	**7.7%**
Mild-Moderate AL	**20%**	**25%**
Moderate AL	**28.3%**	**23.1%**
Moderate-Severe AL	**21.7%**	**38.5%**
Severe AL	**0%**	**5.8%**

Assessment of the mandibular posterior teeth revealed occlusal wear to different degrees in some animals. While no significant attrition was noted for the teeth of the wild-type animals (120 teeth were included), integrin-knockout animals presented with 54 teeth demonstrating ≥ 50% cuspal wear and therefore were given the score 1 (101 teeth included). With all teeth of the 12-month old integrin-knockout animals scoring 1, attrition was 100% in this group. Since occlusal wear into the dentin was observed for some animals, further analysis was necessary in order to assess for possible tooth eruption. This was achieved by measurement of the distance mid-lingually between the CEJ and a straight line connecting the most apical anterior and posterior points of the alveolar process. The measurements were performed using images taken with a dissecting stereomicroscope (Leica MZ6, Switzerland) at X 40 magnification (objective 4, Eyepiece 10). With the exception of the 2nd molar in the 6-month old ($P < 0.005$) and the 1st molar

in the 12-month old age groups ($P < 0.005$), no significant difference was noted (see Figure 10 for 1st molar and Figure 11 for 2nd molar).

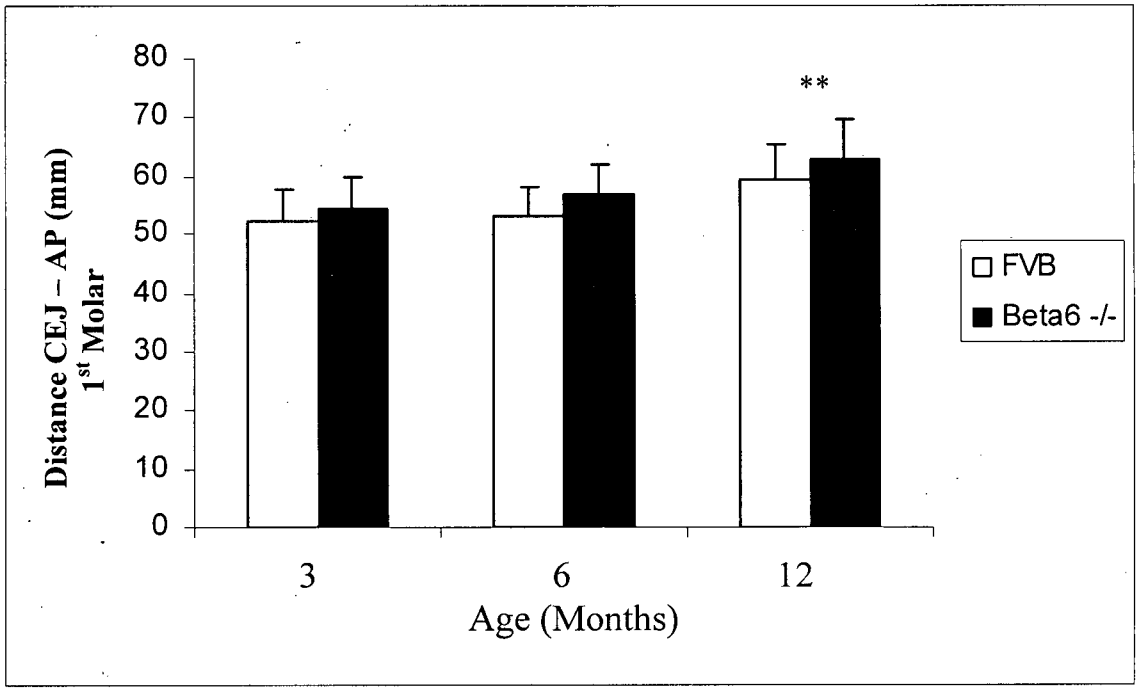


Figure 10. Assessment of eruption of 1st mandibular molar. Measurement of vertical distance from the CEJ to alveolar process (AP) mid-lingually in mm (40 X magnification). Separate measurements from each root (mesial & distal) were combined for each tooth. Mean values of 24 teeth from 3- and 6-month and 63 teeth from 12-month old animals ± SD are presented. Statistical analysis performed using Student t-test. (**): Very significant difference.

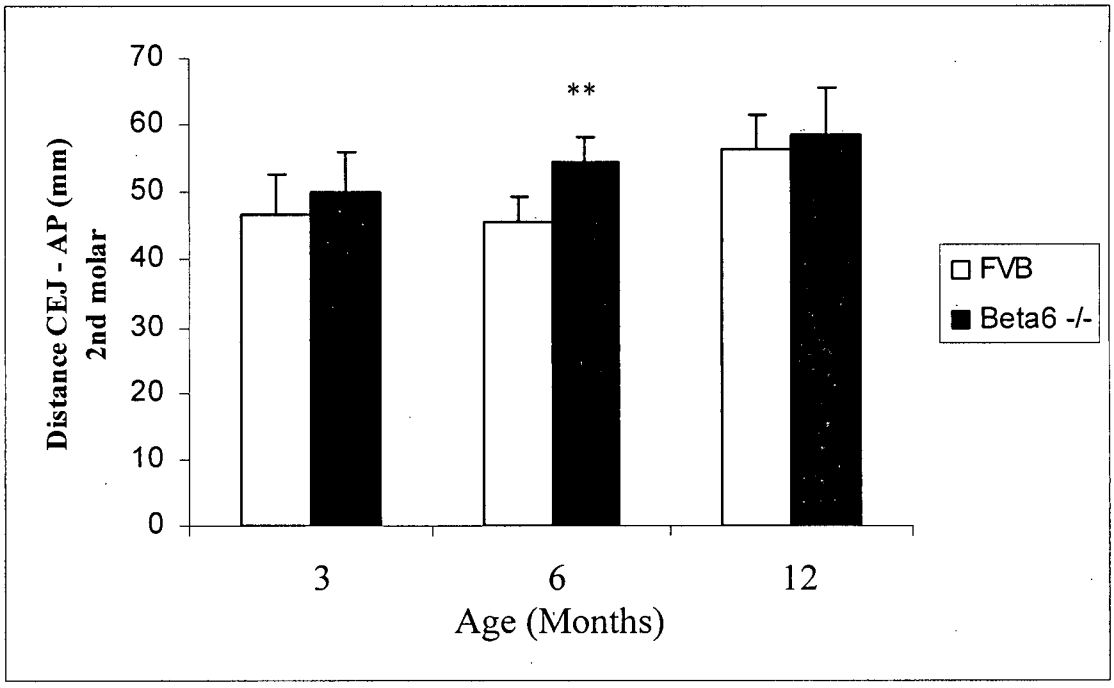


Figure 11. Assessment of eruption of 2nd mandibular molar. Measurement of vertical distance from the alveolar process to the CEJ mid-lingually in mm (40 X magnification). Separate measurements from each root (mesial & distal) were combined for each tooth. Mean values of 24 teeth from 3- and 6-month and 62 teeth from 12-month old animals ± SD are presented. Statistical analysis performed using Student t-test. (**): Very significant difference.

6.2 Maxilla

To explore whether the lack of expression of αvβ6 integrin affected the migration of the JE, paraffin sections of decalcified maxillary jaws of three wild-type (FVB) and three β6 integrin-knockout mice, both 12 months old, were stained with H&E and assessed microscopically. While the specimens of the wild-type mice showed no or minimal JE

migration beyond the CEJ, JE in the knockout animals started to invade the connective tissue apical to the CEJ, resulting in formation of pocket epithelium (PE) (Figure 12).

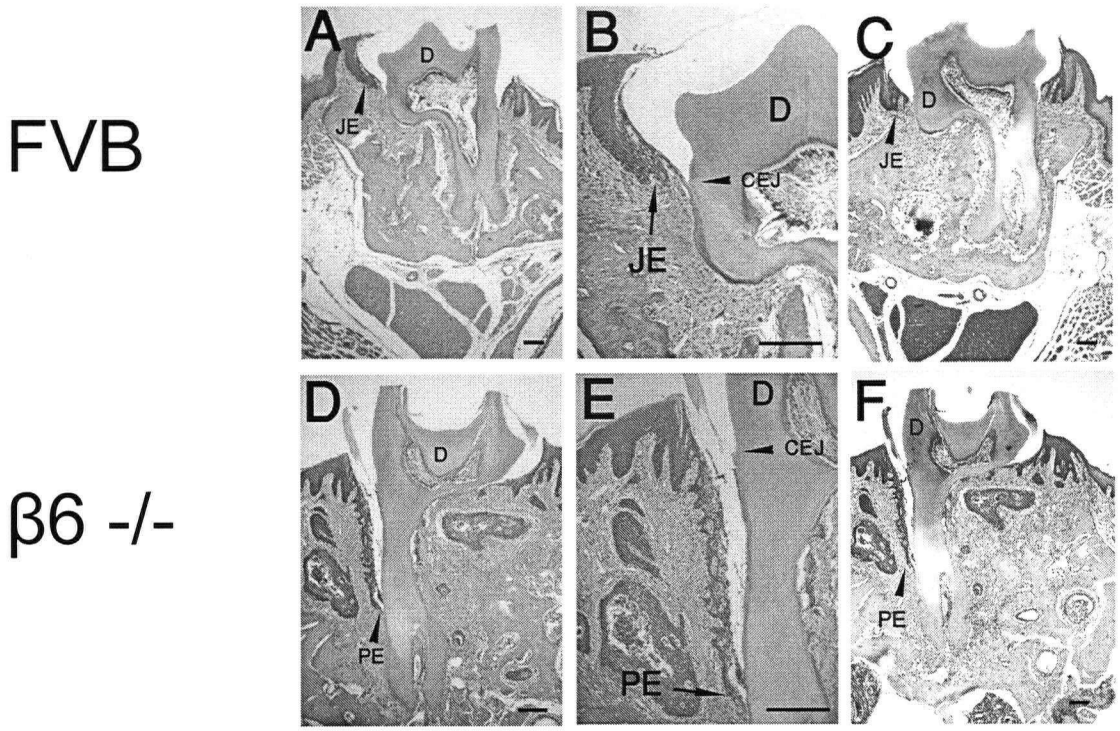


Figure 12. Migration of junctional epithelium (JE) in 12-month old mouse gingiva of wild-type (FVB) and β6 integrin-deficient (β6 -/-) animals. **A)** H&E staining, no migration of JE beyond the CEJ; **B)** Magnification of structures seen in image A); **C)** Pentachrome staining for better visualization of different structures with different degrees of collagen content; **D)** Migration of JE beyond the CEJ forming pocket epithelium (PE); **E)** Magnification of structures seen in image D); **F)** Pentachrome staining for better visualization of different structures with different degrees of collagen content. Bar = 200μm.

When comparing the degree of migration based on measurements from 82 stained sections (FVB: 28 sections, β6 -/-: 52 sections), statistical analysis using Student t-test revealed a significant difference between the groups (P-value < 0.0005). Mean values were 0.059 mm ± 0.040 and 0.379 mm ± 0.142 for FVB & β6 -/-, respectively (Figure 13).

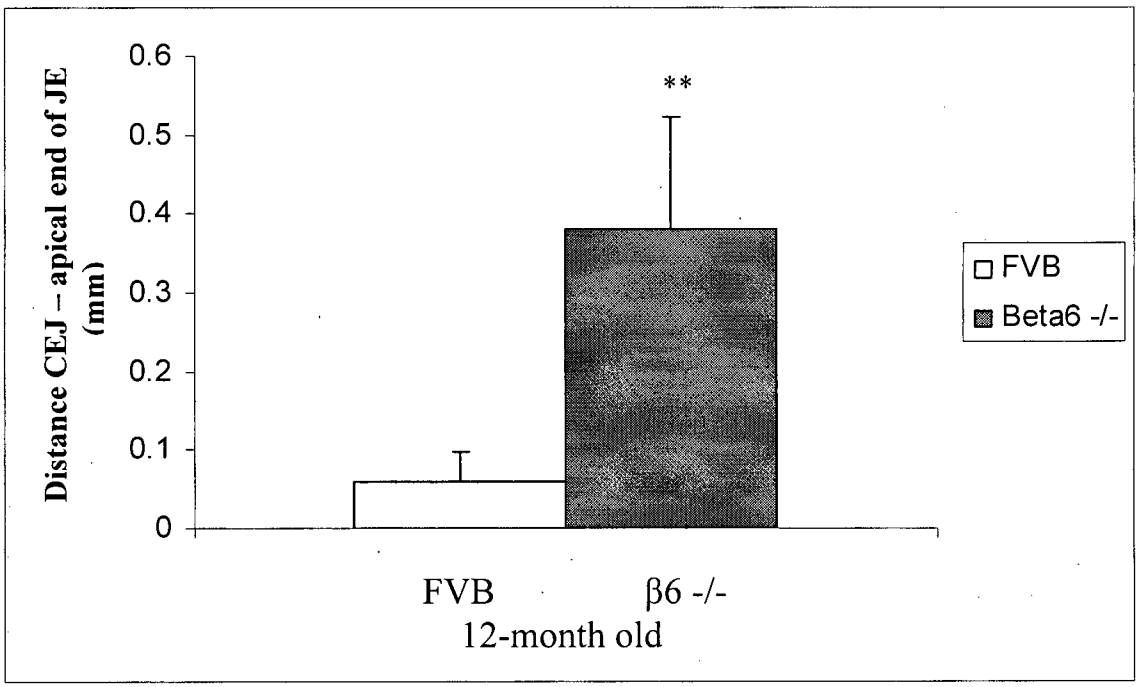


Figure 13. Apical migration of JE from the CEJ in mm. Measurements performed on 82 stained paraffin sections from six12-month old animals using ImageJ software. Mean values ± SD are presented. Statistical analysis performed using Student t-test. (**): Very significant difference, P-value < 0.0005.

Based on the criteria explained in Table 3, the qualitative assessment of inflammation was performed per site using the H&E stained maxillary paraffin sections of 6 animals (3

FVB & 3 β6 -/-). For this purpose 117 sites from wild-type (FVB) animals were compared to 115 sites of knockout (β6 -/-) mice.

Utilizing Mann-Whitney U test, the comparison revealed significantly more inflammation among the knockout mice (β6 -/-) (P-value < 0.005) (Figure 14).

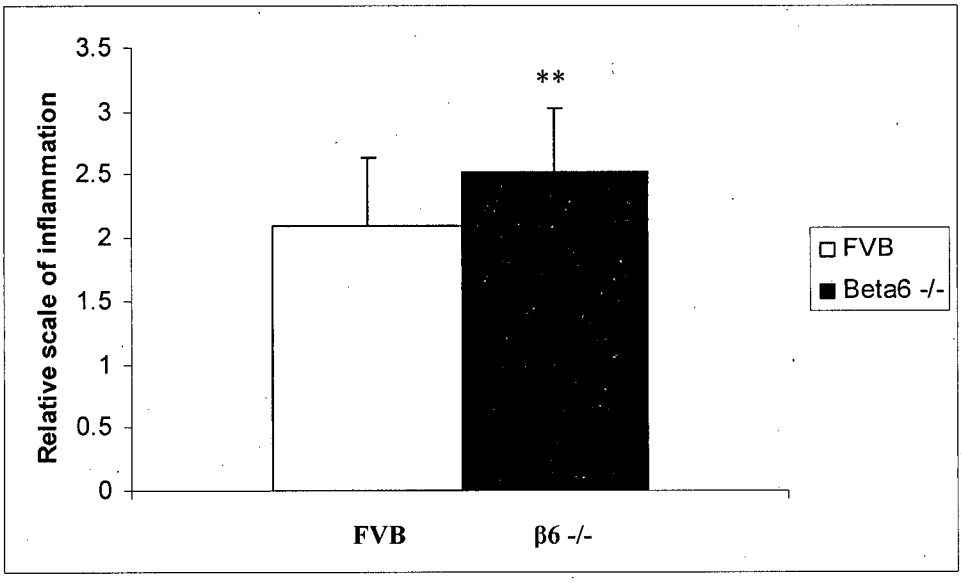


Figure 14. Qualitative assessment of inflammation immediately adjacent to JE and/or PE. Mean values ± SD of 117 sites from FVB group and 115 sites from β6 -/- are presented. Statistical analysis performed using Mann-Whitney U test. (**): Very significant difference, P-value < 0.005.

CHAPTER VII

Discussion

The present study is one of the first studies to compare the initiation and progression of periodontal disease along with the degree of alveolar bone loss between wild-type and αvβ6 knockout mice. As an exclusively epithelial integrin, the αvβ6 integrin found in junctional epithelium appears to exercise a protective function in the development of periodontal disease. As shown previously, induction of αvβ6 expression as a result of tissue injury plays a crucial role in the activation of TGF-β1 via binding LAP at the RGD site and therefore is an important factor in downregulating the inflammatory response to injury (Munger et al., 1999). The significance of this cascade has been demonstrated in TGF-β1 null phenotype animals which died within a few weeks of birth as a result of diffuse mononuclear cell infiltrates (Shull et al., 1992; Kulkarni et al., 1993). Consequently, the findings of the present study suggest that the enhanced bone loss and pocket formation seen in β6 integrin-deficient mice is subsequent to insufficient suppression of inflammation as a result of poor TGF-β1 activation due to lack of αvβ6 integrin.

Furthermore, the apical migration of junctional epithelium associated with increased infiltration of inflammatory cells seen in these animals indicates that avβ6 integrin is possibly one of the key components in regulating the inflammation mediated via TGF-β1 signaling.

As demonstrated previously, αvβ6 integrin has limited distribution in the body. Despite the fact that it is not expressed in oral gingival epithelium, αvβ6 integrin is continuously expressed in junctional epithelium, making it a unique epithelium (Garcia, 2005). In adult tissue, αvβ6 integrin is expressed during inflammation, carcinogenesis, and in wound healing (Breuss et al., 1995).

In β6 integrin knockout mice, alteration in cell response to tissue injury and inflammation has been demonstrated in specific organs such as in skin, intestine and lungs (Huang et al., 1996; Munger et al., 1999; Sheppard, 2001; Morris et al., 2003; Jenkins et al., 2006). Moreover, formation of spontaneous chronic wounds was observed in the skin of transgenic mice overexpressing αvβ6 integrin which found to be associated with elevated TGFβ levels. (Häkkinen et al., 2004).

In a recent experiment, Hahm et al. (2007) demonstrated inhibition of renal fibrosis in β6-deficient Alport mice, indicating the potential regulatory role of αvβ6 integrin as an important mediator in the initiation and maintenance of kidney fibrosis (Hahm et al., 2007). Considering its ability to bind and activate latent precursor TGFβ, it was suggested that misregulation of the αvβ6 function in an existing disease condition could enhance disease-associated tissue damage and inflammation (Hahm et al., 2007).

In the present study, we compared the degree of inflammation in the connective tissue immediately adjacent to the junctional epithelium between the 12-month old αvβ6 integrin deficient and 12-month old wild-type mice. The results showed statistically

significant more inflammatory cells present in the connective tissue of β6 integrin knockout animals. In addition, pocket formation as a result of apical migration of JE was a common observation in αvβ6 integrin-deficient mice. These findings suggest that αvβ6 integrin may be involved in both regulation of cell proliferation and the immune response in the gingiva.

Periodontal inflammation is a multifactorial process that develops as a result of different host factors. Although the bacteria are recognized as the initiating force, the host immune response is considered critical for disease progression (Socransky and Haffajee, 1992; Genco, 1992). IL-1β is one of the most potent cytokines involved in the pathogenesis of periodontal disease (Koide et al., 1995). Synthesized by a variety of cells such as stimulated monocytes, macrophages, and epithelial cells, IL-1β mediates tissue remodelling, repair, and inflammation.

While low levels of IL-1β give rise to protective ability against infection (Graves et al., 2000), increased production of IL-1β leads to tissue destruction through synthesis of collagenase and PGE2 (Graves and Cochran, 2003).

Dayan et al. (2004) have demonstrated proliferation and apical migration of JE in mice overexpressing IL-1β in keratinocytes, independent of the bacterial colonization. Similar morphological changes of JE were reported by Garcia (2005) in αvβ6 integrin-deficient mice. The proliferation and apical migration of JE resulting in formation of pocket epithelium was also seen among the β6 integrin knockout mice in the present study.

The antagonistic functions between TGF-β1 and IL-1β leads to TGF-β1 counteracting IL-1β functions (Benus et al., 2005; Takahashi et al., 2005). Animal studies have shown that TGF-β1 downregulates IL-1β expression in murine hepatocytes (Matsumura et al., 2004). Considering these findings along with the observations made in the present study, it can be speculated that lack of αvβ6 integrin may potentially lead to increased IL-1β expression as a consequence of decreased levels of activated TGF-β1. However, since in the present study cytokine levels were not measured, this has to be interpreted with caution.

TGF-β1 is a potent cytokine in regulating the immune system (Li et al., 2006). Produced in the later phase of inflammation, TGF-β1 with its multifaceted roles controls the initiation and resolution of inflammatory responses (Li et al., 2006).

αvβ6 integrin, with its ability to bind to TGF-β1, is an important component in the activation of this cytokine. Despite the many pathways known to activate TGF-β1, activation via αvβ6 integrin seems to be the predominant mechanism in cells containing this integrin (Jenkins et al., 2006). Several inflammatory problems have been reported due to poor activation of TGF-β1 in animals lacking αvβ6 integrin (Munger et al., 1999; Sheppard, 2001). In addition, the JE of β6 integrin knockout mice was found to be thickened, indicating increased cell proliferation (Garcia, 2005).

In the JE, cell proliferation taking place in the basal layer occurs adjacent to the gingival connective tissue rather than adjacent to the tooth (Watanabe et al., 2004). In the process

of periodontal pocket formation, it seems that basal JE cells move apically towards the root before migrating coronally along the cementum towards the clinical crown (Stern, 1981). Therefore, maintaining the JE in a steady state and providing a balance between numbers of cells undergoing apoptosis, proliferation, and shedding is critical in the process of pocket formation (Watanabe et al., 2004).

Cell cycle progression controlling this dynamic process is regulated by a class of enzyme complexes composed of cyclins and cyclin-dependent kinases (Cdk) (Morgan, 1995; Ekholm and Reed, 2000). Two classes of Cdk inhibitors (CKIs), the Cip/Kip family and the INK4 family, tightly control the activities of Cdk complexes. The Cip/Kip family with its three members (p21, p27, and p57) considered to be broad spectrum CKI and can, therefore, bind to and inhibit both cyclin-D–Cdk4/6 kinases, as well as cyclin-E/A–Cdk2 (Ekholm and Reed, 2000). The INK4 family, composed of four members (p15, p16, p18 and p19), is a group of narrow spectrum CKI and can only bind to and inhibit Cdk4 and Cdk6 (Ekholm and Reed, 2000).

Cyclin-D–Cdk4/6 complexes provide a reservoir for Cip/Kip inhibitors, preventing them from interacting with cyclin-E–Cdk2 and thereby promoting cell cycle progression (Ekholm and Reed, 2000). Disruption of these complexes allows Cip/Kip inhibitors to interact with cyclin-E–Cdk2, resulting in inhibition of cell cycle progression (Reynisdottir et al., 1997).

In a recent study Watanabe et al. (2004) have demonstrated enlarged JE in p21/p27 double knockout mice (dKO), suggesting specific roles for both p21 and p27 in controlling the size and possibly the integrity of the JE. Enlarged JE associated with increased proliferation was also seen in our study among mice lacking the αvβ6 integrin. These similarities could be explained by insufficient TGF-β1 activation as a result of αvβ6 integrin deficiency.

Reviews of cell cycle regulation in mammalian cells indicated that TGF-β1 stimulates p15 induction, which then forms stable complexes with Cdk4/6 leading to disruption of cyclin-D–Cdk complexes (Ekholm and Reed, 2000). In addition, analyses of human prostate epithelium have shown the effect of exogenous TGF-β1 in blocking the cell cycle at G1, and increases in p21 and p27 levels. Consequently, impaired TGF-β1 activation downregulates the accumulation of cyclin-dependent kinase inhibitors p15, p21 and p27, allowing for increased epithelial cell proliferation (Watanabe et al., 2004).

Therefore, αvβ6 integrin deficiency could contribute via poor TGFβ activation to increased epithelial cell proliferation and thereby pocket formation in periodontal disease. Furthermore, the present study showed that lack of αvβ6 integrin leads to increased alveolar bone loss in mice as measured per exposed root surface area. With the exception of the 2nd molars from the 12-month old age group, statistically significantly more bone loss was measured among the knockout animals independent of age or gender. Considering the fact that among 12-month old the β6 -/- mice, three out of fifty-six molars were lost as a result of excessive bone resorption, one might argue that exclusion

of 5% of the data may have contributed to underestimation of the differences between the two groups. In addition, the lack of significant difference might be explained by the fact that from the thirty-six 2nd molars (18 animals) included in the FVB group, seven teeth from 6 different animals presented with severe bone loss similar to the values measured for the β6 -/- group. With the exception of one animal which showed severe bone loss on both sides (left and right) of the mandibular jaw, the extensive alveolar bone destruction was only unilateral for the other five animals with the contralateral side exhibiting values similar to the mean. Comparison of the two groups (FVB & β6 -/-) using Student t-test demonstrated statistically very significant differences (P-value < 0.005) when those seven teeth were excluded from the analysis (data not shown). As known local factors are important etiological factors in initiation and progression of periodontal disease and subsequently alveolar bone loss, the author does not exclude the possibility that the extensive periodontal destruction observed for those 7 teeth might have been as a result of local factors such as animal hair impaction to the gingival sulcus.

When comparing the exposed root surface area, it is critical to rule out compensatory supereruption of the teeth involved, which results naturally as tooth substance is lost when in function. In mice, the three molars present per quadrant complete their eruption within 5 weeks of birth (Page and Schroeder, 1982). When fully in occlusion, natural wear through enamel and dentin will occur. Since alveolar bone follows this secondary tooth eruption only partially (Gilmore and Glickman, 1959), an increase in the distance between the CEJ and the alveolar crest will occur, providing an illusion of periodontal bone loss. To ensure that the increased distance is mainly due to periodontal disease and

not as a result of attrition and consequent supereruption, the distance between the CEJ and a set straight line at the bone of alveolar process was measured for each root of each tooth. The results have indicated significant differences between the two groups (FVB & β6 -/-) among the 1st molars of the 12-month old and 2nd molars of the 6-month old animals. This physiological supereruption might have contributed to overestimation of the degree of bone loss and therefore exaggerated the differences between the two groups. Nonetheless, the fact that knockout mice demonstrated statistically significantly more bone loss among all age groups for both molars suggests increased periodontal bone loss as a result of αvβ6 integrin deficiency. The enhanced periodontal destruction associated with lack of αvβ6 integrin was further confirmed when comparing radiographic images. Since the assessment included the entire alveolar bone surrounding the roots, supereruption could not have affected the results. This was true for visual assessment of 3D EM images which have clearly exhibited crater formation around the teeth of knockout animals.

During the process of collecting the data, it was noticed that β6 -/- mice exhibited generally more attrition of the teeth which increased in severity as the animals aged (data not shown). This could be caused by either increased function due to periodontal disease affecting grinding of the hard mouse food pellets or defects in the enamel itself. Interestingly, it was recently documented that β6 integrin is expressed by ameloblasts during the late enamel organ formation (Moffatt et al., 2006). Therefore, a possible involvement of αvβ6 integrin in the mineralization of the enamel organ cannot be ruled out and requires more in depth investigation.

CHAPTER VIII

Conclusions

Integrins as cell surface receptors mediate cell-to-cell or cell-to-extracellular matrix adhesion. Through many intracellular signaling pathways, they regulate diverse processes such as proliferation, migration, and differentiation. We present here the first evidence that αvβ6 integrins, absent from oral gingival epithelium but continuously expressed in junctional epithelium, play a significant role in the etiology of periodontal disease. This was demonstrated through the comparison of alveolar bone loss as well as the formation of pocket epithelium between wild-type and β6 integrin-deficient mice. Previous studies have indicated the association of αvβ6 integrin in the activation of TGF-β1, a multifactorial immunoregulatory cytokine. Since in periodontal disease the host immune response is considered critical for disease progression, it seems plausible to conclude that the increased attachment loss seen in β6 -/- animals is partly due to lack of TGF-β1 activation as a result of αvβ6 integrin suppression. Furthermore, the regulatory influence of TGF-β1 in the cell cycle dynamic suggests that αvβ6 integrin via its ability to activate TGF-β1 possibly plays an important role in controlling epithelial cell proliferation during pocket formation in periodontal disease.

Recommendation for future studies

Despite the new advances in determining the role of αvβ6 integrin in junctional epithelium, the exact mechanisms by which lack of this integrin leads to alterations in morphology and cellular organization of JE is not clearly understood. More studies are needed to elucidate the dynamics of this process.

It seems that suppression of αvβ6 integrin in mice is a critical factor in the development and progression of periodontal disease during the natural aging process. Therefore, future studies could focus on alterations seen in β6 -/- mice during inductive conditions such as diabetes or bacterial inoculation.

In addition, quantification of proinflammatory cytokines and inflammatory cell infiltrate in animals lacking αvβ6 integrin may be of benefit in determining the immunoregulatory role of αvβ6 integrin in the gingiva.

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