EXPRESSION OF αvβ6 INTEGRIN IN THE JUNCTIONAL EPITHELIUM

by

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Abstract

Cell-cell binding and cell-extracellular matrix adhesion is mediated by cell surface receptors known as integrins, which play important roles during wound healing. Junctional epithelium (JE) that links gingiva to tooth enamel mimics wound epithelium. The αvβ6 integrin is an epithelial integrin that is not normally expressed by oral gingival epithelium but is induced during wound healing, and has also been shown to be expressed in squamous cell carcinomas and in leukoplakia specimens. Furthermore, αvβ6 integrin can bind and activate transforming growth factor-β (TGF-β), suggesting a central role for the integrin in this unique epithelium. Using human gingival specimens from extracted teeth and human gingival specimens attached to decalcified teeth, the presence of JE was confirmed using simple epithelia tissue markers (cytokeratin-19 and laminin-5). The presence of αvβ6 integrin in the JE and some of its putative ligands (such as tenascin, fibronectin and TGF-β) was confirmed using immunohistochemical labelling. Paraffin sections of wild-type (FVB), β6integrin-overexpressing (B6F1) and β6integrin-knock-out mice (β6-/-) were stained with hematoxylin and eosin and observed under the light microscope to analyze the morphology of the JE. Interestingly, the analysis of the β6-/- sections showed striking alterations in the morphology and cellular organization of the JE. The findings of this study suggest that αvβ6 integrin is constitutively expressed in the JE, in which it could function as an immunoregulator via activation of TGF-β1.

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List of Abbreviations

αvβ6	Alphav beta6
BM	Basement Membrane
B6F1	Beta6integrin-Overexpressing
β-/-	Beta6integrin-Knock-Out
CEACAM	Carcinoembryonic Antigen Cell Adhesion Molecule
CK	Cytokeratin
DAT	Directly Attached to Tooth
EA	Epithelial Attachment
EBL	External Basal Lamina
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FGF	Fibroblastic Growth Factor
FN	Fibronectin
FVB	Wild-Type
GTP	Guanosine Triphosphate
HSPG	Heparan Sulphate Proteoglycan
H&E	Hematoxylin and Eosin
IBL	Internal Basal Lamina
ICAM	Intercellular Adhesion Molecule
IGF	Insulin Like Growth Factor
IL	Interleukin
JE	Junctional Epithelium
K-	Keratin-
LM	Laminin
MMP	Matrix Metalloproteinase
OE	Oral Epithelium
PA	Plasminogen Activator
PAI	Plasminogen Activation Inhibitor
PDGF	Platelet Derived Growth Factor
PMN	Polymorphonuclear
REE	Reduced Enamel Epithelium
SE	Sulcular Epithelium
TGF-β	Transforming Growth Factor-beta
TN	Tenascin
TNF	Tumor Necrosis factor
t-PA	Tissue-Type Plasminogen Activator

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DEDICATION

This work is dedicated to my parents Oswaldo and Maria Isabel. Their continuous support made possible my coming to Vancouver, and their example of hard work and perseverance has taught me that giving-up is never an option. No tengo palabras para agradecer todo lo que han hecho y siguen haciendo por mi, los quiero mucho mami y papi.

And to you Gethin, thanks for all your love, care and support. You are an example to follow of how to be a great man and a great professional. I love you.

CHAPTER I

1. Dentogingival epithelium

1.1 Development

The primary epithelial attachment is the junction between the reduced ameloblasts and the enamel surface; this is, the attachment apparatus before eruption. During early maturation of ameloblasts, their cytoplasmic border becomes striated (ie. projections and deep infoldings) and is separated from the enamel surface by a small gap (400-800 Å) containing a fine granular material. This material with the electron-lucent layer adjacent to the cell membrane, will represent the internal basal lamina of the reduced enamel epithelium (Schroeder and Listgarten, 1971).

The proliferating cells of both oral and reduced enamel epithelium will fuse just before crown erupts into the oral cavity, forming the primary epithelial attachment (Schroeder and Listgarten, 1971; Ten Cate, 1996). This primary epithelial attachment will persist for varying periods of time until a reduced enamel epithelium is no longer demonstrable and the permanent secondary epithelial attachment is established (Ten Cate, 1996). During this transition, the ameloblasts will become flat and loose their ability to divide and undergo apoptosis (Shibata et al., 1995).

The origin of junctional epithelium (JE) from reduced enamel epithelium (REE) has been further demonstrated using immunohistochemistry and immunoblotting, where cell cultures of junctional, oral gingival and reduced enamel epithelium were compared for patterns of phenotypic expression. Junctional and reduced enamel epithelium showed similarities between them, supporting the concept of the origin of JE from the REE (Gao and Mackenzie, 1992). However, the cells of the secondary junctional epithelium seem to be derived from the oral gingival epithelium, since they showed to have a similar pattern of co-expression of certain keratins (Mackenzie et al., 1991).

In summary, the JE originates from the enamel organ and both the sulcular and gingival epithelium derive from the oral mucosa (for review refer to Bartold et al., 2000).

1.2 Composition/structure

The JE is a non-keratinized epithelium, forming the base of the gingival sulcus and extending apically towards the enamel surface to form a "seal" between the epithelium and the tooth.

Ultrastructurally, the JE is wider in the sulcular area, tapering apically. It may be composed on its widest areas of up to 15-30 cells (which make 82%), which are mainly flattened parallel to the surface; and about 1-3 cell layers apically. The intercellular space often contains crossing PMN leukocytes and monocytes (Schroeder and Listgarten, 1971; Schroeder et al., 1989). The border with the connective tissue may have slight rete pegs, but is usually straight.

The JE has a high average (4.6 –10.9 days) cell turnover rate, this is 50-100 times faster than for the oral epithelial cells (Skougaard, 1965). The cells of the JE have ovally elongated nuclei, prominent Golgi complexes, extended cisternae of the rough endoplamic reticulum and few cytoplasmic filament bundles (Schroeder, 1986).

The vascular network of the JE is made up of a flat squamous-shaped mesh, comprised mainly of venous capillaries. Almost half the capillaries that make this vascular network are fenestrated blood vessels with pores in the vascular wall, making the JE highly permeable (Matsuo and Takahashi, 2002).

Epithelial attachment (EA) is the union found towards the tooth, consisting of basement lamina (called internal basal lamina) and hemidesmosomes (Stern, 1981; Hashimoto et al., 1986). These hemidesmosomes are more specifically of the type I (Hormia et al., 2001). This internal basal lamina has an average width of 1,190 ±190 Å, but cannot be clearly divided into lamina lucida and densa (Schroeder and Listgarten, 1971). On the connective tissue side, the external basal

lamina can be found. External basal lamina is a typical basement membrane that is linked to the underlying connective tissue via anchoring fibres that are absent from the internal basal lamina. More details regarding the composition of the basement membrane of the JE will be given below.

Cytokeratins characterize different types of epithelia. Cytokeratins are a subfamily of intermediate filament proteins and are characterized by a remarkable biochemical diversity, represented in epithelial tissues by at least 20 different polypeptides (Presland and Dale, 2000). This major protein is used in the formation of unique cell-type specific filament network in the keratinocyte. They range in molecular weight between 40-68 kDa and isoelectric pH between 4.9-7.8. The cytokeratins (CK) are designated 1 to 20. CK-1 has both the highest molecular weight and isoelectric pH, while CK-19 has the lowest ones. Cytokeratins are also divided into subgroups I and II, where subgroup I members (ie. CK 1- CK 8) are acidic-neutral and the latter group members (CK 9- CK 19) are the acidic ones. A keratin filament is composed of one acid and its corresponding basic keratin (Schaafsma and Ramaekers, 1994; Lu, 2000).

The various epithelia in the human body usually express cytokeratins, which are not only characteristic of the epithelium, but also related to the degree of maturation and differentiation within an epithelium. Different types of CK phenotype can be found in different epithelia. Cuboidal and columnar epithelium, or one-layered epithelium (ie. simple epithelium), which is in direct contact with the basement membrane and has a free luminal surface at the apical side of the cell, usually express CK-8 and –18; and often CK-7, -19 and –20 (these three are expressed in certain subtypes of epithelia). Both keratinizing and non-keratinizing stratified epithelia have a basal cell layer, which express CK-5 and –14; whereas the suprabasal cell layers express CK-1, -4, -10 and –13. Transitional stratified epithelia express CK-4 and –13, plus the CK expressed in the simple epithelia. Combined epithelia (ie. a basal cell layer and a layer of columnar cells both in contact with the basement membrane) expresses CK-5, -14 and –17 in its basal cells and a

combination of CK-7, -8, -18, -19 and –20 in its lumical cells. Squamous epithelia with a high cell turnover express CK- 6 and –16 (Schaafsma and Ramaekers, 1994; review by Owens and Lane, 2003).

CK-19 is one of the smallest cytokeratins (Wu and Rheinwald, 1981) and it is expressed in the basal cell layer of non-keratinized epithelia. CK-19 expression is abundant in basal cell layers of primary and secondary junctional epithelium, whereas the suprabasal and basal layers show expression of CK-4, -5, -13, -14 and -16 (Bartek et al., 1986; Juhl et al., 1989; Bampton et al., 1991; Carmichael et al., 1991; Feghali-Assaly et al., 1994 and 1997.) There are different and distinctive patterns of keratin expression in the oral gingival, oral sulcular and junctional epithelium (Mackenzie et al., 1991) (Table 1). Epithelium of alveolar mucosa express CK-4 and –13, while oral epithelium express cytokeratin profile typical to multilayered keratinised epithelium (Ouhayoun et al., 1985). Cytokeratin profile of the junctional epithelium is shared by the sulcular epithelium (Bampton et al., 1991; Feghali-Assaly et al., 1994 and 1997; Pritlove-Carson et al., 1997; Sculean et al., 2001).

Table 1. Localization of different cytokines in different oral epithelia

EPITHELIA	CYTOKERATIN	STUDY
Oral	1, 2, 5, 6, 10, 11, 13, 14, 16, 17	Juhl et al., 1989; Pelissier et al., 1992; Pritlove-Carson et al., 1997
Junctional-basal	5, 14, 19	Bampton et al., 1991; Carmichael et al., 1991; Feghali-Assaly et al., 1994 and 1997; Pritlove-Carson et al., 1997; Lu et al., 2000
Junctional-suprabasal	4, 5, 6, 8, 13, 14, 16, 17, 18	Bampton et al., 1991; Carmichael et al., 1991; Feghali-Assaly et al., 1994 and 1997
Sulcular-basal	5, 13, 14, 19	Feghali-Assaly et al., 1994 and 1997; Pritlove-Carson et al., 1997
Sulcular-suprabasal	4, 5, 13, 14, 17	Feghali-Assaly et al., 1994 and 1997; Pritlove-Carson et al., 1997
Alveolar mucosa	4, 13 (major); 5, 6, 14, 17 (minor)	Ouhayoun et al., 1985

1.3 Role in disease/protection

As described above, the JE is a non-keratinizing, non-differentiating, fast-renewing epithelium. Its intercellular spaces provide a pathway for inflammatory exudates, for foreign molecules and also serves as a temporary residence for lymphocytes and monocytes (see reviews by Schroeder and Listgarten, 2000; Bosshardt and Lang, 2005).

Analyzing cross-sections of gingiva, Schroeder and co-workers determined that the most coronal part of the JE was comparatively loose with widened intercellular spaces, in particular, near the enamel surface. The most enlarged intercellular spaces occur in the central region, orientated corono-apically as open passages lined by smooth cell walls (Schroeder et al., 1989). The presence of wide intercellular spaces may be correlated with the low number of desmosomes, which interconnect cells mechanically in the JE (Hashimoto et al., 1986). Under normal conditions, about 1-2% of these intercellular spaces is occupied by neutrophils, macrophages and lymphocytes (Schroeder and Listgarten, 1997).

Under mild gingival inflammation, the JE serves as a main pathway for the inflammatory exudate. In inflammation, the exudation from adjacent blood vessels creates a pressure differential, forcing fluid from the interstitial connective tissue into the intercellular spaces of the JE, dilating them. These wide intercellular spaces also allow chemotactic substances to diffuse from the sulcus into the tissues. The spaces may fuse with one another, creating wide-open passages that run apico-coronally through the central region of the JE (Schroeder et al., 1989). Mononuclear cells, mainly lymphocytes, are located on the connective tissue side of the JE. These are passively carried into the sulcus as a result of normal JE turnover. On the other hand, neutrophils actively migrate into the sulcus. When the number of these neutrophilic granulocytes increases to occupy about 30% of the intercellular spaces, the disruption of the JE starts to occur (Schroeder et al., 1989; Schroeder and Listgarten, 1997). Therefore, the permeability of the epithelium is related to the outward flow of gingival fluid and to the transmigration of

neutrophilic granulocytes between epithelial cells (Shimono et al., 2003). The normal turnover of the JE is not able to compensate for all this damage during active inflammation (Schroeder et al., 1989).

The apical two-thirds of the junctional epithelium contains sensory nerves rich in dense nerve plexus (Byers and Holland, 1977; Tanaka et al., 1996). The nerve fibers extend from the nerve bundles in the lamina propria and penetrate into the JE, suggesting that this dense network of nerve fibers may serve as a sensory receptor, amongst other functions (Maeda et al., 1994). In 1999, a neurokin-1 receptor was found in the JE cells. Authors suggested that this substance activates substance P, which may modulate the permeability of the blood vessels beneath the JE, as well as regulate the proliferation and endocytic activity of the JE. Despite these studies, its function has not been fully clarified (Kido et al., 1999).

1.3.1 Cell adhesion molecules

Cell adhesion molecules, such as integrins and cadherins, are expressed by the junctional epithelial cells. Integrins and its expression in the JE will be discussed later. On the other hand, cadherins are transmembrane proteins that play a role in developmental processes and in the maintenance of tissue architecture. They are localized in adherence junctions, establishing linkages with the actin-containing cytoskeleton and with intermediate filaments (for review see Juliano, 2002).

Epithelia cadherin (E-cadherin) is found in stratified squamous epithelia and is important in the maintenance of structural integrity and function of adherens and desmosomal epithelial intercellular junctions. Immunohistochemical analysis has shown that both healthy gingiva and advanced periodontal disease, express patterns of E-adherin. A significant reduction in staining intensity was observed from the healthy external oral epithelium to the junctional and pocket epithelium; these changes indicated an impairment of the epithelial structure and moreover

alteration of intercellular adhesion and cell-to-cell communication (Ye et al., 2000). Controversially, E-cadherin was shown not to be expressed in the JE cells at all (Heymann et al., 2001; Ivanov et al., 2001).

A cell adhesion molecule, CEACAM1 (carcinoembyonic antigen cell adhesion molecule 1), that belongs to the immunoglobulin superfamily, has been shown to be present in the JE of rats and mice, regardless of bacterial infiltration (Singer et al., 2000; Juliano, 2002). CEACAM1 has been shown to be a negative regulator of cell proliferation (Singer et al., 2000; Abou-Rjaily et al., 2004). It can also function as a receptor for certain pathogenic bacteria (Kammerer et al., 2004; Hill et al., 2005). This suggests a possible role of CEACAM1 in JE immunomodulation (Heymann et al., 2001).

ICAM's (intercellular adhesion molecules) also belong to the immunoglobulin superfamily expressed on the surface of endothelial cells. They mediate cell-to-cell interactions in inflammatory reactions, allowing for the passage of leukocytes from the capillaries and hence into the underlying tissue. The expression of adhesion molecules is one of the key events in neutrophil migration at sites of infection. ICAM-1 and IL-8 have been shown to be expressed in JE cells, establishing a gradient of ICAM-1 receptors within the JE of healthy gingiva (Tonetti, 1997; Tonetti et al., 1998). This expression increased from the basal cells towards the surface of the JE, and then to the areas of bacterial infiltration; suggesting a role in the routing of PMNs to the site of infection (Tonetti, 1997; Tonetti et al., 1998).

1.3.2 Cytokines

JE expresses a number of cytokines. Cytokines that have been shown to be expressed, especially in the superficial layers of the coronal half of the JE cells are TNF-α, IL-1α and IL-1β. The expression of these cytokines is enhanced in the presence of inflammation, compared to health (Miyauchi et al., 2001). Epidermal growth factor (EGF), on the other hand, has been shown to be

poorly expressed in the JE (Nordlund et al., 1991). Transforming growth factor-beta (TGF-β) has been shown to be present in both junctional and oral gingival epithelium (Lu et al., 1997).

1.3.3 Proteases

Plasminogen activators (PA) are proteases associated with the fibrinolytic system, that play major roles in extracellular proteolysis (review by Skrzydlewska et al., 2005). Plasmin degrades tissues, thus degrading matrix proteins and activating metalloproteinases. PA's are likely to participate in the pathogenesis of periodontal disease, and have been identified in gingival crevicular fluid and in JE of both healthy and diseased gingival tissue. Activity of PA was found in healthy tissue, originating from the junctional epithelium (Schmid et al., 1991). More recently, both t-PA (tissue-type plasminigen activator) and PAI (plasminogen activator inhibitor) were found to be expressed in the outer layers of sulcular and junctional epithelia; showing that their expression increases during experimental inflammation (Lindberg et al., 2001a and b).

As mentioned above, matrix metalloproteinases (MMP's) are activated by plasmin. These MMP's are neutral proteinases that are capable of degrading all components of the ECM, playing a role in physiological ECM remodelling, and also in pathological conditions such as periodontitis. MMP-7 (matrilysin) is one of the smallest MMP's, and it is expressed in many epithelial cells. It has also the capacity to activate antibacterial defensins. In a human gingival model, MMP-7 was localized in suprabasal cells of the JE at the internal basal lamina. Its expression was stimulated by certain oral bacteria. This suggested a possible role in intra-epithelial cell migration process in the JE, and also in the conversion of antibacterial α-defensin peptides to their active forms (Uitto et al., 2002).

1.4 Transition to pocket epithelium

The transition from junctional epithelium to pocket epithelium involves many different factors. Pocket formation seems to be initiated by the degenerative changes in the second or third cell layer, from the innermost cells in the most coronal part of the JE. The result is a very thin and ulcerated epithelium, with a disruption of the epithelial barrier (Takata and Donath, 1988). Periodontopathogenic bacteria and their virulence factors are also responsible for this pocket formation. For example, *P. gingivalis*'s gingipains have been shown to be implicated in the degradation of epithelial junctional complexes (Deshpande et al., 1998; Lamont and Jenkinson, 1998; Potempa et al., 2000; Chen et al., 2001; Hintermann et al., 2002; Katz et al., 2002; Imamura et al., 2003).

In summary, the JE is firmly attached to the tooth and creates an epithelial barrier. It allows the passage of gingival crevicular fluid, inflammatory cells and host defense cells into the gingival sulcus; and its rapid turnover, which helps to the rapid repair of damaged tissue. These characteristics form the first line of defense against antimicrobial invasion into tissue (Pollanen et al., 2003). In inflammation, the JE produces IL-1, IL-8, PDGF and ICAM-1, that cause neutrophil recruitment (Stern, 1981; Crawford, 1992; Tonetti et al., 1998; Bartold et al., 2000), leading to a pathologically altered JE, which commences the earliest formation of a periodontal pocket.

CHAPTER II

2. Basement Membrane

2.1 Composition and functions

Basement membranes that are also called basal laminae, are found throughout the body, as thin layers (40-120 nm) of extracellular matrix separating connective tissues from epithelial, endothelial, muscle cells, fat cells and nervous tissues (reviews by Leblond and Inoue, 1989; Miosge, 2001). They are involved in embryonic development, wound healing, metastasis and tissue remodeling (refer to review by Miosge, 2001).

Electron microscopically, the basement membrane (BM) is divided into three zones or layers: lamina lucida (or lamina rara), lamina densa (or basal lamina) and lamina reticularis (Tsiper and Yurchenko, 2002). The lamina lucida is adjacent to the surface cells and mainly composed of carbohydrates. Its thickness is approximately 27 nm (can vary between 15-65 nm). It is not clearly delimited, apparently continued with the glycocalix of the surface cells. This lamina lucida, which has been shown to contain anchoring filaments, has been also considered as a surface layer of the lamina densa. These filamentous structures extend from the basal keratinocytes' hemidesmosomes to the lamina densa, thus, traversing the lamina lucida; and then, connecting the anchoring fibrils to the sublamina densa, more specifically, to the anchoring plaques (Nievers et al., 1999). One of the major components of the anchoring filaments is laminin-5 (Rousselle et al., 1991; Marinkovich et al., 1992), amongst others such as laminin-6 (Marinkovich et al., 1992), protein p105 (Chan et al., 1993), and BP180 (Masunaga et al., 1997). It has been suggested that laminin-5 is incorporated into the basement membrane via laminin-6 and –7, as they can bind directly to the nidogen (Nievers et al., 1999). Proteoglycans such as perlecan have been located in the lamina lucida, and they seem to participate in the cell connections of the basement membrane (Leblond and Inoue, 1989; Merker, 1994; Aba and Osawa, 1999; Sawada and Inoue, 2001; Tsuprun and Santi 2001).

The thickness of the lamina densa varies depending on its localization and the origin of the tissue (eg. diabetes, age), from 15-125 nm (Mandel et al., 1983; Roy et al., 1994; Gardiner et al., 2003). Using electron- microscopy, Leblond and Inoue (1989) were able to identify a three-dimensional network of irregular strands of an average thickness of 3-4 nm, named "cords". These cords continue into an anchoring fibril (a way of attachment to the extracellular matrix), which is a 2-8 nm filamentous structure traversing the lamina lucida beneath the hemidesmosomes and consists basically of disulfide bond-stabilized dimers of collagen VII (Sakai et al., 1986; Leblond and Inoue, 1989; Ghohestani et al., 2001; Sawada and Inoue, 2001). Anchoring fibrils originate from the lamina densa and form different roots, which will then combine to form trunks; both of its ends contain a cluster of COOH-terminal domains of type VII collagen molecules available for interaction with other matrix components (Hay, 1991; Aba and Osawa, 1999; Ghohestani et al., 2001). The cords, mentioned earlier, join one another to form a three-dimensional network; and between them, an intercordal space which can be either empty or full of tenous material. Cords may be in continuity with simple filaments, as well. Within cords, there may also be "double tracks", which are 4.5 nm thick structures consisting of two parallel running lines with an intermediary lighter band (Leblond and Inoue, 1989; Sawada and Inoue, 2001). These cords are composed of a core of type IV collagen filaments and a sheath containing and integrating the rest of the basement membrane components, which will be discussed later (Inoue and Leblond, 1988; Sawada and Inoue, 2001).

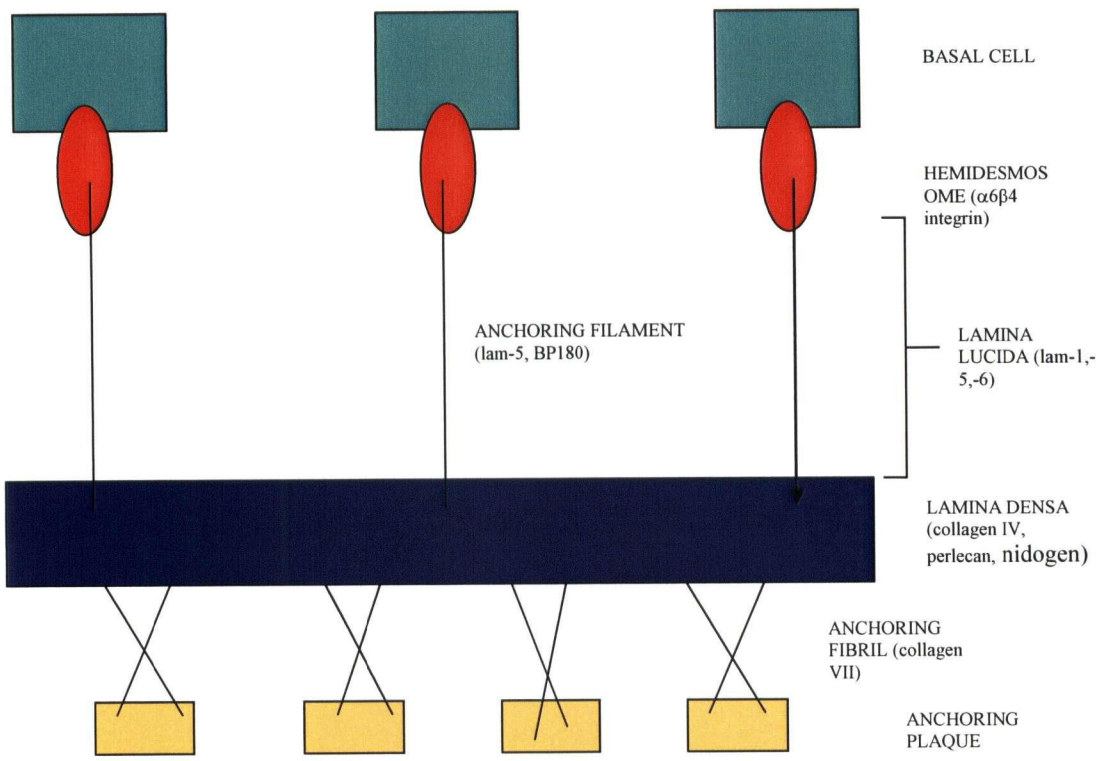

Figure1. Schematic diagram of the basement membrane (adapted from Chan LS. Frontiers in Bioscience, 1997).

The lamina densa will bind to the parenchymal cells by cords extending across the lamina lucida, and to the connective tissue via the lamina or pars reticularis. This is a transitional zone between the lamina densa and the underlying connective tissue, usually containing anchoring fibrils and microfibrils (Sawada and Inoue, 2001).

Therefore, the elements that can be found in the lamina densa of all BM are collagen IV, laminin, nidogen (or entactin) and heparan sulfate proteoglycan. These components can be found in association with each other and with other macromolecules such as fibulin-1, fibronectin, tenascin and growth factors (Leblond and Inoue, 1989; Miosge, 2001; Sawada and Inoue, 2001).

Collagen IV consists of two α1(IV) and one α2(IV) chains. It can be secreted and assembled as a procollagen-like molecule in which each chain has a molecular weight of 160,000-180,000. Under the electron microscope, each molecule looks like a rod of approximately 400 nm with a nonhelical domain at its COOH-terminal. Molecules can be associated with these nonhelical domains; but also, can be associated by their opposite NH_2-terminal. The basic unit consists of four individual molecules that overlap and are joined together at their NH_2-terminal end (also called 7S). At the other end, two molecules associate at their nonhelical domains, and are called NC1. The molecule extending out of the 7S region as one of the four arms forming, is in the form of a collagen triple helix, which is interrupted at some places by nonhelical sequences (Hay, 1991). Therefore, the two linking sites are the 7S and the NC1 domains; where the NC1 tends to join the same type of domain of another molecule and the 7S region may attach to four other monomers in an antiparallel manner, being stabilized by cystein and lysine-hydroxylin residues. The NC1 can also bind to other areas of the molecule; and moreover, other monomers may even twist around one another, creating a more irregular and complicated network for collagen IV (Yurchenco and Schittny, 1990; Merker, 1994). There is a direct attachment of collagen IV to the BM, without need for protein mediators (Aumailley and Timpl, 1986; Saccà et al., 2002).

Type XV collagen has been identified related to the basement membrane as well. This is a non-fibrillar collagen which is expressed by fibroblasts, muscle, endothelial and some epithelial cells; and is detected in most tissues except for the liver and brain. Immunogold localization showed collagen XV as an integral and unique part of the fibrillar collagenous network immediately subjacent to the basement membrane. It has been suggested that it may play a key role in both structural organization, in signal transduction pathway and may even help to protect collagen fibres from proteolysis (Amenta et al., 2005).

The rest of components of the lamina densa, are either anchored in or on the collagen IV; the latter forming a network that determines the biochemical stability and macromolecular organization of the basement membrane and providing a scaffold into which other tissue constituents are incorporated (Saccà et al., 2002). Nidogen, or entactin, is a 148-150 kDa sulphated glycoprotein found in BM. It binds strongly to laminin (another BM component which will be discussed later), and almost all laminin preparations contain nidogen bound noncovalently to laminin, in a 1:1 ratio. It has the shape of a dumbell of approximately 20 nm, with a cystein-rich flexible connecting part of 28 kDa and the two globular ends consist of one 70 kDa terminal (641 amino acid residues) and a 36 kDa terminal (328 amino acid residues) (Hay 1991). The laminin-binding site of entactin is present in the smaller carboxyl-terminal globular domain, and it binds to the short arm of laminin. This laminin-entactin complex binds easily to collagen IV (Hay, 1991; Merker, 1994).

Heparan sulfate proteoglycans have two types of molecules. The larger one contains a 400 kDa core with three-four other smaller chains of about 60 kDa (for review see Hassell et al., 1986). The smaller type has no exact function yet, but the larger heparan sulfate proteoglycan (HSPG) is believed to be the precursor which is converted into the small one by proteolytic processing. HSPG's core proteins are anchored in or at the surface of the lamina densa via specific interactions that contribute to basement membrane architecture. They also influence in other BM's properties such as permeability and anchoring of other molecules (Hay, 1991; Merker, 1994). Perlecan is a typical peripheral membrane HSPG that interacts with the cell surface through its core protein. It is a component of the basement membrane, but is also expressed in other extracellular matrices. Its role is mainly in the assembly and maintenance of the basement membrane, and in the regulation of a wide variety of cellular processes. Perlecan's core protein interacts with a number of extracellular proteins, such as nidogen, fibronectin and it can also bind to cell surface molecules and growth factors. The lack of perlecan can lead to loss of

structural integrity in basement membranes, especially in regions of increased mechanical stress. Recently, it has been shown that perlecan interacts with fibrillin-containing microfibrils in the vicinity of the basement membrane zones; and its role may be involved in anchoring mechanisms of microfibrils to the basement membrane (Tiedemann et al., 2005).

Other components of BM have been described in the literature, such as amyloid, acetylcholinesterase, the von-Willebrand factor, collagen V, fibronectin and tenascin, amongst others. They may be localized in the lamina lucida or even in the lamina fibroreticularis, but their functions in this context are not yet known (review by Merker, 1994). Some growth factors such as TGF-beta, EGF, IGF, FGF, PDGF have been also found bound to the basement membrane; and especially during abnormal function (Vukicevic et al., 1992; Engbring and Kleinman, 2003).

Laminins are major components of basement membranes (reviews by Beck et al., 1990; Tunggal et al., 2000). Laminins are heterodimeric molecules which contain an α-, a β- and a γ-chain (also called A, B1 and B2). It is a cross-shaped small molecule (weight 900 Da and length 115nm) with a large, multilobulated globular domain at the base of the cross (Hay, 1991; Tunggal et al., 2000). To date, five different α-, three different β- and three different γ-chains have been identified, theoretically giving raise for up to 45 different trimeric laminin isoforms. Only 14 isoforms have been shown so far (for details refer to Tunggal et al., 2000; Pakkala et al., 2002). They have domains with different functions on the three smaller arms, and are expressed at different stages of development and in different tissues. On its longer arm, laminin has a binding site for cell matrix receptors, such as integrins, dystroglycan, receptor tyrosine phosphatase, heparan sulfates and other cell surface proteins (Leblond and Inoue, 1989; Colognato and Yurchenko, 2000; Miosge, 2001). The domain for heparin binding is located in the α-chain subunit and the one for collagen IV is located near the end of the short and long arms (Hay, 1991).

Laminins are the most abundant noncollagenous glycoproteins in the basement membranes. They have a role in morphogenesis, development and physiology, forming a network throughout the basement membrane to which other glycoproteins and proteoglycans attach. Their signalling role provides adjacent cells with diverse information by interacting with cell surface components. Laminin chain expression is tissue-specific and developmental stage-specific (Aumailley and Rousselle, 1999; Sasaki et al., 2004).

Laminin-1 ($\alpha 1\beta 1\gamma 1$) is the principal laminin expressed during embryogenesis, and it was also the first one to be identified and analyzed (refer to reviews by Colognato and Yurchenko, 2000; Sasaki et al., 2004). This laminin-1 binds to many cell surface receptors. In addition to its role in early differentiation, laminin-1 may also be required for the development of various organs (refer to reviews). While laminin-1 is essentially required for morphogenesis and embryogenesis, other laminin isoforms are not indispensable for these early stages, but they have tissue-specific functions at later developmental stages (Aumailley and Rousselle, 1999). For instance, laminin-5 has an important role in maintaining the architecture and stability of the basement membrane by interacting with other extracellular and transmembrane molecules of the anchoring complexes (Aumailley and Rousselle, 1999). Mutations in laminin-5 desestabilize the bridge that links the keratin intermediate filament network of the basal keratinocyte to the underlying basement membrane. Therefore, lack of laminin-5 is associated with loss of cohesion between the epidermis and dermis, causing blistering (McGowan and Marinkovich, 2000). Details of laminin-5 in the dento-epithelial junction will be discussed below.

Laminin-6 has been identified in cutaneous basement membrane and in the external basal lamina of the murine dento-epithelial junction. Laminin-7 is present in the basement membrane of fetal and adult bovine skin (Aumailley and Rousselle, 1999; Oksanen et al., 2001).

2.2 Difference of BM in the JE

Two different BMs demarcate the JE. The internal basal lamina (IBL) faces the tooth and the external basal lamina (EBL) faces the connective tissue. The IBL is a homogenous layer 80-120 nm wide, composed of a lamina densa and a thin lamina lucida (Stern, 1981). The lamina densa is 160 nm, considered thick in comparison with other BM; and is composed of thick cords (8 nm compared to 3-5 nm). This relative increase in thickness may contribute to mechanical strength in maintaining a tight seal during mastication (Sawada and Inoue, 2001). The components of the IBL are produced by the cells directly attached to the enamel (DAT cells, Salonen et al., 1989). These cells possess firm adhesive structures that bind to the tooth through laminin-5 (see below) and integrin $\alpha 6\beta 4$ (Hormia et al., 1992 and 2001). The DAT cells share some characteristics with the basal cells such as high proliferative capacity and turnover (Overman and Salonen, 1994). DAT cells are characteristically keratin-19 positive (Salonen et al., 1989). The cytoskeleton and surface structures of the DAT cells show bundles of actin, hemidesmosomes appearing as high density plaques associated with a microfilament network on their surface, and microvilli-like structures. It has been suggested that they migrate in a coronal direction along the enamel surface (Ishikawa et al., 2005). During periodontal disease, the DAT cells demonstrate degenerative changes (Overman and Salonen, 1994).

DAT cells synthesize laminin-5 and collagen VIII into the IBL. Differently from other BMs, the IBL lacks laminin-1, collagen IV and almost all anchoring filaments (Pollanen et al., 2003). Sawada et al. in 1990 confirmed the lack of collagen IV in the IBL. Based on the molecular composition and structure, the IBL is not considered a true BM (Hormia et al., 2001).

One of the main components of the IBL is laminin-5, a laminin isoform specific for epithelial cells (Rousselle and Aumailley, 1994; Hormia et al., 1995 and 1998; Oksanen et al., 2001). Laminin-5 is initially secreted as a heterodimer containing three chains: $\alpha 3A$ (200kDa), $\beta 3$ (140kDa) and $\gamma 2$ (155kDa), where the $\alpha 3A$ and the $\gamma 2$ chains will undergo further processing (for

review refer to Colognato and Yurchenko, 2000). Laminin-5 has also been associated with many epithelial tissues with secretory and protective functions (ie. skin and mucous membranes), as well as migrating keratinocytes (Carter et al., 1991; Larjava et al., 1993; Pakkala et al., 2002); playing an essential role in keratinocyte adhesion and migration (Kainulainen et al., 1998). During tooth formation, laminin-5 has been shown to be present since the secretory stage of the differentiation of the tooth in the ameloblasts and in the enamel, and later it can also be identified in the oral epithelium and BM (Yoshiba et al., 1998 and 2000). Ameloblasts synthesize it during enamel secretion, and it becomes deposited into the enamel matrix (Sahlberg et al., 1998). It has been postulated that laminin-5 expression correlates with the histiogenesis of the dental organ, ameloblast differentiation and enamel formation, in which it might promote cell adhesion between epithelial cells and the extracellular matrix (Yoshiba et al., 1998).

Although laminin-5 is one of the most important adhesive proteins at the IBL, its exact distribution remains to be explored. Despite the fact that laminin-5 can interact with the three integrins $\alpha6\beta4$, $\alpha6\beta1$ and $\alpha3\beta1$ at the surface of IBL keratinocytes (Delwel et al., 1994; Rousselle and Aumalley, 1994; Pakkala et al., 2002), it is likely that the adhesion is mediated mainly through $\alpha6\beta4$ integrin, as this integrin perfectly co-localizes with laminin at the IBL (Niessen et al., 1994; Rousselle et al., 1997; Kainulainen et al., 1998; Tsuruta et al., 2003).

In addition to laminin-5, other laminin isoforms have been identified at the EBL, but not at the IBL, namely, laminin-1 and –10 (Hormia et al., 1998, 2001; Oksanen et al., 2001).

CHAPTER III

3. Integrins

3.1 Concept/role/types

Integrins are transmembrane proteins that bind, sense and respond to the extracellular matrix (Hay, 1991; Longhurst et al., 1998; Martin et al., 2002). Common ligands for integrins include fibronectin, laminins, various collagens, entactin, tenascin, thrombospondin, von Willebrand factor and vitronectin (Hay, 1991; Martin et al., 2002). Integrins are heterodimeric molecules consisting of two subunits, α and β. Each subunit has a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain or tail (Fernández et al., 1998). These two subunits are non-covalently bound to one another by divalent cations (Hay, 1991).

Until now, eighteen different α subunits and 8 β subunits have been identified in humans, making 24 different integrins (Hynes and Zhao, 2000; Plow et al., 2000; van der Flier and Sonnenberg, 2001; Hynes, 2002).

Integrins are different to other cell-surface receptors, in the fact that they bind their ligands with a low affinity and also in that they are usually present at 10-100 fold higher concentration on the cell surface. However, they can only bind their ligands when they exceed a certain minimum number of integrins at certain places. This ligand binding is dependent on extracellular calcium or magnesium (Longhurst and Jennings, 1998; Alberts et al., 2002). More recently, it has been shown that integrins undergo a global conformational change that preceed ligand binding (Liddington and Ginsberg, 2002). The two conformations are known as open and closed. In the open conformation, the integrin either does not bind, or binds with low affinity (Takagi and Springer, 2002).

Integrins can bind simultaneously but weakly to many molecules, being able to explore the environment. After the integrin binds to its ligand, the cytoplasmic tail of the β chain binds to talin and α-actinin, initiating the assembly of a complex of intracellular attachment proteins that

link the integrin to the actin filaments in the cell cytoplasm (Longhurst and Jennings, 1998; Alberts et al., 2002; Hynes, 2002). Integrins can recognize and bind to specific ECM ligands and transduce signals leading to activation of intracellular signalling pathways and to the assembly of actin-based adhesion structures that transmit cellular forces. Therefore, when the integrin binds to the extracellular ligand, it clusters with other integrins, forming highly organized intracellular complexes known as focal adhesions, initiating a cascade of intracellular responses (Qin et al., 2004).

Integrins can trigger calcium fluxes, activate members of the RAS family of guanosine triphosphates (GTPases), therefore, activating tyrosine and serine/threonine protein kinases (which play a role in gene expression and cell cycle progression); and they can activate the Rho family of small GTPases which control the dynamics and structure of actin-based processes. Moreover, they are important in cell survival (Martin et al., 2002; Danen and Sonnenberg, 2003).

3.2 Integrin alphavbeta6 (αvβ6)

Prior to the present study, the expression of αv integrin subunit had been reported by JE cells (Hormia et al., 2001). We will report in this thesis that this integrin subunit forms a complex with the beta 6 subunit to make αvβ6 integrin at the JE.

β6 integrin is composed of 788 amino acids, sharing 38-47% identity with each if the other three β subunits (β1, β2 and β3). The cytoplasmic tail of β6 integrin differs from other related β-subunits having an extra sequence of eleven amino acids at its carboxyl terminal, suggesting different interactions with cytoplasmic components (Sheppard et al., 1990).

Using affinity chromatography in a study involving pancreatic carcinoma cells, fibronectin was identified as an RGD-dependent ligand for αvβ6 integrin (Busk et al., 1992; Weinacker et al., 1994). Another ligand for αvβ6 integrin includes the glycoprotein tenascin/cytotactin. This

attachment occurs at a site dependent on the RGD-containing third repeat (Prieto et al., 1993). It is necessary to mention here, that although αvβ6 integrin attaches to tenascin, the adhesion is not as strong as with other integrins, which will cause cell proliferation as a result (Yokosaki et al., 1996). Vitronectin was identified as another ligand for αvβ6 integrin in migrating keratinocytes (Huang et al., 1998). Although RGD is a binding site for integrins, it may not be the only one. An additional motif for αvβ6 integrin, DLXXL, which is present in several matrix components and in the β chain of many integrins, has been reported (Kraft et al., 1999).

αvβ6 integrin has a limited distribution in the body. It is expressed in epithelial cells of the kidney tubule (more specifically, the macula densa), endometrium in a uterus in secretory phase, salivary gland ducts, gall bladder and epididymis (Breuss et al., 1993).

The expression of αvβ6 integrin is up-regulated in wound healing in subclinical inflammation, and in tumorigenesis (Breuss et al., 1995). Re-epithelialization of human wounds appears to be associated with a switch from integrin αvβ5 to αvβ6 (Clark et al., 1996). In a similar manner, αvβ6 was shown to be critical for keratinocyte migration on both fibronectin and vitronectin (Huang et al., 1998). More recently, αvβ6 integrin co-localized with its ligand tenascin during wound repair (Hakkinen et al., 2000).

Interestingly, β6-knock-out mice showed a significant inflammatory cell infiltration (more specifically, lymphocytes) of their skin and lungs compared to control mice (Huang et al., 1996). Later, it was demonstrated that TGF-β1 is a ligand for αvβ6 integrin (Munger et al., 1999) and that β6-knock-out mice are protected against pulmonary fibrosis. Furthermore, the loss of integrin αvβ6-mediated activation of latent TGF-β1 causes age-dependent pulmonary emphysema. This is related to the increase in expression of the extracellular macrophage metalloproteinase (Mmp)-12 (Morris et al., 2003). In a similar manner, it was demonstrated that interruption of αvβ6integrin–mediated activation of TGF-β1 can protect against tubulointerstitial

fibrosis (Ma et al., 2003). Regulated expression of αvβ6 integrin by epithelium is, therefore considered, important for local activation of TGF-β1 in response to injury and inflammation (Munger et al., 1999; Sheppard, 2001). During nematode infection in the gut, αvβ6 integrin and TGF-β1 showed to be expressed in the jejunal epithelium; and at the same time, it was shown that the presence of αvβ6 integrin was essential for nematode-induced mast cell hyperplasia and for the expression of the mast cell protease1 (mMCP-1) (Knight et al., 2002). Similarly to the lung, αvβ6 integrin seems to play a role in intestine inflammatory response, and it may be involved in mast cell recruitment (Knight et al., 2002).

Integrin αvβ6 has also been implicated in malignant transformation, and it might be useful as a prognostic factor. This notion is supported by a study showing that approximately 50% of tumours showed upregulation of β6 integrin, where the majority were well-differentiated and node-negative (Smythe et al., 1995). The role of αvβ6 integrin in carcinomas has also been shown to be as of a contributor to squamous cell carcinoma's cell spreading and migration via fibronectin matrix (Koivisto et al., 2000; Thomas et al., 2001). In oral leukoplakia, αvβ6 integrin was highly expressed throughout the whole lesion, with a high tendency for progression in αvβ6 integrin-positive specimens (Hamidi et al., 2000). In addition, both tenascin and β6 integrin overexpression in both floor of mouth in situ and squamous cell carcinomas was shown (Regezi et al., 2002). Also, serous epithelial ovarian cancer, showed overexpression of the αv β6 integrin (Ahmed et al., 2002). Contrary reports about the role of αvβ6 integrin in tumours has also been reported (Smythe et al., 1995).

The αvβ6 integrin can also bind viruses. Foot-and-mouth disease virus is believed to use RGD-dependent integrins as cell receptors (Jackson et al., 2000; Miller et al., 2001). Also, coxsackievirus A9 (CAV9) binds through its RGD-motif to αvβ6 integrin, enhancing the cell's susceptibility to infection in presence of the β6 chain (Williams et al., 2004).

CHAPTER IV

Aim of the study

Integrin αvβ6 is an epithelial integrin that is not normally expressed by oral gingival epithelium. Expression of this integrin is induced during wound healing and it has been shown to be expressed in certain carcinomas and in leukoplakia specimens. Junctional epithelium (JE) that links gingiva to tooth enamel mimics wound epithelium in many respects.

The purpose of our study was to investigate whether αvβ6 integrin is expressed in the JE and to analyze if knocking out this receptor would alter the normal morphology of the JE.

Our hypothesis was that integrin αvβ6 is constitutively expressed by JE, in which it could function as an immunoregulator via activation of TGF-β1.

The project is divided in two parts:

1. Expression of αvβ6 integrin and its potential ligands was investigated together with markers for JE in mouse and human specimens with JE.

2. Effect of lack of β6 integrin in the morphology of JE was investigated using paraffin sections of teeth containing JE. Results were compared with JE specimens from wild type mice and those overexpressing β6 integrin under cytokeratin-14 promoter.

CHAPTER V

Material and Methods

5.1 Human Study

5.1.1 Tissue specimens

Human gingiva specimens containing JE were collected during extraction of teeth required for normal treatment. The protocol was approved by the Ethics Board of the University of British Columbia, Vancouver, Canada. Altogether, specimens from ten individuals were analyzed. These specimens included gingival tissue removed from the tooth after tooth extraction and those in which gingival tissue was maintained with the tooth. For the former specimens, gingival tissue attached to the tooth was carefully removed and embedded in tissue-tek® and snap frozen. Cryostat sections (6 microns) were cut and stored at –80 °C until used. For the latter specimens, the tooth substance was carefully removed using a diamond bur, leaving only a thin layer of tooth where the JE was attached. All the samples were embedded in tissue fixative (4% formaldehyde in phosphate buffered saline, pH= 7.2, PBS) and kept at room temperature for two hours. After this, specimens were transferred into a decalcifying solution containing 12.5% EDTA (ethylene-diaminetetra-acetic acid) and 2% formaldehyde in distilled water and kept at +4°C. The solution was changed weekly for several weeks until the tooth was soft. When the decalcification was complete, the specimens were incubated in 2.3 mol/L sucrose in PBS for twenty-four hours and then embedded in tissue-tek® and frozen in liquid nitrogen. Cryostat sections (6 microns) were cut and stored at -80°C until used.

5.1.2 Antibodies

The following antibodies were used for immunolocalization of αvβ6 integrin, its ligands and markers of JE.

As a marker of junctional and sulcular epithelium we used the monoclonal mouse anti-cytokeratin-19 (K-19), at a concentration of 1:800 (Santa Cruz Biotechnology Inc, California, US.). The presence of external and internal basal lamina at the junctional epithelium (JE) in the specimens was confirmed using the monoclonal mouse anti-laminin-5 antibody (GB-3) at a concentration of 1:30 (Abcam Ltd, Cambridge, UK).

The αvβ6 integrin was localized with the use of the monoclonal rabbit anti-β6 integrin antibody (B6B1) at a concentration of 1:20, a generous gift from Dr. Dean Shephard, University of California, San Francisco, US. To localize potential ligands of αvβ6 integrin in the JE, we used monoclonal antibodies for tenascin-C at a concentration of 1:400 (Sigma, Chem. Co, St. Louis, MO, US), fibronectin EDA at a concentration of 1:500 (Harlan Sera Lab, Loughborough, England) and polyclonal rabbit TGF-β-PAN antibody at a concentration of 1:200 (Santa Cruz Biotechnology Inc, CA, US), that recognizes TGF-β1, -2 and –3.

5.1.3 *Immunohistochemical labelling*

The immunostainings were performed using the ABC Vectastain kit (Vector Laboratories, Inc, Burlingame, CA, US) samples were prepared for immunostaining. The protocol used was performed at room temperature, unless otherwise stated. To this end, frozen sections were first fixed for five minutes in -20°C acetone. After leaving the slides to dry for a few minutes, each section was molded apart using a PAP pen (ImmEdge Pen, Vector Laboratories Inc.) and washed for 5 min in PBS containing 0.19% bovine serum albumin (PBS/BSA) (Sigma Chemical Co, St. Louis, MO, US). Sections were then rinsed and incubated with normal blocking serum for 30 min in a humidified chamber. After washing for 5 min in PBS/BSA, the sections were incubated overnight with the primary antibody in PBS/BSA in a humidified chamber at +4°C. Sections were then washed for 5 min in PBS/BSA and then incubated at room temperature with the

corresponding mouse or rabbit biotinylated secondary antibody for 60 min followed by incubation with Vector VIP substrate kit for peroxidase (Vector Laboratories Inc, Burlingame, CA, US). All the reactions were monitored until suitable colour was obtained, after which the samples were gently washed in distilled water for 5 min. The slides were left to dry at room temperature for about 60 min before mounting them with Vecta-Mount solution (Vector Laboratories Inc, Burlingame, CA, US).

Different number of sections were immunostained for the different antibodies used. Three blocks of human gingiva specimens with an average of one-hundred (100) sections per block were available. Five (5) individual specimens stained for K-19 and ten (10) for laminin-5, confirmed the presence of external and internal basal lamina in the JE. The presence of β6 integrin in the JE of these human gingiva samples was demonstrated in twelve (12) individual specimens. Some of the potential ligands for β6 integrin in the JE were shown staining four (4) individual specimens for fibronectin-EDA, four (4) for tenascin-C, two (2) for TGF-β-PAN and two (2) for LTBP.

Seven blocks of human gingiva attached to demineralized tooth, with an average of one-hundred (100) sections per block were available. In these specimens, the presence of external and internal basal lamina in the JE was confirmed by staining forty (40) individual specimens for K-19 and forty (40) other for laminin-5. To confirm the results of the gingival samples regarding the presence of β6 integrin in the JE, ninety (90) individual specimens containing gingival tissue attached to the tooth were stained for β6 integrin. The presence of tenascin-C in these samples containing gingiva attached to tooth, was confirmed staining seventy (70) individual specimens.

Negative controls for all different antibodies were stained as described above, replacing the primary antibody with PBS/BSA.

The immunostainings were then observed under the light microscope (Nikon) and photographed at different magnifications using a Nikon digital microscope camera.

5.2 Animal study

Four 6-month-old male β6 integrin-knock-out mice (β6 -/-, Huang et al., 1996: generous gift from Dr. Dean Shephard, University of California, San Francisco), four 6-month-old male wild type mice (FVB) and four 6-month-old β6 integrin-overexpressing mice (β6F1, Häkkinen et al., 2004) were euthanized and the heads were immersed in a fixative solution containing 4% formaldehyde in PBS (pH=7.2) for 24 hours. The mandibles were then separated and decalcified in 0.4 M EDTA and 2% formaldehyde in PBS (pH=7.2), changing the solution every other day for four weeks. The specimens were then processed for embedding in paraffin and sectioning using a routine protocol. Paraffin sections (5-6 μm) were cut and stained with Hematoxilyn and Eosin (H&E), observed under the light microscope and photographed using a digital camera (Nikon).

Six (6) blocks of the FVB mice, four (4) blocks of the β6F1 mice and eight (8) blocks of the β6-/- mice were included in the study. From these, an average of 100-140 sections were cut per block. Every tenth slide from each block of sections was stained with H&E, giving an average of sixty (60) stained slides for the FVB mice, forty (40) stained slides for the β6F1 mice and one-hundred (100) stained slides for the β6 -/- mice.

CHAPTER VI

Results

6.1 Human Study

The presence of JE in gingiva specimens was confirmed by morphological and immunohistochemical criteria. As shown in Figure 2A, JE was identified as a keratin-19-positive epithelium. The higher magnification view shows that the cells in the JE have a flattened shape and lie parallel at the surface that was facing the tooth (Figure 2A). Because of the collection technique, the most apical cells of the JE are seldom recovered in the specimens. The oral sulcular gingival epithelium (SE) was recognized as a transitional zone between the JE and the oral epithelium. It often contains the epithelial ridges with a few cytokeratin-19 positive cells, especially at the tips of these (Figure 2A).

The presence of internal and external basal lamina was demonstrated using immunolocalization of laminin-5 as a marker. Both sides of the epithelium were positive for laminin-5, confirming that a full thickness junctional epithelium, containing parts of the internal and external basal lamina, was present in the specimens (Figure 2D). Only the external basal lamina area was decorated with the anti-laminin-5 antibody in the oral sulcular epithelium (Figure 2D).

Interestingly, all the keratin-19 positive epithelial cells in the JE were found to express $\alpha v\beta 6$ integrin. Cells against the internal basal lamina, however, appeared more strongly labelled for $\alpha v\beta 6$ integrin compared to those against the external basal lamina (Figure 2E). The sulcular epithelium also stained positive for $\alpha v\beta 6$ integrin, while oral epithelium was negative (Figure 2E).

Next, we localized the expression of three putative ligands of $\alpha v\beta 6$ integrin in the JE, namely fibronectin EDA, tenascin and TGF-$\beta 1$. Most abundant expression of tenascin was localized to the external basal lamina of the JE (Figure 2B). The basal cell layer of the junctional epithelium

showed more abundant staining for tenascin, compared to the SE and oral epithelium (Figure 2B). No connective tissue staining was observed for tenascin in the gingival tissue samples (Figure 2B).

Cellular fibronectin (EDA) was found to be localized throughout the connective tissue (Figure 2C). Compared to the deeper connective tissue and superficial connective tissue under the oral epithelium, stronger expression was seen under the JE (Figure 2C). A weaker staining was observed for the cells in the junctional epithelium (Figure 2C). At higher magnification, the typical flattened appearance of the cells of the JE and its wider intercellular spaces was clearly seen (Figure 2C).

TGF-β was expressed through the JE and SE. The strongest expression was confined to the cells close to the internal basal lamina (Figure 2F). Expression of TGF-β was relatively faint for the cells in the connective tissue, reacting mainly with vascular endothelia (Figure 2F). Control stainings did not produce any specific staining.

Visual scoring of the results for each antibody used in the gingival tissue samples is summarized in Table 2.

Table 2. Immunohistochemical localization of keratin-19 (K-19), laminin-5 (LM-5), tenascin (TN), αvβ6 integrin (B6), fibronectin EDA (FN), and TGF-β (TGF-b) in various compartments of normal human gingiva. JE, junctional epithelium; SE, sulcular epithelium; OE, oral epithelium; CT, connective tissue.

	JE	SE	OE	CT
K-19	+++	++	-	-
LM-5	+++	+	+	-
TN	+	+/-	+ *	-
B6	+++	++	-	-
FN	+	-	-	+
TGF-b	+	+	+	-

- = no staining
+ = light staining
++ = moderate staining
+++ = intense staining
* staining localized to the basal cells

The colocalization of αvβ6 integrin with different molecules in the subcellular level, could be investigated using immunogold labelling and transmission electron microscopy.

Because the most apical extent of the JE is often lost in specimens where the tissue is removed from the tooth, we studied the expression of αvβ6 integrin, its ligands, and JE markers in demineralized frozen sections of human gingiva attached to the tooth (Figure 3). In these samples, the entire JE was cytokeratin-19 positive (Figure 3A). The immunolocalization of laminin-5 in the JE of decalcified frozen sections, was seen both along the external basal lamina facing the connective tissue and in the internal basal lamina facing the tooth (Figure 3C).

The expression of αvβ6 integrin in the JE of decalcified frozen sections of human gingiva attached to tooth showed an evenly distributed staining throughout the whole thickness of the JE (Figure 3D).

Interestingly, tenascin was localized to both the internal and external basal lamina of the JE in the decalcified sections (Figure 3B). Moreover, the periodontal ligament was also decorated with the anti-tenascin antibody, while practically no expression was seen in the gingival connective tissue (Figure 3B). Control stainings did not give any specific staining in the decalcified specimens.

Visual scoring of the expression of αvβ6 integrin, its ligands and JE markers in frozen sections of demineralized teeth with gingiva attached, is summarized in Table 3.

Table 3. Immunohistochemical localization of keratin-19 (K-19), laminin-5 (LM-5), tenascin (TN) and αvβ6 integrin (B6) in various compartments of frozen sections of demineralised teeth with human gingiva attached. JE, junctional epithelium; SE, sulcular epithelium; PDL, periodontal ligament; CT, connective tissue.

	JE	SE	PDL	CT
K-19	+++	++	-	-
LM-5	+++	+	-	-
TN	++	-	++	-
B6	+++	+	-	-

- = no staining
+ = light staining
++ = moderate staining
+++ = intense staining

6.1 Animal Study

To explore whether the lack of expression of αvβ6 integrin affected the morphology of the JE, paraffin sections of decalcified buccal and palatal/lingual gingiva of wild type, β6integrin-overexpressing and β6integrin-knock-out mice were stained with hematoxylin and eosin (H&E).

In the specimens of the wild type mice (FVB), a JE with typical cuboidal cells with their long axis parallel to the tooth surface was observed. The JE appeared to be composed of thin epithelial cells tightly bound together. A clear border between the JE and the underlying connective tissue could be clearly identified, both at the apical and lateral borders (Figure 4). At higher magnification the gingival epithelium showed a typical morphology of murine JE, in which the oral epithelium folds down merging with the coronal part of the JE (Figure 4). Sometimes, when this fold is very narrow it can give the mistaken impression of a thicker epithelium, since the oral epithelium may appear as part of the junctional epithelium. The underlying connective tissue at the most apical area of the JE showed normal characteristics and no visual inflammatory cell infiltration (Figure 4).

JE of the β6integrin-overexpressing mice (B6F1) sections showed similar morphology to the JE of the wild-type mice (Figure 5).

In contrast to the JE of the wild-type (FVB) and β6integrin-overexpressed mice (B6F1), the JE of the β6integrin-deficient mice (β6-/-) showed a disorganized epithelium which appeared loose and thicker than normal (Figure 6). The basal cells looked elongated with disorganized polarization and wide intercellular spaces (Figure 6). The JE in these specimens appeared to have started to "invade" the connective tissue and appeared more apical from its normal position. In addition, sections of the β6integrin-deficient mice revealed an apparent incipient inflammatory cell infiltration in the underlying connective tissue (Figure 6).

A comparison of the wild-type, β6integrin-overexpressing and β6integrin-deficient mice is summarized in Figure 7. Analysis of all specimens revealed outstanding alterations in the morphology and cellular organization of the JE in β6integrin-deficient specimens. These unusual findings were presented as cellular disorganization, overall increase in thickness, elongated cells, and an invasive appearance (Figure 7).

Two blinded investigators were asked to rank the seventeen (17) photographs of different sections of gingiva of three different groups of mice.

Interestingly, the criteria used by the two investigators agreed with each other. These criteria included thickness of epithelium, distance between cells in the junctional epithelium, cell morphology and polarization, width of intercellular spaces, border with the underlying connective tissue and apical extent of epithelium. The photographs containing the JE of the β6integrin-deficient mice were clearly distinguished from the others, while these investigators were not able to make a separation between the wild-type mice and the β6integrin-overexpressing mice.

CHAPTER VII

Discussion

7.1 Human Study

In the present study we show for the first time that αvβ6 integrin is constitutively expressed in the human junctional epithelium. Furthermore, the morphological changes found in the junctional epithelium of mice lacking αvβ6 integrin, suggest a possible role in regulation of inflammation mediated via TGF-β1 signalling.

The expression of αvβ6 integrin in gingiva samples was co-localized with immunoreactivity to cytokeratin-19, that is a unique cytokeratin marker of the JE (Mackenzie et al., 1991; Feghali-Assaly et al., 1994; Pritlove-Carson et al., 1997). Relatively strong expression was observed in DAT cells (Salonen et al., 1989) that were identified by laminin-5 staining.

Laminin-5 is involved in hemidesmosome formation and it is synthetized by JE cells. LM-5 is considered as a major component of the IBL but it is not a ligand for αvβ6 integrin (Hormia et al., 1995; Hormia et al., 1998; Oksanen et al., 2001). Several other integrins, such as α2β1, α3β1, α6β1 and α6β4, have been identified in the JE (Hormia et al., 1990; Hormia et al., 1992) that can potentially bind to laminin-5 (Carter et al., 1991; Rousselle et al., 1991; Sonnenberg et al., 1991). It is likely that α6β4 integrin is the primary LM-5 receptor in the JE, as these two are crucial for the hemidesmosomal adhesions (Sonnenberg et al., 1991; Rousselle et al., 1991; Niessen et al., 1994) that link the JE to the tooth. The expression of αvβ6 integrin by the JE cells is a novel finding.

As explained above, expression of αvβ6 integrin is restricted to very specific sites in adult epithelia (Breuss et al., 1993). Therefore, the JE serves as a unique epithelium where αvβ6 integrin is constitutively expressed. It has been shown previously that αvβ6 integrin is expressed in carcinomas and metastases (Albelda et al., 1990; Pignatelli et al., 1992; Breuss et al., 1995;

Hamidi et al., 2000; Impola et al., 2004), during re-epithelialization (Clark et al., 1996), and with injury and inflammation of skin and lungs (Huang et al., 1996). It has been proposed that JE mimics wound epithelium in many respects. For example, DAT cells do not deposit complete BM and regulate adhesion via LM-5 that is typical for keratinocytes. It is possible, therefore, that expression of αvβ6 integrin in the JE relates to its unique phenotype and serves with a specific role in this epithelium. Subclinical inflammation could also potentially induce αvβ6 integrin expression in the JE, as it is a site of leukocyte crossing even in health (Schroeder and Listgarten, 1997). This is unlikely, however, as αvβ6 integrin is not induced in pocket epithelium at periodontally inflamed sites (Haapasalmi et al., 1995).

The αvβ6 integrin can bind to multiple ligands, including fibronectin (Weinacker et al., 1994; Chen et al., 1996), tenascin (Prieto et al., 1993), vitronectin (Huang et al., 1998) and more recently, TGF-β1 (Zambruno et al., 1995; Munger et al., 1999, Sheppard, 2001). In the present study, two of these ligands, namely tenascin and TGF-β, were identified in the JE.

The presence of tenascin in the internal basal lamina of the JE was observed. Tenascin was only preserved in the JE attached to the tooth, suggesting that it is intimately attached to the enamel and not remained in the basal cells. Tenascin mediates adhesive and repulsive interactions, as well as binding to certain proteoglycans and fibronectin. Its location is mainly at sites of tissue remodeling. It is also present at the edges of healing wounds, especially underneath the migrating and proliferating epithelial cells (Hay, 1991; Häkkinen et al., 2000). Interestingly, in vitro studies have shown that tenascin inhibits cell migration (Tan et al., 1987; Kiernan et al., 1996; Kaninuma et al., 2004).

Tenascin has been shown to be mainly expressed during morphogenesis in embryonal life, but it reappears in adults in malignant tumours and during inflammation and tissue repair (Tiita et al., 1994; Häkkinen et al., 2000). The tenascin group of glycoproteins (Tn-C, Tn-X, Tn-R, Tn-Y, Tn-

W) have shown to have contrary cellular functions, depending on their mode of presentation and the cell types and different states of the target tissues (reviewed by Jones et al., 1990). Tenascin has also been shown to alter the adhesion properties of human monocytes and both B and T cells (Ruegg et al., 1989; Chiquet-Ehrismann, 1990). The majority of tenascin found during odontogenesis is synthesized by pre-odontoblasts and the inner enamel epithelium (Tucker et al., 1993). Tenascin-C has showed higher expression in connective tissue surrounding tumours, in wounds and in inflamed tissues where it may regulate cell morphology, growth and migration. Several integrin signalling pathways are regulated by its presence (refer to review by Chiquet-Ehrismann and Tucker, 2004).

It is possible that tenascin through binding the αvβ6 integrin at the IBL, can be present as a modulator of DAT cell adhesion and migration. Further studies, however, will be needed to elucidate the potential interaction between αvβ6 integrin and tenascin, and its role in cell regulation in the JE. Lack of αvβ6 integrin did not cause dettachment of the JE from the tooth, suggesting that it does not mediate critical adhesive functions at the JE.

Our results also showed the presence of another αvβ6 integrin ligand, namely TGF-β, in the JE of normal human gingiva. TGF-β has been previously identified as a ligand for integrin αvβ6 (Munger et al., 1999; Sheppard, 2001). Studies have localized TGF-β in both healthy and inflamed gingiva (Lu et al., 1997; Steinsvoll et al., 1999). The αvβ6 integrin binds to the RGD motif at the LAP of the small latent TGF-β complex (Munger et al., 1999). This binding requires the complex to be fixed to the LTBP1 (Gleizes et al., 1996; Saharinen et al., 1996; Gleizes et al., 1997). In fact, αvβ6 integrin may prefer LAP-binding as it binds to it with higher affinity than to fibronectin, for example (Munger et al., 1999). TGF-β has a strong immunomodulatory action (refer to reviews by Massague et al., 2000; Werner and Grose, 2003). Animals deficient to αvβ6

integrin have several inflammatory problems due to poor activation of TGF-β1 (Munger et al., 1999; Sheppard, 2001; Knight et al., 2002; Morris et al., 2003; Ludlow et al., 2005).

To test the role of αvβ6 integrin in the JE in transgenic mouse lines that were either lacking or overexpressing the integrin, the morphology and cellular organization of the JE were analyzed.

7.2 Animal Study

Integrin αvβ6 is expressed in specific sites of many organs, for instance, kidney, uterus, ovary, salivary glands and gall bladder (Breuss et al., 1993). In β6integrin-knock-out mice, alteration in cell response to tissue injury and inflammation, has been confined to the skin, intestine and lungs (Huang et al., 1996; Munger et al., 1999; Sheppard, 2001; Knight et al., 2002; Morris et al., 2003). In the present study, we found that human JE constitutively expresses αvβ6 integrin. Murine (both rat and mouse) JE also expresses αvβ6 integrin (Dr. H. Larjava, personal communication).

Therefore, we examined the histology of the junctional epithelium and the adjacent gingiva of 6-month-old αvβ6 integrin-deficient and 6-month-old αvβ6-overexpressing mice. The results showed that the JE in αvβ6 deficient mice was disorganized and thickened, and appeared invasive into the connective tissue. In addition, there were more inflammatory cells present in the connective tissue of the gingiva in the αvβ6 deficient mice, suggesting that αvβ6 integrin may regulate both the cell proliferation and immunoregulation in the gingiva (refer to review by Page and Schroeder, 1976; Irfan et al 2001). The JE of the αvβ6-overexpressing mice did not differ from controls.

Periodontal inflammation has been shown to develop as a result of different host factors. Amongst all the cytokines studied in the context of periodontal disease, IL-1β is one of the most potent ones (refer to reviews by Braddock et al., 2004; Delaleu and Bickel, 2004). IL-1β is

synthesized by macrophages, monocytes, lymphocytes and fibroblasts, amongst others. It mediates tissue remodelling, repair and inflammation. Increased production of IL-1β will result in tissue damage via synthesis of collagenase and PGE2, therefore, it has a key role in the pathogenesis of periodontitis (Hefti, 1993; Page, 2002; Delaleu et al., 2004; Taylor et al., 2004). It has been previously reported that increased levels of IL-1 can contribute to the development of periodontal disease (McDevitt et al., 2000; Delaleu and Bickel, 2004; Taylor et al., 2004). The solely presence of bacteria in the host will not necessarily lead to periodontal disease (Kinane et al., 2003; Bascones-Martínez et al., 2004). Interestingly, mice overexpressing IL-1β in keratinocytes developed alterations in the JE and features of periodontal disease (Dayan et al., 2004). These alterations were independent of the bacterial colonization (Dayan et al., 2004). The JE showed proliferation of epithelium and apical migration of the epithelium (Dayan et al., 2004). These changes were very similar to the morphological changes seen in the αvβ6 integrin-deficient mice, suggesting that lack of αvβ6 integrin could potentially be linked to increased IL-1β production due to lack of counteractive TGF-β. This is highly speculative, as cytokine levels were not measured in our present study. However, in tissue homeostasis, IL-1β and TGF-β have antagonistic functions (Benus et al., 2005; Takahashi et al., 2005), and TGF-β can couteract IL-1β functions (Hefti, 1993; Jacobsen et al., 1995). For example, in murine hepatocytes, TGF-β downregulates IL-1 expression (Matsumura et al., 2004). TGF-β is considered as a multifactorial immunomodulator with immunosuppressive effects. It has been shown to be produced in a later phase of inflammation, therefore, acting as a regulator of inflammation (Matsumura et al 2004).

In the αvβ6 integrin-deficient mice, the JE appeared thickened, indicating increased cell proliferation. The progression of the cell cycle is controlled by cyclin/cyclin dependent kinase (Cdk) complexes (refer to reviews by Morgan, 1995; Ekholm and Reed, 2000), whose activities are regulated by two families of cyclin kinase inhibitors. P21 and p27 are members of the

Cip/Kip family, which can inhibit the cyclin/cyclin dependent kinase (Cdk) complexes, controlling cell cycle progression (Cheng et al., 1999; Sherr and Roberts, 1999). Image analysis of the JE in mice lacking p21 and p27 showed alterations in the morphology of the JE. Enlarged JE in double-knock-out mice (p21/p27) presented an increased area of epithelium associated with connective tissue "islands". The authors used the latter term to describe invaginated areas of epithelium in the underyling connective tissue. There was no inflammatory infiltration associated with any of the study groups (Watanabe et al., 2004). Therefore, the authors concluded that both p21 and p27 are required for controlling the epithelial proliferation in the JE. The alterations in the JE in p21/p27-deficient mice were similar to those of our αvβ6 integrin-deficient mice. These similarities could be explained by impaired TGF-β activation followed by removal of the inhibitory blockage of TGF-β through p21/p27 nuclear signalling pathway. TGF-β regulates, namely inhibits, epithelial cell proliferation through the Smad-p21/p27 nuclear signalling pathway (Watanabe et al., 2004). Inhibition of cell proliferation by TGF-β1 involves downregulation of c-Myc leading to upregulation of cyclin-dependent kinase inhibitors p15, 21 and 27, which inhibit the CDK 4/6-cyclin D and CDK2-cyclin E mediated phosphorylation of the retinoblastoma protein (Robson et al., 1999; Ten Dijke et al., 2002).

Interestingly, development of pocket epithelium in periodontitis is associated with lack of αvβ6 integrin expression (Haapasalmi et al., 1996). This "downregulation" (compared to healthy JE) is likely caused by either inflammatory cytokines or bacteremia. It is possible, therefore, that "natural" deficiency of αvβ6 integrin could contribute to the catabolic downregulation and epithelial cell proliferation in periodontal disease.

CHAPTER VIII

Conclusions

Integrins are adhesion proteins that bind to components of the extracellular matrix, regulating adhesion, migration and differentiation through intracellular signalling cascades. We present here the first evidence of expression of integrin αvβ6 in the human junctional epithelium.

The role of αvβ6 integrin in the JE is still unclear. Previous studies have suggested a possible role of αvβ6 integrin in TGF-β activation. The fact that the JE of the β6-/- specimens presented here showed remarkable alterations in its morphology and cellular organization, suggests that αvβ6 integrin may play a role in controlling JE proliferation and immunoregulation in the gingiva. The suppression of αvβ6 integrin, may thus be important in epithelial proliferation and immunomodulation during pocket formation in periodontal disease.

Recommendations for Future Studies

Future studies are required to elucidate the exact mechanisms of alterations caused by lack of αvβ6 integrin in the JE. Downregulation of αvβ6 integrin may play a role in the development and progression of periodontal disease. Therefore, future studies should focus on the disease of β6integrin-deficient mice and controls during its natural progression (aging) or during inductive conditions (such as diabetes and bacterial inoculation).

In addition, the quantification of inflammatory cell profile and proinflammatory cytokines in specimens lacking αvβ6 integrin may also help to elucidate the role of αvβ6 integrin in the immunomodulation in the gingiva.

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Appendix

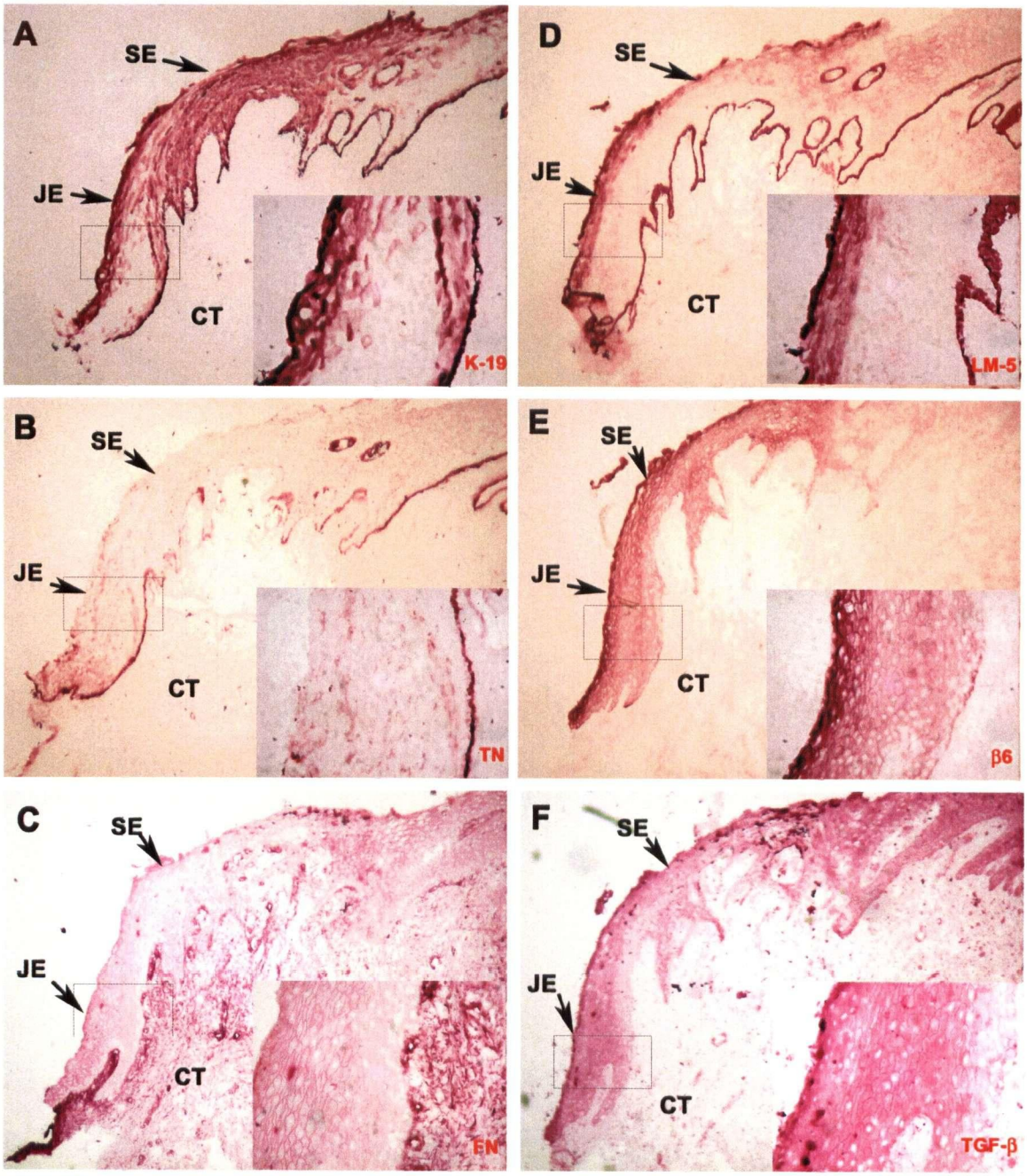

Figure 2. Expression of keratin-19 (panel A, K-19), tenascin-C (panel B,TN EDA-fibronectin (panel C, FN), laminin-5 (panel D, LM-5), αvβ6 integrin (panel E, β6) and TGFβ-PAN (panel F, TGF-β) in sulcular (SE) and junctional (JE) epithelium of human gingiva. For magnified view of the outlined area (box), see bottom right corner in each panel. The most apical part of the JE is list in sample preparation. CT, connective tissue.

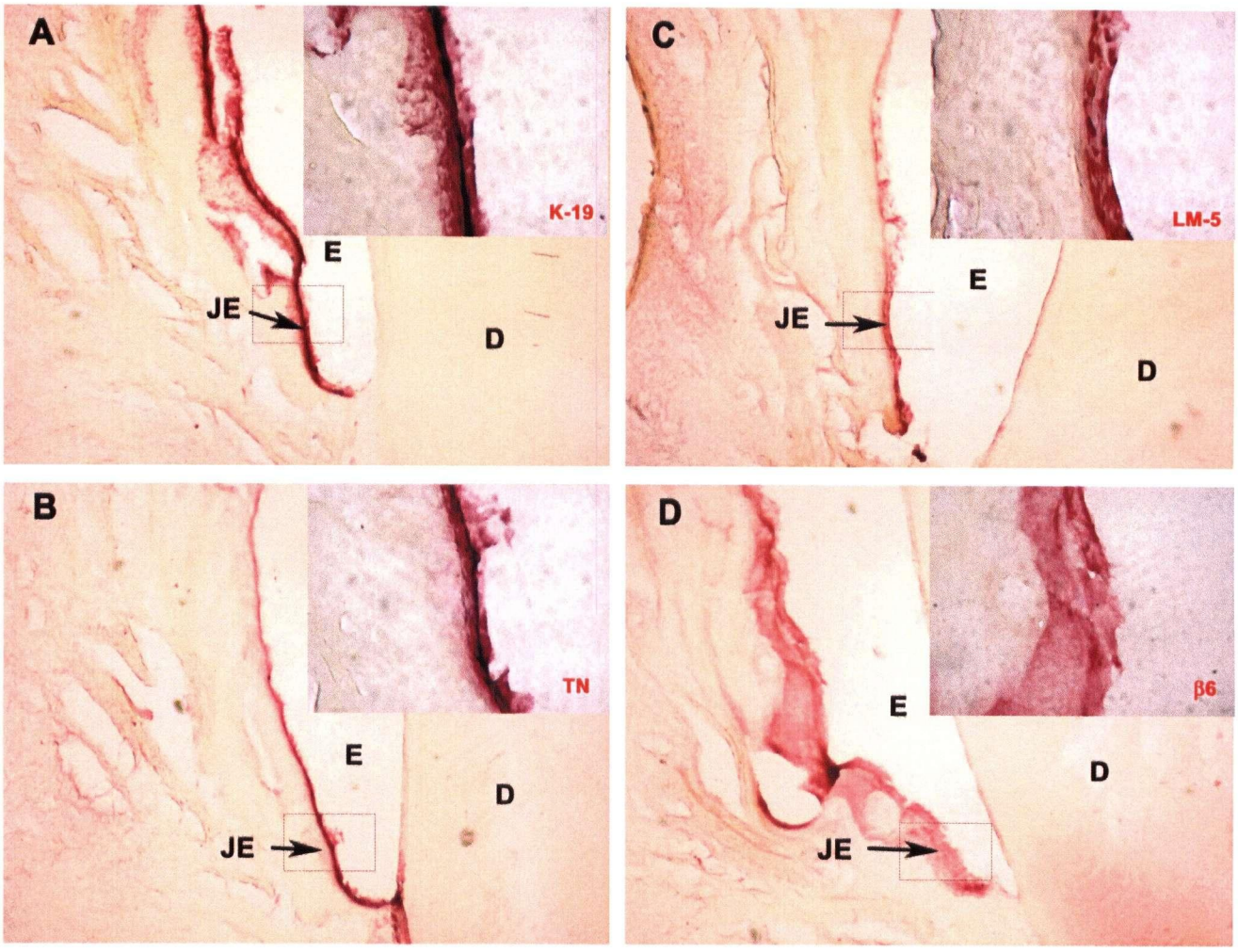

Figure 3. Expression of keratin-19 (panel A, K-19), tenascin-C (panel B, TN), laminin-5 (panel C, LM-5) and αvβ6 integrin (panel D, β6) in junctional epithelium (JE) of human gingiva attached to decalcified tooth enamel (E). For magnified view of the outlined area (box), see top right corner in each panel. D, dentin; E, enamel.

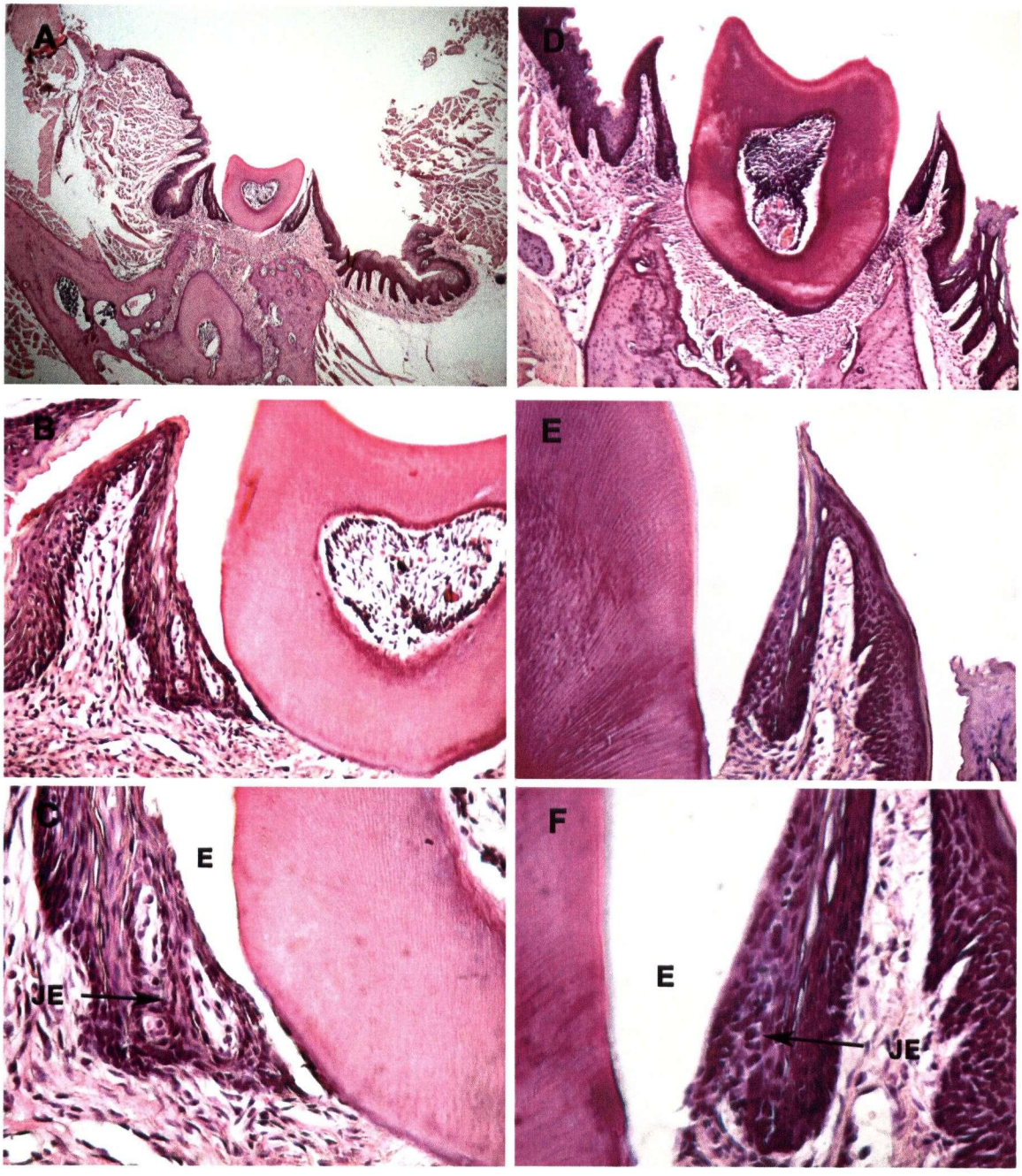

Figure 4. Morphology and organization of the junctional epithelium (JE, arrows) in mouse gingiva of wild type (FVB) animals. Panels A and D show the lowest magnification views (x10). For magnified views, see panels B and E (x20), and panels C and F (x40). E, enamel.

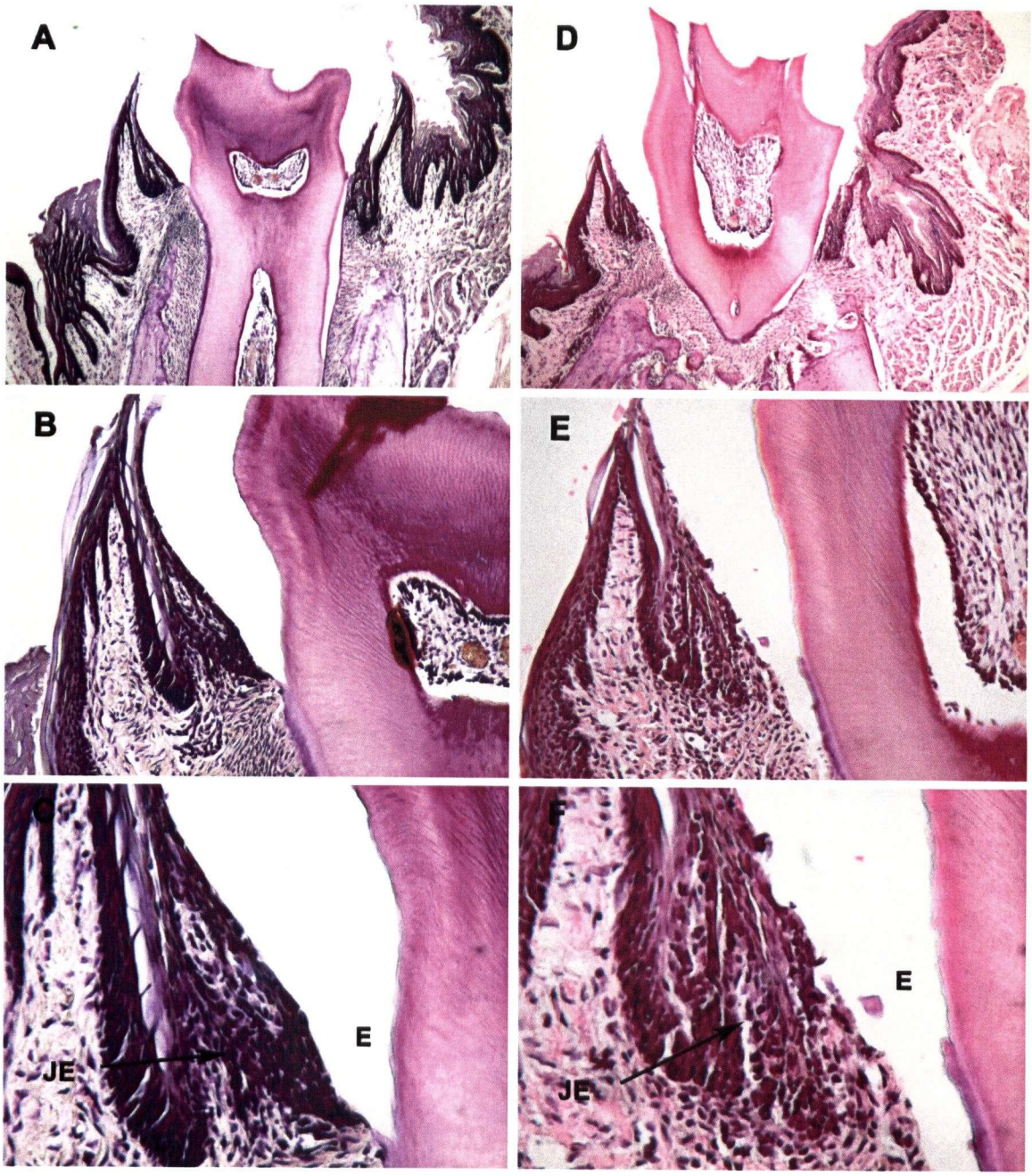

Figure 5. Morphology and organization of the junctional epithelium (JE, arrows) in mouse gingiva of β6integrin-overexpressing (B6F1) animals. Panels A and D show the lowest magnification views(x10). For magnified views, see panels B and E (x20), and panels C and F (x40). E, enamel.

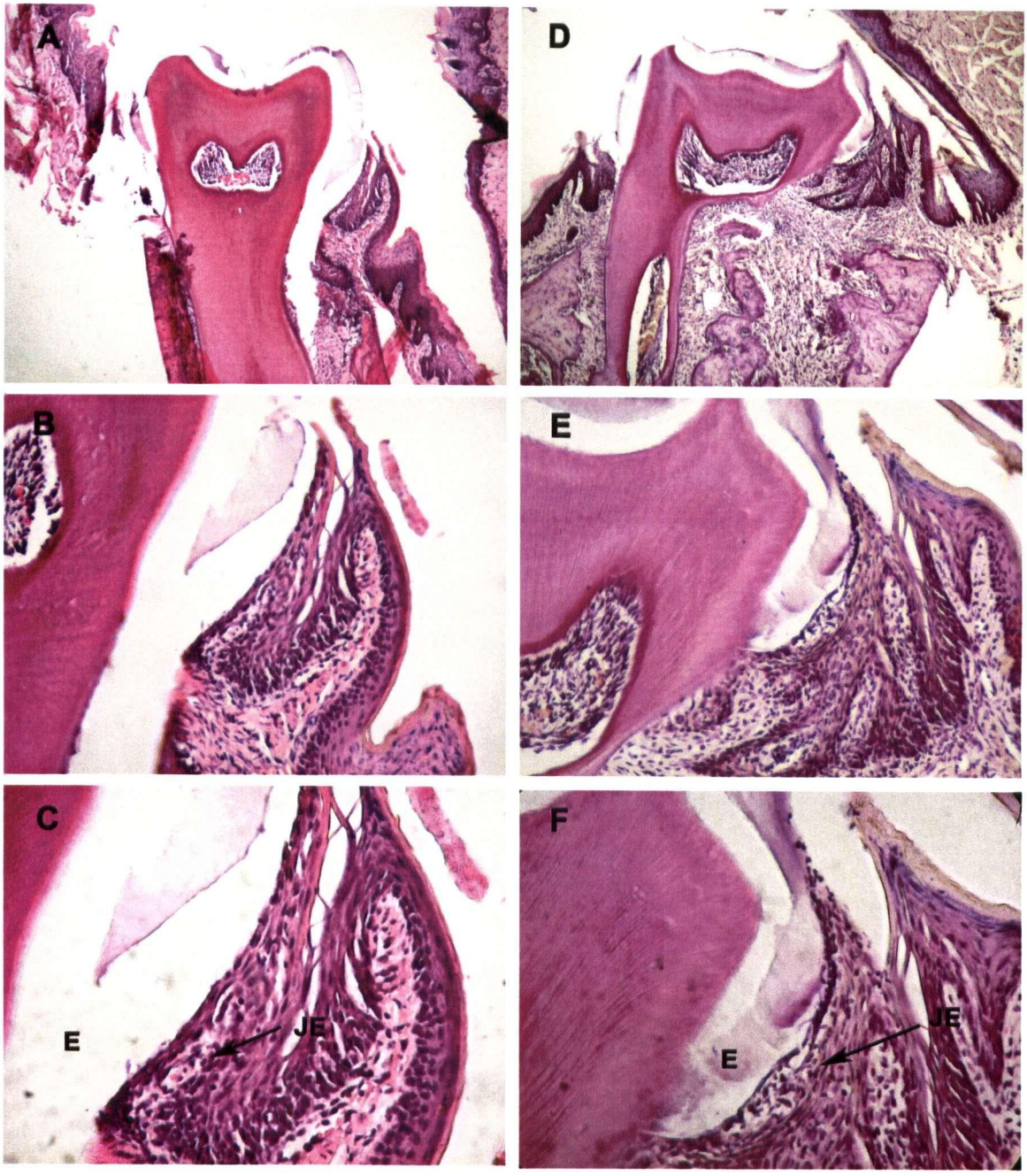

Figure 6. Morphology and organization of the junctional epithelium (JE, arrows) in mouse gingiva of β6integrin-deficient (β6-/-) animals. Panels A and D show the lowest magnification views(x10). For magnified views, see panels B and E (x20), and panels C and F (x40). E, enamel.

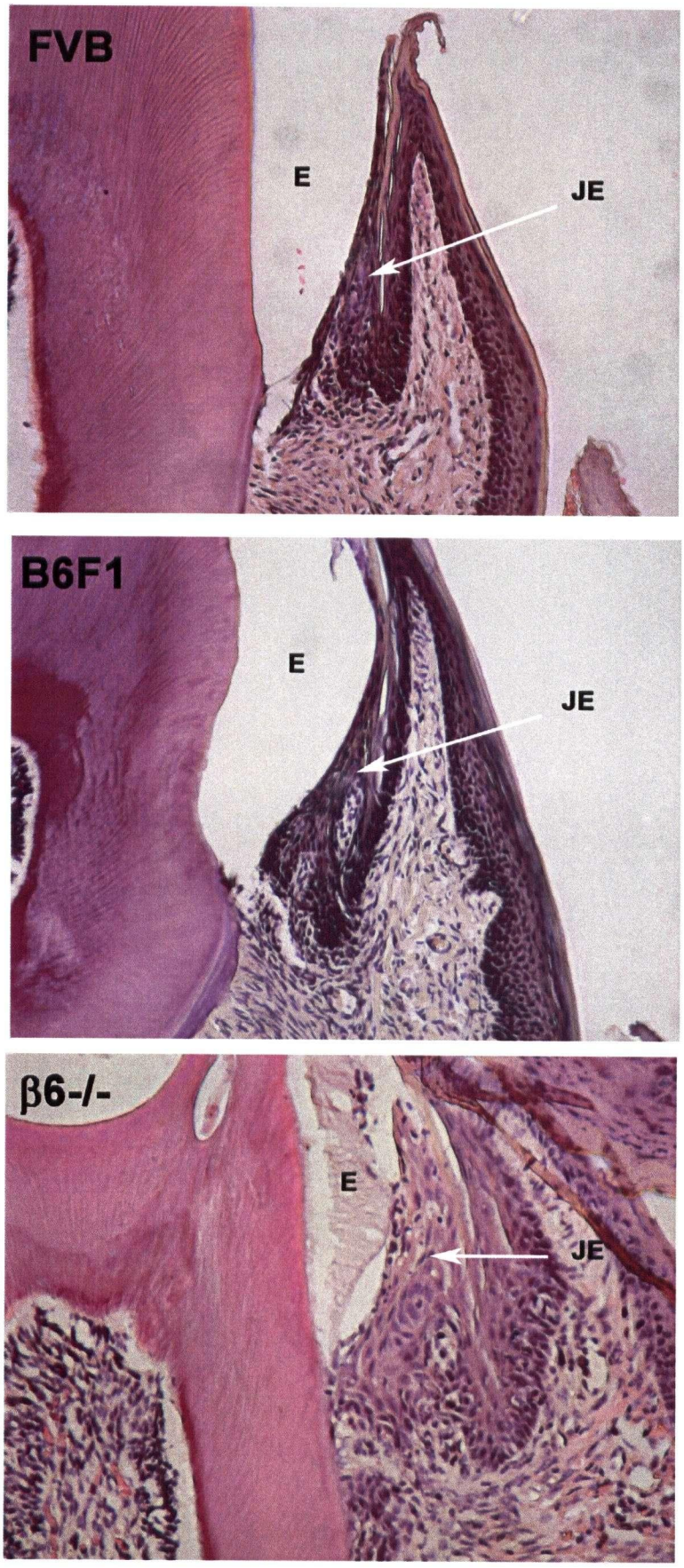

Figure 7. Morphology and organization of junctional epithelium (JE, arrows) in mouse gingiva of wild type (FVB), β6integrin-overexpressed (B6F1) and β6 integrin-deficient (β6-/-) animals. Note that the JE of β6-/- mice appear disorganized with wider intercellular spaces and elongated basal cells that appear to invade the connective tissue. The overall thickness of JE is increased.