**Substratum roughness alters the growth, area, and focal adhesions of epithelial cells,
and their proximity to titanium surfaces**

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Abstract

Epithelial (E) cells were cultured on smooth tissue culture plastic (TCP), TCP-Ti, polishedTi (P) and rough grit blastedTi (B), acid etchedTi (AE), and grit blasted & acid etchedTi (SLA) surfaces and their growth, area, adhesion, and membrane-Ti proximity assessed. Rough surfaces decreased the growth of E cells compared to smooth surfaces in cultures up to 28 days. In general rough surfaces decreased the spreading of E cells as assessed by their area with the most pronounced affect for the SLA surface. On the other hand, the strength of E cells adhesion as inferred by immunofluorescence staining of vinculin in focal adhesions indicated that E cells formed more and larger focal adhesions on the smooth P surface compared to the rougher AE surface. As this finding indicates a stronger adhesion to smooth surfaces, it is likely that E cells on rough surfaces are more susceptible to mechanical removal. An immunogold labeling method was developed to visualize focal adhesions using back-scattered electron imaging with a scanning electron microscope (SEM). On rough surfaces focal adhesions were primarily localized on to the ridges rather than the valleys and the cells tended to bridge over the valleys. Transmission electron microscopy (TEM) measurements of membrane proximity to the Ti surface indicated that average distance of cell to the Ti increased as the Ti surface roughness increased. The size and shape of surface features are important determinants of epithelial adhesive behavior and epithelial coverage of rough surfaces would be difficult to attain if such surfaces become exposed.

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Chapter I- Introduction

Overview

The number of individuals receiving artificial devices to replace damaged or missing tissue or organs is increasing (Griffith and Naughton, 2002). Such devices include total or partial hip and other joint implants or prostheses, cardiovascular shunts and dental implants. The success of an implanted device depends upon the interaction of cells with artificial surface including those interactions involving cell adhesion and migration along the surface. These interactions are influenced by the physical and chemical composition of the substratum such as surface topography and surface energy (Brunette, 1986; Brunette, 1988; Boyan et al., 1996; Curtis and Wilkinson, 1997; Brunette and Chehroudi 1999; Curtis and Riehle, 2001; Brunette et al., 2003).

The surfaces of commercially available implants vary greatly in their topography and physical characteristics (Esposito et al., 2003). Brunette (1986; Brunette et al., 1983; Chehroudi et al., 1990; 1995; Brunette and Chehroudi, 1999; Glass-Brudzinski et al., 2002; Chehroudi and Brunette, 2002; Teixeira et al., 2003; Karuri et al., 2004) investigated the effect of well-defined surface topographical features on cell behavior *in vitro* and *in vivo*. However, the effect of random surface topographical features on epithelial cell behavior has not been as extensively investigated (Hormia et al., 1991; Cochran et al., 1994).

Literature Review

I. Epithelial cells of rests of Malassez

In general, epithelial tissue is one of the major types of tissues (others include: nerve, muscle, and connective tissues) in vertebrates. In epithelial tissues or epithelium, cells are bound tightly together. Epithelium is a coherent cell sheet formed from one or more layers of cells covering external surfaces or lining cavities or surrounding glands (Alberts et al., 1994).

Epithelial cells (E-cells) derived from rests of Malassez were used in this study. The epithelial rests of Malassez represent the fragmented remnants of an epithelial structure, Hertwig's epithelial root sheath, which in early tooth development delineates the shape of the roots (Wentz et al., 1950; Reeve and Wentz, 1962; Simpson, 1965; Listgarten, 1975) and persist as a network or as islands of epithelial cells.

Some differences in the incidence of epithelial rests of Malassez cells between species have been reported. For instance, in pigs the rest cells are numerous (Grant and Bernick, 1969). Also, the prevalence of epithelial rest of Malassez in human periodontal ligament decreases rapidly in the first three decades of life, followed by a slower decrease thereafter (Simpson, 1965). The rest cells are completely surrounded by connective tissue cells (Ten Cate, 1972). Furthermore, the exact function of epithelial rest of Malassez cells *in vivo* is unknown.

A. Epithelial cells of rests of Malassez *in vitro* and *in vivo*

Hong and Brunette (1987) extended the primary findings that cell shape can regulate proteinase secretion in fibroblasts (Aggeler et al., 1982) and investigated the relationship between cell proliferation, cell shape, and proteinase secretion using E-cells

derived from the epithelial rests of Malassez. Hong and Brunette (1987) altered the E-cells' shape by physical means (growth on less adhesive substrata and grooved surfaces and mechanical stretching), application of cholera toxin and dibutyryl cyclic AMP (that induced cell proliferation and flattening), and 12-O-tetradecanoylphorbol-13-acetate (that increased cell rounding). Cell shape influenced the proteinase secretion more than cell proliferation. Neutral proteinase and plasminogen activator secretion were found to correlate with cell shape; the more round the cells, the greater the amount of proteinase secreted.

Uitto et al., (1992) investigated the adhesion of E-cells derived from epithelial rests of Malassez to different extracellular matrix molecules, expression of fibronectin, and integrin receptors by means of immunofluorescent staining, in situ hybridization and time-lapse cinemicrography techniques. E-cells attached and spread well on different surfaces coated with fibronectin, vitronectin, and type I collagen and in some instances laminin-5. Immunofluorescent staining of fibronectin indicated that fibronectin was localized to the extracellular matrix as well as intracellular granules and tracks left behind cells. Treatment with integrin binding peptide prior to culturing inhibited the attachment of receptors to the coated surfaces strongly indicating the involvement of integrin receptors in adhesion structures. Also, β_1 integrin staining was localized to the cell-cell contacts as well as leading cell projections.

Abiko et al., (1994) using immunohistochemistry examined the localization of laminin, a marker protein for basement membrane, in E-cells derived from epithelial rests of Malassez grown on titanium. Gelatin zymography of conditioned medium confirmed the gelatinase activity of epithelial cells. The authors suggested that laminin contributes

to the E-cell attachment to titanium and that gelatinase may be mobilized for E-cell movement on titanium.

Onishi et al., (2002) investigated the immunohistochemical localization of heat shock protein-25 of rat molar teeth (during root formation) and laminin. Heat shock proteins are a group of small molecules that are induced in certain types of cells in response to heat shock or other deleterious stresses, and enable cells to recover from stressful conditions (Lavoie et al., 1993). Onishi et al., (2002) observed heat shock protein-25 immunoreactivity in odontoblasts and cells in close proximity to the acccelular cementum that lacked laminin immunoreactivity. Furthermore, the transmission electron micrographs of heat shock-25 immunoreactive cells showed that cells along the surface of the cementum were round and rich in organelles such as mitochondria and rough endoplasmic reticulum. In contrast, cells at the cervical region were oval and contained few cell organelles. Onishi et al., (2002) suggested that heat shock protein-25 might be involved in shape alteration of epithelial rests of Malassez cells, cementoblasts, and odontoblasts during differentiation. Furthermore, Onishi et al., (2002) confirmed the *in vitro* findings of Abiko et al., (1994) showing that most of the cells of rests of Malassez had expressed laminin.

Uitto et al., (2002) examined the expression and localization of matrilysin, a matrix metalloproteinase, upon exposure to an oral bacteria in gingival epithelium and E-cells derived from epithelial rests of Malassez. Supra-basal cells of junctional epithelium facing the teeth expressed matrilysin. As well, the bacteria exposure elevated the expression of matrilysin by epithelial rests of Malassez *in vitro*. The authors suggested

that the matrilysin might have an active role in the physiology of periodontal ligament in defence as well as normal physiology of junctional epithelium.

Yoshihiro et al., (2003) using reverse transcription polymerase chain reaction compared the profiles of alkaline phosphatase (ALPase), osteopontin (OPN), and bone morphogenetic protein 2 & 4 (BMP-2 & BMP-4) in fibroblasts and E-cells derived from human periodontal ligament. Epithelial rests of Malassez expressed lower levels of ALPase and BMP-4 compared to the fibroblasts. However, the expression level of OPN in E-cells was higher than fibroblast cells with no differences in expression level of BMP-2 between E-cells and fibroblast cells. Yoshiro et al., (2003) suggested that epithelial rests of Malassez in the periodontal ligament may be involved in cementum repair.

Talic et al., (2004) investigated the effect of morphological changes/ shape of periodontal ligament in rats and the effect on the expression of integrins using immunofluorescent staining. The periodontal ligaments were either compressed or tensed using an elastic band placed around molars. β_3 integrin was strongly localized at the membrane of epithelial rests of Malassez within the ligaments suggesting a mechanical (adhesion) or signaling role in response to the conditions of stress or remodeling.

II. Surface topography

There is sufficient published evidence to convince even the most skeptical newcomer to implantology that surface topography and roughness strongly influence cell behavior. Over the years implant materials have been designed with different surface topographies. For example, and of relevance to this work, surfaces with horizontal grooves have been applied to prevent epithelial down-growth (Brunette, 1986; Chehroudi

et al., 1990; Chehroudi and Brunette, 2002). Structural features respectively in the range of 0.1-100 μm and 1-100 nm define micro- and nanotopography (Curtis and Wilkinson, 1997; Teixeira et al., 2003; Karuri et al., 2004). In general, cells are extremely sensitive to microstructures of biomaterials and their behavior and function can be modified by different topographies (Chehroudi and Brunette, 1995; Curtis and Wilkinson, 1997; Brunette and Chehroudi, 1999; Curtis and Riehle, 2001; Brunette et al., 2003).

Topographical features can promote adhesion and spreading of different cell types. Osteoblast cells preferentially attach to roughened surfaces (Abe et al., 1983) as do macrophages (Rich and Haris, 1981, Salthouse T.N., 1984); whereas, gingival fibroblast cells prefer to adhere to smooth polished surfaces (Bowers et al., 1992). Therefore, it has been postulated that surface topography can be designed to select for specific cell populations. Moreover, topographical features can act as directional cues to influence cell orientation and direction of migration of population of cells. Directional cues can be chemical (adhesive) or physical (topographic or mechanical) as reviewed by Curtis and Clark, (1990), Curtis and Wilknison (1997), Brunette and Chehroudi (1999), and Brunette et al., (2003).

Population pressure and the phenomena of contact inhibition of locomotion cause cells to move away from densely populated areas towards more sparsely populated areas. In particular, contact between epithelial cells results in contact induced spreading as first observed by Middleton C.A. (1977). Upon contact epithelial cells increase their area, form stable lateral contacts between adjacent cells and assemble into sheets (Brown and Middleton, 1981).

Weiss (1934; 1945) was the first to coin the term contact guidance following the classical work done by Harrison (1914) who investigated the reaction of tissues from embryos of frog chick of various ages on a fibrin networks of the clotted plasma, spider webs, cover-glasses and the surface film of the fluid drops. Weiss (1945) described the orientation response of neural cells in culture along thin glass fibers, scored surfaces or grooves. By contact guidance (also called topographical guidance) Weiss referred to the tendency of a cell to be oriented and guided in its direction of migration by substratum topography (Brunette et al., 1983; Chehroudi et al., 1990; 1995; Curtis and Clark, 1990; Brunette and Chehroudi, 1999; Glass-Brudzinski et al., 2002; Chehroudi and Brunette, 2002; Teixeira et al., 2003; Karuri et al., 2004).

In vitro and *in vivo*, contact or topographic guidance has been observed in different cell types, such as epithelium, on a variety of substrata including surfaces with grooved features (Brunette et al., 1983; Chehroudi et al., 1988; Chehroudi and Brunette, 2002; Teixeira et al., 2003; Karuri et al., 2004). Oakley and Brunette (1995) using epifluorescence and confocal microscopy examined the effect of titanium coated grooved substrata on the shape, orientation, direction, and distribution of actin filaments and microtubules on single, double, and clusters of E-cells derived from epithelial rests of Malassez. They showed that cell contact increased the spreading. Furthermore, actin filaments and microtubule staining showed the initial alignment of the filaments along the walls and the ridge-groove edges. The data presented also confirmed that the clusters of epithelial cells were less susceptible to the topographical modulation as local topographic effects on the cytoskeleton could be overridden by adjacent cell contacts as suggested by Clark et al., (1990; 1991).

Grooved substrata can be obtained by a variety of techniques and can be defined by groove width, depth, and geometry. On substrata with multiple parallel sets of identical grooves, the groove width, depth, and the number of adjacent grooves is important. The response of cells to grooves depends on factors such as the type of cells, the size of cells, and the dimensions, density, and spacing of the groove features (den Braber et al., 1996; Curtis and Wilkinson, 1997; Teixeira et al., 2003; Karuri et al., 2004)

However, the effects of random topographical features on the behavior of epithelial cells have not been investigated so extensively. Hormia et al. (1991) investigated the effect of random topographical roughness of titanium surface on the growth and adhesion of gingival epithelial cells in vitro. Based on observation of immuno-fluorescent staining of actin, vinculin, and components of desmosomes (DP 1&2) they suggested that epithelial cells on titanium prefer smooth to rough titanium surfaces. Also, they proposed modes of adhesions such as close contacts, extracellular matrix contacts, and hemidesmosomes as observed previously by Gould et al., 1981. Hormia et al., (1991) did not observe staining of vinculin in focal adhesion like structures and instead observed vinculin in cell-cell contacts of epithelial cells grown on titanium surfaces of varying roughness. Cochran et al., (1994) investigated the effect of finely and coarsely blasted titanium surfaces on the growth of gingival epithelial and fibroblast cells as well as fibroblasts from periodontal ligaments. Growth on tissue culture plastic, smooth titanium surface and the two rough surfaces was monitored up to day-9 post seeding. The smooth tissue culture plastic and titanium surfaces supported the growth of epithelial cells in contrast to the blasted surfaces that demonstrated no statistically significant differences in cell numbers between day-1 and day-9. However, the growth of

periodontal ligament fibroblasts was unaffected by the surface topography of the growth substratum i.e. all surfaces supported the growth to the same extent.

III. Titanium

Although there is no agreement on what constitutes the ideal topography, there is some agreement on which materials are biocompatible (Brunette D.M., 1988; Esposito et al., 2003). Titanium and its alloys, Ti6Al7Nb and Ti6Al4V, are among the most commonly used implant materials.

Titanium, as the choice for biomaterial purposes, is known to have a passive surface, low rates of metal ion release, low specific weight, low modulus of elasticity, high tensile strength, poor heat conduction, and minimal affinity to cause adverse cell or tissue responses (Ratner B.D., 2001). Native titanium is a very reactive metal and has a very high affinity for oxygen. Therefore, when titanium and its alloys are exposed to air or moisture a thin (3-7 nm) layer of titanium oxide (TiO_2 , Ti_2O_3 or TiO) spontaneously covers the surface. The thickness of this natural oxide layer can vary according to the conditions and environments under which the metal has been processed. However, the excellent corrosion resistance of titanium and its alloys results from the formation of this stable, continuous, and highly adherent natural barrier. The solubility of this oxide layer is considered to be negligible in water.

In general, different processes that influence the adsorption of biomolecules are dependent on the surface properties of the biomaterial and the general mechanisms of protein adsorption have been reviewed (Wahlgren and Arnebrant, 1991). Titanium oxide has an affinity for proteins, glycoproteins, and glycolipids and other matrix molecules

that in turn promote the adhesion of cells and tissues to the surface of the implanted devices (Brunette, 1986; Winkelmann et al., 2003; Scotchford et al., 2003).

IV. Surfaces

One of the main differences among the commercially available dental implants is their surface topography. The quest for practical and functional implant materials has resulted in a number of commercially available osseointegrated dental implants that differ in materials, body shapes, diameters, lengths, and surfaces properties. Examples include Astra® (TiO₂ blast titanium screws with a homogenous structure), Branemark® (Titanium screws with orientation in form of screw threads), Steri-Oss® (acid-etched titanium screws), ITI® (hollow TPS titanium screws with a rough inhomogeneous structure), and ITI® (coarsely blasted and acid-etched surfaces “SLA”) (Esposito et al., 2003). In recent years, the SLA surfaces have been extremely investigated in a number of *in vitro* and *in vivo* studies. SLA surfaces have produced a high rate of success clinically and appear to promote bone production adjacent to the implant (Cochran et al., 2002; Boyan et al., 2003; Wieland et al., in press; Buser et al., 2004). Therefore, it was of interest to this thesis to evaluate the effect of rough surfaces such as acid-etched, coarsely blasted, and SLA, on epithelial cells *in vitro*. All original surfaces were prepared and analyzed in Institute Straumann and were a generous gift from Dr. M. Wieland. Wieland et al., (2002) evaluated the surfaces using non-contact laser profilometry, SEM, stereo-SEM, and X-ray photoelectron spectroscopy. (For the complete evaluation of surface properties refer to Wieland PhD thesis, 1999).

Non-contact laser profilometry and stereo-SEM were used for the quantitative evaluation of surface roughness values (Wieland et al., 2002). The roughness values were

recorded in form of R_a , R_q , R_t , R_{ZDIN} , S_m , S_k , and L_r . The latter parameters values are described as following:

R_a : The arithmetic average of the absolute values of all points of profile

R_q : The root mean square of the values of all points of profile

R_t : The maximum peak to valley height of the entire measurement trace

R_{ZDIN} : The arithmetic average of the maximum peak to valley height of the roughness values of five consecutive sampling sections over the filtered profile

S_m : The arithmetic average spacing between the falling flanks of peaks on the mean line

S_k : The amplitude distribution skew

L_r : The relation of stretched length of the profile L_0 to the scanned length L_m

X-ray photoelectron spectroscopy analysis indicated that Ti, O, C, and N were present on all Ti surfaces. Ti and O were found to be the dominant elements. The TiO_2 thickness of 4.5-5.5 nm protected the Ti surfaces (Wieland et al., 2002).

V. Cell adhesion to the substratum

A. Morphology

The attachment of cell membrane to the substratum is a complex process. This interaction has been described on the basis of the appearance of the attachment sites in light and electron microscopy. Abercrombie et al. (1971) used transmission electron microscopy technique to reveal electron dense plaques that were associated with actin filaments. In 1976, Izaard and Lochner applied the technique of interference reflection microscopy to distinguish three types of regions depending upon the distance between the substrate and the plasma membrane, extracellular matrix contacts (a distance separation

of 100-140 nm), close contacts (a distance separation of ~ 30 nm), and focal adhesions (a distance separation of 10-15 nm). Heath and Dunn (1978) used the combined techniques of electron microscopy and interference reflection microscopy to confirm that adhesion plaques (reported by Abercrombie et al., 1971) and focal adhesions (reported by Izzard and Lochner, 1976) described the same structures.

B. Focal adhesions

Focal adhesions evolve from small dot-like adhesion sites. Transition of these small dot-like structures into the mature focal adhesions is accompanied by transition of the associated actin mesh into densely packed straight bundles of filaments known as stress fibers (Heath and Dunn, 1978). Bershadsky et al. (1987) showed that accumulation of vinculin in focal adhesions parallels the transition of dot-like adhesion sites to the dash-like mature focal adhesions. Focal adhesions are usually located under peripheral sites of leading lamellae and near edges of non-spreading regions of the cell margins in moving and stationary cells. Focal adhesions play a critical role in the adhesion mediated signaling (through focal adhesion kinase pathway) as well as the transfer of mechanical force (through the stress fibers). However, the small dimensions of individual adhesion sites, their spatial proximity, and the small magnitude of the forces involved, make it challenging to accurately measure and map these sites (Bershadsky et al., 2003). Among the proteins that are concentrated on the cytoplasmic side of focal adhesions are talin, paxillin, vinculin, α -actinin, tensin, filamin, and the signaling molecule focal adhesion kinase (Liu et al., 2000; Zamir and Geiger, 2001).

C. Vinculin

Vinculin is a 130 Kda cytoskeletal protein localized in focal adhesions that has been proposed to be involved in the attachment of microfilaments to the receptors within the plasma membrane (Geiger, 1979; Geiger et al., 1980). The above attachment comprises a complex, and possibly orderly, assembly of proteins at the adhesion plaques. For example, the cytoplasmic domain of β_1 can bind to talin (review by Beckerle and Yeh, 1990; Marti et al., 2003) and α -actinin. In turn, talin can bind to vinculin (Otto, 1990) or nucleate actin assembly and self-associate. Vinculin can bind paxillin and α -actinin that in turn the latter can bind and crosslink actin. Also, tensin can bind to vinculin. Woods and Couchman (1992) reported that vinculin is a late addition of focal adhesions and others (Herman and Pledger, 1985) investigated the persistence of focal adhesion structures that lack vinculin. Avnur et al. (1983) showed that the association of vinculin with focal adhesions is largely actin independent. Furthermore, Herman and Pledger (1985) and others (Feltkamp et al., 1991) showed that presence of vinculin in focal adhesions could precede the presence of stress fibers. Finally, Samuelsson et al. (1993) investigated the direct aggregation and association of integrin heterodimers with stress fibers independent of vinculin and talin.

D. Stress-fibers

i. **Stress-fibers-** Stress-fibers are actin containing contractile structures that also contain a number of actin-associated proteins such as myosin I and II. Myosin I is a single molecule with one globular head and tail that attaches to another molecule and in this way the attached molecule get carried relying on the motor activity of myosin head.

However, Myosin II is composed of a pair of identical myosin molecules thus having two globular heads with tail twisting around each other length.

ii. **Actin** – Actin filaments are found in all eukaryotic cells and are essential for many of their functions including cell shape, movement, contractile activities, phagocytosis of large particles, intracellular transport, cell division, and trans-membrane signaling (Ridley et al., 2003; Engqvist-Goldsrein and Drubin, 2003). Although actin filaments are dispersed throughout the cell, they are most highly concentrated in the cortex, just beneath the plasma membrane (Bray and White, 1988; Ridley et al., 2003). Actin filaments are helical polymers of the protein actin. They appear as flexible structures, with a diameter of about 7 nm, that are organized into a variety of linear bundles, two-dimensional networks, and three-dimensional gels (Alberts et al., 1994). Individual dumbbell-shaped actin subunits polymerize to form globular actin /G-actin microfilaments with an intrinsic polarity, the heads of actin monomers all pointing to the same direction (Bremer and Aebi, 1992; Alberts et al., 1994; Ridley et al., 2003). Actin polymerization in lamellipodia is mediated by Arp 2/3 complex that binds to the sides or tip of a preexisting actin filament and induces the formation of new daughter filament that branches off the mother filament (Pollard and Borisy, 2003). Addition of each monomer of actin to the microfilament is ATP dependant. However the current opinion in the actual event of protrusive, pushing of the membrane, is that the process occurs not by elongation of the actin filaments but by “elastic Brownian ratchet” that means the thermal energy bends the short filaments, storing elastic energy. Unbending of an elongated filament against the leading edge would then provide the force for protrusion (Pollard and Borisy, 2003). The rate and organization of actin polymerization is controlled through the

availability of free available monomers. For example, profilin prevents self-nucleation by binding to actin monomers (reviewed by Ridley et al., 2003). Also, filament elongation is restricted by the action of capping proteins. In addition, ADF/ cofilin family assists the disassembly of older filaments that releases actin monomers that are needed for the polymerization process at the front end (Welch and Mullins, 2002; Ridley et al., 2003). There are a number of other supporting/stabilizing proteins (cortactin, filamin A, and α -actinin) involved in the process of polymerization and de-polymerization (reviewed by Welch and Mullins, 2002).

The interactions between the stress fibers and the extracellular matrix molecules (collagen, laminin, fibronectin, and etc.) on the substratum are regulated by transmembrane integrin receptors.

E. Integrins

Integrins are a family of heterodimeric, transmembrane glycoproteins that act as cell-surface receptors for extracellular matrix molecules including fibronectin, different types of collagen, and laminins and cooperate with growth factor receptors to promote cell survival, cell cycle progression, and cell migration (Anderson and Springer, 1987; Buck and Horwitz, 1987; Hynes, 1987; Gumbiner, 1996; Giancotti and Ruoslahti, 1999; Giancotti, 2003). Integrins are composed of a single α and a β subunit. There are several subfamilies of integrins defined by their common β chains. At least 18 different α and 8 β subunits are currently known. These subunits can combine to form at least 22 different cell surface receptors that have distinct ligand binding specificities (Larjava et al., 1996; Giancotti and Ruoslahti, 1999; Giancotti, 2003). Integrins are responsible for most cell

extracellular matrix interactions in various cell types including epithelial cells (review by Watt and Jones, 1993).

Integrins are important in many aspects of tissue organization and cell migration during development. The only cell types that lack expression of integrins are red blood cells. A single cell may express multiple integrins some of which have similar binding specificity. For example, Kovisto et al. (1999) showed that a human epithelial keratinocyte can express three different fibronectin-binding integrins namely $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_6$. Some integrins are expressed in only one cell type; however, many integrins are expressed by more than one cell type. For example, leukocytes are the only cell type expressing $\alpha_L\beta_2$ integrin; however, many cell types express various B_1 integrins (review by Hakkinen et al., 2000).

F. Extracellular matrix

Extracellular matrix or basal lamina is a mat of bio-molecules supporting the epithelium. Mechanical stability between epithelium and the under-lying basal lamina is brought about by the interaction between the basal cells and the specialized extracellular matrix molecules forming a thin bio-mat otherwise known as basement membrane zone. The basement membrane zone provides structural support, partitions tissue into specific divisions, and influences cellular behavior (Paulsson, 1992). Basement membrane components are involved in cell migration and influence stratification as well as differentiation (Fine, 1994). The basal cell layer is responsible for the synthesis of a number of basement membrane zone components including different types of collagen (collagen IV), laminins (Laminin-5), fibronectin, and etc. (Burgeson and Christianson, 1997).

i. **Collagen IV-** Collagen IV is the major structural component of the basement membrane that possibly influences epithelial adhesion (Reviewed by Bou-Gharios et al., 2004). Collagen IV is a hetero-trimer consisting of two α_1 chains and one α_2 chain, and in large parts it adopts the collagen characteristic triple-helical fold. (Fraser et al., 1979). The triple-helical fold is known to be essential for higher affinity binding of $\alpha_1\beta_1$ integrin to collagen type IV. (Kuhn Eble, 1994).

ii. **Laminin-5-** Laminin-5 is characterized as a component of anchoring filaments in the basement membrane (Carter et al., 1991). Laminin-5 is composed of three subunits called α_3 , β_3 , γ_2 (Matsui et al., 1995). Both $\alpha_3\beta_1$, $\alpha_6\beta_4$ integrins interact with laminin-5 (Carter et al., 1991). Previous investigations have shown that laminin-5 is expressed by migrating keratinocytes during re-epithelialization of human full-thickness wounds (Larjava et al., 1993). In vitro, laminin-5 promotes the formation of hemidesmosomes (Hormia et al., 1995).

iii. **Fibronectin-** Fibronectin is a critical early component of the clot and the forming granulation tissue (Review by Hakkinen et al., 2000). Fibronectins are a family of closely related adhesive glycoproteins, and are key components of the basement membrane and the provisional wound matrix. Fibronectin is also an integral extracellular matrix that serves as the substratum during cell migration in wound healing (Yamada et al., 2003). Since fibronectin contains multiple binding sites, it is capable of linking various components of basement membrane to each other as well as to the receptors on the cell surface. Furthermore, fibronectin molecules can bind to each other, further enhancing their capacity to form a stable network of basement membrane components and their anchorage to the cell surface (Alberts et al. 1994; Yamada et al., 2003).

Plasma fibronectin is the soluble form of the fibronectin found in blood. Fibrin is a blood clotting protein that interacts with plasma fibronectin. The ability to bind fibrin makes fibronectin an important component of the plasma clot found in wounds. However, the blood clot initially contains plasma fibronectin that is later replaced by cellular fibronectin produced by keratinocytes, fibroblasts, and macrophages (Clark, 1996).

A fibronectin molecule consists of two large polypeptides linked near their carboxyl ends by a pair of disulfide bonds consisted of three types of homologous repeating modules termed I, II, and III (Peterson et al., 1983). Each polypeptide contains about 2500 amino acid residues and has a mass of approximately 250Kda. Polypeptides are non-identical and each of the polypeptides is folded into a series of globular domains connected by short, flexible segments of the polypeptide chain. The capability of some of the domains to recognize various components of the basement membrane, including several types of collagen (I,II, and IV) and specific proteoglycans allows specific binding interactions between fibronectin and basement membrane. Other domains recognize and bind cell surface receptors via a specific tetrapeptide sequence, RGDS (arginine-glycine-aspartate-serine). The RGDS sequence is a common motif recognized by integrins such as $\alpha_5\beta_1$, "fibronectin receptor" (Alberts et al., 1994; Yamada et al., 2003).

The capacity for the large number of variants of a gene to exist is based on differential splicing pattern of the mRNA. There are more than 20 different isoforms of fibronectin recognized. Fibronectin can vary structurally in a tissue specific manner by alternative splicing of three regions namely EIIIA, EIIIB, and V120 (Yamada and Clark, 1996). For example, wound healing in adult rat skin is accompanied by a re-expression of cellular fibronectin that contains both EIIIA and EIIIB (Buck and Horwitz, 1987).

The Aim of the thesis

My hypothesis is that titanium implant surfaces with different surface roughness will differ in their effects on epithelial cell behavior. The specific aims of this thesis are to compare and evaluate the effects of random surface topographical roughness features on the growth, area, focal adhesion formation and proximity of epithelial cells to the titanium surfaces. In particular:

- The primary purpose of rough surfaces in dental implants was meant to optimize the behavioral response of bone cells in respect to growth and adhesion. Epithelial cells can also come in contact with rough surfaces (Deporter et al., 1986); therefore, it is of interest to determine the effect of these rougher surfaces, such as SLA, on the proliferation of epithelial cells. As a working hypothesis based on the data of Cochran et al., (1994) one would expect smooth surfaces to promote the growth of E-cells to a greater extent than rough SLA surfaces. The above hypothesis could be tested by monitoring the growth of epithelial cells on surfaces of varying roughness as a function of time.
- Contact-induced spreading (increase in projected cell area upon contact with another cell of same origin) of epithelial cells *in vitro* has been shown to be characteristic of epithelial cells. Oakly and Brunette (1995) and Clark et al., (1991) evaluated the contact-induced spreading behavior of epithelial cells on smooth surfaces and grooved surfaces. Besides other findings Oakly and Brunette (1995) as suggested by Clark et al., (1991) reported a less responsive behavior of epithelial cells in clusters to the topographical cues i.e. the

topographical effects on the cytoskeleton could be overridden by adjacent cell contacts on the groove surfaces. Therefore, a working hypothesis would be that isolated epithelial cells would spread well on smooth surfaces and exhibit contact-induced spreading whereas spreading and contact-induced spreading would be less evident on rough SLA surfaces. In addition one would expect that clusters of epithelial cells on rough surfaces to be less responsive to the topographical cues. The above hypotheses could be tested by collecting morphological data on the projected area of epithelial cells in singlets, doublets, as well as clusters on smooth and rough SLA surface using SEM microscopy.

- A critical aspect of cell behavior on the biomaterial surfaces for many applications is the adhesion of cells to the surfaces. Previously it has been observed that epithelial cells adhere and spread well on smooth surfaces. However, the response of epithelial cells on the rough SLA surfaces in terms of adhesion has not been studied. As a working hypothesis one could expect that epithelial cells develop more and/or larger focal adhesions on the smooth surfaces compared to the rougher surfaces. The latter hypothesis could be tested by quantitative assessment of area of focal adhesions/ area of cell using immunofluorescence staining of vinculin in focal adhesions.
- To resist bacterial invasion a tight seal between the soft tissue, in particular epithelial tissue, and the dental implants is considered to be important in the long-term success. As a working hypothesis one expects that epithelial cells adhere more closely to the smooth surfaces compared to the rough SLA

surfaces. The hypothesis could be tested by direct measurement of epithelial tissue proximity to the underlying supporting surfaces using thin cross sections and TEM microscopy.

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Chapter II- Manuscript

Substratum roughness alters the growth, area, and focal adhesions of epithelial cells, and their proximity to titanium surfaces

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Introduction

The surface topography of an endosseous dental implant is an important determinant of cell behavior and implant performance. The quality of the seal between the dental implant and the surrounding gingival tissue, among other things, contributes to the success of an oral implant (Cranin et al., 1977; Brunette and Chehroudi, 1999; Brunette D.M., 1986) because a tight seal between the epithelium and the surface is required to protect the underlying tissues from bacterial invasion. However, a tight seal itself is not a sufficient condition for success of an implant device that penetrates the oral mucosa. The down-growth of epithelial tissue along an implant can result the failure of devices penetrating epithelium (von Recum A.F., 1990). Surface topography has been used to directly influence the response of connective tissue and epithelium and to inhibit the down-growth of epithelium at the device interface (Brunette D.M., 1986; Chehroudi et al., 1995).

However, surface topography can also affect cell adhesion (Harris A.K., 1984). One important class of cell adhesion structures are focal adhesions that are specialized regions of the cell membrane and are separated from the substratum by 10-15 nm. Bundles of actin filaments terminate at focal adhesions where they attach to integrins. Integrins comprise a large family of cell-surface matrix receptors with an extra-cellular domain that binds to a protein component of the extra-cellular matrix and an intra-cellular domain that indirectly binds to actin filaments via a complex of attachment proteins, including talin, α -actinin, and vinculin (Anderson and Springer, 1987; Buck and Horwitz, 1987; Hynes R.O, 1987). Localization of vinculin by immunofluorescence microscopy is often used to demonstrate the presence of focal adhesions (Geiger 1979; Geiger et al.,

1983). Richards et al., (2001) and others (Tan et al., 2003) have argued that the area of vinculin staining is an indication of the strength of cell adhesion.

Titanium (Ti) coated acid etched (AE), grit blasted (B), and grit blasted & acid etched (SLA) surfaces decreased the growth and the area of epithelial (E) cells compared to smooth tissue culture plastic (TCP), tissue culture plastic Ti coated (TCP-Ti), and polished Ti coated (P) surfaces. Moreover, E cells formed more and larger focal adhesions on P than on rougher AE surface. TEM measurements of membrane proximity to the Ti surface indicated that membrane-Ti distance increased as the Ti surface roughness increased. In summary the findings in this study indicated that rough surfaces decreased the growth, spreading, membrane-Ti proximity, and adhesion of epithelial cells on Ti surfaces indicating that epithelial coverage of rough surfaces would be difficult to attain if such surfaces become exposed.

Methods and Materials

I. Cell culture

The epithelial (E) like cells were derived from epithelial rests of Malassez as described in Brunette et al., (1976). E cells between the 4th and 6th subculture were maintained in the alpha Minimal Essential Medium (α MEM)¹, plus 15% fetal bovine serum (FBS)², and antibiotics (penicillin G³, 100 μ g/ml; gentamicin³, 50 μ g/ml; amphotericin B⁴, 3 μ g/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The media was changed on day one and subsequently at 48 hours intervals. For counting, cells were detached from the surfaces using 0.25% citrate saline trypsin³ (1:250) solution (pH 7.8) containing 0.1% glucose³ and then numbers were determined using a Z1 Coulter®⁵ cell counter instrument.

¹ StemCell Technologies Inc., Vancouver, B.C.

² MEDICORP, Montreal, Quebec

³ Sigma, St. Louis, Mo.

⁴ Fungizone, Gibco, Grand Island, N.Y.

⁵ Coulter Electronics Limited, Luton, Beds.

⁶ (Thermonax), Nalge Nunc Int., Rochester, N.Y.

⁷ (PROVIL® novo Light), Heraeus Kuzler GmbH & Co., Hanau

⁸ (EPO-TEK 302-3), Epoxy Technologies, Billerica, MA

⁹ (METASERVE), Buehler UK Ltd., Coventry

¹⁰ ICN Biomedicals Inc., Aurora, Ohio

II. Surface topography

E cells were cultured on TCP⁶, TCP-Ti, P, AE, B, and SLA Ti coated replicas. Replicas of these surfaces were prepared according to Wieland et al., (2002). In brief, impressions of P, AE, B, and SLA surfaces were made with vinyl polysiloxane⁷ impression material. Vinyl polysiloxane negative replicas were then used to cast epoxy-resin⁸ positive replicas. The epoxy replicas were cured at room temperature for 24 hours followed by a 36 hours curing in the oven at 58°C. Surfaces were then ground on the back (i.e. flat) side of the replicas to final even thickness of 1mm on a grinder-polisher machine⁹. All surfaces were cleaned by ultrasonication in 7X-detergent¹⁰ and sputter coated¹¹ with 50 nm of Ti. Final, cleaning and sterilization was accomplished by glow discharge treatment for 4 minutes in an argon chamber prior to culturing of the E cells. SEM images of the rough surfaces are shown (Figure 1). The R_a , average roughness, values of the epoxy-resin replica surfaces follow: P: 0.06 μm , AE: 0.58 μm , B: 5.09 μm , and SLA: 4.33 μm (complete review characterization of these surfaces is given in Wieland et al., 2002).

¹¹ Randex 3140 Sputtering System, Palo Alto, CA.

¹² (Axioskop 2), Zeiss, Jena

¹³ Empix Imaging Inc., Mississauga, ON

III. Propidium Iodide (PI) staining

To determine the effect of surface roughness on cell proliferation, cells were seeded at an initial density of ~ 35 cells/mm² and cell numbers assayed on days 1,2,3,4, and 5 post seeding. Also, high-density (~ 500 cells/mm²) cultures were seeded and observed on days 1, 3, 7, 14, and 28 post seeding. Modification of the protocol described by Mazzini et al., (1980) was used to stain E cells with the PI. In brief, cells were rinsed thoroughly with warm (37°C) plain α MEM prior to fixation. Cells were then fixed in absolute ethanol for 2 minutes. Cells were hydrated in graded (95%, 90%, 70%, 50%, 30%) ethanol series, 2 minutes each, followed by a final hydration in distilled water. Each surface was then inverted on a 50 μ l drop of freshly diluted PI dye at the final concentration of 1g/L in phosphate saline buffer (PBS), pH 7.3, for 20 minutes in dark. Cells were rinsed thoroughly in distilled water followed by a final rinse in 100% ethanol in order to remove any unbound dye. Glass coverslips were mounted on the surfaces with immersion oil and examined using epifluorescence microscope¹² equipped with rhodamine filter set ($\lambda_{\text{emission}} = 540\text{nm}$) and image analysis software, Northern Eclipse¹³.

IV. Scanning electron microscopy (SEM) determination of cell area

To determine cell morphology, E cells were seeded at an initial density of ~ 100 cells/mm² on the surfaces of differing roughness. Cell morphology was prepared for examination in the scanning electron microscope (SEM) on day-2 post seeding according

to Wieland et al., (2002). In brief, cells were rinsed thoroughly with warm (37°C) α MEM, fixed for 1 hour in 2.5% glutaraldehyde³ in PBS at 4°C, and rinsed in 0.1M PBS. The subsequent treatment steps were performed using a microwave technique¹⁴. Cells were irradiated in the microwave at 37°C with a power of 750 W for post-fixation (2% osmium in PBS, followed by 1% tannic acid enhancement of osmium fixation) and at 45°C with 750 W for dehydration (50%, 70%, 90%, 95%, 100% ethanol, 1 minute each). Cells were further critical point dried with CO₂ in a semi-automatic critical point dryer¹⁵, and sputter-coated¹⁶ with 10 nm of gold. Cells were observed with a Cambridge Stereoscan 260 at an accelerating voltage of 12 KeV. Singlets (single cell), doublets (2 cells in contact), and clusters of E cells (3 and more cells in contact) were examined separately. Micrographs of ten cells in each class on each surface were collected. Cells were traced individually using a PC computer station equipped with a WACOM drawing plate and analyzed for cell area using software ImageJ from NIH.

V. Immunofluorescence

To eliminate the background fluorescence caused by the epoxy replicas, original metal surfaces were used in these experiments. Focal adhesion formation was visualized on day-2 post seeding at which time the cells were prepared for incubation with fluorescent hydrazide to stain cell components including the cell membrane and vinculin in focal adhesions using anti-Vinculin antibody. Samples were prepared for immunofluorescence observation according to Oakley and Brunette (1993) with minor

¹⁴ (PELCO™ 3470 Hornet Microwave System), Pelco International, Redding, CA

¹⁵ (SAMADRI®-795), Tousimis, Rockville, MD

¹⁶ (Hummer VI Sputtering System), Technics

modifications. In brief, cells were rinsed twice in 37°C plain α MEM and 37°C cytoskeleton-stabilization (CS) buffer, fixed for 10 min in 3.7% formaldehyde at 37°C and rinsed in 37°C CS buffer twice, oxidized in 4.2mM sodium periodate¹⁷ in PBS for 30 minutes at 4°C and rinsed in 0.1M PBS. Samples were inverted on a 50 μ l drop of 10 mM fluorescein-5-thiosemicarbazide¹⁸, filtered with 0.45 μ m syringe filter, for 2 hours in 37°C to stain the cell membrane and rinsed in PBS with 0.1% bovine serum albumin (BSA) 5 times. This was followed by 3 min extraction in 0.5% Triton X-100 at 4°C in CS buffer and 5 rinses in CS buffer at room temperature. Samples were then quenched in fresh 0.05% sodium borohydride¹⁷ in PBS for 10 min at room temperature and 5 rinses in CS buffer. All the rinses were performed on a rotator¹⁹ for 2 min each in dark. Nonspecific binding was blocked with 3% BSA in PBS at 37°C for 30 min. Incubation of the mouse monoclonal primary anti-Vinculin antibody²⁰ was performed overnight at 4°C in a humidified sealed chamber in the dark. Cells were then rinsed five times with 1% BSA in PBS and then inverted on a 50 μ l drop of Texas-Red goat anti-mouse IgG secondary antibody¹⁸ for 1 hour at room temperature in the dark, rinsed in 0.1% BSA in PBS for 5 times. Glass coverslips were mounted onto the surfaces with 1:1 glycerol: PBS solution containing 0.02% sodium azide and 1,4-diazabicyclo [2.2.2] octane. Twenty randomly selected cells that were not in contact with any other cell on each of the surfaces (P and AE) were examined with 40x objective on a confocal laser-scanning microscope (CLSM)¹². The outer boundary of the cell membrane, as visualized with

¹⁷ BDH Inc., Toronto, Ont.

¹⁸ Molecular Probes Inc., Eugene, Oreg.

¹⁹ (Rotomix 48200), Thermolyne, Dubuque, Iowa

²⁰ Chemicon International Inc., Temecula, CA

fluorescent hydrazide staining was traced using a PC computer station equipped with a WACOM drawing plate and analyzed for cell area using software ImageJ from NIH for each cell. The total area of focal adhesions as assessed by anti-Vinculin staining was analyzed in the same manner.

VI. Immunogold staining and detection of vinculin in focal adhesions using back-scattered electron (BSE) imaging on SEM

To visualize vinculin in focal adhesions at a higher resolution and to evaluate the relationship between topography and the focal adhesion sites an immunogold labeling technique using Alexa Flour-488 Fluoronanogold- Fab' 1.4 nm gold particle conjugate²¹ was developed. Cells were seeded at an initial density of ~ 100 cells/mm² on P, AE, B, and SLA epoxy replicas. The staining protocol was identical to that of immunofluorescence staining of vinculin in focal adhesions prior to the application of secondary antibody incubation. At this time, cells were incubated with 5% goat serum in PBS to block nonspecific binding sites for 30 min at 37°C. Cells were incubated with Alexa Flour-488 Fluoronanogold- Fab' 1.4 nm gold particle conjugate (diluted 1: 200 in 0.1% BSA in PBS) for 1 hour at room temperature and rinsed in 1% BSA in PBS 5 times. Cells were then fixed in 1% gluteraldehyde³ in PBS for 10 minutes at room temperature, and rinsed 5 times with 50 mM glycine²² in PBS to remove aldehydes. Samples were then rinsed in 1% BSA in PBS with 0.5% Tween-20²³ 5 times and rinsed with distilled water.

²¹ Nanoprobes Inc., Stony Brook, N.Y.

²² EM Sciences, Gibbstown, NJ

²³ Fisher Scientific, Edmonton, AB

The 1.4nm gold particles were enhanced to ~ 15 nm in diameter using the gold enhance kit²¹. Specimens were then carbon coated with 10 nm of carbon using a vacuum evaporator²⁴. Specimen examination was performed on a Hitachi S-4700 SEM field emission microscope with modifications to parameters set by Richards and ap Gwynn (1995). BSE image of vinculin in focal adhesions in an E cell on P is shown in Figure 2.

VII. Transmission electron microscopy (TEM) evaluation of membrane-Ti proximity

To investigate the effect of surface roughness on the proximity of E cells' basal membranes with the Ti surface, cells were seeded at an initial density of ~ 500 cells/mm² on P, AE, B, and SLA epoxy replicas. Samples were prepared for TEM evaluation on day-2 post seeding using a modification to the technique of Gould et al., (1981). In brief, samples were treated in the same manner as described above for SEM preparation prior to the application of critical point drying. At this time samples were infiltrated in a mixture of ethanol: plain Araldite epon-resin²⁵ without accelerator in a 2: 1, 1: 1, 1: 2 ratio for 1 hour each at room temperature on the rotator and 100% resin over night at room temperature. Further, samples were infiltrated with resin-accelerator, DMP-30²⁵, mixture for 1 hour at room temperature on the rotator, and embedded in vinyl polysiloxane impression molds. The resin was cured for 24 hours at 37°C, 24 hours at 45°C, and 24 hours at 58°C. Ultrathin sections perpendicular to the surface were cut using a diamond knife from each of the surfaces. The sections were then stained with alcoholic uranyl acetate and aqueous lead citrate, and five cells from each surface were viewed under an

²⁴ Mikros Inc., Portland. Oreg.

²⁵ Canemco Inc., St. Laurent, Quebec

electron microscope²⁶. Five randomly selected locations on each cell were imaged at 12000x magnification and 10 different measurements at each location were taken. The micrographs were developed and scanned into a PC computer station equipped with a WACOM drawing plate and analyzed for membrane Ti proximity using software ImageJ from NIH.

VIII. Statistical Analysis

A one-way analysis of variance (ANOVA) with Tukey HSD (honesty significant difference) test was employed using JMP_{IN}²⁷. A level of $P \leq 0.05$ was accepted as statistically significant.

²⁶ (EM 300), Philips, Holland

²⁷ SAS Institute Inc.

Results

I. Epithelial Growth

E cell interactions with surfaces of differing roughness were studied using low population density cultures that were assessed on days 1,2,3,4, and 5 and high population density cultures that were observed on days 1, 3, 7, 14, and 28.

Figure 3 shows the PI staining of E cells at low population density on day-1 and day-5 on surfaces with different roughness. The cell numbers were plotted against the time for the different roughness topographies (Figure 4). A tissue culture plastic (TCP) surface was included as a standard for comparison with other studies. To investigate the effect of Ti coating relative to this standard, a TCP surface coated with Ti (TCP-Ti) was also employed. It was found that there was a 10-fold increase in E cell numbers on day-5 (~ 100 cells/mm²) compared to day-1 (~ 10 cells/mm²) on TCP whereas there was a 5-fold increase in the cell number on day-5 (~ 50 cells/mm²) compared to day-1 (~ 10 cells/mm²) on TCP-Ti. Although there were no significant differences in E cell numbers on different surfaces on day-1 ($P>0.05$), on day-2 there was a significant increase, 2-fold increase, ($p\leq 0.05$) in E cell numbers on TCP (~ 30 cells/mm²) compared to the TCP-Ti (~ 15 cells/mm²). The same trend was observed on day-3, day-4 and day-5. Furthermore, on day-3 there was a significant increase in E cell numbers on TCP-Ti and P (smooth topography) compared to the AE, B, and SLA (rough topography). The same trend was observed on day-4 and day-5. There were no statistically significant differences ($P>0.05$) in E cell numbers on the rough surfaces (AE, B, and SLA) on day-1 through day-5.

High population density cultures were used to assess the effects of the rough surfaces on sheets of E cells that could be considered analogous to epithelial tissue. On

day-1 E cells were uniformly distributed on all the surfaces and had formed a confluent layer of cells. On day-3 E cell cultures on TCP, TCP-Ti, and P surfaces had formed multi-layers whereas the cells on the AE, B, and SLA surfaces remained a confluent monolayer. However, on day-7 some of the E cells cultured on B and SLA had detached and patchy areas were evident that lacked any cells. On day-14 the growth of E cells on AE surface had increased so that small densely packed islands of E cells could be observed in the confluent monolayer; however, on B and SLA E cells formed distinct colonies with large gaps between the colonies. The cultures were followed until day-28 at which time E cells grown on TCP, TCP-Ti, and P surfaces had undergone exfoliation cycles, in which confluent sheets of E cells detached from the surface leaving a few cells behind which multiplied to re-establish confluence. On AE surface the highly densely packed islands of E cell islands had grown in area and increased in numbers. E cells on B and SLA surfaces in colonies grew in area but still did not contact other colonies.

II. Cell area

As previous studies (Middleton C.A., 1977; Oakley and Brunette 1995) have shown that epithelial cells exhibit contact-induced spreading, it was necessary to measure the cell area of three different categories (singlets, doublets, and clusters). Images comparing the three different classes of E cells are shown (Figure 5).

Areas of E cells on different surfaces were measured and are plotted by surface (Figure 6). In agreement with previous reports E cells in contact with other E cells exhibited a threefold increase in cell area. There were no significant difference ($P>0.05$) in area/cell for each cell category between TCP and TCP-Ti surfaces. However, the area/cell of isolated E cells on TCP, TCP-Ti, P, AE, B surfaces was significantly

($P \leq 0.05$) greater than on the SLA surface. Furthermore, the area/cell of E cell doublets was significantly higher ($P \leq 0.05$) on TCP and TCP-Ti compared to those on the AE, B, and SLA. The area/cell of doublets was significantly ($P \leq 0.05$) higher on P compared to the SLA and no significant ($P > 0.05$) difference in area/cell was observed among P, AE, and B. Finally, the area/cell of E cells in clusters was significantly ($P \leq 0.05$) higher on TCP, TCP-Ti, P, and AE compared to the B and SLA. In general, E cells were more flattened and well spread on TCP, TCP-Ti, P, and AE in contrast to their more cuboidal morphology on the B and SLA surfaces.

III. Focal adhesions

There were more and larger focal adhesions on the P surface compared to the AE surface (Figure 7). The % area of focal adhesion/cell on the P (smooth surface) was 10.1 ± 4.52 (S.D.), which was significantly ($P \leq 0.05$) higher than the AE (rough surface) of 3.38 ± 1.51 (S.D.).

IV. Immunogold staining and detection of vinculin in focal adhesions using BSE imaging on SEM

An immunogold labeling technique of vinculin in focal adhesions using Fab'-gold (Au) probes was developed to investigate relationships of the focal adhesions with topographic features at higher resolution. BSE images of vinculin in focal adhesions on different surfaces are shown (Figure 8). As found earlier by fluorescence microscopy the focal adhesions were larger and more prominent on the P surface compared to the rough surfaces (AE, B, and SLA). Moreover, the focal adhesions on the rough surfaces (AE, B,

and SLA) tended to be localized to the ridges rather than the valleys of these surfaces (Figure 9).

V. TEM evaluation of membrane-Ti proximity

In agreement with the BSE imaging on SEM immunogold labeling, cell contact with the surfaces was most frequently found on the protruding portions of the rough surfaces (Figure 10). The distance in nm between the basal membrane and the Ti surface topography is given in Figure 11. On average, E cells were significantly ($P \leq 0.05$) closer to the Ti on the P and AE surfaces compared to the B and SLA surfaces.

Discussion

This study examined the effect of Ti surface roughness on the growth, spreading, adhesion, and membrane-Ti proximity of E cells. Our results demonstrated that surface roughness is an important determinant of these epithelial cell behaviors. In general, the smoother surfaces of TCP, TCP Ti, and P best supported the growth, spreading, and attachment of E cells.

Our results agree with those of Hormia et al., (1991), who used immunofluorescence techniques to observe that gingival epithelial cells attached and spread more readily on smooth than on rough, sandblasted Ti surfaces. Furthermore, Cochran et al., (1994) investigated the attachment and growth of periodontal cells on smooth and rough Ti surfaces and observed that after a lag period of attachment human gingival epithelial cells proliferated on control and smooth Ti, but not on rough Ti surfaces. The growth studies reported here extend those findings by examining the growth in more detail and for high population density at times up to 28 days. As expected the smooth surfaces supported growth to a greater extent than the rough surfaces. In the long term, 28-day, cultures of E cell on TCP, TCP-Ti and P surfaces underwent cycles of exfoliation in which sheets of cells were sloughed off but E cells recolonized the underlying denuded surfaces. On rougher surfaces some E cell exfoliated but the cells that remained were much slower to grow and growth limited to patches of isolated E colonies.

A novel aspect of this study is that we quantitatively evaluated the effect of roughness on cell area as a measure of cell spreading in three categories of isolated E cells, doublets, and clusters of E cells. The SEM analysis of E cell areas on different

surfaces indicated that E cells were in general more spread and flattened on the smoother surfaces (TCP, TCP-Ti, and P) and AE as compared to the roughest surfaces (B and SLA). Isolated E cells spread more on TCP, TCP-Ti, P, AE, B compared to the SLA surface, that is the result of both etching and blasting. The same trend was observed for E cells in doublets. As well, spreading of E cells was enhanced on contact with other E cells on smooth surfaces (TCP, TCP-Ti, and P) a phenomena first observed and named contact-induced spreading by Middleton C.A. (1977). Contact-induced spreading was not evident on the AE, B, and SLA surfaces as on the smooth surfaces. These findings along with the growth data suggest that epithelial coverage of rough surfaces will be difficult to attain if such surfaces become exposed because the growth of E cells as well as spreading is decreased on rough surfaces.

Although Hormia et al., (1991) did not observe the localization of vinculin in focal adhesions, several other laboratories have reported vinculin stained in focal adhesions in other types of epithelial cells on a variety of substrata (Teixeria et al., 2003; Shiraiwa et al., 2002).

Focal adhesions can be classified by areas to immature ($\text{area} < 2 \mu\text{m}^2$) (Galbraith et al., 2002), mature ($\text{area } 2\text{-}6 \mu\text{m}^2$) (Tamariz and Grinnell 2002), and super-mature ($\text{area} > 6 \mu\text{m}^2$) (Dugina et al., 2001). Immunofluorescence staining of vinculin in focal adhesions showed that E cells expressed more and larger focal adhesions on the P surface compared to the rougher AE surface. The quantification of adhesion by means of vinculin immunofluorescence staining specific to focal adhesions indicated that the total % area of focal adhesions/cell was greater on the P compared to AE surface. As the area of focal adhesions, is thought to be related to the strength of cell adhesion (Lotz et al., 1989; Hinz

and Gabbiani, 2003; Richards et al., 2001), it seems likely that epithelial tissue attaches more strongly to the smooth Ti surfaces by promoting the formation of mature to super-mature focal adhesions in comparison to the rougher AE surface. These observations suggest that epithelial tissue could be more susceptible to mechanical removal from a rough surface compared to a smooth surface.

This study involved the development of an immunogold labeling technique using Alexa Fluor-488 Fluoronanogold- Fab' 1.4 nm gold particle conjugate to visualize vinculin in focal adhesions. The technique is novel in that it involves smaller size 1.4 nm gold probes (nanogold) and application of Triton-X post fixation in contrast to that of Richards et al., (2001), who used larger gold particles in an application of conventional immunogold staining of vinculin in focal adhesions in fibroblasts and Triton-X extraction prior to the fixation. In agreement with the data using immunofluorescence to visualize vinculin in focal adhesions the immunogold method revealed fewer and smaller focal adhesions on the rough surfaces. The focal adhesions on rough surfaces tended to be localized to ridges (i.e. protruding structures) and were less evident in the valleys.

The ultrastructural analysis of the proximity of the cell membrane to the Ti showed that E cells were significantly closer to the smooth Ti surface (P) and AE compared to the rougher B and SLA surfaces. However, there was a high variation in membrane-Ti proximity on B and SLA as the protuberances on the rougher surfaces were in close contact with the cell membrane while the cells bridged over valleys. These observations are similar to those of Rakhi and von Recum (2003) who evaluated the proximity of fibroblasts on Ti surfaces using TEM, and also observed the bridging of cells between surface protrusions. The bridging behavior has the consequence that both

the mean and the standard deviation increase on the rough surfaces; nevertheless, statistically significant differences in membrane-Ti proximity were observed between rough and smooth surfaces.

Overall this study supports the view that smooth Ti surfaces are better suited for interfacing devices with epithelium than rough surfaces with random features for the smooth surfaces promote better adhesion, growth, and close adaptation of E cell membrane to the Ti surface. However, this conclusion is dependant on size and shape of the features and is limited to sand blasted and acid etched features that were examined in this study. Microfabricated surfaces with grooves of varying dimensions, whose walls, ridges, and floors are smooth, are also well suited for interfering with epithelial tissue although their R_a values may be high (Chehroudi et al., 1990). Thus both the size and shape of surface topographic features are potential determinants of epithelial cell adhesive behavior.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

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Chapter III- Conclusions and Future studies

Conclusions

In contrast to orthopedic implants, which are fully enclosed in the body, dental implants are exposed to the environment of a germ-laden oral cavity. The problem of integrating a foreign body in bone has largely been solved; but a second major problem, the maintenance of peri-implant soft tissue around the implant has not been addressed adequately. In my thesis, I addressed one aspect of the latter problem through evaluation of the effect of surface roughness on the growth, area, focal adhesion formation, and proximity of epithelial cells to titanium surfaces.

E cells responded to surface topography by decreasing their growth on the rougher surfaces of AE, B, and SLA compared to the smooth surfaces. During a 28-day culture period, cultures of E cells on smooth surfaces of TCP, TCP-Ti, and P underwent cycles of exfoliation, in which confluent sheets of cells detached from the surfaces and underlying remaining cells covered the exposed surfaces, whereas on the rougher AE, B, and SLA surfaces growth was much slower and limited to the isolated colonies spaced relatively far apart.

Furthermore, projected cell area was measured using SEM in the three classes of isolated, doublets, and clusters of E cells indicated that E cells were in general more spread and flattened on the smoother surfaces (TCP, TCP-Ti, and P) and AE compared to the rougher surfaces of B and SLA. Contact-induced spreading was observed on smooth surfaces (TCP, TCP-Ti, and P) but not on AE, B, and SLA. Also, E cells in isolation and doublets spread the least on SLA surface compared to the other surfaces.

E cells appeared to adhere more tightly to the smooth P surface as compared to the AE surface using “mature to super mature” focal adhesions (terminology used to classify focal adhesions by their size by Galbraith et al., 2002 and Tamariz and Grinnell, 2002). The data in my thesis showed that focal adhesion numbers and total area were decreased on rough surfaces. As the number and size of focal adhesions is thought to be related to the strength of adhesion, these observations suggest that epithelial tissue could be more susceptible to mechanical removal from a rough surface compared to a smooth surface.

Another novel aspect of my thesis was the development of an immunogold labeling technique using Alexa Fluor-488 Fluoronanogold- Fab' 1.4 nm gold particle conjugate to visualize vinculin in focal adhesions using BSE imaging on SEM. The spatial visualization of vinculin, which is one of the 50 proteins found at focal adhesion sites (reviewed by Zamir and Geiger, 2001), in relation to the underlying substratum topography at high magnification with the resolution of EM was an interesting and promising application of the technique. Focal adhesions on rough surfaces were concentrated to the ridge tops i.e. the protrusions of the rough surfaces. This finding will be more fully explained in the future studies section.

Finally the ultrastructural analysis of the E cells proximity to the Ti showed that epithelial tissue adhered much more closely to the smooth P surfaces and the AE surface compared to the rough B and SLA surfaces. On rougher surfaces of B and SLA E cells bridged between surface protrusions. These findings suggest that it would be problematic to use rough surfaces to form a successful soft tissue seal to the implant neck.

Future studies

The application of 1.4 nm nanogold particles in visualization of vinculin was demonstrated for the first time in my thesis (chapter-2, submitted for publication). Other applications of nanogold include labeling of antigens (CAV-1 α in capillary endothelial cells of placenta (Takizawa and Robinson, 2003); actin layer in frozen rabbit testis sections (Guttman et al., 2001)). The Alexa Fluor- FluoroNanogold is a dual-purpose probe that consists of an affinity-purified Fab' fragment conjugated covalently through a hinge thiol to 1.4 nm gold. The Fab' fragment carries amine groups that carry positive charges. Also, the gold itself is positively charged during the process of enhancement.

During the development of the technique, one of the difficulties I faced was the high background resulting from non-specific adhesion of the secondary probe. The background could be seen both on the titanium oxide surface (hydrophilic and negatively charged) as well as E cells. Four different methodological modifications were introduced to decrease the unspecific background staining: a) addition of a secondary blocking step i.e. goat serum, b) an increase in duration of blocking incubation of both primary blocking agent as well as secondary blocking agent, c) an increase in concentration of the BSA-PBS (primary) blocking agent, and d) an increase in washing time with PBS buffer. The modifications helped in reducing the non-specific background staining on Ti surfaces as well as reduction in non-specific staining observed in cells. The remaining non-specific staining can possibly be attributed to the positive charge on the gold particles (personal communication with Nanoprobes Inc.) or free vinculin in the cytoplasmic pool. Addition of non-fat milk powder at 5% concentration to the bathing buffer (PBS) has shown promise in quenching the interaction between gold and cells (personal

communication with Nanoprobes Inc.). More recently others have used Alexa fluor streptavidin FluoroNanogold particles (Takizawa and Robinson, 2003). Streptavidin has been added as it is thought to carry the required negative charge to suppress the positive charge on the gold particles. Therefore, the next logical step in enhancement of the immunogold staining procedure discussed in my thesis would be the use of milk proteins as well as use of streptavidin conjugated Nanogold probes to see if the non specific staining caused by the secondary antibody can be further reduced. Another approach would be, staining of actin filaments (using mild osmium fixation (Owen et al., 2001)) that are associated with focal adhesions as means to distinguish free vinculin from vinculin specific to focal adhesions.

The magnification and resolution power of an EM as well as successful and specific staining of vinculin in focal adhesions is of potential interest because other antigens pertaining to the adhesion sites (paxillin, talin, α -actinin, ARP 2/3, integrins and etc.) could be investigated in terms of spatial distribution in respect to the surface roughness characteristics, as a function of time and in relation to one another (in terms of the order of arrival at the adhesion sites). The latter could possibly be accomplished by sequential enhancement. In brief enhancement of Nanogold particles was performed using a gold-enhance kit. The 1.4 nm Nanogold particles could be developed to the particle sizes of up to 50 nm in diameter as the function of time (the longer the particle is enhanced the larger the particle size would be). Therefore, it is possible to form a standard curve relating the enhancement time to the particle size. The manufacturer states that "The time period for optimum gold enhancement varies with application; but 3-20 minutes has been found to be optimal for enlarging the 1.4 nm Nanogold particles to 3-20

nm or larger in size... and larger development times will give larger particles up to 50 nm in size" (Nanoprobes Gold-enhance kit, Cat. # 2113). Thus one could stain antigens sequentially with enhancement procedures between each step. The first antigen stained would be associated with the largest gold particles and the last antigen stained with the smallest gold particles. In this way one could study the sequence of addition of specific proteins in focal adhesions. Therefore, one could possibly propose a mechanism for the formation of focal complexes, dot shape structures maturing to focal adhesions, (reviewed by Wozinal et al., 2004) as well as fibrillar focal adhesions, responsible for matrix organization, (reviewed by Wozinal et al., 2004), and the regular focal adhesions, ranging from immature to super mature, (Galbraith et al., 2002; Tamariz and Grinell, 2002; Dugina et al., 2001). A further possible, application of the technique could be directed towards the investigation of adhesion structures expressed by E cells in 3-D, culturing the cells in collagen gels, (following the classical work done by Emerman and Pitelka, 1977; Cukierman et al., 2001; and more recently Glass-Brudzinski et al., 2002;).

Integrin expression of osteoblasts on the biomaterial surfaces has been investigated previously (Sinha and Tuan, 1996; Krause et al., 2000; and recently reviewed by Paine and Wong, 2003). It has been shown that profile of integrin expression by primary osteoblasts is dependent on underlying substratum topography and the type of material (Sinha and Tuan, 1996). Sinha and Tuan showed that hetro-dimeric functional integrin receptors are specific in terms of preference for proteins found in matrix on biomaterial surfaces. Findings relating to osteoblasts could be further investigated in terms of epithelial cells. Profile of integrin expression of epithelial cells on smooth and rough titanium surfaces could be studied by coating these surfaces with fibronectin,

vitronectin, laminin and collagen. Comparison of integrin expression of epithelial cells on different topographies coated with different matrix proteins as well as control surfaces could help elucidate the putative the role of surface features on differential expression of integrins.

Previously it has been shown that surface topography can modulate cell shape and secretion of proteins by epithelial cells (Hong and Brunette, 1987). Also, the data in my thesis showed that rough surface topographies modulate the morphology of epithelial cells. Therefore, it would be interesting to further investigate the effect of surface topography on production of different matrix proteins such as fibronectin, laminin, and collagen with which epithelial cells come into contact *in vivo*. One means to quantify the amount of protein produced by epithelial cells as influenced by underlying surface topography would be to use Western blots. However, the choice of the antibodies, monoclonal or polyclonal, used for the detection of the antigens and the commercial availability of the antibodies could be limiting. The major advantage of monoclonal antibodies is their specificity; however, the denaturation of antigens that can occur during the preparation can potentially make monoclonal antibodies useless in an application of Western blotting. Also, the specific cross-reactions between proteins can produce false positive bands when monoclonal antibodies are used. Polyclonal antibodies are widely used in immunoblotting as they bind to a number of denaturation-resistant epitopes on the antigen; however, polyclonal antibodies could also produce false positive bands due to presence of contaminating antibodies. Another frequently encountered problem in immunoblotting is the presence of diffuse background staining due to the nature of the secondary antibody (when indirect immunoblotting is performed). However, serial

dilution of the secondary antibody, usage of other alternative secondary antibodies as well as direct immunoblotting could reduce the diffuse background. In any case, it would be of interest to determine whether surface topography can stimulate epithelium to alter their microenvironment of attachment proteins.

In summary there are several possibilities to extend the findings of this thesis from the current base of morphological characterization to the molecular level of protein production and specific mechanisms of binding to Ti surfaces.

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