Effects of Extracellular Matrix Constituents on the Attachment of Human Oral Epithelial Cells at the Titanium Surface

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This in vitro study attempts to delineate the role of extracellular matrix (ECM) constituents at the epithelial tissue–implant interface. To know which ECM constituents have a beneficial influence on the behavior of epithelial cells, the attachment, proliferation, morphologic pattern, and differentiation or cytoskeletal organization of human oral epithelial cells on ECM-coated (type IV collagen, fibronectin, type I collagen, laminin, and vitronectin) and noncoated titanium surface have been evaluated and compared. In each experiment comparing commercially pure titanium and oxygen plasma-cleaned titanium, the same ECM constituents were used. In this study, type IV collagen could provide an excellent substratum for epithelial cell attachment on titanium surface, but vitronectin-coated titanium revealed lower effectiveness for attachment of epithelial cells than noncoated titanium. These results suggested that type IV collagen could be used as a means for obtaining good epithelial seal, whereas vitronectin could be used to restrain the attachment of epithelium to dental implants.
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Key words: cell attachment, cell proliferation, extracellular matrix protein, implant, oral epithelium, titanium

Obtaining a perimucosal seal of soft tissue to the implant surface should be one of the prerequisites for successful treatment with endosseous dental implants. Components of the soft tissue cover, ie, the epithelium as well as the connective tissue, act as an important barrier between the internal and external environment.[1] If the seal is lost and/or the tissues are not held close to the implant neck by the circular fibers encircling the structure in the gingiva, the periodontal pocket will extend to the osseous structures and the crest of the bone around dental implants.[2–4] Therefore, one must be concerned over breakdown between oral soft tissue adjacent to implanted materials with potential apical epithelial migration to the ultimate point of implant exfoliation.[5]

With respect to biomaterials such as commercially pure titanium and titanium alloy,[6–8] several additional factors that may influence cell substrate interactions must be considered. These parameters include composition, topography, protein adsorption and conformation, charge density, corrosion and ion release, oxide layer formation, and other physical factors.[9–11] The biomaterial surface, which regulates extracellular matrix (ECM) protein adsorption and conformation, may modify the signaling ability of the ECM molecules. Thus, it is likely that the surface characteristics of a substrate provide cells and tissue with a variety of signals and environmental cues that subsequently influence cellular behavior.

A possible practical application of this information may lie in coating biomaterials with substances that promote the attachment of one desired population of cells at the expense of others.[12] For dental implants, it may be advantageous to coat the coronal part of an

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implant with a factor that promotes epithelial attachment; whereas more apically, where fibroblast attachment is more desirable,[13,14] different coating might be applied. However, this philosophy may be overly simplistic for connective tissue because connective tissue is composed of many cell types and most attachment proteins show differing activities on diverse cell types. Moreover, since different cells have a variety of receptors for different attachment proteins, it may be difficult to obtain a surface that excludes other cell types while promoting the adhesion of fibroblasts. However, at the epithelial tissue–implant interface, it may be easier to control the epithelial cells, because epithelial tissue is composed of only one cell type. Therefore, the use of ECM molecules to coat dental implant materials at the epithelial tissue–implant interface has also been considered as a means to promote the adhesion or differentiation of epithelial cells to ideal sites on the implant surface, thus facilitating the perimucosal seal and ultimately the success of the dental implant.

The molecules involved in cell adhesion include ECM molecules, transmembrane receptors known as integrins, and intracellular cytoskeletal elements. In part, these effects may be mediated through signal transduction pathways that are coupled to the cell surface–binding proteins, which function as receptors for extracellular matrix proteins; but additionally, these effects may also be mediated by receptor-linked effects on cytoskeletal organization.[15] There are many studies to suggest that cell attachment plays a critical role in coordinating and integrating cellular differentiation, shape, movement, and biosynthetic activity.[16,17]

This in vitro study attempts to delineate the role of ECM constituents with epithelial cells at the epithelial tissue–implant interface. To know which ECM constituents have a positive influence on the behavior of epithelial cells, the attachment, proliferation, morphologic pattern, and differentiation or cytoskeletal organization of human oral epithelial cells (HOEC) on ECM-coated and noncoated titanium surfaces have been evaluated and compared. Titanium was used as a control and the following ECM constituents were employed: Type IV collagen (CIV), fibronectin (FN), Type I collagen (CI), laminin (LN), and vitronectin (VN). The results of using commercially pure titanium (cp titanium) were also compared with those obtained from using oxygen plasma–cleaned titanium (opc titanium). Evaluation of these effects on human oral epithelial cells is intended to serve as a reference for determining attachment proteins on titanium and to provide information concerning the achievement of an adequate perimucosal seal around the dental implant.

Materials and Methods

Serum-free culture media, keratinocyte growth medium (KGM) supplemented with pituitary extract, was purchased from Clonetics (San Diego, CA). Trypsin, ethylenediaminetetraacetic acid (EDTA), Hank's balanced salt solution (HBSS), penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). The 0.4% dispase and N-acetyl-ß-D-hexosaminidase were obtained from Sigma (St. Louis, MO). ECM constituents for cell attachment were obtained from the following sources: CIV (EHS sarcoma, powdered, Sigma); FN (human plasma, lyophilized, Sigma); CI (calfskin, 0.1% solution, Sigma); LN (human placenta, solution, Sigma); VN (human plasma, lyophilized, Sigma). A microbicinchoninic (BCA) protein assay kit was purchased from Pierce (Rockford, IL). Methyl ^{3}H-thymidine was secured from Amersham Life Science (Arlington, IL). Tissue culture plastic ware was obtained from Falcon (Cockeysville, MD) and Nunc (Rockslide, Denmark). Monoclonal antipan cytokeratin antibody and antimouse gamma G immunoglobin (IgG) (Fc specific) TRITC-conjugate were from Sigma. Cytokeratin AE-1 monoclonal antibody and AE-3 monoclonal antibody were from Vector (Burlingame, CA). Commercially pure titanium discs were purchased from Friatec (Kontich, Germany). The titanium target for transmission electron microscopy was made from cp titanium discs which were melted at the Department of Prosthetics, College of Dentistry, Chosun University, Kwang-ju, Korea.

Cell Culture and Maintenance. Primary human oral epithelial cells were obtained using modified protocols described by Oda and Watson.[18] Briefly, healthy gingiva overlying the impacted third molar teeth of adult humans was used as a source of tissue. These biopsies were obtained with the informed consent of the patients in accordance with the ethical standards of the Review Committee on the use of Human Subjects at Seoul National University, Korea. Specimens were processed within 1 hour after excision and were kept at 4°C until processing. Before dispase treatment, the specimens were washed several times with calcium and magnesium-free phosphate buffered saline containing antibiotics (penicillin-streptomycin and fungizone). The tissues were trimmed of any ragged edges, cut into 0.3 × 0.3 cm small pieces and subsequently were incubated overnight at 4°C with 0.4% dispase grade II.[19] The epithelium was collected by mechanical separation and treated with 0.05% trypsin and 0.53 mmol/L EDTA for 10 to 15 minutes at 37°C, centrifuged, and suspended in KGM supplemented with pituitary extract. The cells were incubated at 37°C in a humid-

Table 1 Profilometric Analysis of Randomly Selected Commercially Pure Titanium Discs, IMZ Implants, and Oxygen Plasma–Cleaned Titanium Discs

Material	Ra
IMZ (n = 16)	0.12 ± 0.05
Cp titanium (n = 8)	0.17 ± 0.08
Opc titanium (n = 7)	0.13 ± 0.05

Cp = commercially pure; opc = oxygen plasma–cleaned. Results were arithmetic mean deviation of the profile (Ra, µm) and expressed as mean ± SD. ANOVA with Duncan multicomparison test was performed; no significant difference among them appeared ($P > .05$).

ified incubator with 5% CO_2 and fed every 2 days. When the monolayers were confluent, they were subpassaged using routine methods. Cells used for the experiments were in the third generation.

Preparation of Titanium Substrates. Commercially pure titanium having a disc shape with a diameter of 10 mm, a thickness of 3 mm, and a smooth surface were utilized. According to the manufacturer, these substrates were processed in a manner similar to their clinical implants. They were polished using 600, 800, 1000, 1200, 2400, and 4000 grit; this was completed using a polishing cloth (Struers, Salzburg, Austria) with aluminum paste with a diameter of 0.25 µm. The titanium substrates were then washed in mild soap solution and extensively rinsed in water purified by reverse osmosis. Prior to use, the specimens were sonicated 3 times in distilled water for 5 minutes each time, washed in deionized water, passivated in 29% nitric acid for 2 to 4 hours, again extensively rinsed in deionized water, dried overnight in the laminar flow, and then sterilized under ultraviolet light for 15 minutes. After the above processing, some samples were treated with oxygen plasma cleaner (Anatech, Springfield, VA) for 30 minutes at 200 W. These surfaces are referred to as cp titanium and opc titanium. For standardization of the specimens, surface roughness was measured for some of the discs using a roughness tester (Shimadzu, Tokyo, Japan), and surface roughness of titanium specimens was compared with that of the titanium insert element (TIE) portion of IMZ implants (Friatec) shown in Table 1.

Measurement of ECM Protein Bound to Titanium Specimens. Titanium discs in the 4-well plate were coated with ECM constituents in the following concentrations according to the manufacturer's instructions: CIV, 6.0 µg per disc; FN, 2.0 µg per disc; CI, 7.0 µg per disc; LN, 1.0 µg per disc; and VN, 0.2 µg per disc. The above-listed concentrations were selected because they are reported by the suppliers to be active at those concentrations. The discs were rinsed twice with calcium- and magnesium-free phosphate-buffered saline (PBS), after which binding protein was resolved with 1% sodium dodexyl sulfate in PBS solution. Microtiter wells received 0.1 mL samples of ECM proteins. The protein content of each well was measured using the Pierce micro-BCA assay[20] according to the manufacturer's instructions, except that the absorbance was read at 490 nm in a Biotek enzyme-linked immunosorbent assay (ELISA) reader. Standard curves for each protein were established by linear regression. The data were obtained by calculating the percentage of attached proteins to total applied proteins. Experiments were also carried out using sixplicate wells with cp titanium and opc titanium in each.

Cell Attachment Assay. Titanium discs were coated with the 5 ECM constituents as experimental groups, and titanium only was used as a control group. Epithelial cells were displaced using 0.05% trypsin and 0.53 mmol/L EDTA in HBSS, resuspended in KGM, counted, and collected by centrifugation. The cells were added on the titanium disc in each well, 4×10^4 per ml, and incubated for 30, 60, and 180 minutes in each. Following the incubations, the unattached cells were removed by aspirating the medium and rinsing the wells with CMF-PBS. The attached cells were dissolved in 0.5% triton X-100, transferred into microtiter wells, and quantitated by measuring endogenous N-acetyl-ß-D-hexosaminidase.[21,22] The absorbance was read at 405 nm on a Biotek ELISA reader. To relate absorbance to cell number, enzyme activity was measured in wells to which known numbers of cells were added and processed. The data were obtained by calculating the percentage of attached cells to total applied cells. Experiments were carried out using triplicate wells and repeated twice. These assays were also done with cp titanium and opc titanium in each.

Cell Proliferation Assay. In a control group and 5 experimental groups, the epithelial cells were added on titanium discs in each well (4×10^4 per ml). If the cell confluency was 70 to 80%, 5 millicuries of ^{3}H-thymidine were added. The cells were then further incubated for 3 hours, removed from the medium, fixed with ice-cold 5% trichloroacetic acid at 4°C for 30 minutes, lysed with 0.5 mol/L sodium hydroxide, the cocktail solution added, and the radioactivity was measured with a liquid scintillation counter. Experiments were carried out using triplicate wells and repeated twice. These assays were also done with cp titanium and opc titanium in each.

Scanning Electron Microscopy (SEM). The cells were added to titanium discs in each well (4×10^4 per ml) and incubated for 30 minutes, 60 minutes, 180 minutes, and 24 hours in each. Following the incubations, specimens were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.3,

for 30 minutes and 1% osmium tetroxide in 0.1 mol/L cacodylate buffer, pH 7.4, for 1 hour. After rapid dehydration through a series of ethanol baths, critical-point drying, and sputter-coating with gold, the dorsal surface of the cell was observed with a scanning electron microscope (Jeol 840A, Jeol, Tokyo, Japan). After detaching the attached cells using adhesive tape, the ventral surface and the remnant of an attached area of cells in some of the specimens were observed.

Transmission Electron Microscopy (TEM). For TEM, titanium substrates were obtained using modified protocols described by Gould et al[23] and Jansen et al.[24] Briefly, titanium substrates were obtained by depositing a thin film, approximately 30 to 50 nm thick, on the inner surfaces of polystyrene tissue-culture 4-well dishes using an ion-sputtering coater and titanium target and sterilized with acetone and 70% alcohol. The cells were grown on titanium thin film in each well (4×10^4 per ml) and incubated for 24 hours. After the incubation period, the attached cells were fixed in situ using 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.3, for 30 minutes and 1% osmium tetroxide in 0.1 cacodylate buffer, pH 7.4, for 1 hour. The fixed cultures were dehydrated through a graded series of alcohols, infiltrated using hydroxypropyl methacrylate, and embedded in Epon (Polyscience, Washington, PA). The titanium-Epon complex containing substrate, as well as the cells attached to them, were cut on an ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined and photographed using a transmission electron microscope (Jeol 1200EXII, Japan).

Indirect Immunofluorescence Using Cytokeratin Antibody. Epithelial cells were grown on cp titanium in a control group and 5 experimental groups and coated with various proteins for 60 minutes, 180 minutes, and 24 hours in each. Cells were fixed in methanol (5 minutes at 4°C) and permeabilized in acetone (10 minutes at –20°C). They were preincubated with goat IgG, incubated with primary antibody (monoclonal antibody anti-pancytokeratin and the antiepidermal keratin molecules AE-1 and AE-3) for 1 hour at room temperature, then with fluorescent-labeled secondary antibody (Fc-specific antimouse tetramethyl rhodamine isothiocyanate-[TRITC] conjugate) for 30 minutes. After rinsing, the samples were mounted with glycerol-PBS and examined with fluorescence microscope (Olympus, Tokyo, Japan).

Statistical Analysis. The experimental design was a between-group comparison. Between groups, the data were analyzed using analysis of variance (ANOVA) with Duncan's multicomparison procedure at the 5% level. Student's *t* test was also performed between cp titanium and opc titanium at the 5% level. The SAS statistical software (Cary, NC) designed for personal computers (release 6.03) was used for all statistical procedures.

Table 2 Binding of ECM Protein to Commercially Pure and Oxygen Plasma–Cleaned Discs

Substrate	Cp titanium	Opc titanium
Type IV collagen	46.5 ± 2.6	51.9 ± 10.1
Fibronectin*,†,††	66.3 ± 3.4*,†	68.1 ± 5.2*,†,††
Type I collagen†	58.6 ± 11.2	53.5 ± 1.1†
Laminin*,†,††	69.6 ± 2.5*,†	71.4 ± 4.9*,†,††
Vitronectin	43.4 ± 3.7	42.6 ± 1.9*

Cp = commercially pure; opc = oxygen plasma–cleaned. Results were obtained from sixplicate wells. All values were binding percentages of extracellular matrix proteins and expressed as mean ± SD. Student's *t* test was performed; no significant difference between cp titanium and opc titanium was observed ($P > .05$). ANOVA with Duncan multicomparison test was also performed: statistically significant relative to type IV collagen (*$P < .05$); to vitronectin (†$P < .05$); to type I collagen (††$P < .05$).

Results

Binding of ECM Protein to Titanium Substrate. The amount of each ECM constituent that bound to titanium substrates and had actual influence on epithelial cells in culture media was calculated. Both cp titanium and opc titanium followed the same rank order of protein binding to all substrates in the range of 42 to 72%: LN>FN>CI>CIV>VN as shown in Table 2. Opc titanium revealed higher binding of matrix protein than cp titanium in CIV, FN, and LN but showed lower binding in CI and VN. Statistically significant differences were not observed between cp titanium and opc titanium in all groups.

Cell Attachment. In the cp titanium and opc titanium, CIV-coated titanium demonstrated a high attachment of human oral epithelial cells to titanium, whereas VN-coated titanium revealed a low attachment of epithelial cells to titanium (Table 3). Attachment of epithelial cells in the CIV-coated group, expressed as percentages, were 73.4:75.7, 79.7:78.9, and 90.2:89.4, respectively, according to the incubation time, which was significantly different from those in FN, CI, LN, and VN groups; whereas attachment of epithelial cells in the VN-coated group, expressed as percentages, were 58.5:60.5, 65.1:66.7, and 73.1:77.4, respectively, according to the incubation time, which was significantly different from those in the CIV, control, FN, CI, and LN groups. A significant difference between cp titanium and opc titanium was not found with the exception of the FN-coated group at 30 minutes after incubation and the LN-coated group at 30 minutes after incubation. There was not direct correlation between the binding per-

Table 3 Attachment of Human Oral Epithelial Cells on Titanium Discs Coated with Extracellular Matrix Protein According to the Incubation Time

Substrate	Time (min)	Commercially pure titanium		Oxygen plasma–cleaned titanium	
Type IV collagen*	30	73.4 ± 5.9		75.7 ± 4.0	
	60	79.7 ± 2.2	*	78.9 ± 1.5	*
	180	90.2 ± 2.1		89.4 ± 6.0	
Control*	30	69.4 ± 4.4		72.4 ± 1.1	
(titanium only)	60	76.1 ± 4.4	*	77.6 ±6.8	*
	180	84.7 ± 1.5		83.7 ± 2.0	
Fibronectin*,†	30††	66.1 ± 1.2		75.3 ± 1.7	
	60	67.5 ± 12.2	†	76.9 ± 1.3	*
	180	76.4 ± 3.5		82.3 ± 7.5	
Type I collagen*,†	30	66.9 ± 8.1		73.5 ± 4.0	
	60	70.9 ± 14.8	†	73.1 ± 10.8	*
	180	78.2 ± 6.6		82.6 ± 1.1	
Laminin*,†	30††	63.9 ± 3.8		73.1 ± 4.2	
	60	70.4 ± 4.9		73.1 ± 2.6	†
	180	84.4 ± 4.1		75.8 ± 1.1	
Vitronectin*,†	30	58.5 ± 3.7		60.5 ± 2.8	
	60	65.1 ± 0.9	†	66.7 ± 3.0	†
	180	73.1 ± 4.4		77.4 ± 4.6	

Results were obtained from sixplicate wells. All values were percentages of attached cells and expressed as mean ± SD. ANOVA with Duncan multicomparison test was performed: statistically significant relative to vitronectin (*$P < .05$); to Type IV collagen (†$P < .05$). Student's *t* test was performed; commercially pure titanium versus oxygen plasma–cleaned titanium (††$P < .05$).

Table 4 Proliferation of Human Oral Epithelial Cells on Titanium Discs Coated with Extracellular Matrix Protein

Substrate	Commercially pure titanium	Oxygen plasma–cleaned titanium
Type IV collagen	3076 ± 1019	3233 ± 170
Control (titanium only)*,†	939 ± 493*,†	1412 ± 222*
Fibronectin*	1685 ± 714*	1915 ± 926*
Type I collagen*	1878 ± 373*	1990 ± 669*
Laminin*	1291 ± 735*	1406 ± 806*
Vitronectin	1728 ± 348*	1708 ± 241*

Results were obtained from sixplicate wells. All values were count per minute (CPM) and expressed as mean ± SD. Student's *t* test was performed; no significant difference between commercially pure titanium and oxygen plasma–cleaned titanium was observed ($P > .05$). ANOVA with Duncan multicomparison test was also performed: statistically significant relative to type IV collagen (*$P < .05$); to type I collagen (†$P < .05$).

centage of ECM protein and HOEC attachment at the concentration of ECM protein recommended by the suppliers.

Cell Proliferation. The growth of epithelial cells was increased, especially by CIV, in cp titanium and opc titanium discs (Table 4). For the CIV-coated group, values were higher (3076:3233) than those of the control, FN, CI, LN, and VN-coated groups. Statistically significant differences were not observed between cp titanium and opc titanium in all groups.

Scanning Electron Microscopic Findings. Thirty minutes after plating, all epithelial cells were spherical, with numerous microvilli and blebs, and were rimmed by a thin lamella in contact with the titanium substrates (Fig 1a). After 60 minutes, a lamellar rim of cytoplasm often spread out radially from the round cell body containing the nucleus (Fig 1b). After 180 minutes, with increasing time of attachment, the area of lamellae increased, and the cells became more flattened, with fewer microvilli and blebs evident (Figs 2 and 3). Often lamellae were edged by filopodia, and the filopodia were increased with time of spreading. After 24 hours, the cells became polarized and spread extensively across the titanium substrates (Figs 4 to 6). Cells were elongated and well spread out, indicating good attachment, but showed no preferred orientation. In the control (titanium only) group and especially in the VN-coated groups epithelial cells spread slowly compared with other groups. When the epithelial cells

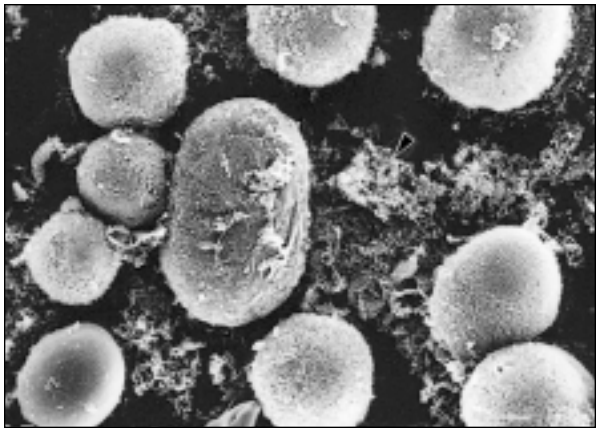

Fig 1a Scanning electron micrograph showing ventral surface of epithelial cells after incubation for 30 minutes on CIV-coated titanium (magnification ×350). Note the meshwork/network structure of the type IV collagen *(arrowhead)* on titanium.

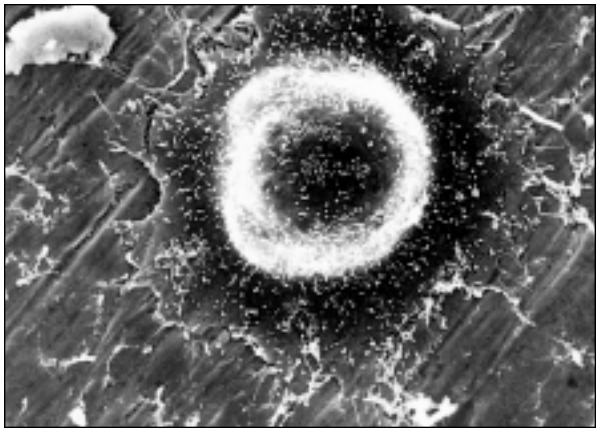

Fig 1b Scanning electron micrograph showing dorsal surface of epithelial cells after incubation for 60 minutes on CIV-coated titanium (magnification ×500).

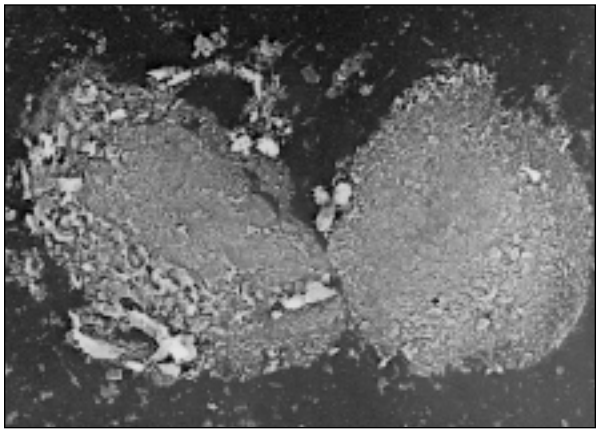

Fig 1c Scanning electron micrograph showing the remnant of the attached area of epithelial cells after detaching the attached cells following incubation for 60 minutes on CIV-coated titanium (magnification ×700).

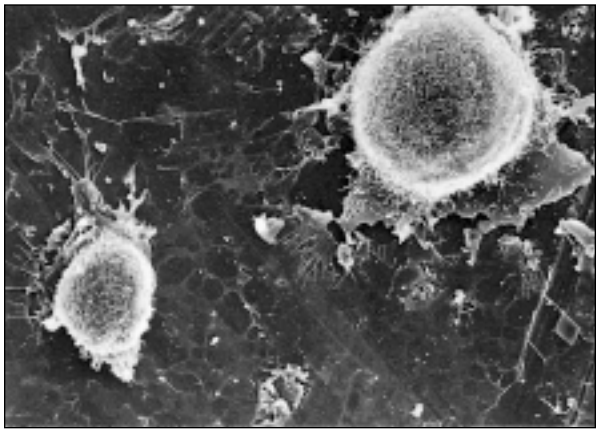

Fig 2 Scanning electron micrograph showing dorsal surface of epithelial cells after incubation for 180 minutes on VN-coated titanium (magnification ×950).

approached the substrate 24 hours after incubation, each cell process terminated on a globular accretion in FN (Fig 4), LN, CI, and CIV-coated substrates.

After detaching the attached cells using adhesive tape, the ventral surface and the remnant of attached area of cells were observed (Figs 1a, 1c, and 6). In that case, epithelial cells on CIV-coated substrates showed even and smooth or flat contact with the titanium surface; epithelial cells on other protein-coated substrates showed irregularities and small or large vacuole-like structures in their cytoplasm (Fig 1c).

Transmission Electron Microscopic Findings. Multilayers of cells were found on all substrate surfaces. The cells appeared elongated and desmosomes were present between cells. The cells had a normal

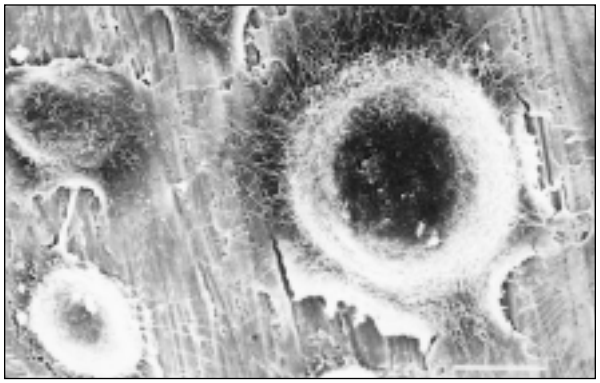

Fig 3 Scanning electron micrograph showing dorsal surface of epithelial cells after incubation for 180 minutes on CI-coated titanium (magnification ×500).

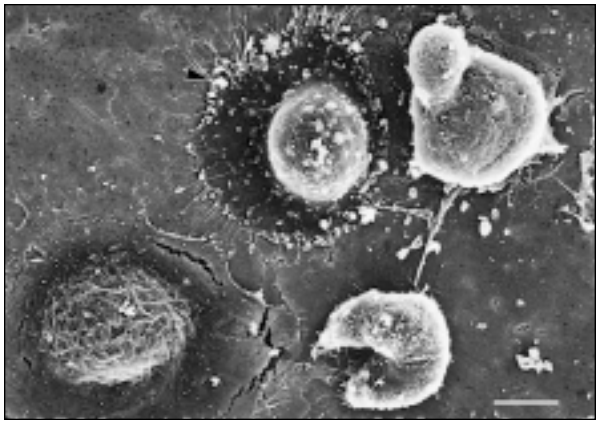

Fig 4 Scanning electron micrograph showing dorsal surface of epithelial cells after incubation for 24 hours on FN-coated titanium (magnification ×1,500). Note the globular accretions *(arrowhead)* in the cytoplasm of the attached epithelial cell.

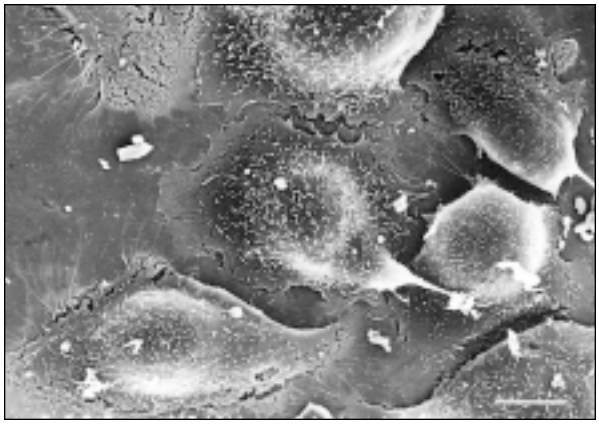

Fig 5 Scanning electron micrograph of LN-coated group. Dorsal surface of epithelial cells after incubation for 24 hours on LN-coated titanium (magnification ×750).

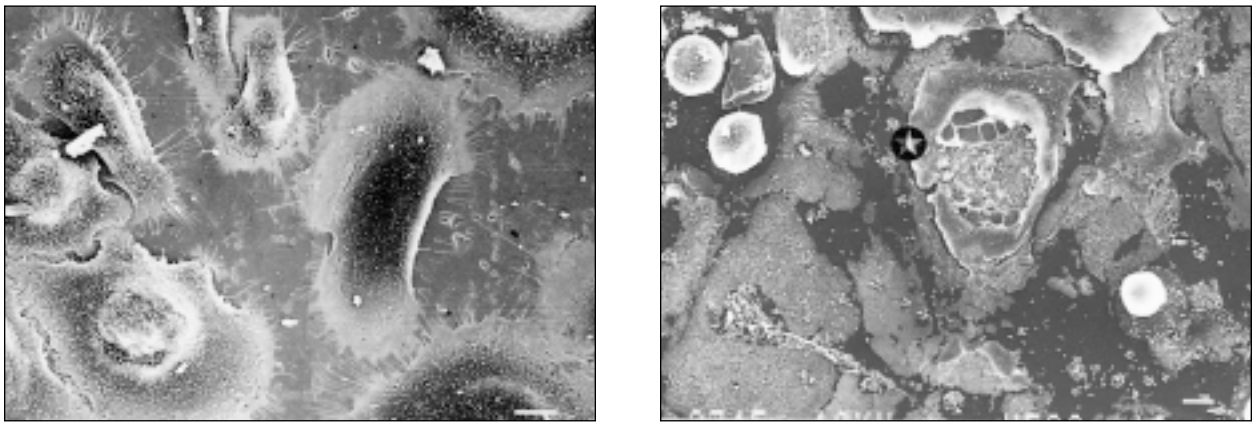

Fig 6 Scanning electron micrograph of control group (titanium only). *(Left)* Dorsal surface of epithelial cells after incubation for 24 hours on titanium (magnification ×1,875). *(Right)* The remnant of attached area of epithelial cells after detaching the attached cells after incubation for 24 hours on titanium (magnification ×750). Note the vacuole-like structure *(star)* of remnant of attached area on titanium.

appearance characterized by the presence of ribosomes, surface pseudopodial projections, tonofilament bundles, vesicles, and a nucleus. Lysosomes were observed in the cells, and numerous vacuoles containing large particles were also seen. Some of the cells contained coated vesicles, and occasionally, in the plasma membrane, coated regions were found on surfaces facing the substratum. The titanium was visible as an irregular, dense black line covering the surface of the culture substrate, having a thickness of about 30 to 50 nm (Fig 7).

There were 3 distinctive types of cell-substrate contacts. The first type of cell-substrate contact was close contact between the cell membrane and the substratum. Close contact between cells and substrate surface occurred in a strikingly high frequency. In most of these areas, a gap of 20 to 40 nm could be found between the cell and the substrate, and these regions were also identified as a focal contact. Microfilament bundles were in intimate association with these sites of close contact (Fig 8). The second type of cell-substrate contact was ECM contact between the cell membrane and the substratum, having a gap of about 100 to 150 nm. Strands of ECM appeared to connect the plasma membrane and the substratum surface. The third type of cell-substrate contact was hemidesmosome contact, which is characterized by an electron-dense plaque inside the cytoplasm and by peripheral filament extending from the plasma membrane to the substrate surface bridging a gap approxi-

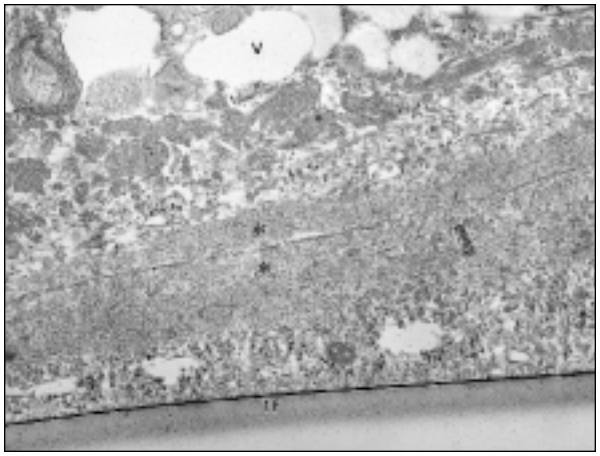

Fig 7 Transmission electron micrograph of control group after incubation for 24 hours on the thin titanium film (TF). An epithelial cell is attached to the titanium surface by close contact or ECM contact. Cell organelles are prominent throughout the cytoplasm, and there are lamella bodies, vacuoles (V), cytokeratin filament bundles, and numerous actin filaments *(asterisk)* associated with intercellular junction of the epithelial cells (magnification ×30,000).

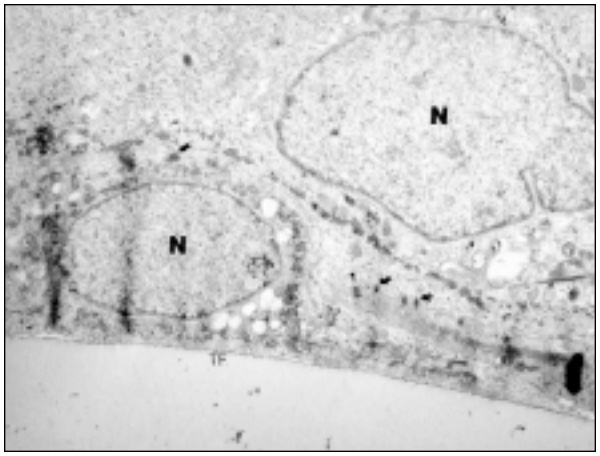

Fig 8 Transmission electron micrograph of CI-coated group after incubation for 24 hours on the thin titanium film (TF). Epithelial cells are attached to the titanium surface by close contact. Note the 2 epithelial cells including nuclei (N) and intact desmosomes *(arrow)* along the cell junction (magnification ×8,000).

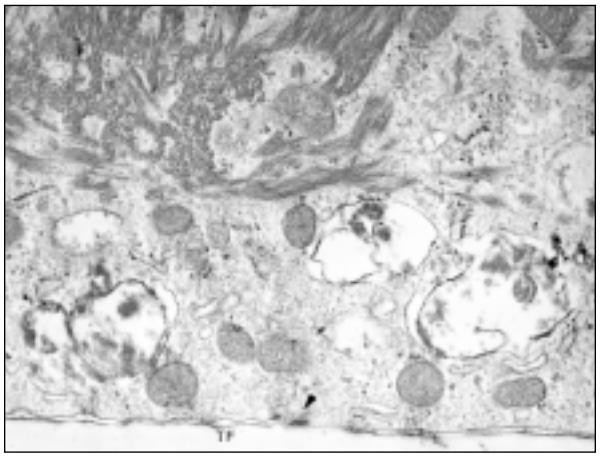

Fig 9a Transmission electron micrograph of CIV-coated titanium after incubation for 24 hours on the thin titanium film (TF). An epithelial cell is attached to the titanium surface by hemidesmosome contact *(arrowhead)* (magnification ×40,000).

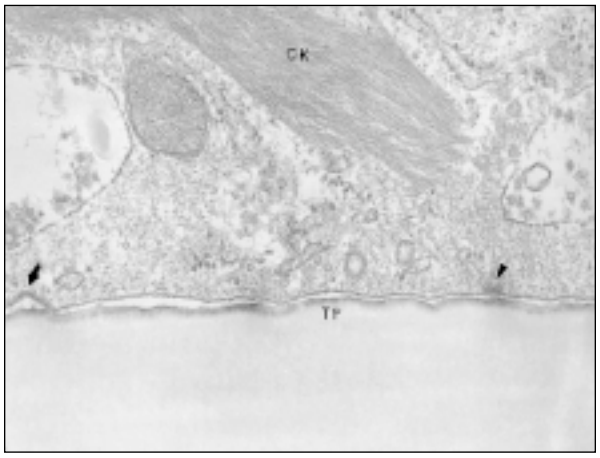

Fig 9b Transmission electron micrograph of CIV-coated group after incubation for 24 hours on the thin titanium film (TF). Note the cytokeratin filament bundle (CK) in the cytoplasm, the coated pits *(arrow)* and hemidesmosome *(arrowhead)* in the contact area of epithelial cell with titanium (magnification ×30,000).

mately 10 to 20 nm wide (Figs 9a and 9b). These 3 types of contact were observed in all specimens. Hemidesmosome contact, 24 hours after incubation, was not clearly distinguished in the control, FN, CI, LN, and VN-coated titanium discs but was definitely observed in the CIV-coated titanium discs.

Cytoskeletal Organization. All cultured epithelial cells reacted strongly with anti-pancytokeratin monoclonal antibody, which is specific to stratified squamous epithelium. They reacted with AE-1, which is specific to cytokeratin filaments with low molecular weight and intermediate molecular weight, and they also reacted with AE-3, which is specific to cytokeratin filaments with high molecular weight. They demonstrated a network of filamentous staining.

Four distinct cytoskeletal morphologies were observed. Type I cells displayed central staining with faint cytokeratin filament formation in the central area of the cell. Type II cells showed strong central staining and faint peripheral staining with some radi-

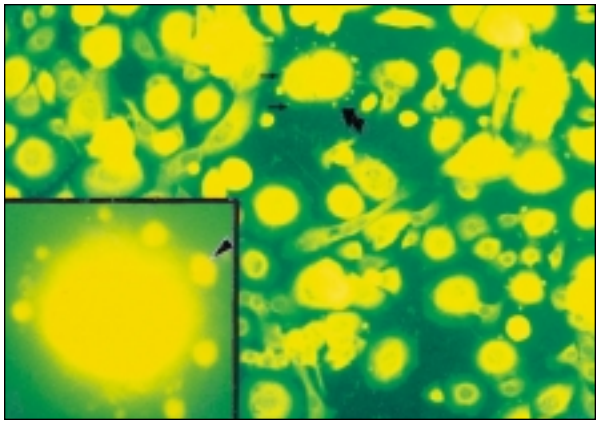

Fig 10 Immunofluorescent staining of CIV-coated titanium using pancytokeratin antibody after incubation for 24 hours on titanium (magnification ×100). Left inset is magnification of epithelial cell (magnification ×400). Note the regional intense globular fluorescent granule *(arrow and arrowhead)* in the periphery of the cytoplasm (magnification ×400).

ally oriented filaments. Type III cells had distinct, well-formed cytokeratin filaments that were located in all of the cytoplasm. These 3 types each represented a different stage in the degree of cytoskeletal reorganization according to the attachment process. Type IV cells revealed strong staining in all cytoplasm and characteristically small multiple globular staining along the periphery of the cell. This type was clearly observed in FN, CI, and CIV-coated substrates after 24 hours incubation (Fig 10). Except for this globular staining, there was no difference in the cytoskeletal organization or differentiation of attached epithelial cells on the titanium substrate noncoated or coated with various ECM constituents.

Discussion

In the natural dentition, the junctional epithelium (JE) provides a seal at the base of a periodontal sulcus, protecting against the penetration of periodontally pathologic chemical and bacterial substances. Disruption of this seal or lysis of the connective tissue fibers inserted into root cementum apical to the JE leads to rapid migration of the crevicular epithelium, forming a pathologic pocket.[25] As no cementum or fiber insertion is reported on the surface of titanium perimucosal abutments, an epithelial perimucosal seal could provide the only barrier against pathologic insults to deeper tissues.[26,27] Therefore, most studies of cell attachment have been designed to determine whether certain proteins can function selectively as ligands for epithelial cell attachment. In these studies, researchers usually used ECM proteins such as FN, CI, LN, and occasionally VN, but CIV was not widely used as an attachment protein for epithelial cells. It may be because of the fact that CIV can interact with cells indirectly through LN and also binds to heparin and heparin sulfate proteoglycans.[28]

FN is widely distributed in basal lamina and periodontal connective tissue. It has been found to promote the substrate attachment of both fibroblasts and epithelial cells.[29] LN is found in the basal membrane and has also been shown to be involved in a multitude of other functions, such as attachment of epithelial cells, wound healing, neurite growth, and bacterial adhesion. It has also been reported that laminin is a potent chemoattractant and theoretically could prevent epithelial cell migration. VN has been isolated from human serum and has been shown to promote the attachment and spreading of cells grown in culture. CI is distributed in the basal layer of epithelial cells and provides the proper environment for cellular migration, attachment, and differentiation. CIV is a major constituent of basal membrane and is also distributed and present in a rather homogenous meshwork or network-forming structure.[30–32] It is involved in interactions with cells and possesses 2 specific recognition sites for the integrins α1ß1 and α2ß2. To function in vivo, the cellbinding sites have to be accessible if the molecules are incorporated into the macromolecular network of CIV. In this respect, the binding sites are located within the N-terminal area of the CIV molecule, which is more exposed and less protected than the C-terminal half of the triple-helical domain.[33]

In the present study, CIV-coated titanium revealed increased attachment of human oral epithelial cells to titanium, whereas VN-coated titanium revealed less attachment of epithelial cells to titanium. Epithelial cell attachment to CIV-coated titanium was measurably better compared with the other groups, especially after 180 minutes of incubation (unpublished data). This result could be affected by 2 factors. First, other ECM proteins such as laminin can be actively synthesized and secreted on CIV by epithelial cells with the passage of appropriate time; and second, CIV is contributed in a manner by which cell receptors are controlled by or interact with substrates secreted by its own epithelial cells, not by applied substrates in the early process of cell attachment. VN-coated titanium revealed low attachment of epithelial cells to titanium and had a statistically significant difference compared to the other groups. This could have resulted from the fact that the cell receptor for VN was not found in epithelial cells. From this outcome of VN, it was suggested that VN be used as a medium to restrain the attachment of epithelium in dental implants.

Types I, III, and V collagen increased the growth of osteoblast-like cells or dermal fibroblasts on tita-

nium or titanium alloy in the steady-state level.[34] FN also promoted fibroblastic proliferation in several studies involving laboratory animals.[34] However, the growth of epithelial cells on titanium resulted from a significant increase in the CIV-coated titanium, as shown in the present study.

In general, the surface chemistry, surface energy, and surface topography govern the biologic response to implanted material.[35] The cellular response to implant materials may also be affected by adsorbed surface species that affect the surface composition and charge. These adsorbed species may be, initially, contaminant films arising from preparatory procedures that result in low-energy surfaces and adverse responses.[36] For example, the plasma cleaning of dental implants to obtain a high-energy surface has been recommended.[37,38] The difference of protein attachment, cell attachment, and cell growth between cp titanium and opc titanium were investigated but no significant differences were detected, except with the FN-coated group at 30 minutes after incubation and the LN-coated group at 30 minutes after incubation in case of cell attachment. Why is it that plasma cleaning improved the effectiveness of cell attachment only in FN-coated titanium and LN-coated titanium? Further investigations to resolve this question will be necessary.

In the SEM observations, cells were elongated and well spread out, indicating good attachment, but they showed no preferred orientation according to incubation time. Epithelial cells on CIV-coated substrates also showed even or smooth contact with the titanium surface. Each cell process terminated on a globular accretion in CIV-, FN-, CI-, and LN-coated substrates, which correlated with intense spherical immunofluorescent staining to cytokeratin antibody.

SEM is usually used to observe the morphologic characteristics of a cell, especially the dorsal surface. However, to investigate the cell contact between certain substrates and cells thoroughly, it was necessary to see the ventral or attached surface of the cells.[39] In this study, the ventral surface and the remnant of attached area of cells could be observed using adhesive tape, as well as the dorsal surface. This method could contribute to the discovery of the attachment mechanism of cells to substrate.[39]

It is well known that epithelial cells attach to the surface of the titanium in much the same manner as they attach to the surface of a natural tooth, with a basal lamina and the formation of hemidesmosomes.[40] However, hemidesmosomes have appeared to develop by 2 days, 3 days, or 2 weeks according to conditions.[41,42] The hemidesmosome was well established on CIV-coated titanium within 1 day in our investigation. Differences in cell population, culture conditions, or the treatment of titanium substrates may have influenced the different results.[24] The substrate surface was covered with an adsorbed layer of serum proteins, so that direct cell-substrate contact is unlikely to occur. Therefore, the hypothesis that can be put forward is that the substrate's influence on the nature and/or conformational state of the serum components adsorbed determines whether hemidesmosome or focal- and ECM-type contacts only will be formed.[23]

The ECM constituents can act as a regulator of cellular processes such as differentiation, cytoskeletal organization, and phenotypic expression.[43,44] But only three types of HOEC morphology, each representing a different stage in the degree of cytoskeletal reorganization as studied by indirect immunofluorescence, were found. To delineate the effect of ECM constituents on cell differentiation at the titanium surface, the specific gene expression of epithelial cells will be examined by other methods such as in situ hybridization.

Conclusion

These experiments suggested that CIV could provide an excellent substratum for cell attachment on titanium surfaces, but we were unable to detect the basic cause and/or mechanism by which CIV showed the greatest effectiveness in epithelial cell attachment compared to FN, CI, LN, and VN. Therefore, further research will focus on the molecular biologic approach to uncover this and will also consider the influencing factors including concentration, cultivation time, and application of CIV produced by HOEC.

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References

1. Berglundh T, Lindhe J, Ericsson I, Marinello CP, Liljenberg B, Thomsen P. The soft tissue barrier at implants and teeth. Clin Oral Implants Res 1991;2:81–90.
2. Schluger S, Yuodelis R, Page RC, Johnson RH (eds). Periodontal Diseases. Philadelphia: Lea and Febiger, 1990:207–211.
3. Donley TG, Gillette WB. Titanium endosseous implant–soft tissue interface: A literature review. J Periodontol 1991;62:153–160.
4. Bauman GR, Rapley JW, Hallmon WW, Mills M. The periimplant sulcus. Int J Oral Maxillofac Implants 1993;8:273–280.

5. Tonetti MS, Schmid J. Pathogenesis of implant failures. Periodontology 2000 1994;127–138.
6. Bruneel N, Helsen JA. In vitro stimulation of biocompatibility of Ti-Al-V. J Biomed Mater Res 1988;22:203–214.
7. Gross UM. Biocompatibility: The interaction of biomaterials and host response. J Dent Educ 1988;52:798–803.
8. Steinberg AD, Willey R, Drummond JL. In-vivo comparisons of clot formation on titanium and hydroxyapatite-coated titanium. J Periodontol 1992;63:990–994.
9. Squier CA, Collins P. The relationship between soft tissue attachment, epithelial downgrowth and surface porosity. J Periodont Res 1981;16:434–440.
10. Baier RE, Meyer AE. Future direction in surface preparation of dental implants. J Dent Educ 1988;52:788–791.
11. Brunette DM. The effect of implant surface topography on the behavior of cells. Int J Oral Maxillofac Implants 1988;3:231–246.
12. Majeska RJ, Port M, Einhorn TA. Attachment to extracellular matrix molecules by cells differing in the expression of osteoblastic traits. J Bone Miner Res 1993;8:277–289.
13. Lowenberg BF, Aubin JE, Deforter DA, Sodek J, Melcher AH. Attachment, migration and orientation of human gingival fibroblasts to collagen-coated, surface-demineralized and non-demineralized dentin in vitro. J Dent Res 1985;64:1106–1110.
14. Von Recum AF, Schreuders PD, Powers DL. Basic healing phenomena around permanent percutaneous implants. In: Van Steenberghe (ed). Proceedings of the International Congress of Tissue Integration in Oral and Maxillofacial Reconstruction. Amsterdam: Excerpta Medica, 1986:159–169.
15. Steffesen B, Duong AH, Milam SB, Potempa CL, Winborn WB, Magnuson VL, et al. Immunohistochemical localization of cell adhesion proteins and integrins in the periodontium. J Periodontol 1992;63:584–592.
16. Hay ED. Cell biology of extracellular matrix. New York: Plenum Press, 1991:343–359.
17. Bruce A, Dennis B, Julian L, Martin R, Keith R, James DW. Molecular biology of the cell. New York: Garland, 1994:963–1006.
18. Oda D, Watson E. Human oral epithelial cell culture I. Improved conditions for reproducible culture in serum free medium. In vitro. Cell Dev Biol 1990;26:589–595.
19. Stenn KS, Link R, Moellmann G, Madri J, Kuklinska E. Dispase, a neutral protease from Bacillus polymyxa, is a powerful fibronectinase and type IV Collagenase. J Invest Dermatol 1989;93:287–290.
20. Redinbaugh MG, Turley RB. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal Biochem 1986;153:267–271.
21. Landegren U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase applications to detection of lymphokines and cell surface antigens. J Immunol Methods 1984;67:379–388.
22. Oldberg A, Franzen A, Heinegard D. Cloning and sequence analysis of rat bone sialprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell binding sequence. Proc Natl Acad Sci USA 1986;83:8819–8823.
23. Gould TRL, Brunette DM, Westbury L. The attachment mechanism of epithelial cells to titanium in vitro. J Periodont Res 1981;16:611–616.
24. Jansen JA, de Wijn JR, Wolters-Lutgerhorst JML, van Mullen PJ. Ultrastructural study of epithelial cell attachment to implant materials. J Dent Res 1985;64:891–896.
25. Tencate AR. The gingival junction. In: Brånemark P-I, Zarb GA, Albrektsson T (eds). Tissue Integrated Prosthesis. Osseointegration in Clinical Dentistry. Chicago: Quintessence 1985:145–153.
26. Meffert RM. The soft tissue interface in dental implantology. J Dent Educ 1988;52:810–811.
27. Meffert RM, Langer B, Fritz ME. Dental implants: A review. J Periodontol 1992;63:859–870.
28. Magloire H, Bouvier M, Exbrayat P, Andujar MB, Couble ML, Joffre A, et al. Transmission electron microscopical identification of extracellular matrix components using immunocytochemistry. In: Davies JE (ed). The Bone-Biomaterial Interface. Toronto: Univ of Toronto, 1990:229–240.
29. Cannas M, Denicolai F, Webb LX, Gristina AG. Bioimplant surfaces: Binding of fibronectin and fibroblast adhesion. J Orthop 1988;6:58–62.
30. Marchisio PC, Bondanza S, Cremona O, Cancedda R, Luca MD. Polarized expression of integrin receptors (α6ß4, α2ß1, α3ß1, and αvß5) and their relationship with the cytoskeleton and basement membrane matrix in cultured human keratinocytes. J Cell Biol 1991;112:761–773.
31. Mariotti A. The extracellular matrix of the periodontium: Dynamic and interactive tissues. Periodontology 2000 1993;3:39–63.
32. Thomas K, Ronald V. Guidebook to the Extracellular Matrix and Adhesion Proteins. Oxford: Sambrook and Tooze, 1993:56–105.
33. Klaus K. Basement membrane (type IV collagen) collagen. Matrix Biol 1994;14:439–445.
34. Goldring SR, Wang JT. Molecular biological approaches to investigate cell/biomaterial interactions. In: Davies JE (ed). The Bone-biomaterial Interface. Toronto: Univ of Toronto, 1991;242–247.
35. Bowers KT, Keller JC, Randolph BA, Wick DG, Michaels CM. Optimization of surface micromorphology for enhanced osteoblast response in vitro. Int J Oral Maxillofac Implants 1992;7:302–310.
36. Dennison DK, Hürzeler MB, Quinones C, Caffesse RG, Contaminated implant surfaces: An in vitro comparison of implant surface coating and treatment modalities for decontamination. J Periodontol 1994;65:942–948.
37. Baier RE, Meyer AE. Implant surface preparation. Int J Oral Maxillofac Implants 1988;3:9–20.
38. Young FA. Future directions in surface preparation of dental implants. J Dent Educ 1988;52:770–774.
39. Chung YM, Kim HM, Hwang SM, Ko JS. Scanning electron microscopic observation on the resorbing apparatus of rabbit osteoclast in vitro. J Coll Dent Seoul National Univ 1991;15:219–232.
40. Buser D, Weber HP, Donath K, Fiorellini JP, Paquette DW, Williams RC. Soft tissue reactions to non-submerged unloaded titanium implants in beagle dogs. J Periodontol 1992;63:226–236.
41. Listgarten MA, Lai CH. Ultrastructure of the intact interface between an endosseous epoxy resin dental implant and the host tissue. J Biol Buccale 1975;3:13–28.
42. McKinney JRV, Steflik DE, Koth DL. Evidence for a junctional epithelial attachment to ceramic dental implants: A transmission electron microscopic study. J Periodontol 1985;56:579–591.
43. Carmichael RP, McCulloch CAG, Zarb GA. Quantitative Immunohistochemical analysis of keratines and desmoplakia in human gingiva and peri-implant mucosa. J Dent Res 1991;70:899–905.
44. Sinha RK, Morris F, Shah SA, Tuan RS. Surface composition of orthopedic implant metals regulates cell attachment, spreading, and cytoskeletal organization of primary human osteoblasts in vitro. Clin Orthop 1994;305:258–272.