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Adherence of Alveolar Fibroblasts and Microorganisms on Titanium Implants

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Abstract**—**An implant elicits a biological response in the surrounding tissue which determines the acceptance and long-term function of the implant. Dental implants have become one of the main therapy methods in clinic after teeth lose. A successful implant is in contact with bone and soft tissue represent by fibroblasts. In our study we focused on the interaction between six different chemically and physically modified titanium implants (Tis-MALP, Tis-O, Tis-OA, Tis-OPAAE, Tis-OZ, Tis-OPAE) with alveolar fibroblasts as well as with five type of microorganisms (*S. epidermis, S.mutans, S. gordonii, S. intermedius, C.albicans*). The analysis of microorganism adhesion was determined by CFU (colony forming unite) and biofilm formation. The presence of $\alpha_3\beta_1$ and vinculin expression on alveolar fibroblasts was demonstrated using phospho specific cell based ELISA (PACE). Alveolar fibroblasts have the highest expression of these proteins on Tis-OPAAE and Tis-OPAE. It corresponds with results from bacterial adhesion and biofilm formation and it was related to the lowest production of collagen I by alveolar fibroblasts on Tis-OPAAE titanium disc.

Keywords**—**titanium disc, alveolar fibroblasts, microorganisms, adhesion

I. Introduction

DENTAL implant has become one of the main therapy methods in clinic. A successful implant is in contact with bone and soft tissue. Early studies with oral implants were mostly focused on implant-bone interface, but rarely on implant-soft interface. Although the success rate of dental implant is high today, postoperative implant failure has often been reported. One of the reasons leading to implant failure may be marginal infection resulting from implant penetration of the oral mucosa. Some of which may be related to an absence of attachment of gingival soft tissue to the implant [1]. Studies of the interface between various non-osteogenic cell types and implant surfaces showed that fibroblasts are adjacent and in contact with the titanium surface of implants. Titanium surface coatings or textures lead to differences in implant fibroblast cell attachment but few studies have focused on the molecules required for this attachment process. Recent studies have shown the extracellular matrix molecule have a functions in fibroblast attachment to an implant surface. Periodontal ligament has been shown to express multiple integrin protein subunits including 4, 5, 6 and 1. Integrin proteins bind to many different ligands including extracellular matrix proteins, plasma proteins, and integral membrane proteins but the role of integrin molecules in periodontal ligament attachment to titanium implant materials has not been studied [2].

Many studies showed that collagen, especially type collagen 1, is very important extracellular matrix protein. Under physiological conditions, for collagen-1, a dynamic balance between its synthesis and degradation, is essentials [3]. Collagen is the most abundant extracellular protein in mammals, responsible for maintenance of architecture and integrity of connective tissue. It also plays an important role in interaction with cell surface integrin receptors, through which it may participate in regulation of numerous physiological and pathological processes. The integrin receptors are responsible for recognition and adhesion of cells to the elements of extracellular matrix as well as cell–cell interaction. The interaction is involved in cytoskeleton reorganization, intracellular ion transport, lipid metabolism, kinase activation, gene expression, cell cycle regulation and cancer metastasis [4].

Human dental plaque is a complex biofilm that is present on tooth tissues as well as on restorative material. Oral biofilm may harbour many bacteria that are involved in the development of disease conditions such as secondary caries and demineralisation process of marginal enamel and dentin. A number of studies have well documented that biofilm formation occurs on the surface of materials of different chemical nature shortly after placement in oral cavity. The formation of superficial biofilm on a dental surface is a complex phenomenon and different key factors are involved [5]. Dental diseases are among the most prevalent afflictions of humankind. These diseases are associated with the formation of biofilms harbouring pathogenic bacteria. Adhesion of oral bacteria to the tooth surface is facilitated by physical, chemical and biological mechanisms. The controlling the dental biofilm is one of the major approaches to reducing dental caries and periodontal diseases [6].

In our study we focused on the comparing the adhesive properties of alveolar fibroblasts and five type of microorganisms to different modified titanium implants.

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II. MATERIALS AND METHODS

A. Titanium disc

Six different modified titanium disc (Tis-MALP, Tis-O, Tis-OA, Tis-OPAAE, Tis-OZ, Tis-OPAE) were tested in our study (Table I). The titanium discs were sterilized 48 hrs in ethanol and after that 15 min by UV irradiation.

TABLE I
MODIFICATION OF TITANIUM DISC

Labeling	Surface description
Tis-MALP	glazed
Tis-O	unglazed
Tis-OA	unglazed and alkali etched
Tis-OPAAE	unglazed, sand blasted, acid and alkali etched
Tis-OZ	unglazed and coated with ZrN
Tis-OPAE	unglazed, sand blasted and acid etched

B. Cell isolation, seeding and culture

Human normal alveolar fibroblasts were obtained during surgical procedures (before insert dental implants) from a healthy volunteer according to the informed consent agreement of the Ethical committee of the Faculty Hospital in Olomouc. The human alveolar fibroblasts were cultivated in DMEM medium supplemented with 10 % fetal calf serum (FCS), penicillin and streptomycin at 37°C and 5% CO_2. The cells were seeded on titanium surfaces (put in cultivation plates) at the final concentration of $1x10^4$ cells/cm^2. The cells without disc were used as a control.

C. Phospho-Specific Antibody Cell Based ELISA

The cells were seeded onto different materials on 96-well culture plates. After incubation period, the cells were washed with cold PBS and fixed with 4% formaldehyde in PBS for 30 min. The cells were washed with 1% Triton X-100 in PBS (PBS-T) and the endogenous peroxidase activity was quenched using 0.3% H_2O_2 in PBS-T for 30 min. Cells were three times washed with PBS-T, followed by blocking with 10% fetal calf serum (FCS) in PBS-T for 1 h. Primary mouse antibody against vinculin or $\alpha_3\beta_1$ integrin were added in 5% FCS-PBS-T and incubated over night at 4°C with rocking. After primary antibody incubation, cells were washed three times with PBS-T and incubated with secondary goat antibody IgG (H+L), HRP linked antibody with a dilution 1:3000 in PBS-T. The cells were washed three times and then ultra sensitive TMB was added and incubated 20 min in dark. The oxidation reaction was stopped using equal volume of 2M H_2SO_4 and coloured products were transfer to a new 96 well plates with standards protein of vinculin and $\alpha_3\beta_1$ and absorbance was measured at 450 nm with reference at 655 nm (Sunrise Remote, Tecan, Austria).

D. Collagen production

The production of type collagen I was assayed using an indirect ELISA. Briefly, the supernatant of media was collected after 24 hours. The supernatant was incubated on special 96 well plates over night at 4°C with rocking. After incubation period plate was three times washed with wash buffer, followed by blocking with 1% BSA in washing buffer for 2 h. Primary mouse antibody against collagen I was added in 1% BSA washing buffer and incubated over night at 4°C with rocking. After primary antibody incubation, cells were washed three times with wash buffer and incubated with secondary goat antibody IgG (H+L), HRP linked antibody with a dilution 1:3000 in washing buffer. The cells were washed three times and then ultra sensitive TMB was added and incubated 20 min in dark. The oxidation reaction was stopped using equal volume of 2M H_2SO_4 and coloured products were transfer to a new 96 well plates with standards protein of collagen I and absorbance was measured at 450 nm with reference at 655 nm (Sunrise Remote, Tecan, Austria).

E. Used strains

Staphylococcus epidermidis CCM 7221 (Czech Collection of Microorganisms, CCM),
Streptococcus mutans CCM 7409 (Czech Collection of Microorganisms, CCM),
Streptococcus gordonii CCM 4045 (Czech Collection of Microorganisms, CCM),
Streptococcus intermedius CCM 4044 (Czech Collection of Microorganisms, CCM),
C. albicans GDH 2346, the strain was kindly provided by L. J. Douglas, University of Glasgow, Scotland.

The strains were stored in cryoprotective medium glycerol-serum broth at -76°C. Before each experiment, the strains were thawed quickly at 37 °C and cultivated on blood agar (Bio-Rad, Marnes La Coquette, France) at 37 °C overnight (18 hrs). *C. albicans* was cultivated on Sabouraud dextrose agar (Hi-Media, Mumbai, India) for 48 hrs.

F. The biofilm formation on tested discs

The microbial cultures were resuspended in Physiological Saline Solution (PSS) (pH 7.2) to the optical density 1 of the McFarland scale. Wells of the 24-well flat-bottomed polystyrene microtiter plates containing tested discs and 900 µl of Brain Heart Infusion (Hi-Media, Mumbai, India) with 4 % glucose were inoculated with 100 µl of microbial suspension. The negative control wells were inoculated only with 100 µl PSS. After 24 hrs of incubation at 37 °C, the discs were washed three times with 10 ml PSS. The biofilm layer on the surface of each disc was fixed by air-drying and subsequently stained with 1% crystal violet (1 ml) for 20 min. For the spectrophotometric assessment the bound dye was eluted with 1 ml of 33% glacial acetic acid per well. After 30 min 100 µl of destaining solution was transferred to a new well and the optical density was measured at 595 nm using Anthos Labtec Instruments 2001 reader (Salzburg, Austria).

G. Adhesion of the microorganisms on the surface of tested discs

The microbial cultures were resuspended in PSS (pH 7,2) to the optical density 1 of the McFarland scale. The tested disks were submerged in the wells of the 24-well flat-bottomed polystyrene microtiter plates containing 1 ml of microbial suspension. The negative control wells contained only 1 ml PSS. After 1 h of incubation at 22 °C, the discs were transferred into wells of the 6-well microtiter plate and washed with 5 ml PSS. The plate was stirred with an orbital shaker at 500 rpm. Washed disks were subsequently transferred into test tubes containing 1 ml of PSS, sonicated for 1 min (Powersonic PS3000A, Vrable, Slovakia) and vortexed. The mechanical effect of the ultrasound and vortexing disrupted the biofilm and released the microbial cells from the biofilm layer. Obtained bacterial suspension was serially diluted ($1:10^1$ to $1:10^9$) by transferring of 100 µl of the suspension into 0.9 ml of sterile PSS. 100 µl of all dilutions was inoculated on Muller-Hinton's agar (Bio-Rad, Marnes La Coquette, France) and incubated for 24–48 hrs at 37 °C. A number of colony forming units (CFU) surviving on discs was counted.

H. Statistical analysis

All experiments were repeated at least three times, performed in duplicates. The cell culture experiments were analyzed by t-test. The data were expressed as mean ±SD, significant differences were note at a P-value of <0.05.The differences in bacterial adhesion and biofilm formation on the tested discs were analysed ANOVA in statistic program Statistica CZ, version 8.0 (StatSoft, Inc., Tulsa, USA).

III. Results

A. Expression of vinculin and $\alpha_3\beta_1$ integrin

Titanium implants affected the integrin and vinculin expression in alveolar fibroblasts only in some titanium disc. Vinculin expression was increased on Tis-OPAE and Tis-OPAAE (Fig. 1). Level of integrin $\alpha_3\beta_1$ expression was significant higher on Tis-OPAAE and Tis-OPAE on titanium disc after 24 hrs (Fig. 2).

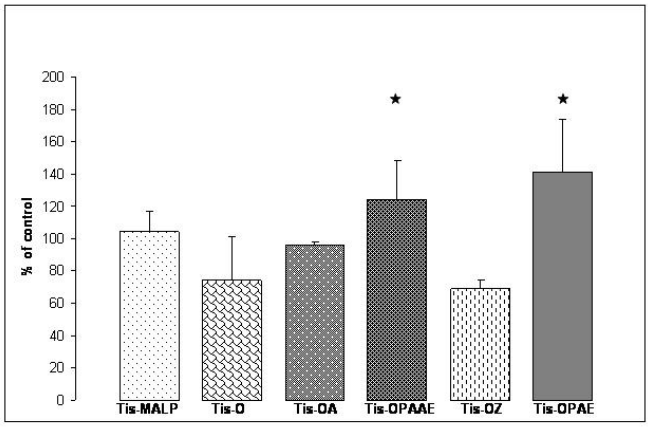

Fig. 1 Expression of vinculin after 24 hrs

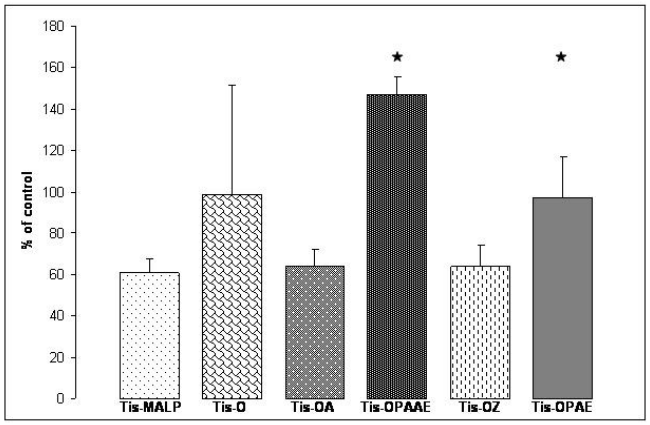

Fig. 2 Expression of $\alpha_3\beta_1$ integrin after 24 hrs

B. Biofilm production

Similar pattern to bacterial adhesion was also demonstrated by testing the biofilm production. The production was significantly increased on Tis-OPAAE and Tis-OPA titanium disc (Table II).

C. Microorganisms adhesion

Significant differences in the bacterial adhesion to the six different titanium discs were evident (P>0.001), with the highest adhesion to Tis-OPAAE and Tis-OA for all tested strains (Table III).

D. Collagen I production

Collagen I synthesis was measured by indirect ELISA after 24 hrs in the culture medium. The collagen I production was increased after 24 hrs on Tis-OZ disc compare with control (Fig. 3).

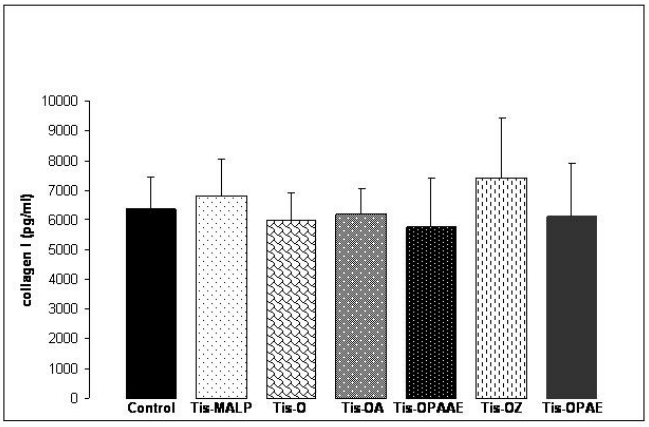

Fig. 3 Collagen I production after 24 hrs by alveolar fibroblasts

TABLE II
BIOFILM PRODUCTION BY TESTED MICROORGANISMS ON TITANIUM DISC

Titanium disc	Absorbance (595 nm) ± SD				
	S.epidermis	*S. mutans*	*S.gordonii*	*S.intermedius*	*C.albicans*
Tis-OPAAE	3.575 ± 0.174	1.918 ± 0.231	2.979 ± 0.068	0.843 ± 0.331	2.771 ± 0.368
Tis-OA	3.384 ± 0.095	1.761 ± 0.065	2.976 ± 0.134	0.683 ± 0.421	2.912 ± 0.434
Tis-O	2.876 ± 0.255	0.742 ± 0.273	2.478 ± 0.143	0.341 ± 0.162	2.526 ± 0.341
Tis-OPAE	3.494 ± 0.106	1.293 ± 0.336	2.575 ± 0.142	0.397 ± 0.072	2.751 ± 0.404
Tis-MALP	3.022 ± 0.102	0.37 ± 0.004	2.353 ± 0.087	0.264 ± 0.012	2.477 ± 0.658
Tis-OZ	3.544 ± 0.071	0.278 ± 0.029	2.258 ± 0.118	0.234 ± 0.019	2.722 ± 0.362

TABLE III
MICROORGANISMS ADHESION ON TITANIUM DISC

Titanium disc	Amount of CFU (colony forming units) on titanium disc ± SD				
	S.epidermis	*S. mutans*	*S.gordonii*	*S.intermedius*	*C.albicans*
Tis-OPAAE	$4.62 \times 10^7 \pm 1.59 \times 10^7$	$4.27 \times 10^5 \pm 5.51 \times 10^4$	$1.27 \times 10^6 \pm 1.24 \times 10^5$	$7.11 \times 10^4 \pm 1.01 \times 10^4$	$2.12 \times 10^5 \pm 3.15 \times 10^4$
Tis-OA	$2.19 \times 10^7 \pm 4.35 \times 10^7$	$2.14 \times 10^5 \pm 5.73 \times 10^4$	$6.70 \times 10^5 \pm 6.28 \times 10^4$	$1.25 \times 10^4 \pm 8.27 \times 10^3$	$3.45 \times 10^5 \pm 4.33 \times 10^4$
Tis-O	$7.14 \times 10^4 \pm 1.23 \times 10^4$	$1.26 \times 10^3 \pm 4.24 \times 10^2$	$2.36 \times 10^5 \pm 1.24 \times 10^4$	$6.38 \times 10^2 \pm 9.31 \times 10^1$	$9.45 \times 10^4 \pm 1.44 \times 10^4$
Tis-OPAE	$3.76 \times 10^4 \pm 8.06 \times 10^3$	$2.28 \times 10^3 \pm 8.16 \times 10^2$	$2.95 \times 10^4 \pm 6.41 \times 10^3$	$1.03 \times 10^3 \pm 2.16 \times 10^2$	$2.36 \times 10^4 \pm 1.21 \times 10^3$
Tis-MALP	$1.35 \times 10^5 \pm 4.72 \times 10^4$	$1.02 \times 10^3 \pm 1.23 \times 10^2$	$3.95 \times 10^5 \pm 8.44 \times 10^4$	$7.79 \times 10^2 \pm 3.43 \times 10^1$	$6.71 \times 10^4 \pm 1.03 \times 10^4$
Tis-OZ	$9.72 \times 10^4 \pm 2.32 \times 10^3$	$7.51 \times 10^2 \pm 4.33 \times 10^1$	$1.11 \times 10^6 \pm 1.18 \times 10^5$	$1.42 \times 10^2 \pm 8.22 \times 10^1$	$1.33 \times 10^3 \pm 1.22 \times 10^3$

IV. Conclusion

The highest adhesion of alveolar fibroblasts as well as microorganisms was on Tis-OPAAE titanium disc.

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