CELLULAR ATTACHMENT AND GENE EXPRESSION OF OSTEOBLAST-LIKE CELLS ON ZIRCONIA CERAMIC SURFACES

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INTRODUCTION

The material of choice for dental implants is commercially pure titanium because of its high biocompatibility and suitability of tooling. This biocompatible material[1] enables direct bone anchorage called osseointegration.[2] In an attempt to achieve stable bonding between the titanium implants and bone, the surfaces of titanium dental implants have been modified by additive methods (titanium plasma spray) or by subtractive methods (acid etching, sandblasting) to increase the surface area and promote cell attachment .[3,4]

Healing around implants can be influenced by (1) physicochemical properties of the implant material, (2) mechanical properties of the implant, (3) surface topography of the material ; macrotopography and microtopography, (4) overall shape and design of the implant.[5] The surface topography of implant material can influence adherence, attachment, spreading of cells and modify and control the osseointegration process. Recent studies on the effect of various surface topographies on cell adhesion and proliferation have already been reported or are in progress.[6-9]

One drawback of the titanium as implants material from an esthetic point of view is that the dark color of titanium can shine through the thin bone and mucosa.[10] Also, soft tissue shrinkage, gingival recession, and peri-implant lesions may leave the implant fixture top or titanium abutment visible. One possible solution to these problems with titanium would be to make implants and transgingival abutments from tooth-colored materials such as zirconia.

Zirconia (ZrO_2) has adequate mechanical properties for use in medical and dental purposes. Its mechanical properties are similar to those of stainless steel and its ivory color which is similar to that of natural teeth, makes it useful in the esthetically important area of the oral cavity.[11] Its ability to transmit light renders it a suitable material in esthetic restorations. Zirconia exists in three phases (monoclinic, tetragonal and cubic) depending on the temperature. By mixing ZrO_2 with other metallic oxides, such as MgO, CaO, or Y_2O_3, greater molecular stability can be obtained.[11] Yttrium-stabilized zirconia, also known as

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tetragonal zirconia polycrystal (TZP), is the combination with the best mechanical properties and is presently commercially available. Every transition between the different crystalline phases is due to stress on the zirconia surface, and this produces a volumetric change in the crystal where the compressive force is applied. When stress occurs on zirconia surface, cracking energy creates T-M (tetragonal-monoclinic) transition. This crystalline modification is followed by a 4 % volumetric expansion that seals the crack.[12,13]

Biomaterial properties of zirconia compared to titanium proved to have more advantages. Bacterial adhesion, which is an important aspect in order to maintain zirconia restorations without periodontal alterations, proved to be satisfactorily slight.[14,15] Scarano *et al.* reported a degree of coverage by bacteria of 12.1 % on zirconia as compared to 19.3 % on titanium.[16] Rimondini *et al.* confirmed these results with an *in vivo* study, in which Y-TZP accumulated fewer bacteria than titanium in terms of the total number of bacteria and presence of potential putative pathogens as rods.[17] Inflammatory infiltrate, microvessel density, and vascular endothelial growth factor expression were found to be higher around the titanium caps than around the ZrO_2 ones.[18] Zirconium oxide is also able to modulate immunity and cell cycle regulation.[19] Additionally, allergic reactions and sensitivities to titanium have been reported.[20,21]

Zirconia is a biocompatible material and has the highest mechanical properties among oxide ceramics. Its biocompatibility as dental implant material has been demonstrated in several animal investigations.[22-27] Also, biological response of osteoblast-like cells between zirconia/alumina ceramic and pure titanium has been proved to be comparable.[28]

In this study, we discuss zirconia surfaces provided with micrometer-sized grooves. Such microgrooves influence cell behavior: the cells align themselves, and migrate guided by the surface grooves. This phenomenon is known as “contact guidance”.[29] The microgrooves create a pattern of mechanical stress, which influences cell spreading and causes cell alignment. Matsuzaka *et al.* confirmed that the ‘contact guidance’ behavior of cells on microgrooved surfaces, i.e. on smooth surfaces cell position is at random, whereas on any type of grooves cells will align themselves towards the groove direction.[30]

Anselme reported that cellular proliferation decreased as surface roughness increased,[31] while Mustafa and colleagues demonstrated that proliferation and differentiation were enhanced by surface roughness.[32,33] Surface of material not only regulates bone growth but also osteoblast differentiation by modulating the expression of key osteoblast genes in osteogenesis. These studies indicate that zirconium oxide can be suitable for implant materials.

This study was performed to define attachment and growth behavior of osteoblast-like cells cultured on grooved surfaces of zirconium oxide by MTT assay and SEM and evaluate the genetic effect of grooves on zirconium oxide surfaces using the RT-PCR.

MATERIAL AND METHODS

1. Specimen preparation

The commercially pure titanium (c.p. Ti) disks (Osstem, Pusan, Korea) used for the cell culture were machined from grade 2 commercially pure Ti (Dynamet, Inc. Carpenter Co., Washington, PA, USA). The disks were prepared to be 12 mm in diameter and 4 mm thick and used as the culture substrate in the control group (T group). Zirconia disks (LAVA™, 3M ESPE, St. Paul, MN, USA) of Y-TZP (yttria-stabilized tetragonal zirconia polycrystal) were prepared to be 12 mm in diameter and 4 mm thick also. Two types of disc surfaces were prepared of the zirconia disks. One was Y-TZP with smooth surface (ZS group) and the other was Y-TZP with 100 μm grooves (ZG group). Disc samples were rinsed twice in absolute alcohol and once in demineralized water in ultrasonic, before sterilization by autoclave for testing using MC3T3-E1 cells.

2. Cell culture

MC3T3-E1 cells are osteoblast-like cells from rat calvaria. MC3T3-E1 cells were cultured at 37℃ in a CO_2 incubator (5 %). Cells were cultured using alpha-Eagle’s minimal essential medium (alpha-MEM, Gibco, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS) and antibiotics.

3. MTT assay

The proliferation of cells was examined with MTT test assay (Sigma, St. Louis, MO., USA) after culturing the cells on the titanium and zirconia disks. The substance used for MTT test was a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium salt, which turns into blue formazan product due to the viable mitochondria in active cells. E1 cells were seeded at a density of 1×10^5 cells/ml. Cells were incubated at 37° C in 5% CO_2 for 24 hrs and 48 hrs. The disks were moved to well plates after 24 hours and 48 hours incubation and new media were added after which the media were removed and added by diluted MTT (5 mg/ml) solution and incubation was continued at 37° C in 5% CO_2 for 4 hours. The incubation medium was then removed and 400 μl of isopropanol with 0.04N HCl was added in each well and the resulting formazan crystals were dissolved. The absorbance of produced formazan at 490 nm was measured on a microplate reader (Bio-kinetics reader, EL312e, Winooski, VT, USA). Experiments were repeated independently in triplicate.

4. Scanning electron microscopy

Surfaces were analyzed using scanning electron microscopy (SEM) to determine the cellular attachment and morphology. E1 cells were seeded at a density of 1×10^5 cells/ml. The cultured cells were incubated for 4 h and 24 h at 37℃ in 5 % CO_2. The loosely adherent or unbound cells from the experiment wells were removed by aspiration, the wells were washed twice with a 0.1 M Phosphate Buffered Saline (PBS) buffer (pH 7.4), and the remaining bound cells were fixed with 4 % glutaraldehyde for more than 6 hours. The excess glutaraldehyde solution was removed and cells rinsed once more in PBS before being dehydrated progressively in higher concentration of ethanol baths (50, 60, 70, 80, 90, 95, and 100 %, 10 min in each bath). After critical point drying, the samples were sputtered with gold at the thickness of 100 nm using Ion coater (Eiko IB-type 3). Cells on the discs were observed by FE-SEM (Japan, Hitachi S-800). Images were recorded at 300× and 1000× magnification.

5. RT-PCR (reverse transcriptase-polymerase chain reaction) analysis

The osteoblastic differentiation of E1 cells was evaluated by RT-PCR examination of Runx2, alkaline phosphatase, osteocalcin, IGF-1, TGF beta, and G3PDH. The cells were seeded at a density of 1×10^5 cells/ml. Cells were incubated for 24 hours. Total RNA extraction was performed with RNeasy mini kit (Qiagen, Chatsworth, CA, USA). The extracted total RNA samples were converted to cDNA. The amplifications were performed using AmpliTaq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR products were fractionated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The intensity of the bands was quantified under UV transillumination (Eagle eye II, Stratgene, La Jolla, CA, USA). The sequences of the specific primers

Table I. Gene-specific primer sequences used in RT-PCR of MC3T3-E1 cells

	Primer	Molecular weight
Runx2	5'-GTATGAGAGTAGGTGTCCCG-3' 5'-ACATCCCCATCCATCCACTC-3'	183bp
Alkaline phosphatase	5'-GATCGGGACTGGTACTCGGATAA-3' 5'-CACATCAGTTCTGTTCTTCGGGTAC-3'	155bp
TGF-beta 1	5'-CTCTCCACCTGCAAGACCAT-3' 5'-CTGCWTACAACTWAGTGA-3'	679bp
Osteocalcin	5'-GGGGAAGGGACAACACATGA-3' 5'-TCCTGGACATGGGGATTGAC-3'	412bp
IGF-I	5'-GCAAGCTTCAGCCACCTTAC-3' 5'-GGGTCGTTTACACAWAGGT-3'	511bp
G3PDH	5'-GATTTGGCCGTATCGGACGC-3' 5'-CTCCTTGGAGGCCATGTAGG-3'	977bp

used are listed on Table I.

6. Statistical analysis

Test mean values and standard deviations (SD) were computed for MTT test and the analysis of variance (ANOVA) was used to assess the significance level of the differences between the experimental groups. All statistical analyses were performed using SPSS software (Version 12.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P< 0.05$.

RESULT

1. Cellular proliferation

The density of MC3T3-E1 cells were measured over 2 different time-periods; 24 hours and 48 hours by using the MTT assay. Figure 1 shows the MTT assay results of MC3T3-E1 cells. After 24 hours of adhesion, the osteoblast-like cell density on titanium surfaces and zirconia surfaces showed no significant difference. Grooves of zirconia had no significant effect on the proliferation of osteoblast-like cells. But after 48 hours of adhesion of MC3T3-E1 cells, the optical density of smooth zirconia did not increase significantly, but the optical density of titanium group and grooved zirconia significantly increased ($P<0.05$). Therefore, it is suggested that the microgrooves of the titanium disks proved to be as effective as grooves of the zirconia on the proliferation rate and both groups increased compared to the smooth zirconia group after 48 hours of cell incubation. Overall, the osteoblast-like cells seeded onto titanium and zirconia showed similar vitality and proliferation rate.

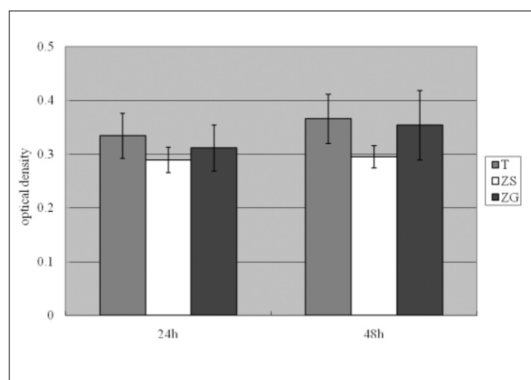

Fig. 1. Evaluation of cellular viability by using MTT assay during 24 hours and 48 hours of MC3TC-E1 cells on titanium group, smooth zirconia group and grooved zirconia group.

2. Cellular attachment and morphology

The general shape and growth pattern of the osteoblast-like cells were observed using scanning electron microscopy for each group. Figure 2, 3, 4 and 5 show representative scanning electron micrographs of MC3T3-E1 cells cultured for 4 hours and 24 hours on the T group, ZS group, and ZG group. Orientation of osteoblasts of T group and ZG group were observed to be parallel to the direction of the microgrooves, whereas the cells in ZS group were observed to be oriented in random directions. Majority of the cells were found inside the microgrooves with increased formation of filopodia.

On machined titanium, SEM images show that the cells were irregularly triangular or elongated in shape. They were primarily oriented along the grooves and appeared flattened, with some long protoplasmic processes that were well attached to the substrate. However, cells cultured on zirconia disks showed higher initial adhesion properties compared to the titanium discs in the first 4 hours. After 24 hours of cell culture, osteoblast-like cells both showed more increased formation of filopodia and the cells showed more contact with each other and firm adhesion to the surface of the specimen.

3. Cellular differentiation

After incubation of E1 cells for 24 hours, the mRNA expression of alkaline phosphatase, osteocalcin, IGF-1, TGF-β, Runx2, and G3PDH on the titanium and grooved zirconia group showed similar activity (Fig. 6). Factors related to the quality of calcification; alkaline phophatase, osteocalcin, IGF-1, and TGF-β increased only very slightly on the smooth zirconia group compared to titanium and grooved zirconia group. Overall, the gene expression analysis of E1 cells cultured showed no significant difference between the three groups.

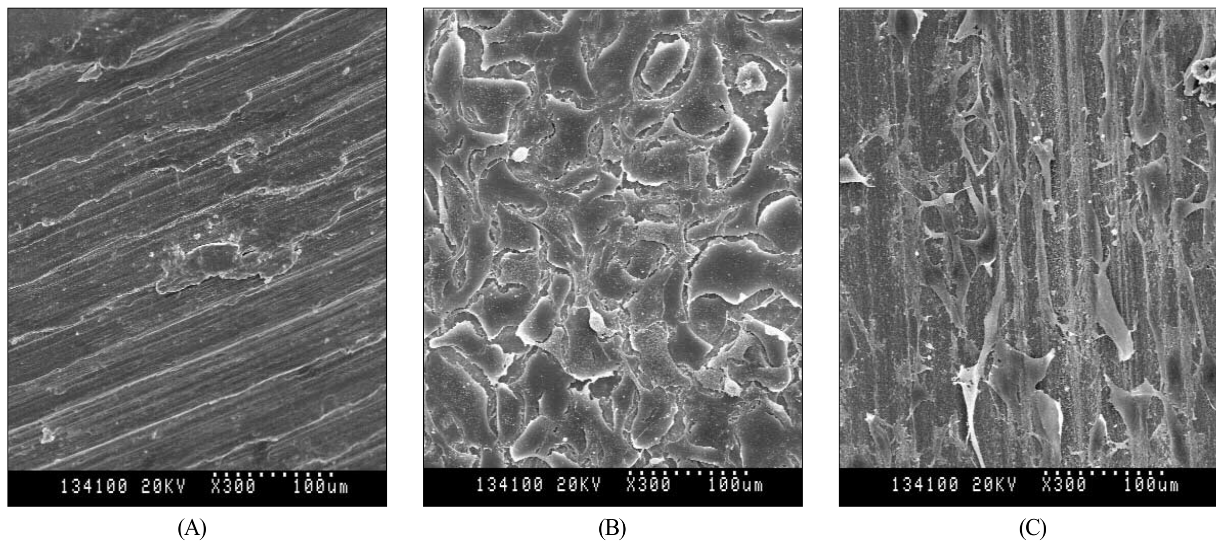

Fig. 2. SEM images of cultured osteoblast-like cells after 4 hours (×300).
(A) titanium group, (B) smooth zirconia group, (C) grooved zirconia group
Osteoblasts of T group and ZG group were observed to be oriented parallel to the direction of the grooves.

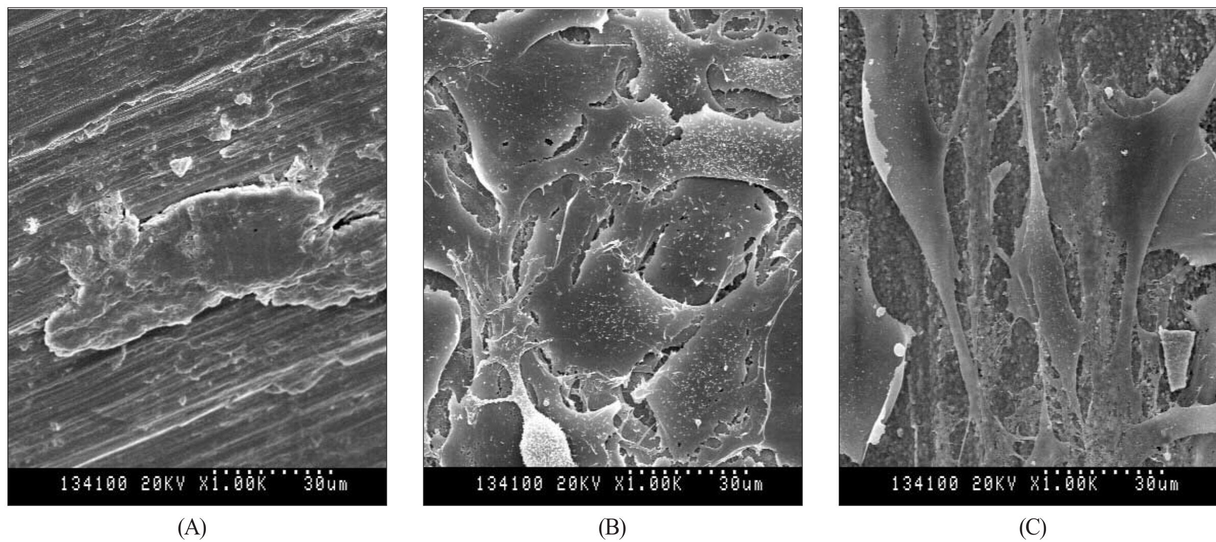

Fig. 3. SEM images of cultured osteoblast-like cells after 4 hours (×1000).
(A) titanium group, (B) smooth zirconia group, (C) grooved zirconia group
Osteoblasts of T group and ZG group were found inside the microgrooves with increased formation of filopodia.

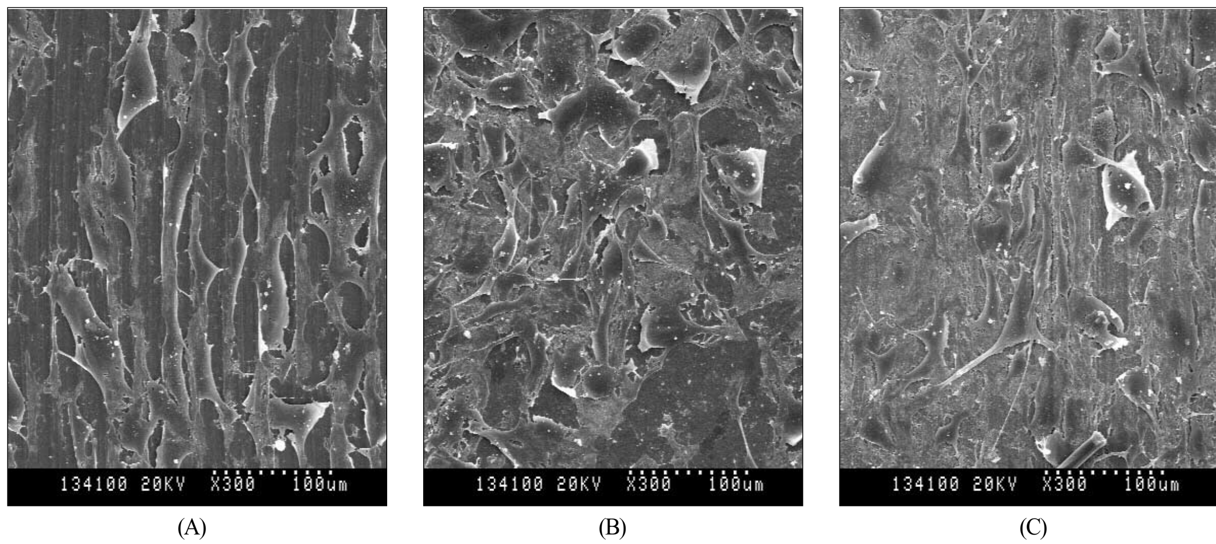

Fig. 4. SEM images of cultured osteoblast-like cells after 24 hours (×300).
(A) titanium group, (B) smooth zirconia group, (C) grooved zirconia group
Osteoblasts of T group and ZG group were observed to be oriented parallel to the direction of the grooves.

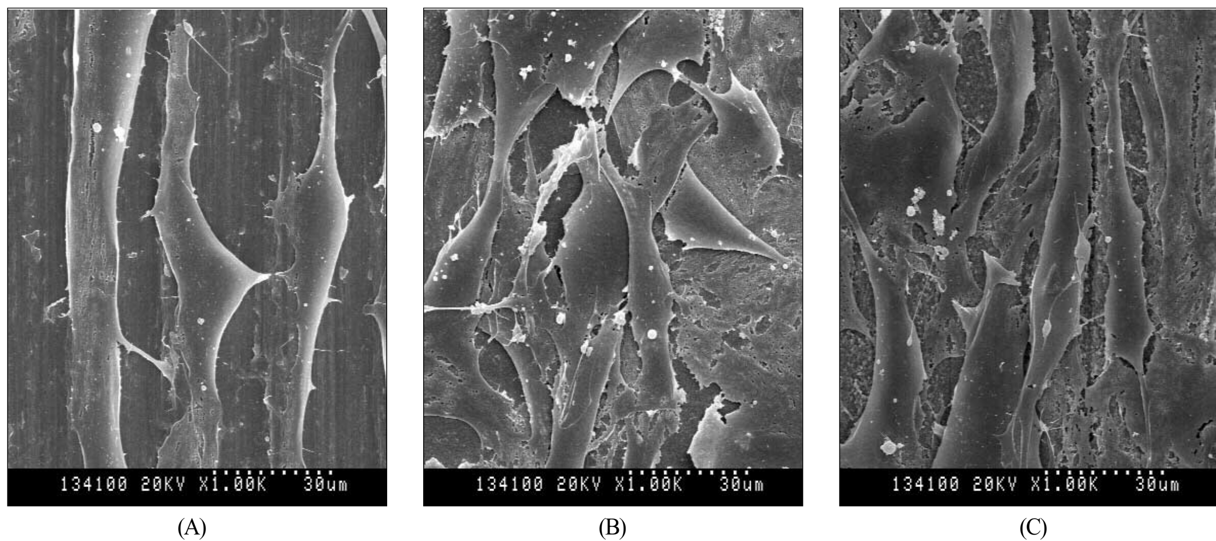

Fig. 5. SEM images of cultured osteoblast-like cells after 24 hours (×1000).
(A) titanium group, (B) smooth zirconia group, (C) grooved zirconia group
Osteoblasts after 24 hours of culture showed more contact with each other and appeared more flattened.

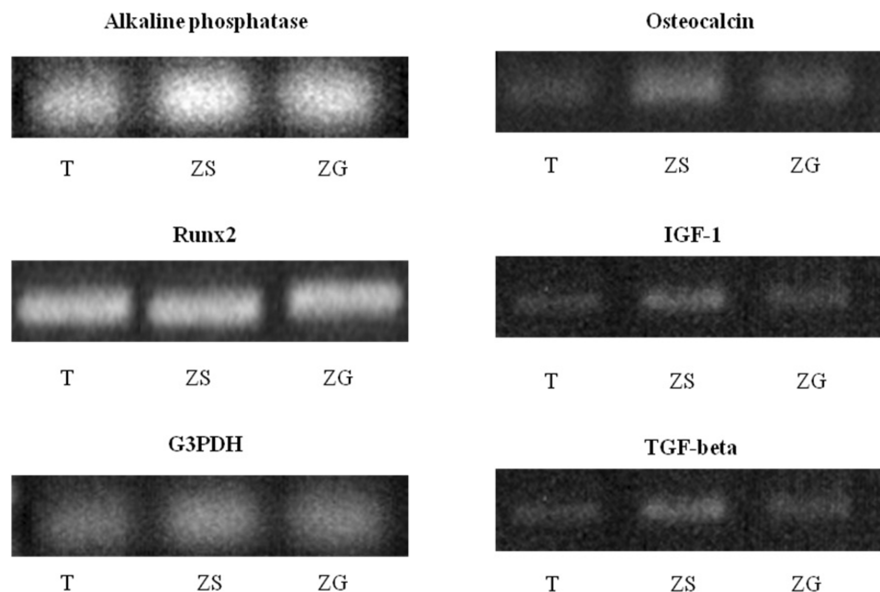

Fig. 6. Expression of alkaline phophatase, osteocalcin, Runx2, IGF-1, G3PDH, TGF-β.

DISCUSSION

Alteration in surface morphology can be used to influence cell and tissue responses to implants. Surface morphology and biomaterial affects the osteoblastic-specific gene expression. Also, these surface characteristics determine how biological molecules will adsorb on the surface. Cell-material interaction occurs in two phases, the first phase involves the attachment, adhesion and spreading of the cells and it is the quality of this phase that influences the second phase, the capacity of the cell to proliferate and differentiate itself on contact with the implant.[31]

Smooth surfaces are considered to not favor cell adhesion, whereas micromachined surfaces inhibit epithelial downgrowth. Other investigations report that smooth surfaces favor human oral fibroblast attachment and soft tissue growth,[34-36] whereas rough surfaces favor osteoblast attachment and ingrowth of bone.[2,37,38] From the results of this study, we can suggest that grooves can favor the proliferation of osteoblasts compared to the smooth surface.

In this study, we examined the difference in cellular attachment and proliferation between titanium and zirconia. From the results of SEM, after 4 hours of cell culture, we could observe that titanium group showed the lowest cell adhesion. The machining of Ti6Al4V alloys has previously been shown to induce the formation of a concentration of aluminum oxides on the outermost surface.[39] This phenomenon can be explained by the concentrations of Al on the machined implant surfaces since they constitute a potential risk of Al dissolution in the biological fluids surrounding alloyed Ti surgical implants. In this study, the Al dissolution from the surface may also explain the lower cell adhesion at 4 hours of cell culture.

Cell proliferation was comparable between the two materials and grooves proved to have similar cell proliferation effect as smooth surfaced material after a short-term cell culture period. However, osteoblast-like cells of the grooved zirconia group showed to be more flattened and spread evenly over the disks. Cells in a rounded configuration divide at a lower rate than those flattened and well spread on a substratum. Consequently, cells which attach to materials but spread little will show lower proliferative rates than those materials which allow greater spreading.[40] Cell morphology, as well as cell numbers, also affects the degree of cell attachment. Aligned cells are said to demonstrate more favorable adhesion behavior than a

spherically shaped cell.[41] In consequence, surface topography, such as grooves as in this study, may influence the cell spread and growth especially in the early phase of cellular proliferation.

Runx2 is known to be factors for cell differentiation, alkaline phosphatase is an enzyme related to calcification. Osteocalcin is a protein which absorbs calcium on bone surfaces and is specifically synthesized by differentiated osteoblasts. IGF-1 is known to have stimulatory effect on osteoblast proliferation. TGF-β is a well-known bone growth factor and G3PDH is a transcription factor which regulates RNA formation.[42]

Osteoblast differentiation generally implies alkaline phosphatase activity (ALP) and specific protein expression like osteocalcin, osteopontin, type I collagen and *in vitro* mineralization capacity. *In vitro* mechanical stimulation has shown various effects on ALP activity of cells.[31] The results show that no significant difference in the expression levels of Runx2, G3PDH was observed between the titanium group and zirconia group. In addition, grooves of zirconia surface have no effect on the mRNA expression of the osteoblast-like cells or HGFs. However, factors related to the quality of calcification; alkaline phophatase, osteocalcin, IGF-1, and TGF-β increased very slightly on the smooth zirconia group compared to titanium and grooved zirconia group. This suggests that zirconia might have effects on enhancement of mineralization capacity of osteoblastic cells after a long-term cell incubation.

Recent studies of surface roughness have focused on cell attachment of titanium surfaces and showed better attachment on rough surfaces compared to smooth surfaces.[43] On the contrary, it was found that on titanium disks with various degrees of roughness, proliferation and alkaline phosphatase activity was reduced when roughness increased.[31] In an experiment with the beagle dog, the different surface characteristics of abutment made of c.p. titanium; rough or smooth surface failed to influence soft tissue reactions.[44]

To further improve the esthetic aspect for dental implants, efforts are undertaken to develop systems with tooth-colored implants and tooth-colored abutments that are biocompatible and able to withstand masticatory forces. Zirconia implants are in clinical experiment and available on the market because of the demand for more esthetic results.[45] Sollazzo *et al.* reported a study in which implants treated with zirconium oxide coating showed significantly higher bone-implant contact percentage than in untreated titanium.[46] According to a finite element analysis[47] and animal experiments,[23,24] zirconia implants seem to be able to withstand occlusal forces for a long period. Zirconia implants with rough surface can achieve higher stability in bone than zirconia implants with machined surface. Roughening the turned zirconia implants enhances bone apposition and has a beneficial effect on the removal torque values.[48] However, in a recent study of Y-TZP with different surface topographies, cell attachment and cell proliferation proved to be independent of the surface treatments and even machined Y-TZP disks showed to be rough enough to enable the cells to fix onto the biomaterial.[9]

The results of this study show that the overall cell response to c.p. titanium and zirconia material was comparable. Further investigation is needed to identify the influence of depth and thickness of grooves on the zirconia surface. In general, zirconium oxide can be suitable for implant materials, but more clinical and mechanical trials are necessary for complete understanding of behavior of zirconia as implant materials throughout a long-time period.

CONCLUSION

The present *in vitro* study showed that surface topography and material of implant abutments can play an important role in expression of osteoblast phenotype markers. We evaluated the initial osteoblast-like cell response to titanium and zirconia ceramic material.

1. Zirconia ceramic showed comparable biological responses of osteoblast-like cells with titanium during a short-time cell culture period.
2. Grooves of implant material can be more effective on the cellular proliferation of osteoblast-like cells compared to the smooth surface after 48 hours of cell incubation.
3. Machined titanium surface, smooth zirconia ceramic, and grooved zirconia ceramic showed comparable osteoblast-specific gene expression However, expression of factors related to the quality of calcification; alkaline phophatase, osteocalcin, IGF-1, and TGF-β of the E1 cells increased only very slightly

on the smooth zirconia group compared to titanium and grooved zirconia group.

4. Grooves influence cell spreading and guide the cells to be aligned parallel within surface grooves.

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CELLULAR ATTACHMENT AND GENE EXPRESSION OF OSTEOBLAST-LIKE CELLS ON ZIRCONIA CERAMIC SURFACES

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STATEMENT OF PROBLEM: Zirconium oxide can be a substitute to titanium as implant materials to solve the esthetic problems of dark color in the gingival portion of implant restorations. **PURPOSE:** This study was performed to define attachment and growth behavior of osteoblast-like cells cultured on grooved surfaces of zirconium oxide and evaluate the genetic effect of zirconium oxide surfaces using the reverse transcriptase-polymerase chain reaction (RT-PCR). **MATERIAL AND METHODS:** MC3T3-E1 cells were cultured on (1) commercially pure titanium discs with smooth surface (T group), (2) yttrium-stabilized tetragonal zirconia polycrystal (Y-TZP) with machined surface (ZS group), and (3) Y-TZP with 100μm grooves (ZG group). Cell proliferation activity was evaluated through MTT assay and cell morphology was examined by SEM. The mRNA expression of Runx2, alkaline phosphatase, osteocalcin, TGF-β 1, IGF-1, G3PDH in E1 cells were evaluated by RT-PCR. **RESULTS:** From the MTT assay, after 48 hours of adhesion of MC3T3-E1 cells, the mean optical density value of T group and ZG group significantly increased compared to the ZS group. SEM images of osteoblast-like cells showed that significantly more cells were observed to attach to the grooves and appeared to follow the direction of the grooves. After 24 hours of cell adhesion, more spreading and flattening of cells with active filopodia formation occurred. Results of RT-PCR suggest that T group, ZS group, and ZG group showed comparable osteoblast-specific gene expression after 24 hours of cell incubation. **CONCLUSION:** Surface topography and material of implants can play an important role in expression of osteoblast phenotype markers. Zirconia ceramic showed comparable biological responses of osteoblast-like cells with titanium during a short-time cell culture period. Also, grooves influence cell spreading and guide the cells to be aligned within surface grooves.

KEY WORDS: Zirconia ceramic, MC3T3-E1 cells, Contact guidance, Cell proliferation, Gene expression

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