SURFACE CHARACTERISTICS OF ANODIC OXIDIZED TITANIUM ACCORDING TO THE PORE SIZE

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Statement of problem. The success of osseointegration can be enhanced with an implant that has improved surface characteristics. Anodic oxidation is one of the surface modifying method to achieve osseointegration. Voltage of anodic oxidation can change surface characteristics and cell activity.

Purpose. This study was performed to evaluate MG63 cell responses such as affinity, proliferation and to compare surface characteristics of anodic oxidized titanium in various voltage.

Material and method. The disks for cell culture were fabricated from grade 3 commercially pure titanium, 1 mm in thickness and 12 mm in diameter. Surfaces of 4 different roughness were prepared. Group 1 had a machined surface, used as control. Group 2 was anodized under 220 V, group 3 was anodized under 300 V and group 4 was anodized under 320 V.

The microtopography of specimens was observed by scanning electron microscope (JSM-840A, JEOL, Japan) and atomic force microscope(Autoprobe CP, Park Scientific Instrument, USA). The surface roughness was measured by confocal laser scanning microscope(Pascal, LSM5, Zeiss, Germany). The crystal structure of the titanium surface was analyzed with x-ray diffractometer(D8 advanced, Bruker, Germany). MG63 osteoblast-like cells were cultured on these specimens. The cell morpholgy was observed by field emission electron microscope(Hitachi S-4700, Japan). The cell metabolic and proliferative activity was evaluated by MTT assay.

Results and conclusion. With in limitations of this in vitro study, the following conclusions were drawn.

1. In anodizing titanium surface, we could see pores which did not show in contol group. In higher anodizing voltage, pore size was increased.
2. In anodizing titanium surface, we could see anatase. In higher anodizing voltage, thicker oxide layer increased crystallinity(anatase, anatase and rutile mixed).
3. MG63 cells showed more irregular, polarized and polygonal shape and developed more lamellipodi in anodizing group as voltage increased.
4. The activity of cells in MTT assay increased significantly in group 3 and 4 in comparison with group 1 and 2. However, there was no difference between group 3 and 4 at $P<0.05$. Proliferation of MG63 cells increased significantly in pore size(3-5.5 ㎛) of group 3 and 4 in comparison with in pore size(0.2-1 ㎛) of group 2.

Key Words
Anodic oxidation, pore size, anatase, MG63 cell, MTT assay

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Cellular behaviors such as adhesion, morphologic change, functional alteration, and proliferation are greatly affected by surface properties, including hydrophilicity, roughness, texture, and morphology.[1] Osteoblast-like cells demonstrate significantly higher levels of cell attachment on rough surfaces than they do on smooth surfaces.[2] It is well known that titanium is one of the best materials for inducing osseointegration, while a number of other materials, such as stainless steel, tend to promote fibrous tissue formation.[3,4] In the air at room temperature, the surface of titanium is covered spontaneously by oxide layer which is 1.5-10 nm in thickness.[5] It was defined that the oxide layer has low level of electronic conductivity, great thermo-dynamical stability, and low ion-formation tendency in aqueous environments.[6] The excellent biocompatibility of titanium implants is related to the TiO_2 surface. Albrektsson et al.[7] proposed six factors which have been generally accepted as especially important for the establishment of reliable osseointegration : implant material, implant design, surface quality, status of the bone, surgical technique, and implant loading condition. Further modifications of the surface oxide properties of an implant have potential to ensure clinically favorable performance.[8] A prospective randomized clinical study by Rocci et al.[9] found a 10% higher survival rate following immediate loading of oxidized implants in the posterior mandible compared with the outcome of machined implants(95.5% and 85.5%, respectively; p=0.0575). Glauser et al. performed two prospective clinical studies on immediate loading using oxidized and machined implants.[10,11] They reported a failure rate of 3% for oxidized implants and 17.3% for machined implants. Although these were two separate studies, the results indicate a difference.

The success of osseointegration can be enhanced with an implant that has improved surface characteristics. Although the range of biomechanical properties which promote an optimal bone-implant interface are not all known, surface roughness is thought to be one of the more important considerations for investigation. Clearly, the optimal degree and type of surface roughness has not been well defined. The pattern, size and distribution of peaks and valleys that compose the surface roughness may significantly influence the overall intimacy and mechanical interlocking of the bone-implant interface.[12] Larsson[13] concluded that an increased oxide thickness and roughness on the sub-micrometer scale were advantageous surface properties for early bone tissue response. Knowledge is still lacking about the role of surface oxide thickness during the dynamic build-up of an osseointegration process. Very few in vivo studies have investigated bone tissue responses to surface oxide thickness of C.P. titanium implants. Anodic oxidation of the electropolished surfaces, which produced areas of increased roughness and a thicker surface oxide, had an enhancing effect on the rate of bone formation. Increasing the oxide thickness of rough machined implants only had no significant effect on the bone response. The results show that both surface topography on the submicrometer scale and oxide thickness influence the bone response to titanium.[14] Sul et al.[15] showed that implants with oxide thickness of approximately 600, 800, and 1000 nm demonstrated significantly stronger bone responses in the evaluation of removal torque value than did implants that had an oxide thickness of approximately[17] and 200 nm. The surface topography relates to the degree of surface roughness and orientation of surface irregularities. Buser et al.[16] reported that increased bone to metal contact correlated to increased surface roughness.

There are basically 2 ways to modify the surface layer, ie, creation of a convex texture or a concave texture. Additive treatments such as plasma spray-coating of hydroxyapatite particles or titanium beads, or physical or chemical vapor deposition, are performed to create convex surface morphology. It is possible that deposited particles can fracture from the convex surface. In contrast, mechanical treatment such as sandblasting or chemical treatment with acid or alkaline can create concave surface texture.[17] Anodic oxidation also create concave surface texture.

Anodic oxidation is an electrochemical process that increase the TiO_2 surface layer and roughness. The implant is immersed in a suitable electrolyte and becomes an anode in an electrochemical cell. When a potential is applied to the sample, ionic transport of charge transfers through the cell, and an electrolytic reaction takes place at the anode, resulting in the growth of an oxide film.[18] Niki et al.[19] and Ishizawa et al.[20] reported strong bone response to a new implant surface anodized in a sulfuric acid and phosphoric acid(H_2SO_4 + H_3PO_4) mixed electrolyte system, resulting in an oxide thickness of about 4 ㎛. Hall and Lausmaa introduced the TiUnite implant(Nobel Biocare, Göteborg, Sweden), which was also anodized in a mixed electrolyte system containing H_2SO_4 + H_3PO_4. The oxide thickness of the TiUnite implant is claimed to be 1 to 2 ㎛ at the coronal end, including the first threads, and 7 to 10 ㎛ at the apical ends.[21]

Electrochemically oxidized implants are currently being used clinically, and improved experimental and clinical performance has been reported. Significantly more bone-implant contact and bone inside the thread area were found for the oxidized implants than for the machined ones. The reasons for the stronger bone reaction to the oxidized implants compared to the machined controls might be single or multiple. The thicker oxide layer itself might lead to a stronger bone response. The change in the morphology of the oxidized implants(size and distribution of pores) might be another reason, whereas the machined surface lacks such features. The surface enlargement and increased surface roughness for the oxidized implants may be a relevant factor for the strong bone reaction.[22] Anodic oxidation is efficient to control the thickness, composition and topography of oxide film on titanium.[23] With such an increase of the oxide thickness, the microstructural properties and crystallinity of the titanium oxide varied substantially with the oxide thickness. In oxides thicker than 600 nm, porous microstructures appeared due to voltage surge and micro arcing(breakdown phenomenon). The crystal structures of the titanium oxide revealed different oxide structures at different thickness: thermal oxide was amorphous while anodic oxidation produced mainly the anatase phase.[24] The thickened titanium oxide layer is highly crystalline containing anatase and rutile, which are the common crystalline forms of titanium oxide and phosphates.[17] Titanium dioxide occurs in four forms: rutile, tetragonal mineral usually of prismatic habit, often twinned; anatase or octahedrite, a tetragonal mineral of octahedral habit; and brookite, an orthorhombic mineral. Titanium dioxide(B) or TiO_2(B), a monoclinic allotrope of titanium dioxide has a density lower than that of the other three allotropes. A certain amount of titania of anatase and/or rutile structures on the oxidized titanium surfaces was required for the apatite formation. The structure of rutile is matching to the structure of apatite.[25] It was reported that the matching structure could be the nuclei for crystal growth.[26] During implantation titanium releases corrosion products into the surrounding tissue fluid, even though it is covered by a thermodynamically stable oxide film.[27]

The purpose of this study was to evaluate

MG63 cell morphology, proliferatve response and viability and to compare surface characteristics of anodic oxidized titanium in various conditions.

MATERIALS AND METHODS

1. Preparation of titanium disk

The disks for cell culture were fabricated from grade 3 commercially pure titanium, 1 mm in thickness and 12 mm in diameter. Surfaces of 4 different roughness were prepared.

Group 1 : machined surface, used as control
Group 2 : anodized under 220 V
Group 3 : anodized under 300 V
Group 4 : anodized under 320 V

Disks were anodized with the pulse power. The Electrolyte solution contained 0.25M H_2SO_4 and 0.25M H_3PO_4. After anodizing, they were rinsed with distilled water and sterilized with ethylene oxide gas(130℃, 10psi, 3hours).

2. Surface characterization

The microtopography of specimens was observed by scanning electron microscope(JSM-840A, JEOL, Japan) and atomic force microscope (Autoprobe CP, Park Scientific Instrument, USA). The surface roughness was measured by confocal laser scanning microscope(Pascal, Zeiss, Germany). The crystal structure of the titanium surface was analyzed with x-ray diffractometer(D8 advanced, Bruker, Germany).

3. Cell culture

MG63 osteoblast-like cells were used for these studies because of resemblances to human osteoblast cell. MG63 cells were originally isolated from an osteosarcoma and they display numerous osteoblastic traits that are typical of immature osteoblasts, including stimulation of alkaline phosphatase specific activity and osteocalcin production in response to treatment with 1,25-$(OH)_2D_3$.[28,29]

MG63 cells were purchased from the American type culture collection(Rockvill, MD) and cultured in Dulbeccco' s modified eagle medium(DMEM) containing 10% fetal bovine serum(FBS) and 1% antibiotic-antimycotic(10,000 unit/ml penicillin, 10 mg/ml streptomycin, 25 ㎍/ml amphotericin B) at 37℃, 5% CO_2 for 12 hour, 24 hour, 48 hour for MTT assay on each group samples.

4. Scanning electron microscopy

For the observation of cell morphology, the samples were fixed with 4% paraformaldehyde 1 ml and reserved at 4℃.

The cell morpholgy was observed by field emission electron microscope(Hitachi S-4700, Japan).

5. Cell proliferation assay

Examination of cell viability and proliferation forms the basis for numerous in vitro assay of a cell population' s response to external factor. The reduction of tetrazolium salts is a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

The plated cells with medium were left 1 ml per well and 250 ㎕ of 2 mg/ml MTT solution(M2128,

Sigma-Aldrich, USA) was added. After incubation at 37℃ for 4 hours, the media were removed with needle and syringe and detergent agent (dimethylsulfoxide) 300 ㎕ were added to each well and pipette up and down to dissolve crystals for 10 minutes. 200 ㎕ was transferred to 96 well and absorbance was measured micro reader(Eliza 540 nm).

6. Statistics

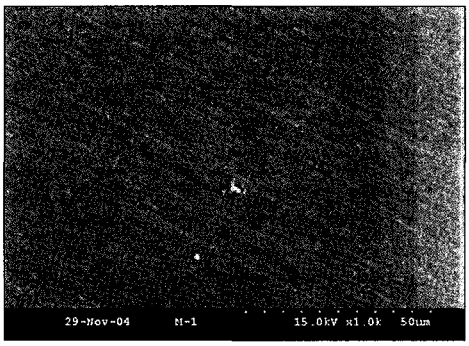
SPSS 12.0 for windows was used to carry out statistical analysis. One-way analysis of variance (ANOVA) was used for statistical analysis of the MTT assay data.

RESULTS

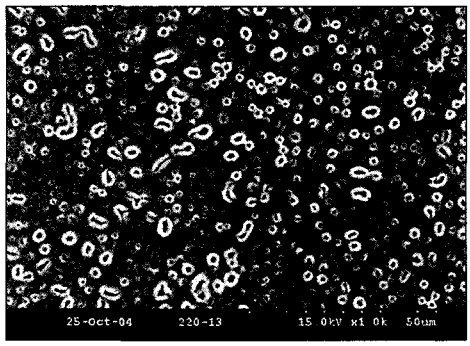
1. Surface morphology and roughness

The machined sample showed the appearance of machined grooves and ridges. In the anodized samples showed the porous oxide layer, the pore size increased with change of voltage. Pore size were 0.2 - 1.0 ㎛ in 220 V, 3.0 - 5.5 ㎛ in 300 V and 320 V.(Fig. 1)

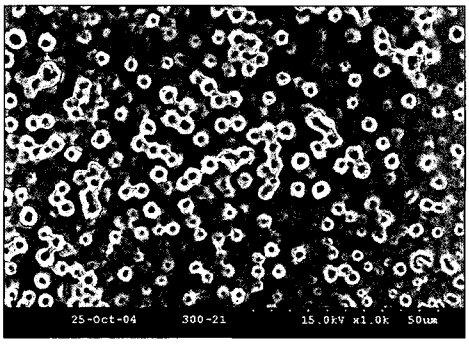
Confocal laser scanning microscopy images are shown in Fig. 2. The roughness of the oxidized titanium surface was characterized by average roughness(Ra). As the applied voltage increased, Ra values decreased. Ra value were 2.84(±0.89) ㎛ in machined surface, 3.29(±0.37) ㎛ in 220 V, 1.78(±0.14) ㎛ in 300 V, and 1.74(±0.14) ㎛ in



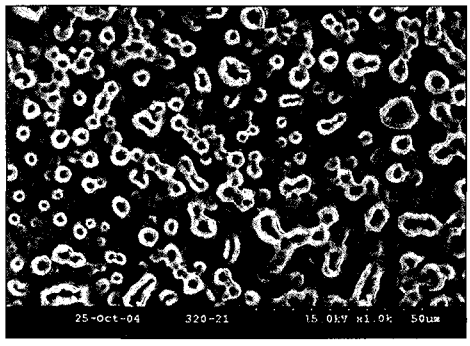
a) Group 1 : machined



b) Group 2 : 220 V

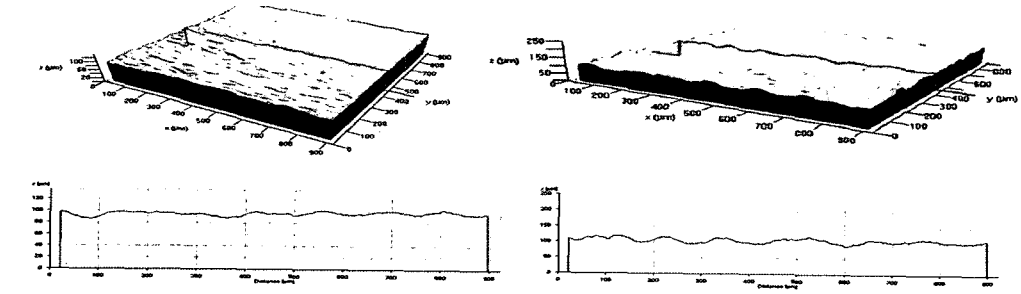


c) Group 3 : 300 V



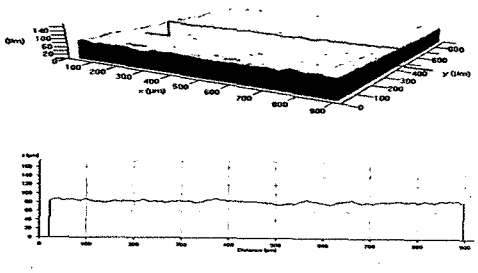
d) Group 4 : 320 V

Fig. 1. SEM images of each group.

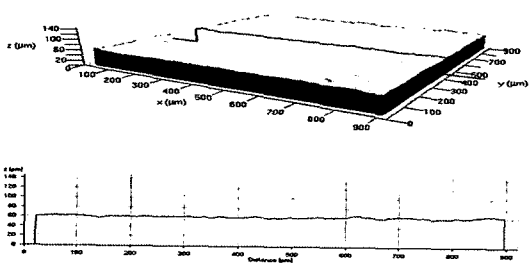


a) Group 1 : machined

b) Group 2 : 220 V



c) Group 3 : 300 V



d) Group 4 : 320 V

Fig. 2. Confocal laser scanning microscopy images.

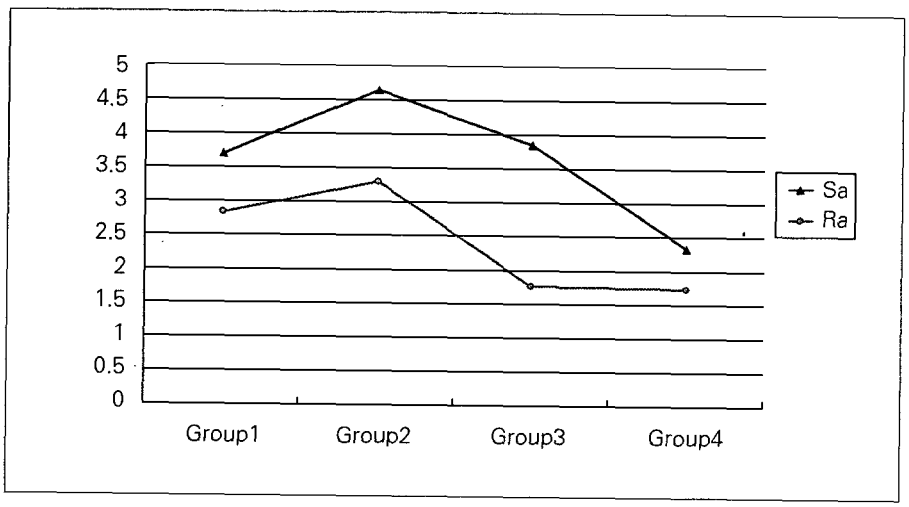


Fig. 3. The graph of Ra and Sa values.

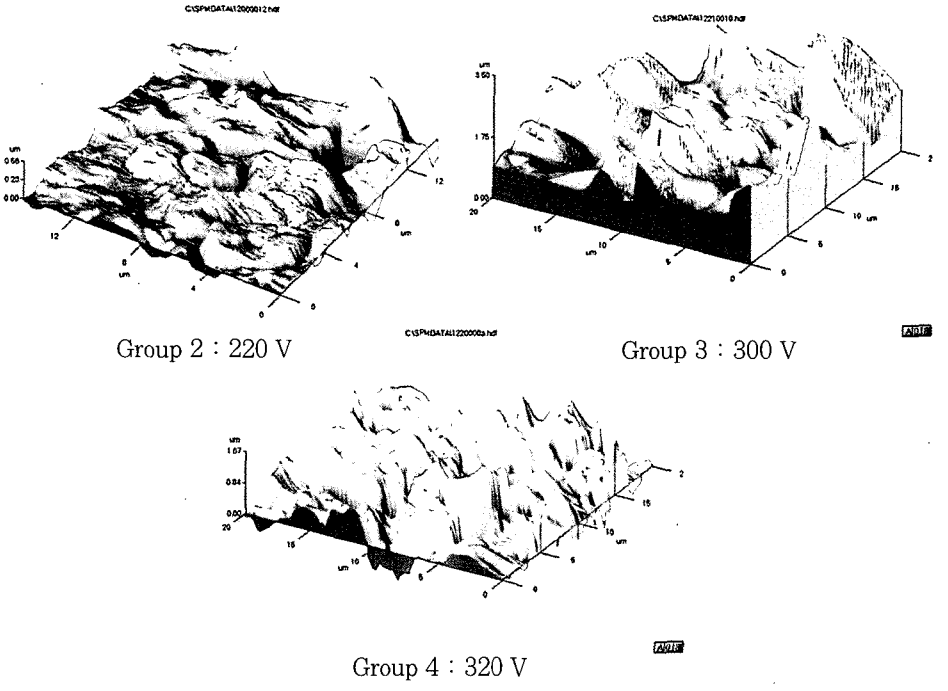


Fig. 4. Atomic force microscopy images.

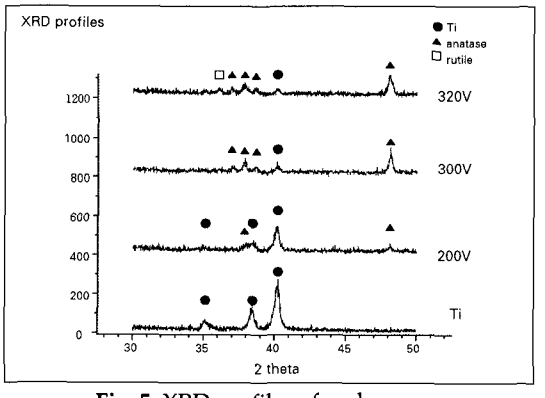


Fig. 5. XRD profiles of each group.

320 V. The graph of Ra and Sa values of each group are shown in Fig. 3. AFM imaging demonstrated the discontinuity of pores and the distance between the peak and the valley.(Fig. 4)

2. Crystal structure of oxide layer

XRD patterns indicated that the anodic oxide films containing anatase, rutile, and amorphous oxides. The amount of anatase increased with change of voltage. Rutile observed at 320 voltage group. The absence of rutile in other groups might be due to the sensitivity of XRD. The results are shown in Fig. 5.

3. Cell morphology

Three kinds of fully spread cell morphology were shown, a polygonal shape, a polarized shape and a round shape. Flat morphology of MG63 cells were adhered to machined titanium surface. As the surface changed more roughly, MG63 cells

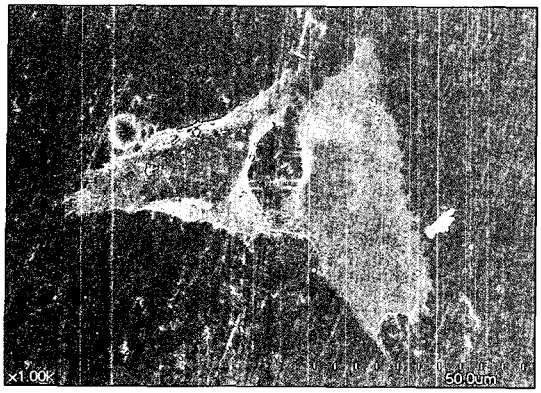


Fig. 6. Group 1, 24 hour.

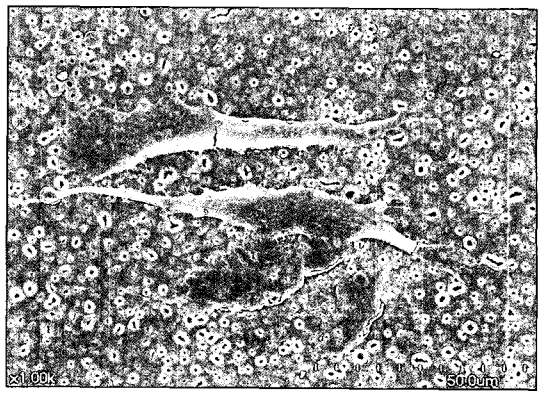


Fig. 7. Group 2, 24 hour.

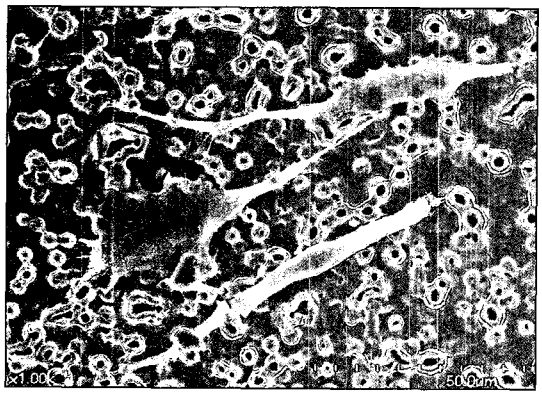


Fig. 8. Group 3, 24 hour.

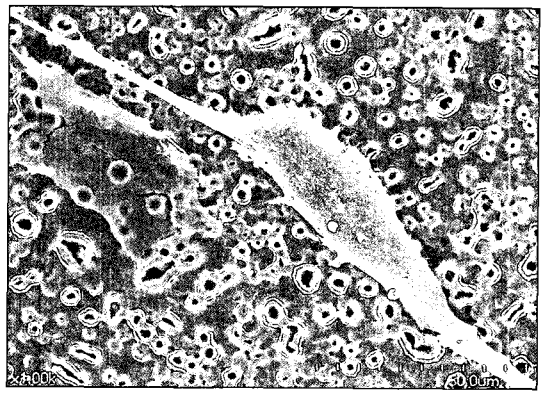


Fig. 9. Group 4, 24 hour.

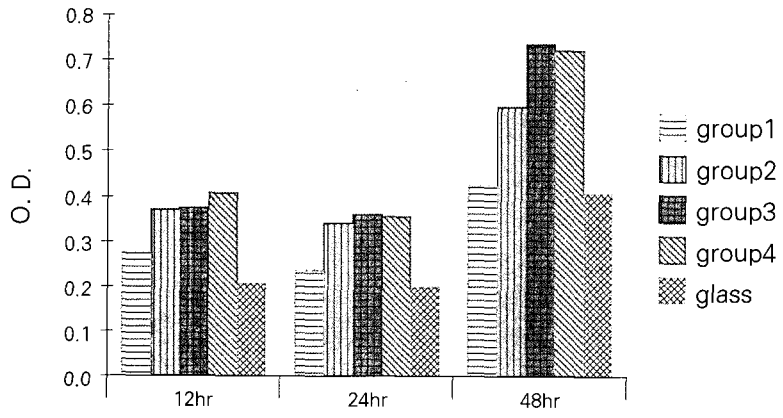


Fig. 10. MTT assay.

Table I. Data of MTT assay

culture time (hours)		group1	group2	group3	group4	glass
12	average	0.275	0.371	0.375	0.409	0.207
	SD	0.037	0.065	0.043	0.071	0.058
24	average	0.237	0.341	0.361	0.356	0.200
	SD	0.032	0.072	0.045	0.074	0.047
48	average	0.427	0.598	0.737	0.725	0.408
	SD	0.080	0.098	0.166	0.089	0.068

showed more irregular, polarized, and polygonal growth and developed more lamellipodi connected to the surface and stretched to the pores.(Fig. 7-9)

4. MTT assay

The disks of machined surface(Group 1) were used as control whereas glass disks were used for comparison. Statistical analysis revealed that the proliferation of MG63 cells was increased in anodized groups, there was significant difference compared with machined and glass, but no significant difference was shown between the group 3 and 4 at $P<0.05$.(Fig. 10. and Table I) Also, there was no significant difference between Group 1 and glass.($P<0.05$) The same results were shown in each time of observations.

DISCUSSION

Anodic oxidation of titanium implants demonstrates changes of various oxide properties, not only oxide thickness, but also surface morphology, pore configuration, crystallinity, chemical composition, and surface roughness.[20] Anodized titanium surface had porous oxide layer, and there were increase in both size and number of pores, as the anodizing voltage became higher.[30]

Cell attachment, spreading and subsequent proliferation are closely related to the surface properties of the substrate, e.g. composition, roughness, wettability and morphology.[20] The absorption concentration of complement C3, which is related to the cellular attachment, increases with an increase in the thickness and/or crystallinity of the titanium oxide. Oxide crystallinity seems to be a more significant factor than oxide thickness.[31] After the spherical cells attach on the surfaces, the following event for cell-substrate interaction is cell spreading. The substratum surface topography alters cell shape and modulates fibronectin at the transcriptional and post-transcriptional levels, as well as the amount of fibronectin assembly into the extracellular matrix.[32] It was reported that the surface texture of the Ti substrates can also affect the expression of fibronectin and vitronectin integrin receptor, modify their clustering or aggregation, and therefore determine variations in shape and spreading of cells.[33,34] Fibronectin, a cell-surface protein, enables cells to interact with the extracellular matrix. Fibronectin consists of two 250-kd polypeptide chains that are linked by a disulfide near their carboxyl termini. This highly elongated protein, 600 Å long and 25 Å wide, contains a linear array of domains, each able to specifically bind certain molecules outside the cell, such as fibrin, collagen, and heparin. In addition, fibronectin has a cell-binding domain. This cell-surface protein is important for cell migration in development and for wound healing. Vitronectin is an adhesive gly-

coprotein with a molecular weight of 75-kd found in human plasma, tissue and extracellular matrices. It tends to promotes the attachment and spreading of cells. It has implications for thrombosis since it binds to heparin, protecting thrombin and factor Xa from heparin-dependent inactivation by antithrombin. Vitronectin is also involved in inflammation and has been identified as the carrier protein of beta-endorphin and has also been found to protect bystander cells from lysis by complement. We could see in our experiment that cell proliferation was enhanced by oxide surfaces leading to more irregular and distinct polygonal spreading of the cells. Many more lamellipodia involved in cell migration were observed in cells on the more intensively anodized titanium than on the machined titanium. These phenomena indicated that the ability of cell migration on anodic oxidized titanium could be higher than that on machined titanium and increased as anodizing voltage increased. Focal contacts on the machined titanium were more intensive than on the oxidized groups. Focal contacts act as a special structure of cell adhesion on the substrates, but in general, cells that form strong focal adhesions are less migratory. More stress fibers but fewer lamellipodia were formed on the control or weakly anodized surfaces. As a highly organized cytoskeleton with stress fibers is often associated with strong cell adhesion, the reorganization of the actin cytoskeleton, together with the results of the amount and distribution of focal contacts, further confirm the assumption that the cells on anodic oxides may have higher motility in comparison with those on the control.[23,35] Although the initial cell response to different surface topographies is unclear, it were known that an increase in calcium and phosphorus deposition in physiological fluids and increase in protein production and calcium uptake by osteoblast-like cells.[36] The titanium oxide itself could have promoted mineralization owing to its ability to bind calcium and thereby stimulate bone formation.[37]

Oxides, formed on surfaces and behaved in a hydrophobic nature were identified as rutile-type titanium oxide only, while oxides formed on surfaces behaving in a hydrophilic character were identified as a mixture of dominant rutile and anatase oxides. It is not clear that this structural difference between rutile and anatase oxides will contribute to the observed differences between hydrophilic and hydrophobic behaviors in terms of the relationship between surface roughness and contact angle.[17] Sul et al.[8] suggested that surface properties of implants directly influence bone responses. Based on the bone response in their study, which was expressed as a function of quantitative changes in the surface oxide properties. TiO_2 in a crystalline phase, ie, a mixture of anatase and rutile phase rather than amorphous, seemed to be optimal. The optimal oxide thickness of a porous surface structure appeared to be in the range of 1,000 to 5,000 nm. The optimum surface chemistry of magnesium incoporated, oxidized implants consisted of approximately 9% magnesium at relative atomic concentration in TiO_2 matrix. An optimum porosity of open pores was in the range of 19% to 30%, ie, approximately 24%; with a pore size of $\leq$ 2.0 ㎛. Surface roughness values of 0.7 to 1.0 ㎛ for Sa, 0.9 to 1.4 ㎛ for Sq, and 27% to 46% for Sdr seemed to be optimal. Because roughness of the surface plays a predominant role in cell adhesion during the implant healing phases, this factor should be considered in the manufacturing of endosseous implants. However, surface roughness is not as important as other surface properties in biologic responses. Furthermore, primary stability could be negatively influenced by an increase in surface roughness, which could counteract the stabilization of the implant known to be essential for implant fixation.[38] Other surface

properties should be also considered important in the biologic response and may be more critical parameters of biocompatibility than surface roughness.[17]

The presence of porous surfaces on the anodic oxide was suggested to increase the surface roughness and energy and may cause microscopic tissue-cell ingrowth, thereby improving implant fixation.[39]

The size of the pores, which originate from sparks on the interface of the oxide and electrolyte, was related to the nature and concentration of ions in the electrolyte.[40] Previously published studies had suggested an optimal pore size for bone ingrowth in the range of [50] to around 400 ㎛.[41,42] Schupbach et al.[43] suggested that the bone can be formed into smaller pores with diameters of less than 2 ㎛. It is not necessary for osteoblasts to enter into the pores to form bone. Osteoblasts are polarized cells, and the findings of the investigation indicate that these cells stay at the surface and deposit bone matrix into the pores of oxidized surface. Wong et al.[44] reported the push out tests revealed small deposits of bone in these 1-2 ㎛ pores. One explanation for the increased pushout strength of this group is likely the increased mechanical interlocking that occurs between bine matrix and these small pores. Their study described the fixation of implants by direct apposition of bone during the early healing stages in trabecular bone. Porous structures supply positive guidance cues for anchorage-dependent cells to attach, leading to enhance cell attachment. In contrast, the cells attached to a smooth titanium surface by focal contacts around their periphery as predominant adhesion structures, since repulsive signals from the environment led to retraction of the filopodia back to the cell bodies. These cells showed well-organized stress fibers, which exert tension across the cell body, resulting in flattened cells.[45] In our study, The pore sizes of group 3 and 4 which were good results in MTT assay were various from 3.0 to 5.5 ㎛. The shapes of pores were various from round to oval. The difference in diameter may be due to the method of measurement in oval shape pore.

The surface modification of the titanium with anodic oxidation enhances cellular adhesion with minor change in the gene expression of osteoblast cells. Thus the enhanced cell adhesion produced by anodic oxidation might result in increased bone growth, and contribute to the achievement of a tight fixation within a shorter period of time after surgery.[6] The cell reaction to an implant surface is a very complex situation. This study was aim to find adequate surface characteristics of initial cell attachment and proliferation.

CONCLUSIONS

Surface characteristics of anodic oxidized titanium influence osteoblast response. The good response of osteoblast can result in reinforcement of osseointegration.

There are various factors which affect the surface characteristics in anodic oxidization of titanium such as electrolyte, time of anodizing, and applied voltage. The changing voltage is one of the easy way to control the anodic oxidation.

We inspect the surface characteristics of anodic oxidized titanium and the response of MG63 cell. The following conclusions were drawn.

1. In anodizing titanium surface, we could see pores which did not show in contol group. In higher anodizing voltage, pore size was increased.
2. In anodizing titanium surface, we could see anatase. In higher anodizing voltage, thicker oxide layer increased crystallinity(anatase, anatase and rutile mixed).
3. MG63 cells showed more irregular, polarized,

polygonal shape and developed more lamellipodi in anodizing group as voltage increased.

4. The activity of cells in MTT assay increased significantly in group 3 and 4 in comparison with group 1 and 2. However, there was no difference between group 3 and 4 at $P<0.05$. Proliferation of MG63 cells increased significantly in pore size (3-5.5 ㎛) of group 3 and 4 in comparison with in pore size (0.2-1 ㎛) of group 2.

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The 2006 Spring Scientific Meeting of the Korean Academy of Prosthodontics

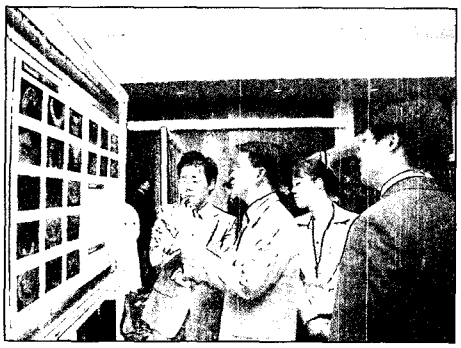
April 28-29, 2006, Seoul, Korea

Agreement for academic cooperation between KPA and JPS

Coffee break

International session

Officers and Directors of KAP with the president of JPS



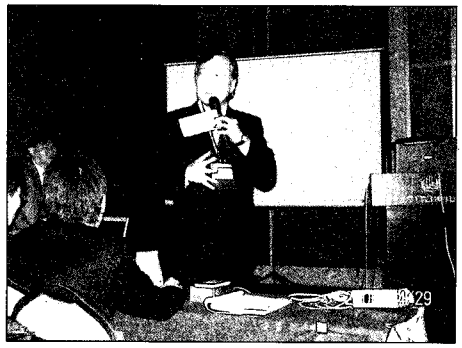
Poster presentation

President' s Welcome Party

Reception desk

Sales of Academy official tie

Special lecture by Prof. C.E. Besimo



Symposium