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Effect of Al_2O_3 blasted titanium surface roughness on the cell adhesion

조선대학교 대학원

치 의 학 과

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AI_2O_3 분사 처리한 티타늄 표면 거칠기가 세포 부착에 미치는 영향

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지도교수 강 동 완

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조선대학교 대학원

치 의 학 과

황 인 택

황인택의 박사학위 논문을 인준함

위원장 부산대학교 교 수 전 영 찬 인

위 원 조선대학교 교 수 정 재 헌 인

위 원 조선대학교 교 수 정 문 진 인

위 원 조선대학교 교 수 국 중 기 인

위 원 조선대학교 교 수 강 동 완 인

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조선대학교 대학원

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Al_2O_3 분사 처리한 티타늄 표면 거칠기가 세포 부착에 미치는 영향

황 인 택
(지도교수: 강 동 완)
조선대학교 대학원 치의학과

서로 다른 크기의 Al_2O_3입자를 분사한 후 형성된 티타늄 표면에 전조골세포인 MC3T3-E1 세포를 배양하여 이들 표면처리방법들이 조골세포의 반응에 미치는 영향을 비교해 보고자 한 본 실험에서 주사전자현미경 관찰, MTT 분석, 역전사 중합효소 연쇄반응 분석 등을 통해 다음과 같은 결론을 얻었다.

1. 알칼리성 인산분해효소 활성도(Alkaline phosphatase activity)측정에서 세포의 부착 초기단계에서 조골세포의 ALP 활성을 분석한 결과 6시간 배양이 24시간 배양보다 ALP활성도가 높은 것으로 나타나 24시간 이상 배양에서 ALP의 활성이 저하되고 특히 70㎛ sand 처리한 티타늄 표면에서 ALP 활성도가 높게 나타남을 확인하였다.
2. MTT 분석에서 24시간 후에는 70㎛, 150㎛, 300㎛ 분사 처리한 티타늄 표면에서는 세포 증식에 많은 차이가 보이지 않았으나 혼합 분사 처리한 티타늄 표면에서는 70㎛, 150㎛, 300㎛ 분사 처리한 것에 비하여 1.1, 1.7, 1.3배 정도의 세포 증식이 활발하게 일어났음을 확인할 수 있었다.
3. 역전사 중합효소연쇄반응 분석에서 osteopontin 발현은 각각의 서로 다른 티타늄 표면에서 균등하게 발현되었지만 70㎛와 혼합 분사 처리한 표면에서 배양한 세포에서 약간 높게 발현되었다.

4. 주사전자현미경 관찰에서 24시간 배양 후에는 세포부착 단계를 지나 세포 퍼짐, 그리고 증식되었고 150㎛와 300㎛로 분사 처리한 티타늄표면에서는 70㎛에 비해 세포가 비대해졌음을 확인할 수 있었다.

티타늄 표면을 처리하는 방법 중 분사 입자의 크기에 따른 반응에서 다양한 크기의 표면 거칠기에 따른 조골세포의 부착 후 증식을 측정한 결과 분사 입자의 크기가 70㎛ 로 처리한 표면과 혼합한 군에서 전조골세포의 증식이 활발한 것으로 나타났다.

그러나 본 실험은 티타늄 표면에 전조골세포가 초기 부착과 증식 단계에 대한 실험이었으므로 최상의 세포반응을 유도할 수 있는 거칠기의 표준치를 얻기 위해서는 좀 더 장기적인 실험 및 관찰 그리고 동물실험이 필요할 것으로 사료되었다.

Ⅰ. INTRODUCTION

One definition of osseointegration (a term originally proposed by Branemark et al[1]) was provided by Albrektsson et al[2] who suggested that this was "a direct functional and structural connection between living bone and the surface of a load carrying implant". Osseointegration is attained by cellular processes that contribute to bone formation at the alloplastic surface[3,4].

Maintaining long-term osseointegration is one of the important factors to increase the success rate of implants[5]. To have a successful osseointegration in the primary stage of implant, primary stability, blood flow, and mechanical integration of the new tissue between the implant and the bone should be achieved. Having established that affect, osseointegration must be recognized as implant biocompatibility, fixture design, surface characteristics, surgical techniques, state of host, biomechanical status, and time[2].

Schwartz et al[6] stated that during the process of integration between implant and the bone tissue, the factors such as surface energy, surface roughness, surface components, and the surface shape are intimately related, and among these, the surface energy and the surface roughness play especially important roles.

The bone formation from osteoblasts in bone to implant contact happens through the complicated processes of cell attachment, movement, proliferation, differentiation, substrate production, and calcification[7,8]. The results of many experiments[9,10,11,12] stated that the roughness of titanium surface affects the process of

osteoblasts growth, and as the roughness increases, the beneficial conditions for bone formation on implant surface enhance as well[13,14,15]. The surface roughness affects attachment, form, and proliferation of osteoblasts[9,16,17], and it also affects alkaline phosphatase (ALP) activity[9,11], the formation of ECM, such as type I collagen, osteocalcin, matrix Gla protein(MGP), osteopontin, bone sialoprotein(BSP), and the formation of proteins such as bone morphogenic proteins(BMPs), transforming growth factor-ß (TGF-ß).

Although sand-blasting to increase the surface roughness shows good clinical results, the experiments about the effect of the size variation in Al_2O_3 particles used in sandblasting were lacking. This experiment shows the variation of Al_2O_3 particle sizes sandblasted on the titanium surface, analyzing the surface roughness and the effect of osteoblasts.

Ⅱ. MATERIALS AND METHODS

1. Preparation of titanium surfaces

Grade V Ti-6Al4V was used to create a titanium disc 5mm thick and 10mm in diameter. The different sized Al_2O_3 particles were sandblasted 10cm away from the blasting machine in 3.2 bar pressure, creating 4 different titanium discs. After the surface was sandblasted, the discs were soaked in distilled water and anhydrous alcohol, and ultrasonic cleaning was done. High-pressure vapor sterilization was done in 135℃, and the discs were stored away from the UV rays.

Group 1 (SA 70) : Disc with Al_2O_3 sandblasted in 70㎛ diameter
Group 2 (SA 150) : Disc with Al_2O_3 sandblasted in 150㎛ diameter
Group 3 (SA 300) : Disc with Al_2O_3 sandblasted in 300㎛ diameter
Group 4 (SA mix) : Disc with Al_2O_3 sandblasted with three different sizes mixed in same ratio

2. Cell culture

MC3T3-E1 mouse osteoblastic cells were used for these studies because they are differentiate into osteoblastic cells and retain osteoblastic defferentiative phenotype well such as high alkaline phosphatase (ALP) activity, type I collagen synthesis, mineralization[18]. The MC3T3-E1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in α

-MEM (without ascorbic acid, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah, USA), 100/100 ㎍/㎖ penicillin G sodium/streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA), and 0.25㎍/㎖ amphotericin B (Sigma-Alderich) in a 5% CO_2 at 37°C. When the cells are growing confluently, cells are detached with 0.25% trypsin/1ethylene-diamine tetra aceticacid (EDTA), counted and suspended in a culture medium at a density of 2×105cells/㎖.

3. Assay of Alkaline phosphatase activity

To determine the activity of alkaline phosphatase (ALP), StemTAG Alkaline Phosphatase activity assay kit (Cell Biolabs, San Diego, CA, USA) was used with minor modification of manufacture's protocols. Briefly, MC3T3-e1 cells (1.6 x107 cells/㎖) were grown on different rough-surface of titanium discs in complete α-MEM medium for 6 hrs or 24hrs, independently. After cultured indicated times, cells are washed twice with cold-PBS (phosphate buffered saline) and lysed in Cell Lysis Buffer (Cell Biolabs, San Diego, CA, USA), incubated at 4° for 10 min, and centrifuged at 12,000 x g for 10 min. The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Berkeley, CA). The 10㎍/50㎕ of cell lysates are added to a 96-well plate, initiated by adding 50 ㎕ of StemTAG AP Activity Assay Substrate (Cell Biolabs, San Diego, CA, USA), and incubated for 30 min at 37℃. The reaction is stopped by adding 50 ㎕ of 1X Stop Solution (Cell Biolabs, San Diego, CA, USA) and mixed by placing the plate on an orbital plate shaker for

30 sec. The ALP activity was normalized to the total protein content of each sample using AP activity assay standards (Cell Biolabs, San Diego, CA, USA). The reactants are read testing the absorbance of each well at 405 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instrument, Winooski, VT, USA).

4. Cell proliferation assay

Cell proliferation was measured with a CellTiter 96 Non-radioactive cell proliferation assay kit, which is based on measuring changes in absorbance at a specific wavelength using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], according to the manufacturer's directions (Promega, Madison, WI)). Briefly, MC3T3-E1 cells were plated in different rough titanium surface at a density of 5,000 cells/titanium-discs in 50 ㎕ of medium. Cells were allowed to grow up to 24 hrs then combined MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-phenozine methosulfate solution (15 ㎕/well) was added. After incubation for 4h at 37°C in a humidified 5% CO_2 atmosphere, 100ul of Solubilization Solution/Stop Mix (Promega) was added. The absorbance was measured at 5700 nm by using an ELISA reader (BioTek Instrument). Data presented the average of three wells in one experiment which was repeated four times.

5. Scanning electron microscopy

To determine the effect of the different rough titanium surfaces for osteoblast growing, the cell morphology was observed by scanning electron microscopy (SEM, S-4300, Hitachi, Japan). MC3T3-E1 cells were cultured on different rough titanium surface at a density of 1x 10^4 cells/titanium-discs for 6 and 24 hrs, respe- ctively. After culturing the cells the indicated times, the disc were rinsed four times with PBS and then fixed with 2.5% parafor- maldehyde-glutaraldehyde (in PBS, pH7.2) at 4°C for 1 hour. After fixation, the discs were rinsed three times with cold-PBS and post-fixation with 1% osmium tetraoxide solution for 2 hours. After post-fixation the discs were rinsed with PBS, sequentially incubated for 30 minutes each in 50%, 70%, 90%, 95%, and 100% ethanol for dehydration. The dehydrated discs were dried in air and platinum coated using E-1030 ion sputter coated (Hitachi, Japan). Cells were examined with S-4300 scanning electron microscope (Hitachi, Japan). Disc surfaces without cells were also platinum coated for scanning electron microscopy.

6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

To examine the cell adhesion and spreading in different surface roughnesses of titanium, we analyzed gene expression of osteopo- ntin. Briefly, MC3T3-E1 cells were grown on different titanium discs for 6 hrs at 37°C in a CO_2 incubator and total RNA was

extracted with Trizol reagent (Gibco BRL) according to the manufacturer's directions. After extraction, the RNA was converted to cDNA and amplified. Reverse transcription (RT) was perfo-rmed using 2 ㎍ of denatured RNA and 100 pmol of random hexamers (Gibco BRL) in a total volume of 20 ㎕ containing 50U Superscript reverse transcriptase (Gibco BRL). PCR was performed in a GeneAmp PCR System 9700 (applied biosystems, Carlsbad, CA, USA), using 2.5 ㎕ of the cDNA (equivalent to 0.25 ㎍ RNA) and AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) in a 25-㎕ reaction according to the manufacturer's instructions. The DNA sequences of primers used for RT-PCR were; Glyceraldehydes phosphate dehydrogenase (GAPDH, 587 bp): Sense 5'-AGCCGCATC TTCTTTTGCGTC -3', Antisense 5'-GCATGGACTGTGGTCATGAGT-3', Sense 5'-AAGC GAGGAGTTGAATGG -3', Antisense 5'-GGAAAGTTCCTGACTATC -3'. PCR condition is denaturation (94°C, 2 min); Amplification and quantification (94°C, 15sec 58°C for GAPDH and 56°C for osteopontin 30 sec followed by 72℃ for 5 min). The PCR products size was confirmed using 1 kb DNA ladder (Fermentas Inc., Glen Burnie, Maryland).

Ⅲ. RESULTS

1. Alkaline phosphatase activity

It was reported that alkaline phosphatase (ALP) is an early marker of osteoblasts (Mosmann, 1983). We examined the ALP activity of MC3T3-E1 cells which are grown on different roughnesses of titanium surfaces (70 ㎛, 150 ㎛, 300 ㎛ and mixed micro-sandblastedon titanium surface) for 6 and 24 hours, respectively (Fig. 1). As shown figure 1, ALP activity was higher in 70 ㎛ surface roughness in both 6 and 24 hours, but surface roughness larger than 70㎛ was slightly lowered. Also, cells grown 6 hours at 70㎛ roughness showed significantly higher ALP activity than those grown for 24 hours. These results suggested that ALP is associated with early osteoblast adhesions.

2. Cell proliferation assay

The effect of roughness of Titanium surfaces on MC3T3-E1 osteoblast was evaluated by using the MTT colorimetric assay methods. This method was broadly used for mammalian cell survival and proliferation (Mosmann, 1983). The cells were grown for 24 hours on different titanium discs and analyzed for cell proliferation (Fig. 2). As shown in Figure 2, cells are grown on mixed micro-sandblasted treatment of titanium surface showing higher cell proliferation when compared with 70, 150 and 300㎛, although 70㎛ was slightly higher than 150 ㎛. This result

implies that surface roughness affected proliferation

3. Scanning electron microscopy

To confirm the ALP and MTT activity assay, we examined the cell proliferation by using scanning electron microscopy. The results of SEM analyses of surface attachment and growth are displayed in Figure 3. SEM images showed that 70㎛ sandblasted at 6 hours cultured cells are more adhesive to clustering on Titanium surface, but less attachment on 150㎛ sandblasted surface.

When sandblasted larger than 70㎛, cells showed irregular phenotype and cell bodies linked to each other like cytoplasmic bridges. Additionally, the cell bodies are thick compared with 70㎛ sandblasted surfaces. These results are similar to a previous reported paper (Marinucci et al., 2006). They showed that cells grown on macro-sandblasted surfaces were thicker than micro-sandblasted surfaces.

4. RT-PCR analysis

To examine the gene expression of MC3T3-E1 cells on different sandblasted Titanium surface, cells were cultured for 6 hours and analyzed by using RT-PCR methods. It was reported that osteopontin expressed the initial stage of cell adhesion (Marinucci et al., 2006; Yamate et al., 1997); therefore, we examined osteopontin expression at early stages of osteoblast adhesion. Figure 4 shows the gene expression of osteopontin in MC3T3-E1 cells. GAPDH

mRNA levels served as controls. As shown in Figure 4, osteopontin expression was slightly increased on the 70㎛ sandblasted titanium surface, and this result correspond with ALP activity and cell proliferation assay. From these results, we confirmed that the 70㎛ sandblasted titanium surface increased the osteoblast adhesion, attachment and cell proliferation.

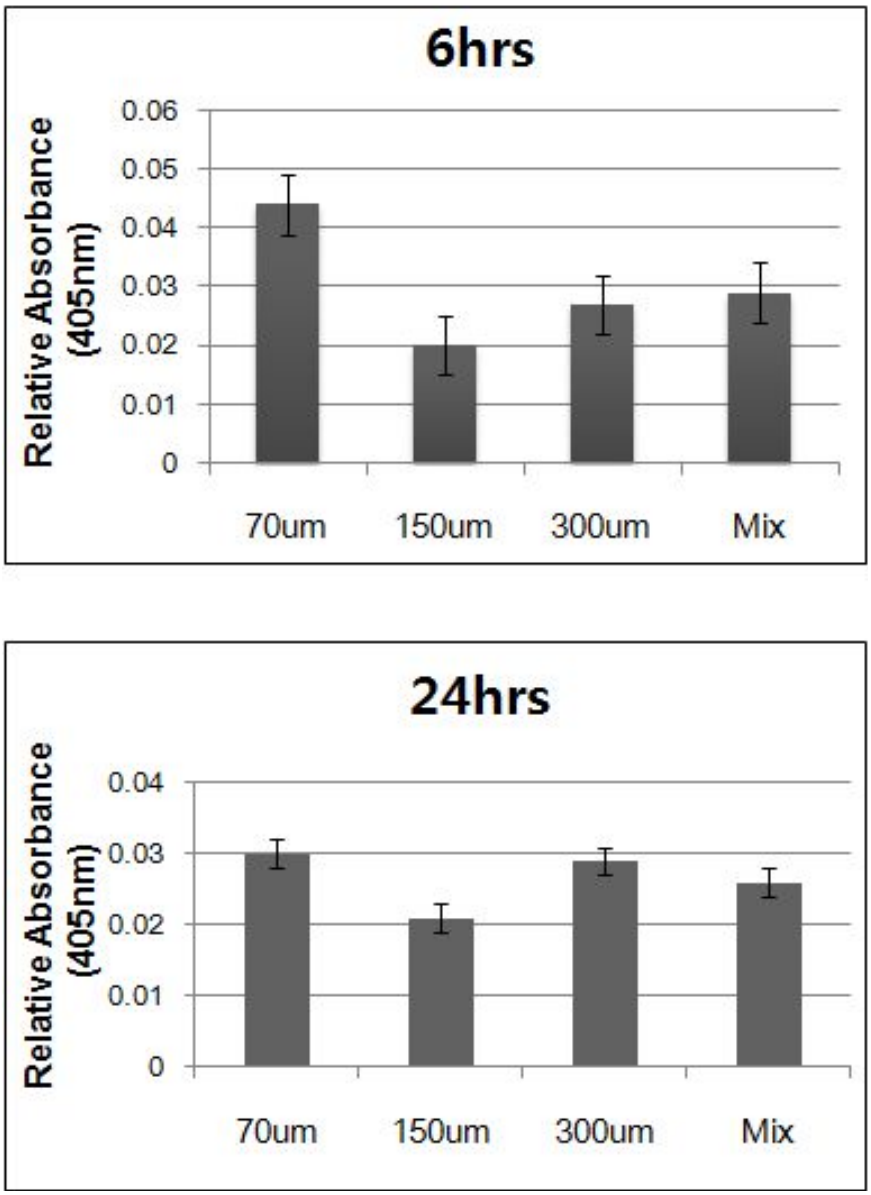

Fig. 1. Alkaline phosphatase activity of different sandblasted titanium surfaces. MC3T3-E1 cells were cultured on 70㎛, 150㎛, 300㎛ and mixed-sandblasted titanium surfaces and the alkaline phosphatase activity was compared.

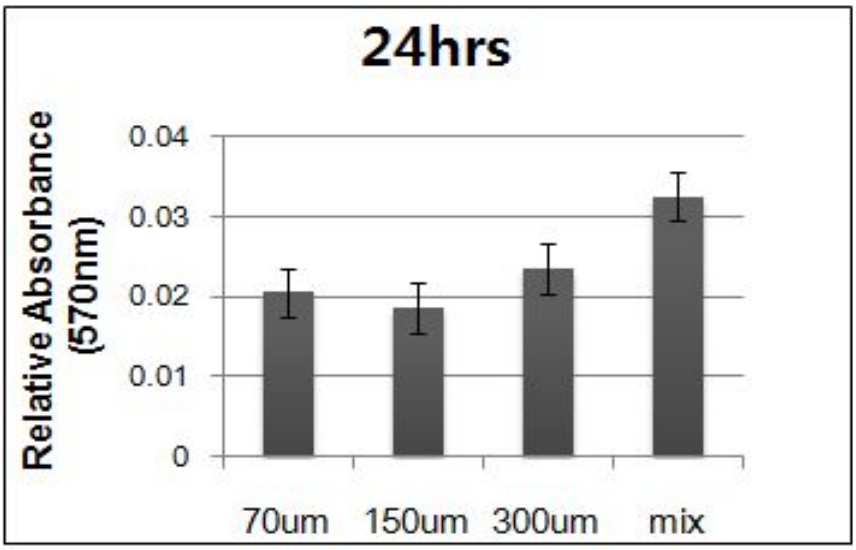

Fig. 2. Cell proliferation of MC3T3-E1 cells on different sandblasted titanium surfaces. MC3T3-E1 cells were plated in 70㎛, 150㎛, 300㎛ and mixed-sandblasted titanium surfaces at a density of 5,000 cells/titanium-discs in 50 ㎕ of medium. Cells were allowed to grow up to 24 hrs and then the MTT assay was performed.

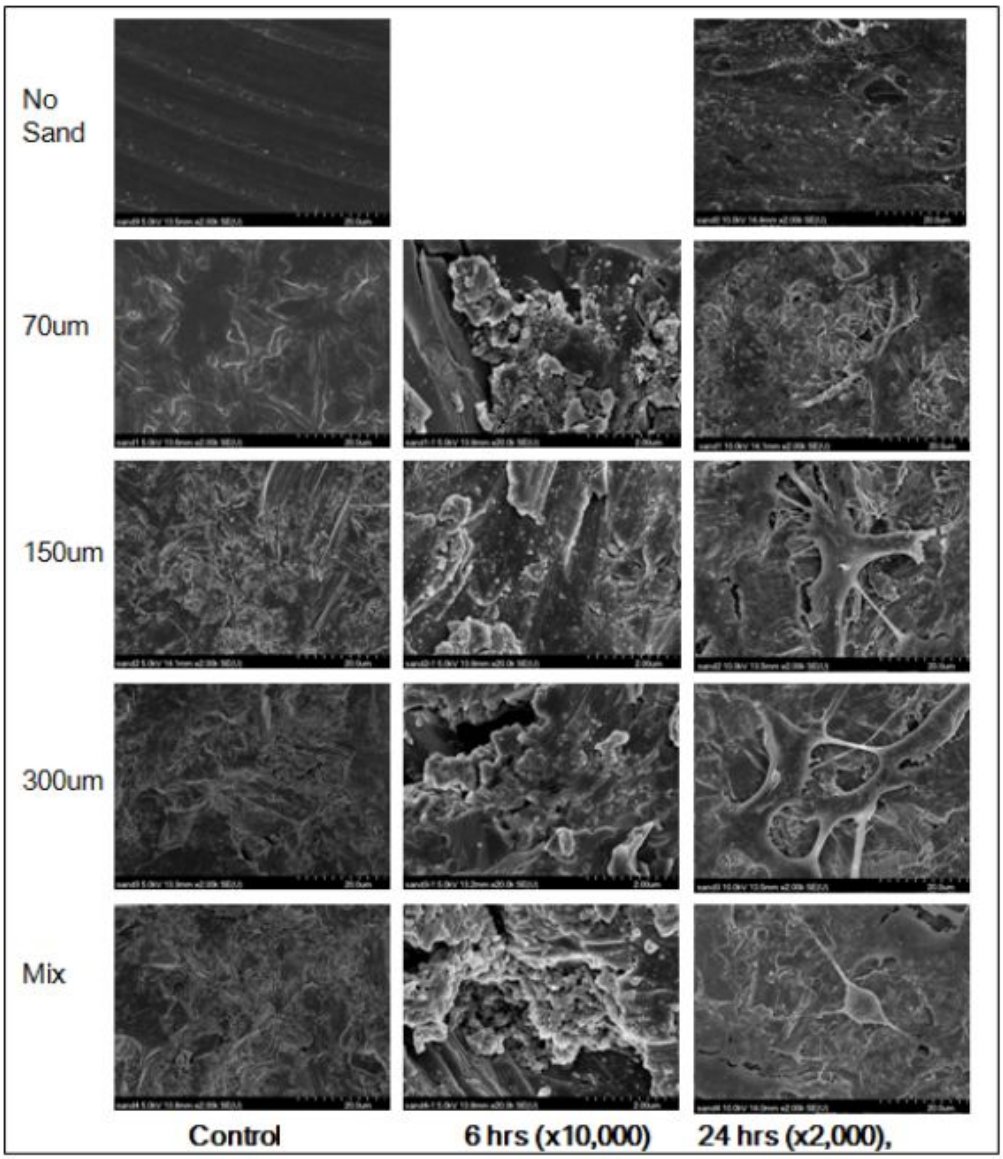

Fig. 3. Scanning electron microscopy images of different sandblasted titanium surfaces. MC3T3-E1 cells were cultured for 6 and 24 hours on 70㎛, 150㎛, 300㎛ and mixed sandblasted titanium surfaces. After fixation, cells were coated and examined by using SEM.

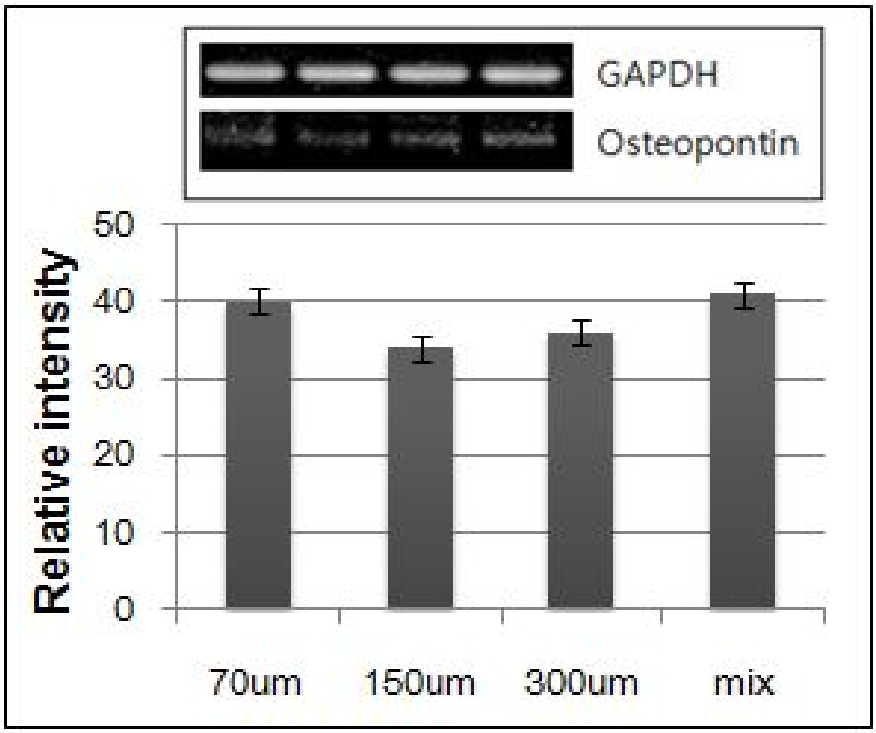

Fig. 4. Expression of osteopontin mRNA in MC3T3-E1 cells on different sandblasted titanium surfaces. Equal amounts of total RNA were analyzed by RT-PCR method. GAPDH is normal expression controls.

Ⅳ. DISCUSSION

In dental implants, osseointegration is very important, and osteoblasts play an important role in the beginning stage of osseointegration. The effects of osteoblasts based on the characteristics of implant surface are varied, so the surface properties influence the attachment, form, proliferation, and differentiation of osteoblasts greatly[7,8,9,10,11,12,16,17,19].

MC3T3-E1 cells are one of the widely known types of osteoblasts and were proved to be appropriate in titanium surface research regarding attachment, proliferation, and differentiation of osteoblasts[18,20].

In this experiment, the different sizes of Al_2O_3 particles were sandblasted, MC3T3-E1 cells were cultured on the titanium surface, and the effect on attachment, proliferation, and genetic expression of osteoblasts, depending on the different sizes of surface treatment, were analyzed.

From the analysis of alkaline phosphatase (ALP) activity in the primary stage of cell attachment, 70㎛ sandblasted titanium had the highest ALP activity at 6 and 24 hours, which indicated high attachment of MC3T3 cell at 70㎛. As Bancroft et al[21] and Shin et al[22] also reported, alkaline phosphatase (ALP) activity is manifested well in the primary stage of osteoblastic differentiation, and the activity is thought of as a primary indicator of bone formation, and matrix mineralization. Therefore, in comparing the activity levels, the primary expression in 70㎛ sandblasted titanium was prevalent.

When Mustafa et al[17] increased the size of the particle to 300 on the human mandible bone, the initial attachment of cell was not increased, which produced the similar result with alkaline phosphatase (ALP) activity manifestation. From the alkaline phosphatase (ALP) activity experiment by Martin et al[12] using MG63 osteoblasts, the results showed that increasing roughness of surface decreased the expression of ALP.

Using MTT analysis, the results of MC3T3 cells cultivated for 24 hours show little difference in the cell proliferation for 70㎛, 150㎛, 300㎛ sandblasted titanium surface. However, the mixed sandblasted titanium surface showed 1.1, 1.7, and 1.3 folds higher in cell proliferation than 70㎛, 150㎛, and 300㎛ sandblasted titanium discs, respectively.

In comparison with the surface roughness, the cell proliferation increased in order of SA 150, SA 300, and mixed groups. From the results of many experiments, the direct relationship between surface roughness and differentiation of osteoblasts is shown, which demonstrate that increasing surface roughness will promote beneficial conditions for bone formation on the implant surface [14,23].

The result of an experiment by Kiewswetter et al[19] shows the influence of prostaglandin E2 (PGE2) and transforming growth factor beta1 (TGF-ß1) depending on the method of surface treatment of titanium. As the surface roughness increased, the attachment of osteoblasts on titanium surface decreased. The increase in PGE2 and TGF-ß1 were observed, and the reason was because the cells are in a more differentiated form on the rough surface than the smooth surface. The decrease in cell proliferation occurs preceding the expression of phenotypes in

differentiated osteoblasts[24]. The cell proliferation decreases as the cell differentiation occurs, and the differentiation is accelerated on the micro개ugh surfaces with the bone-formation accelerating factors such as 1 alpha,25 (OH)2D3. Simultaneously, PGE2 and TGF-ß1 also increases, provoking the 1 alpha, 25(OH)2D3, and synergy effect [14,25].

After cultivating MC3T3-E1 cells in the 4 different groups with the varied roughness of the titanium surfaces and observing with a Scanning Electron Microscope(SEM), in all groups, the osteoblasts were well-attached to the titanium surfaces. After 6 hours of cultivation, the result of observing MC3T3 attachment through SEM shows that cells were attached to the titanium surface, and after 24hours of cultivation, the cells were not only attached, but they had also spread and proliferated. Especially after 24 hours of cultivation, 150㎛ and 300㎛ sandblasted titanium surface showed more enlarged cells compared to the 70 ㎛ sandblasted titanium surface, while ALP and MTT analysis showed that cell proliferation decreased.

For the SA 70㎛ group, which had the lowest surface roughness, thin, flat preosteoblasts were well-spread and almost covered the whole titanium surface. However, as the roughness increased, the preosteoblasts branched out on the irregular, small concaves. SA 300 had longer projections than the SA 150. The distance between the cells became wider as the roughness increased, decreasing the area covered on the titanium surface. These results show that the surface roughness affected the form and projection of the cells.

Through RT-PCR analysis, the expression of mRNA proteins related to bone formation was observed. The expression of oste-

opontin[24,25], a protein involved in processes from the differentiation to the transcription of osteoblast, was analyzed. Osteopontin expression was higher as the surface roughness increased, and SA 70 had higher expression of osteopontin in preosteoblasts than SA 150 or SA 300. SA mix had higher expression of osteopontin in preosteoblasts than SA 70, SA 150, or SA 300.

Ku et al[15] reported that osteopontin, a glycoprotein affecting the photochemistry of bone, substrate-cell reciprocity, and collagen bonding on the rough titanium surface, affects the expression of preosteoblast forms and characteristics greatly. Ayukawa et al[26]also stated that glycoproteins, such as osteocalsin and osteopontin, are distributed widely on the new bone, and it appears often around the bone near the titanium. From these views, because this experiment was about the initial attachment and proliferation of preosteoblasts, the consideration was shown for the necessity of animal and long-term cell experiments to obtain the surface roughness that will accelerate new bone formation.

In the case of mixed Al_2O_3 particle with 1:1:1 ratio of 70㎛, 150㎛, and 300㎛ groups, the group affecting the difference was unknown; therefore, the necessity of another experiment with varying ratio was considered. Also, the necessity of sandblasting the particles with varied sizes simultaneously and sandblasting the particles with varied sizes in respective order was considered to observe the difference in development of surface roughness.

V. CONCLUSION

After sandblasting Al_2O_3 particle and culturing MC3T3-E1 cell on the titanium surface, Alkaline phosphatase activity, MTT analysis, SEM observation, RT-PCR were used to analyze the effect on osteobalsts due to surface treatments. The following results were obtained.

1. From the measure of alkaline phosphatase activity, the initial attachment phase of cell had high ALP activity when cultured for 6 hours and 24 hours. 70㎛ sandblasted titanium surface showed the highest ALP activity.

2. MTT analysis showed not much difference among the 70㎛, 150㎛, 300㎛ sandblasted titanium surface after 24 hours. However, the mixed sandblasted titanium surface showed 1.1, 1.7, and 1.3 folds higher cell proliferation than 70㎛, 150㎛, 300㎛ sandblasted titanium surface, respectively.

3. RT-PCR analysis showed higher expression of osteopontin in the cultured cells on 70㎛ and mixed sandblasted surface.

4. From SEM observation, the cells passed the attachment phase and proliferated after 24 hours of cell culture. Compared to 70㎛, the cells were more enlarged in 150㎛ and 300㎛.

Among the titanium surface treatments, depending on the

particle sizes, the 70㎛ and mixed sandblasted surface showed more active proliferation of preosteoblasts. However, because this experiment was about initial attachment and proliferation phase of preosteoblasts on the titanium surface, a long-term cell and animal experiment, which shows the acceleration of new bone formation after the initial attachment, proliferation, and differentiation, may be necessary.

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저작물 이용 허락서

학 과	치의학과	학 번	20087557	과 정	박사
성 명	한글 : 황 인 택 한문 : 黃 仁 澤 영문 : Hwang, In taek				
주 소	전주시 덕진구 송천동 1가 291-2 늘사랑치과의원(063-277-7575)				
연락처	010-3684-3266	E-MAIL	hit337@korea.com		
논문제목	한글 : Al_2O_3 분사 처리한 티타늄 표면 거칠기가 세포 부착에 미치는 영향				
	영문 : Effect of Al_2O_3 blasted titanium surface roughness on the cell adhesion				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

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저작자: 황 인 택 (서명 또는 인)

조선대학교 총장 귀하