A STUDY ON THE RESPONSES OF OSTEOBLASTS TO VARIOUS SURFACE-TREATED TITANIUM

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Statement of problem. The long-term success of implants is the development of a stable direct connection between bone and implant surface, which must be structural and functional. To improve a direct implant fixation to the bone, various strategies have been developed focusing on the surface of materials. Among them, altering the surface properties can modify cellular responses such as cell adhesion, cell motility and bone deposition.

Purpose. This study was to evaluate the cellular behaviors on the surface-modified titanium by morphological observation, cellular proliferation and differentiation.

Material and methods. Specimens were divided into five groups, depending on their surface treatment: electropolishing(EP) anodizing(AN), machining(MA), blasting with hydroxyapatite particle(RBM) and electrical discharge machining(EDM). Physicochemical properties and microstructures of the specimens were examined and the responses of osteoblast-like cells were investigated. The microtopography of specimens was observed by scanning electron microscopy(SEM). Surface roughness was measured by a three-dimensional roughness measuring system. The microstructure was analyzed by X-ray diffractometer(XRD) and scanning auger electron microscopy(AES). To evaluate cellular responses to modified titanium surfaces, osteoblasts isolated from neonatal rat were cultured. The cellular morphology and total protein amounts of osteoblast-like cell were taken as the marker for cellular proliferation, while the expression of alkaline phosphatase was used as the early differentiation marker for osteoblast. In addition, the type I collagen production was determined to be a reliable indicator of bone matrix synthesis.

Results.

1. Each prepared specimen showed specific microtopography at SEM examination. The RBM group had a rough and irregular pattern with reticulated appearance. The EDM-treated surface had evident cracks and was heterogeneous consisting of broad sheet or plate with smooth edges and clusters of small grains, deep pores or craters.
2. Surface roughness values were, from the lowest to the highest, electropolished group, anodized group, machined group, RBM group and EDM group.
3. All groups showed amorphous structures. Especially anodized group was found to have increased surface oxide thickness and EDM group had titaniumcarbide(TiC) structure.
4. Cells on electropolished, anodized and machined surfaces developed flattened cell shape and cells on RBM appeared spherical and EDM showed both. After 14 days, the cells cultured from all groups were formed to be confluent and exhibited multilayer proliferation, often overlapped or stratified.
5. Total protein amounts were formed to be quite similar among all the group at 48 hours. At 14 days, the electropolished group and the anodized group induced more total protein amount than the RBM group($P<.05$).
6. There was no significant difference among five groups for alkaline phosphatase(ALP) activity at 48 hours. The AN group showed significantly higher ALP activity than any other groups at 14 days($P<.05$).
7. All the groups showed similar collagen synthesis except the EDM group. The amount of collagen on the electropolished and anodized surfaces were higher than that on the EDM surface($P<.05$).

Key Words
Titanium, Surface treatment, Surface analysis, Cellular responses, Collagen production

The long-term success of implants is the development of a stable direct connection between bone and implant surface, which must be structural and functional (Osseointegration). Successful osseointegration of endosseous implant results from a favorable interaction between implant geometry, surface texture and the tissue at bone site.[1] In general, only the surface of an implant is in direct contact with the host tissue, and thus this portion of the material plays a central role in determining its biocompatibility.[2] Mineralizing tissues have demonstrated the ability to bond directly to titanium (Ti) and the overall success of cellular attachment, whether occurring *in vitro* or *in vivo*, is dependent on many parameters, including surface roughness, chemical composition and oxide thickness and other surface properties.[3-6]

Some researchers investigated the influence of surface roughness on bone integration of implants.[3,6-13] *In vivo*, rough surfaces were found to produce better bone fixation than smooth surfaces suggesting that this surface property might have a substantial effect on the attachment of osteoblasts and their subsequent proliferation and differentiation. Buser et al[14] found that the extent of bone contact was positively correlated with an increasing roughness of implant surface and Gotfredsen et al[15] showed a significant higher bone-to-implant contact for TiO_2 blast implants in comparison with only machined implants. However, in the study of Larsson et al,[3] a reduction of surface roughness of titanium using electropolishing and anodic oxidation had no influence on the amount of bone after 12 weeks in rabbit cortical bone, and a high degree of bone contact and bone formation was achieved by titanium implants, which are modified with respect to oxide thickness and surface topography. Recently, some studies investigated the surface roughness of implants which affected bone cell response examined with cell culture.[16-18] Most of these results suggested that cell proliferation, differentiation, protein synthesis and matrix production be affected by surface roughness and chemical composition *in vitro*.

In the field of metallurgy, these surface treatments are known to induce chemical modifications of the implant, associated with the modifications of surface topography.[19] In the field of biomaterials, many studies have independently studied the surface topography and surface chemistry effects on in vitro and in vivo biocompatibility with bone but few have investigated their simultaneous biological effects.[7-11,14] Anselme et al[20] attempted to perform a concomitant analysis of the roughness and chemistry of polished and sandblasted titanium alloy surfaces and demonstrated the significance of chemical surface analysis after any surface treatment of titanium-based implants. Morra et al[21] also suggested that when comparing different topographies, it should be taken into account that surface chemistry might be a variable as well.

In vitro studies with cells from the target tissue are useful tools for the investigation of implant behavior, because these cells react differently on various surface structures and chemical properties. In vitro study has two major advantages; first, the control of the physicochemical environment (pH, temperature, O_2 and CO_2 tension) is possible; second, tissue samples are invariably heterogeneous after one and two passages. Therefore, culture cell lines assume a homogeneous (or at least uniform) constitution.

This study was attempted to perform a concomitant analysis of roughness and chemistry of some types of surface-treated titanium and the cell behaviors on that was investigated by morphological observation, cell proliferation and differentiation.

MATERIAL AND METHODS

2.1. Titanium discs preparation and characterization

Titanium discs were fabricated by Osstem (Busan, Korea) using commercially pure titanium. The discs

were cylinders of 10mm in diameter and 2mm in height (Fig. 1).

The discs were prepared to produce five different surface textures are followings;

1. EP : Electropolished;
2. AN : Anodized;
3. MA : Machined;
4. RBM : Blasted with hydroxyapatite (HA) particles(resorbable blast media)
5. EDM : Electric discharge machined.

All specimens were washed with alcohol and distilled water and sterilized with ethylene dioxide gas.

The prepared titanium discs were examined by following. At the first, the surface morphology of the discs was examined using a scanning electron microscopy(SEM) and surface roughness was measured by a three-dimensional roughness measuring system. Average surface roughness, maximum surface roughness, and skewness values were taken. X-ray diffractometer (XRD) analysis was performed to identify the surface composition and structures of the differently treated samples. Surface elemental composition of five kinds of specimen was analyzed with scanning Auger electron microscopy (AES).

2.1.1. Surface microtopography

SEM (JSM-840A, JEOL, Japan) was used to obtain an overall picture of the surface finish and the topography of the specimens. The SEM micrographs were taken at several randomly chosen areas on each specimens (× 2000).

2.1.2. Surface roughness

A confocal laser scanning microscope (CLSM), PASCAL LSM5 (ZEISS, Germany), was used for three dimensional roughness measurements and more detail topographic characterization of one sample of each preparation. The area of measurement was 450 × 450 ㎛. Following parameters were measured.

Sa : This is the arithmetic mean of the absolute values of the surface departures from a mean plane within the sampling area. The parameter is measured in ㎛ and is a general and commonly used parameter.

St : The value is arithmetic mean deviation of the distance of five highest peaks and five lowest valleys within the sampling area five lowest valleys within the sampling area and is measured in ㎛.

Ssk : Skewness is the measure of the symmetry of surface deviations about the mean plan.

2.1.3. Surface composition and structure

XRD (M18XHF^{22}SRA, MAC Science Co. Japan) was carried out to identify the high atomic number composition and structure. Using Cu-Kα radiation, the specimens were analyzed from 10 degrees(2θ) to 80 degrees(2θ). The tube voltage was 50kV, the tube current was 100 mA, the scanning width was 0.0200deg and scanning speed was 5deg/min.

2.1.4. Surface elemental composition

The surface elemental composition of each specimen was analyzed with AES (Perkin-Elmer, PHI660, USA). The oxide thickness was estimated by measuring the number of cycles for the titanium-oxygen (Ti-O) crossover point. The oxide thickness of all surfaces was approximated by multiplying the sputtering rate by the cross over time obtained from the Auger depth profile during continuous sputtering. For a Ti surface, the crossover time is the time corresponding to equal intensities of the Ti and oxygen (O) Auger signals.[22] The sputtering rate was 11.5㎚/min for TiO_2.

2.2. Cell culture and morphology

For all experiments calvarial osteoblast-like cells were used. Cells were isolated from calvaria of 1-2 day old rats. Rats were anesthetized with ether

and sacrificed. Parietal bones and frontal bones were removed by scissors, the periostea stripped off and the bone minced with scissors. Isolated calvaria were washed several times with Hanks Balanced Salts Solution (HBSS), and then digested with 0.05% Trypsin-4ml ethylenediaminetetraacetic acid (EDTA) with 150U/㎖ collagenase (type I). Supernatants were collected and centrifuged. These procedures were repeated six times. And the cells of 4-6th procedures were collected and cultures on discs placed in 24 well plates (Falcon, Becton Dickson, UAS).

Sterilized discs were placed in the 24-well plate, 1㎖ α-minimal essential medium (α-MEM : Gibco, USA) were added to the well plate. Cells were cultured 24 hours at 37℃, 5% CO_2, 95% air, and 100% humidity incubator (Forma Scientific, USA). After removing the culture media, the harvested rat calvarial osteoblast-like cells were resuspended in the medium. The cells were plated at 10,000 cells/discs in a α-MEM containing 10% fetal bovine serum (FBS) and 1% antibiotics-antimycotics (Gibco, USA). The cultures were incubated at 37℃, 5% CO_2, 95% air, and 100% humidity incubator (Forma Scientific, USA), and the media were changed every third day throughout the experiment.

To determine whether cell morphology varied as a function of the surface roughness and chemical composition, the culture were examined by SEM. Cell morphology studies can provide information concerning the cell interaction with the treated disc and cell-to-cell contact. At 6 hours, 48 hours, 14 days, the samples were harvested and the culture media were removed. The discs were then rinsed three times with HBSS and fixed for 60 minutes with 2.5% glutaldehyde and 0.1% cacodylate buffer (pH 7.4). After fixation, the discs were rinsed with 0.1M cacodylate buffer, sequentially incubated for 30-45 minutes each 50, 75, 90 and 100% ethyl alcohol. And then discs were dried by CO_2 critical point drier (Bio-Rad, England) and sputter-coated with gold (36nm thickness). Morphologic analysis was performed by SEM (JSM-840A, JEOL, Japan).

2.3. Total protein measurement

To determine cellular proliferation, cellular protein concentrations were measured with a microBCA (bicinchoninic acid) protein assay (Pierce). After 48 hours culture and 14 days culture, the osteoblast-like cells on each specimens were treated with a 0.05% Trypsin-0.025M EDTA solution several times. Cell suspensions from trypsination were centrifuged at 1300rpm (500×g) for 10 minutes. Cell pellets were washed with trishydroxymethyl aminomethane (Tris) buffer (Trisma base, Sigma, USA) and resuspended in Trisbuffer. Isolated cells and titanium discs were washed protein lysis buffer. Their protein contents were determined by the method of Lowry et al.[23] Extracted protein production was determined using Pierce BCA reagent (BCA Protein Reagent Kit, Pierce, USA) and the absorbance read was performed using spectrophotometer (UV=1601, Shimadzu, Japan).

2.4. Alkaline Phosphatase activity

After 48 hours culture and 14 days culture, alkaline phosphatase (Orthophosphoric monoester phosphohydrolase alkaline) activity was assayed as the release of ρ-nitrophenol from ρ-nitrophenylphosphate at pH 10.4.

A 20㎕ aliquot of the cell lysate used was added to 200㎕ ρ-nitrophenylphosphate solution (NPP : Sigma, USA). Cells were incubated for 30min at 37℃ in 0.1% Triton X-100/saline, 0.1M glycin-NaOH buffer. The reaction was stopped using 2N sodium hydroxide and the absorbance read was performed at 405nm using microplate reader (Themomax, Molecular devices, USA). This value was compared with the values of a standard series. The specific activity was calculated referring to protein concentration of lysates.

2.5. Type I collagen measurement

Collagen assay was based on the binding of a dye, Sirius Red F3BA (Chroma, stuttgart, Germany), to the triple helical collagen fibril.[24] After 14 days culture, 50 μl /well of cell lysate was dispersed into 24-well plates, incubated at 37℃ for 16 hours in humidified atmosphere and then for 24 hours in dried atmosphere. The staining was performed with 100 μl /well of 0.1% Sirius Red F3BA in saturated picric acid for 1 hour at room temperature. The plates were rinsed five times with 200 μl of 10mM HCl, then washed with 0.1M NaOH and absorbance was read at 540 nm. The optical density values were compared to the standard curve of type I collagen samples.

2.6. Statistical analysis

For the cell responses, all data presented were from 3-6 replicate experiments, which yield comparable results. Results were expressed as mean values and standard deviation of each group of samples. Statistics were performed by analysis of variance (ANOVA), followed by Scheffe' s test to determine the significance between groups. P values < 0.05 were considered to be significant.

RESULTS

3.1. Specimen characteristics

3.1.1. Surface microtopography

(1) Electropolished discs

The electropolished surface appeared to have a shallow large crater, wavy morphology, with some porosity and spherical pits. Traces of machining grooves were not observed on the electropolished surfaces(Fig. 2A).

(2) Anodized discs

The anodized surfaces and the machined surfaces had a relatively similar appearance, with machining grooves in the 1-10㎛ range as a main feature. The anodized surfaces also showed a rather smooth appearance and more homogeneous than machined surface(Fig. 2B).

(3) Machined discs

Typical machining grooves, as produced by manufacturing instruments, were observed in the surface of titanium discs with a rather irregular surface topography consisting of microgrooves, small pits and elevated area of dimensions approximately 10 ㎛ and smaller(Fig. 2C).

(4) RBM discs

The RBM surfaces had a rough and irregular pattern. Reticulated appearance with undermining deformation of the resorbable hydroxyapatite material blasted under pressure on the surface was observed (Fig. 2D).

(5) EDM discs

The EDM surfaces were rough and very irregular pattern. The surface of the EDM implant was heterogeneous, consisting of areas with a broad sheet or plate with the smooth edges associated with some globules or grains which looked like the result of melting and resolidification(Fig. 2E) and areas made up of clusters of small grains in deep pore or crater(Fig. 2F). EDM had evident substrate cracks and showed many deep pore.

3.1.2. Surface roughness

Table I and figures 3, 4, 5 showed that results of three dimensional roughness measurements.

The lowest surface roughness was observed in the EP group and highest was in the EDM group.

The Ssk values of the EP, AN, MA and RBM were negative shift, which had more valleys than peaks. The Ssk value of EDM showed positive shift, which had more peaks than valleys.

Table I. Result of three dimensional measurement

Group	Roughness		
	Sa(㎛)	St(㎛)	Ssk
EP	0.66	13.11	-0.89
AN	0.77	14.44	-0.67
MA	0.89	11.59	-0.54
RBM	1.66	23.52	-0.03
EDM	4.20	56.97	0.47

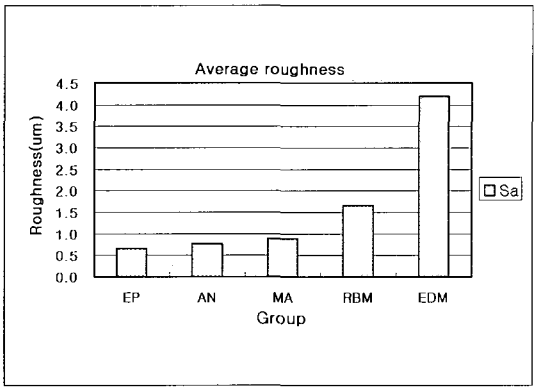


Fig. 3. Average roughness measurement.

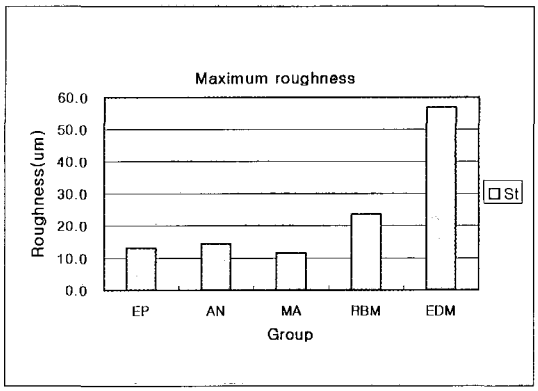


Fig. 4. Maximum roughness measurement.

3.1.3. Surface composition and structure

Fig. 6 showed the result of XRD analysis. In the EP, AN, MA and RBM group, the X-ray diffraction spectroscopy expressed only Ti peaks, amorphous in crystal structure.

EDM group showed titaniumcarbide(TiC) peaks and one Ti peak in the XRD pattern.

3.1.4. Surface composition and oxide thickness

The relative concentrations of the elements detected in AES survey spectra were represented graphically in Fig. 7. The spectra were in all cases dominated by strong Ti, O and C signals. In RBM groups, trace amount of Ca, P and S were detected and in the AN groups, weak S signal was detected.

The depth profile for Ti, O, and C showed a different qualitative behavior for all samples. In EP, MA and EDM groups, O signals rapidly disappeared. This decrease was accompanied by an increase in Ti signals. The sputtering time required for the Ti signal crossover with O signal was taken as a measurement of the oxide thickness. EP, MA and EDM group had similar oxide thickness, and RBM group and AN group had thick oxide films. The oxide thickness were approximately 8.6 ㎚ in EP group, 126.5 ㎚ in AN group, 7 ㎚ in the MA group, 115 ㎚ in the RBM group and 11.5 ㎚ in the EDM group.

In RBM and EDM, carbon(C) signals showed no decrease but constantly existed.

3.2. Cell morphology

3.2.1. Cell morphology after 6 hours

More cells were observed on EP and AN and MA compared to RBM surfaces. The cells did not

have regular orientation; they looked scattered in all directions. On EP, AN, MA surfaces, osteoblast-like cells attained partial confluence(Fig. 8A). Cells on EP, AN and MA surfaces were observed to be flattened and have polygonal shape. The cell body formed such intimate contact with the implant surface that the underlying topography of the surface was visible beneath the cell(Fig. 8B and C).

The cells on the RBM surface were more irregularly shaped, and adaptation to surface irregularities was not observed(Fig. 8D). After 6 hours, on the RBM the osteoblasts appeared to be thicker than on the smooth discs. The attaching ends were well spread. Sometimes the osteoblasts also possessed many thin, capillary-like extensions. The flattening and spreading cells spanned across pits and pores, mostly contacting prominent features of the surface.

On the EDM surface, cells had unique morphology. Cells attached to all areas with atypical and abnormal appearance but most of those on the granulated or porous surface were spherical appearance, while on the relative flat areas the cells were most frequently flatten but thick cytoplasm(Fig. 8E).

3.2.2. Cell morphology after 48 hours

The osteoblast cells on EP, AN and MA surfaces were fully spread and confluent(Fig. 9A) and adjoining cells were oriented with preference directions(Fig. 9B). The cells exhibited such close contact with each other that detection of the complete cell perimeter was difficult(Fig. 9C). In some regions the osteoblasts started to form a multilayer (Fig. 9D).

The cells on the RBM and EDM surfaces were partial confluent and separated from each other(Fig. 9E and F). They had no particular orientation.

3.2.3. Cell morphology after 14 days

The cells cultured on all groups were observed to be confluence and exhibit multilayer proliferation, often overlapped/stratified.

3.3. Total protein measurement

The proliferation pattern described as total protein amount was observed to be quite similar among all the group at 48 hours but not at 14 days. The mean values of total protein contents were, from the highest to the lowest, MA, EDM, EP, AN and RBM after 48 hours culture. There was no significant difference among five groups for total protein amount at 48 hours. Total protein amounts of 14 days cultivation were, from the highest to the lowest, AN, EP, MA, EDM and RBM. At this time point, The AN group induced a statistically more total protein amount than the RBM group ($P<.05$)(Table II and Fig. 10).

3.4. Alkaline phosphatase activity

Table III and figure 11 showed ALP activity of five groups. After 48 hours, the mean values of ALP activity were, from the highest to the lowest, RBM, AN, EP, MA and EDM. There was no significant difference among five groups for ALP activity at 48 hours. The AN group was significantly higher ALP activities than any other group at 14 days ($P<.05$). After 14 days, the mean values of ALP activity were, from the highest to the lowest, AN, RBM, MA, EP and EDM.

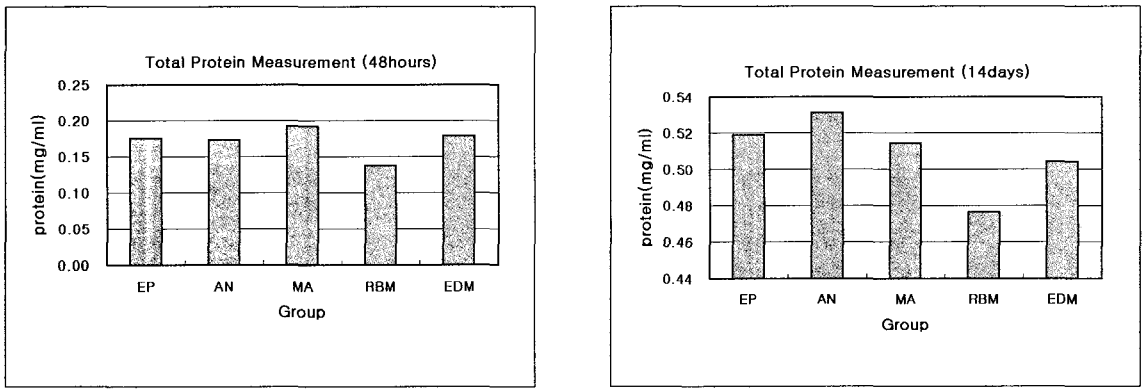
3. 5. Type I collagen measurement

Table IV and figure 12 showed the amounts of type I collagen produced by osteoblast-like cells at 14 days cultures. All four groups showed similar collagen synthesis except the EDM group. The type I collagen productions were, from the highest to the lowest, AN, EP, RBM, MA and EDM. The amounts on the EP and AN surfaces were higher those on the EDM surface ($P<.05$).

Table II. The result of total protein measurement (mg/ml)

		EP	AN	MA	RBM	EDM
Cultivation for 48h	Mean	0.176	0.174	0.193	0.138	0.179
	S.D.	0.084	0.094	0.089	0.038	0.085
Cultivation for 14d	Mean	0.519*	0.531*	0.515	0.477*	0.504
	S.D.	0.004	0.014	0.010	0.006	0.006

Data were expressed as mean values and standard deviation(S.D.).
Significant difference between values marked with asterisk(P<.05).



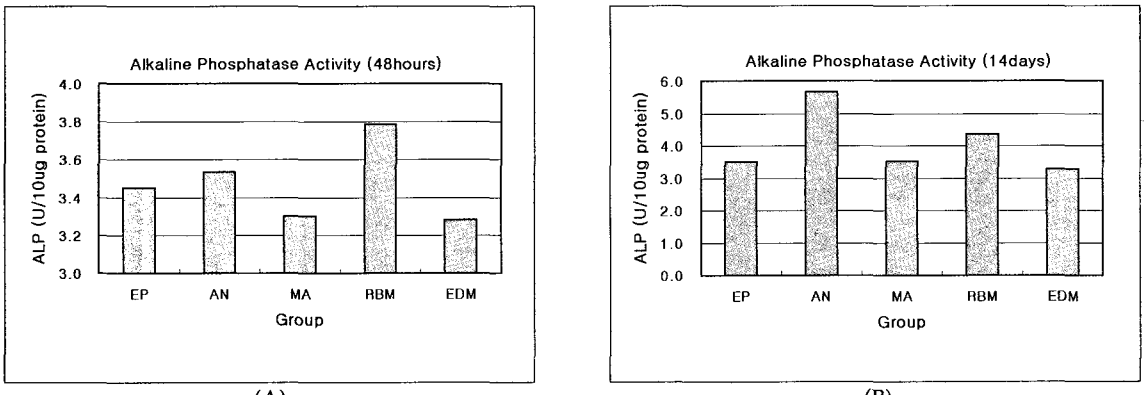
(A) (B)

Fig. 10. The result of total periodontal measurement: (A) after 48 hours, (B) after 14 days.

Table III. The result of alkaline phosphatase activity(Unit/10㎍ protein)

		EP	AN	MA	RBM	EDM
Cultivation for 48h	Mean	3.454	3.536	3.303	3.786	3.284
	S.D.	0.583	0.554	0.579	0.533	0.546
Cultivation for 14d	Mean	3.520	5.670*	3.534	4.355	3.288
	S.D.	0.150	0.049	0.534	0.098	0.056

Data were expressed as mean values and standard deviation(S.D.).
Significant difference between values marked with asterisk(P<.05).



(A) (B)

Fig. 11. Alkaline phosphatase specific activity: (A) after 48 hours, (B) after 14 days.

Table IV. The result of type I collagen production (mg/10ml)

		EP	AN	MA	RBM	EDM
Cultivation for 48h	Mean	4.440*	4.673*	4.180	4.367	3.457*
	S.D.	0.145	0.218	0.200	0.123	0.154

Data were expressed as mean values and standard deviation(S.D.).
Significant difference between values marked with asterisk (P<.05).

DISCUSSION

The aim of the present study was to examine the cellular response and the interaction between neonatal rat calvarial osteoblasts and several surface-treated implants. The cell morphology using SEM and total protein amount were taken as a marker for cell proliferation while the expression of alkaline phosphatase, an enzyme thought to be involved in preparing the extracellular matrix for an ordered deposition of mineral, was used as a early differentiation marker for osteoblast. The type I collagen production was determined, which constitutes the main structural protein of the extracellular matrix and thus may be a reliable indicator of bone matrix synthesis.

The purpose of surface treatment is to alter the surface properties that enable more favorable cellular responses at implant-tissue interface. *In vitro* studies using osteoblast-like cell culture had examined the effects of surface roughness on cell attachment.[16-18] They concluded that rougher surface was more favorable than smoother one for the cell attachment. In addition some *in vivo* studies supported those observation.[14,15] Chemical composition, oxide thickness, surface microtopography also might play roles. Several studies showed that even subtle difference in surface composition, including Ti oxide crystallinity could modify cell responses, even when surface roughness was held constant.[25,26] Stanford et al.[27] and Lim et al.[12] reported that roughness per se did not promote cell attachment. The works described that roughness was not as important as other surface properties in biologic responses, other surface properties should also be considered important in the biologic response and might be more critical parameters of biocompatibility than surface roughness.

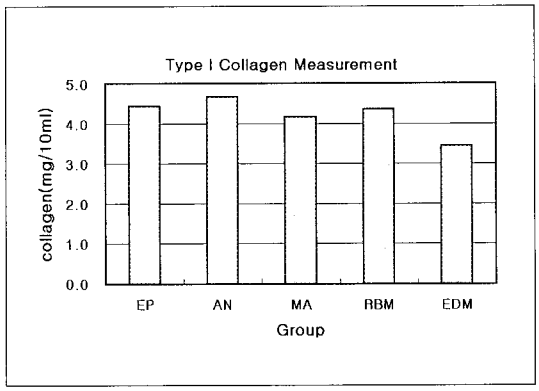


Fig. 12. Type I collagen production after 14 days.

Titanium naturally forms a thin oxide layer in air. The biocompatibility of titanium is largely related to the its oxide layer.[2] Oxide depth can be estimated by using the titanium-oxygen(Ti-O) crossover point or by measuring the number of cycles for the oxygen peak to reach half the maximum concentration in Auger electron spectroscopic analysis(AES).[28] The two methods of determining oxide thickness showed similar trends, though thickness by Ti-O crossover point is attained relative thin oxide. The EP, MA, EDM discs had thin oxides of similar thickness, while RBM and AN discs had much thicker oxides. In AES analysis of RBM and EDM, carbon (C) signals showed no decrease but constantly existed. In EDM, TiC structure seems to be responsible for prolonged C signal and in RBM, carbon appeared to be interstitial structure. These car-

bon structure could affect oxide layer quality and cellular responses.

There were controversies about the relationship of oxide thickness and cellular reponses. Ellingen and Videm[29] showed that there was no significant correlation between oxide thickness an titanium implants and the bony responses. They explained the reason why the outermost molecular layer of the biomaterials is the most important part for the bone response, because the surface chemistry of these layer is exposed to the tissue. But Hazan et al[30] found recently that heat treatment of titanium alloy implants, leading to increased oxide-thickness, leads to higher removal torque and higher degree of calcification of the bone around implants in rats. It was not known where or not the oxide thickness alone affected the cellular responses. In this study, thicker oxide film groups showed higher alkaline phosphatase activity and collagen production. It could be thought that thicker oxide thickness groups showed higher cell differentiation.

SEM and roughness measuring system identified significant differences also in the surface microtopography and roughness of the different groups of discs. The microtopography of anodized disc in this study is similar to those of Larsson et al[31] and Velten et al.[32] In the case of the anodic oxide film, surface properties have been shown to be sensitive to oxide growth conditions depended on a particular electrolyte used and on the employed anodic process parameters such as the applied current density, the electrolyte concentration, the electrolyte temperature, agitation speed, and cathode to anode surface area ratios. In general, it has been shown that various surface characteristics of anodic films are accompanied with an increase of oxide thickness.

When cell morphology is concerned, generally cells on the smooth surfaces are flatted shape and cells on the rough surfaces are spherical shape. Lauer et al[33] found that cells on polished surfaces developed an extremely flat cell shape, but on sandblast surfaces a more cuboidal shape. Sequential morphologic changes of osteoblast-like cells are a function of time, and detailed descriptions of cellular morphology have been reported in several previous studies.[34-36]

Whereas on the relative smooth surfaces (EP, AN and MA) of this study, the cells showed a flattened fibroblast-like morphology and well spreaded on discs, on the rough surface (RBM), the cells were three-dimensionally oriented and filled all pits and crevices provided. The cells formed focal attachments at the end of surface protrusions, permitting them to span the space between surface peaks. This have been described by other authors as well.[35-37] On the cellular level, it is known that a rough surface promotes the differentiation of osteoblasts, whereas the proliferation mostly is downregulated. On a rough surface cells already have these cell-to-cell contacts at lower cell concentrations because of the three-dimensional network to the substrate and so they mineralize even without a noticeable multilayering growth and nodule formation of osteoblast. On EDM surface, the peculiar form of cell attachment was shown. It seems that cell morphologic behavior may be influenced by irregular heterogeneous topography and chemical composition of TiC on EDM disc.

The cell proliferation described as total protein amount with occurred equally well on all substrates at 48 hours. However, at 14 days RBM group showed less cell proliferation than anodized group revealing significant differences. The relative weak proliferative responses, in conjunction with increased alkaline phosphatase activity on the rougher surfaces, suggest that osteoblast-like cells grown on rough surfaces are at a more advanced stage of development than their counterparts on the smooth surfaces.[10] The other possible explanation is such that the sharp angularity of rough surface hinders anchorage of cell processes on adjacent regions and thus slows down the process of attachment.[35]

ALP is glycoprotein associated with the formation

of calcified tissues. ALP activity is expressed early along the maturational pathway of bone cells and is retained until the formation of early osteocytes and then lost. ALP is present in the osteoblast and in the matrix vesicle membrane and is very good indicator of osteogenic differentiation, bone formation and matrix mineralization. Various studies already demonstrated that the alkaline phosphatase activity expressed by osteoblast-like cells on titanium and titanium alloys was much influenced by surface roughness. Ong et al[17] found that rougher surface showed prolonged ALP specific activity and a more rapid osteocalcin production as compared with the polished titanium surface. Hong et al[38] reported that the level of ALP activity was higher in the AN group with higher roughness and thicker oxide layer than in EP and MA groups and described that the anodized surface was more favorable than other surface in osteoblast differentiation.

In this study, different specific alkaline phosphatase activity was found depending on the type of surface and on the period of culture. In detail, at 48 hours, there was no significant difference among five groups for ALP activity. At this time point, RBM group the highest ALP enzyme activity. Whereas RBM group showed only slight increase in alkaline phosphatase activity at 14 days, surprisingly increasement of enzymatic activity on AN group exhibited a more prolonged effect on the time, which induced a significant level. This suggested that rough surface became more effective in earlier stage and surface with thick oxide film was more favorable than other surfaces in osteoblast differentiation. Montanaro et al[39] described that roughness appeared to cause to its effects only at an early stage. This behaviour could be explained by the growth in multilayer of cells and by the progressive apposition of extracellular matrix on the surface, both possibly capable to mask the initial effects of the surface.

In present study, collagen production on EDM surface was significantly lower than those on AN and EP surfaces. It seems that collagen synthesis was influenced by surface chemical composition and was negatively affected by the TiC rich layer of EDM group. As far as proliferation and collagen secretion are concerned, similar responses have already been reported for osteoblast-like cells.[40-42] The cellular growth and type I collagen production was influenced either by the chemical nature of the substratum or by the configuration of its surface.

Electrical discharge machining is available in cp Ti and Ti-alloy process. Bigerelle et al[43] found EDM surfaces are more favourable to osteoblast adhesion and proliferation than polished or machined surfaces. Authors described that EDM process transformed the surface to a thin film of titanium oxide (mainly TiO_2) that was not detected on the polished surfaces. However, the EDM discs of this study had strong TiC peaks on XRD spectra and this TiC could have negative influence on the osteoblast-like cell responses, even if EDM discs had favorable surface topography. The presence of TiC was explainable through the study of Lee et al.[44] They fabricated a porous-coated Ti-6Al-4V implant by electrodischarge compaction(EDC), which consisted of a complex of TiO_2 as well as small amounts of TiC and TiN. They proposed that after oxide film breakdown, the alloy repassivation by oxidation, nitrification and carburetion with atmospheric components is responsible for the altered surface composition.

The present study demonstrated that the surface treatment could affect the surface properties and the surface roughness, oxide thickness and surface chemical composition have an effect on osteoblast-like cell proliferation, differentiation and matrix production. Biocompatibility test such as those performed in this study could only quantify particular aspects of cell behavior. The cell reaction to an implant is however a very complex situation and can only be partially understood using standard biological assays. Further studies such as histomorphometric analysis in long-term periods are required.

CONCLUSIONS

1. Each prepared specimen showed specific microtopography at SEM examination. The RBM group had a rough and irregular pattern with reticulated appearance. The EDM-treated surface had evident cracks and was heterogeneous consisting of broad sheet or plate with smooth edges and clusters of small grains, deep pores or craters.
2. Surface roughness values were, from the lowest to the highest, electropolished group, anodized group, machined group, RBM group and EDM group.
3. All groups showed amorphous structures. Especially anodized group was found to have increased surface oxide thickness and EDM group had titaniumcarbide structure.
4. Cells on electropolished, anodized and machined surfaces developed flattened cell shape and cells on RBM appeared spherical and EDM showed both. After 14 days, the cells cultured from all groups were formed to be confluent and exhibited multilayer proliferation, often overlapped or stratified.
5. Total protein amounts were formed to be quite similar among all the group at 48 hours. At 14 days, the electropolished group and the anodized group induced more total protein amount than the RBM group($P<.05$).
6. There was no significant difference among five groups for alkaline phosphatase activity at 48 hours. The AN group showed significantly higher ALP activity than any other groups at 14 days($P<.05$).
7. All the groups showed similar collagen synthesis except the EDM group. The amount of collagen on the electropolished and anodized surfaces were higher than that on the EDM surface($P<.05$).

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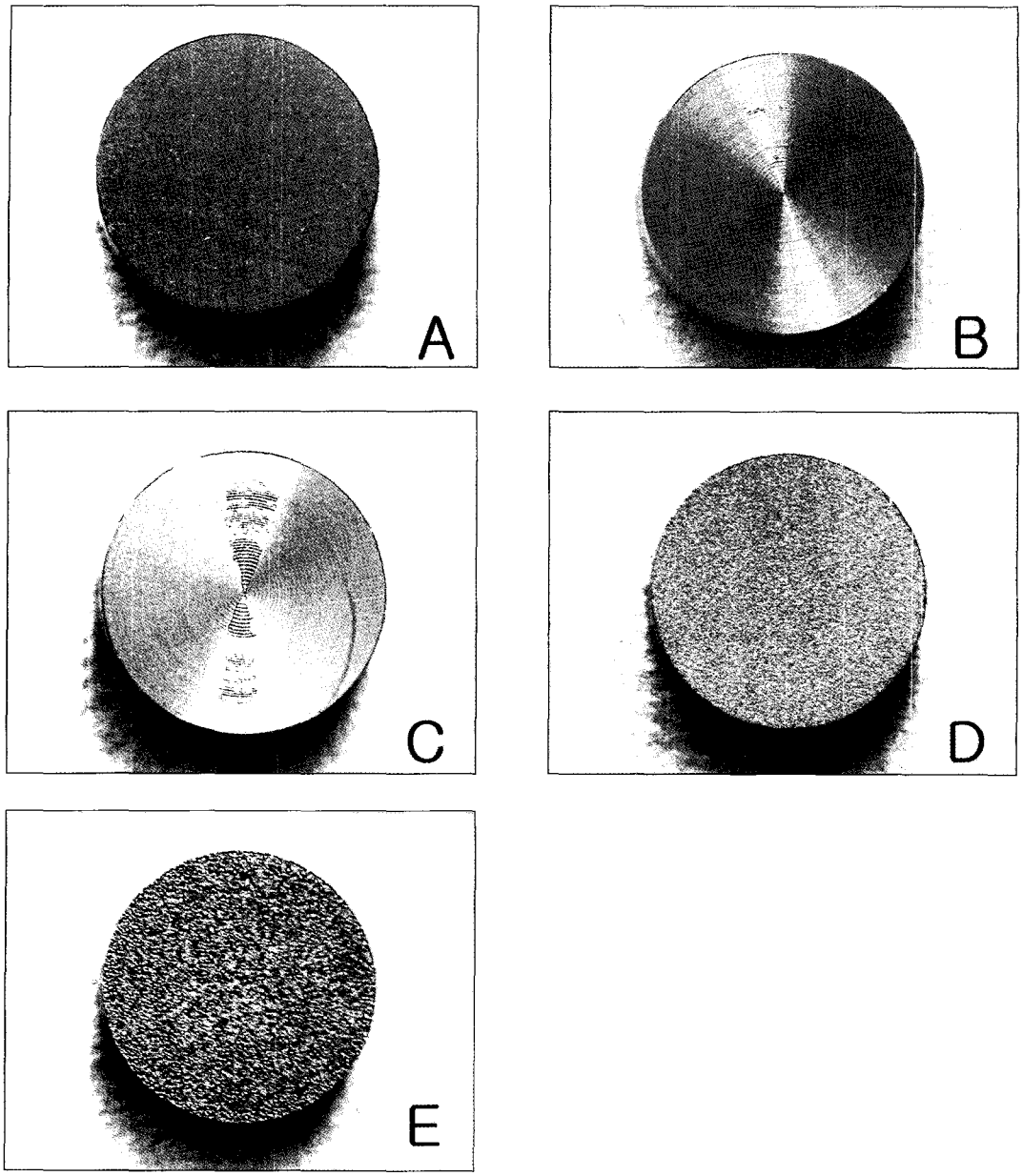

Fig. 1. Photographs of each discs.
A. Electropolished surface.
B. Anodized surface.
C. Machined surface.
D. RBM surface.
E. Electrodischarged surface.

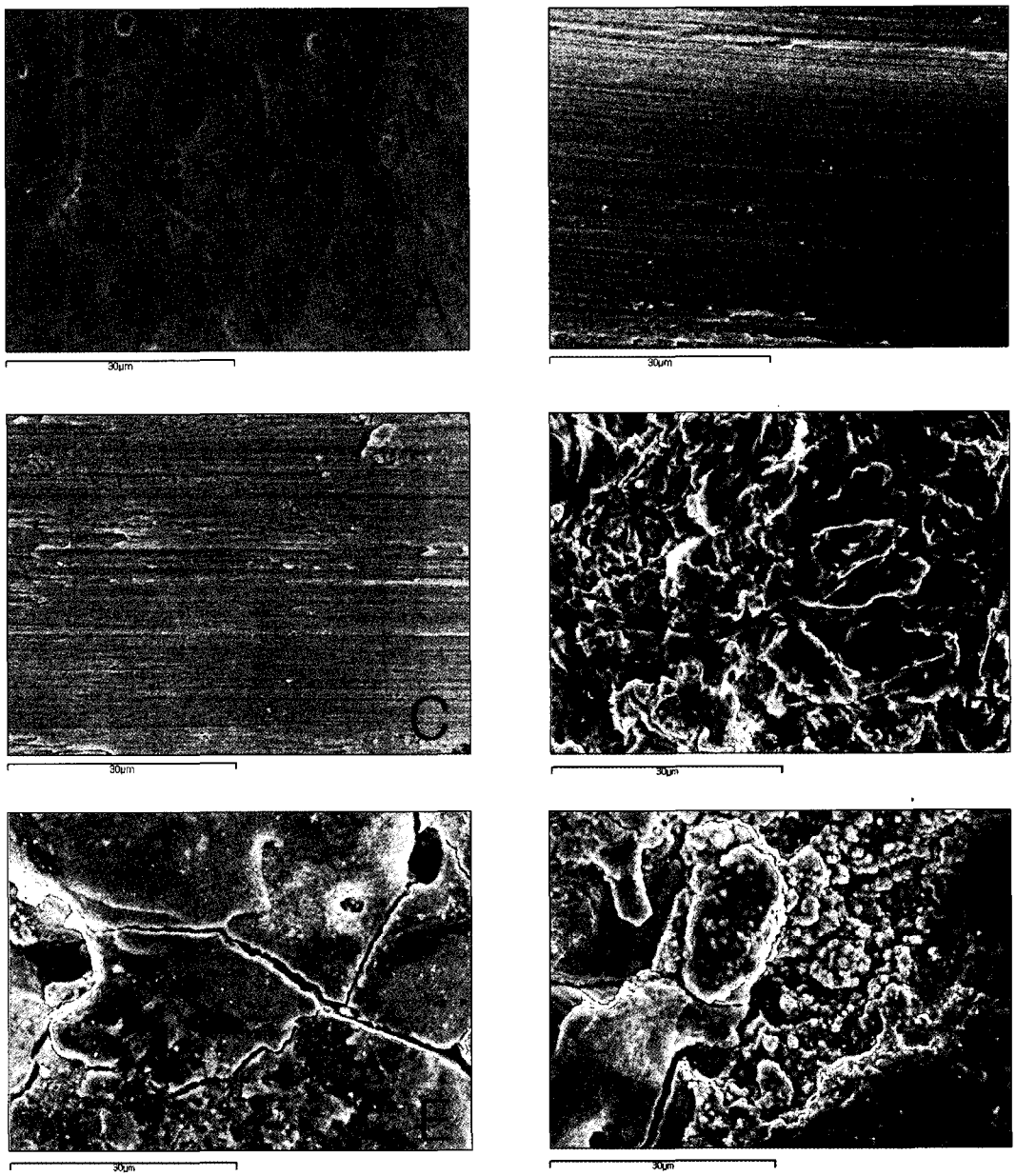

Fig. 2. SEM photographs of each specimens.
A. Electropolished surface (×2000).
B. Anodized surface (×2000).
C. Machined surface (×2000).
D. RBM surface (×2000).
E. Electrodischarged surface (×2000).
F. Electrodischarged surface (×2000).

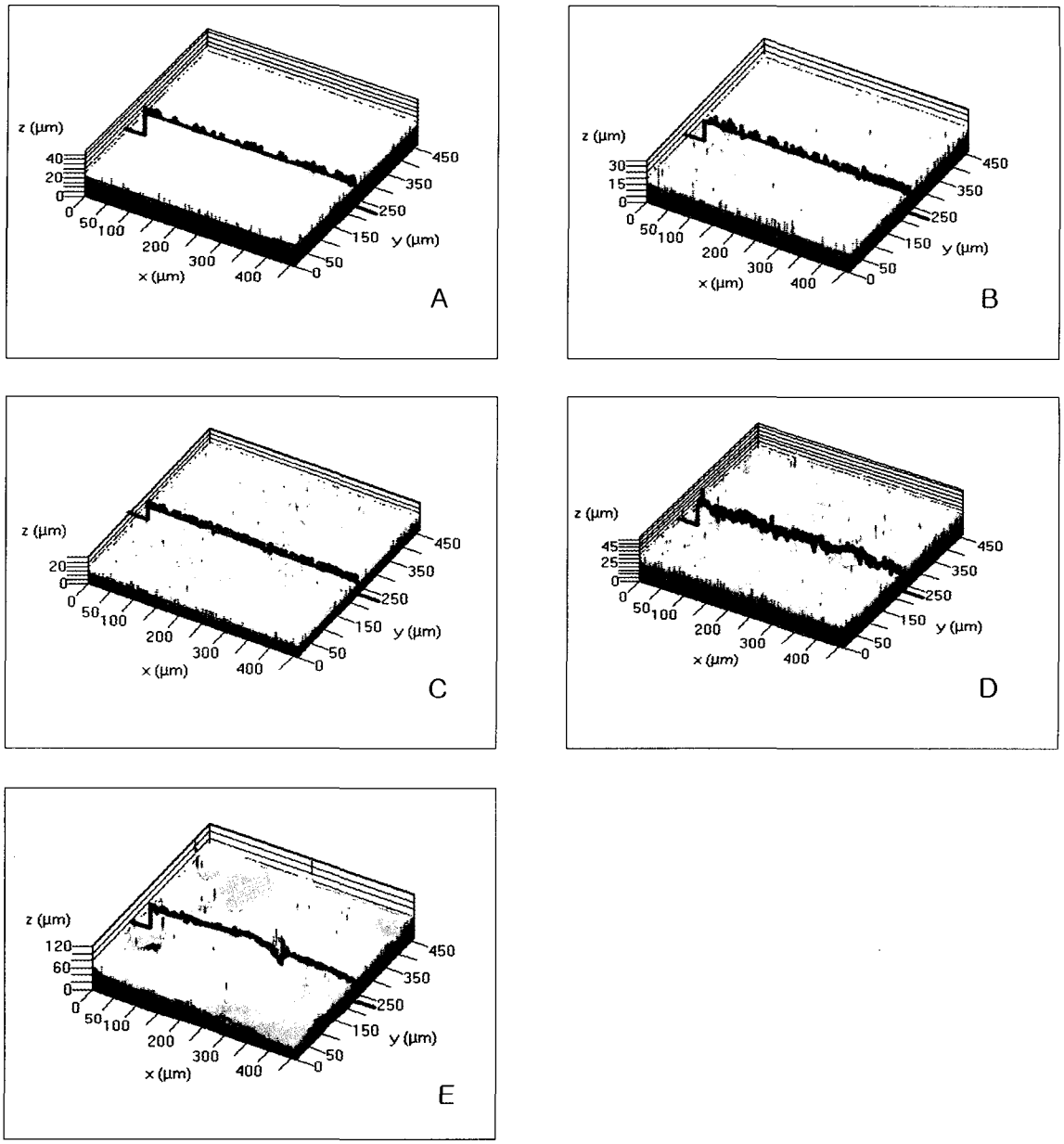

Fig. 5. The results of surface roughness measurement.
A. Electropolished surface.
B. Anodized surface.
C. Machined surface.
D. RBM surface.
E. Electrodischarged surface.

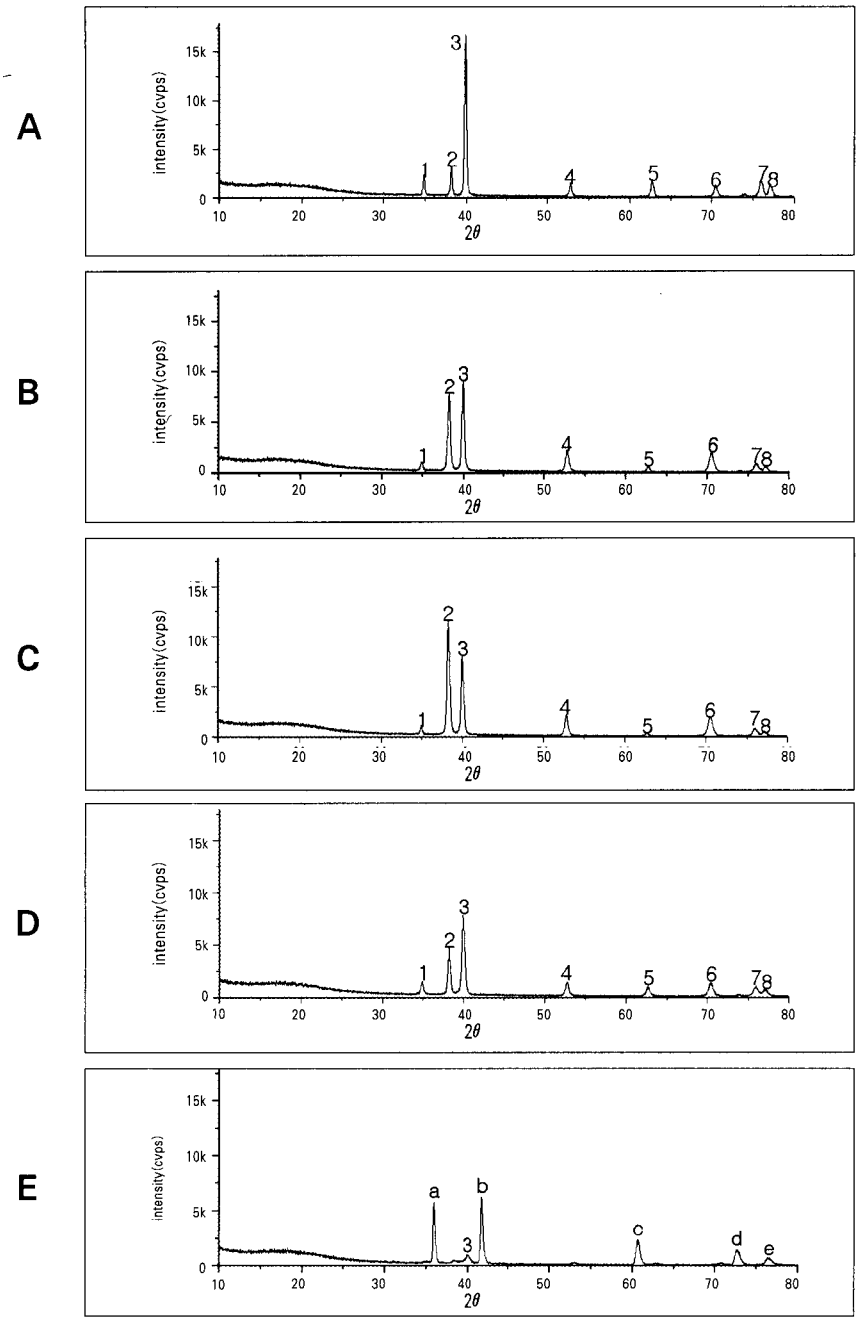

Fig. 6. The results of XRD analysis.
A. Electropolished surface. B. Anodized surface. C. Machined surface.
D. RBM surface. E. Electrodischarged surface.
Every peak in the A, B, D, E is Ti.
The a, b, c, d, e peaks in the E are TiC and the 3 peak is Ti.

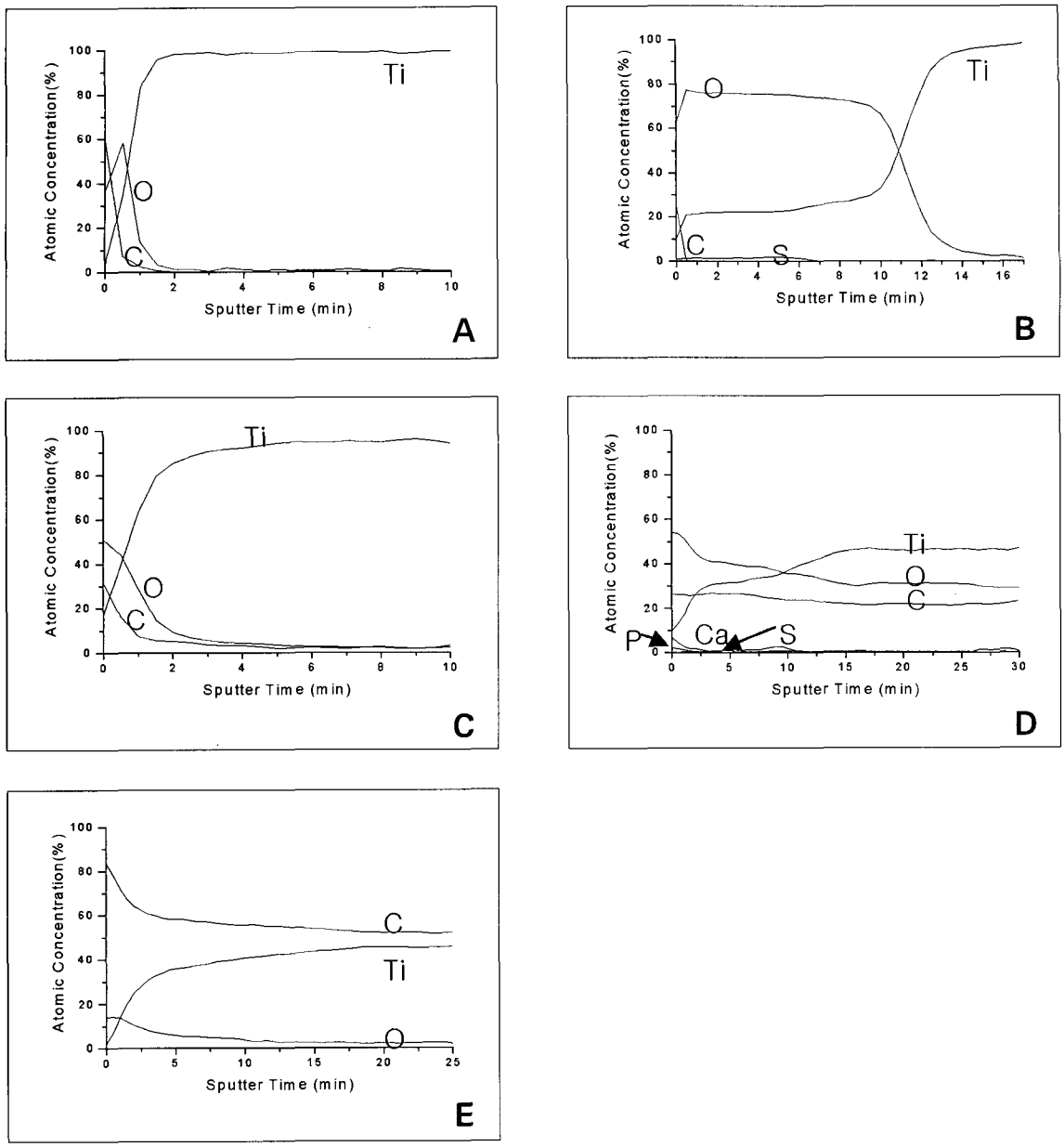

Fig. 7. The results of an auger electron scanning microscopy.
A. Electropolished surface.
B. Anodized surface.
C. Machined surface.
D. RBM surface.
E. Electrodischarged surface.

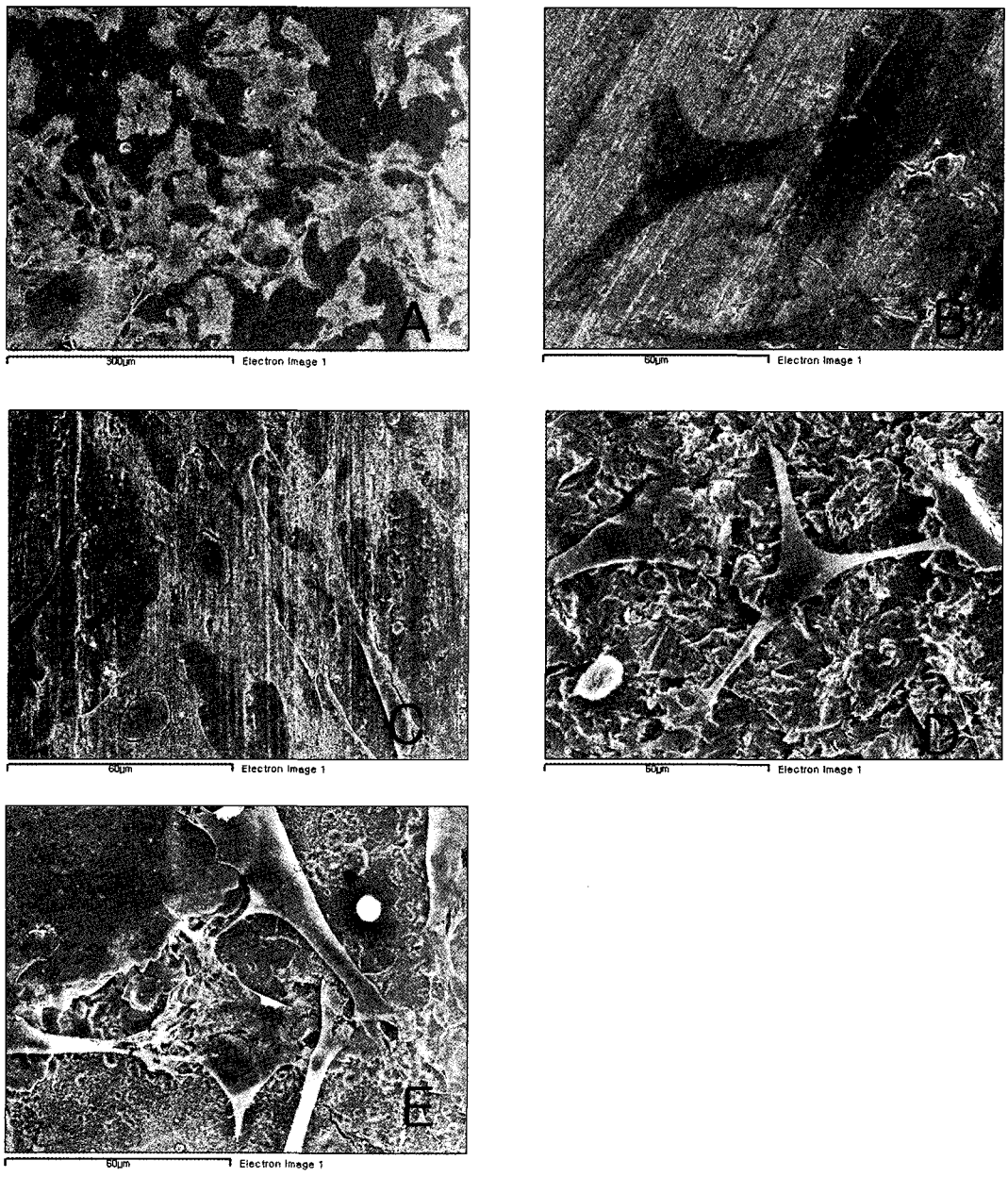

Fig. 8. SEM photographs cells cultured for 6 hours.
A. Electropolished surface (× 200).
B. Anodized surface(× 1000).
C. Machined surface (× 1000).
D. RBM surface(× 1000).
E. Electrodischarged surface(× 1000).

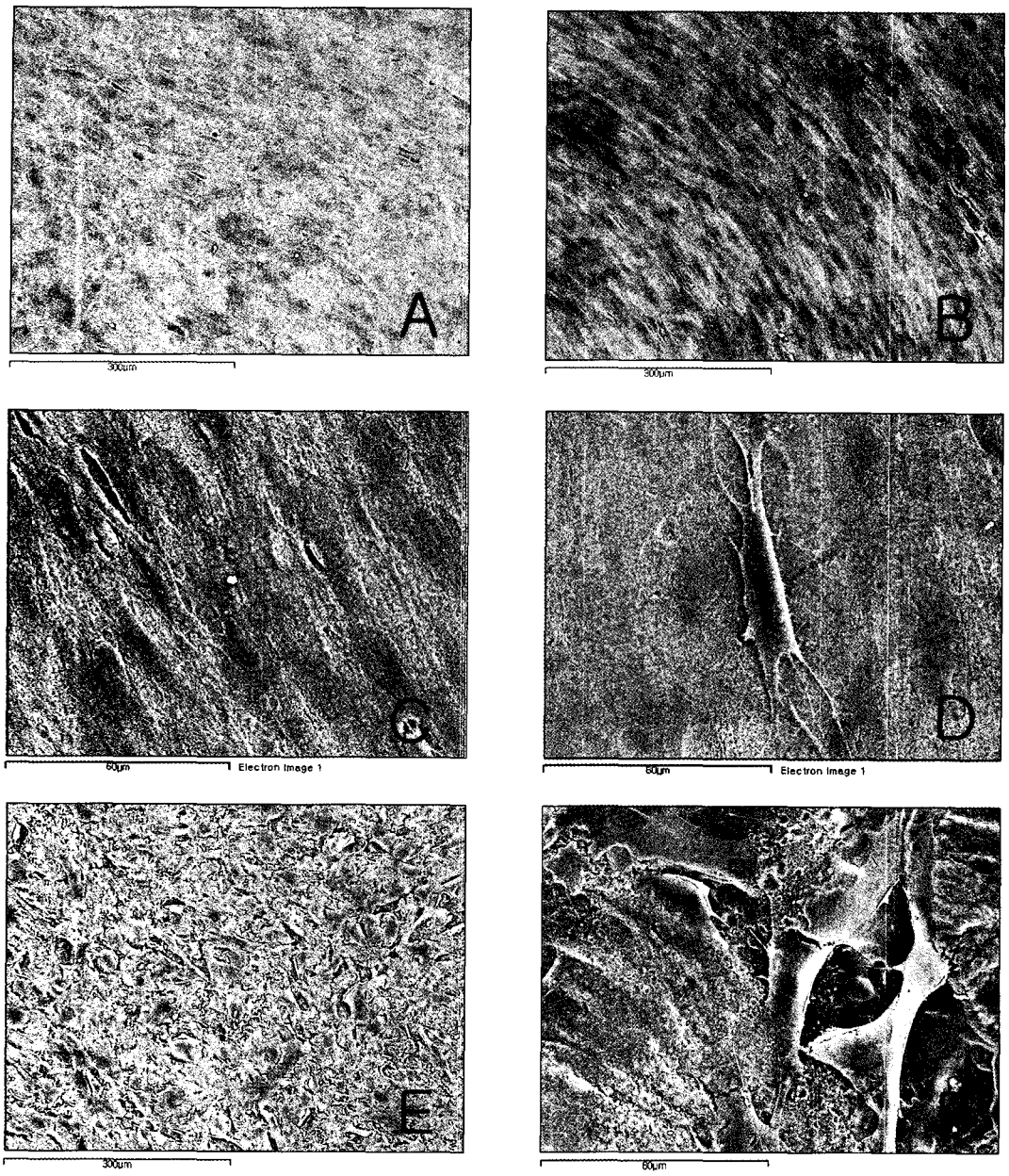

Fig. 9. SEM photographs cells cultured for 48 hours.
A. Electropolished surface (×200).
B. Machined surface (×200).
C. Anodized surface (×1000).
D. Electropolished surface (×1000).
E. RBM surface (×200).
F. Electrodischarged surface (×1000).