The Otto Aufranc Award

Enhanced Biocompatibility of Stainless Steel Implants by Titanium Coating and Microarc Oxidation

Young Wook Lim MD, Soon Yong Kwon MD,
Doo Hoon Sun MD, Yong Sik Kim MD

Published online: 9 October 2010
© The Association of Bone and Joint Surgeons® 2010

Abstract

Background Stainless steel is one of the most widely used biomaterials for internal fixation devices, but is not used in cementless arthroplasty implants because a stable oxide layer essential for biocompatibility cannot be formed on the surface. We applied a Ti electron beam coating, to form oxide layer on the stainless steel surface. To form a thicker oxide layer, we used a microarc oxidation process on the surface of Ti coated stainless steel. Modification of the surface using Ti electron beam coating and microarc oxidation could improve the ability of stainless steel implants to osseointegrate.

Questions/purposes The ability of cells to adhere to grit-blasted, titanium-coated, microarc-oxidated stainless steel in vitro was compared with that of two different types of surface modifications, machined and titanium-coated, and microarc-oxidated.

Each author certifies that he/she has no commercial associations (eg, consultancies, stock ownership, equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article.

This work was performed at Seoul St Mary's Hospital and St. Mary's Hospital.

Y. W. Lim, Y. S. Kim (✉)
Department of Orthopaedic Surgery, Seoul St Mary's Hospital,
505, Banpo-dong, Seocho-gu, Seoul, Korea
e-mail: yongsik@korea.com

S. Y. Kwon
Department of Orthopaedic Surgery, St Mary's Hospital,
Seoul, Korea

D. H. Sun
Department of Orthopaedic Surgery, Sun Hospital,
Daejeon, Korea

Methods We performed energy-dispersive x-ray spectroscopy and scanning electron microscopy investigations to assess the chemical composition and structure of the stainless steel surfaces and cell morphology. The biologic responses of an osteoblastlike cell line (SaOS-2) were examined by measuring proliferation (cell proliferation assay), differentiation (alkaline phosphatase activity), and attraction ability (cell migration assay).

Results Cell proliferation, alkaline phosphatase activity, migration, and adhesion were increased in the grit-blasted, titanium-coated, microarc-oxidated group compared to the two other groups. Osteoblastlike cells on the grit-blasted, titanium-coated, microarc-oxidated surface were strongly adhered, and proliferated well compared to those on the other surfaces.

Conclusions The surface modifications we used (grit blasting, titanium coating, microarc oxidation) enhanced the biocompatibility (proliferation and migration of osteoblastlike cells) of stainless steel.

Clinical Relevance This process is not unique to stainless steel; it can be applied to many metals to improve their biocompatibility, thus allowing a broad range of materials to be used for cementless implants.

Introduction

The essential requirements of cementless stems are high biocompatibility, high mechanical strength, high corrosion resistance, and durability [11]. Presently, titanium (Ti) alloy is widely used in cementless arthroplasty implants because it satisfies these requirements [6, 11, 14]. However, Ti alloy is difficult to work with during manufacturing, and it is expensive since the demand for Ti has increased due to its use in various industrial fields. Thus, in developing

countries especially, cemented stems are more commonly used than cementless stems [23].

Stainless steel is one of the most frequently used biomaterials for internal fixation devices because of its favorable combination of mechanical properties, corrosion resistance, cost effectiveness, and ease of manufacturing [4, 21]. However, stainless steel is not used in cementless arthroplasty implants because of its high modulus of elasticity and low biocompatibility. After implantation of a stainless steel implant, a fibrous capsule forms around the implant, rather than direct bone-implant contact (osseointegration) [4, 11]. Biologic fixation (osseointegration) is considered a prerequisite for implant-supported prostheses and their long-term durability. The rate and quality of osseointegration in implants are related to their surface properties (surface chemical composition, hydrophilicity, roughness) [11]. A thicker oxide layer leads to an enhanced rate and quality of osseointegration [7]. Therefore, a stable oxide layer on the surface of the metal is essential for biocompatibility [7, 9, 11, 22]. However, an oxide layer does not readily form on the surface of stainless steel because of its chemical characteristics [6], and the biocompatibility of stainless steel is lower than that of Ti alloy and cobalt-chromium alloy [4, 6].

Electron beam deposition is a simple process that allows a dense and uniform film to be generated on almost any substrate [20, 24]. It has been used to treat high-temperature components of gas-turbine engines for aircraft, such as airfoils and vanes, with metallic and ceramic coatings to enhance performance and reliability [20]. Because the surface of stainless steel is hard to modify, a Ti coating was added to the stainless steel surface by electron beam deposition, combining the surface properties of Ti with the mechanical properties of stainless steel. Then, a microarc oxidation (MAO) process was used to form a rough TiO_2 layer on the Ti surface to enhance osteoblast-like cell integration [2, 12–14, 26].

We assessed whether these surface modifications enhanced the biologic and morphologic responses of an osteoblastlike cell line (SaOS-2) to stainless steel in vitro based on (1) morphology; (2) proliferation (cell proliferation assay); (3) differentiation (alkaline phosphatase [ALP] activity); and (4) attraction ability (cell migration assay) of the cells, compared to machined stainless steel.

Materials and Methods

We studied the biologic and morphologic responses of an osteoblastlike cell line (SaOS-2) to stainless steel using three types of Ti-coated surface-modified stainless steel discs. We performed energy-dispersive x-ray spectroscopy (EDS) and scanning electron microscopy (SEM)

investigations to assess the chemical composition and structure of the surfaces and the morphology of the SaOS-2 cells. We also measured the proliferation (cell proliferation assay), differentiation (ALP activity), and attraction ability (cell migration assay) of the SaOS-2 cells.

We performed an a priori power analysis for differences between the groups at an alpha level of 0.05. We considered the following differences biologically important: greater than 0.1 (nmol/min/ug) in alkaline phosphatase activity and greater than 0.1 (absorbance at 450 nm) in cell proliferation and cell migration assay. The standard deviations were 0.075 in alkaline phosphatase activity, 0.062 in cell proliferation rate, and 0.057 in cell migration assay. These analyses indicated sample sizes of 10, 7, and 6 were required for each group to achieve significance based on the above differences.

Three types of stainless steel 316L discs measuring 12 mm in diameter and 10 mm in thickness were manufactured ($n = 174$): (1) machined stainless steel ($n = 58$); (2) surface-modified stainless steel with a Ti coating and MAO ($n = 58$); and (3) surface-modified stainless steel with grit blasting, Ti coating, and MAO ($n = 58$) (Fig. 1). Grit blasting was achieved with Al_2O_3 particles (diameter, 200–500 μm) moving in a high-velocity air stream (KSSA-5FD; Kum-Kang Tech, Incheon, Korea). The roughness of the grit-blasted specimens was in the range of 5 to 7 μm . For electron beam deposition, commercially pure-grade Ti plates with dimensions of 10 $\times$ 10 $\times$ 1 mm were prepared as a target material. A Ti layer up to 10 μm thick was then coated on the substrates at a rate of 0.1 nm per second. During the coating process, the substrate holders were rotated at 20 rpm to achieve a uniform layer. After Ti coating, the specimens were oxidized electrochemically using the MAO process. The MAO treatment of the prepared specimens was carried out in an aqueous electrolyte containing Ca and P sources, applying a pulsed DC field. As an electrolyte, a solution of 0.15 mol/L calcium acetate monohydrate (Sigma-Aldrich Corp, St Louis, MO) and 0.02 mol/L glycerol phosphate calcium salt (Sigma-Aldrich) was used. The applied voltage, frequency, and duty of the pulsed DC power were 230 V, 660 Hz, and 60%, respectively. All MAO treatments were carried out in a water-cooled glass bath using a stainless steel plate as a counterelectrode for 3 minutes. The surface roughness was measured using an optical interferometer (Accura 2000[®]; Interplus Co, Seoul, Korea). The average surface roughnesses (Ra values) were $6.2 \pm 0.32 \mu\text{m}$ (mean $\pm$ SD) for the grit-blasted, Ti-coated, MAOed group, $0.5 \pm 0.15 \mu\text{m}$ for the Ti-coated, MAOed group, and $0.2 \pm 0.11 \mu\text{m}$ for the machined group. The three surfaces were characterized using an SEM (JEOL JSM-6700F; JEOL, Ltd, Tokyo, Japan) after the test specimens had been coated with platinum. SEM results showed different surface characteristics

Fig. 1 A flow diagram shows the study design. Three different types of stainless steel 316L specimens were made: (1) machined stainless steel (SUS) ($n = 32$), (2) surface-modified stainless steel with Ti coating and MAO (SUS + Ti coated + MAOed) ($n = 32$), and (3) surface-modified stainless steel with grit blasting, Ti coating, and

MAO (SUS + GB + Ti coated + MAOed) ($n = 32$). Biologic and morphologic responses of an osteoblastlike cell line (SaOS-2) were examined by measuring cell morphology, proliferation, ALP activity, and migration.

(Fig. 2). Compared to the machined surface (Fig. 2A), the Ti-coated, MAOed surface had a remarkable multilayered porosity of 0.5 to 9.0 μm (average, 3.1 μm) (Fig. 2B). These measurements were obtained from three randomly selected areas. Scanned areas were $26 \times 20 \mu\text{m}$. Pore characteristics in the present study were confined to the “opened pores” on the surface (not including the “buried pores” in the anodic oxide film). The porosity is presented as a total area of opened pores per scanned area ($3 \times 26 \mu\text{m} \times 20 \mu\text{m}$). The grit-blasted, Ti-coated, MAOed surface showed a rugged, multilayered porosity in the macropores, like the Ti-coated, MAOed surface, as a result of the grit blasting (Fig. 2C).

EDS (JEOL JSM-6700F) was performed to evaluate the chemical composition of the stainless steel surfaces of the three groups. Compared to the machined stainless steel (Fig. 3A), the Ti-coated, MAOed group showed a completely different surface, with high peaks of Ti, oxygen, calcium, and phosphate (Fig. 3B). The MAO was able to cover contaminating alumina particles impacted by the grit blasting. This was also confirmed by the EDS spectrum results of the surface chemical characterization (Fig. 3C).

To prepare specimens for morphologic assessment using SEM, 0.5 mL SaOS-2 cells (5×10^4 cells/mL) in a medium containing 1% fetal bovine serum (FBS) were seeded on each of the 16 stainless steel samples. After 6 hours of incubation, the medium was removed. Phosphate-buffered saline (PBS) was added, and the cells were washed with PBS two to three times. After adding 2% glutaraldehyde-PBS solution, the cells were allowed to stabilize for

2 hours. The fixative solution was removed, and the cells were washed with distilled water three times. At 30-minute intervals, the cells were dehydrated through 50%, 70%, 90%, and 100% ethanol solutions. Finally, the ethanol was removed, and the cells were left at room temperature to evaporate the ethanol completely. We determined cell morphology using a SEM (JEOL JSM-6700F) after the test specimens had been coated with platinum.

For the cell proliferation assays, 0.5 mL SaOS-2 cells (5×10^4 cells/mL) in a medium containing 1% FBS were seeded on each of the 16 stainless steel samples and incubated (37°C , 5% CO_2 , 95% humidity) for 24, 48, 72, and 96 hours. The medium was exchanged with fresh medium before measuring cell proliferation using the CellTiter 96® nonradioactive cell proliferation assay (Promega Corp, Madison, WI) according to the manufacturer’s protocol. This assay is a colorimetric method for determining the number of viable cells. The amount of formazan formed can be measured by its absorbance at 450 nm using a plate reader and is directly proportional to the number of viable cells in the culture. Using this kit, 15 μL dye solution was placed on the stainless steel sample and the cells were incubated in 5% CO_2 at 37°C for 4 hours. After 100 μL stop solution was added, 100 μL pipetted cells was transferred to a 96-well plate (Nunc A/S, Roskilde, Denmark). Cell proliferation was measured at 450 nm using a spectrophotometer (EL 312e; BioTek Instruments, Inc, Winooski, VT).

ALP activity was measured by seeding 0.5 mL SaOS-2 cells (5×10^4 cells/mL) in medium containing 1% FBS on

Fig. 2A–C SEM images of the surfaces of (A) machined ($\times 2000$), (B) Ti-coated, MAOed ($\times 2000$), and (C) grit-blasted, Ti-coated, MAOed ($\times 2000$) stainless steel 316L specimens showed different surface characteristics. (A) Compared to the machined surface, (B) the Ti-coated, MAOed surface showed a remarkable multilayered porosity of 0.5 to 6.4 μm and (C) the grit-blasted, Ti-coated, MAOed surface showed a multilayered porosity due to MAO in the macropore because of grit blasting.

each of the 16 stainless steel samples, followed by incubation for 7, 14, and 21 days. The medium was removed, and the cells were washed with PBS three times to remove as much of the serum in the culture fluid as possible. Next, 1 mL 0.02% Triton[®] X-100 was placed on the stainless steel sample to lyse the cells. Cytolytic solution was transferred into a 1.5-mL tube, and the cells were sonicated. The tube was centrifuged (14,000 rpm, 4°C, 15 minutes), and the supernatant was transferred to a new

1.5-mL tube. Then, 100 μL 1 mol/L Tris-HCl, 20 μL 5 mmol/L MgCl_2 , and 20 μL 5 mmol/L p-nitrophenyl phosphate were added to the supernatant. The mixture was left to react at 37°C for 30 minutes, and 50 μL 1 N NaOH was added to stop the reaction. Using p-nitrophenol as a standard, the absorbance was measured at 410 nm using a spectrophotometer. The measured ALP activity was expressed as the p-nitrophenol production quantity divided by the reaction time and the protein synthesis quantity, as measured by the Bio-Rad Protein assay kit (Bio-Rad Laboratories, San Jose, CA).

For the cell migration assay, we placed each of 10 stainless steel samples in a Transwell[®] chamber in 24-well plates (Costar, Cambridge, MA) and put 500 μL medium with 1% FBS in the lower chamber (Fig. 4). We prepared 0.5 mL SaOS-2 cells (5×10^4 cell/mL) in serum-free medium, added this cell suspension plus culture fluid to the upper chamber above the polycarbonate filter (8- μm pore size), and cultured the cells for 24 hours at 37°C in 5% CO_2 . We then removed the medium from the upper chamber and removed the membrane and nonmigrated cells using a swab. To count the migrated cells, we placed the chamber in a new well, put 200 μL extraction solution in the chamber, shook the plate on an orbital shaker for 10 minutes, transferred 100 μL of each sample to a 96-well plate, and measured the absorbance at 560 nm using a spectrophotometer (EL 312e).

We compared the mean cell proliferation assay and ALP activity of the cells on the three different surfaces using the Kruskal-Wallis test and compared the cell migration assay results using the Wilcoxon signed-rank test. Statistical analyses were performed using SPSS[®] software (Version 11.5; SPSS Inc, Chicago, IL).

Results

The morphologic assessment of the cells after 6 hours of incubation using SEM showed the machined stainless steel (Fig. 5A) and Ti-coated, MAOed (Fig. 5B) surfaces were covered with small, slender osteoblastlike cells, whereas the grit-blasted, Ti-coated, MAOed surface was largely covered with lamellipodia from the osteoblastlike cells (Fig. 5C). Additionally, the lamellipodia largely covered and the filopodia extended into the micropores on the MAO surface. Thin cytoplasmic processes branched out from the filopodia and entered the interior of the micropores (Fig. 5C).

After 24-, 48-, 72-, and 96-hours incubations, the proliferations of the SaOS-2 cells on the Ti-coated, MAOed stainless steel and grit-blasted, Ti-coated, MAOed stainless steel surfaces were higher ($p < 0.001$) than that on the machined stainless steel surface, and the cell proliferation

Fig. 3A–C EDS spectra of (A) machined, (B) Ti-coated, MAOed, and (C) grit-blasted, Ti-coated, MAOed stainless steel 316L specimens. (A) Compared to the machined specimen, (B) the Ti-coated, MAOed specimen was completely different, showing high peaks of Ti and O. (C) The grit-blasted, Ti-coated, MAOed specimen showed high peaks of aluminum due to grit-blasting materials

Fig. 4 A diagram illustrates the Trans-well[®] cell migration assay. The cells were loaded in the upper chamber above the 8- μ m pore membrane filter, with different types of the test specimen in the bottom chamber.

on the grit-blasted, Ti-coated, MAOed stainless steel surface was higher ($p = 0.01$) than that on the Ti-coated, MAOed surface (Fig. 6).

We observed no differences in the ALP activity of the SaOS-2 cells on the surfaces among the groups after 7-, 14-, and 21-day incubations (Fig. 7). The ALP value reached a maximum at 14 days (Fig. 7).

The migrations of the SaOS-2 cells on the Ti-coated, MAOed stainless steel and grit-blasted, Ti-coated, MAOed stainless steel surfaces were higher ($p < 0.001$) than that on the machined stainless steel surface, but the cell migration on the grit-blasted, Ti-coated, MAOed stainless steel surface was similar to ($p = 0.609$) that on the Ti-coated, MAOed stainless steel surface (Fig. 8).

Discussion

To enhance the potential for stainless steel to osseointegrate, we introduced a Ti coating using electron beam deposition, which has been successfully used in the space, turbine, optical, biomedical, and auto industries. We then developed a modified coating process using grit blasting followed by Ti coating and MAO. We asked whether this grit-blasting, Ti-coating, MAO process could improve the morphology, proliferation, differentiation, and attraction ability of SaOS-2 cells to stainless steel in vitro compared with two other surface modifications, machined and Ti-coated, and MAOed.

We note several limitations of our study. First, this study assessed only in vitro effects on cell morphology, proliferation, and differentiation. Further studies are needed to determine whether Ti coating and MAO actually improve osseointegration, which has not yet been studied in vivo. Second, we used SaOS-2 cells, an osteosarcoma cell line, which may behave differently from primary osteoblasts, so caution must be exercised in drawing any conclusion regarding osseointegration. SaOS-2 cell line revealed a 2 to

3-fold greater mean doubling-time and a 15 to 20-fold higher saturation density than osteoblasts [17]. However, to study interactions of osteoblastic cells with biomaterials, osteoblastlike cell lines from human osteosarcoma (SaOS-2) are often used [1, 3, 15, 16]. SaOS-2 cells in particular exhibited the most mature osteoblastic phenotype compared with other osteosarcoma-derived cells line [17]. That is why we used SaOS-2 cells in this study. Third, a DNA study was not performed. A DNA study could enhance the impact of these findings by also assessing the levels of Type I collagen and osteocalcin. Further study is needed. Fourth, the assessment of biocompatibility was limited by considering only alkaline phosphatase activity and cell proliferation rate, and we did not perform the necessary normalization experiments.

Electron beam deposition is a simple process in which a focused high-energy electron beam is directed to melt the evaporant material in a vacuum chamber [20]. The evaporating material condenses on the surface of the substrates or components, resulting in the formation of a coating [20]. During deposition, external heating is often applied to the substrate to enhance metallurgical bonding between the coating and the substrate. Electron beam deposition technology produces a new material with controlled microstructure and microchemistry in the form of coatings. This technology is being used to form coatings for many applications, including space, turbine, optical, biomedical, and auto industries. Coatings are often applied on components to extend their performance and life under severe environmental conditions, such as thermal, corrosion, wear, and oxidative stress. Performance and properties of the coating depend on its composition, microstructure, and deposition conditions [20, 24].

The cell morphology and proliferation assay indicated all the surfaces examined were cytocompatible. This is consistent with many studies indicating Ti and stainless steel 316L are biocompatible substrates for cell culturing [19]. In our study, cell proliferation was elevated on the

Fig. 5 A–C SEM images show SaOS-2 cells after 6 hours of incubation on (A) machined ($\times 1000$), (B) Ti-coated, MAOed ($\times 1000$), and (C) grit-blasted, Ti-coated, MAOed stainless steel 316L specimens ($\times 1000$). The (A) machined and (B) Ti-coated, MAOed surfaces were weakly covered with small, slender osteoblastlike cells (arrows). (C) The grit-blasted, Ti-coated, MAOed surface was largely and strongly covered with healthy lamellipodia of the osteoblastlike cells, and a thin cytoplasmic process branched out from the filopodia to enter the inside of a micropore.

Ti-coated, MAOed groups compared with the machined group. Moreover, we noticed osteoblastlike cells on the grit-blasted, Ti-coated, MAOed stainless steel spread and formed lamellipodia on mirror-polished stainless steel surfaces comparable to those observed on the machined and Ti-coated, MAOed stainless steel surfaces. These findings indicate the grit-blasting, Ti-coating, MAO process can enhance cell integration on a stainless steel surface.

Fig. 6 A graph shows the results (mean + SD) of SaOS-2 cell proliferation assays at 24, 48, 72, and 96 hours for machined (SUS), Ti-coated, MAOed (SUS + MAOed, Ti), and grit-blasted, Ti-coated, MAOed (SUS + GB + MAOed, Ti) stainless steel specimens. Cell proliferations were higher for the Ti-coated, MAOed and grit-blasted, Ti-coated, MAOed specimens than for the machined specimens.

Fig. 7 A graph shows the ALP activity (mean + SD) of SaOS-2 cells cultured for 7, 14, and 21 days on machined (SUS), Ti-coated, MAOed (SUS + MAOed, Ti), and grit-blasted, Ti-coated, MAOed (SUS + GB + MAOed, Ti) stainless steel surfaces. Secretion of ALP was similar in the three groups.

ALP activity is an indicator of osteogenic differentiation, bone formation, and matrix mineralization [11]. Several studies have demonstrated the influence of surface roughness on ALP activity [5, 12, 25]. However, we did not find ALP activity increased with roughness parameters, in contrast to the results reported by Kim et al. [10]. This discrepancy may be attributed to the use of SaOS-2 cell lines in this experiment, and SaOS-2 cells may lift off after achieving high confluence. ALP activity peaked at about 14 days in bone-derived cell cultures because the cells were slowly dividing over 3 weeks [8, 17, 25].

To assess whether the stainless steel attracted osteoblastlike cells, we used a Transwell® migration assay system. The cell migration results were higher with

Fig. 8 A graph shows the results (mean + SD) of SaOS-2 cell migration assays for the machined (SUS), Ti-coated, MAO (SUS + MAOed, Ti), and grit-blasted, Ti-coated, MAOed (SUS + GB + MAOed, Ti) stainless steel specimens. Cell migrations were higher for the Ti-coated, MAOed and grit-blasted, Ti-coated, MAOed specimens than for the machined specimens.

Ti-coated, MAOed stainless steel than with machined stainless steel; thus, these surface modifications enhanced attraction of osteoblastlike cells.

A stable oxide layer on the surface of metals is essential for good biocompatibility [7, 9, 11, 22]. On stainless steel, such an oxide layer cannot readily form, in contrast to Ti. Thus, electron beam Ti deposition was used to make a stable oxide layer on the stainless steel surface. We also wanted to improve the biocompatibility of the Ti-coated stainless steel, so we introduced the MAO process to make a uniform oxide layer on the stainless steel [11–14]. However, not all metals can be treated by the MAO process. For the MAO process, passivation at the anode side should occur, and the oxide layer formed on the metal surface should be stable in the working electrochemical environment. For these reasons, stainless steel cannot be MAOed [18]. The minimum thickness required for stable MAO treatment is dependent on the applied voltage; with increasing voltage, the minimum required Ti coating thickness increases [6]. For stable MAO treatments with 230 V, the Ti coating layer should be thicker than 3 μm [6]. The coating thickness in this research was in the range of 3 to 5 μm .

To increase surface roughness, we modified the surface of the stainless steel 316L by grit blasting followed by Ti coating and MAO. We then assessed the advantages of MAO treatment. This modification did enhance the cell proliferation and migration compared to machined stainless steel. Moreover, this process can overcome the

disadvantages of grit blasting, such as the release of blasting particles. In this method, the blasting material is also embedded in the surface, as it is with grit blasting. MAO is not a mechanical phenomenon, but a chemical bond to the surface of the Ti alloy, in which a stable oxide layer surrounds embedded particles [14].

We used the grit-blasting, Ti-coating, MAO process on stainless steel 316L. These surface modifications enhanced the biocompatibility (proliferation and migration of osteoblastlike cells) of stainless steel. This process is not unique to stainless steel; it can be applied to many metals and should also improve their biocompatibility. More generally, it should allow a broad range of materials for cementless arthroplasty components, which today are largely limited to Ti and cobalt-chromium alloys.

Acknowledgments We thank Hyoun Ee Kim, Seung Soo Kim, and Shang Joong Lee for providing editorial assistance.

References

- Ahmad M, Mc Carthy MB, Gronowicz G. An in vitro model for mineralization of human osteoblast-like cells on implant materials. *Biomaterials*. 1999;20:211–220.
- Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials*. 2000;21:667–681.
- Declercq H, Van den Vreken N, De Maeyer E, Verbeeck R, Schacht E, De Ridder L, Cornelissen M. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. *Biomaterials*. 2004;25:757–768.
- Disegi JA, Eschbach L. Stainless steel in bone surgery. *Injury*. 2000;31:2–6.
- Giordano C, Sandrini E, Busini V, Chiesa R, Fumagalli G, Giavaresi G, Fini M, Giardino R, Cigada A. A new chemical etching process to improve endosseous implant osseointegration: in vitro evaluation on human osteoblast-like cells. *Int J Artif Organs*. 2006;29:772–780.
- Han CM, Kim HE, Kim YS, Han SK. Enhanced biocompatibility of Co-Cr implant material by Ti coating and micro-arc oxidation. *J Biomed Mater Res B Appl Biomater*. 2009;90:165–170.
- Hazan R, Brenner R, Oron U. Bone growth to metal implants is regulated by their surface chemical properties. *Biomaterials*. 1993;14:570–574.
- Hoemann CD, El-Gabalawy H, McKee MD. In vitro osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathol Biol*. 2009;57: 318–323.
- Kim HK, Jang JW, Lee CH. Surface modification of implant materials and its effect on attachment and proliferation of bone cells. *J Mater Sci Mater Med*. 2004;15:825–830.
- Kim MJ, Choi MU, Kim CW. Activation of phospholipase D1 by surface roughness of titanium in MG63 osteoblast-like cell. *Biomaterials*. 2006;27:5502–5511.
- Le Guehennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. *Dent Mater*. 2007;23:844–854.
- Li LH, Kong YM, Kim HW, Kim YW, Kim HE, Heo SJ, Koak JY. Improved biological performance of Ti implants due to surface modification by micro-arc oxidation. *Biomaterials*. 2004; 25:2867–2875.

13. Li Y, Lee IS, Cui FZ, Choi SH. The biocompatibility of nano-structured calcium phosphate coated on micro-arc oxidized titanium. *Biomaterials*. 2008;29:2025–2032.
14. Lim YW, Kwon SY, Sun DH, Kim HE, Kim YS. Enhanced cell integration to titanium alloy by surface treatment with microarc oxidation: a pilot study. *Clin Orthop Relat Res*. 2009;467:2251–2258.
15. Lo H, Kadiyala S, Guggino SE, Leong KW. Poly(l-lactic acid) foams with cell seeding and controlled-release capacity. *J Biomed Mater Res*. 1996;30:475–484.
16. Matsuura T, Hosokawa R, Okamoto K, Kimoto T, Akagawa Y. Diverse mechanisms of osteoblast spreading on hydroxyapatite and titanium. *Biomaterials*. 2000;21:1121–1127.
17. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, Milz S. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. *Anti-cancer Res*. 2004;24:3743–3748.
18. Schmuki P. From Bacon to barriers: a review on the passivity of metals and alloys. *J Solid State Electrochem*. 2002;6:145–164.
19. Schuler M, Trentin D, Textor M, Tosatti SG. Biomedical interfaces: titanium surface technology for implants and cell carriers. *Nanomed*. 2006;1:449–463.
20. Singh J, Wolfe DE. Nano and macro-structured component fabrication by electron beam-physical vapor deposition (EB-PVD). *J Mater Sci*. 2005;40:1–26.
21. Steinemann SG. Metal implants and surface reactions. *Injury*. 1996;27:16–22.
22. Sul YT. The significance of the surface properties of oxidized titanium to the bone response: special emphasis on potential biochemical bonding of oxidized titanium implant. *Biomaterials*. 2003;24:3893–3907.
23. Unnanuntana A, Dimitroulias A, Bolognesi MP, Hwang KL, Goodman SB, Marcus RE. Cementless femoral prostheses cost more to implant than cemented femoral prostheses. *Clin Orthop Relat Res*. 2009;467:1546–1551.
24. Wolfe DE, Singh J, Narashimhan K. Synthesis and characterization of multilayered TiC/TiB₂ coatings deposited by ion beam assisted, electron beam-physical vapor deposition (EB-PVD). *Surf Coat Tech*. 2003;165:8–25.
25. Xavier SP, Carvalho PS, Beloti MM, Rosa AL. Response of rat bone marrow cells to commercially pure titanium submitted to different surface treatments. *J Dent*. 2003;31:173–180.
26. Zhu X, Kim KH, Jeong Y. Anodic oxide films containing Ca and P of titanium biomaterial. *Biomaterials*. 2001;22:2199–2206.