Enhanced osteoblast response to electrical discharge machining surface

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The purpose of this study is to investigate the surface characteristics and biocompatibility of titanium (Ti) surfaces modified by wire electrical discharge machining (EDM). EDM surface characteristics were evaluated by scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), thin-film X-ray diffractometry (XRD) and contact angle measurements. MC3T3-E1 cell morphology, attachment and proliferation, as well as analysis of osteoblastic gene expressions, on machined surfaces and EDM surfaces were also evaluated. EDM surfaces exhibited high super hydrophilicity, due to high surface energy. XPS and XRD revealed that a passive oxide layer with certain developing thickness onto. EDM surfaces promoted cell attachment, but restrained proliferation. Counted cell numbers increased significantly on the machined surfaces as compared to the EDM surfaces. Real-time PCR analyses showed significantly higher relative mRNA expression levels of osteoblastic genes (ALP, osteocalcin, Runx2, Osterix) in cells cultured on the EDM surfaces as compared to cells cultured on the machined surfaces.

Keywords: Titanium, Electrical discharge machining, Attachment, Proliferation, Differentiation

INTRODUCTION

Titanium (Ti) and Ti alloys have been widely used as primary bio-metals in orthopedic and dental procedures due to their high corrosion resistance and biocompatibility. Their performance is mainly attributed to a passive oxide film that spontaneously forms on the Ti surface. Considerable in vitro and in vivo studies have drawn much attention for a variety of successful Ti surface modifications that have demonstrated enhanced biocompatibility and/or early, reliable osseointegration.

Modifications of implant surfaces can affect many cellular responses, including cell attachment, adhesion, proliferation and differentiation. Sand-blasting, a method that creates surface micro-roughness, has been reported to stimulate decreased cell proliferation associated with increased differentiation, while micro-roughness with a superposition of submicron scale structures on Ti surfaces has been shown to accelerate the osteoblastic phenotype without decreasing cell attachment, spreading or proliferation.

Surface energy of Ti surfaces has also been reported to have an important role in osteogenesis. The thickness of the Ti surface oxide film modulates protein adsorption, which regulates cell adhesion, spreading and proliferation as a result of the high surface energy. It has been reported that developing thickness of surface oxide films increases surface hydrophilicity even on a thermally-oxidized Ti surface.

We have developed a surface modification of Ti by means of wire electrical discharge machining (EDM). EDM enables to process the electro-conductive materials by spark discharge generated between a wire electrode (Ti wire) and the material through running pure distilled water. The wire and the material are noncontact. This computer associated technique enables extremely accurate, complex sample shaping. It also yields an optimal micro-textured surface during the processing. EDM surfaces allow a unique surface topography and chemistry. Complicated micro-scale topography with a superposition of submicron scale structure and an oxidation layer are formed on the Ti surface during such processing. Thus, we expected EDM modifications will support superior osteoblastic phenotypes on the modified surfaces in comparison with machined-Ti surfaces.

In a previous report, we analyzed the osteoblastic gene expressions of mesenchymal stem cells cultured on EDM surfaces over time. In the present study, to further explore the effect of EDM on osteoblast responses, we examined osteoblastic MC3T3-E1 cell gene expressions by real-time polymerase chain reaction on these surfaces.

MATERIALS AND METHODS

Ti plate preparation
JIS grade 2 Ti plates (KS-50, Kobe Steel, Kobe, Japan) were used in this study. EDM of the Ti plates (10×10×1.0 mm) was prepared by wire electrical discharge machining (ROBOCUT α-0iA, FANUC LTD, Yamanashi, Japan) using Ti electrode, deionized water as dielectric fluid. EDM samples of the same dimensions were processed (machined surface). The samples were cleaned in acetone, ethanol and sterile water using an ultrasonic washing machine.

Surface analysis
The surface morphologies of the Ti plates were observed by scanning electron microscopy (SEM, S-2360N, HITACHI, Tokyo, Japan). The crystalline structure and
chemical composition of each oxide layer was investigated by thin-film X-ray diffractometry (XRD-6100, SHIMADZU, Kyoto, Japan) with CuKα radiation. XRD was performed at 40 kV and 40 mA with a scanning speed of 0.5°/s and scanning range of 20–50°. EDM surfaces were analyzed by X-ray photoelectron spectroscopy (ESCA-3400; SHIMADZU). High resolution spectra of Ti2p and O1s were analyzed by Mg Kα radiation. A 20 mA emission current and 8 kV of accelerated voltage were applied in the analysis (UHV conditions at<5.0×10⁻¹¹ mbar). The binding energies for each spectrum were calibrated based on the C1s spectra of 285.0 eV. The surface energy of each prepared sample was evaluated by the contact angle of 3.6 μL H2O using a contact angle measuring device (Simage02V, Excimer Inc., Atsugi, Japan).

Cell culture
Osteoblastic cells (MC3T3-E1) were obtained from RIKEN Cell Bank and maintained using α-Modified Eagle’s Medium (Wako, Osaka, Japan) and 10% fetal bovine serum (Sigma, Tokyo, Japan), 500 U/mL penicillin and 500 U/mL streptomycin. The cells were cultured under 100% humidity and 5% CO2 at 37°C. The medium was changed every other day, and before confluence, cells were passaged using 0.05% trypsin/0.02% EDTA.

Cell morphology
The cells were seeded onto each sample in 24-well culture plates at an initial seeding density of 1.0×10⁴ cells/well and cultured for 3 days. Samples were fixed in 3% glutaraldehyde, washed twice in PBS and dehydrated using a graded series of ethanol. After critical point drying and platinum coating, the cell morphologies of adherent cells on the Ti surfaces were observed by SEM.

Cell attachment
The cells were seeded onto each specimen in 24-well cultured plates at an initial seeding density of 4.0×10⁴ cells/well. After 2, 6, 12 and 24 h, cell numbers were counted using a Coulter counter (Model Z1 Coulter Counter, Beckman Coulter Inc., Miami, FL, USA).

Cell proliferation
The cells were seeded onto each specimen in 24-well cultured plates at an initial seeding density of 1.0×10⁴ cells/well. The medium was refreshed every 3rd day. After 3, 5 and 7 days, cell numbers were counted using a Coulter counter.

Real-time polymerase chain reaction (PCR)
The cells were seeded onto each specimen in 24-well culture plates at an initial seeding density of 4.0×10⁴ cells/well and cultured for 3 and 7 days. Induction of osteoblastic differentiation was initiated 24 h after plating of cells onto the Ti plates by changing the culture medium to differentiation medium containing ascorbic acid and β-glycerophosphate. Differentiation medium was changed every 3rd day. Osteoblastic genotypic markers tested included alkaline phosphatase (ALP), osteocalcin, Runt-related gene 2 (Runx2) and Osterix. Total RNA was extracted with an RNaseasy Plus Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. Cells were homogenized (QIAshredder column, Qiagen), applied to a gDNA eliminator to remove genomic DNA and then applied to an RNasey mini-spin column, rinsed and eluted. Using the extracted RNA as a template, reverse transcription reactions were carried out with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA), and the RT reactions were performed in a Gene Amp PCR System 9700 (Applied Biosystems). The reactions were performed using TaqMan Gene Expression Master Mix (Applied Biosystems), and thermal cycling was performed in an ABI 7500 Fast Real-Time PCR System. The relative mRNA was determined by the 2−ΔΔCT method. GAPDH was used for normalization. The primers and probes (TaqMan(R) Gene Expression Assays) used for real-time PCR were as follows:

GAPDH (Mm99999915_g1), ALP (Mm01187117_m1), osteocalcin (Mm03413826_mH), Runx2 (Mm03003491_m1), Osterix (Mm00504574_m1); undisclosed by Applied Biosystems.

RESULTS
SEM analysis
The SEM analyses of the surfaces studies are displayed in Figs. 1a–d. The machined surfaces showed grooves by surface abrasion defects (Fig. 1a). On the EDM surfaces, complicated crater-shaped irregularities created by the electric discharge were observed (Fig. 1b). In particular, the image of Fig. 1c, which is the same region of 1b viewed from a diagonal direction, shows the characteristic topography of an EDM surface. Figure 1d shows a submicron-scale electric discharge groove that was formed in the micron-scale electric discharge groove. Figure 1d is a high-power image of Fig. 1b.

XRD analysis
XRD patterns of the specimens are shown in Fig. 2. Typical XRD patterns of titanium were detected similarly on machined and EDM surface. The 2θ value of TiO2 suboxide peaks on EDM surface were more distinctive in comparison with machined surface.

XPS analysis
The binding energy of O1s on EDM surface was detected at 530.500 eV while Ti2p was detected at 458.750 eV. The most outer layer TiO2 was able to be confirmed in the result of XPS, though clear peaks of TiO2 was not admitted in XRD.

Contact angle
The contact angle of H2O on the machined surface was 39.9±2.5 degrees (Fig. 3a). EDM surfaces showed a super hydrophilicity and were not able to provide a contact angle measurement (Fig. 3b). Figure 3c and 3d exhibit images taken from diagonal directions of Fig. 3a and 3b,
respectively.

Cell morphology
After 3 days of culture on the Ti plates, MC3T3-E1 cells exhibited flattened morphologies along the abrasion grooves on the machined surfaces (Fig. 4a), while the cells were more elongated in various directions on the EDM surfaces (Fig. 4b).

Cell attachment
At 2, 6 and 12 h, cell numbers increased significantly on the EDM surfaces compared to those on the machined surfaces (Fig. 5). There were no differences in cell numbers at 24 h.

Cell proliferation
At 3, 5 and 7 days, cell numbers increased significantly on the machined surfaces as compared to the EDM surfaces.
Fig. 4 (a): A representative SEM image showing the morphology of spread MC3T3-E1 cells cultured for 3 days on a machined surface. The cells appeared flattened along the abrasion grooves, aligned in the same direction. The cells had fewer cytoplasmic extensions. (b): Morphological assessment of cells cultured on an EDM surface. Cells were elongated in all directions. Pseudopodium-like structures were observed. The cells were polygonal in shape, with many thin filopodia attached to the Ti surfaces. All magnifications were ×1,000; bar=50 µm.

Fig. 3 Contact angle measurements. (a): Machined surfaces showed an angle of 39.9±2.5 degrees. (b): H2O spread on the EDM surfaces was not able to be measured. (c, d): Images taken from diagonal perspectives. The EDM surface exhibited a super hydrophilicity.
Fig. 5 Cell attachment assay over 24 h. At 2, 6, and 12 h, the cell numbers increased significantly on the EDM surfaces compared to the machined surfaces ($p<0.01$). There were no differences in cell numbers at 24 h. Values are expressed as mean±SD from three independent cultures and were analyzed by one-way ANOVA.

Fig. 6 Cell proliferation assay over 7 days. At 3, 5, and 7 days, cell numbers increased significantly on the machined surfaces compared to cells on the EDM surfaces ($p<0.01$). Values are expressed as mean±SD from three independent cultures and were analyzed by one-way ANOVA.

Fig. 7 The effect of EDM modification on cell differentiation. (a): The level of ALP mRNA expression was significantly higher in cells on the EDM surfaces at 3 days ($p<0.05$) and 7 days ($p<0.01$) than on the machined surfaces. (b): Osteocalcin gene expression was significantly higher in cells on the EDM surfaces at 3 days ($p<0.01$) than on the machined surfaces. (c): The expression of Runx2 was significantly increased on the EDM surfaces at 3 days ($p<0.01$). There were no differences in Runx2 expression at 7 days in cells on the two surfaces. (d): The expression of Osterix was significantly increased in cells on the EDM surfaces at 3 days ($p<0.01$) compared to the machined surfaces. There was no difference in Osterix expression levels at 7 days on the two surfaces. Values are expressed as mean±SD from three independent cultures and were analyzed by one-way ANOVA.
surfaces, indicating that the EDM modification did not enhance cell proliferation (Fig. 6).

**Osteoblastic gene expression**

Gene expression markers associated with osteoblastic cells were present on both surfaces at all time points (Figs. 7a–d). The relative mRNA level for ALP was significantly higher in cells on the EDM surfaces compared to levels detected on the machined surfaces at 3 and 7 days (Fig. 7a). For osteocalcin, gene expression increased significantly in cells on the EDM surfaces at 3 and 7 days (Fig. 6b). Runx2 and Osterix expression levels were higher on the EDM surfaces at 3 days, and there were no significant differences at 7 days (Figs. 7c and d).

**DISCUSSION**

EDM modification was found to promote super hydrophilicity as a result of high surface energy. Surface hydrophilicity increases the adsorption of cell adhesion-promoting proteins (fibronectin, vitronectin, and laminin, etc) on such surfaces, which is known to contribute to subsequent enhanced cell responses, such as osteoblastic cell attachment and proliferation compared with hydrophobic Ti surfaces.

Although binding energy if Ti2p and O1s revealed a TiO2 outermost layer on the EDM surfaces, developing thickness of passive oxide layer on the EDM surface was mainly due to TiO since an increase of TiO rather than TiO2 was detected by XRD.

We investigated representative genes in each osteoblast differentiation stage. ALP is regarded as an early differentiation marker gene, while osteocalcin is the most abundant non-collagenous matrix protein that is a known osteoblastic late differentiation marker gene. ALP and osteocalcin relative expression levels of adherent cells on the EDM surfaces showed significantly higher levels as compared with those on the machined surfaces at 3 and 7 days.

Runx2 is a transcription factor that is essential for the maturation and differentiation of osteoblasts. Osterix is a protein needed for osteoblast differentiation and acts downstream of Runx2. Runx2 and Osterix function in the osteoblast to control osteoblast-specific gene expression of target genes such as ALP, type I collagen, osteopontin and osteocalcin. The greater expression of these transcription factors in cells cultured on the EDM surfaces rather than on the machined surfaces at 3 days culture further confirmed the enhanced osteoblastic phenotype on the EDM surfaces.

Submicron-scale pits were found on the superposition of the micron-scale roughness structure of EDM surface. In a particularly high-magnification image, many fine pits were observed, whereas simple, linear grooves were observed on the machined Ti surfaces. Such a complicated surface structure may also contribute to the adherent cell morphology, resulting in the enhanced osteoblast phenotype on the EDM surfaces, as well as speculated by previous research.

In conclusion, our results indicate that EDM surfaces results in improved MC3T3-E1 cell responses as compared to machined surfaces. Specifically, EDM surface modification appeared to promote cell attachment and differentiation on the Ti surfaces, but did not affect cell proliferation. This result is most likely attributable to characteristic chemical structure of the surface, as well as to the complicated topography. Such a modified surface exhibits super hydrophilicity, a property that is known to promote cell attachment and differentiation.

**REFERENCES**


