The Effect of Surface Processing of Titanium Implants on the Behavior of Human Osteoblast-Like Saos-2 Cells

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ABSTRACT

Background: The surface qualities of dental implants appear to modulate osteoblasts' growth and differentiation, affecting bone healing. During manufacturing of implants, the surface quality is affected by industrial processes.

Purpose: To examine the effect of manufacturing procedures on the growth and differentiation of human osteoblast-like cells, Saos-2.

Materials and Methods: Saos-2 cells were cultured on titanium (Ti) disks. Cell growth was examined using the XTT assay, and cell differentiation was tested by alkaline phosphatase (ALP) activity and osteocalcin secretion. The following variables were examined: roughening of the surface by sandblasting and acid-etching, aging of the acid used for etching, fluoride modification of the surface, and the type of the packaging material.

Results: An inverse relationship was noted between Saos-2 growth and ALP activity on the tested surfaces. Roughening of the surface tended to decrease cell proliferation and to increase differentiation. Immersion of up to 200 cycles in acid decreased proliferation and increased differentiation. Cells grown on fluoride-modified surfaces exhibited more ALP activity as compared to the unmodified surfaces. No difference was noted between the three packaging materials tested.

Conclusions: The data suggests that industrial processes may affect the behavior of osteoblast-like cells around titanium implants and should be monitored carefully by bioassays.

KEY WORDS: cell differentiation, osteoblast, surface properties

INTRODUCTION

Interrelations between biomaterial structure and its surface characteristic and the cell phenotypic behavior are of importance in understanding the interaction of bone cells with titanium dental implants. The complex process of cell adherence to surfaces is governed by four basic mechanisms: protein adsorption, cell-surface contact, cell-surface attachment, and cell spreading.[1–5] Considerable differences have been observed in the behavior of osteoblasts grown on different dental implant surfaces in vitro.[6,7] These differences have been attributed to varying surface chemistries and topographies. In general, increased surface roughness is associated with decreased cell proliferation and increased differentiation. However, the cell type as well the culture conditions may affect the results. Protein adsorption is a complex process that lasts a relatively short time compared with the whole-cell adhesion process and is strongly affected by the surface physical and chemical properties. In culture, the initial cell-surface contact is basically gravitational and brings the cells to a distance of 50 nm from the surface, at which physical and/or chemical forces close the cell-surface gap. Attached cells then slowly (typically within hours) spread over the surface at a rate that is governed by the compatibility of the surface material and its physical properties. Needless to say, the protein composition of the initial phase can greatly affect the entire bioadhesion process.[8–11] Thus, comprehensive assessment of cytocompatibility requires

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a combination of assays that are sensitive to the various stages of cell-material interaction.

Titanium is by far the material of choice for dental implants. Various surface treatments of titanium have been proposed for the enhancement of the implant osseointegration. Blasting of the surface was found to increase bone cell differentiation.[12] Improved bone bonding and accelerated bone formation were noted with blasted surfaces further modified by acid[13] or fluoride.[14]

Sarcoma osteogenic (Saos-2) cells are a nontransformed cell line derived from the primary osteosarcoma of an 11-year-old Caucasian girl in 1973. It was later determined that Saos-2 cells possess several osteoblastic features and could be useful as a permanent line of human osteoblast-like cells and as a source of bone-related molecules.[15] Saos-2 cells are characterized by osteoblastic properties such as alkaline phosphatase (ALP) activity, expression of parathyroid hormone and $1,25(OH)_2D_3$ receptors, and secretion of osteocalcin.[15]

ALP is a glycoprotein plasma membrane enzyme found on the surface of osteoblasts (liver and intestine) and serves as a biochemical indicator of bone turnover. Osteocalcin is a biochemical indicator of bone turnover, and its level serves as a parameter for osteoblast differentiation. It is synthesized by osteoblasts and considered the most abundant noncollagenous protein of bone matrix. After synthesis in the bone, a small amount of osteocalcin is released in the circulation.

The aim of the present study was to examine the effect of some manufacturing procedures of the titanium surface on the growth and differentiation of the human osteosarcoma osteoblast-like cells, Saos-2. The effects of the following variables were examined: machined versus rough (sandblasted/acid-etched) surfaces, the number of implants previously immersed in the acid during etching, fluoride modification of the surface, and the packaging material of the implants.

MATERIALS AND METHODS

Titanium Samples

Disks (6 mm in diameter and 2 mm thick) made of grade 5 titanium (prepared by MIS Ltd., Shlomi, Israel) were used in this study. The disks were prepared with machined or rough surfaces. The surface roughness and microgeometry of the titanium were achieved by surface blasting with large particles (300–400 μm) of Al_2O_3 followed by etching with hydrochloric/sulfuric acid (the roughness index, Ra, with this method is 1.8–2.2 microns). This process increases the surface envelope of the implant. During manufacturing of implants, the acid used for etching is used for several cycles of immersions. In order to test the effect of the "aging" of the acid, the disks were immersed in acid that was used previously to etch a determined number of implants. Because of the hypothesis that fluoride modification can improve osseointegration,[14] in some experiments, one group of disks was treated with hydrofluoric acid (HF) after the standard blasting/acid-etching protocol. In order to test the effect of packaging material, the disks were inserted into vials made of polycarbonate, polystyrene, or glass and were sterilized by gamma radiation.

Cell Culture

The osteoblast-like cell line Saos-2 (kindly provided by Ben Basat's laboratory, Department of Experimental Medicine, The Hebrew University, Jerusalem, Israel) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (2 mmole/L) and penicillin/streptomycin (25 μg/mL). Experiments were carried out in eight replicates for each sample, in 96-well culture plates. Saos-2 cells were harvested by trypsin-ethylenediaminetetraacetic acid (EDTA), seeded into the culture wells with the titanium disks at a density of 5,000 cells/200 μL/well, and cultured (5% CO_2 and 37.5°C). At the end of the culture (72 hours), the cells were harvested for proliferation analysis, ALP activity, secretion of osteocalcin, and scanning electron microscopy (SEM).

Cell Proliferation

To determine relative cell numbers attached to the titanium discs, we used a cell proliferation colorimetric assay (Biological Industries, Beit Haemek, Israel). Two pilot runs and one final experimental run were executed. The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt (XTT) to orange compounds of formazan. The intensity of the dye is proportional to the activity of mitochondria enzymes and thus reflects the number of active cells in the well. Standard 96-well cell culture plates were used for the XTT assay. From each of the eight duplicate wells, 100 μL of media was taken and frozen at −80°C. The stored media would

later be used for quantitation of osteocalcin production. The remaining 100 μL was used for the analysis of cell proliferation patterns by adding 100 μL of XTT reagent for two hours at room temperature and measuring absorbance at wavelengths of 450 to 650 nm (reference absorbance at wavelength of 630–690 nm), as specified by the manufacturer's protocol.

ALP Specific Activity and Osteocalcin Secretion

At the termination of the culture, media were carefully taken out of each well. Cells were washed twice with phosphate buffer saline (PBS), followed by the addition of 150 μL prewarmed lysis Triton-buffered saline, and incubated for 1 hour at room temperature. The cell lysate was used for the measurement of ALP activity. This assay is based on the ability of the sample to degrade a specific ALP substrate. Briefly, 10 μL of the cell lysate was diluted at a ratio of 1:5 with lysis buffer. An amount of 150 μL of phosphatase substrate was added to the samples on ice and incubated on a shaker for 10 minutes in a 37°C water bath. P-nitophenol was used as a standard in the range from 10 to 1600 nmol/mL. The reaction was stopped by placing the plate on ice and adding 150 μL of ice-cold NaOH-EDTA solution. Optical density was measured at 405 nm by using a microplate enzyme-linked immunosorbent assay (ELISA) reader (kinetic microplate reader, Molecular Devices, Palo Alto, CA, USA).

A number of immunoassays have been developed to measure serum osteocalcin. In the present study, 100 μL of supernatant fluid that was previously taken from the supernatant fluid of each well and frozen at −80°C was assayed for osteocalcin levels by using a commercial ELISA kit from BioSource (Camarillo, CA, USA). ELISA was therefore performed according to the manufacturer's protocol. The concentration of osteocalcin in the sample is proportional to the absorbance, and values are obtained by comparison to a standard curve prepared on the same plate.

SEM

The media were taken out from the wells, and 300 μL of gluteraldehyde was added to each well for 24 hours, followed by washing with PBS. Cells were covered with 4% osmium tetraoxide in a 2% buffer for 1 hour, then dehydrated by passing them through a series of alcohols, and dried in an Emitech K850 Critical Point Drier (Gatan Biomed, Munich, Germany). The samples were then mounted onto stubs by using C Conductive Carbon Cement (SPI Supplies/Structure Probe, Inc., West Chester, PA, USA) and coated with gold (15 nm thick). The stubs were viewed with a Quanta 200 scanning electron microscope (FEI, Eindhoven, the Netherlands). The specimens were viewed at varying magnifications (×1.0k, ×2.0k, ×4.0k) at secondary electron mode.

Data Analysis

All experiments were carried out in eight replicates for each sample. Statistical analysis was performed by a one-tail analysis of variance at alpha set to 0.05, by using Systat software (San Jose, CA, USA). Paired samples were analyzed by a *t*-test.

RESULTS

Effect of Rough versus Machined Surfaces on Cell Behavior

The numbers of cells on the titanium surfaces were determined by using the XTT assay. Cell proliferation tended to be higher on the machined surfaces than on the rough surfaces but did not reach statistical significance (Figure 1A, $p = .06$). ALP activity, however, tended to be higher in the cells on the rough surfaces than on the machined surfaces but again did not reach statistical significance (see Figure 1B, $p = .07$). No difference was noted for the secretion of osteocalcin between the two surfaces (see Figure 1C, $p = .22$).

By using SEM (Figure 2), differences in the cellular morphology were noted when the cells were grown on different surfaces; the machined surface was covered by a uniform monolayer of cells (see Figure 2A), while layers of nonhomogeneously shaped cells were observed on the rough surfaces (see Figure 2B).

Effect of the Number of Previous Immersions during the Etching Process

During manufacturing of rough-surface implants, the acid is used for several etching cycles, as determined by the manufacturer. We tested the biologic effect of the etching process using disks that were etched in acid that had been used previously for a known number of implants. The results showed that an increased number of previous immersions up to 200 decreased cell proliferation in a dose-dependent manner (Figure 3A, $p < .05$). However, the change noted between 200 and

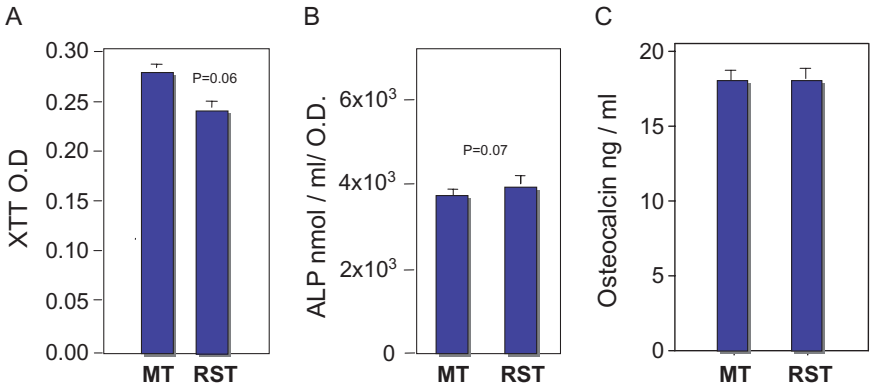

Figure 1 The effect of surface modification on growth and phenotypic expression of Saos-2 osteoblasts. Saos-2 cells were grown on machined-surface Ti disks (MT) or on rough-surfaced titanium disks (RST) for 24 hours. Growth of Saos-2 osteoblasts was measured by the XTT assay (A). Alkaline phosphatase (ALP) activity (B) and osteocalcin secretion (C) were used as markers of differentiation (bars represent means ± SE). (XTT = tetrazolium salt; Saos-2 = Sarcoma osteogenic; O.D. = optical density; SE = standard error.)

2000 previous immersions did not reach statistical significance ($p = .08$). In contrast, ALP activity was increased in a dose-dependent manner (see Figure 3B, $p < .05$). Again, the increase shown between 200 and 2000 previous immersions did not reach statistical significance ($p = .08$). Osteocalcin secretion was not affected by changing the number of implants previously treated in acid (see Figure 3C, $p = .24$).

Effect of Fluoride Modification

At the end of the culturing process, the number of cells grown on disks treated with HF was not statistically different from the number of cells grown on the control disks (not HF treated) (Figure 4A, $p = .09$). However, ALP activity was significantly higher in cells attached to fluoride-modified titanium surfaces (see Figure 4B, $p < .05$), while no difference was found for osteocalcin secretion (see Figure 4C, $p = .09$).

Effect of the Packaging Material

In order to test the biologic effect of different packaging materials, the disks were inserted into vials made from polystyrene, polycarbonate, or glass prior to gamma sterilization. No statistical differences were noted between the three materials on cell growth, ALP activity, or osteocalcin secretion (Figure 5, $p = .24$).

DISCUSSION

The present study demonstrates that some processes of implant surface preparation may affect the biologic activity of osteoblast-like cells in vitro. ALP activity and osteocalcin production are accepted markers for cell

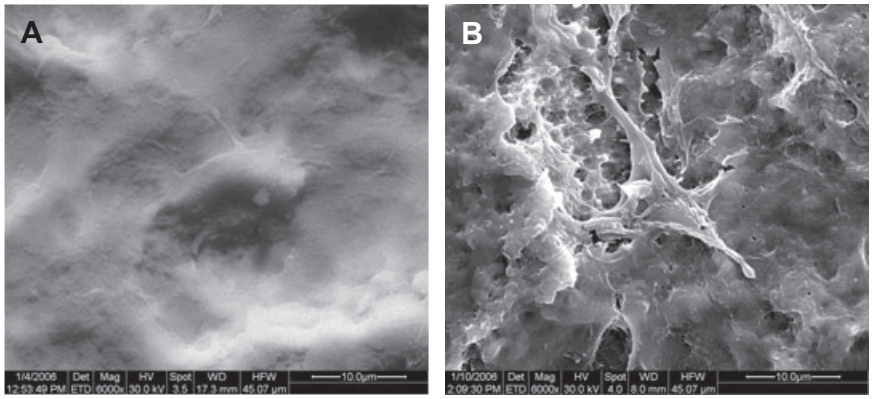

Figure 2 Demonstration of Saos-2 cells grown on the machined (A) or roughened (B) titanium surfaces, by scanning electron microscopy at ×6,000 magnification.

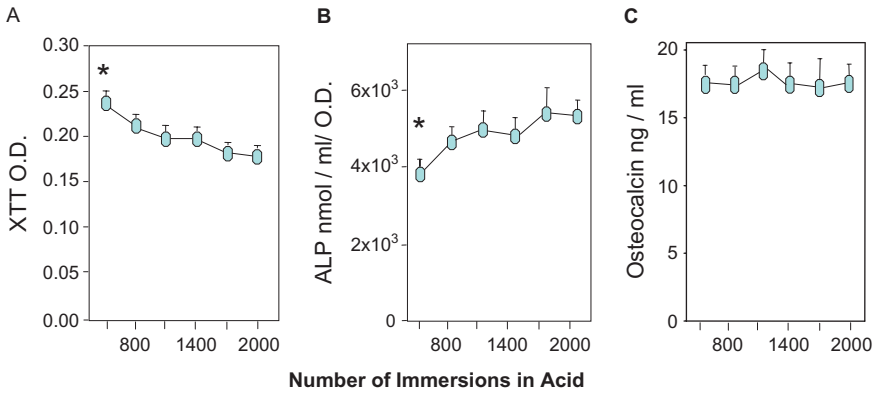

Figure 3 The effect of acid treatment on growth and phenotypic expression of Saos-2 osteoblasts. Disks were etched in acid for a known number of immersions. These disks were used for cell culture. Growth of Saos-2 osteoblasts was measured by the XTT assay (A). Alkaline phosphatase (ALP) activity (B) and osteocalcine secretion (C) were used as markers of differentiation (points represent means ± SE, $^*p < .05$). (XTT = tetrazolium salt; Saos-2 = Sarcoma osteogenic; O.D. = optical density; SE = standard error.)

differentiation toward osteoblastic phenotype. Previous studies with different cell culture models including Saos-2 cells,[16] MC3T3-E1 cells,[17] or 2T9 osteoblast progenitor cells[18] have shown that the implant surface influences osteoblast proliferation, differentiation, matrix synthesis, and growth factor production.[19–21] Additionally, it could be shown that the phenotypic expression of osteoblasts is sensitive to the topography and topology of titanium surfaces.[22] The above studies had shown that cells cultured on rougher surfaces tended to exhibit attributes of more differentiated osteoblasts (e.g., reduced cell numbers and increased ALP-specific

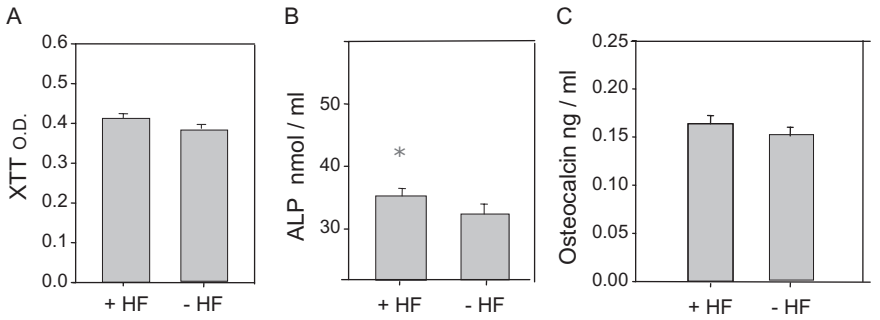

Figure 4 Growth and differentiation of Saos-2 osteoblasts on titanium disks with or without hydrofluoric (HF) treatment. Disks were exposed to HF acid, followed by cell culture for 24 hours. Growth of Saos-2 osteoblasts was measured by the XTT assay (A). Alkaline phosphatase (ALP) activity (B) and osteocalcine secretion (C) were used as markers of differentiation (bars represent means ± SE, $^*p < .05$). (XTT = tetrazolium salt; Saos-2 = Sarcoma osteogenic; O.D. = optical density; SE = standard error.)

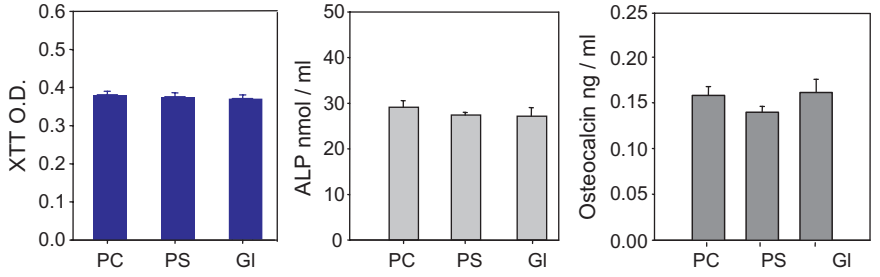

Figure 5 Growth and differentiation of Saos-2 osteoblasts on titanium disks stored in different vial materials. Growth of Saos-2 osteoblasts was measured by the XTT assay (A). Alkaline phosphatase (ALP) activity (B) and osteocalcine secretion (C) were used as markers of differentiation (bars represent means ± SE). (PC = polycarbonate vial; PS = polystyrene vial; Gl = glass vial; XTT = tetrazolium salt; Saos-2 = Sarcoma osteogenic; O.D. = optical density; SE = standard error.)

activity) than those cells cultured on smoother surfaces for comparable periods of time.[23–25] The present results are in agreement with the previous studies, showing that the Saos-2 cells grown on sandblasted and acid-etched rough surfaces that were produced for the present study tended to present a more differentiated phenotype than cells grown on machined surfaces. However, in the present conditions, this tendency failed to show statistical significance. By using SEM, differences in the cell structure were noted when the cells were grown on titanium surfaces with different treatments, thus demonstrating morphological effect of the surface topography on the adhered osteoblast-like cells.

Fluoride modification of the surface was suggested to enhance osteoblastic activity. A clinical study in dogs showed that the fluoride-modified implants promoted new bone formation and increased bone-to-implant contact in the early stages of healing.[26] Using a culture model, Isa and colleagues[14] have found that fluoride modification of the surface has no effect on cell attachment, ALP, and osteocalcin expression compared with blasted surfaces. In contrast, the present study demonstrated that a fluoride-modified surface increased ALP activity compared with unmodified surfaces, suggesting an upregulation of the genes already expressed by the osteoblast-differentiated Saos-2 cells. A possible explanation is that this difference between the two studies represents the differences in the manufacturing processes of the titanium disks. While Isa and colleagues[14] used disks that were blasted with 125 μm TiO_2 particles, we used disks that were blasted with 300 to 400 μm of Al_2O_3, followed by acid etching. The use of large particles for blasting and the additional treatment with the acid prior to fluoride modification may have an effect on the surface topography of the disk. Another possible explanation is the differences in the source of the studied osteoblasts (Saos-2 cells vs embryonic palatal mesenchimal cells).

An interesting finding is the effect of aging of the acid used for surface treatment. This manufacturing variable has not yet been studied, and the results suggest a decrease in proliferation and an increase in differentiation with an increased number of implants previously immersed in acid up to a cycle of 200. This result suggests that the biologic activity of the implant surface is at least not harmed by using the acid, even for up to 2,000 cycles. Moreover, this finding is in agreement with other reports on the inverse relationship between cell proliferation and cell differentiation.[23–25] As for the packaging materials tested, we can conclude that this variable does not have a measurable effect on the biologic activity of the implant surface.

Three of the variables tested showed that surface modifications can enhance upregulation of differentiation markers in osteoblastic-like cells. These processes are beneficial for the biologic response of osteoblastic-like cells to the surface and, therefore, should be encouraged.

However, the present study tested the behavior of osteoblast-like cells in vitro, and any application of the results in vivo should be made cautiously. In vivo studies using animal models do contribute a higher level of scientific evidence. However, in vitro studies using osteoblast-like cells are an efficient screening method prior to in vivo studies and are being extensively used as a scientific tool in the understanding of the surface biology of implant materials.[6–12]

CONCLUSIONS

The results showed an inverse correlation between cell proliferation and differentiation. Modification of the sandblasted and acid-etched surface with fluoride may enhance the differentiation of the cells. The data suggest that industrial processes may affect the behavior of osteoblasts around titanium implants and should be monitored carefully by bioassays.

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