EFFECTS OF SURFACE MICROSTRUCTURE AND NANOSTRUCTURE ON OSTEOBLAST-LIKE MG63 CELL NUMBER, DIFFERENTIATION AND LOCAL FACTOR PRODUCTION

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EFFECTS OF SURFACE MICROSTRUCTURE AND NANOSTRUCTURE ON OSTEOBLAST-LIKE MG63 CELL NUMBER, DIFFERENTIATION AND LOCAL FACTOR PRODUCTION

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DEDICATION

This thesis is dedicated to my husband, Chenyu Yan. I give my deepest expression of love and appreciation for the encouragement that you gave and the sacrifices you made during this graduate program. Thank you for the support and company during late nights of typing.

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SUMMARY

Surface roughness affects bone formation around orthopaedic implants *in vivo* and osteoblast functions *in vitro*. Osteoblast-like MG63 cells cultured on rough surfaces exhibited decreased cell number, increased differentiation and increased local factor production when compared to cells grow on smooth surfaces. In these experiments, roughness was characterized as average peak to valley height (Ra) which is not equal throughout the surface. Other features of roughness, including peak and valley area distributions and curvature of the valleys, will affect cell functions. In this study, novel titanium surfaces were prepared by photolithography to produce well designed microstructure and nanostructure. Smooth disks were made by producing craters of 10μm, 30μm and 100μm diameters on titanium disks with constant curvatures. Craters were placed sparsely (10/1, 30/1, 100/1) or compactly (10/6, 30/6, 100/6). Smooth disks were also acid etched to make an overall roughness of Ra 0.7 μm or anodized to produce volcano-like nanostructure of Ra 0.4 μm. The results revealed the distinguishing contributions of microcrater size, crater spacing and nanostructures to surface effect on cell number, differentiation (alkaline phosphatase; osteocalcin) and local factor levels (TGF-β1; PGE_2). Cell attachment depends on crater spacing; cell growth and aggregation depend on crater dimension and cell morphology depends on the presence of nanostructural features. Cell differentiation and local factor production are

modulated by acid etched roughness in concert with microstructure, and active TGF-β1 level depends on nanoscale roughness.

CHAPTER 1

INTRODUCTION

Ti implants are widely used in orthopaedics and dentistry because of their good biocompatibility, resistance to corrosion and mechanical properties. Osseointegration of Ti implants depends on implant surface morphology.[1] *In vitro* studies show that surface morphology affects cell adhesion, proliferation, differentiation and local factor production. These *in vitro* observations correlate with the clinical outcome of implantation. The purpose of this thesis is to use defined Ti surfaces to better understand the response of osteoblast-like cell to surface morphology. The possible mechanism mediating these effects will be discussed in this review.

A. Osteoblast Response to Titanium (Ti) Surface Morphology

The MG63 cell line is a well established model for studying the effects of surface morphology on osteoblast-like cells. This cell line was originally derived from a male human osteosarcoma and represents a less differentiated stage of osteoblastic maturation.[2,3] MG63 cells exhibit increased alkaline phosphatase activity and osteocalcin level in respond to 1α,25 dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$].[4] However, they do not mineralize the osteoid they synthesize.

Thus, MG63 cells are used to assess the effects of surface morphology on early differentiation events and not to assess effects on terminal differentiation with respect to matrix calcification.

The surfaces used in the studies reviewed here were commercial pure Ti disks with different types of surface morphology. Surface roughness was characterized by average peak to valley distance (Ra). One surface prepared by greasing and acid pickling was designated as smooth (PT, Ra = 0.6μm). Two rough Ti surfaces with differing morphologies were used. The SLA surface was prepared by grit blasting the PT surface and then etching the surface with heated mixture of HCl and H_2SO_4, resulting in an Ra of 3.97 μm. The TPS surface was prepared by coating the PT surface with irregular projections of Ti via Ti plasma spraying. This surface had an Ra of 5.21μm. All of the surfaces had an oxide layer of comparable thickness about 200Å, which attributes to stability of Ti surfaces.

When MG63 cells are cultured on smooth Ti surfaces, they display a flattened morphology, while on rougher surfaces with Ra of 4-7μm, they display a more cuboidal morphology characterized by prominent cytoplasmic extensions.[5] MG63 cells exhibit decreased cell number and [^{3}H]-thymidine incorporation when grown on rough Ti surfaces. In contrast, those cells show increased alkaline phosphatase activity, osteocalcin, prostaglandin E_2 (PGE_2) and transforming growth factor-β1 (TGF-β1) when they were grown on rough Ti surfaces. Moreover, there is a synergistic increase when the cells are treated with $1\alpha,25\text{-}(OH)_2D_3$.[6]

To overcome the consideration that MG63 cells are transformed and may not respond to surface morphology in the same manner as normal osteoblasts, fetal rat calvarial cells (FRC) and normal human osteoblasts (NHOst) were also plated on these Ti surfaces and showed a similar response to the surface morphology as MG63 cells did. The NHOst cells demonstrated a more mature phenotype on rougher surfaces with increased levels of osteocalcin, PGE_2 and TGF-β1. However, in contrast to MG63 cells, alkaline phosphatase activity decreased with increasing surface roughness.[7]

The difference in alkaline phosphatase activity between MG63 cells and NHOst cells in these two studies may reflect the fact that alkaline phosphatase is an early marker of osteoblast differentiation. Activity of this enzyme increases before mineralization really occurs and then decreases. The relatively immature MG63 cells may have been at a different state of osteoblastic maturation than the NHOst cells, such that at the time of assay MG63 cell activity was on its way up whereas NHOst cell activity had already peaked and was in its way down.

This hypothesis is supported by studies using other cell culture models. Previous reports showed that mature osteoblast-like cells and osteocyte-like cells also behave differently from MG63 cells.[8] As the surface becomes rougher, all the cells exhibit a more differentiated phenotype with decreased cell proliferation, and increased osteocalcin, PGE_2 and TGF-β1. In contrast FRC cells, which also like MG63 cells are in the early stage of osteoblastic linage, exhibit increased alkaline phosphatase activity; OCT-1 cells, a more differentiated osteoblast-like

cell line, respond with decreased alkaline phosphatase activity; and the terminally differentiated MLO-Y4 cells exhibit no change in alkaline phosphatase activity. The effect of surface roughness was also examined using rat costochondral cartilage cells.[9] Resting zone cells, which are less mature in terms of endochondral differentiation than growth zone cells, responded to surface roughness like the MG63 cells. However, growth zone chondrocytes exhibited a different response with respect to alkaline phosphatase and collagenase-digestible protein production. These observations indicate that response of cells to surface roughness is dependent on cell maturation state.

The osteoblast response to implants *in vivo* is more complicated than the *in vitro* response. The defect sites are first filled with a clot and/or hematoma. The chemical composition and physical aspects of the material dictate the composition and conformation of serum components that adsorb to the surface.[10,11] The first cells that actually come in contact with the implant are neutrophils and macrophages. Bone may be formed by the osteoblast progenitors that migrate toward the implant from the endosteal surface of the surrounding bone bed or differentiated progenitor cells migrating from vasculature and marrow.

Though most of the osteoblasts will not contact the implants directly, there is abundant evidence that surface morphology does play an important role through autocrine, paracrine and endocrine signaling pathways. Previous studies showed that when Ti was implanted into marrow ablated rat tibia, matrix vesicle production was increased while maturation was delayed. Interestingly, similar effects were

also observed in the contralateral limb, which indicates there was a material specific effect both locally and systematically.[12] The cells may interact either directly with the surface, releasing bioactive factors that act both locally and systemically, or indirectly with the surface by responding to factors released by cells at the bone/implant interface.[1] Surface roughness increases the integration of Ti implants with bone *in vivo*. The surfaces with greater micro-roughness exhibit greater pullout strength both in animal experiments and in clinical trials.[13,14] These effects are caused by cell response to the implant material and surface roughness rather than by mechanical interlock.

B. Osteoblast Response to Regulatory Factors Modulated by Surface Roughness

The response of osteoblast-like cells to circulating hormones is also affected by surface roughness. Treatment of to MG63 and FRC cells with $1\alpha,25(OH)_2D_3$[6,8] caused a synergistic increase in alkaline phosphatase specific activity, osteocalcin levels and local factor levels on rougher Ti surfaces. In contrast, $1\alpha,25(OH)_2D_3$ did not affect proliferation and alkaline phosphatase activity in more mature cells (OCT-1 and MLO-Y4 cells), but it increased osteocalcin levels. The results indicate that the surface roughness promotes osteogenic differentiation of less mature cells and enhances their responsiveness to $1\alpha,25(OH)_2D_3$. Osteoblast response to implant surface morphology was also modulated by estradiol.[7] On smooth

surfaces, estradiol affected only alkaline phosphatase in female NHOst cells, but on rough surfaces, estradiol increased levels of osteocalcin, TGF-β1 and PGE_2.

Surface roughness also modulates the cell response to local factors including bone morphogenetic protein-2 (BMP-2) and shear force. Ong *et al.* reported that when osteoblast progenitor 2T9 cells were plated on polished and 600 grit Ti surfaces, BMP-2 prolonged alkaline phosphatase specific activity and caused more rapid osteocalcin production on 600 grit Ti surfaces.[15] Shear force did not affect cell proliferation and differentiation on smooth surfaces but caused reversal of the increase in osteoblast differentiation seen in cultures on rough surfaces.[16]

In addition to modulating cell proliferation and differentiation responsive to surface texture, surface roughness affects production of local regulatory factors by the cells, including PGE_2 and TGF-β1. PGE_2 modulates normal osteoblast differentiation in a concentration dependent manner. At low concentrations, PGE_2 stimulates alkaline phosphatase activity and osteocalcin production, however, PGE_2 inhibits osteoblast function at high concentrations. TGF-β1 is associated with the matrix through latent TGF-β1-binding protein-1.[17] In bone remodeling, osteoclasts resorb bone mineral by decreasing local pH, resulting in activation of latent TGF-β1.[18,19] The increase in local concentration of active TGF-β1 causes an inhibition of bone resorption and contributes to bone formation by action on osteoblast.[20,21] MG63 cells release increased levels of PGE_2 and TGF-β1 into conditioned medium.[22] Moreover, $1\alpha,25(OH)_2D_3$ increases PGE_2 and TGF-β1 in a synergistic manner on rough surfaces.[6] A similar effect was also observed on

NHOst cells. Estradiol synergistically increases PGE_2 and TGF-β1 in cultures on rough surfaces.[7] Again, the production of PGE_2 and TGF-β1 and their response to $1\alpha,25(OH)_2D_3$ depend on the maturation state of the osteoblast.[8]

C. Mechanisms Mediating Osteoblast Response to Surface Morphology

The osteoblasts must first attach to the surface before they produce and mineralize their extracellular matrix. The number of osteoblasts that actually adhere to the Ti surface appears to be less than that on plastic surface.[23] The cell morphologies on Ti surface depend on surface roughness. The cells cultured on rough Ti surfaces appear to attach to the surface through multiple cytoplasmic extensions and show a cubical morphology typical of differentiated osteoblasts. The results indicate that cytoskeleton in the cells cultured on rough surface is rearranged and may have important consequences downstream.

Integrins are important membrane receptors that mediate the cell attachment and adhesion to the extracellular matrix and regulate the arrangement of cytoskeletons, followed by cell functions. Inhibition of integrin-associated PKC activity has no effect on proliferation of MG63 cells regardless of surface roughness, but it blocks the surface-dependent increase in alkaline phosphatase activity.[25] The results indicate that intergrin may mediate the surface dependent osteoblast differentiation. In contrast, $1\alpha,25(OH)_2D_3$-dependent alkaline phosphatase is

mediated by PKA instead of PKC,[26] so that the $1\alpha,25(OH)_2D_3$ may regulate osteoblast differentiation in a different signaling pathway.

Prostaglandins may also mediate the initial response of MG63 cells to Ti surfaces.[27] Indomethacin, a general cyclooxygenase (Cox) inhibitor that blocks prostaglandin production, inhibits the response of nonconfluent MG63 cells to surface roughness, and the effect vanishes when cells reach confluence. In further studies, both constitutive Cox-1 and inducible Cox-2 were found to be involved in mediating the cell response to surfaces.[28] The results indicate that both forms of the enzyme are involved and cell response to prostaglandins is complex.

D. Investigational Objective and Aims

The studies reviewed above were performed using micro-rough surfaces with relatively complex morphologies. Surface topography is composed of various features, including distances between the peaks, peak and valley area distribution and curvatures of the valleys. The average peak to valley distance is not sufficient to describe the complex structures on the surface. It is not known yet which feature of the surface structure contributes to cell proliferation, differentiation or local factor production. In this study, novel Ti surfaces were designed with precisely controlled microscale and nanoscale topographies. Hemispherical craters were produced on Ti surfaces with different diameters; and the craters were distributed on the disks with different distances. The nanostructure was overlaid on microstructured disks

by electropolishing, acid etching or anodizing. The microstructure and nanostructure are replicable and the chemical composition is consistent on all the disks. The MG63 cells were plated on these designed disks and cell morphology, cell number, differentiation and local factor production were examined.

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CHAPTER 2

OSTEOBLASTS ARE DIFFERENTIALLY REGULATED BY SPECIFIC SUBSTRATE MICROSTRUCTURAL FEATURES

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A. INTRODUCTION

Osteoblast behavior is sensitive to biochemical and structural features of their substrate (for a review, see [1-3]). We have used titanium disks with defined surface chemistry and microtopography as a model system to study the response of osteogenic cells to the microarchitecture of a substrate. These studies show that cells cultured on Ti surfaces with microrough features exhibit reduced proliferation and enhanced differentiation when compared to cells grown on tissue culture plastic or smooth Ti substrates. In addition, the effects of regulatory factors including estrogen [4], 1α,25-dihydroxyvitamin D_3 [5], and bone morphogenetic protein 2 (BMP-2) [6], as well as shear force [7], are altered.

The mechanisms involved in the differential cell response to surface microarchitecture are not well understood. Autocrine/paracrine regulatory factors such as prostaglandin E_2 (PGE_2), transforming growth factor beta-1 (TGF-β1), and osteoprotegerin (OPG) are elevated on rougher surfaces [8]. These factors regulate differentiation of osteoblasts and modulate the activation of osteoclasts, indicating that surface-dependent changes in the cell are important to cell response at a local level. Inhibition of prostaglandin production blocks the effects of surface microarchitecture on many of the parameters described above [9,10]. Similarly, inhibition of integrin-associated PKC-dependent signaling pathways also inhibits cell response [11,12]. Because there is a marked change in osteoblast morphology

associated with surface microarchitecture [13], it is possible that subsequent alterations in cytoskeletal architecture are responsible as well.

The use of substrate morphology on medical implants was initially based on the empirical observation that there was greater bone to implant contact when rougher surfaces were used [14]. More recent studies using dental implants with well controlled microarchitectural features demonstrated that pull-out strength was increased when the surface had a microtopography that included a mixed morphology of craters created by grit blasting overlaid with micropits produced by acid etching [15,16]. This surface has structural features that are similar to an osteoclast resorption pit [17], which is the preferred surface used by osteoblasts in vivo. Many of the responses observed when osteoblasts are cultured on bone wafers that have been conditioned by osteoclasts are also seen when osteoblasts are cultured on Ti disks with these surface features [18,19].

It is unclear, however, which aspects of the mixed microtopography are responsible for the effects of the substrate on bone cell proliferation, differentiation or local factor production. By identifying these features, new materials can be designed that optimize specific cell behavior. To accomplish this goal, we used photolithography to produce Ti surfaces with precise and reproducible features and examined the effects of these substrates on the behavior of human osteoblast-like cells in vitro.

B. MATERIALS AND METHODS

MG63 human osteoblast-like cells were cultured on 15 mm Ti disks in 24 well plates, such that in each experiment, each variable was tested using six separate disks with one disk per well. Six culture wells were used as tissue culture plastic controls. Smooth Ti disk surfaces (polish) were prepared by degreasing and acid-prepickling 1 mm thick sheets of grade 2 unalloyed Ti. After washing in acetone and processing through a 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55 ^{o}C for 30 sec, surfaces were mechanically polished to a mirror finish with an average roughness (Ra)< 0.05 μm. Ti disks with mixed microtopography (SLA) were prepared by grit blasting the smooth surface with 0.2 to 0.5 μm corundum grit at 5 bars until the surface reached a uniform gray tone. This process produced 100 μm diameters craters, although smaller diameter craters were present. Disks were then acid-etched using a mixture of concentrated HCl and H_2SO_4 heated above 100 ^{o}C, producing micropits 1-3 μm in diameter, resulting in an overall Ra of approximately 4.5 μm [4].

To prepare templates with individual structural features of the SLA surface, circular concavities were created by anodic dissolution of the polish disks through a patterned photoresist in a methanol-based 3 M sulfuric acid electropolishing electrolyte [20]. The surface outside the cavities was the original polish surface, and the inside had an electropolished surface finish. By using this electrochemical micromachining (EMM) method, well-defined cavities were produced with diameters of 10, 30 or 100 μm (Figure 1). To mimic the distribution of craters on the SLA

disk, the cavities were distributed over the surface of the polish disk resulting in a ratio of the cavity surface over the flat outside surface of either 1(Figure 1a,b,c) or 6 (Figure 1 d,e,f). EMM microstructured disks as well as polish disks that had not been grit blasted were also acid-etched as described above, resulting in pits approximately 700 nm in depth covering the entire surface (Figure 1f).

After processing, all samples were subjected to a passivation treatment by sequential sonications for 15 min each in concentrated nitric acid. They were then ultrasonically cleaned in ultra pure water (3 x 5 min) and dried under nitrogen. Prior to cell culture, all disks were sterilized in an oxygen plasma cleaner. The plastic surface of the tissue culture plates and an additional group of SLA disks that had been sterilized by steam autoclaving were also included in the experiments.

C. RESULTS

MG63 osteoblast-like cells (American Type Culture Collection, Rockville, MD) were cultured as described previously [21-23]. Cell number was determined 5 days after plating by counting the number of cells released from the substrate by two sequential digestions of the extracellular matrix with 0.25% trypsin in Hank's balanced salt solution containing 1 mM EDTA [24]. The number of cells on the surfaces at five days after plating depended on the surface microstructure (Figure 2). In cultures grown on smooth 10/1 and 30/1 surfaces, the number of cells was comparable to tissue culture plastic, but on smooth 100/1 surfaces, cell number was reduced to levels seen

on SLA (Figure 2a). When cells were grown on smooth surfaces with cavities close together, cell number on the 10/6 disks was similar to cell number on SLA; cell number on 30/6 was comparable to plastic but greater than SLA; and cell number on 100/6 was greater than plastic (Figure 2b). In contrast when cells were grown on polished Ti or on polished Ti that was acid etched as well as on acid etched 10/1, 30/1 and 100/1 surfaces, cell number was reduced approximately 10% compared to plastic but was still almost twice as great as seen on SLA (Figure 2c). Changing the spacing between cavities on the etched surfaces resulted in an inverse relationship between cavity diameter and cell number. As the cavity diameter became larger, cell number was reduced and on 100/6 disks, was comparable to the cell number on SLA (Figure 2d).

Effects of surface microstructure on cell morphology were assessed by scanning electron microscopy (SEM). Cultures were fixed in phosphate buffered 4% formaldehyde/1% glutaraldehyde for 1 hour and post-fixed with 1% OsO4 in Zetterqvist's buffer. Following dehydration in a graded series of alcohols, samples were vacuum dried. A thin layer of gold palladium was sputter-coated onto the samples prior to examination.

Cell morphology was comparable on autoclaved and plasma cleaned SLA surfaces (data not shown). When cultured on SLA, MG63 cells did not form a closely contacted monolayer but small cellular extensions were seen and the cells appeared to grow preferentially into the cracks and crevice of the surface created by the grit blasting process. Smooth polish surfaces were covered with a monolayer of

smooth edged cells (data not shown), whereas on cells grown on polish surfaces that had been etched had a morphology more typical of cells grown on SLA (Figure 3d). Individual cells extended across grain boundaries other etched surfaces in a manner similar to cells cultured on 30/6 surfaces that were etched (Figure 3e).

Cells on microstructured disks exhibited variations in morphology that were surface-dependent. The shape of the cell depended on cavity size. Cells aggregated in the cavities of the etched 100/6 disks and clusters of multilayered elongated cells were found in some areas (Figure 3a). On etched 30/6 surfaces, individual cells were found in the cavities and they had extended filopodia across the cavity wall to reach neighboring cells (Figures 3b and 3e). On etched 10/6 surfaces, MG63 cells spread above the cavities, sometimes covering 7-10 cavities (Figures 3C and 3f).

Cavity distribution was a factor as well. Osteoblasts on the outside surface of etched 100/1 disks exhibited the flat morphology typical of cells on polish surfaces, but did not form a contiguous monolayer (data not shown). Cells that were in the cavities of these disks had a more differentiated and elongated phenotype. On the etched 30/1 surface, cell bodies were found within the cavity and extending to the outside surface and those cells that were only on the outside surface exhibited morphology similar to cells on the polish disks (data not shown). On etched 10/1 surfaces, cells attached to the outside surface and covered the cavities without entering them (data not shown).

When MG63 cells were grown on the microstructured disks in the absence of the etched overlay, they exhibited a flat phenotype without forming a monolayer, regardless of whether the disk was of the 1 or 6 type. Disks that had 100 μm diameter cavities (100/6 and 100/1) supported the growth of a small number of elongated cells wit hin the cavities.

Differentiation was sensitive to the microstructural features of the surface. Specific activity of alkaline phosphatase (orthophosphoric monoester phosphonydrolase, alkaline; EC 3.1.3.1) was measured in lysates of the cell layer, which included any alkaline phosphatase-enriched extracellular matrix vesicles [23,25]. Activity was increased on SLA surfaces compared to plastic. Part of this effect was due to cavity dimension. When MG63 cells were grown on 10/1 and 30/1 smooth surfaces, enzyme activity was comparable to that seen in cells grown on plastic (data not shown). When the cavity diameter and distribution was 100/1, enzyme activity was greater than on plastic and comparable to the polished Ti surface, but still lower than on SLA. However, cavity distribution also played a role. Activity on 100/6 surfaces was comparable to plastic and activity on 10/6 surfaces that was comparable to the polished Ti surface (Figure 4a). Osteocalcin levels in the conditioned media varied with cavity size and distribution in a similar manner. On the 1-series surfaces, osteocalcin was greater than on plastic but reduced in comparison to SLA (data not shown). On the 6-series surfaces, osteocalcin was elevated over plastic in cultures grown on 10/6 only (Figure 4c).

Addition of the acid etch altered cell response regardless of cavity dimension or distribution. Alkaline phosphatase specific activity was greater than on plastic for all Ti cultures and when MG63 cells were grown on 100/1 surfaces, enzyme activity was greater than on smooth Ti with no microstructural features (Figure 5a). By moving the cavities closer together, enzyme activity was increased in a diameter-dependent manner (Figure 5b). On 10/6 surfaces, alkaline phosphatase activity was greater than on the polished Ti $\pm$ acid etch and on 30/6 and 100/6, it was comparable to SLA.

Substrate microarchitecture modulated levels of osteocalcin in a similar manner to its effect on alkaline phosphatase activity (Figure 5c,d). However, levels were comparable to SLA only on the 100/6 acid etched surfaces. In these experiments, osteocalcin in the conditioned media was measured using a radioimmunoassay kit (Human Osteocalcin RIA Kit; Biomedical Technologies, Stoughton, MA), as described previously [24].

Local factor levels in the conditioned media were also sensitive to the substrate microstructure. Total TGF-β1 was measured following acidification of the conditioned media using a commercially available enzyme-linked immunoassay kit specific for active human TGF-ß1 (Promega Corp., Madison, WI) [26]. Levels of this growth increased on polished Ti disks and to a much greater extent on SLA. On the smooth 1-series surfaces, TGF-β1 levels were comparable to plastic for both the 10/1 and 30/1 microstructured disks, and in cultures grown on 100/1, TGF-β1 levels were comparable to those of cells grown on polished Ti (data not shown). If the cells

were grown on 6-series surfaces, TGF-β1 levels were comparable to plastic regardless of cavity size (Figure 4b). In contrast, addition of the etch resulted in an increase on all Ti surfaces. On 30/1 and 100/1, the levels of TGF-β1 were elevated over polished Ti ± acid etch, but still not as high as seen on SLA (Figure 6a). However, when the cavities were in closer proximity, TGF-β1 levels were elevated on 10/6 and 30/6 surfaces, and on 100/6, were comparable to those seen on SLA (Figure 6b).

PGE_2, measured using a radioimmunoassay kit (NEN Research Products Boston, MA)[27], was modulated by surface microstructure as well. Regardless of the structural features, however, no cultures produced PGE_2 at levels comparable to those seen on SLA (Figures 4d; 6c,d). The highest levels of PGE_2 were found in cells cultured on 100/6 surfaces that had been acid etched. PGE_2 levels were dependent on cavity size rather than whether or not etch was present. Levels produced in smooth 10/1 and 10/6 cultures were greater than on smooth Ti disks and similar to those seen in cultures grown on Ti disks that were acid etched. Cells grown on smooth 30/1 and 100/1 produced PGE_2 at levels comparable to those produced by cells grown on acid etched 30/1 and 100/1 (Figures 6c), as well as on smooth 30/6 and 100/6(Figure 4d). Levels were only slightly higher on 30/6 and 100/6 etched surfaces (Figure 6d).

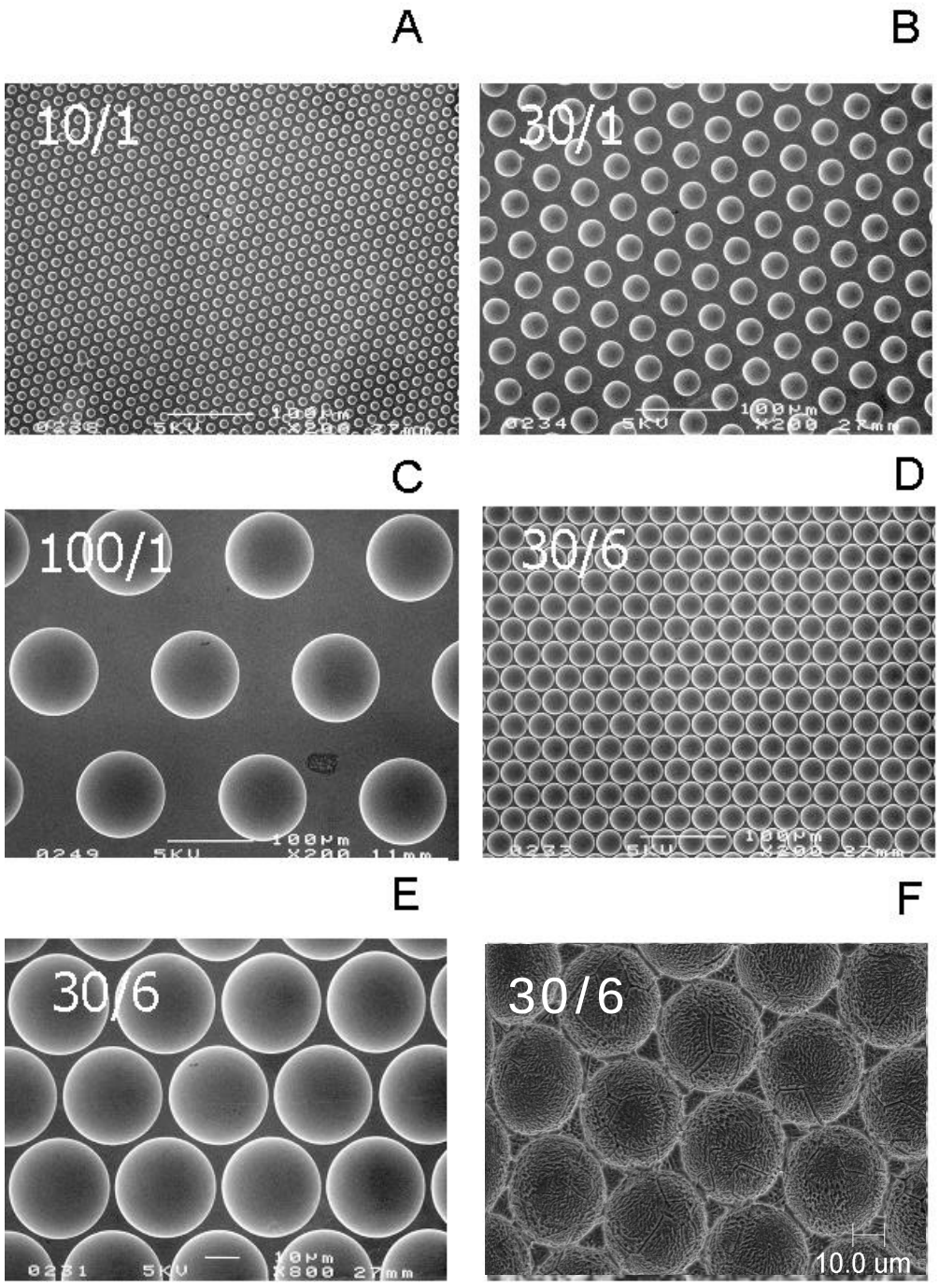

Figure 2-1 Surface microtopography of titanium disks.

Surface microtopography of titanium disks prepared using electrochemical micromachining. Polished Ti surfaces with specific microstructural features were prepared by electrochemically dissolving Ti through a patterned photoresist. Cavities were created with diameters of 10, 30 or 100 μm. Cavities were arrayed such that the ratio of the diameter to the intercavity space was either 1 or 6. Surfaces with intercavity space equal to the cavity diameter are shown in Panels A, B and C. Examples of surfaces with a cavity diameter to intercavity space diameter ratio of 6 is shown in Panel D at the same magnification as the 30/1 surface shown in Panel B, and at higher magnification in Panel E. 30/1 surfaces were treated with acid to produce a nano-etch with an Ra of 0.7 μm, shown in Panel F.

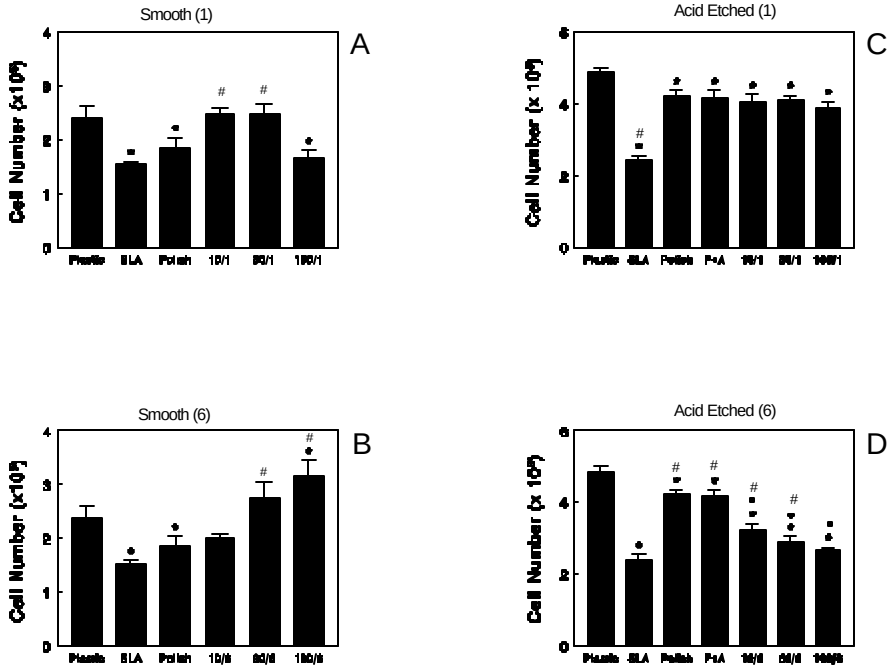

Figure 2-2 Effect of surface microtopography on cell number.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), electrochemically polished Ti (Polish), and polished surfaces that were acid etched (P+A), as well as on microstructured surfaces prepared using electrochemical machining (EMM). Smooth EMM surfaces had cavities of 100, 30 and 10 μm with a cavity diameter to intercavity space ratio of 1 (Panel A) or 6 (Panel B). EMM disks were acid etched resulting in 1-series (Panel C) and 6-series (Panel D) surfaces. Cell number was determined five days after plating. Values are

means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. polish or P+A.

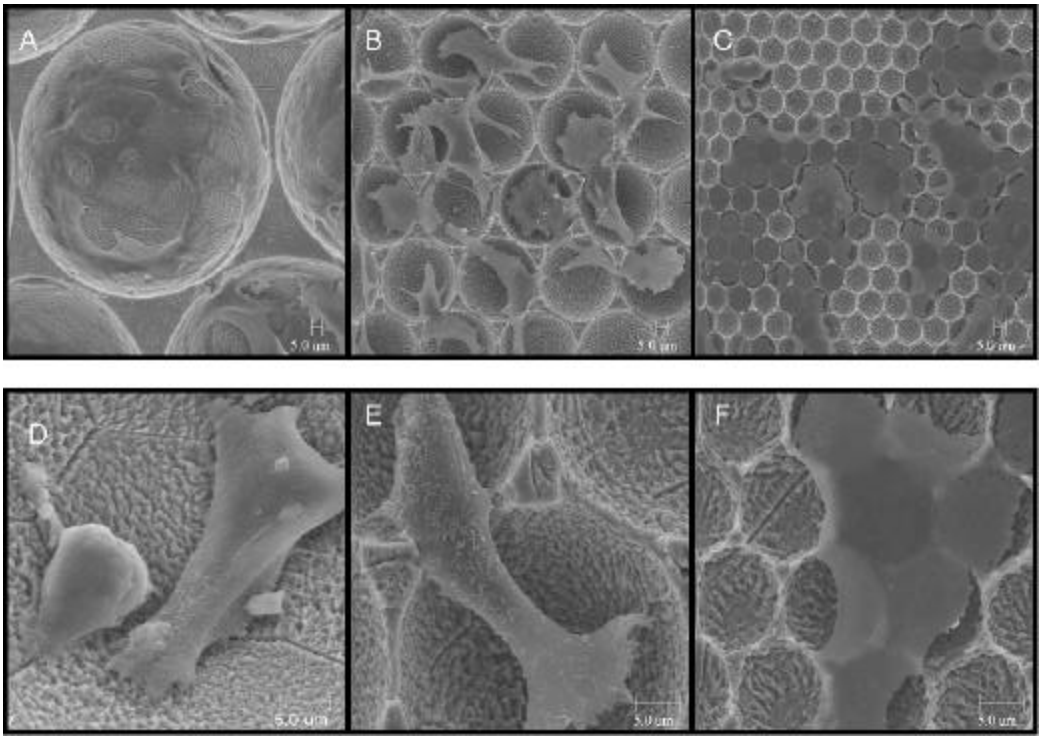

Figure 2-3 Morphology of MG63 osteoblast-like cells cultured on microstructured titanium surfaces.

Osteoblasts tended to aggregate in cavities with diameters of 100 μm. Panel A shows cells in the cavities on 100/6 etched surfaces. Cells on the 30/6 etched surfaces tended to anchor in adjacent cavities (B and E). The morphology of cells on the 100/6 etched surfaces was comparable to morphology on etched surfaces in the absence of cavities (D). On 10/6 etched surfaces, cells exhibited a flattened and spread morphology extending over adjacent cavities (C and F).

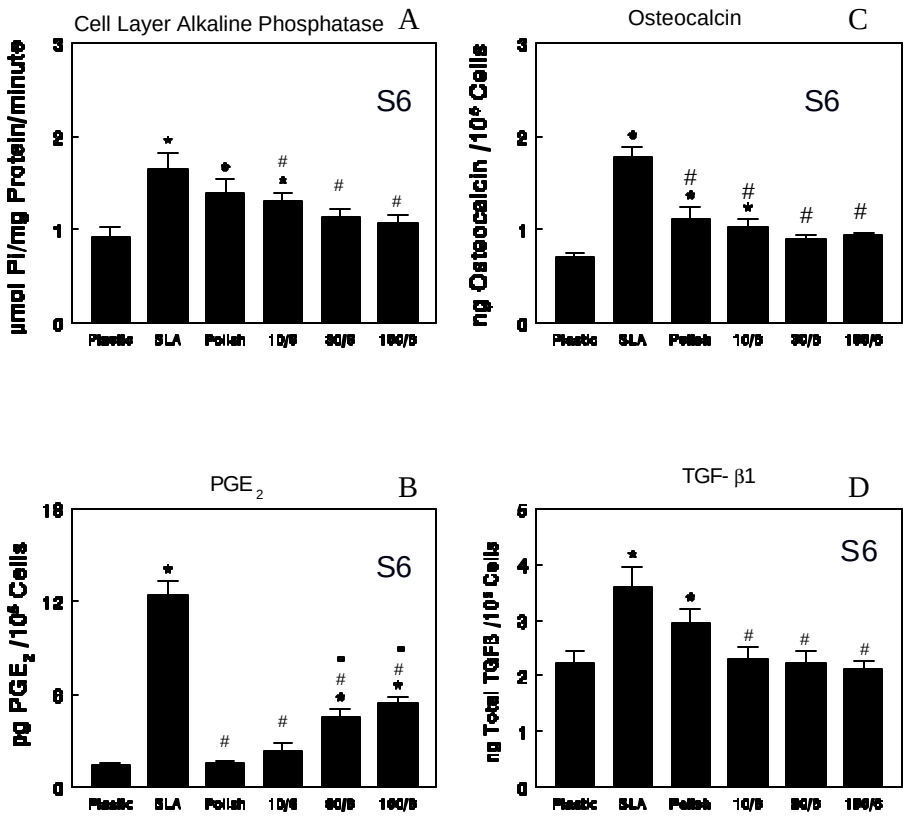

Figure 2-4 Effect of microstructured smooth surfaces on osteoblast differentiation and local factor levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), and electrochemically polished Ti (Polish), as well as on microstructured surfaces prepared using electrochemical machining (EMM). Smooth EMM surfaces had cavities of 100, 30 and 10 µm with a cavity diameter to intercavity space ratio of 6. Alkaline phosphatase specific activity was measured in

cell layer lysates (A). TGF-β1 was measured using an ELISA kit specific for active human TGF-β1 following acidification of the conditioned media (B). Osteocalcin levels were measured in the conditioned media using an RIA kit (C). PGE_2 was measured in the conditioned media using an RIA kit (D). Values are means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. polish.

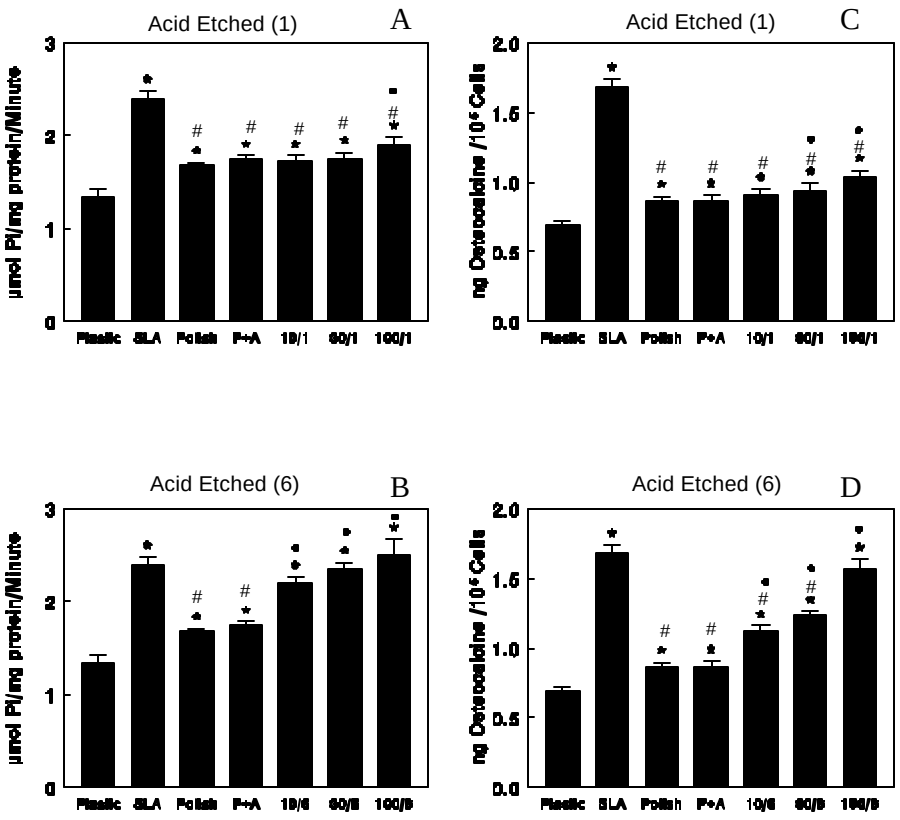

Figure 2-5 Effect of surface microtopography on osteoblast differentiation.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), electrochemically polished Ti (Polish), and polished surfaces that were acid etched (P+A), as well as on microstructured surfaces prepared using electrochemical machining (EMM). EMM surfaces with cavities of 100, 30 and 10 µm that had a cavity diameter to intercavity space ratio of 1 (Panels A and C) or 6 (Panels B and D) were acid etched. Alkaline phosphatase specific activity was

measured in cell layer lysates (Panels A and B). Osteocalcin content of the conditioned media was determined by RIA (Panels C and D). Values are means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. polish or P+A.

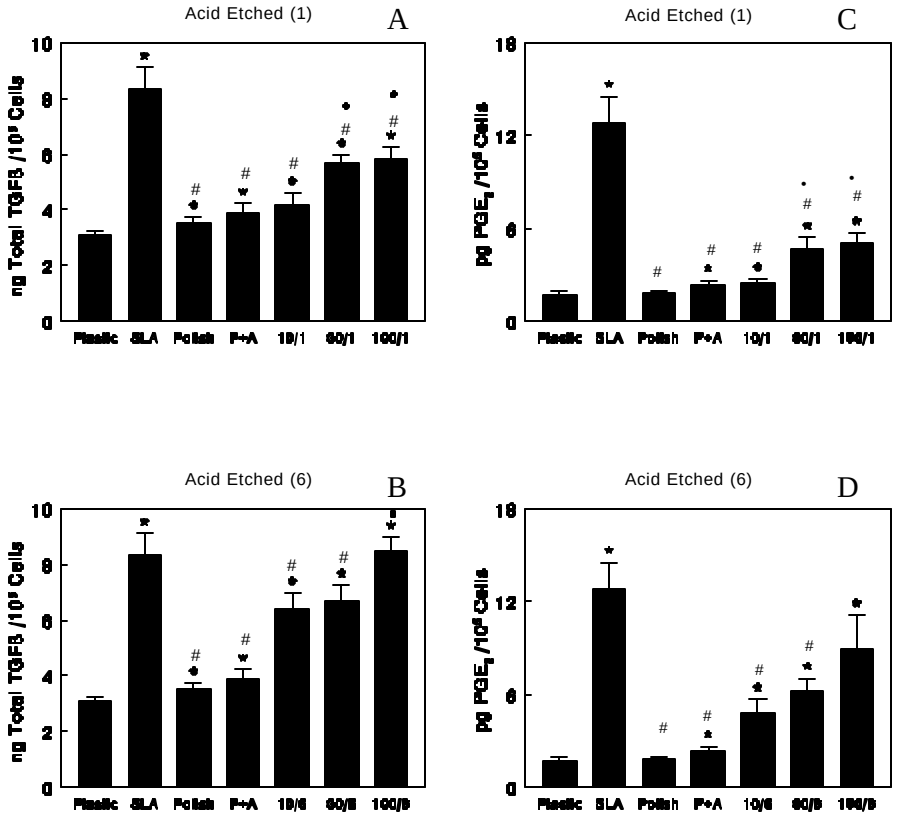

Figure 2-6 Effect of surface microtopography on local factor levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), electrochemically polished Ti (polish), and polished surfaces that were acid etched (P+A), as well as on microstructured surfaces prepared using electrochemical machining (EMM). Surfaces with cavities of 100, 30 and 10 μm that had a cavity diameter to intercavity space ratio of 1 (Panels A and C) or 6 (Panels B and D) were acid etched. TGF-β1 content was measured following acidification

of the conditioned media using and ELISA kit specific for active human TGF-β1 (Panels A and B). PGE_2 content of the conditioned media was determined by RIA (Panels C and D). Values are means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. polish or P+A.

D. DISCUSSION

Cell responses to surface microtopography are the sum of their ability to attach, proliferate, and differentiate. It is difficult to determine which structures are responsible for individual phenotypic traits. The mixed microtopography that characterizes the SLA surface is a complex one. By using the EMM fabrication technology to produce Ti surfaces with specific microstructures, we were able to determine the contributions of three surface design components to osteoblast response: microcrater size, microcrater spacing, and the presence of nano-pits.

The results confirmed the utility of the model since only MG63 cells that were cultured on microstructured surfaces with closely packed 100 μm cavities overlaid with etch approximated the responses of MG63 cells to SLA. The grit-blasting process produces an SLA surface with craters that have an average diameter of 100 μm, but both smaller and larger craters are present. Moreover, the acid etch on the SLA surface produces pits with an average diameter of 1 to 3 μm, which is larger than the dimensions of the etch produced on the polished and microstructured surfaces used in the present study. In addition, the etch on the SLA surfaces creates relatively sharp edges on the pits and when laid on top of the grit blasting results in craters adjacent to craters with no smooth interface. The surfaces prepared by the EMM method did not duplicate this morphology as even in the 6-series of surfaces, inter-cavity space still existed. These differences in surface microstructure may have

accounted for differences in the response of osteoblasts on 100/6 etched surfaces and SLA.

Other laboratories have used micropatterned tissue culture plastic to understand how specific structural features of a surface can modulate cell response [28-30]. These studies support our observation that surface microtopography modulates behavior of attachment-dependent cells. There are a number of differences in the experimental approach, however. While studies on tissue culture plastic can address how specific microarchitecture affects cells on tissue culture plastic, they cannot predict the response of cells to other substrates such as Ti. We and others [30,31], have shown that osteoblast response to bone wafers, which more closely approximate their natural surface in vivo, also differs from plastic. Thus, any discussion of microarchitecture must also consider surface chemistry.

Architectural dimensions are an important consideration. Our results indicated that osteoblasts tended to congregate in cavities, whether or not the cavities were etched. Similarly, osteoblasts tend to colonize osteoclast resorption pits on dentin and bone wafers [18,19] before they migrate onto the non-resorbed surface, and in vivo, osteoblasts synthesize osteoid on surfaces of the cutting cone created by osteoclastic resorption. It was presumed that the nano-architectural components of the osteoclast resorption pit, including collagen tufts and attachment proteins, contributed to this phenomenon [17,32,33]. It is possible that the "pit" itself is an equally important factor. MG63 cells within 100/1 and 100/6 pits had an elongated morphology more typical of differentiated osteoblasts. These cells exhibited phenotypic characteristics such as

elevated alkaline phosphatase specific activity and osteocalcin production that reflected a more differentiated physiology. In addition, morphology within the cavity was distinctly different from morphology on the inter-cavity space.

When cells were cultured on 10/6 surfaces, whether or not the etch was present, they tended to anchor to the inter-cavity space and extend over the cavities with a flattened, spread morphology. Given that the average diameter of an osteoblast is approximately 10 μm [34], it is likely that they simply could not fit into the small cavities, but it is of note that they did not extend filopodia into these spaces. This observation suggests that filopodia attachment is favored by the smaller 1-3 μm pits on SLA and nano-etch on the microstructured surfaces examined in the present study.

Clearly, the presence of the nano-etch is an important contributor to the overall response of the osteoblasts to the microstructured surfaces. It is only when this feature was present that differentiation of the cells was comparable to differentiation on SLA. TGF-β1 may play a role, as it varies in concert with alkaline phosphatase activity and has been shown previously to stimulate this enzyme activity, particularly at early stages of differentiation [22].

PGE_2 production was tied to cavity dimensions, however, and not to the spacing of the cavities or to the presence of the nano-etch. Exactly why this might be is not clear. Studies using smooth Ti and SLA surfaces show that the response of osteoblastic cells to surface microrugosity depends on prostaglandin production via both cyclooxygenase-1 and cyclooxygenase-2 [10,35]. Prostaglandins other than PGE_2

may be involved [36-38]. In addition, structural features present in the more complex mixed microtopography of SLA that were not modeled via the nano-etch method used here, may also modulate prostaglandin production.

In this study, we did not examine the relative rates of proliferation on each surface, but the total number of cells that were present five days after plating. The differences in cell number noted at that time are the product of differences in attachment, survival and doubling rate. The surface design modifications resulted in variations in surface area, which may have been a factor in phenotypic expression. Certainly, there was greater surface area on the microstructured disks in comparison with plastic or even polished Ti, particularly on etched surfaces, but the number of cells was fewer and their differentiation was enhanced. This indicates that the cells did not conform to the surface but formed focal attachments that allowed them to suspend across structures, as was seen on 10/6 surface. Thus, the changes in cell number are complex and not easy to explain simply as a function of surface microarchitecture alone. It is likely that the variations in substrate design resulted in modifications in protein adsorption from the media or in the conformation of the adsorbed proteins [39-41], thereby altering the cell-substrate interaction.

In summary, the use of well controlled surfaces with defined microarchitectures to model how cells interact with their surface are essential to design materials that modulate cell response in a predictable way. Attachment-dependent cells, like osteoblasts, live in three-dimensional environments that include a complex physical substrate. Our results show that the microdimensions and spacing of cavities are

important signals for osteoblast proliferation and differentiation and suggest that changes in local factor production modulate these responses. Cell attachment depends on cavity spacing, cell growth and aggregation depends on cavity dimensions, and cell morphology depends on the presence of nanostructural features. The nanoarchitecture also modulates differentiation in concert with the microarchitecture, suggesting synergistic mechanisms are involved. Finally, TGF-ß1 and PGE_2 levels are differentially regulated by micro and nano structural features of the surface. Previous studies showing that TGF-ß1 levels depend on prostaglandin production [10], suggest that either prostaglandins other than PGE_2 are responsible, or that the effects of cavity dimension are a pre-requisite to confer sensitivity to the nano-etch.

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CHAPTER 3

OSTEOBLASTS ARE SENSITIVE TO SURFACE NANOSTRUCUTRE

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A. INTRODUCTION

Osseointegration of titanium implants *in vivo* depends on surface morphology. The microtopography of the surface affects cell attachment, adhesion, proliferation, differentiation and local factor production *in vitro*.[1-3] This affects the clinical outcome of the implant *in vivo*. Surfaces with greater micro-roughness exhibit greater pullout strength both in animal experiments and in clinical trials.[4,5]

The MG63 cell line, originally derived from a male human osteosarcoma, is a well established model for studying the effects of surface microtopography on osteoblast-like cells *in vitro*.[6] MG63 cells cultured on rough Ti surfaces exhibit decreased attachment and proliferation and increased phenotypic differentiation compared to cultures on plastic and smooth Ti surfaces. These effects are also seen in normal human osteoblasts (NHOst).[7] Surface microtopography also affects cell response to systemic hormones (estradiol,[7] $1\alpha,25(OH)_2D_3$[8,9]), mechanical stimuli (shear force[10]), and local regulatory factors (bone morphogenetic protein-2[11]). Studies comparing osteoblast cell lines at different states in the osteoblast lineage show that response of cells to surface roughness depends on cell maturation state as well.[12]

In addition to modulating cell proliferation and differentiation, surface roughness affects the ability of cells to produce local regulatory factors, including PGE_2 and TGF-β1. MG63 cells release increased levels of PGE_2 and TGF-β1 into their conditioned medium;[13] and $1\alpha,25(OH)_2D_3$ increases PGE_2 and TGF-β1 in a

synergistic manner on rough surfaces.[14] A similar effect was also observed in culltures of NHOst cells in response to estradiol.[7] Again, production of PGE_2 and TGF-β1 and their responses to $1\alpha,25(OH)_2D_3$ depend on the maturation state of osteoblast.[12]

The mechanisms mediating the cell response to surface morphology are not clear. Previous studies reveal that integrin signaling may be involved. Inhibition of integrin-associated protein kinase C (PKC) activity has no effect on proliferation of MG63 cells regardless of surface roughness, but it blocks the surface-dependent increase in alkaline phosphatase activity.[15] Prostaglandins also play an important role. Inhibition of prostaglandin production inhibits the response of MG63 cells to surface roughness.[15] Both constitutive cyclooxygenase-1 (COX-1) and inducible COX-2 are involved. Studies examining the time course of the prostaglandin effect show that prostaglandins may mediate the initial response of MG63 cells to Ti surfaces.[16]

Although Ti surface topography is important in implant design, the optimal surface texture is not yet known, because surface roughness has not been well characterized in previous studies. In general, roughness was defined as the average peak to valley height (Ra). Many of these surfaces have had very complex microtopographies but how specific features impacted cell response were not described, including distance between peaks, curvature of the valleys, and relative distribution of flat and smooth regions. In this study, we used chemical etch and electrochemical machining to produce reproducible nanoscale structures on Ti

surfaces. The nanoscale structures were also superimposed on microscale structures to examine MG63 cell responses.

B. MATERIALS AND METHODS

a. Preparation and Characterization of Ti Disks

Three different nanostructural features were used on two different surface preparations: mechanically polished titanium (PT) and titanium with a hexagonal patterned overlay of cavities (30/6) prepared by photolithography as described below. Ti disks were prepared from 1 mm thick sheets of grade 2 unalloyed Ti (ASTM F67 'Unalloyed titanium for surgical implant applications') and supplied by Institut Straumann AG (Walderburg, Switzerland)[17]. The disks were punched to be 15 mm in diameter so as to fit into the well of a 24-well tissue culture plate, and were processed as follow. Disks were degreased by washing in acetone, processing through 2% ammonium fluoride / 2% hydrofluoric acid / 10% nitric acid solution at 55°C for 30 s, and mechanically polished to obtain a mirror finish surface with R_a = 0.05μm. The mechanically polished Ti disks were then coated with a polyisoprene based negative photoresist (HNR80, Arch Chemicals Inc., CT), which was exposed to UV light to develop the initial patterns. Cavities were made by anodic dissolving of titanium through the patterned photoresist in an ethanol-methanol based 3M sulfuric acid electropolishing electrolyte. (Figure 1A)

Nanometer structures were produced by two different methods: acid etching and porous anodization. Acid etching was performed by immersing the Ti disks into a mixture of HCl and H_2SO_4 heated above 100°C for several minutes. (Figure 1B,C) Porous anodization was performed in a jacketed glass vessel containing 1M H_2SO_4 under sweeping potential at 20V/s from 0V to 125V.(Figure 1E,F)

Control surfaces included tissue culture plastic and a clinically used Ti surface with mixed micro and nanotopography (SLA). SLA disks were first degreased as pretreatment disks, then course grit-blasted with 0.25-0.50 mm corundum grit at 5 bar until the surface became a uniform gray, followed by acid etching as described above.[6-8] (Figure 1D)

After mechanical, electrochemical or chemical treatments described above, all samples were subjected a passivation treatment in 65% nitric acid for 2 times 15 min in an ultrasonic bath. They were then ultrasonically cleaned in ultra pure water for 3 times 5 min and dried with nitrogen. Prior to cell culture, all the samples were plasma cleaned 2 min for each side (Plasma Cleaner PDC-32G, Harrick, NY).

The average surface roughnesses (R_a) measured by non-contact laser profilometry were 700, 400, 60 and 40 nm on acid etched, porous anodized, mechanically polished and electropolished surfaces, respectively. The average pore diameter produced by anodization was 82±35 nm, based on measurements of scanning electron micrographs. X-ray photoelectron spectroscopy survey spectra measured after the final cleaning procedure were similar for all treatments. This may due to the spontaneous formation of a surface oxide film in air, and TiO_2 was

the most important component of the oxide layer. The SLA surfaces had an overall roughness R_a of 3.97±0.04μm as shown previously.[8]

b. Cell Culture

MG63 osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD). This osteoblastic cell line was originally isolated from an osteosarcoma and exhibits numerous osteoblastic traits, including a high level of $1\alpha,25(OH)_2D_3$ responsive alkaline phosphatase specific activity and osteocalcin synthesis. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2 and 100% humidity. The medium was exchanged every 48 hours. When the cells reached confluence in T-75 flasks, they were subcultured onto the disks 24-well tissue culture plates at a plating density of 9,600 cells/cm^2, based on the surface area of the tissue culture plastic wells. For each experiment, there were six wells with no disks (plastic surface) and six wells each for kind of Ti disks.

c. Cell Morphology

Cell morphology on the test surfaces was examined by scanning electron microscopy (SEM). At harvest, the culture media were removed and samples were fixed by phosphate buffered 4% formaldehyde / 1% glutaraldehyde for more than one hour. The samples were then rinsed with 0.1M phosphate buffered saline

(PBS) and fixed with 1% osmium tetroxide in Zetterqvist's buffer (30mM sodium barbital, 40mM sodium acetate, 0.1M NaCl, 4mM KCl and 1mM $CaCl_2$ adjusted to pH 7.4) for 30 min. After fixation, the disks were rinsed with PBS, followed by sequential dehydration in 50, 75, 90% *tert*-butyl alcohol for 20 min, and 100% *tert*-butyl alcohol 3 times, followed by drying under vacuum on ice. A thin layer of gold-palladium was sputter-coated onto the samples for 1.5 min prior to examination using a Hitachi S800 FEG scanning electron microscope.

d. Cell Number

At harvest, cells were washed twice with DMEM and then were released from the culture wells by 0.25% trypsin in Hank's balanced salt solution (HBSS) containing 1mM ethylenediamine tetraacetic acid (EDTA) for 10 min at 37°C, followed by addition of DMEM containing 10% FBS to stop the reaction. This procedure was repeated because second digestion was important for releasing the cells completely from rough Ti surfaces.[6] Cell suspensions were centrifuged at 500 x g for 15 min at 4°C. Supernatant was discarded and cell pellets were resuspended in 10mL 0.9% NaCl. Cell number was determined by counting the particles between 5.02 μm and 17.02μm in 500μL suspension (Z1 cell and particle counter, Beckman Coulter). Cell viability was proved to be >95% in this method previously based on trypan blue dye.

e. Cell Differentiation

Cellular alkaline phosphatase specific activity

Cell pellets were lysed in 500 µL 0.05% Triton X-100 for protein quantification and cellular alkaline phosphatase specific activity assay. Protein content was determined by detecting of colorimetric cuprous cation in biuret reaction (BCA Protein Assay Kit, Pierce Biotechnology Inc., Rockford, IL) at 570nm (Microplate reader, BioRad Laboratories Inc., Hercules, CA). Alkaline phosphatase [orhtophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1] specific activity was assayed as the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2 and specific activity was determined.[18]

Osteocalcin

Osteocalcin levels in the conditioned media were measured using a commercially available radioimmunoassay (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA) as described previously.[8] Media were concentrated fivefold by speed vacuuming (DNA110 Speedvac, Thermo Savant Corp., Milford, MA) and incubated with 100µL [I-125] osteocalcin tracer and 100µL of rabbit antihuman osteocalcin serum at 37°C for 2.5 h. Goat anti rabbit IgG, polyethylene glycol (100µL each) and 1mL PBS were then added, followed by centrifuging at 500 x g for 15 min at 4°C. The supernatant was decanted and the pellets were counted for 1 min in gamma counter.

f. Local Factor Levels

Prostaglandin E_2

The amount of PGE_2 produced by the cells and released into the media was assessed using a commercially available competitive binding radioimmunoassay kit (NEK020A Prostaglandin E_2 RIA kit, Perkin Elmer, Wellesley, MA).[19] Media were acidified by 50μL 0.5M HCl for 250μL sample at -20°C to preserve PGE_2. In the assay, unlabeled PGE_2 in the sample and radiolabeled PGE_2 were incubated overnight at 4°C with PGE_2 antibody. Antigen-antibody complexes were separated from free antigen by precipitation with polyethylene glycol and counted for 1 min in gamma counter. Sample PGE_2 concentrations were determined by correlating the percentage bound over unbound counts to a standard curve.

Transforming growth factor b1

TGF-β1 was measured using a commercially available enzyme-linked immunoassay (ELISA) kit specific for human TGF-β_1 (G7591 TGF-β1 E_{max} immunoassay system, Promega Corp., Mandison, WI).[13] To determine the TGF-β1 content of the media, immediately prior to assay, conditioned media were diluted 200-fold. An aliquot of the diluted media was assayed for active TGF-β1. The remaining media were acidified by the addition of 1M HCl till pH lower than 3 for 15 min to activate any latent TGF-β1. Following neutralization with 1M NaOH, the assay was performed according to the manufacturer's direction. Latent TGF-β1 was defined as total TGF-β1 minus active TGF-β1.

g. Statistical Analysis

The data presented here are from one of two separate experiments. Both experiments yielded comparable observations. For any given experiment, each data point represents the mean ± standard error of six individual cultures. Data were first analyzed by analysis of variance; when statistical differences were detected, the Student's *t*-test for multiple comparisons using Bonferroni's modification was used. Additionally, data were analyzed using two-factor analysis of variance with equal replication using the SPSS program for PCs. *P*-values < 0.05 were considered to be significant.

C. RESULTS

Cell morphology was sensitive to surface nanostructure. The MG63 cells cultured on 30/6 smooth surfaces tended to form a continuous monolayer (Figure 2A, D), which was similar to cells cultured on PT surfaces (data not shown). When cultured on 30/6 acid etched surfaces, MG63 cells exhibited an enlonged morphology and did not form a closely contacted monolayer. Individual cells extended across the cavity boundaries and attached to the surfaces by cellular extensions (Figure 2B, E). Anodized 30/6 surfaces were covered with a continuous monolayer of MG63 cells as were smooth 30/6 surfaces, but part of the cells behaved like those on 30/6 acid etched surfaces, extending across the cavities (Figure 2C, F).

Cell number was affected by surface nanostructure superposed on microstructure (Figure 3). Cell numbers were lowest on SLA surfaces and 30/6 acid etched surfaces. The numbers of cells on all the other Ti surfaces were between the levels on plastic and SLA surfaces, and there was no significant difference between them.

Alkaline phosphatase specific activity varied with surface microstructures and nanostructures (Figure 4). Enzyme activity in the cell lysates was comparable in cultures grown on plastic, PT and acid etched PT surfaces. Cell cultures grown on the other surfaces resulted in reduced alkaline phosphatase specific activity.

Osteocalcin level was affected by surface structure in an opposite way to cell number (Figure 5). Cells on all Ti surfaces produced more osteocalcin than those on plastic surfaces. The highest osteocalcin levels were seen in the cultures on SLA and 30/6 acid etched surfaces.

Production of PGE_2 was sensitive to surface microstructure and nanostructure (Figure 6). MG63 cells cultured on SLA surfaces produced more PGE_2 than those on all the other surfaces. PGE_2 levels in cultures on PT surfaces were comparable to those on plastic surfaces. Cells cultured on 30/6 acid etched surfaces produced more PGE_2 than those on the other Ti surfaces except SLA.

Surface nanostructure had a significant effect on the level of both active (Figure 7) and latent (Figure 8) TGF-β1 in the conditioned media. The active TGF-β1 levels increased in cultures on all Ti surfaces, except on PT surfaces. The effects were most significant on 30/6 acid etched, followed by SLA and 30/6 anodized

surfaces. The latent TGF-β1 levels in the media of cells cultured on SLA, 30/6 acid etched and 30/6 anodized surfaces were higher than in cultures grown on others surfaces. The lowest levels of TGF-β1 were in cultures on plastic, PT and acid etched PT surfaces.

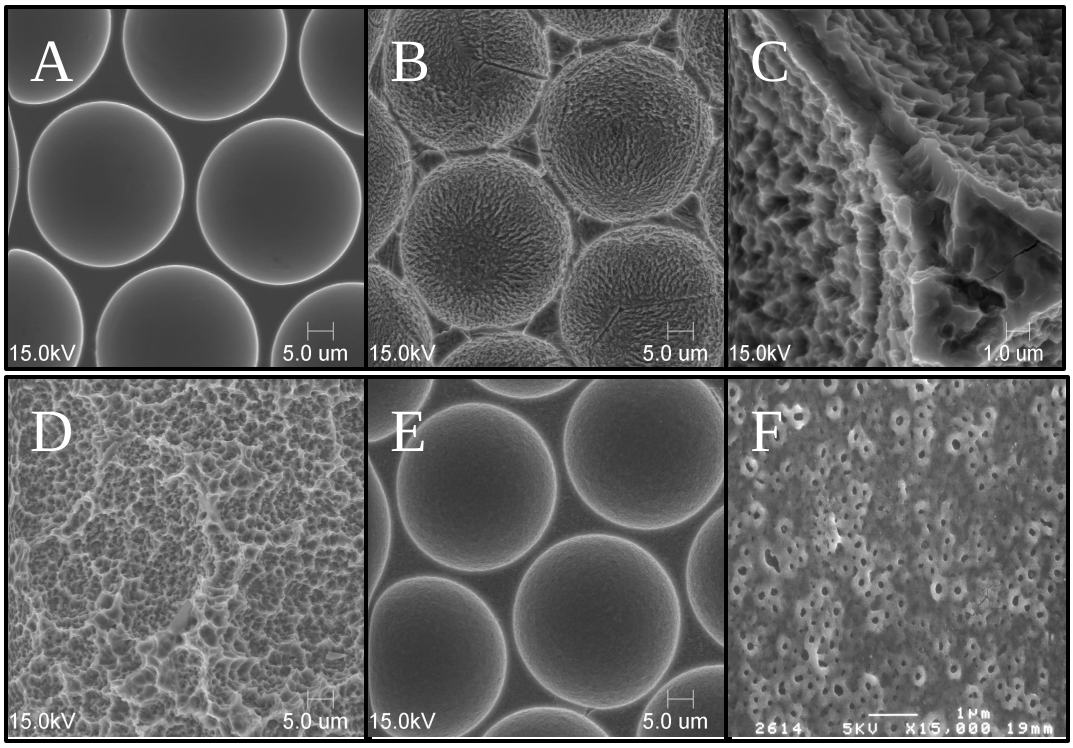

Figure 3-1 Surface nanoscale structures of titanium disks prepared using acid etching and electrochemical machining.

Mechanically polished Ti surfaces with 30μm diameter hemispherical craters were prepared by dissolving Ti in electropolishing electrolyte through a patterned photoresist.(A, x 500) These surfaces were further treated with acid to produce a nano-etch with an Ra of 0.7 μm.(B, x 500; C, x 10,000) SLA surfaces were produced by grit blasting and acid etching with an overall Ra of 4 μm.(D, x 500) The surfaces shown in (A) were also anodized to produce porous surface with an Ra of 0.4 μm.(E, x 500, F, x 15,000).

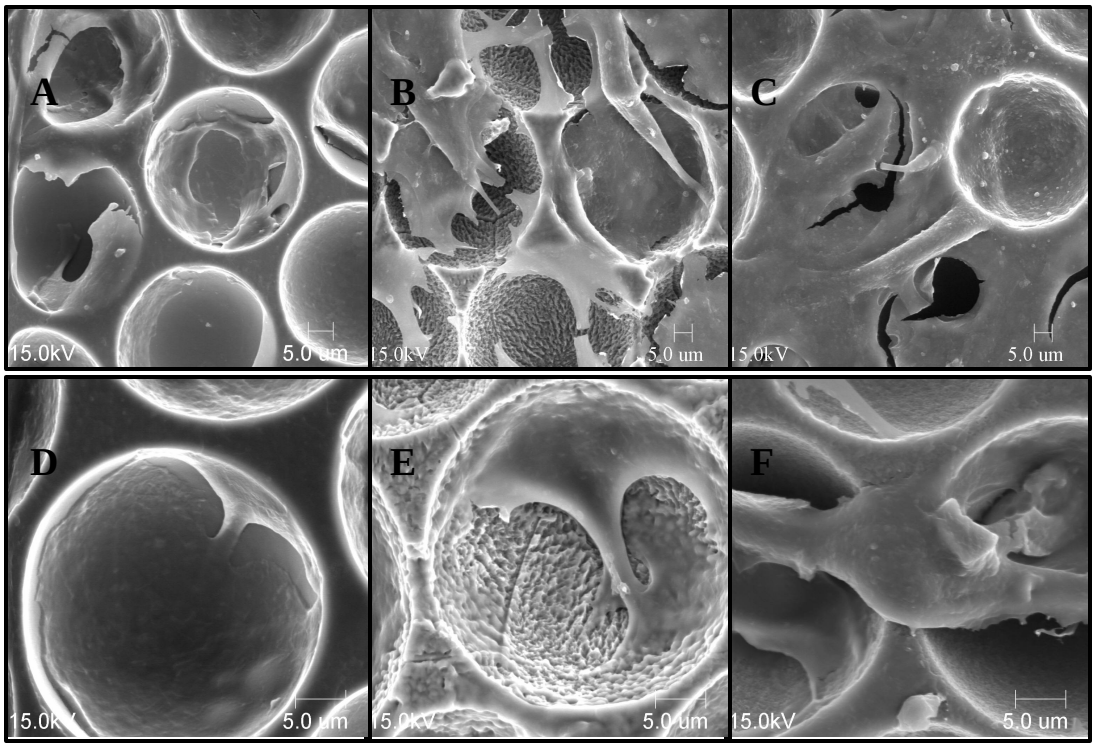

Figure 3-2 Morphology of MG63 osteoblast-like cells cultured on micro and nanostructured Ti surfaces

Osteoblasts cultured on 30/6 smooth surfaces exhibited flattened morphology and composed a confluent monolayer.(A, x 500, D, x 1,000) Cells on the 30/6 ethced surfaces tended to anchor in adjacent cavities and exhibited a more cytoplasmic extensions.(B, x 500; E, x 1,000) On 30/6 anodized surfaces, cells exhibited a confluent monolayer and spread across the cavities.(C, x 500; F, x 1,000)

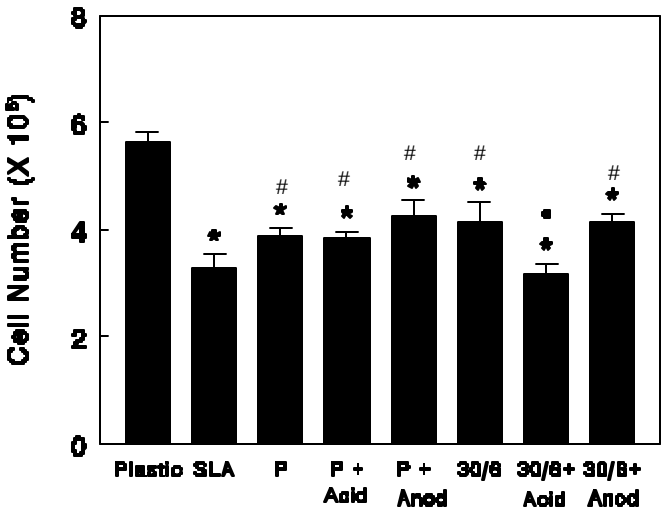

Figure 3-3 Effect of surface nanotopography on cell number.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+Acid) or anodized (+Anod) resulting in nanotopographies. Cell number was determined five days after plating. Values are means $\pm$ SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. other surfaces.

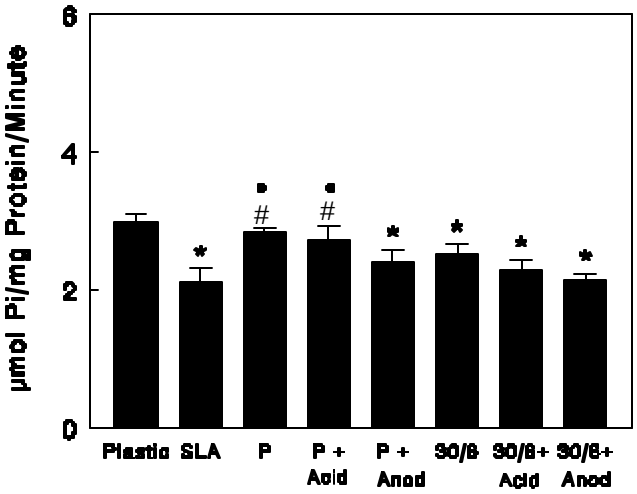

Figure 3-4 Effect of surface nanotopography on cellular alkaline phosphatase specific activity.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+Acid) or anodized (+Anod) resulting in nanotopographies. Alkaline phosphatase specific activity was measured in cells released by trypsin. Values are means $\pm$ SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. 30/6 + Acid and 30/6 + Anod surfaces.

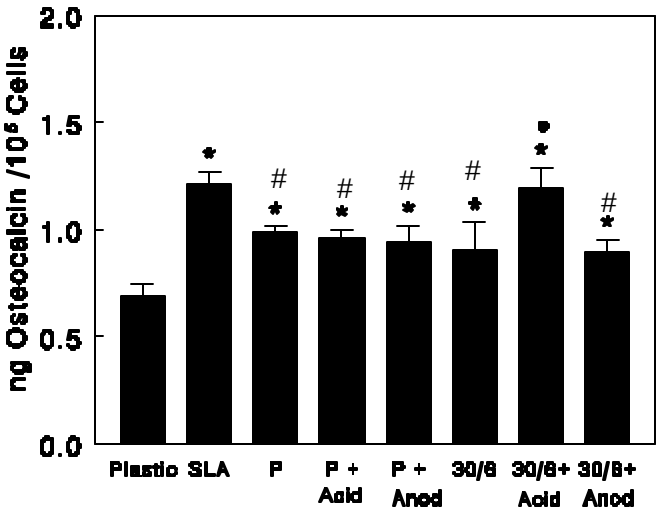

Figure 3-5 Effect of surface nanotopography on osteocalcin levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+Acid) or anodized (+Anod) resulting in nanotopographies. Osteocalcin levels were measured in the conditioned media using an RIA kit. Values are means $\pm$ SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. other surfaces.

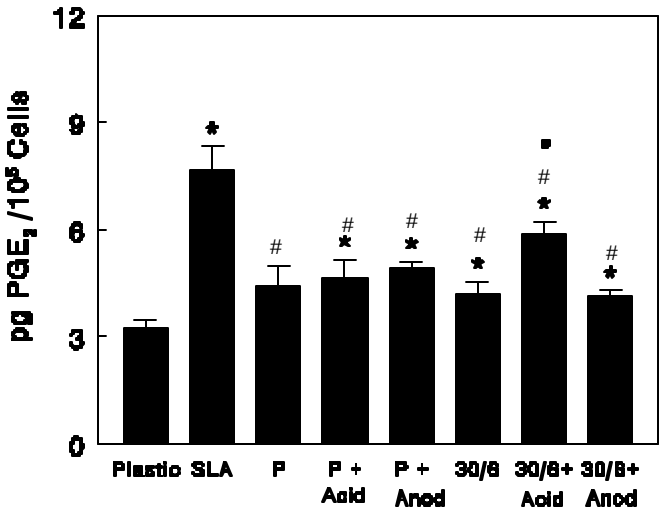

Figure 3-6 Effect of surface nanotopography on PGE_2 levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+ Acid) or anodized (+ Anod) resulting in nanotopographies. PGE2 levels in the conditioned media were determined by using an RIA kit. Values are means $\pm$ SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. other surfaces.

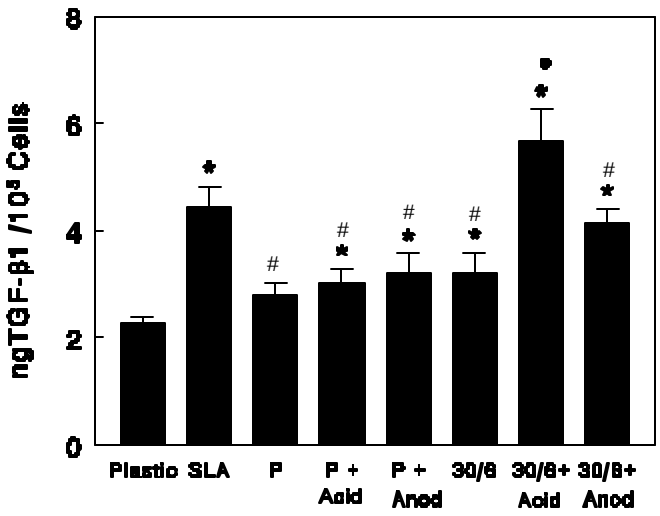

Figure 3-7 Effect of surface nanotopography on active TGF-β1 levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+ Acid) or anodized (+ Anod) resulting in nanotopographies. Active TGF-β1 levels were measured in the conditioned media using an ELISA kit specific for human TGF-β1. Values are means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. other surfaces.

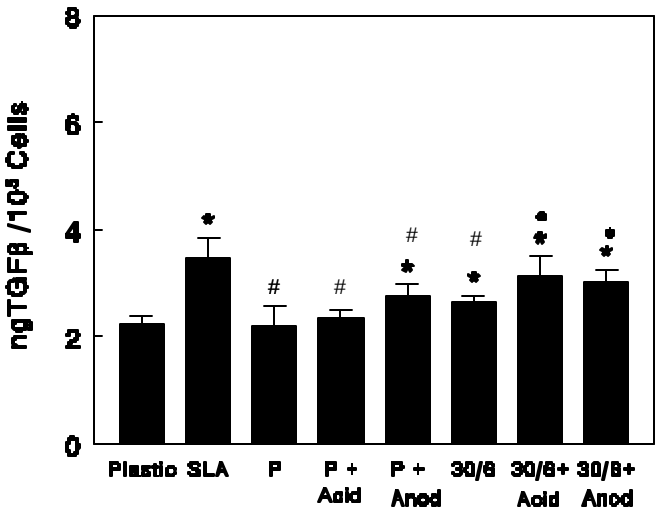

Figure 3-8 Effect of surface nanotopography on latent TGF-β1 levels.

MG63 cells were cultured on tissue culture plastic (plastic), grit blasted and acid etched Ti (SLA), polished Ti (P) and microstructured surfaces prepared by using electrochemical machining (30/6). The polished surfaces and microstructured surfaces were acid etched (+ Acid) or anodized (+ Anod) resulting in nanotopographies. Latent TGF-β1 levels were measured by subtracting the active TGF-β1 levels from acidified total TGF-β1. Values are means ± SEM of six independent cultures. Data are from one of two separate experiments, both with comparable results. Data were analyzed by ANOVA and significant differences between groups determined using the Bonferroni modification of Student's t-test. *$p<0.05$, surface v. plastic; #$p<0.05$, surface v. SLA; •$p<0.05$, surface v. P and P+ Acid.

D. DISCUSSION

Surface morphology affects osteoblast-like cell behavior. MG63 cells respond to SLA surfaces with decreased cell number, more differentiated phenotype and increased local factor production. Previous studies failed to discriminate which distinct surface features contribute to specific cell responses. The present study used Ti surfaces with controllable microscale and nanoscale structures to resolve the role of nano structure in modulating osteoblast behavior. The results show MG63 cells are sensitive to nanostructure, they can discriminate between nanostructure morphologies, and microstructure modifies the response to nanostructure.

MG63 cells cultured on 30/6 acid etched surfaces exhibit a similar phenotype to cells on SLA, characterized by decreased cell number and alkaline phosphatase activity, increased osteocalcin levels and increased local factor production, whereas cells cultured on PT behaved like cells cultured on plastic. However, the different structures between SLA and 30/6 acid etched surfaces need to be noted. The grit-blasting procedure to produce SLA results in irregularly shaped craters with varied diameters from 10 to 100 μm. In contrast, variances in designed microstructured surface are smaller. The depth of the craters on SLA are much more shallow than those on 30/6 surfaces. The acid etching on SLA produces pits with an average diameter of 1-3 μm, and there are no significant boundaries between craters. On 30/6 surfaces, the acid etching produces an overall Ra of 0.7 μm. The

nanoscale roughness on 30/6 appears more regular than on SLA. These differences may contribute the differences in cell response to SLA and 30/6 acid etched surfaces.

Three different nanostructures were compared in this study: electropolished (smooth), acid etched and anodized surfaces. Although the Ra of the anodized surface was close to that of the acid etched surface, the anodized surface topography presented to the all was more like that of the PT surface. Acid etching produced a peaky and pointy morphology, whereas anodizing produced a sponge-like surface with belt-like rings around the pore openings. No peaky structures were seen on anodized surfaces. The results confirmed that Ra is not sufficient to describe the surface morphology. Other components of surface features affect the cell behavior.

When MG63 cells were cultured on the PT surface, they formed a continuous monolayer regardless of overlaid nanostructure (data not shown). On 30/6 Ti surfaces, cells tended to aggregate in the cavities. The cellular extensions, which are universally observed on SLA surfaces, were seen on 30/6 acid etched and anodized surfaces, but were not evident on smooth surfaces. This indicates that filopodia attachment is favored by the nanoscale structures on the microstructured surfaces.

When nanostructures were superposed on PT, there were not effects on cell number, differentiation phenotype or local factor production. In addition, there was no differences between PT and smooth 30/6 Ti surfaces. However, when acid etched nanostructure was overlaid on 30/6 surfaces, the cell responses were modulated most significantly. The effect of anodized 30/6 surfaces is moderate.

These results indicate that cell responses are modulated by the combination of microscale and nanoscale structure, and the responses are dependent on the nanoscale roughness and shape. Combining with previous data that osteoblast-like cells may attach to implant materials through integrins,[21] we suggests that not only the focal adhesion distribution, but integral conformation of the cytoskeleton may mediate cell response to surface topographies.

The changes in MG63 cell phenotype on the 30/6 acid etched surfaces may be related to the increased production of PGE_2 and TGF-β1 noted on the same surfaces. In MG63 cells, the roughness-dependent increase in osteocalcin is consistent with the increase in PGE_2, which has been shown to stimulate differentiation of osteoblasts in other systems.[19] TGF-β1 also activates osteoblast dfferentiation[23], specifically targeting matrix vesicle alkaline phosphatase activity. We did not measure this aspect of phenotypic expression in this study due to Ti disk limitations. The fact that alkaline phosphatase activity was reduced in cells grown on SLA and acid-etched 30/6 surfaces whereas active TGF-β1 was specifically increased suggests that enzyme was incorporated into the extracellular matrix typical of differentiated cells.

In summary, the Ti surfaces with controllable nanostructure superposed on microstructure were used in this study to distinguish the surface features contributing to osteoblast response. Only when nanoscale roughness produced by acid etching was superposed on microscale structures, the cells respond to surface with

significant differentiated phenotype, and this effect is dependent on nanoscale morphology.

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CHAPTER 4

CONCLUSION

By using of Ti surfaces with well controlled microstructure and nanostructures, osteoblast-like cell response to specific surface features were distinguished in this study. The osteoblast-like cells tends to aggregate into the microscale cavities and the effect was modulated by cavity diameter and spatial distribution. The morphology of the cells depends on the combination of micro and nanostructures. The cells exhibited an elongated appearance in larger cavities and cytoplasmic extensions were observed on the rough nanostructures overlaid on microscale cavities. Cell growth depends on cavity dimension. Cell differentiation is modulated by nanostructural features in concert with the microstructures. Cell number, alkaline phosphatase specific activity and osteocalcin level in cultures on structured 100/6 acid etch surfaces are comparable to those on the complex grit blasted SLA surface, confirming that our model surfaces adequately mimicked these specific design features. Local factor production was also modulated by micro and nanostructures. The cells cultured on cavities with the roughest nanostructure produced the highest level of local factors. The fact that the PGE_2 level depended on cavity dimensions indicates either that cavity dimension is a prerequisite confering sensitivity to the nano-etch, or that prostaglandins other than PGE_2 are involved in the cell response.

These results reveal that cell morphology, proliferation, differentiation and local factor production are modulated by microscale cavity dimension, cavity spacing and nanoscale roughness. The study of surface structural features will contribute to optimize the surface design of implants in clinical application. However, the mechanisms of cell response to these structural features are no yet clear. A possible candidate mediating the response is integrins. Integrins are cell membrane receptors that bind to extracellular matrix and modulate cell functions through the formation of focal adhesion complexes and intracellular signaling pathways. The surface structures might determine the absorbed proteins, affecting the distribution of focal adhesion complexes. Further studies will be performed to examine the effect of specific integrin in mediating the cell response to surface structural features.