Formation of Mineralizing Osteoblast Cultures on Machined, Titanium Oxide Grit–Blasted, and Plasma-Sprayed Titanium Surfaces

Lyndon F. Cooper*/Takayuki Masuda**/S. William Whitson***/
Paula Yliheikkilä****/David A. Felton*****

Altering osseous responses at implant surfaces to enhance bone is a current goal of clinical therapy. Cell culture may be used to investigate surface-dependent responses of bone-forming cells. In this report, the ability of primary fetal bovine mandibular osteoblast cultures to form a mineralizing matrix on machined, titanium plasma-sprayed, and titanium oxide grit–blasted surfaces has been compared. Immunohistochemical markers associated with bone formation were used to define the differentiated state of the formed matrix using qualitative light microscopy, and von Kossa staining was used to demonstrate the presence of mineralization within this matrix. Compared to either titanium oxide grit–blasted or machined surfaces, titanium plasma-sprayed surfaces displayed a unique pattern of mineralized matrix formation. Scanning electron microscopy further revealed that each surface accumulated unique organic and inorganic deposits during matrix formation, suggesting that surface-dependent physicochemical and biochemical conditioning of implant surfaces takes place. Surface topographic features of commercially pure titanium substrates can alter cultured osteoblast extracellular matrix formation and mineralization. Similar molecular and cellular assessment of in vivo responses to implant surface topography may contribute to improved engineering of endosseous implants.
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Key words: differentiation, osseointegration, osteoblast culture, surface topography

Current efforts to improve bone formation at endosseous titanium implant surfaces include the recent introduction of clinical dental implants with unique surface topographies. In addition to existing titanium implants bearing machined or plasma-sprayed surfaces, there is an array of endosseous titanium implants that offer surfaces altered by grit blasting and/or acid etching as a parameter that may affect bone formation at the implant-bone interface.

There exist few clinical guidelines for the selection of the surface type of a titanium endosseous implant. A limited number of fundamental studies considering surface topography effects on bone formation at implants provide insight into bone formation as a function of implant surface topography.[1–3] In addition, in vitro studies using osteoblast cell culture have examined the effects of surface roughness on cell attachment.[4] These studies indicate that roughness per se does not promote cell attachment. The effects of surface roughness on the terminal differentiation of osteoblasts and the associated formation of a differentiated matrix have not been determined in cell culture.

*Associate Professor, Department of Prosthodontics and Dental Research Center, University of North Carolina School of Dentistry, Chapel Hill, North Carolina.
**Visiting Research Associate, Dental Research Center, University of North Carolina, Chapel Hill, North Carolina; Department of Prosthodontics, Tokyo Medical and Dental University, Tokyo, Japan.
***Professor, Southern Illinois University School of Dental Medicine, Carbondale, Illinois.
****Prosthodontic Resident, Department of Prosthodontics, University of North Carolina School of Dentistry, Chapel Hill, North Carolina.
*****Associate Professor and Chair, Department of Prosthodontics, University of North Carolina School of Dentistry, Chapel Hill, North Carolina.

Reprint requests: Dr Lyndon F. Cooper, 302 Brauer Hall CB #7450, University of North Carolina, Chapel Hill, NC 27599.

Osteoblast activity is responsible for bone formation at implant surfaces. The production and mineralization of an extracellular matrix by osteoblasts occurs following the surgical placement of titanium implants in bone.[5,6] A number of osteoblast cell culture models have been developed to study the production and mineralization of extracellular matrix at implant surfaces.[7] The bovine mandibular osteoblast model has been described as one that produces an abundant, mineralizing extracellular matrix when cultured on titanium implant surfaces.[8]

The authors have considered the hypothesis that if osteoblastic cells recognize differences in cellular topography, then the formation of bone matrix by adherent cells will be altered in ways specific to each surface. In this report, the bovine mandibular osteoblast model was used to study the potential role of titanium implant surface topography on the process of extracellular matrix formation and mineralization. After 14 and 21 days of culture, the mineralizing matrices formed on TiO_2 grit–blasted, titanium plasma-sprayed, and machined surfaces were compared by histologic methods.

Materials and Methods

Titanium Disk Preparation. A commercially pure (cp) titanium rod 12.5 mm in diameter was provided by Teledyne (Alvac/Vasco, Monroe, NC), and 2.5-mm thick disks were lathe-cut and then manually finished to a 600-grit paper roughness (machined Ti). Finished disks were also prepared by titanium plasma-spraying (TPS, Interpore, Irvine, CA) or titanium oxide (TiO_2) grit–blasting (TiO-blast, AstraTech, Waltham, MA). Machined disks were ultrasonically cleaned using methyl ethyl ketone, ethanol, and deionized distilled water.[8] Prior to seeding cultures, all disks were rinsed in 70% ethanol and dried by evaporation under ultraviolet exposure in a tissue culture hood.

Surface Analyses. The prepared titanium disks were examined by atomic force microscopy (Auto Probe CP, Park Scientific Instruments, Sunnyvale, CA). Scans (25 μm × 25 μm) were made of each surface. Packaged algorithms provided calculations of area statistics, which included median and mean height, peak to valley measurements, surface area, volume, root mean square (RMS) roughness, and average roughness.

Cell Culture. The culture of fetal bovine mandibular osteoblasts has been described in detail.[9,10] Second passage cells were briefly plated at a subconfluent density of 50,000 cells per 12.5-mm disk in growth media. On day 3, confluent cultures were fed using ascorbate-supplemented media. On day 5, multilayering cultures were maintained in mineralization media. Media was changed daily during the 21-day experiment. Cultures were collected at 14 or 21 days for analysis.

Light Microscopy and Immunohistochemistry. All cultures were rinsed 3 times in phosphate-buffered saline (PBS) and fixed for 1 hour on the culture surface disks with 3.7% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.3). The cultures were cut with a scalpel blade, and sections were lifted from the surface, transferred to a glass coverslip, and embedded in paraffin; 5-μm thick sections were then cut and mounted on gelatin-coated slides. After staining with hematoxylin and eosin, Periodic Acid Schiff (PAS) Alcian blue, and von Kossa's 5% aqueous silver nitrate stains, serial sections were selected for immunocytochemical analysis. The expression of bone sialoprotein (BSP) and osteocalcin (OC) was examined in this way. After blocking sections with normal goat serum (1:20 dilution in PBS) for 20 minutes, sections were incubated for 60 minutes at room temperature with either anti-BSP or anti-OC rabbit antisera (1:250 dilution in PBS). Immunocomplex detection was facilitated by use of horseradish peroxidase-conjugated goat-antirabbit antibody (Vector, Burlingame, CA; 1:250 dilution in PBS). After incubation for 60 minutes at room temperature, peroxidase activity was demonstrated using diaminobenzidine. Stained sections were evaluated and photographed using an Olympus BH2 photomicroscope (Olympus America, Lake Success, NY).

Scanning Electron Microscopy. Samples from 14- and 21-day cultures were rinsed 3 times with PBS and fixed for 60 minutes with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1% cacodylate buffer (pH 7.4). Scanning electron microscopy (SEM) samples were postfixed with osmium tetroxide, critical point–dried and sputter-coated with gold-palladium. Morphologic analysis and elemental analysis (KEVEX) was performed by SEM (Etec Autoscan, Etec, Haywood, CA).

Results

Titanium disks of different topographies were prepared. The 600-grit finish replicates the machined Ti screw surface[9] and the TPS surface and TiO-blast surfaces were prepared by the manufacturers of the titanium plasma-spray IMZ and the TiO_2 grit–blasted AstraTech dental implants. Each surface displayed a unique topography (Table 1, Fig 1). All prepared surfaces appeared to be clean and free of debris by SEM examination. Surface analysis

Table 1 Surface Topography of Different Specimens

Implant type	Median height (μm)	Peak-to-valley (μm)	Surface area ($μm^2$)	Volume ($μm^3$)	RMS roughness (Å)	Average roughness (Å)
CpTi	0.36	0.73	644.4	223.03	1064	838
TiO-blast	1.5	2.73	758.9	864.12	4686	3809
TPS	1.89	3.78	741.53	1154.35	6366	5048

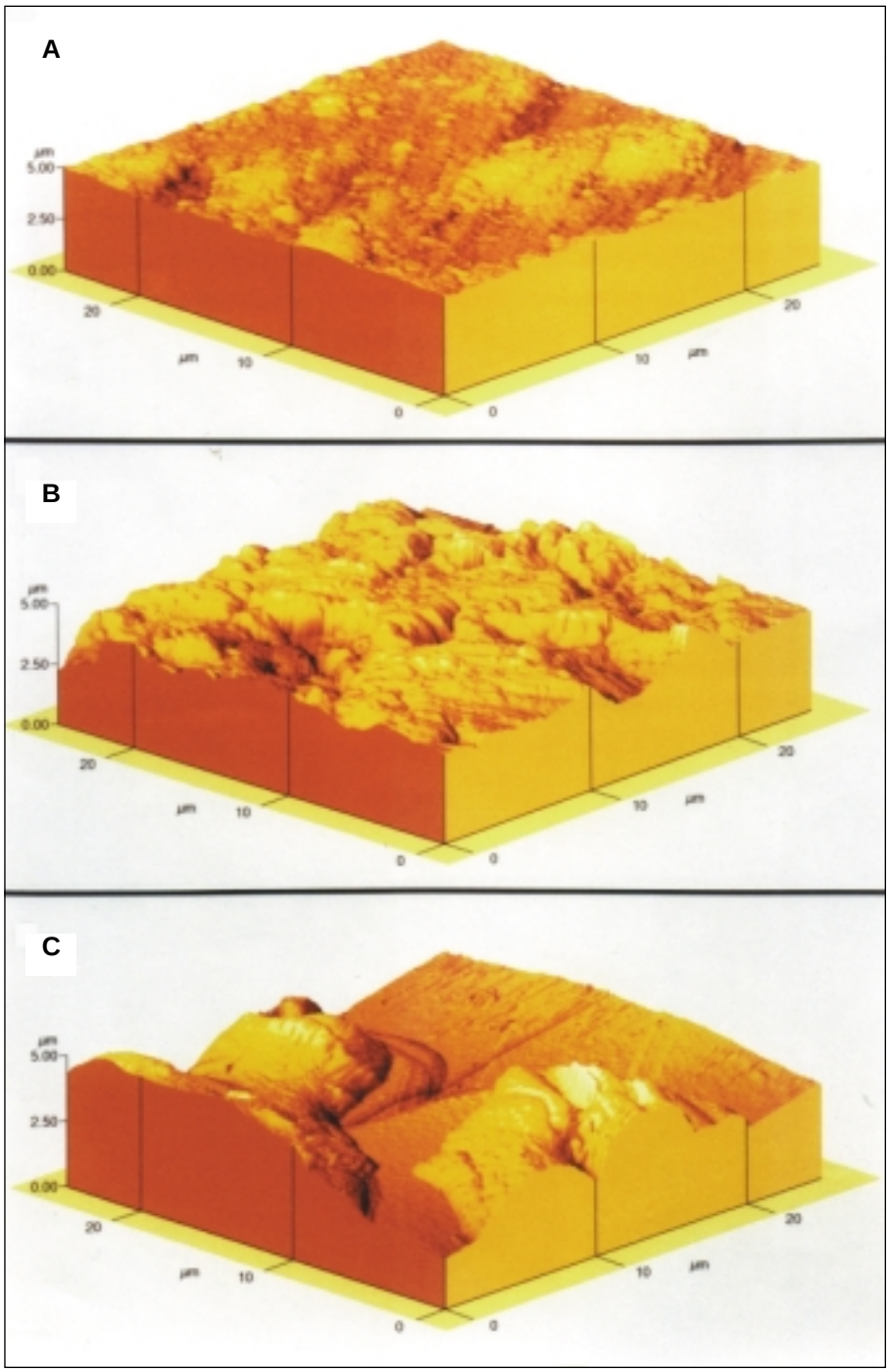

Fig 1 Atomic force microscopy surface topography analyses of representative 25 3 25 μm areas of test substrates: (A) machined Ti surface; (B) TiO-blast surface; (C) TPS surface.

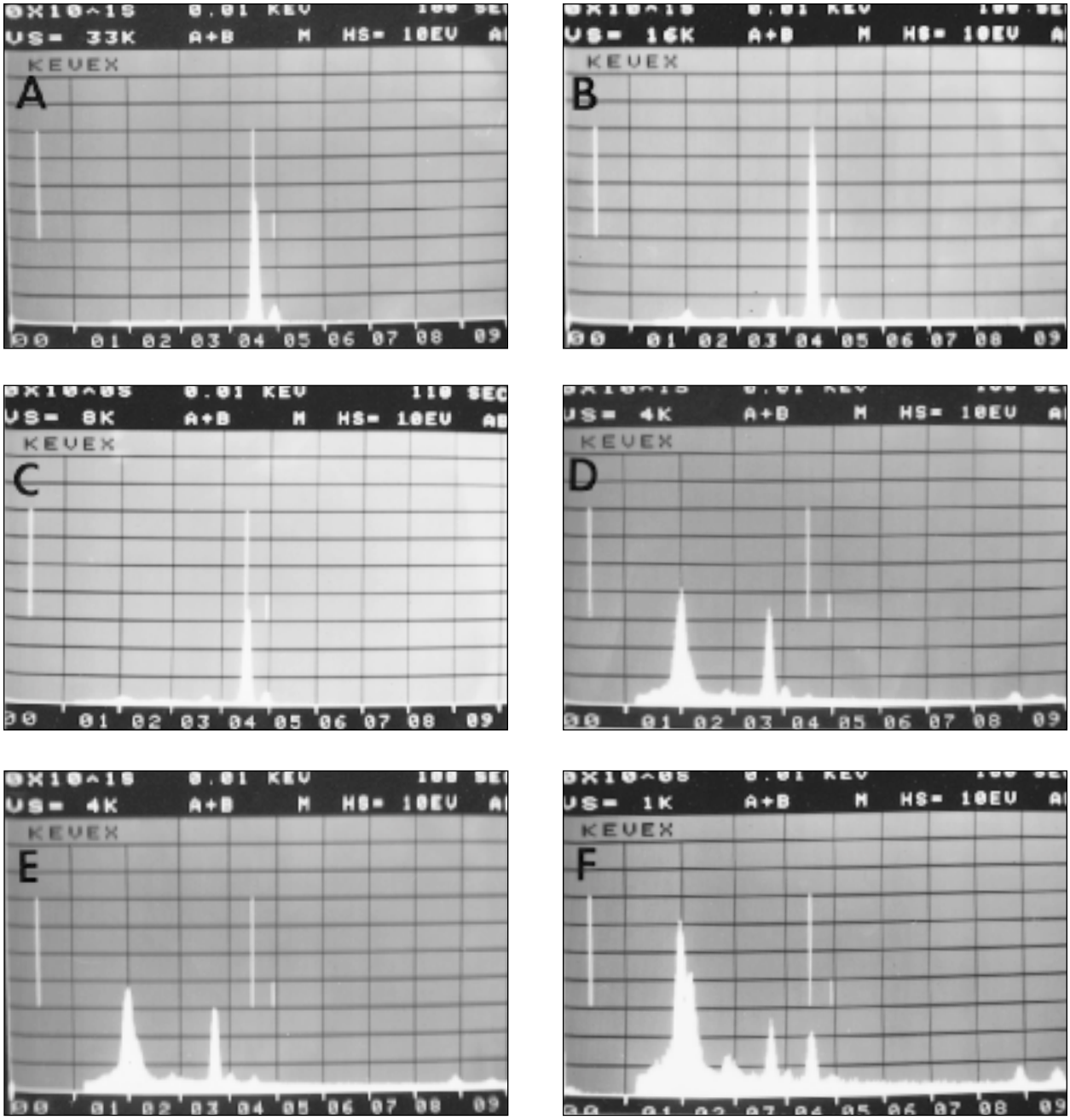

Fig 2 Elemental analysis of test substrates prior to and following 21-day cultures using KEVEX. All substrates show a predominance of titanium and oxygen prior to the initiation of the experiment. (A) machined Ti; (B) TiO-blast; (C) TPS. The titanium peak is completely obscured by calcium phosphate matrix formed at 21 days formed on machined Ti (D) and TiO-blast (E) surfaces. The titanium surface was still visible by this technique when culture layers on TPS surfaces were examined (F).

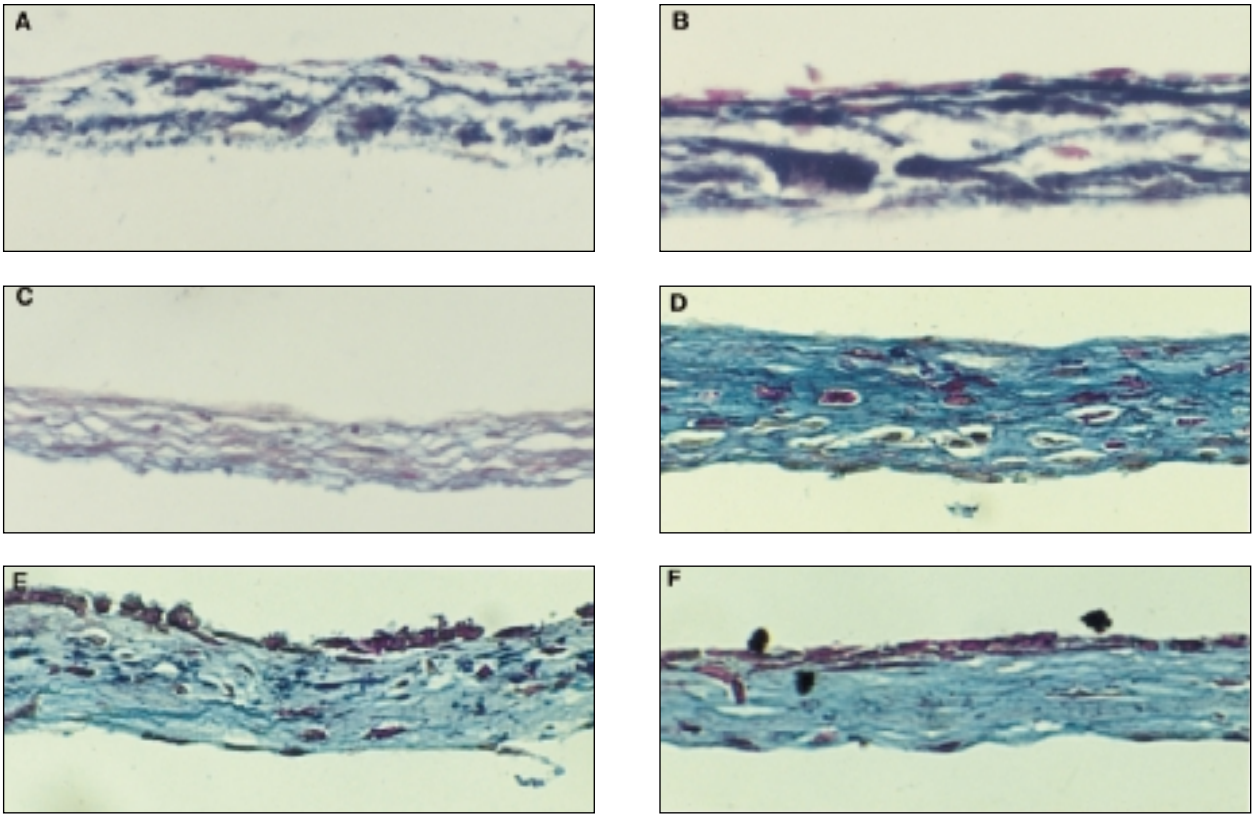

Fig 3 Histologic analysis of cultures grown on test substrates. PAS Alcian blue staining of 5-μm cross sections of cells grown for 14 days (A to C) or 21 days (D to F) on machined Ti (A and D), TiO-blast (B and E), or TPS (C and F).

revealed the predominance of titanium and oxygen species for all surfaces (Fig 2). Machined surfaces had relatively low peak-to-valley measurements compared to rough surfaces. Several AFM tips were broken in attempts to image the highly irregular TPS surface. While surface areas did not vary appreciably, measured volumes and RMS roughness measurements were much greater for both the TiO-blast and TPS surfaces.

The formation of mineralizing multilayering cultures occurred during the first 14 days of culture. However, at the macroscopic level, far less opacification of the culture layer occurred on the TPS surfaces than on the other surfaces. During the culture period, cultures grown on TPS substrate displayed a greater tendency for spontaneous culture layer contraction or reflection than the cultures grown on other substrates. In one trial, where 4 cultures were grown on each surface, all of the titanium plasma-sprayed surface cultures were lost at day 5 to spontaneous contraction or reflection, while the other surfaces were grown to completion. In 2 other separate trials, 1 and 2 of 4 cultures grown on the titanium plasma-sprayed surfaces were lost at day 5 because of contraction or reflection from the surface.

Light microscopic evaluation of the cultures was performed in cross section following removal of the cultures from the test surfaces. On all surfaces, the formed culture layers demonstrated evidence of proliferation and multilayering within an endogenous collagenous matrix (Fig 3). Hematoxylin and eosin and PAS Alcian blue staining revealed prominent PAS staining of cells in the superior aspects of the machined Ti and TiO-blast cultures, whereas the TPS cultured cells failed to demonstrate this staining pattern. The TPS culture layer included cells that were also flattened or spindle-shaped.

Mineral content within these cultures was qualitatively assessed by von Kossa staining. A lack of von Kossa staining of matrix formed on TPS surfaces was noted (Fig 4). While abundant mineral was observed throughout the TiO-blast surfaces, representations of mineral formation were greatest for the machined Ti surfaces.

Immunohistochemical analysis also demonstrated the distinct behavior of cells grown on the TPS surface, when compared to cultures formed

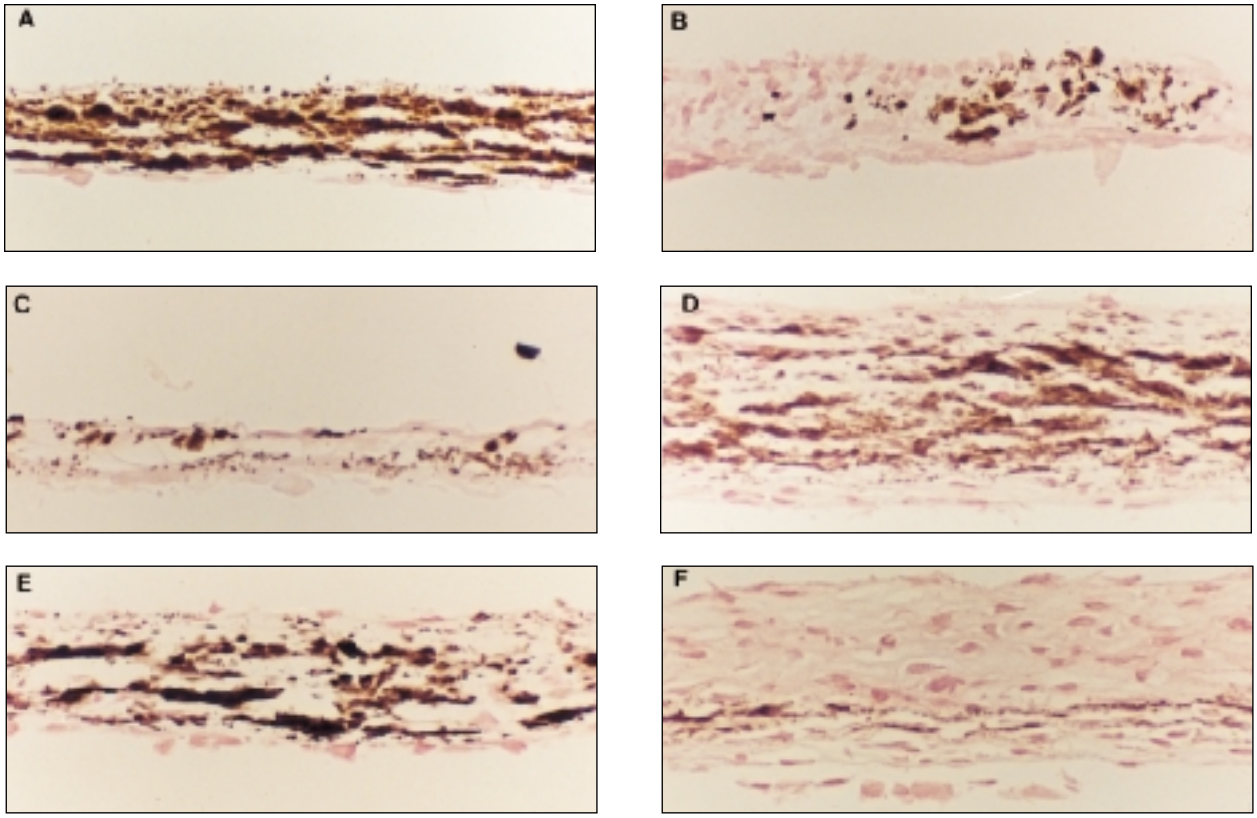

Fig 4 Histologic analysis of mineral accumulation in cultures grown on test substrates. Von Kossa staining of 5-μm cross sections of cells grown for 14 days (A to C) or 21 days (D to F) on machined Ti (A and D), TiO-blast (B and E), or TPS (C and F). Note the relatively limited and restricted staining on cultures grown for 21 days on TPS (F).

on the machined Ti and TiO-blast surfaces. Two immunohistochemical markers associated with the process of osteoblast differentiation and mineral accumulation within forming matrix are BSP and OC.[11,12] Bone sialoprotein expression was observed only in the superior aspects of the machined Ti and TiO-blast cultures. This pattern is identical to previous observations made regarding this culture system's behavior on glass and hydroxyapatite substrates.[13] In contrast, TPS-associated cultures demonstrated BSP expression within the cells adjacent to the substrates. Osteocalcin expression was coincident with von Kossa staining. Minimal staining of TPS-associated cultures by either the BSP or OC antibodies (Figs 5 and 6) was consistent with the pattern of (relative lack of) von Kossa staining.

The KEVEX analysis of the adherent surfaces provided evidence on all surfaces of calcium phosphate accumulation within the mineralizing multilayering cultures formed (Fig 2). However, far less calcium and phosphate was identified within the TPS surface culture layers than within either the TiO-blast or machined Ti surface culture layers. The TPS scan suggests that titanium surfaces were not masked by an intact, mineralizing cell-derived layer.

Scanning electron microscopy was used to examine the material remaining adherent to the culture surfaces following culture layer removal. Each surface tested revealed a unique remnant material upon inspection (Fig 7). The machined Ti surfaces demonstrated an extremely limited number of adherent cells and little remnant material of any kind. The 2 prepared rough surfaces each presented unique remnant material. The TiO_2-blasted surface revealed the greatest number of adherent cells following reflection and had fine globular and individual fibrils of varying lengths interspersed between the elevated aspects of the rough surface. The TPS surface displayed the greatest abundance of remnant material and no residual cellular attachment. The material adhering to the

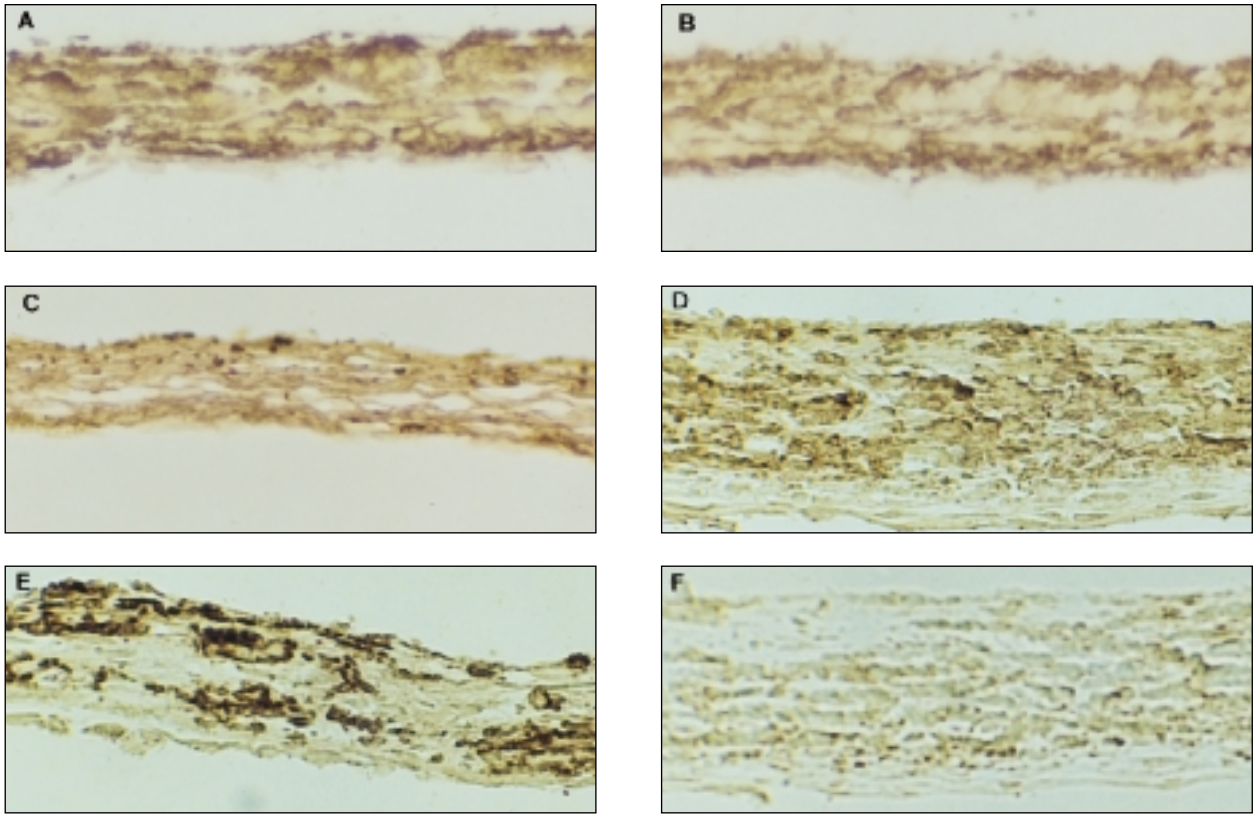

Fig 5 Immunohistologic analysis of bone sialoprotein (BSP) expression in cultures grown on test substrates. Anti-BSP antibody (1:250 dilution) was used to stain 5-μm cross sections of cells grown for 14 days (A to C) or 21 days (D to F) on machined Ti (A and D), TiO-blast (B and E), or TPS (C and F). Note the relatively limited reactivity of BSP staining limited to the inferior portion of cultures grown for 21 days on TPS (F). This is in contrast to the general absence of BSP expression in cells adherent to substrates that has been previously reported.

surface was similar to that of the TiO-blast remnant material but was present in greater abundance. The globular material was much more dense, globules were larger, and networks of many fibrils were commonly observed. This dense array of material was most evident within the relatively large recessed areas created by the plasma-spraying process.

Discussion

This cell culture investigation of the effects of titanium surface topography on osteoblast behavior focused on the relatively delayed responses of the culture system, namely the formation of a multilayered, collagen-enriched, and mineralized matrix. The bovine mandibular osteoblast culture system produces a well-differentiated culture layer within 14 days and offers the advantage of expedience. In a previous study, the similar outcome of culture growth on chemically dissimilar surfaces could have reflected similarities in the topography of the substrates.[14] Here, the effect of surface topography was examined.

The main finding of this study was that the process of osteoblast matrix formation and mineralization in a multilayering culture system is modified by surface topography. The qualitative differences in matrix protein expression and mineralization indicated the processes were altered by changes in substrate topography.

Atomic force microscopy was used to provide a topographic comparison of the test substrates. Table 1 indicates that surfaces with R(a) values ranging from approximately 0.5 μm to 5.0 μm were created. These test substrates reflect the spectrum of dental implant surfaces available and in use in clinical dentistry. This study does not include a complete evaluation of the surface characteristics presented by the prepared culture substrates. While a general measure of topography has been provided along with assurances that gross

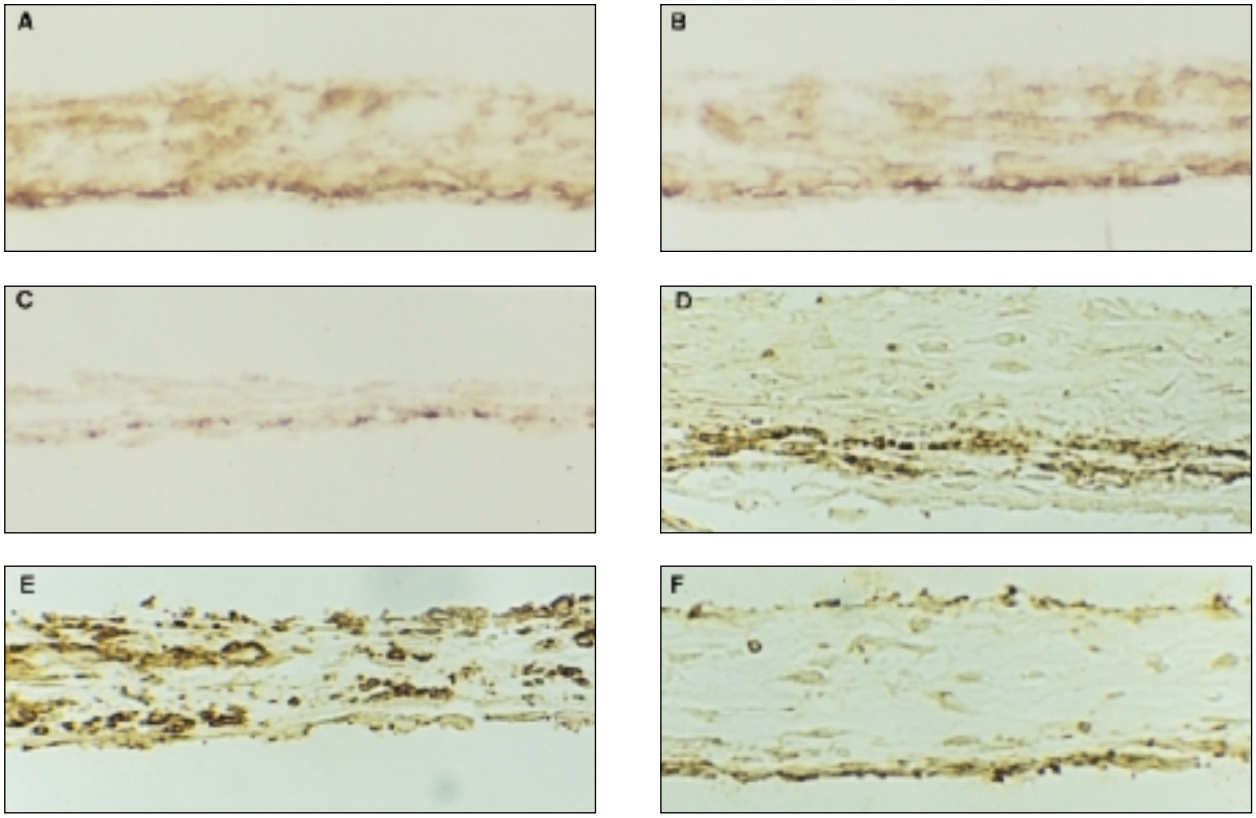

Fig 6 Immunohistologic analysis of osteocalcin (OC) expression in cultures grown on test substrates. Anti-OC antibody (1:250 dilution) was used to stain 5-μm cross sections of cells grown for 14 days (A to C) or 21 days (D to F) on machined Ti (A and D), TiO-blast (B and E), or TPS (C and F). Note coincidence of OC staining with von Kossa staining in Fig 4. TPS substrates at 14 and 21 days demonstrate the relative absence of OC expression.

contamination of the surfaces did not occur, the precise molecular properties of the surfaces were not considered. Thus, the interpretation of these results is limited by further consideration of potential organic and inorganic ionic materials present on these test surfaces.

The bovine mandibular osteoblast culture system displays differentiation-dependent osteopontin, bone sialoprotein, and osteocalcin expression and evidence of matrix-vesicle associated collagenous matrix mineralization.[8] The spatial and temporal expression of matrix molecules during the formation of multilayering cultures has been described in detail.[9] This spatial array of matrix molecules is well suited for examining surface effects on osteoblast matrix formation and mineralization.

Differentiation and the process of matrix formation by osteoblasts in vivo is associated with osteoblast expression of BSP,[15] osteopontin,[16] and osteocalcin[17] within a collagenous matrix. The concomitant cell-mediated mineralization of matrix is indicated by the elaboration of enzyme-specific extracellular matrix vesicles.[18] Three observations support the conclusion that the process of osteoblast matrix formation and mineralization on TPS surfaces differed from that process occurring on either TiO-blast or machined Ti surfaces. At the 14- and 21-day timepoints, TPS adherent cultures displayed: *(1)* diminished and perturbed expression of BSP; *(2)* diminished OC expression; and *(3)* less mineralization within the culture layer (von Kossa staining).

Surface-adherent cells were directly affected by the experimental variable, cp titanium topography. The superior layers of the culture are indirectly related to the cp titanium substrates. Any differences in culture layer formation may reflect either varied culture conditions or alterations in adherent cell physiology. When cultures formed on the TPS surface without catastrophic failure, a unique pattern of differentiation was evident within the culture layer. The perturbed localization of BSP (expression in adherent cells) suggests that TPS-adherent cells are altered in their progression from

Fig 7 Scanning electron microscopy evaluation of cell culture–derived material adherent to culture surfaces following culture layer removal.

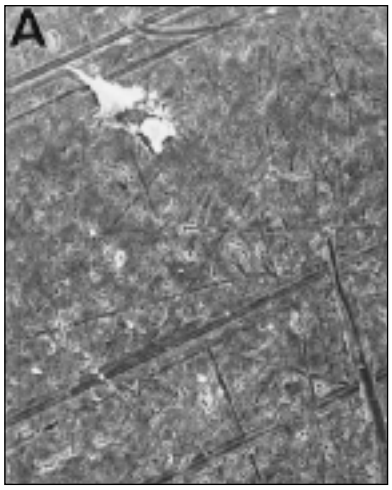

Fig 7a Machined Ti surface displayed little matrix and few cells (×40).

Fig 7b Cells spread along machined Ti surface following 21-day culture displaying intimate adherence and little surrounding matrix (×1,000).

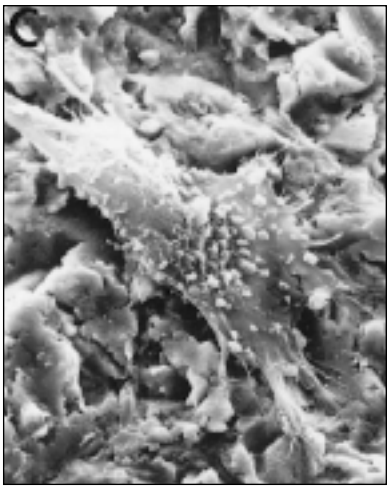

Fig 7c Cell remaining adherent to TiO-blast surface after 21-day culture; cell attachment is mediated by processes associated with peak topographic elements (×1,000).

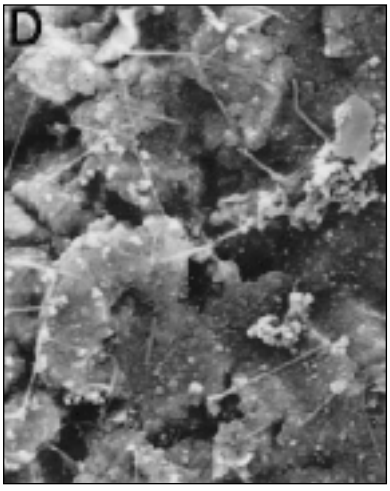

Fig 7d High-magnification evaluation of TiO-blast surface reveals small (< 1 μm) globular accretions and few linear filamentous structures (×2,000).

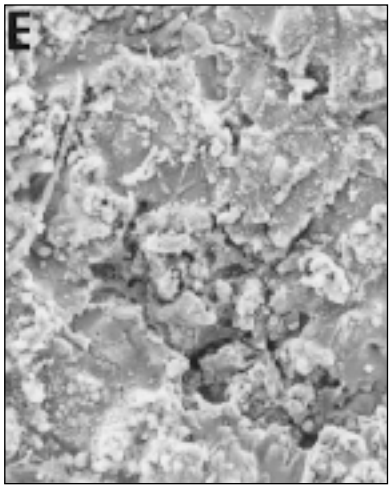

Fig 7e Relatively dense accumulations of rough globular accretions on rough TPS surface (×1,000).

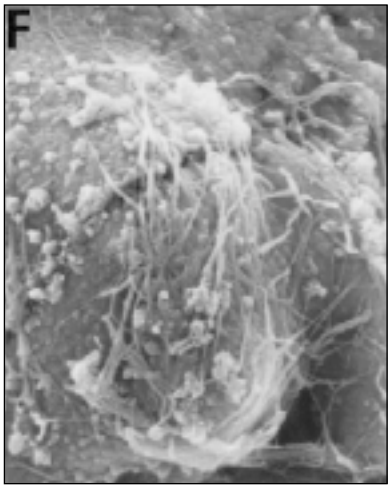

Fig 7f Abundant globules and fibers directly adherent to the rough TPS surface are observed (×4,000).

osteoblastic precursors to osteocytic cells. The superior culture layers were less mineralized than the corresponding layers of the machined or TiO-blast cultures. Further study is needed to evaluate the possibility that surface-directed mineralization and BSP expression occurs uniquely at TPS surfaces.

The loss of culture layer attachment to many of the TPS substrates may reflect a biochemical difference in cell-mediated adhesion to TPS versus other surfaces, differences in the physical properties among the surfaces, or important geometric differences represented by these topographically distinct substrates. In other experiments, we have infrequently observed the partial contraction and spontaneous lifting of culture layers from the edges of test substrates and have attributed this to the process of collagen-gel contraction. This does not reflect cell death; separate studies using metabolic labeling indicate that titanium-adherent cells are metabolically active throughout the culture period

(not shown). The importance of cytoskeletal integrity to the extracellular matrix is currently considered a significant factor in the control of cell phenotypes.[19] The means by which extracellular matrix–mediated events may be altered by topography merits further attention.[20]

There are several possibilities that may be excluded as possible causes for the different behavior of cultures grown on the TPS surfaces. In each of 3 trials, all cultures were grown from single animal-derived primary cultures; variability related to genotype should be excluded. These cultures are homogenous with respect to cell type; thus the type of cell-initiating growth on one or another surface should be similar. Initial experiments demonstrated efficient seeding of cells onto the 3 test surfaces. Thus, this result does not indicate failure to initiate adherence-dependent growth or reflect cell density (Fig 4f). In addition, the fact that growth of cells on all 3 surfaces resulted in a collagen- and cell-rich culture layer of similar thickness and cell density suggests that the primary effect of surface topography was not on cell proliferation. In another investigation of TPS surfaces in which proliferation was directly measured, surface topography did not significantly alter proliferation.[21]

Other studies of surface topography–dependent effects on osteoblast cell behavior have intentionally examined proliferation, collagen synthesis, and alkaline phosphatase expression.[22,23] When an osteosarcoma cell line[22] or rat calvaria–derived osteoblasts[23] were studied, increasing surface roughness was associated with increased proliferation and alkaline phosphatase expression. Although osteoblastic differentiation of rat stromal cells occurred on all surfaces, osteoblast differentiation was modified by substrate chemistry as measured by the temporal pattern of select gene expression.[24] The present study confirms the general observation that osteoblastic cells recognize different alloplastic substrates and that cultured osteoblast physiology is affected by a substrate's topographic parameters.

A second set of observations made in this study were derived from the SEM analysis of residual cell layer–derived materials adherent to the surface of culture substrates following removal of the culture layers. Scanning electron microscopy observations indicate that each test surface bears a morphologically unique postculture period adsorbate. For the titanium surfaces, the presence of both globular particles and fibrillar material was observed. A limited number of cells remained adherent to TiO-blast and machined Ti, but not to the TPS substrates.

In previous studies of mineralizing rat osteoblast cultures grown on machined Ti surfaces, globular accretions of similar size and morphology formed on Ti substrates.[23] Previous investigations by the authors of the bovine mandibular osteoblast culture system failed to display these accretions. The topography of the TPS surface may allow access for the media constituents necessary for globule formation that may not be allowed by the tightly adherent cultures on machined surfaces. The possible differential "biologic" conditioning of implant surfaces in culture may produce novel culture substrates that direct cell behavior. It is equally possible that the adherent material is cell-derived and reflects surface-dependent cellular responses instead of media-dependent adsorbates. These initial observations of surface-specific residual adherent cell-derived material suggest that physicochemical or biologic conditioning of an implant surface involves alloplastic surface–dependent events that might be exploited in tissue engineering and implant design.

Conclusion

Surface topography contributes to the modulation of cultured cell behavior. The perturbation of bone-specific extracellular matrix protein expression and alteration in matrix mineralization in cultures grown on TPS versus TiO-blast or machined surfaces suggests that topography may modulate osteoblast differentiation. Biologic and physicochemical events that condition the alloplastic surface in culture and modify the interface apparently occur as a function of surface topography.

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