Cells and Materials

Volume 2 | Number 2 Article 1

1992

The Role of Implant Surface Geometry on Mineralization In Vivo and In Vitro; A Transmission and Scanning Electron Microscopic Study

B. Chehroudi
University of British Columbia, Vancouver

J. Ratkay
University of British Columbia, Vancouver

D. M. Brunette
University of British Columbia, Vancouver

Cells and Materials, Vol. 2, No. 2, 1992 (Pages 89-104) 1051-6794/92$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

THE ROLE OF IMPLANT SURFACE GEOMETRY ON MINERALIZATION *IN VIVO* AND *IN VITRO*; A TRANSMISSION AND SCANNING ELECTRON MICROSCOPIC STUDY

B. Chehroudi, J. Ratkay, and D.M. Brunette*

Department of Oral Biology, University of British Columbia
2199 Wesbrook Mall, Vancouver, BC, Canada V6T 1Z3

(Received for publication February 2, 1992, and in revised form June 10, 1992)

Abstract

The purpose of this study was to examine the effect of substratum surface topography on bone formation *in vivo* and *in vitro*. Precise control over substratum topography was achieved using micromachining, a technique developed from the fabrication of microelectronic components. In the *in vivo* studies, titanium-coated epoxy replicas of micromachined surfaces were implanted subcutaneously in the parietal area of rats. After 6 weeks, bone-like tissue was found adjacent to some micromachined surfaces. Detailed observation of this tissue with the transmission electron microscope revealed osteoblast/osteocyte-like cells and a fully or partially mineralized collagenous matrix. Mineralized matrix and collagen bundles were found contacting the titanium coating without any intervening material. Mineralized tissue was not found adjacent to smooth surfaces. *In vitro*, enzymatically released osteogenic cells from calvarial bone produced large ($\geq$ 10 μm) and small ($\leq$ 0.5-3 μm) mineralized globules on the micromachined surfaces, whereas only small mineralized globules formed on the smooth control surfaces after 4 weeks of culture. The mineralized nature of the globules was confirmed by energy dispersive X-ray analysis. In a second osteogenic culture system, micromachined or smooth control surfaces were placed on calvarial explants. After 4 weeks, partially mineralized globules ($\approx$ 5 μm) were noted interspersed between cells and extracellular matrix on the micromachined surfaces but not on the smooth surfaces. This study suggests that the surface topography of an implant influences bone formation *in vivo* and *in vitro* and that micromachined surfaces of the dimensions used in these experiments promote mineralized tissue formation.

Key Words: Mineralization, implant, geometry, titanium, electron microscopy, subcutaneous, grooves, micromachining, calvaria, osteoblast.

*Person to contact:
D.M. Brunette
Telephone/FAX no.: (604) 822 2994 / 822 6698

Introduction

Although many synthetic bone-contacting biomaterials are available (Damien and Parsons, 1991), the continuing introduction of new materials indicates that an ideal material has yet to be found. Synthetic bone-contacting biomaterials have surface characteristics which support the adhesion, differentiation, growth and matrix production of osteogenic cells. However, the mechanisms whereby they promote these effects may differ. Synthetic bone-contacting implants made of bioactive glass and hydroxyapatite act by means of specific chemical properties (Hench, 1988; Damien and Parsons, 1991); the function of porous titanium implants is influenced by their mechanical or physical characteristics that promote tissue attachment (Pilliar, 1986). Finally porous ceramic (hydroxyapatite) implants may act by both of the above mechanisms (Ripamonti, 1991).

While it is generally accepted that the surface topography of an implant affects its performance, few systematic studies have dealt with this important feature. Micromachining, a process that was originally developed for the fabrication of microelectronic components, enables precise control over surface topography to be achieved, and thus the effects of surface topography on cell behavior can be studied systematically. Previously we have studied the effects of surface topography on epithelial downgrowth and connective tissue ingrowth in titanium-coated epoxy percutaneous devices (Chehroudi *et al.*, 1990, 1992). The purpose of this study was to examine, in detail, the nature of mineralized tissue formed at the micromachined implant surfaces *in vivo*, and to test the effects of micromachined surfaces on bone formation in a more controlled environment produced by *in vitro* culture systems. In agreement with the *in vivo* study, our current observations suggest that micromachined surfaces enhance mineralized tissue formation *in vitro*.

Materials and Methods

Micromachined substrata

Micromachining is a technique that produces

grooves, slots or pits with precise dimensions in silicon or gallium arsenide wafers. This technology has been extensively utilized in diverse applications such as solar cells, high value capacitors, solid state inductors and miniature gas chromatography systems (Kendall, 1979). The particular micromachining techniques employed in this study were those developed at the University of British Columbia by Camporese *et al.* (1981) for the fabrication of high quality photomasks for solar cells. In brief, micromachining begins with the production of a master pattern which is reduced to the desired dimensions by a step-and-repeat photographic process to produce photomasks. The pattern on the photomask is transferred onto the silicon wafer by photolithography. The pattern on the silicon wafer is then etched to the desired depth by anisotropic etchants. The depth and spacing of an anisotropically-etched surface can be regulated by the time of etching and the crystalline orientation of the silicon wafer. This study employed grooved and tapered pitted surfaces. The grooves were V-shaped with walls forming a 55° ± 3° angle with the surface and a depth of 19 ± 4 μm standard deviation (SD) or 30 ± 8 μm SD, and a repeat spacing of 39 ± 5 μm. The tapered pits formed a square at the surface, with sides of 270 ± 1 μm SD, and had walls that tapered at an angle of 55° to the surface and were 120 ± 1 μm SD deep. These micromachined surfaces were similar to those used in a previous study which produced connective tissue ingrowth (Chehroudi *et al.*, 1992).

Impressions of the micromachined surfaces were made in a vinyl silicone impression material (Exaflex, G-C Dental, Tokyo, Japan), and the impressions were used to cast replicas of the original patterns in epoxy resin (Epotek, Epoxy Technology, Billerica, MA, USA). Then the replicas were used to make implant surfaces for *in vivo* and *in vitro* studies. All implant surfaces were coated with ≈ 50 nm titanium using a sputter coater (Randex 3140 Sputtering System, Palo Alto, CA, USA). Randomly chosen samples from different batches of implants were observed under the scanning electron microscope (SEM) to determine the fidelity of the replica to the original silicon micromachined surfaces, as well as the smoothness and continuity of the titanium coating.

In vivo experiments

Epoxy replicas of micromachined surfaces were used to fabricate implants which were placed subcutaneously in the parietal region of male Sprague Dawley rats. The fabrication process of these implants has been described elsewhere (Chehroudi *et al.*, 1990, 1992). In brief, implants were U-shaped and had two protruding parts connected to each other by a flat pedestal. Each protruding part was 2 mm tall and had an outer surface which faced laterally and an inner surface which faced medially (Fig. 1). Epoxy implants were baked at 60 °C for 3 days and cleaned by ultrasonication for 20 minutes in a detergent specifically formulated for tissue culture (7X Cleaning Solution, Flow Laboratories Inc., McLean, VA, USA). After being rinsed 20 times with deionized water, they were dried overnight in a tissue-culture laminar-flow hood. All implants were coated with ≈ 50 nm titanium and treated for 3 minutes in an argon-gas glow-discharge chamber using a Randex 3140 sputtering system, then immediately stored in deionized, degassed, sterile water in cleaned, water-tight teflon vials.

Implantation: The implantation procedure has been described in detail previously (Chehroudi *et al.*, 1990, 1992). In brief, inbred Sprague Dawley rats, weighing from 400-450 g, were intubated and anesthetized using halothane. The fur above the parietal area was shaved and treated with depilatory, and the underlying skin scrubbed with Betadine (Purdue Frederich Inc., Toronto, Canada) and 70% ethanol. An access incision in the parietal area was made distally from one ear to the other. The epidermal and dermal layers were elevated, and were removed by a combination of blunt and sharp dissection to form a pouch sufficient for the implant. In addition, the periosteum was completely removed and the discontinuity of the bone under the implant was smoothed by means of a bone file. The surgical wound was irrigated by sterile saline and dried by sterile gauze. Implants were then placed on the parietal bone and the access incision was sutured using 4-0 silk. Animals received a prophylactic intramuscular injection of antibiotics (Pen-Di Strep, Roger/STP, London, Ontario, Canada).

Tissue preparation: Two animals were killed every week up to the tenth week after placement, by an overdose of sodium pentobarbital. Prior to complete collapse of the heart, perfusion was carried out through the left ventricle with a solution of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, preceded by a 5 minute flush of warm heparinized normal saline into the blood circulation. The implant and the tissue around it were gently removed using a No. 15 scalpel blade and placed in Karnowsky's fixative for 24 hours at 4 °C. A secondary fixation was accomplished by treating the samples with 2% buffered OsO_4 on a rotator for 3 hours. At this stage, the implants were cut in two, and fixation was allowed to continue for a further 2 hours. Implant/tissue blocks were dehydrated in graded Aquembed (Ladd Res. Ind., Burlington, VT, USA) and embedded in Epon (J.B. EM Inc., Pointe-Claire/Dorval, Quebec, Canada) as described previously (Chehroudi *et al.*, 1991).

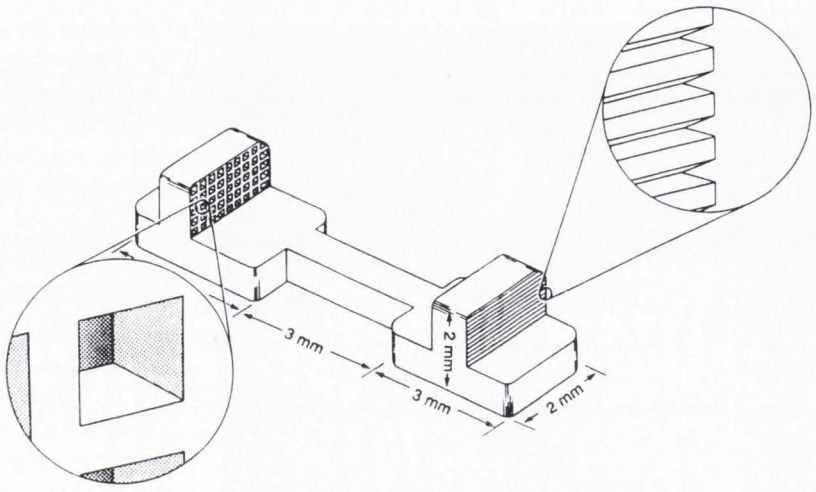


Figure 1. Schematic diagram of the implant used for the *in vivo* experiments.

Transmission electron microscopy (TEM)

Sections were taken throughout the length of the implant using a Sorvall MT2 microtome (Ivan Sorvall Inc., Norwalk, Conn., USA). Two-μm-thick sections were cut in an orientation such that the grooves or pits were cross-sectioned (parallel to the long axis of the implant), stained with Toluidine Blue and observed under light microscopy at 500X magnification. Selected areas of the tissue/implant block were trimmed for transmission electron microscopy (TEM), and 50-60-nm-thick sections were cut with a diamond knife from the tissue/ implant interface. The sections were then stained with alcoholic uranyl acetate and aqueous lead citrate, and viewed under a Philips 300 transmission electron microscope.

In vitro experiments

To test the effects of implant surface topography on bone formation in a more controlled environment, we decided to use two osteogenic cell culture systems; [a] calvaria osteogenic cell culture (COC), and [b] calvaria explant culture. These culture systems have been shown to produce mineralized tissues on standard culture dishes reproducibly (Bellows *et al.*, 1986; Davies *et al.*, 1989).

Calvaria osteogenic cell culture (COC): The method used for cell isolation was similar to that described by Hasegawa *et al.* (1986). In brief, frontal, parietal and occipital bones (calvaria) of newborn or 1-3 day old Sprague-Dawley rats were carefully dissected, rinsed in copious amounts of sterile phosphate buffered saline (PBS), and placed in tissue culture medium (α minimum essential, α-MEM) supplemented with 15% fetal calf serum (FCS) and antibiotics (100 μg/ml Penicillin G, 50 μg/ml Gentamycin sulphate and 0.3 μg/ml Fungizone). Eighteen calvariae were dissected and minced into pieces approximately 1 mm^3. The minced tissue was then incubated in 5 ml of a digestion mixture containing 180 U/ml of clostridial collagenase (type Ia, Sigma, St. Louis, MO, USA), and 0.5 mg/ml trypsin (Gibco, Burlington, Ontario, Canada) in PBS. The suspension was digested for 20 minutes at 37 °C with stirring in a Pierce "Reacti-vial" (Pierce Chemical Company, Rockford, IL, USA). After 20 minutes the supernatant was mixed with an equal volume of cold fetal calf serum (FCS) and centrifuged at 150 g. Then the supernatant was discarded and the cells were resuspended in α-MEM containing 15% FCS and antibiotics. This population of cells is designated population I. The procedure was repeated at 20 minute intervals to yield populations (P) II - V. The cells were counted electronically and plated at 2 X 10^3 cells/cm^2 in either 75 cm^2 tissue culture flasks (if they were to be subcultured later) or into six-well culture plates (35 mm diameter each well).

Two days after primary plating of calvarial cells on standard culture dishes, the medium along with the dead cells was discarded, and adherent cells of PII and PIII were trypsinized and replated at 2 X 10^3 cells/cm^2 onto titanium-coated 6-well culture plates on which the micromachined or smooth control surfaces were replicated. These plates carried epoxy replicas of micromachined surfaces and a smooth control surface similar to those implanted *in vivo*. These surfaces were installed such that 1 square centimeter of each surface was produced side by side at the bottom of each well of a 6-well culture plate. The entire 6-well plate was then coated with 50 nm titanium. When the cells became confluent 5-6 days after plating, the medium was supplemented with 50 μg/ml ascorbic acid and 10 mM Na-β-glycerol-phosphate over the subsequent 30 days. Na-β-glycerol-phosphate acts as a source of organic phosphate, and ascorbic acid is required for collagen synthesis; both have been shown to be necessary for the mineralization process *in vitro* (Bellows *et al.*, 1986; Tenenbaum, 1985). Tissue cultures were subsequently fixed with buffered 2.5% glutaraldehyde for 1 hour at 4°C, and processed for SEM.

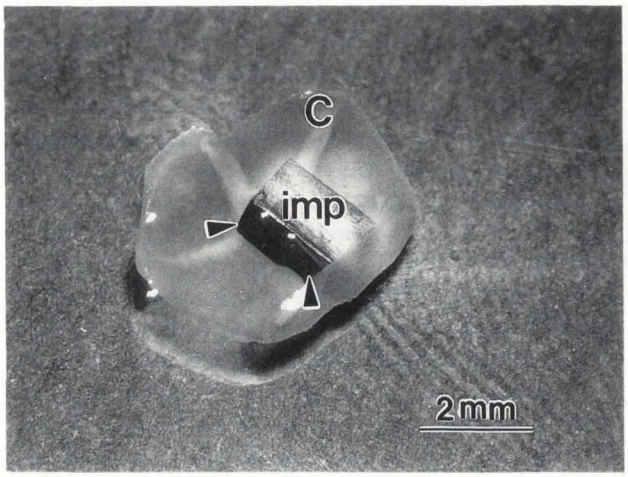


Figure 2. Photograph showing an implant (imp) placed on the inner surface of a calvarial explant (C). Arrows indicate the location of the micromachined or smooth control surfaces.

Calvaria explant culture (CE): The calvarial explant system used in these experiments was adapted from that described by Davies *et al.* (1989). In brief, whole calvaria of newborn rats were carefully dissected, and the periosteum was removed with a sharp scalpel. The calvariae were then placed in a 24-well culture dish with the internal side up. Micromachined test surfaces (4 x 2 x 2 mm) as well as smooth control surfaces were replicated in epoxy resin, coated with ≈ 50 nm titanium and placed on the calvarial bone (Fig. 2). A total of 6 surfaces were placed on the calvaria and cultured for 30 days in α-MEM supplemented with FCS 15%, antibiotic, 50 μg/ml ascorbic acid and 10 mM Na-β-glycerol-phosphate. The whole calvarial culture system provides a source of osteogenic cells from bone and a source of fibroblastic cells mainly from the sutures between individual bones. It has been speculated that this method may provide an environment which closely approximates to *in vivo* conditions (Davies *et al.*, 1989), where implants are interacting with a mixed population of cells. After 30 days of culture, the entire calvaria and the test surface were fixed in buffered glutaraldehyde and processed for SEM.

Scanning electron microscopy: Glutaraldehyde-fixed samples collected from both *in vitro* culture systems were post-fixed in 2% buffered osmium for 1 hour, briefly dehydrated with graded alcohol (50%-100%, 1 hour total), critical-point dried with CO_2, sputter-coated (Hummer VI Sputtering System, Technics, Alexandria, Virginia, USA) with palladium or carbon (≈ 10 nm-thick) and observed in a Cambridge Stereoscan 100 (Leica, Canada) SEM. Our previous experience and the observations of this study on epoxy replicas indicated that brief dehydration with alcohol limited to one hour did not distort micromachined replicas, nor did it lift the titanium coating. Energy dispersive X-ray (EDX) analysis (using a Link AN5500 system) was performed on selected portions of the cultured surfaces.

Results

Surface preparation

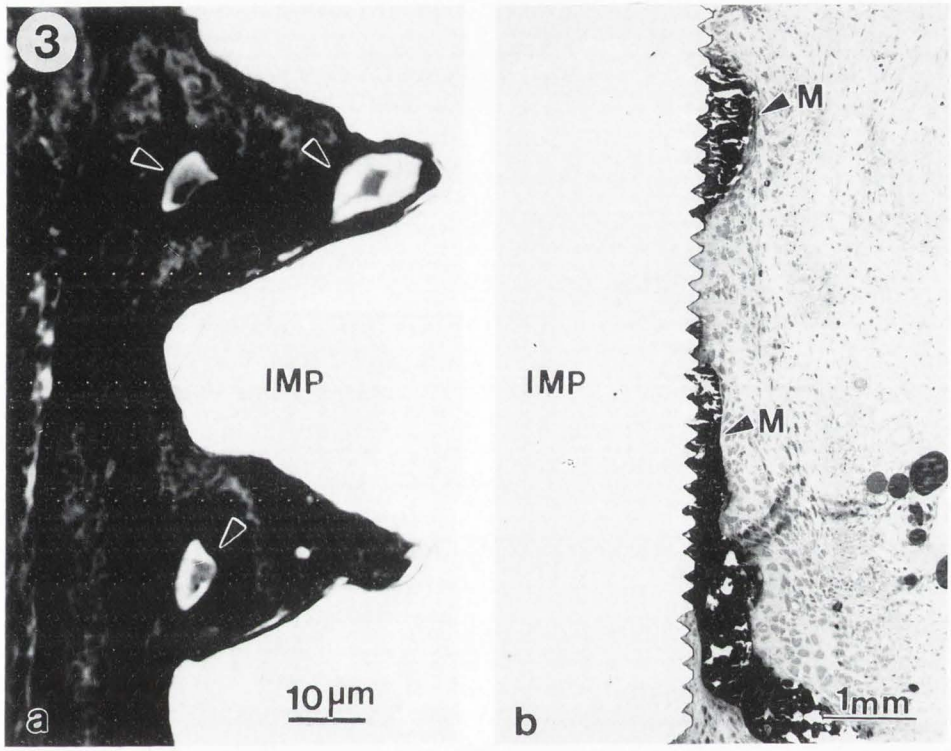
SEM observations of the test surfaces indicated that micromachined surfaces were replicated with high fidelity to the master pattern on the silicon wafers, and the titanium coating was smooth and continuous with no evidence of cracks.

In vivo experiments

The histological processing used in this study was similar to that previously reported and produced acceptable semi-thin sections (2 μm thick) for light microscopic observations. However, the slight differences in the hardness of epoxy, titanium, epon, soft tissue and mineralized tissue made obtaining ultrathin sections (50-60 nm thick) a difficult task. Nevertheless, acceptable ultrathin sections from the tissue adjacent to the implant surface could be obtained.

Light microscopic observations of the healing process at the implant interface have been described before (Chehroudi *et al.*, 1992; Brunette *et al.*, 1991). In brief, an immature granulation tissue was formed at one week postimplantation which gradually matured to a thick connective tissue capsule on the smooth surface by three weeks. Implants with micromachined surfaces, however, demonstrated varying degrees of connective tissue ingrowth starting at two weeks post implantation. After six weeks distinct densely stained foci appeared half way up the surface of the implant on some 30 μm-deep grooved surfaces. The cells around these foci had the appearance of osteoblasts. By seven weeks these foci became mineralized, and by 9 weeks they extended on the implant surface and osteocyte-like cells could be found within the lacunae which were positioned inside the grooves (Fig. 3a, b). Mineralization was also observed on some pitted surfaces after 8 weeks of implantation and on a few of the 19 μm-deep grooved surfaces after 10 weeks of implantation. Mineralized tissue was not observed adjacent to smooth surfaces.

TEM examination indicated that the mineralized foci formed adjacent to micromachined surfaces contained osteoblast-like and osteocyte-like cells as well as mineralized extracellular matrix. Cells surrounding the mineralized foci had the characteristics of osteoblasts (Rodan and Rodan, 1983) including cuboidal shape, abundant rough endoplasmic reticulum, Golgi apparatus, secretory vesicles and cellular processes (Fig. 4a). Cells



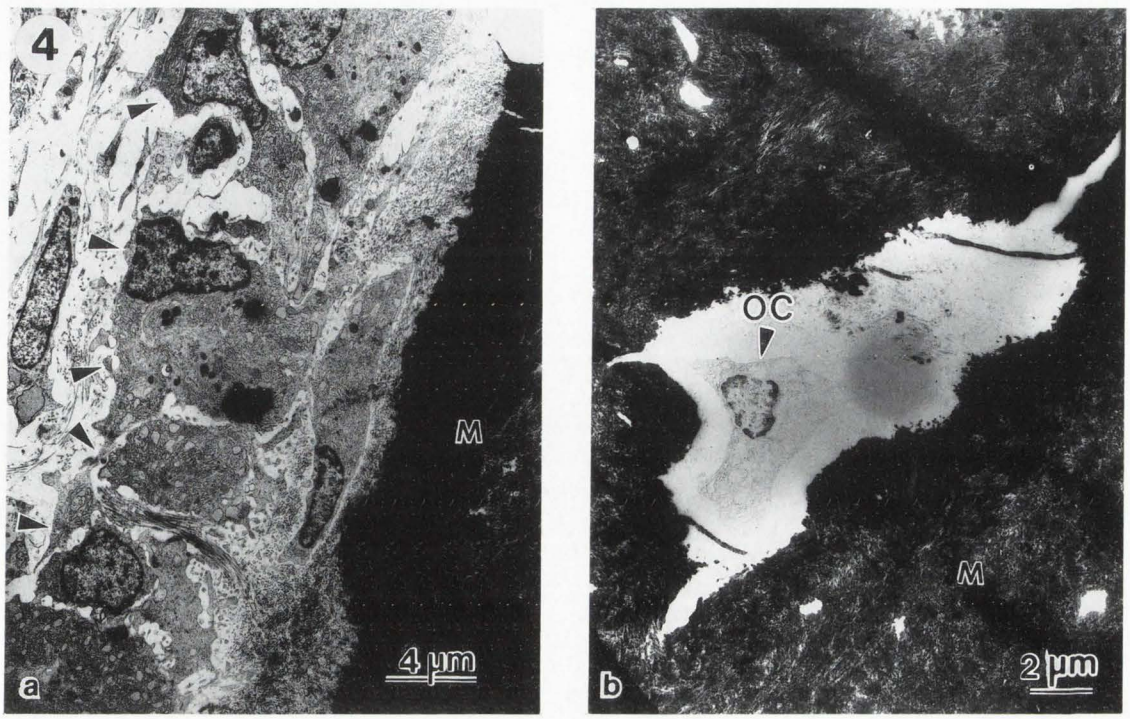


Figure 3. Photomicrographs obtained from an implant (IMP) with 30-μm-deep grooves that had been placed subcutaneously for 6 weeks. Note osteocyte-like cells within the grooves (arrows) and mineralized foci (M) adjacent to the implant.

Figure 4. Electron micrographs of osteoblast-like cells (arrows) close to the mineralized matrix (M) [a], and osteocyte-like cells (OC) within the mineralized matrix [b].

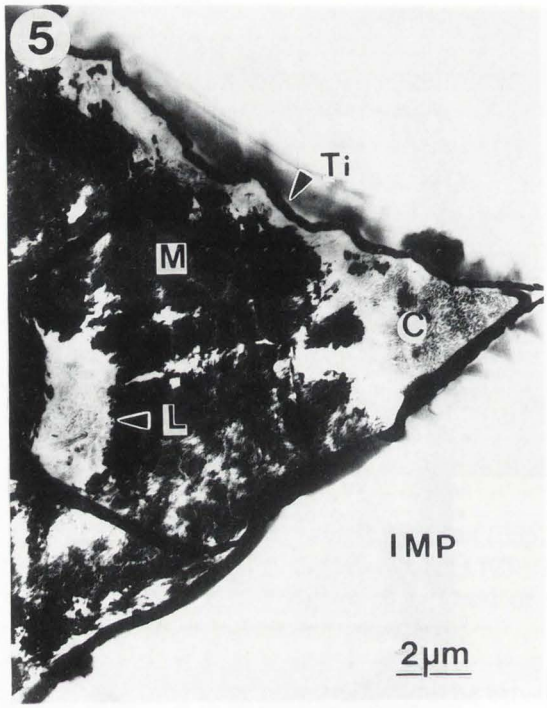
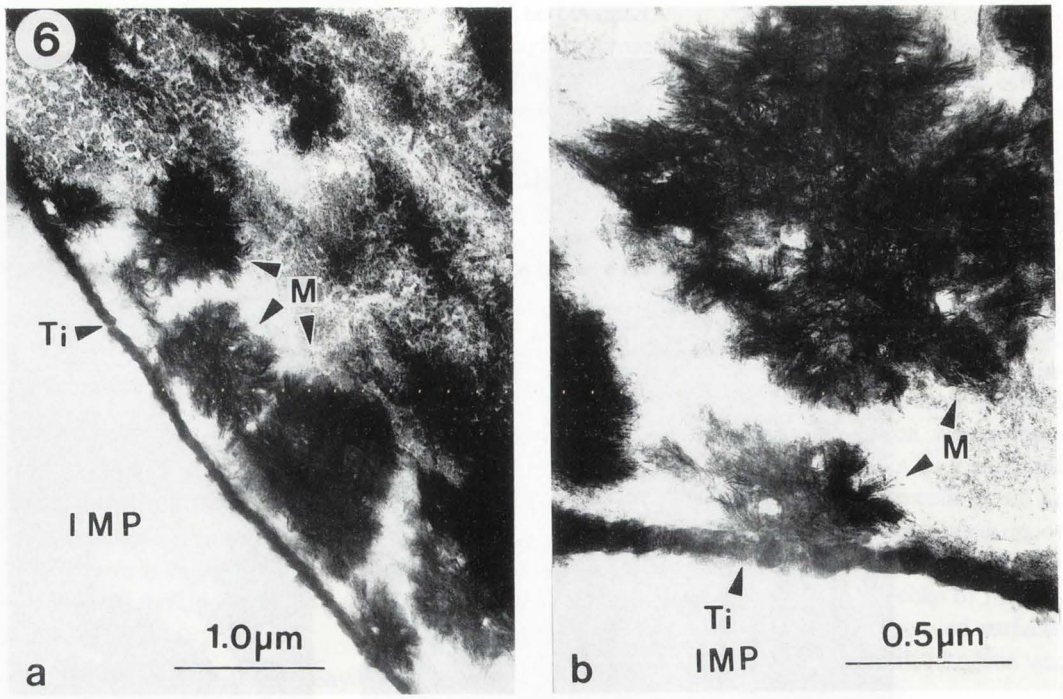


Figure 5 (at left). Electron micrograph showing mineralization (M) in a 30-μm-deep groove. Note an empty lacuna (L) close to the titanium surface (Ti) and collagen bundles (C) within the groove.

Figure 6 (below). Electron micrographs showing clusters containing needle-like structures in mineralized areas (M) in close proximity [a] or in contact [b] with the titanium surface (Ti).

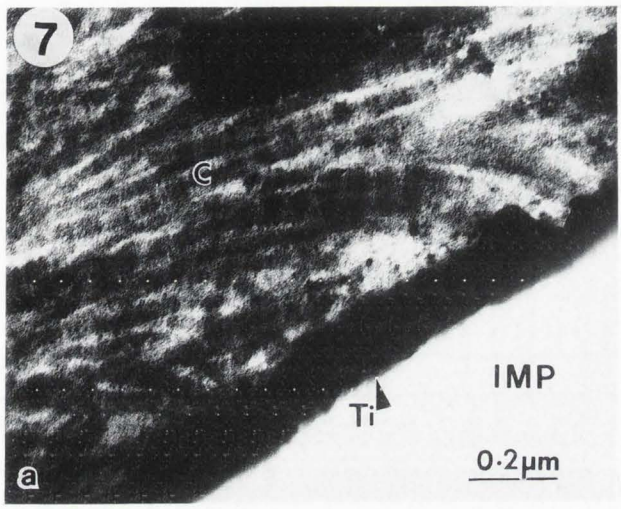
Figure 7 (facing page, top). Electron micrographs showing collagen bundles (C) contacting the titanium (Ti) coating within a 30-μm-deep groove.

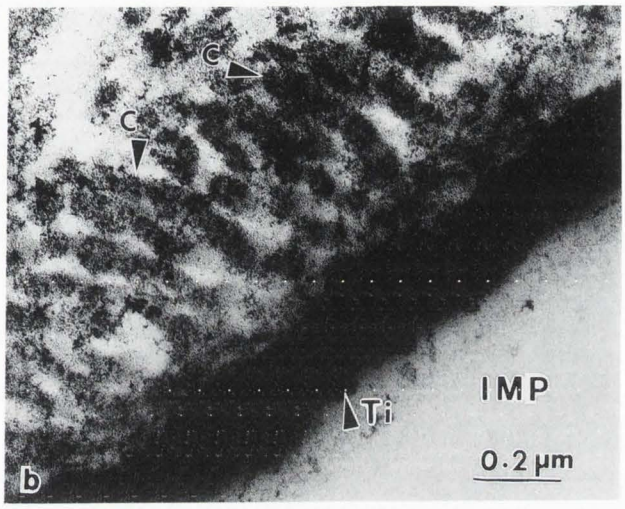
Figure 8 (facing page, bottom). Composite figure of electron micrographs obtained from mineralization sites adjacent to micromachined surfaces. Mineralization areas (M) are found over collagen bundles (C) in close proximity to the titanium (Ti) surface [a, b]. Mineralized tissue approaching the surface of the implant (IMP) and contacting the titanium coating in some areas [c]. Mineralized tissue contacting titanium coating directly, arrows mark the borders of the titanium coating [d].

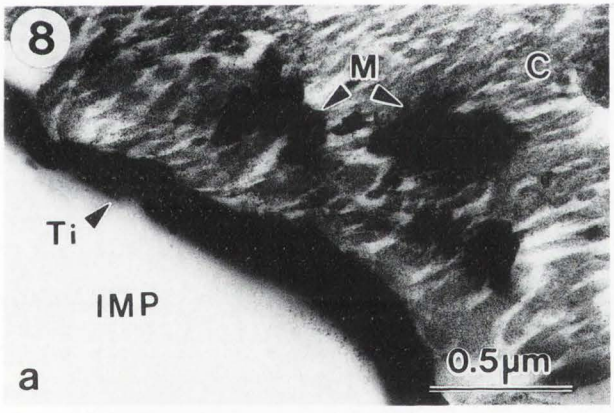


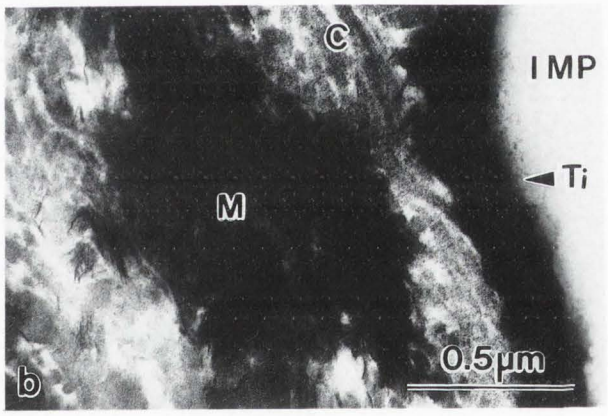
within the mineralized foci resembled osteocytes, as they resided in distinct lacunae and had several extended cell processes (Fig. 4b, Fig. 5). Because of the morphological resemblance to naturally occurring bone, the mineralized areas are referred to as bone-like tissue. Osteoblast-like cells were located approximately 10-50 μm away from the implant surface at this time, whereas bone-like tissue and osteocyte-like cells were found in close proximity to the grooves or pits ($\approx$ 2 μm) and occasionally contacted the titanium surface.

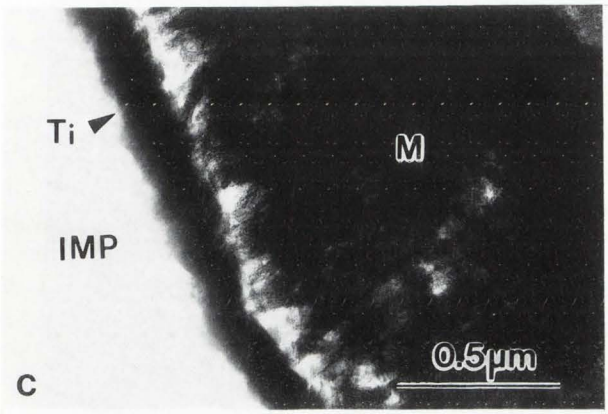
The extracellular matrix abutting the micromachined surfaces mainly comprised collagen fibrils which could easily be identified from their size and typical banding pattern. In some areas of the mineralized foci the

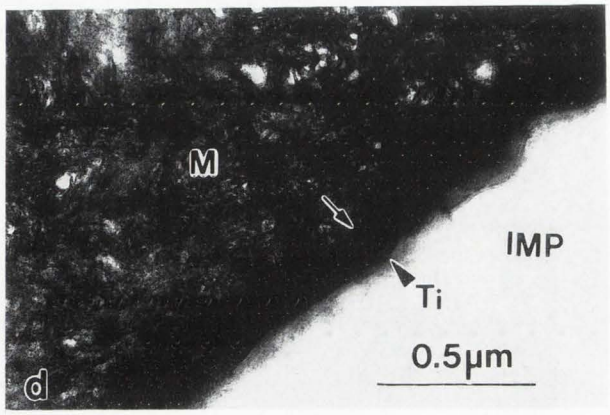




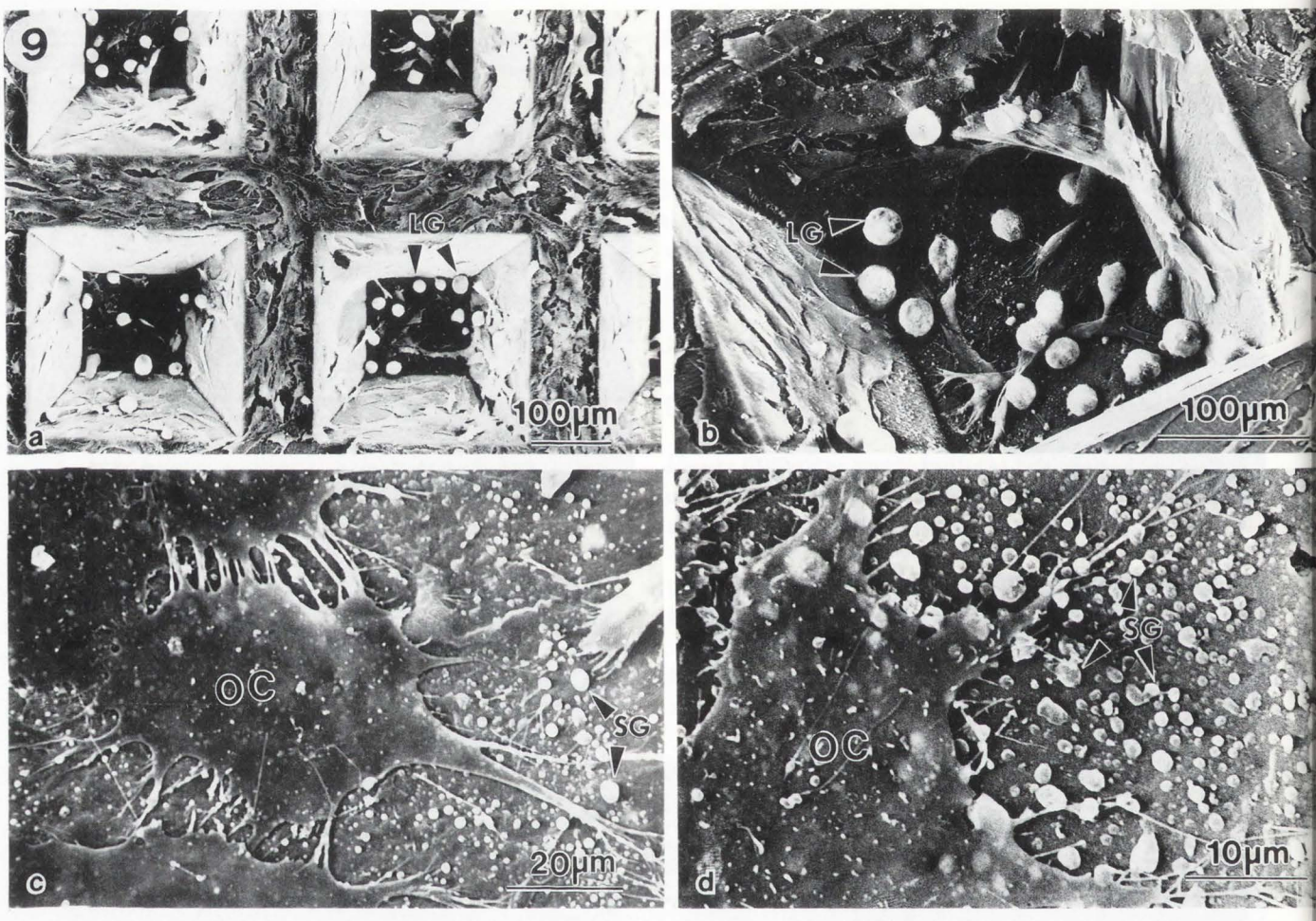








periodicity was indistinct, probably as a result of either the plane of section or incipient mineralization. The orientation of collagen fibrils varied markedly with location; as a general rule, fibrils located at a distance from the implant surface ($\geq$ 50 μm) were oriented mainly parallel with the implant long axis. Closer to the implant surface, the orientation changed to oblique, and within the grooves, fibrils appeared in cross-section, indicating that they are probably oriented along the long axis of the grooves. At the periphery of the grooves, however, short ($\approx$ 2-3 μm long) bundles of collagen fibrils appeared to abut on the titanium coating without a noticeable afibrilar or amorphous layer (Fig. 6a, b). On the implants with tapered pitted surfaces, bundles of collagen extended from one edge of the pit to an opposite edge forming a hammock-like structure which sagged



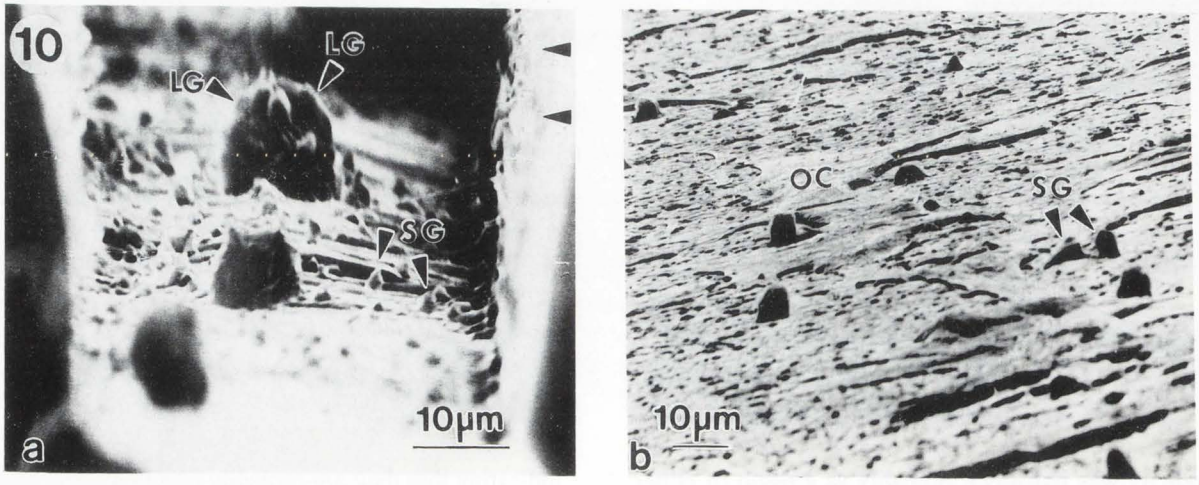


Figure 9. Scanning electron micrographs of osteogenic calvarial cells cultured on tapered pitted surfaces. Note large mineralized globules (LG) [**a**, **b**], and small mineralized globules (SG) which were most frequently formed at the periphery of osteocyte-like cells (OC) located at the bottom of a pit [**c**, **d**].

Figure 10. Scanning electron micrographs of osteogenic calvarial cells cultured on micromachined grooved [**a**, side view] and smooth control [**b**] surfaces. Note large globules (LG) and small globules (SG) at the bottom of a groove, as well as small globules at the periphery of an osteocyte-like cell (OC) on the smooth surface.

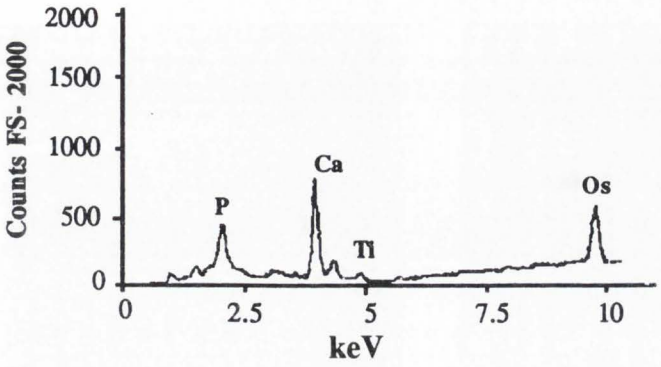


Figure 11. Energy dispersive X-ray (EDX) spectrum (counts, full scale = 2000, versus keV) of large mineralized globules similar to those shown in Figures 9 and 10, demonstrating major peaks for Ca, P, and Os (from the fixation process), and a small peak for titanium.

---

toward the pit bottom. The orientation of collagen fibrils at the bottom of the pit, however, appeared to be random. Areas of tissue undergoing mineralization exhibited roughly circular aggregates of electron-dense crystalline needle-like material scattered in the collagen matrix, and in some areas the needle-like aggregates contacted the titanium coating (Fig. 7a, b).

The mineralization front appeared to overlay the collagenous network, and at many sites projections of the mineralized tissue approached the titanium-coated micromachined surfaces. At the most mineralized sites mineralized tissue was in direct contact with the titanium-coating, without any detectable zone of non-mineralized material (Fig. 8).

In vitro **experiments**

Calvaria osteogenic cell culture: Cells from PII and PIII, the populations associated with the highest frequency of mineralized-nodule production (Bellows *et al.*, 1986), were cultured on titanium-coated replicas of micromachined and smooth surfaces. At the light-microscopic level the development of mineralized tissues on these surfaces can be readily revealed using Von Kossa stain (Culling *et al.*, 1985). At the electron-microscope level, in addition to morphological features, EDX elemental analysis was used to identify mineral deposits. To expose the tissue closest to the implant surface, compressed air was used to blow away the superficial cells. Then the tissue samples were again coated with either palladium or carbon. In agreement with Davies *et al.* (1991a) and Lowenberg *et al.* (1991), SEM observations revealed globular accretions that appeared to be attached to the substratum. However, these microglobules were found to be of two sizes; large globules $\geq$ 10 μm in diameter, and small globules of $\leq$ 0.5 - 3 μm in diameter. Occasionally, large globules were located at the bottom of the tapered pits or grooves (Fig. 9, 10a); whereas small globules were observed primarily at the periphery of individual osteoblast/osteocyte-like cells (Fig. 9c, d). Small globular accretions were the only type of mineralization found on the smooth control surfaces during the time period of this study (Fig. 10b). EDX analysis of both large and small globules revealed substantial amounts of Ca and P, indicating the mineralized nature of these structures (Fig. 11).

Calvaria explant culture: The implant surfaces remained on the calvarial bone during the experiment and SEM observations indicated that in all cases the surfaces were covered by a lawn of cells that migrated from the calvarial explant. These migrating cells were interspersed with extracellular matrix and on the micromachined surfaces numerous mineralized globules ($\approx$ 5 μm) were embedded within the cells and matrix (Fig. 12). EDX analysis of these globules revealed Ca and P peaks as well as a large peak of Os. Globules were also found on the calvarial bone that was not in contact with the implant surface. The amounts of Ca and P were greater in these globules than in globules formed adjacent to the micromachined implants (Fig. 13a, b). On the smooth surfaces we were unable to identify mineralized globules. Although sporadic nodular accumulations of tissue were noted, they failed to show any evidence of calcification when examined by EDX.

Discussion

There have been earlier suggestions that surface geometry may play an inductive role in mineralization. For example, Selye *et al.* (1960) demonstrated that ectopic mineralized tissue could be formed within the lumen of glass tubes, 20 mm long and 30 mm wide, implanted subcutaneously in rats. Subsequently, Reddi and Huggins (1973) reported chondrogenesis and mineralization within the conical roots of teeth when the roots were implanted subcutaneously in rats. They inferred that transformation of fibroblasts to osteogenic cells was the possible cause of the mineralization. Schoen *et al.* (1986, 1988) also documented several cases of biomaterial-related mineralization, and more recently Ripamonti (1991) speculated that bone formation within the porous matrix of a hydroxyapatite replica could be attributed partly to the geometrical configuration of the porous framework. The *in vivo* and *in vitro* observations of the present study demonstrated the importance of surface topography in mineralization, because bone-like tissue formed on grooved and tapered pitted surfaces but was not observed on the smooth surfaces.

In this study, unlike many instances of ectopic mineralization (Schoen, 1988; Rolland *et al.*, 1989), the tissue response to the grooved or pitted implants was not

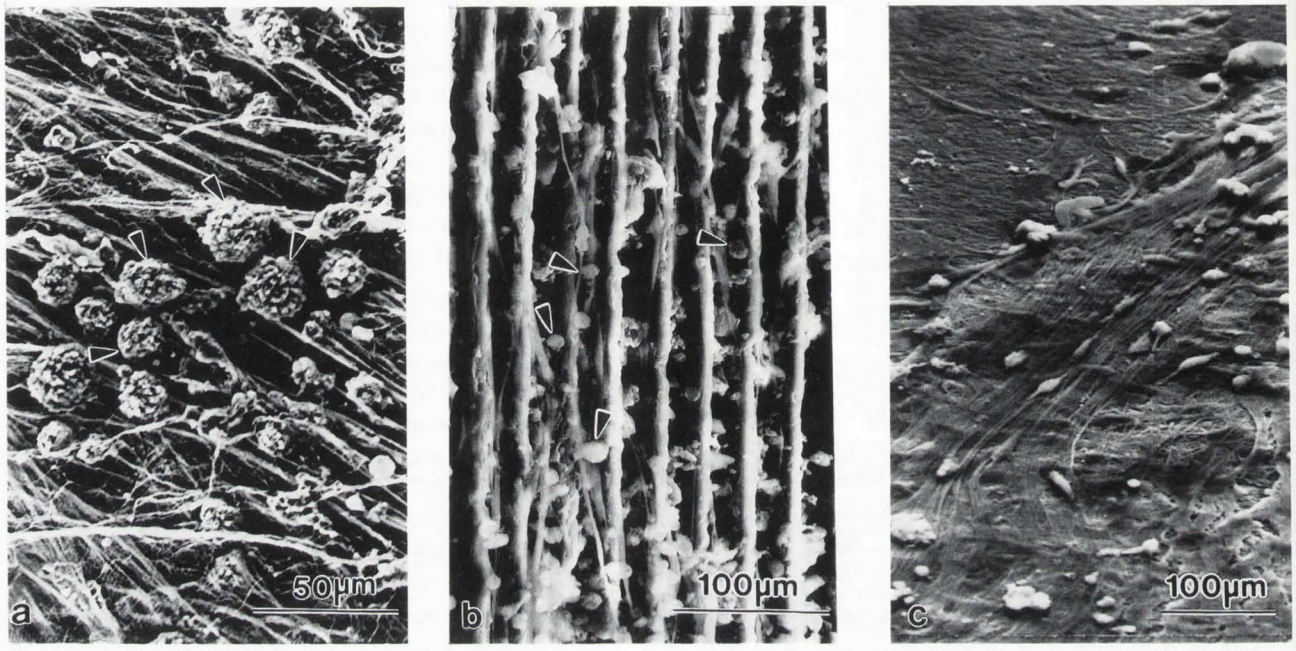


Figure 12. Scanning electron micrographs from the inner side of the calvarial bone cultured for 4 weeks [a]. Arrows indicate highly mineralized globules. Colonizing cells migrated from a calvarial explant onto the micromachined [b] and smooth control [c] surfaces. Arrows indicate partially mineralized globules that were observed on the grooved surfaces.

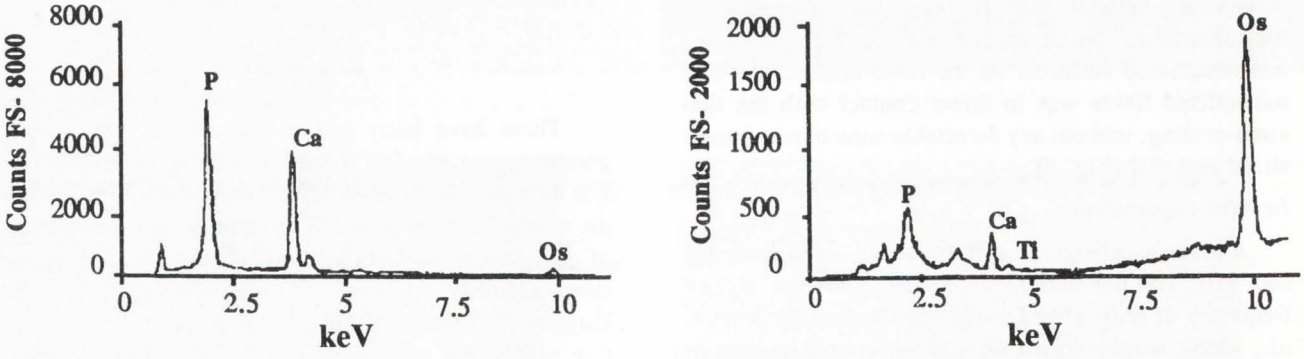


Figure 13. Energy dispersive x-ray (EDX) spectra of mineralized globules formed on the calvarial bone, similar to those shown in Figure 12a [a], and partially mineralized globules similar to those shown in Figure 12b [b].

limited to the development of crystalline material, but rather resulted in the production of bone-like tissue. Detailed TEM observation indicated osteoblast-like and osteocyte-like cells, as well as mineralized matrix and collagen fibrils in the vicinity of the implant. In some areas mineralized matrix and collagen fibrils contacted the titanium surface without a noticeable amorphous zone. This finding contrasts with previous reports on the bone-titanium interface which have noted a collagen free zone of amorphous biomolecules, perhaps glycoproteins or sulphur-containing ground substance, in the immediate vicinity of the titanium (Albrektsson *et al.*, 1983; Davies *et al.*, 1991b,c). Differences in the animal model, surgical technique and surface properties of the implant could explain the differences in observations. However, it should be equally acknowledged that obtaining suitable sections for TEM investigation of the titanium-tissue interface is a challenging technical task; it is possible that close contact between collagen and titanium surfaces in other systems has not been observed or reported because of the small number of sections studied. Finally, it should be noted that the resolution of our TEM study is such that it does not rule out the presence of substratum adhesion molecules, such as fibronectin,

being present at the interface, where they might mediate the attachment of collagen to the titanium. We are currently undertaking immunohistochemical studies to investigate this possibility.

In agreement with Davies *et al.* (1991a) and Lowenberg *et al.* (1991), we have observed small globules produced by cultured osteogenic cells which appeared to be attached to the implant surface. However, in addition to the small globules, large mineralized globules ($\geq$ 10 μm) were observed only on micromachined surfaces and not on smooth surfaces. The large globules may be formed by the fusion of small adjacent accretions as speculated by Lowenberg *et al.* (1991). Moreover, the formation of large globules primarily on the micromachined surfaces and not on smooth surfaces may indicate that the micromachined surfaces produce a more suitable microenvironment for mineralization than smooth surfaces.

In the calvarial explant culture system where cells had to migrate to colonize the implant surface, no mineralized globular accretions were noted on smooth surfaces, whereas on the micromachined surfaces partially-mineralized globules were readily apparent. This difference may arise from the manner in which the cells from the calvarial explant, which comprise a mixture of fibroblasts and osteogenic cells, colonized the surfaces. In particular, it is possible that osteogenic cells selectively populated micromachined surfaces. Another possible explanation may be that fibroblasts that migrated from sutures between calvarial bones selectively populated smooth surfaces and inhibited mineralization by a mechanism similar to that demonstrated by Ogiso *et al.* (1991) who showed that the production of prostaglandins by fibroblasts inhibited mineralization.

The process by which mineralization is initiated and regulated *in vivo* or *in vitro* is poorly understood (Gerstenfeld *et al.*, 1988; Wiess, 1988). A number of commercially available bone-contacting dental or orthopaedic implants are offered that have a variety of surface configurations including porous, rough or textured surfaces, and machined surfaces (Brunette, 1988). Such textured substrata could act through several mechanisms, but the following possibilities are of particular interest with respect to the micromachined surfaces used in this study:

1. Bone-inductive microenvironment. Evans and Potten (1991) have proposed that regulation of stem cell activity *in vivo* depends upon complex interactions that include restriction of the distribution of regulatory factors. The micromachined surfaces may act by constricting osteogenic cells into a confined volume in which regulatory factors achieve concentrations that promote calcified tissue formation. Such a possibility is given credence by the *in vitro* experiments of Nijweide (1975) as well as Tennenbaum and Heersche (1982), who demonstrated that periosteal sheets have to be folded to produce a bone-inductive microenvironment. Similarly, Nakahara *et al.* (1991) have shown that periosteal cells enzymatically liberated from chick tibia produce bone and hypertrophic cartilage only in a microenvironment produced by cells plated in high density.

2. Orientation of collagen bundles. It is generally agreed that a prerequisite for mineralization is the synthesis and assembly of an extracellular matrix onto which crystals of mineral can be formed (Gerstenfeld, 1988). It has been suggested that the spatial arrangement of the collagenous matrix, the major component of the extracellular matrix of calcified tissue, may play an important role in initiation of mineralization *in vivo* (Weiss, 1988). Because the orientation and spatial arrangement of the collagen fibrils appeared to differ on smooth and micromachined surfaces, it may be speculated that micromachined surfaces produce an arrangement of collagen that promotes mineralization. However, serial histological sections and three-dimensional reconstructions of the implant/tissue interface are required to document the arrangement precisely.

3. Cell shape and polarity. The relationship of cell shape to gene expression and cell differentiation have been thoroughly discussed by Watt (1986), Solursh (1989), and Bissell and Barcellos-Hoff (1987). For example, Solursh (1989) demonstrated that induction of a round shape in mesenchymal cells promoted chondrogenesis, while induction of a flattened shape on the same cells promoted fibroblast differentiation. For several cell types establishment of cell polarity has influenced certain cell functions, such as secretion (Gottlieb *et al.*, 1986; Mauchamp, 1987). Similarly, osteogenic cells *in vivo* polarize to become osteoblasts. Therefore bone formation could be enhanced by producing a cell orientation that aids in the development of a functional cell polarity. Micromachined grooved surfaces could promote bone formation by their known property of orienting cells.

Although a plethora of surface topographies have been introduced to enhance the performance of bone-contacting implants (Brunette, 1988), the possible surface geometries are limited by the fabrication processes used. Micromachining is a versatile technology that can produce specifiable topographies over a large range of precisely controlled shapes, depths and spacings. Combined with the *in vivo* and *in vitro* experimental procedures described in this study, micromachining could be a valuable method of investigating the topographical control of bone formation on artificial implants.

Acknowledgements

This research was supported by the British Columbia Health Care Research Foundation and by Medical Research council of Canada Grant #MA7617. We thank Mrs. Lesley Weston, Mr. Andre Wong and Mr. Hiroshi Kato for their excellent technical assistance. We also express our gratitude to Drs. N. Jeager, L. Young and D. Pulfrey of the Department of Electrical Engineering, University of British Columbia (UBC) for providing access to their laboratories for the preparation of micromachined surfaces, and to Dr. Keith Mitchell and Mr. Phil Wong of the Department of Chemistry, UBC, for XPS characterization of the titanium surfaces, and to Mr. Micheal Weis of the Department of Botany, UBC, for EDX analysis of mineralized tissue.

References

Albrektsson T, Branemark P-I, Hansson H-A, Kasemo B, Larsson K, Lundstrom I, McQueen DH, Skalak R (1983). The interface zone of inorganic implants *in vivo*: Titanium implants in bone. Annals Biomed. Eng. **11**, 1-27.

Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986). Mineralized bone nodules formed *in vitro* from enzymatically released rat calvaria cell populations. Calcif. Tissue Int. **38**, 143-154.

Bissell MJ, Barcellos-Hoff H (1987). The influence of extracellular matrix on gene expression: Is structure the message? J. Cell Sci. Suppl. **8**, 327-343.

Brunette DM (1988). The effects of implant surface topography on the behavior of cells. Int. J. Oral Maxillofac. Implants **3**, 231-246.

Brunette DM, Ratkay J, Chehroudi B (1991). The behaviour of osteoblasts on micromachined surfaces. In: The Bone-Biomaterial Interface, Davies JE (ed.), University of Toronto Press, Toronto, pp. 170-180.

Camporese DS, Lester TP, Pulfrey DL (1981). Development of fine line silicon shadow masks for the deposition of solar cell grids. In: Proceedings of the Fifteenth IEEE Photovoltaic Specialists Conference, IEEE, New York, pp. 527-529.

Chehroudi B, Gould TRL, Brunette DM (1990). Titanium-coated micromachined grooves of different dimensions affect epithelial and connective-tissue cells differently *in vivo*. J. Biomed. Mater. Res. **24**, 1203-1219.

Chehroudi B, Gould TRL, Brunette DM (1991). A light and electron microscopic study of the effects of surface topography on the behavior of cells attached to titanium-coated percutaneous implants. J. Biomed. Mater. Res. **25**, 387-405.

Chehroudi B, Gould TRL, Brunette DM (1992). The role of connective tissue in inhibiting epithelial downgrowth on titanium-coated percutaneous implants. J. Biomed. Mater. Res. **26**, 493-515.

Culling CFA, Allison RT, Barr WT (1985). Cellular Pathology Technique, 4th ed., Butterworths & Co., London, pages 295, 417-418.

Damien CJ, Parsons JR (1991). Bone graft and bone graft substitutes: A Review of current technology and applications. J. App. Biomat. **2**(3), 187-208.

Davies JE, Price NM, Matsuda T (1989). *In vitro* biocompatibility assays which employ bone derived cells. In: Oral Implantology and Biomaterials, Kawahara H (ed.), Elsevier Science Publishers, pp. 197-203.

Davies JE, Chernecky R, Lowenberg B, Shiga A (1991a). Deposition and resorption of calcified matrix *in vitro* by rat marrow cells. Cells & Materials. **1**(1), 3-16.

Davies JE, Ottensmeyer FP, Shen X, Hashimoto M, Peel SAF (1991b) Early extracellular matrix synthesis by bone cells. In: The Bone-Biomaterial Interface, Davies JE (ed.), University of Toronto Press, Toronto, pp. 214-228.

Davies JE, Nagai N, Takeshita N, Smith DC (1991c) Deposition of cement-like matrix on implant materials. In: The Bone-Biomaterial Interface, Davies JE (ed.), University of Toronto Press, Toronto, pp. 285-294.

Evans GS, Potten CS (1991). Stem cells and the elixir of life. Bioessays. **13**(3), 135-138.

Gerstenfeld LC, Chipman SD, Kelly CM, Hodgens KJ, Lee DD, Landis W (1988). Collagen expression, ultrastructural assembly and mineralization in cultures of chicken embryo osteoblasts. J. Cell Biol. **106**, 979-989.

Gottlieb TA, Beaudry G, Rizzolo L, Colman A, Rindler M, Adesnik M, Sabatini DD (1986). Secretion of endogenous and exogenous proteins from polarized MDCK cell monolayers. Proc. Natl. Acad. Sci. **83**, 2100-2104.

Hasegawa S, Sato S, Saito S, Suzuki Y, Brunette DM (1985). Mechanical stretching increases the number of cultured bone cells synthesizing DNA and alters their pattern of protein synthesis. Calcif. Tissue Int. **37**, 431-436.

Hench LL (1988). Bioceramics: Material characteristics versus *in vivo* behavior. Ann. New York Acad. Sci. **523**, 54-71.

Kendall LD (1979). Vertical etching of silicon at very high aspect ratios. Ann. Rev. Mater. Sci. **9**, 373-403.

Lowenberg B, Chernecky R, Shiga A, Davies JE (1991). Mineralized matrix production by osteoblasts on solid titanium *in vitro*. Cells & Materials. **1**(2), 177-185.

Mauchamp J, Marianne C, Verrier B, Gabrion J, Chabaud O, Gerard C, Penel C, Pialat B, Anfosso F (1987). Epithelial cell polarization in culture: Orientation of cell polarity and expression of specific functions,

studied with cultured thyroid cells. J. Cell Sci. Suppl. **8**, 345-358.

Nakahara H, Dennia JE, Bruder SP, Haynesworth SE, Lennon DP, Caplan AI (1991). *In vitro* differentiation of bone and hypertrophic cartilage from periosteal-derived cells. Exp. Cell Res. **195**, 492-503.

Nijiweide PJ (1975). Calcium and strontium metabolism of bone *in vitro*. Proc. Kon. Ned. Akad. Wet. **C78**, 416-417.

Ogiso B, Hughes FJ, Davies JE, McCulloch AG (1991). Inhibition of bone nodule formation *in vitro* by prostaglandins: Mechanism for fibroblast regulation of osteogenesis. J. Cell Physiol. **146** (3), 442-450.

Pilliar RM (1986). Implant stabilization by tissue ingrowth. In: Tissue Integration in Oral and Maxillofacial Reconstruction, van Steenberghe D (ed.), Excerpta Medica, Amsterdam, pp. 60-76.

Reddi AH, Huggins CB (1973). Influence of geometry of transplanted tooth and bone on transformation of fibroblasts. Proc. Soc. Exp. Bio. Med. **143**, 634-637.

Ripamonti U (1991). The induction of bone in osteogenic composites of bone matrix and porous hydroxyapatite replicas: An experimental study on the baboon *(Papio ursinus)*. J. Oral Maxillofac. Surg. **49**, 817-830.

Rodan GA, Rodan SB (1983). Expression of the osteoblastic phenotype. In: Bone and Mineral Research, Annual 2, Peck WA (ed.), Elsevier, pp. 244-285.

Schoen FJ (1986). Special considerations for pathological evaluation of explanted cardiovascular prostheses. In: Handbook of Biomaterials Evaluation. Scientific, Technical and Clinical Testing of Implant Materials. von Recum AF (ed.), Macmillan Publishing Co.

Schoen FJ (1988). Biomaterial associated calcification: Pathology, mechanisms and strategies for prevention. J. Biomed. Mater. Res., **22** (A1), 412-435.

Selye H, Lemire Y, Bajusz E (1960). Induction of bone, cartilage and hemopoietic tissue by subcutaneously implanted tissue diaphragms. Roux' Archiv. fur entwicklungsmechanik. **151**, 572-585.

Solursh M (1989). Extracellular matrix and cell surface as determinants of connective tissue differentiation. Am. J. Med. Genet. **34**, 30-34.

Tenenbaum HC, Heersche JNM (1982). Differentiation of osteoblasts and formation of mineralized bone *in vitro*. Calcif. Tissue Int. **34**, 76-79.

Tenenbaum HC, Heersche JNM (1985). Dexamethasone stimulates osteogenesis in chick periosteum *in vitro*. Endocrinology **117**, 2211-2217.

Watt FM (1986). The extracellular matrix and cell shape. TIBS. **11**, 482-485.

Weiss L (1988). The skeletal tissues. In: Cell and Tissue Biology, A Textbook of Histology, 6th ed., Weiss L (ed.), Urban & Schwarzenberg, Baltimore, pp. 218-246.

Discussion with Reviewers

J. Soderstrom: How can micromachining techniques be applied to current implant technology?

Authors: We have used micromachined surfaces only for research purposes. This technique has the advantage of producing surfaces that have precise and repeatable dimensions. Application of this technology to implants on commercial bases would require considerable technological development. However, prior to considering such development, more studies are necessary to characterize the optimal surface topographies required for the different cell populations contacting the implant surface.

J. Soderstrom: How do epoxy replicas coated with ≈ 50 nm titanium differ from the titanium implants currently available on the market today? What is the molecular make up of the sputter coated titanium surface versus conventional cast titanium? Is an oxide layer present?

Authors: We have not attempted to characterize differences between our model and commercial implants. Although there are possible physical differences, the analysis of our surfaces using X-ray photoelectron spectroscopy (XPS) has demonstrated that the surface resembles pure titanium implants in that titanium oxide is the dominant molecular species (Chehroudi *et al.*, 1991).

J. Soderstrom: How does the term "osseointegration" relate to this study?

Authors: The term osseointegration was introduced initially by Branemark (1985, see also Am. Acad. Implant Dentistry, 1986) to specify direct contact between bone and biomaterials as observed at the level of light microscopy. Our study indicated that a direct contact between a bone-like tissue and sputter coated titanium surface can be established at the resolution of the electron microscope. Similar results also have recently been reported by Listgarten *et al.* (1992) with titanium coated epoxy dental implants placed in dogs. Other studies, have not found direct bone/titanium contact, but as in most electron microscopic studies, the actual area observed is small, and since obtaining such areas from the implant/bone interface intact is difficult, it is possible that areas of direct contact have been missed. In future, osseointegration may be viewed not as an all or nothing process, but rather as a condition that varies in degree and extent.

J. Soderstrom: The terms "bone-like" tissues, "osteoblast-like" cells, and "osteocyte-like" cells were used in this paper. Does this mean that these tissues and cells could not be positively identified as bone or osteoblasts? If the time factor was increased could a positive identification be made?

F.J. Schoen: Please define the criteria for "appearance of osteoblasts", "osteocyte-like cells", and "bone-like tissues". Could the osteoblasts be activated macrophages?
Authors: The mineralized tissue was formed in a subcutaneous area which normally does not mineralize. Moreover, our characterization is based on the morphological features (electron microscopic appearances as described in the text) alone. In the absence of detailed criteria and in view of the unusual location, we prefer to be conservative and label the tissue and its cells with a suffix "-like". Biochemical procedures, elemental analysis and specific molecular probes would be required, to definitely characterize this tissue as bone proper.

J. Soderstrom: Can an estimate be made of the amount of mineralized foci that developed adjacent to the implant in relation to square area covered?
Authors: In this study the difference in bone-like tissue formation was absolute; bone formed on the micromachined surfaces but not on the smooth control surfaces. Thus a clear cut difference in the response of cells to the surfaces was observed. However, we agree that it would certainly be interesting to quantify the amount of bone-like tissue formed so that the best micromachined surfaces could be selected.

F.J. Schoen: Please define criteria for "connective-tissue ingrowth"; be more specific, in terms of blood vessels, collagen, etc. Were the morphologic results the same for grooved and taper-pitted surfaces?
Authors: The histological details of the connective tissue ingrowth as well as detailed comparisons of grooved and tapered-pitted surfaces have been published in our previous papers (Chehroudi *et al.*, 1990, 1991; Brunette *et al.*, 1991). There was a similar trend in histological observations. We observed condensation of the connective tissue cells and matrix within the grooves or pits and subsequent formation of mineralized foci after 6-8 weeks of subcutaneous implantation.

F.J. Schoen: Where did the initial mineral form: in cells, cell-derived fragments (i.e., matrix vesicles) or extracellularly in both *in vivo* and *in vitro* studies? Was the observed mineralization physiologic mineralization, in that it was an orderly regulated process, or was it dystrophic, forming within dead or injured cells?
Authors: It is premature at this time to comment on the mechanisms of mineralization in micromachined surfaces confidently. However, in a previous report (Brunette *et al.*, 1992) we reported that matrix vesicles formed adjacent to the titanium coating and in some cases matrix vesicles were even in direct contact with the titanium surfaces. The mineralized tissue formed adjacent to micromachined surfaces contained cells that resembled osteoblasts and osteocytes and did not resemble dystrophic deposition.

H.-A. Hansson: Micromachining of the implant improved bone formation, according to the presented results. Is such conclusion limited to larger metal implants or does it also apply to the design of membranes and tubes used by orthopaedics and orthodontists to promote bone growth?
M.S. Bapna: How do the micromachined surfaces differ from other surfaces, such as controlled sintered surface in relation to mineralization?
Authors: We have no information on these points, nor are aware of any studies comparing micromachined surfaces with other surfaces, such as, sintered surfaces, in the same model system. These matters certainly deserve to be studied.

J.E. Davies: In this, as with much of your previous work, you provide convincing evidence of the effect of micromachined surfaces on cell migration. Have you found any evidence of such orientation effects on bone tissue rather than just bone cells?
Authors: The ability of the micromachined grooved surfaces to direct the migration of locomoting bone cells *in vitro* has been documented in a previous report (Brunette *et al.*, 1991). In addition, our preliminary results indicate that bone tissue formed *in vitro* is oriented along the long axis of grooves. We are currently in the process of quantifying this orientation effect.

D.E. Steflik: The authors conclude that no amorphous material exists between the mineralization front and the titanium coating on these epoxy implants. In extensive ultrastructural investigations involving conventional transmission and high voltage electron microscopy, we have identified a 20 to 50 nm electron dense deposit at the bone-implant interface (Steflik *et al.*, 1992). This is in agreement with the reports of Albrektsson *et al.* (1983), van Blitterswijk *et al.* (1991), and De Lange *et al.* (1989). Even within the resolution limits or the magnification for the submitted micrographs, my interpretation of Figures 6 and 8 suggests that there is an amorphous material present at the interface. Could the authors comment on the relative merit for having or not having either an electron-dense deposit at the interface or amorphous material suggestive of extracellular glycoproteins. Further, could the 50 nm electron dense titanium coating have masked any associated electron dense deposit?

The implants placed in this study were placed subcutaneously, and not directly into bone. Could the authors comment on the transferral of their data to implants placed directly into bone, which, after all, is the

location of choice. Could this factor explain the absence of the electron-dense or amorphous deposit at the implant interface?

Authors: We have documented in various electron micrographs of the current paper as well as in a previous paper (Brunette *et. al.*, 1991), that mineralized matrix and collagen fibers contact titanium-coating directly in some areas without an amorphous layer. In agreement with our morphological findings, Listgarten *et al.* (1992) have recently reported similar electron microscopical findings with titanium-coated dental implants in dogs. These findings are obviously in contrast to previous reports which did not observe such contacts. As suggested in our discussion and by Dr. Listgarten in his discussion, there could be many reasons for these differences and certainly more studies are required to explain the differences. But the possibility of the titanium coating masking the amorphous material appears unlikely. However, the function of this layer and why it should be present, cannot be answered at the present time. To answer such questions, a detailed characterization of the molecules present in the bone/implant interface is required. Perhaps immunohistochemical procedures will prove pivotal in such characterizations.

In Figure 6 as well as Figures 7 and 8, structures such as needle-like crystal of minerals and cross or longitudinal sections of collagen bundles contact the surface of the titanium. It is also true that we have observed and published micrographs demonstrating that amorphous material occurs at the titanium/connective tissue interface (Chehroudi *et al.*, 1991). In summary, there is evidence for various structures occurring at the titanium/tissue interface, and their relative frequency can be determined only by more studies.

D.E. Steflik: The authors comments on the relative importance of textured versus smooth surface to mineralization events are important. Current commercially available implants attempt to use this difference for their design. That is, smooth coronal regions and textured apical regions. Could the authors comment, utilizing their previous reports, concerning epithelial and connective tissue models with similar pitted and grooved implants, on the ideal implant design to enhance ideal tissue migration and modeling. Further, what kind of alterations in the surgical insertion armamentaria would be required for such an implant?

Authors: Our long term goal is to design an implant which would be less technique-sensitive. One can conceive of an ideal implant that has surfaces engineered so that osteogenic cells, connective-tissue cells and epithelial cells attach at specific locations and function optimally. Unfortunately, the optimal surface is not known for any of these cell populations at the current time.

D.E. Steflik: My one major concern is that the authors must specifically state that for the *in vivo* studies, the implant used is a "model" implant manufactured not of metal, but of plastic (epoxy replica coated with 50 nm of titanium). This model system has been used by others for TEM observations (e.g., Listgarten *et al.*, 1992) and may not represent analogous tissue responses *in vivo* as commercially available metal or ceramic dental implants due to applied forces and implant stiffness and strength. I am appreciative of the difficulty in obtaining TEM observations for commercially available implants. I have no problem with the authors important research outcomes if they place the *caveat* that the results obtained may be transferable to the true *in vivo* arena, but that these model implants may not undergo similar biomechanical alterations to commercial implants. Could the authors comment on the transferral of their data with this implant model to an *in vivo* model utilizing metallic or ceramic implants?

Authors: In this paper, we have not tried to compare this implant model with the solid metal or ceramic implants. Nevertheless, we have done detailed X-ray photoelectron spectroscopy (XPS) analysis and found that the surface of our titanium-coating is covered by a layer of titanium oxide which is similar to most commercially pure titanium implants available in the market. Clearly we do not have all the information, and appreciate that this should be evaluated in a similar experimental system in future.

D.E. Steflik: The implants were sectioned in two prior to embedment, but after fixation. Was there any disruption of the tissues from the implants when the implant was so sectioned?

Authors: The histological processing that was developed in our laboratory has been discussed in detail in a previous paper (Chehroudi *et al.*, 1991). The implants were bisected by a thin diamond disk after final stage of fixation. At this stage, the tissue/implant interface is relatively firm and there is little chance of tissue detachment from the implant surface. Nevertheless, any implant/tissue sample with minute evidence of detachment was discarded. The number of samples discarded was less than 20% of the total as reported previously (Chehroudi *et al.*, 1992).

H.-A. Hansson: In our experience tilting of specimens in the transmission electron microscope with the aid of a goniometer often revealed details on the border zone between the mineralized tissue and the implant. Has that been performed? Whether it would change the

authors conclusion or not is difficult to predict. The authors state that sections revealing the details of the interface could be difficult to obtain, which certainly is true. The risks for deformations including compression and distortion is, in my experience, high.

Authors: We did not tilt the specimens as our electron microscope is not fitted with a goniometer; in future, we plan to do this.

J.E. Davies: Why did you choose the groove profiles that you report?

Authors: These surfaces were chosen as a result of a previous experiment (Cheroudi *et al.*, 1992) in which connective tissue was found to grow into grooves and pits of these dimensions.

J.E. Davies: You state: "the epidermal and dermal layers were elevated, and were removed by a combination of blunt and sharp dissection to form a pouch sufficient for the implant. In addition, the periosteum was completely removed and the discontinuity of the bone under the implant was smoothed by means of a bone file. The surgical wound was irrigated by sterile saline and dried by sterile gauze. Implants were then placed on the parietal bone and the access incision was sutured using 4-0 silk". Clearly bone-chips must have formed when you filed the underlying bone. How can you be sure that **all** bone chips were removed?

Authors: As stated in the text (and repeated above), the surgical wound was irrigated with sterile saline and dried with sterile gauze before placement of the implant. It would be difficult to determine whether all chips were removed or not. Nevertheless, all surfaces of the implant including smooth control surfaces, were placed in a similar environment created by this type of surgical technique.

Additional References

American Academy of Implant Dentistry (1986) Glossary of implant terms. J. Oral Imp. Special Issue 2 (XIV), 284-292.

Branemark P-I (1985) Introduction to osseointegration. In: Tissue-Integrated Prostheses, Branemark P-I, Zarb G, Albrektsson T (eds.), Quintessence Publishing Co., Chicago, **1**: 11-76.

Brunette DM, Chehroudi B, Gould TRL (1992) Electron microscopic observations on the effects of surface topography on the behavior of cells attached to percutaneous and subcutaneous implants. In: Proceeding of the 2nd International Congress on Tissue Integration in Oral, Orthopedic and Maxillofacial Reconstruction, Laney WR, Tolman DE (eds.), Quintessence Publishing Co. Chicago, pp. 21-31.

De Lange GL, Donath K (1989) Interface between bone tissue and implants of solid hydroxyapatite or hydroxyapatite coated titanium implants. Biomaterials **10**: 121-125.

Listgarten MA, Buser D, Steinemann SG, Donath K, Lang NP, Weber HP (1992) Light and transmission electron microscopy of the intact interfaces between non-submerged titanium-coated epoxy resin implants and bone or gingiva. J. Dent. Res. **71**(2): 364-371.

Steflik DE, Hanes PJ, Sisk Al, Parr GR, Song MJ, Lake FT, McKinney RV (1992) Transmission electron microscopic and high voltage electron microscopic observations of the bone and osteocyte activity adjacent to unloaded dental implants placed in dogs. J. Periodontol. **63**(5): 443-452.

van Blitterswijk CA, Bukker D, Hessling SG, Koerter HK (1991) Reaction of cells at implant surfaces. Biomaterials **12**: 187-193.