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Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
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in Partial Fulfillment
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By
Richard Charles Batzer, B.S., D.D.S.

San Antonio, Texas

May 1998

THE EFFECT OF TITANIUM SURFACE ROUGHNESS ON MG63 OSTEOBLAST-LIKE CELL DIFFERENTIATION AND RESPONSE TO 1,25-DIHYDROXYVITAMIN D_3

Richard Charles Batzer

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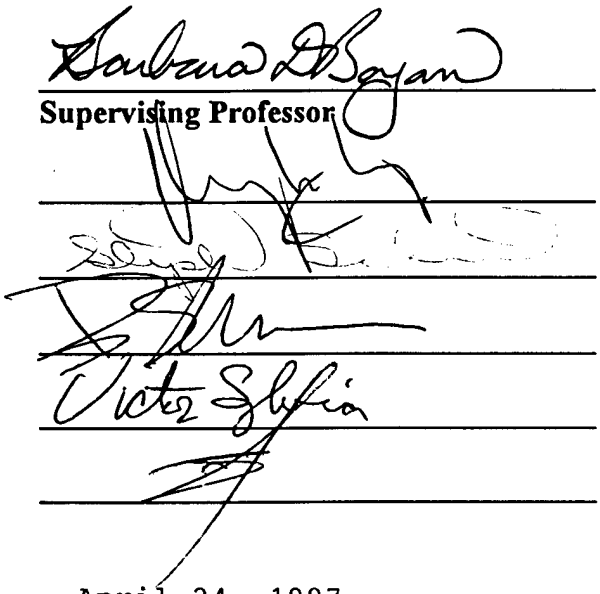

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DEDICATION

I dedicate this thesis to the light of my life, my wonderful wife Kay, whose unwavering love, patience, sacrifice, and support has sustained me through a very demanding and at times difficult time in our lives. To my children, Ashley, Brenton, and Sean; you are truly the greatest gifts I have and for which I count my blessings each and every day. Like your mother, you have made great sacrifices. I also dedicate this work to my parents, Richard and Joan Batzer; you have supported my every endeavor and have instilled in me the confidence to achieve whatever I set out to do. To all I am forever grateful.

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THE EFFECT OF TITANIUM SURFACE ROUGHNESS ON MG63 OSTEOBLAST-LIKE CELL DIFFERENTIATION AND RESPONSE TO 1,25-DIHYDROXYVITAMIN D_3

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Surface roughness has been shown to affect cell differentiation and local factor production of MG63 osteoblast-like cells. The first specific aim of this study was to examine whether surface roughness alters the response of MG63 cells to circulating systemic hormones like 1,25-$(OH)_2D_3$ (1,25). Unalloyed titanium (Ti) disks were pretreated with HF/HNO_3 (PT) and then machined and acid-etched (MA). Ti disks were also sandblasted (SB), sandblasted and acid-etched (CA), or plasma-sprayed with Ti particles (PS). The surfaces, from smoothest to roughest, were: PT, MA, CA, SB, and PS. MG63 cells were cultured to confluence on standard tissue culture polystyrene (plastic) or the Ti surfaces and then treated for 24 hours with either 10^{-8}M or 10^{-7}M 1,25-$(OH)_2D_3$ or vehicle (control). Cellular response was measured by assaying cell number, cell layer alkaline phosphatase specific activity, and the production of osteocalcin, latent transforming growth factor-β (LTGFβ), and prostaglandin E_2 (PGE_2). Alkaline

phosphatase specific activity was affected by surface roughness; as the surface became rougher, the cultures showed a significant increase in alkaline phosphatase specific activity. Addition of 1,25-$(OH)_2D_3$ to the cultures caused a dose-dependent stimulation of alkaline phosphatase specific activity which was synergistic with the effect caused by surface roughness alone. 1,25-$(OH)_2D_3$ also caused a synergistic increase in osteocalcin production, as well as local factor (LTGFβ and PGE_2) production, on the rougher CA, SB and PS surfaces, but had no effect on the the cultures grown on the smoother surfaces. The inhibitory effect of surface roughness on cell number was not affected by 1,25-$(OH)_2D_3$, except on the SB surface. 1,25-$(OH)_2D_3$ decreased cell number, increased alkaline phosphatase specific activity and osteocalcin production, and had no effect on LTGFβ or PGE_2 production by MG63 cells grown on tissue culture polystyrene.

The second specific aim of this study was to determine whether the combined effect of surface roughness and 1,25-$(OH)_2D_3$ on MG63 cell response is mediated by PGE_2 produced by the same cells. MG63 cells were cultured to confluence on the Ti disks in the presence or absence of 10^{-7}M indomethacin, a specific inhibitor of PGE_2 production. In other cultures, cells were exposed to 10^{-7}M 1,25-$(OH)_2D_3$, 10^{-7}M indomethacin, or a combination of the two during the 24 hour culture period after confluence was achieved. At harvest, cell number, cell layer alkaline phosphatase specific activity , and LTGFβ and osteocalcin production were determined. On the rougher surfaces, cell number was decreased and alkaline phosphatase specific activity, osteocalcin production, and LTGFβ production were increased. 1,25-$(OH)_2D_3$ had a significant synergistic effect on all parameters examined in cultures on the rougher surfaces (SB, CA, and PS). When indomethacin was added with 1,25-$(OH)_2D_3$, for 24 hours the synergistic effect on cell

number, osteocalcin and LTGFβ production was abolished; however, this was not found with alkaline phosphatase specific activity. When indomethacin was present throughout the culture period, the effect of surface roughness on all parameters was abolished. However, the addition of indomethacin to confluent cultures for 24 hours had no effect.

This study indicates that bone cell response to systemic hormones such as 1,25-$(OH)_2D_3$ is modified by implant surface roughness. The responsiveness of MG63 osteoblast-like cells to surface roughness is enhanced by the systemic hormone, 1,25-$(OH)_2D_3$. This effect is mediated, at least in part, by PGE_2 produced by the same cells. PGE_2 directly affects differentiation of MG63 osteoblast-like cells on material surfaces.

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I. Introduction and Literature Review

Dental extraction is one of the most frequent injuries incurred by the human skeleton. During the past twenty years, endosseous implants have been used extensively to achieve osseointegration for prosthetic rehabilitation of edentulism. It is estimated that by 1992 there were over 300,000 dental implants placed in the United States (NIH, 1988).

One of the main reasons that endosseous implants have eventually become a major influence within oral implant surgery is the work of P.I. Branemark, a Swedish orthopaedic surgeon. Branemark (1985) proposed the concept of "osseointegration", which he defined as a "direct structural and functional connection between ordered, living bone and the surface of a load carrying implant." Through intense basic research and clinical follow-up examinations, Branemark was able to achieve previously unseen, consistent long term success rates with oral endosseous implants (Adell *et al.*, 1981). Because of Branemark's work (Branemark *et al.*, 1977; Branemark, 1985), dental endosseous implants have gained considerable credibility (Driskell, 1987).

The ultimate goal of an implant when placed in a bony host site is to achieve functional integration of the implant material with the living tissue and fulfill a load-bearing function (Weinlander, 1996). In addition to host factors, such as physical condition of the patient, available bone volume, initial period of unloaded healing, and careful atraumatic host site preparation, the surface of the implant material is of paramount importance for the initial interaction with the surrounding tissues.

The search for the ideal implant surface has recently intensified and is truly multidisciplinary. Not too long ago, researchers focused on the implant material at the macroscopic level and considered the mechanical properties of the material as one of the most important factors for selection of a certain material or surface preparation technique. More recently, however, the molecular and atomic nature of a material has been considered of greater importance. Material properties, such as bioadhesion, surface tension, surface energy, surface wettability, and surface roughness, have been examined to an increasing degree in the development of a biocompatible implant surface. Further, issues related to surface contamination and decontamination have also been viewed as critical to implant success.

A whole spectrum of reactions can occur when a foreign material is implanted into human tissues. On the one hand, the host may mount a massive immunologic response against the specific material and outright rejection may occur. At the other end of the spectrum, complete compatibility may occur between the material and the host resulting in functional longevity. Biocompatibility can be defined as that portion of the spectrum in which the response between the host and the implant material is so minimal that neither is detrimentally affected to a significant degree (Schroeder, 1991). For a material to be biocompatible, three basic requirements must be fulfilled: 1) it must be non-toxic and noncarcinogenic; 2) it must not interfere with the healing of host tissues that were damaged during their surgical placement; and 3) host tissues should tolerate the biomechanical properties of this material (Binderman, 1991).

The most commonly used biocompatible implant materials are metals and their alloys. The use of implantable metals for reconstructive purposes was first popularized in

the early 1900s. Gold, aluminum, silver, bronze, iron, steel, copper, zinc, and nickel were tried in various combinations, but most of these metals had inadequate mechanical properties or unacceptable tissue responses, and were subsequently discontinued (Carruthers, 1941). Cobalt-chromium based alloys became available in the 1930s, and they are still used in a variety of applications today because of their favorable strength characteristics and their general tissue biocompatibility. Further advances in biomaterial science, over recent decades, has led to the development and implementation of 316L stainless steel, titanium, and titanium based alloys for a variety of reconstructive purposes (Kent and Misiek, 1991).

1. Titanium as an Implant Material

Titanium was initially discovered in the 1700s, but it was not until the 1930s that the first commercially feasible extraction process was developed. In 1940, Bothe *et al.*, (1940) studied various metals and reported that titanium had more of a tendency for bone to grow into contact with it than stainless steel or cobalt-chromium alloys. Further studies in the early 1950s demonstrated that titanium had excellent corrosion resistance (Clarke and Hickman, 1953) and excellent tissue tolerance (Leventhal, 1951). Since that time, further improvements in processing and purification techniques have led to the widespread use of this metal for various industrial and medical applications. Commercially pure titanium and titanium alloys are the most widely used metals in implant dentistry today.

Titanium metal adequately fulfills the strength requirement for long-term implant function. It has been found to have excellent biocompatibility (Beder and Eade, 1956; Albrektsson *et al.*, 1981; Williams, 1981; Branemark, 1985; Donley and Gillette, 1991)

due to its high reactivity, which causes the spontaneous production of an inert oxide layer on its surface upon exposure to air (Kasemo and Lausmaa, 1986). This surface oxide layer is approximately 2-5 nanometers thick and is believed to be responsible for the high corrosion resistance and biocompatibility exhibited by titanium (Kasemo and Lausmaa, 1986; Kasemo and Lausmaa, 1988a; Kasemo and Lausmaa, 1988b; Stanford and Keller, 1991). At this time, commercially pure titanium is the material of choice for uncoated implants because it is lightweight and durable, and can be easily prepared in many different shapes and textures without affecting its biocompatibility (Meachim and Pedley, 1981; Rae, 1981; Holgers *et al.*, 1992).

2. Surface Properties of Implants

The surface properties of an implant will ultimately influence the initial cellular events occurring at the cell-material interface. The initial interactions occurring at the bone-implant interface largely determine the success or failure of the implant. The outcome at this site is dependent not only on successful wound healing, but also on successful bone formation. Bone formation on an implant surface requires recruitment of osteoblast precursor cells, their differentiation into secretory osteoblasts, production of unmineralized extracellular matrix (osteoid), and calcification of the extracellular matrix (Schwartz and Boyan, 1994).

The literature is clear that titanium surface properties play an integral role in the reaction of tissue to an implant surface (Andrade, 1973; Ramsey *et al.*, 1984; Doundoulakis *et al.*, 1987; Baier *et al.*, 1988; Kasemo and Lausmaa, 1988a; Carlsson *et al.*, 1989; Hartman *et al.*, 1989). Furthermore, surface properties of an implant play a

major role in the ultimate success of the implant (Albrektsson *et al.*, 1981). When discussing interactions that promote growth and apposition of bone tissue to an implant surface, the surface properties of the implant, both chemical and physical, must be considered (Albrektsson *et al.*, 1981). Those surface properties that influence the behavior of cells of mesenchymal origin like osteoblasts, including matrix production and calcification, can be grouped into four interrelated categories: composition, surface energy, topography, and surface roughness (Schwartz and Boyan, 1994). Surface texture, a combination of topography and roughness, has been hypothesized to be more significant than other variables, such as composition and elastic modulus (Thomas and Cook, 1985). All of these properties may be modified, either intentionally or unintentionally, during the fabrication of the device.

3. Effect of Titanium Surface Roughness

In several *in vivo* studies, rough surfaces were found to produce better bone fixation than smooth surfaces (Thomas and Cook, 1985; Buser *et al.*, 1991; Carlsson *et al.*, 1988; Wilke *et al.*, 1990), suggesting that this surface property might have a direct effect on attachment of osteoblasts and their subsequent proliferation and differentiation. Cell culture studies have supported this hypothesis. *In vitro,* osteoblasts appear to be sensitive to surface roughness and exhibit greater initial attachment to rough titanium surfaces (Michaels *et al.*, 1989; Bowers *et al.*, 1992).

Moreover, osteoblast-like cells can discriminate not only between surfaces with different roughness, but also between surfaces with comparable roughnesses but different topographies (Martin *et al.*, 1995). In these studies, MG63 human osteoblast-like cells

were grown on titanium disks of varying roughness. These cells exhibited differential responses with respect to cell proliferation, alkaline phosphatase specific activity, and matrix production. In general, when compared to MG63 cells grown on the smoother surfaces, cells cultured on the rougher surfaces exhibited decreased levels of cellular proliferation, resulting in lower cell numbers. In contrast, matrix production was increased, as was expression of alkaline phosphatase. These observations suggested that cells cultured on rougher surfaces express a more differentiated phenotype. In addition, cells on the rougher surfaces released higher levels of prostaglandin E_2 (PGE_2) and latent transforming growth factor β (LTGFβ) (Kieswetter *et al.*, 1996), two factors involved in regulation of bone formation. These latter data implicate surface roughness in influencing how cells at the implant-bone interface can modulate local regulatory events.

4. Markers of Osteoblast Differentiation

Two indices of osteoblastic cell activity are alkaline phosphatase activity and the production of osteocalcin. Both alkaline phosphatase and osteocalcin are recognized as important markers of osteoblast differentiation.

Alkaline phosphatase. This enzyme has been established as having a critical role in mineralization and bone formation (Majeska and Wuthier, 1975; Ali, 1980). It is considered the marker enzyme for matrix vesicles, extracellular organelles associated with mineralization. The exact role of alkaline phosphatase is unclear, but it has been found to promote hydrolysis of phosphate-containing substrates into orthophosphate, which is essential for crystal formation (Ali *et al.*, 1977). Alkaline phosphatase is also thought to

promote the uptake of calcium and block the inhibitory effects of ATP on hydroxyapatite formation (Ali and Evans, 1981).

Osteocalcin. Osteocalcin or bone gla protein is one of the major, noncollagenous, bone-specific proteins produced by cells of osteoblastic phenotype (Beresford *et al.*, 1984; Price, 1985). Osteocalcin accounts for 10 to 20% of the noncollagenous protein present in bone and is specifically produced by cells which mineralize their matrix (Beresford *et al.*, 1984; Finkelman and Butler, 1985; Gerstenfeld *et al.*, 1987). 1,25-$(OH)_2D_3$ has been shown to induce osteocalcin production in human primary bone cell cultures, fetal rat calvarial cells, ROS17/2.8 rat osteosarcoma cells and MG63 cells (Price and Baukol, 1980; Beresford *et al.*, 1984; Lian *et al.*, 1985; LaJeunesse, 1990). While the function of this bone-specific protein is not known, it is thought to play a role in the assembly of bone mineral via the regulation of crystal growth, and in bone breakdown (Bonewald *et al.*, 1992a). Osteocalcin is considered to be a terminal marker of osteoblast differentiation as increases in osteocalcin production have been shown to correlate with the onset of calcification (Aronow *et al.*, 1990).

5. Local Factor Regulation of Bone Metabolism

The process of bone formation is very complex and known to involve a variety of local growth factors, cytokines and hormones. Study of these hormonal and local modifiers of bone metabolism has been extensive in recent years. Two local factors produced by osteoblasts that are important in both wound healing and bone formation are prostaglandin E_2 (PGE_2) and transforming growth factor-β (TGF-β) (Dworetzky *et al.*,

1990; Joyce *et al.*, 1990; Bonewald *et al.*, 1992a; Schwartz *et al.*, 1992a; Bonewald *et al.*, 1992b).

Prostaglandin E_2. Prostaglandins, in contrast to systemic hormones, are produced locally by bone cells. While the exact role of PGE_2 in bone metabolism is unknown, it appears to have varying effects on both bone formation and bone resorption. A major role for PGE_2 in bone formation is well established (Chyun and Raisz, 1984). Raisz and Martin (1983) have suggested that prostaglandins stimulate proliferation and differentiation of osteoblast precursors. Prostaglandins of the E-series are known to be potent stimulators of bone resorption (Klein and Raisz, 1970; Tahjian *et al.*, 1972; Dietrich *et al.*, 1975). In general, at lower concentrations, PGE_2 enhances osteoblastic activity, while at high concentrations PGE_2 inhibits osteoblasts and stimulates osteoclasts (Chyun and Raisz, 1984; Raisz and Fall, 1990).

Transforming growth factor β. The most concentrated source of TGF-β in the body is found in platelets (Assoian *et al.*, 1983), but the largest source is in bone (Seyedin *et al.*, 1986). Sporn *et al.*, (1986) have suggested that TGF-β works in conjunction with other cytokines to modulate their effects, be it stimulation or inhibition. The effects of TGF-β are wide ranging and vary according to cell type (Bonewald and Mundy, 1990). Bone formation appears to be strongly related to the presence of TGF-β in the bone matrix (Joyce *et al.*, 1990). TGF-β is believed to be important in initial proliferation, differentiation, and matrix production of osteoblasts (Dworetzky *et al.*, 1990), and has been shown to induce osteoblasts to produce extracellular matrix and increase alkaline phosphatase activity (Bonewald *et al.*, 1992a; Harris *et al.*, 1994).

As previously noted, Kieswetter *et al.* (1996) have demonstrated that the production of TGF-β and PGE_2 by osteoblasts is affected by titanium surface roughness. In general, rougher surfaces enhanced the production of these factors.

6. Hormone Regulation of Bone Metabolism

Vitamin D is known to be essential for proper bone metabolism. Bone matrix synthesis and cartilage growth are inhibited, linear growth is halted, and bone formation is stunted as a result of vitamin D deficiency (Raisz and Kream, 1983b and c). Treatment with vitamin D_3 restores mineralization. An active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$, appears to regulate alkaline phosphatase activity, which is clinically important in the mineralization of skeletal tissues (Raisz and Kream, 1981). The MG-63 cell line responds to 1,25-$(OH)_2D_3$ in a number of ways. The hormone has been shown to: 1) inhibit cell growth and tritiated thymidine incorporation; 2) increase alkaline phosphatase activity two-fold; 3) increase cellular and media levels of fibronectin; 4) increase collagen type I synthesis, while no effect is seen on collagen type III; and 5) stimulate the production of human osteocalcin four-fold (Franceschi *et al.*, 1985,1987). Franceschi *et al.* (1985,1987) have suggested that 1,25-$(OH)_2D_3$ stimulates MG-63 cells to differentiate into a more osteoblastic phenotype.

Previous studies by Boyan and co-workers have revealed wide-ranging effects of 1,25-$(OH)_2D_3$ stimulation on cells. In chondrocyte cultures, Schwartz *et al.* (1992a) demonstrated that PGE_2 production is regulated by 1,25-$(OH)_2D_3$. Furthermore, production appears to be cell-maturation-dependent. The effects of 1,25-$(OH)_2D_3$ on osteoblasts have been hypothesized to occur via autocrine or paracrine action of PGE_2 on

the cell membranes (Schwartz *et al.*, 1992b). When TGF-β is combined with 1,25-$(OH)_2D_3$, a synergistic increase in alkaline phosphatase activity is seen, but 1,25-$(OH)_2D_3$ dependent increases in osteocalcin (an indicator of terminal differentiation of osteoblasts) production are inhibited (Bonewald *et al.*, 1992a; Ingram *et al* ., 1994). The observation that 1,25-$(OH)_2D_3$ regulates PGE_2 production by osteoblasts (Schwartz *et al.*, 1992b) suggests that there may be an interrelationship between TGF-β and PGE_2.

7. Investigation Purpose and Aims

Titanium implants, currently in clinical use, vary with respect to surface roughness. It is imperative to determine the role that implant surface roughness plays not only in osteoblast-like cell differentiation and local factor production, but also on cellular response to endocrine factors such as 1,25-$(OH)_2D_3$. The purpose of this investigation was to examine the effect of surface roughness on cellular response to 1,25-$(OH)_2D_3$ with respect to cell proliferation, differentiation, and local factor production.

The first specific aim of this study was to examine whether surface roughness alters the response of MG63 cells to circulating systemic hormones like 1,25-$(OH)_2D_3$. The second specific aim was to determine whether the combined effect of surface roughness and 1,25-$(OH)_2D_3$ on MG63 cell response is mediated by PGE_2 produced by the same cells. This investigation tested the hypothesis that the interactive effect of surface roughness on response of MG63 cells to 1,25-$(OH)_2D_3$ is mediated by PGE_2.

II. Materials and Methods

1. Titanium Disk Preparation

The Ti disks (15mm in diameter) for cell culture were prepared from 1 mm-thick sheets of grade 2 unalloyed, commercially pure titanium (ASTM F67 "Unalloyed Titanium for Surgical Implant Applications") and were supplied by Institut Straumann AG (Waldenburg, Switzerland). The disks were processed as described below to produce surfaces of varying roughness.

1. PT: Prior to use, the disks were degreased and acid prepickled by washing in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55°C for 30 seconds, and pickling in 2% hydrofluoric acid/10% nitric acid at room temperature for 30 seconds.

2. MA: PT disks were machined and acid-etched in hydrochloric acid/sulfuric acid using a proprietary process of Institut Straumann AG.

3. SB: PT disks were coarse grit-blasted with 0.25 to 0.50 μm corundum grit at 5 bars.

4. CA: PT disks were coarse grit-blasted with 0.25 to 0.50 μm corundum grit at 5 bars until the surface reached a uniform gray tone and then acid-etched in hydrochloric acid/sulfuric acid using a proprietary process of Institut Straumann AG.

5. PS: PT disks were coarse grit-blasted as above and then plasma spray-coated with titanium hydride powder using a proprietary process of Institut Straumann AG.

After preparation of the surfaces was complete, the disks were rinsed with deionized water, neutralized in 5% sodium bicarbonate solution, ultrasonically rinsed in deionized water for three 5-minute periods, wrapped in gauze sponges, and then sterilized by steam autoclaving. For all experiments, cells were cultured on Ti disks placed in 24-well plates (Corning Costar, Cambridge, MA); control cells were cultured directly on tissue culture polystyrene ("plastic") in 24-well plates.

2. Titanium Disk Surface Analysis

After autoclaving, representative disks from each group were subjected to surface analysis as described below.

Profilometry. Surface roughness of the Ti disks was determined by profilometry using a Taylor-Hobson Surtronic 3 profilometer (Leicester, U.K.) at a high sensitivity setting. Six disks were measured from each group to obtain an average roughness value (R_a). R_a values were not determined for the plastic surfaces.

Scanning electron microscopy. Surface characteristics were examined with a JEOL 6400 FEC cold field emission scanning electron microscope (SEM) (JEOL USA, Inc. Peabody, MA) with a nonthermally assisted tip with secondary and backscattered electron capability. Disks were sputter-coated with palladium-gold prior to analysis.

More extensive surface characterization using laser confocal scanning microscopy, energy dispersion X-ray analysis, and Auger electron spectroscopy was performed in a prior study (Martin *et al.,* 1995) with similarly prepared disks and was not repeated for this study.

3. Cell Model and Culture

MG63 human osteoblast-like cells were used for these experiments. The MG63 cell line is from a human osteosarcoma (Franceschi *et al.*, 1985), originally derived from the femur of a 14 year old male. It was first isolated by Billiau in 1975. It is a homogeneous cell line which enables us to achieve cell-specific results. This cell line is desirable because it remains viable and maintains its osteoblastic phenotype while proliferating through many cell passages. MG63 have been well-characterized and show numerous osteoblastic traits, including the production of high levels of alkaline phosphatase activity and osteocalcin synthesis in response to 1,25-$(OH)_2D_3$ (Franceschi *et al.*, 1985; Franceschi *et al.*, 1987).

MG63 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2 in air at 37°C and 100% humidity. Cells were seeded onto plastic or Ti disks of varying roughness at a density of 9,300 cells/cm^2. The media were changed every 48 hours until the cells reached confluence on plastic. Because of the opacity of the Ti disks, there was no practical way to assess confluency of the cultures on them. As a result, when cells reached visual confluence on plastic, cultures on all other surfaces were treated exactly as those grown on plastic. Thus, it is possible that the cultures were not at the same degree of confluence when they were treated as described below. At confluence, the media were replaced with 0.5 ml fresh media containing either 10^{-8}M or 10^{-7}M 1,25-$(OH)_2D_3$, or vehicle. For studies examining the effect of PGE_2, 10^{-7}M 1,25-$(OH)_2D_3$,

10^{-7}M indomethacin, or a combination of the two were added to the cultures at the time of seeding or at the time the cultures were confluent. Indomethacin inhibits cyclooxygenase, an enzyme involved in the production of PGE_2. All cultures were harvested 24 hours after they had achieved confluence.

4. Determination of Cell Number

At harvest, the cultures were washed twice with DMEM and then released from the surfaces by addition of 0.25% trypsin in Hank's balanced salt solution (HBSS) containing 1 mM ethylenediamine tetraacetic acid (EDTA) for 10 minutes at 37°C. The reaction was terminated by the addition of DMEM containing 10% FBS. A second trypsinization of the surfaces was performed in order to assure that any remaining cells were completely removed. Cell suspensions from both trypsinizations were combined and centrifuged at 500 x g for 10 minutes. The supernatant was decanted and the cell pellet washed with phosphate buffered saline (PBS) and resuspended in 0.9% NaCl. Cell number was determined using a Coulter Counter (Hialeah, FL). Cells harvested in this manner exhibit >95% viability based on trypan blue dye exclusion.

5. Cell Differentiation

Alkaline phosphatase activity of cell layers. Cell layers were prepared following the method of Hale *et al.*, (1986) as described previously (Martin *et al.*, 1995). At harvest, culture media were decanted, cell layers washed twice with PBS, and then removed with a cell scraper. After centrifugation, the cell layer pellets were washed once more with PBS and resuspended by vortexing in 500µl deionized water plus 25µl 1%

Triton-X-100. Pellets were further disrupted by freeze/thawing three times. The protein content of these lysates was determined using commercially available kits (Micro/Macro BCA, Pierce Chemical Co., Rockford, IL). Alkaline phosphatase activity [orthophosphoric acid monoester phosphohydrolase, alkaline (E.C. 3.1.3.1)] was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2 (Bretaudier and Spillman, 1984).

Osteocalcin production. The production of osteocalcin by the cultures was measured using a commercially available radioimmunoassay (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA). Culture media were concentrated five-fold by lyophilization in a speed-vacuum and then reconstituted in 100µl normal rabbit serum, 10µl rabbit anti-human osteocalcin antibody, 100µl [^{125}I]-human osteocalcin, and 200µl Tris-saline buffer and placed overnight on an orbital platform shaker (approximately 80 rpm) at room temperature. Goat anti-rabbit antibody and polyethylene glycol (100µl each) were added to each tube the following morning. After vortexing, the samples were placed on an orbital shaker for 2 hours at room temperature. One ml of Tris-saline buffer was added to each sample. The solution was then vortexed and centrifuged at 500 x g for 20 minutes at 4°C. The supernatant was decanted and the pellet placed in scintillation cocktail. Counts were determined using a Beckman LS 61000C liquid scintillation counter. Osteocalcin concentrations were determined by correlating the percentage bound over unbound counts to a standard curve.

6. Local Factor Production

Transforming growth factor-β (TGFβ) production. In order to obtain a quantitative assessment of growth factor production, the level of total TGFβ in the conditioned media was determined using a commercially available ELISA Kit (Promega Corp., Madison, WI) specific for human TGFβ. Immediately prior to assay, samples were diluted 1:10 in DMEM. The 1:10 dilution was further diluted by adding four volumes of PBS. The media were acidified by the addition of 5μl 1M HCl for fifteen minutes to activate latent TGFβ (LTGFβ), followed by neutralization with 5μl 1M NaOH. The assay was conducted by placing the treated samples in microtiter plates coated with monoclonal antibody to active TGFβ for 90 minutes. The unbound proteins were removed and the wells incubated with a polyclonal rabbit anti-TGFβ antibody for two hours, washed, and then incubated with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG antibody for two hours. This step was followed by a wash and color development. Color development was stopped by the addition of 1M phosphoric acid. Intensity measurements were conducted at 450nm using a BioRad Model 2550 EIA Reader (Hercules, CA). Sample concentrations were determined by comparing the absorbance value to a standard curve. We did not measure active TGFβ prior to acidification of the media because prior studies had shown that MG63 cells do not produce measurable levels of active TGFβ when grown on titanium disks (Kieswetter *et al.*, 1996). Moreover, preliminary studies showed that no active TGFβ was produced by MG63 cells cultured on these same disks when treated with $1\alpha,25\text{-}(OH)_2D_3$.

Prostaglandin E_2 (PGE_2) production. The amount of PGE_2 produced by the cells and released into the media was assessed using a commercially available competitive binding radioimmunoassay (NEN Research Products, Boston, MA). In this assay, unlabeled PGE_2 in the sample was incubated overnight with radiolabeled PGE_2 and unlabeled anti-PGE_2 antibody. Antigen-antibody complexes were separated from free antigen by precipitation with polyethylene glycol. Sample PGE_2 concentrations were determined by correlating the percentage bound over unbound counts to a standard curve (Schwartz *et al.*, 1992).

7. Statistical Management of Data

For cell culture studies, data are presented from one of two replicate experiments, both of which yielded comparable results. For any given experiment, each data point represents the mean $\pm$ SEM of six individual cultures. The data were analyzed by analysis of variance and statistical significance determined by comparing each data point to the plastic control using Bonferroni's *t* test. Bonferroni's *t* test was also used for comparisons between two different surface roughness characteristics. P values ≤ 0.05 were considered significant.

III. RESULTS

1. Surface Morphology

Scanning electron microscopy confirmed the smooth appearance of disks in the PT group (Figure 1A). The surface was characterized by the presence of smooth, undulating regions. Pitting of the surface was evident only at high magnification (data not shown). MA surfaces had a uniform appearance, with pits averaging 1-2 μm in size; grain boundaries were seldom observed (Figure 1B). CA surfaces contained pits and craters (Fig. 1C). The pits averaged 1 μm in diameter and appeared to coalesce and form large craters of 10 μm in diameter. Very little evidence of grain boundaries was observed. SB-treated surfaces contained large areas of sheets and globules; pits were frequently observed, as well as fissures and cracks (Fig. 1D). PS-treated surfaces contained large areas of sheets and globular outcroppings, as well as deep pits, frequent fissures, and cracks (Fig. 1E). Regions of irregular, sharp material (<0.1 μm) as well as large, smooth globules (10-20 μm) could be found over the entire surface. The surface was also covered by small crystallites which had a dendritic appearance. While this was representative of the PS disks used in the present study, PS-treated surfaces used in a previous study (Martin *et al.*, 1995) did not display this crystalline material (Fig. 1F).

2. Surface Roughness

PT disks were smoother than any of the other surfaces. The surface roughness values increased in the following order: MA, CA, SB, and PS, with PS being the roughest. No significant difference in roughness was observed between the CA and SB surfaces (Table 1).

3. Biochemical Analyses - Specific Aim #1

Aim #1 results pertain to the first experimental objective which was to examine the effect of surface roughness on the cellular response of MG63 osteoblast-like cells to treatment with 1,25-$(OH)_2D_3$.

A. Cell Number

Cell number decreased as surface roughness increased (Fig. 2). Although the number of cells on the two smooth surfaces, PT and MA, appeared to be less than on the tissue culture plastic, the differences were not significant. All three of the rougher surfaces, CA, SB, and PS, had significantly ($p < 0.05$) fewer numbers of cells on the surface than were seen on plastic. In addition, the number of cells on CA, SB, and PS surfaces were less than on the PT surface; and there were fewer cells on SB and PS surfaces than on the CA surface.

Treatment with either 10^{-8} or 10^{-7}M 1,25-$(OH)_2D_3$ resulted in a significant decrease in cell number on the plastic, PT, and SB surfaces. The effect of 1,25-$(OH)_2D_3$ was not dose-dependent. The number of cells on the SB surface was significantly

decreased following treatment with $1,25\text{-}(OH)_2D_3$. Significant differences were not observed for the other cultures.

B. Alkaline Phosphatase Specific Activity

There was a surface roughness-dependent increase in alkaline phosphatase activity which was significant with respect to plastic on MA, CA, SB, and PS surfaces (Fig. 3). Alkaline phosphatase specific activity on the SB and PS surface was greater than on PT and MA surfaces. $1,25\text{-}(OH)_2D_3$ caused a dose-dependent increase in alkaline phosphatase activity on all the surfaces examined, including plastic. Further, cells grown on rougher surfaces produced a greater relative increase in enzyme activity than did cells on smoother surfaces. When stimulated with 10^{-7} M $1,25\text{-}(OH)_2D_3$, cultures grown on the PT surface showed a 2.1-fold increase compared to untreated control, while those on the PS surface had a 4.2-fold increase.

C. Osteocalcin Production

Osteocalcin production on the SB and PS surfaces was greater than on plastic or the smoother Ti surfaces (Fig. 4). $1,25\text{-}(OH)_2D_3$ (10^{-7}M) increased osteocalcin production on all surfaces. Moreover, cultures on rougher surfaces (CA, SB, PS) displayed a greater relative increase in osteocalcin production than did those on smoother surfaces. This synergistic increase was greatest on the SB and PS surfaces. The addition of 10^{-8} M $1,25\text{-}(OH)_2D_3$ also caused an increase in osteocalcin production, but the increase was only significant on the PT and plastic surfaces.

D. LTGFβ Production

The production of LTGFβ also increased with surface roughness (Fig. 5). Production of this growth factor was significantly greater on MA, CA, SB, and PS surfaces than on the smoother plastic and PT surfaces; LTGFβ production by cultures on the SB and PS disks was greater than those on MA and CA disks, but the differences were only significant between cultures on SB and PS disks compared with those on MA disks.

Treatment with 10^{-8} or 10^{-7} M 1,25-$(OH)_2D_3$ did not affect LTGFβ production on either the plastic or PT surfaces. However, on MA, CA, SB, and PS surfaces, addition of 10^{-7} M 1,25-$(OH)_2D_3$ significantly increased LTGFβ production. On the PS surface, the effect of 1,25-$(OH)_2D_3$ was dose-dependent, with significant increases being observed in cultures treated with 10^{-8} M hormone. As surface roughness increased, the effect of 1,25-$(OH)_2D_3$ became more pronounced. On MA surfaces, 10^{-7} M 1,25-$(OH)_2D_3$ caused a 1.5-fold increased in LTGFβ production, whereas on the PS surface, there was a 3.1-fold increase. The synergistic effect of surface roughness and hormone treatment was seen on all the rough surfaces (MA, CA, SB, PS) when 10^{-7} M 1,25-$(OH)_2D_3$ was used.

E. PGE_2 Production

PGE_2 production was greater in cultures grown on the MA, CA, SB, and PS surfaces, than those cultures grown on the plastic or PT surfaces (Fig. 6). Treatment with 1,25-$(OH)_2D_3$ had no effect on cultures grown on plastic or PT surfaces. However, 1,25-$(OH)_2D_3$ stimulated PGE_2 production on all other surfaces. For cells cultured on MA and CA, only the highest concentration of hormone was effective. On the SB and PS surfaces,

the effects of 1,25-$(OH)_2D_3$ were dose-dependent, being significant at 10^{-8} M and 10^{-7} M hormone. The increase in PGE_2 production in response to 1,25-$(OH)_2D_3$ was synergistic with the effect of surface roughness.

4. Biochemical Analyses - Specific Aim #2

Aim #2 results pertain to the second experimental objective which was to examine whether the combined effect of surface roughness and 1,25-$(OH)_2D_3$ on MG63 cell response is mediated by PGE_2 produced by the same cells.

A. Cell Number

Cell number in the control cultures decreased as surface roughness increased (Figs. 7 and 8). Although the number of cells on the two smooth Ti surfaces, PT and MA, appeared to be less than on the tissue culture plastic, the differences were not significant. All three of the rougher surfaces, CA, SB, and PS, had significantly ($p < 0.05$) fewer numbers of cells on the surface than were seen on plastic. In addition, the number of cells on CA, SB, and PS surfaces were less than on the PT surface; and there were fewer cells on SB and PS surfaces than on the CA surface.

Treatment with 1,25-$(OH)_2D_3$ for 24 hours after the cells reached confluence on plastic resulted in further decreases in cell number for all treatment groups (Fig. 7). Significant decreases were observed for cultures grown on the rougher surfaces CA, SB, and PS as compared to cultures grown on plastic in the presence of the hormone. Treatment of the cultures for 24 hours with a combination of 1,25-$(OH)_2D_3$ and

indomethacin after the cells had reached confluence on plasic abolished the observed decrease in cell number with 1,25-$(OH)_2D_3$ alone.

Treatment with indomethacin alone for the last 24 hours of the culture period had no significant effect on cells cultured on any of the surfaces (Fig. 8). However, when the cultures were treated with indomethacin throughout the culture period, the effect of surface roughness was abolished.

B. Alkaline Phosphatase Specific Activity

There was a surface roughness-dependent increase in alkaline phosphatase specific activity which was significant with respect to plastic on CA, SB, and PS surfaces (Figs. 9 and 10). Alkaline phosphatase specific activity on the SB and PS surfaces was greater than on PT and MA surfaces.

Treatment with 1,25-$(OH)_2D_3$ for 24 hours after the cells reached confluence on plastic resulted in a significant increase in alkaline phosphatase specific activity for all cultures when compared to their respective controls (Fig. 9). Cultures grown on the rougher surfaces CA, SB, and PS displayed significantly greater activity than that observed on plastic. Treatment of the cultures for 24 hours with a combination of 1,25-$(OH)_2D_3$ and indomethacin after the cells had reached confluence on plastic was without effect compared with 1,25-$(OH)_2D_3$ treatment alone.

Treatment with indomethacin alone for the last 24 hours of the culture period resulted in no significant effect for all groups (Fig. 10). However, when the cultures were

treated with indomethacin throughout the culture period, the effect of surface roughness was abolished for all treatment groups.

C. Osteocalcin Production

There was a surface roughness-dependent increase in osteocalcin production which was significant with respect to plastic on the CA, SB, and PS surfaces (Figs. 11 and 12). Osteocalcin production on the CA, SB, and PS surfaces was greater than on PT and MA surfaces.

Treatment with 1,25-$(OH)_2D_3$ for 24 hours after the cultures had reached confluence on plastic resulted in a significant increase in osteocalcin production for all cultures (Fig. 11). However, a synergistic increase was observed on the roughest surface, PS. Cultures grown on the rougher surfaces (CA, SB, and PS) produced significantly more osteocalcin than the cultures grown on plastic. Treatment with the combination of 1,25-$(OH)_2D_3$ and indomethacin after the cells had reached confluence on plastic abolished the increase observed with 1,25-$(OH)_2D_3$ alone for all groups.

Treatment with indomethacin alone for the last 24 hours of the culture period had no significant effect on any of the cultures compared with the untreated control (Fig. 12). However, when the cultures were treated with indomethacin throughout the culture period, the effect of surface roughness was abolished for all treatment groups.

D. Latent TGFβ Production

Latent TGFβ production on the MA, CA, SB, and PS surfaces was significantly greater than on the plastic control surface (Figs. 13 and 14).

Treatment with 1,25-$(OH)_2D_3$ for 24 hours after the cells reached confluence on plastic resulted in a significant increase in latent TGFβ production for those cultures grown on the MA, CA, SB, and PS surfaces (Fig. 13). A synergistic increase was noted on the PS surface. Treatment with a combination of 1,25-$(OH)_2D_3$ and indomethacin abolished the increased effect for all groups except plastic.

Treatment with indomethacin alone for the last 24 hours of the culture period had no significant effect on any of the cultures compared with the untreated control (Fig. 14). However, when the cultures were treated with indomethacin throughout the culture period, the effect of surface roughness was abolished for all treatment groups.

Figure 1.

Scanning electron micrographs of the different Ti surfaces used in this study. (A) PT, (B) MA, (C) CA, (D) SB, (E) PS, with the crystalline layer, used in the present study, (F) PS, without crystalline layer, used in a previous study (Martin *et al.*, 1995). Bar = 10 μM; magnification x 1000.

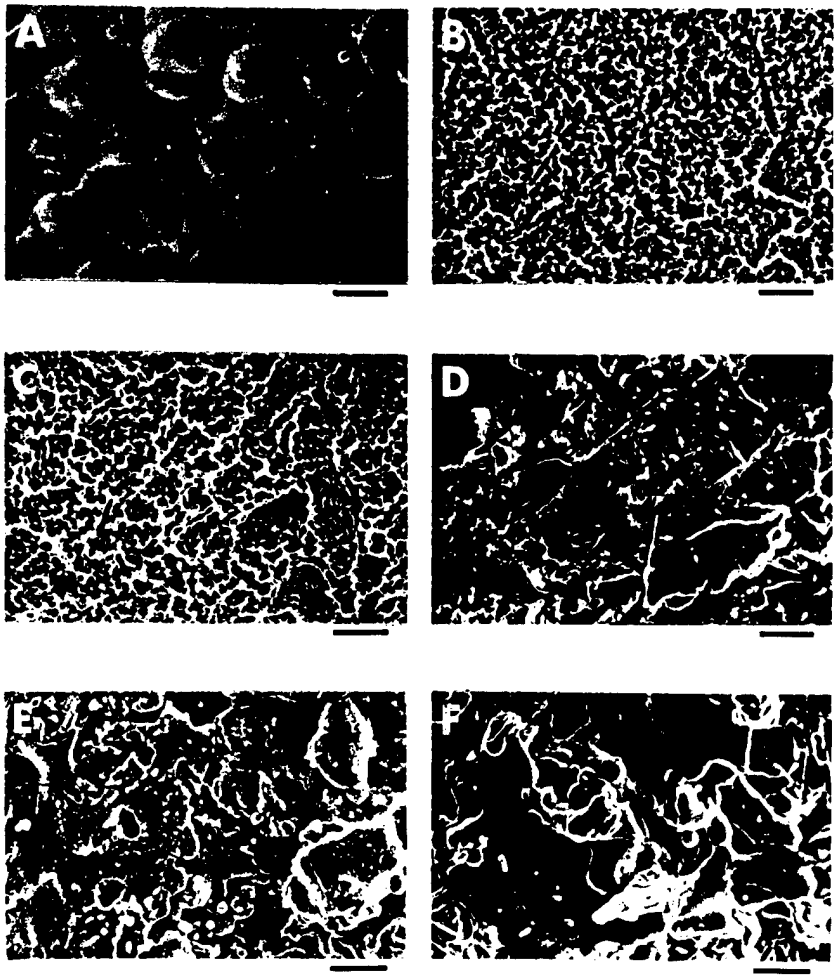

A
B
C
D
E
F

Table 1.

Average Surface Roughness Values for the Ti Disks

Surface	R_a Value
PT	$0.60 \pm 0.02^{*}$
MA	$0.87 \pm 0.03^{*}$
CA	$3.68 \pm 0.12^{\#}$
SB	$3.90 \pm 0.09^{\#}$
PS	$6.81 \pm 0.20^{@}$

The surface roughness of the different groups of disks was determined by profilometry. All R_a values are expressed as the mean ± SEM in μm for n=6. $^{*}P < 0.05$, vs. CA, SB, or PS; $^{\#}p < 0.05$, CA or SB vs. PT, MA, or PS; $^{@}p < 0.05$, vs. all other surfaces.

Figure 2.

Number of MG63 cells released from plastic or Ti disks by trypsinization. MG63 cells were cultured to confluence on plastic or Ti disks and then treated for 24 hours with 10^{-8}M or 10^{-7}M 1α,25-$(OH)_2D_3$ or vehicle. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic. $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

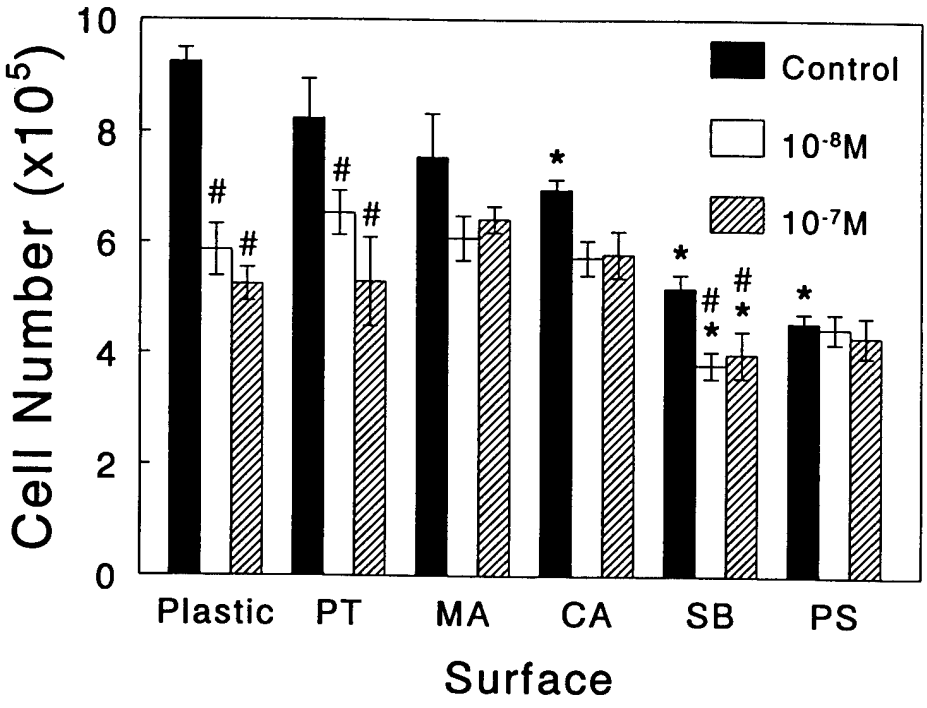

Cell Number (x10^5)
10
8
6
4
2
0
Control
10^{-8}M
10^{-7}M
#
*
Plastic
PT
MA
CA
SB
PS
Surface

Figure 3.

Alkaline phosphatase specific activity of MG63 cell layers after culture on plastic or Ti disks of varying roughness. After cells reached confluence on plastic, cultures were treated for 24 hours with 10^{-8}M or 10^{-7}M 1α,25-$(OH)_2D_3$ or vehicle. At harvest, the cell layer was released by scraping and then assayed for enzyme activity. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

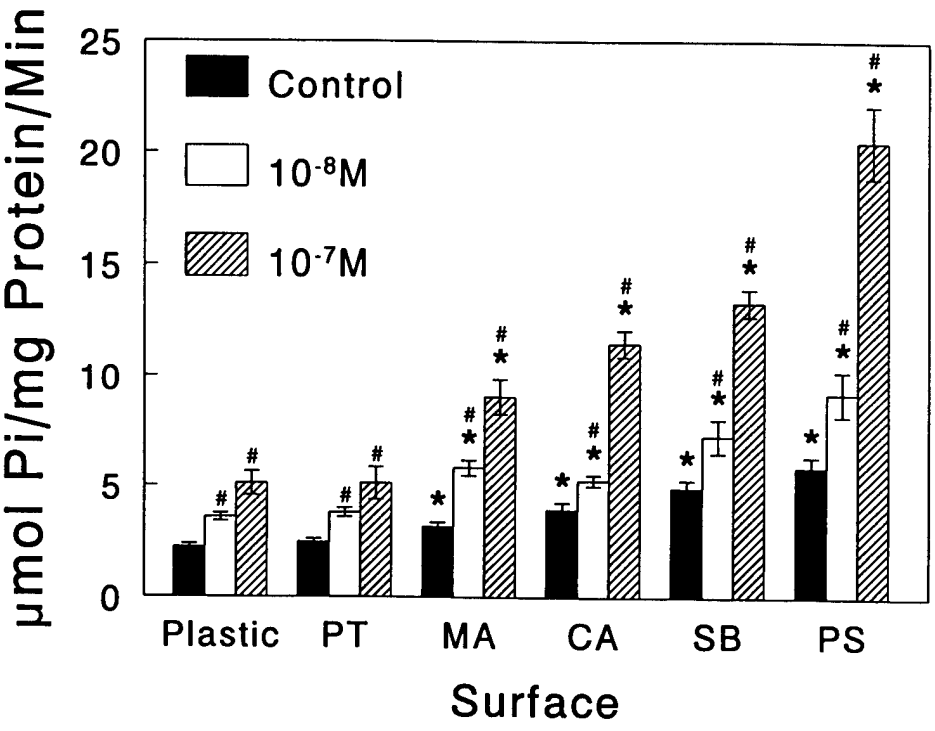

µmol Pi/mg Protein/Min
25
20
15
10
5
0
Control
10-8M
10-7M
Plastic
PT
MA
CA
SB
PS
Surface

Figure 4.

Osteocalcin production by MG63 cells during culture on plastic or Ti disks of varying roughness. After cells reached confluence on plastic, cultures were treated for 24 hours with 10^{-8}M or 10^{-7}M 1α,25-$(OH)_2D_3$ or vehicle, the media collected, and osteocalcin content measured by RIA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

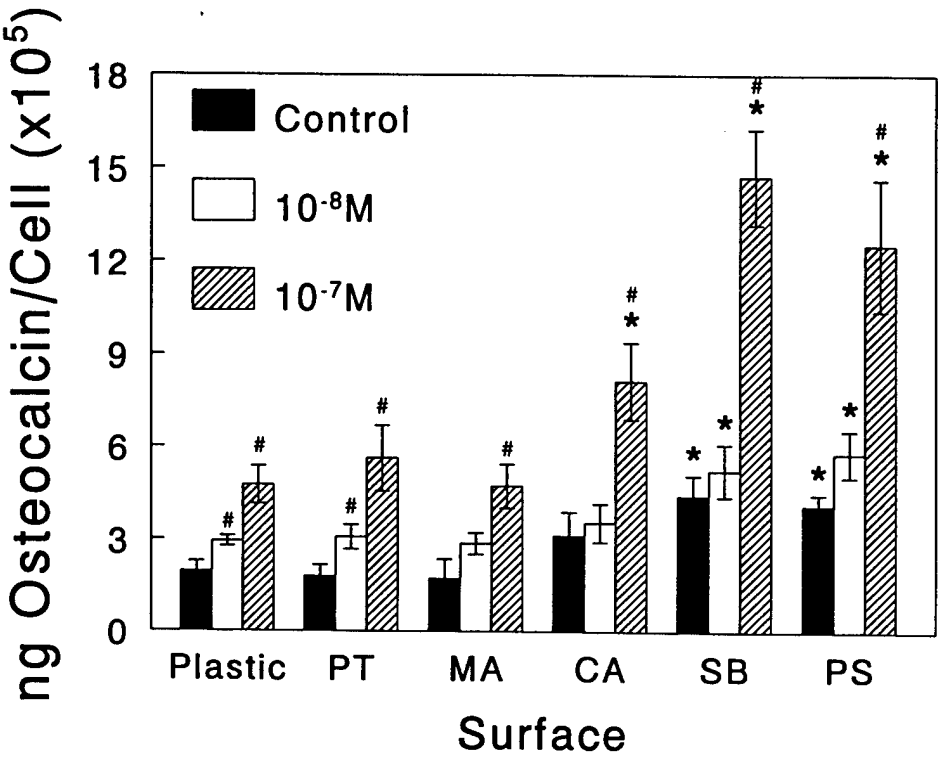

ng Osteocalcin/Cell (x10^5)
18
15
12
9
6
3
0
Control
10^{-8}M
10^{-7}M
Plastic
PT
MA
CA
SB
PS
Surface

Figure 5.

Latent TGFβ production by MG63 cells during culture on plastic or Ti disks of varying roughness. After cells reached confluence on plastic, cultures were treated for 24 hours with 10^{-8}M or 10^{-7}M 1α,25-$(OH)_2D_3$ or vehicle, the media collected, and TGFβ content measured by ELISA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

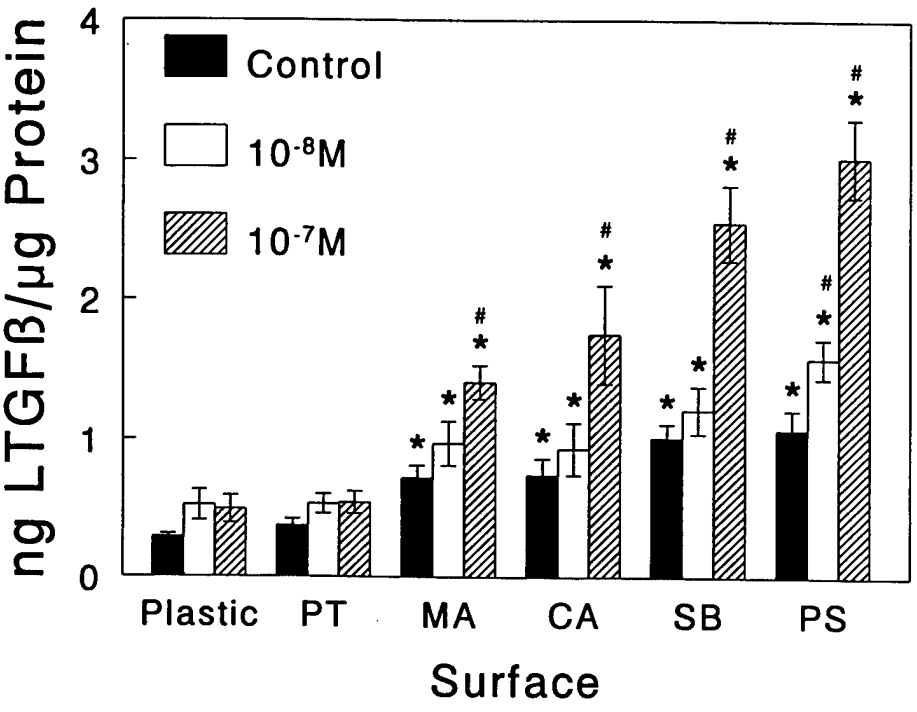

ng LTGFß/µg Protein
4
3
2
1
0
Control
10-8M
10-7M
Plastic
PT
MA
CA
SB
PS
Surface

Figure 6.

PGE_2 production by MG63 cells during culture on plastic or Ti disks of varying roughness. After cells reached confluence on plastic, cultures were treated for 24 hours with 10^{-8}M or 10^{-7}M $1\alpha,25\text{-}(OH)_2D_3$ or vehicle, the media collected, and PGE_2 content measured by RIA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disks vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

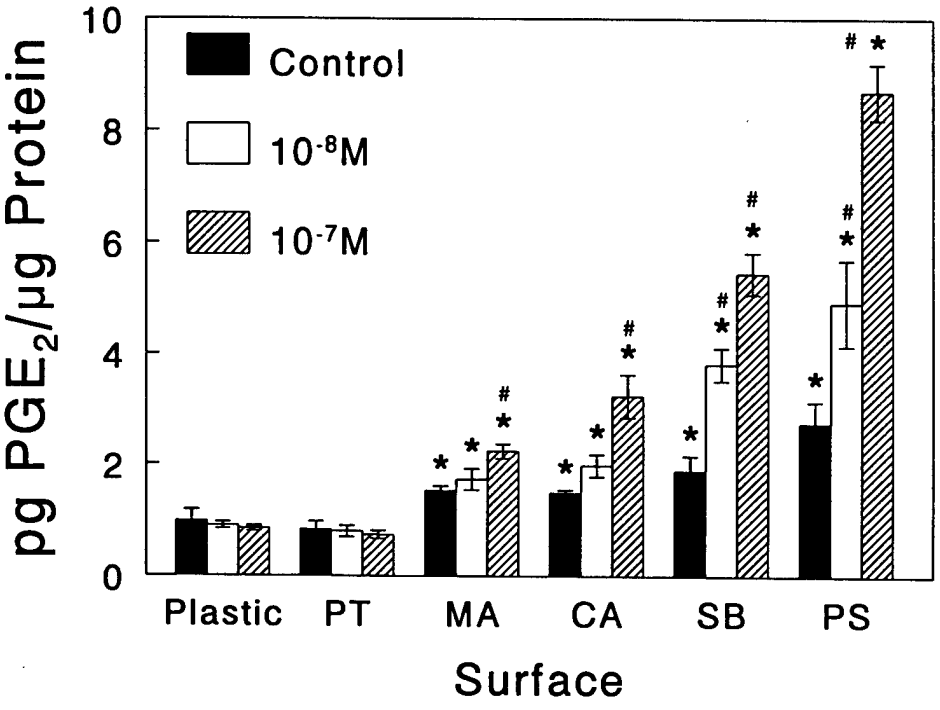

pg PGE2/µg Protein
10
8
6
4
2
0
Control
10-8M
10-7M
Plastic
PT
MA
CA
SB
PS
Surface

Figure 7.

Number of MG63 cells released from plastic or Ti disks by trypsinization. Confluent cultures were treated for 24 hours with 10^{-7}M 1α,25-$(OH)_2D_3$ or 10^{-7}M 1α,25-$(OH)_2D_3$ plus 10^{-7}M indomethacin (1,25 + IND). Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic. $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

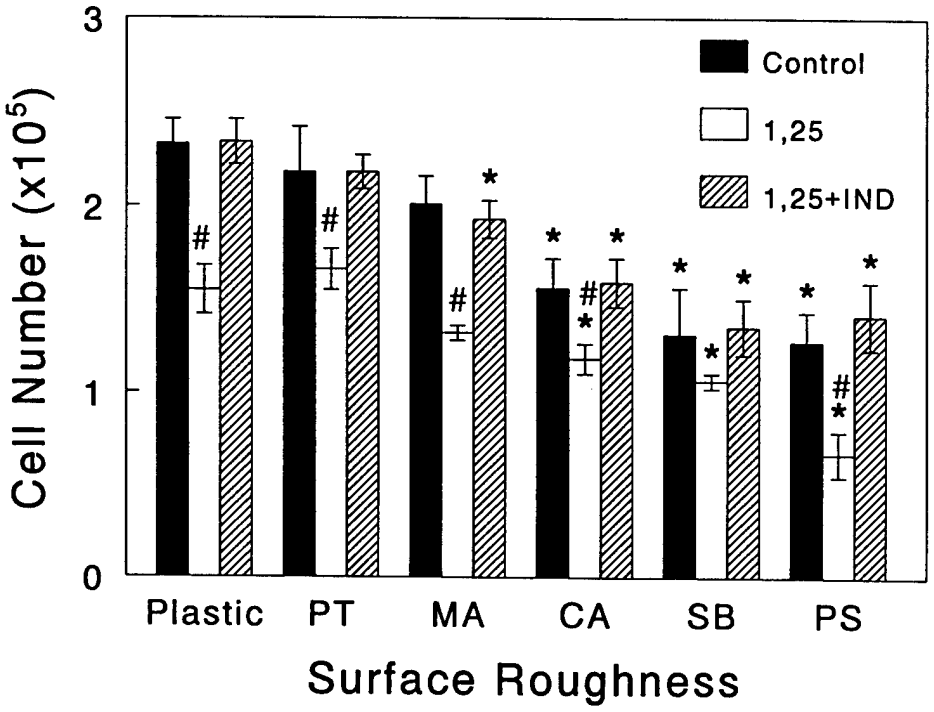

Cell Number (x10^5)
3
2
1
0
Control
1,25
1,25+IND
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 8.

Number of MG63 cells released from plastic or Ti disks by trypsinization. Cultures were treated with indomethacin (10^{-7}M) during the entire culture period or for 24 hours after the cells reached confluence. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic. $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

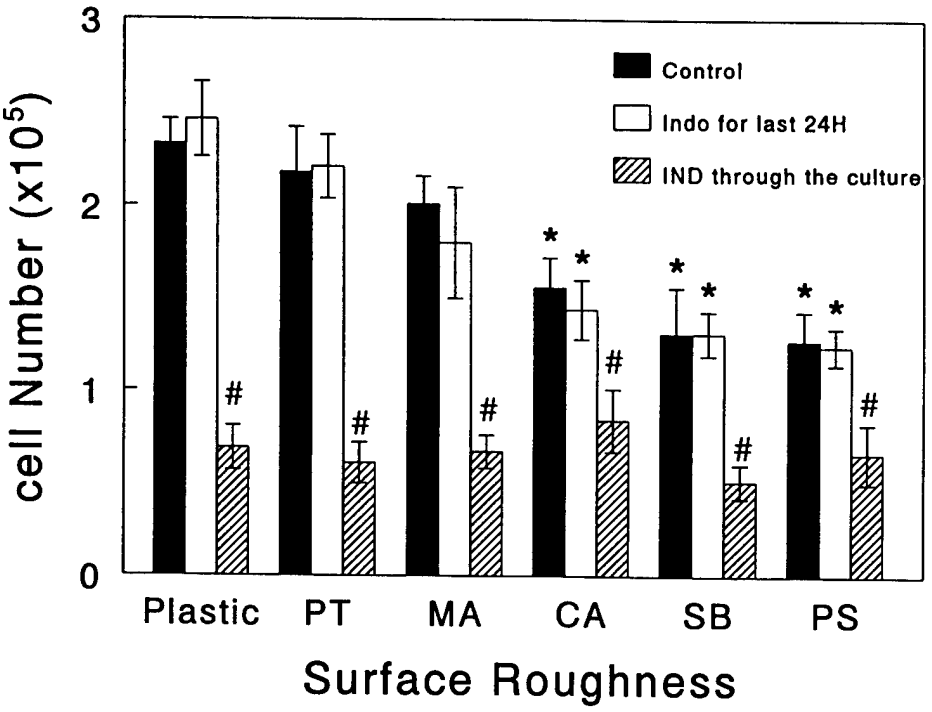

3
2
1
0
cell Number (x10^5)
Control
Indo for last 24H
IND through the culture
*
#
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 9.

Alkaline phosphatase specific activity of MG63 cell layers after culture on plastic or Ti disks of varying roughness. Confluent cultures were treated for 24 hours with 10^{-7}M 1α,25-$(OH)_2D_3$ or 10^{-7}M 1α,25-$(OH)_2D_3$ plus 10^{-7}M indomethacin (1,25 + IND). At harvest, the cell layer was released by scraping and then assayed for enzyme activity. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

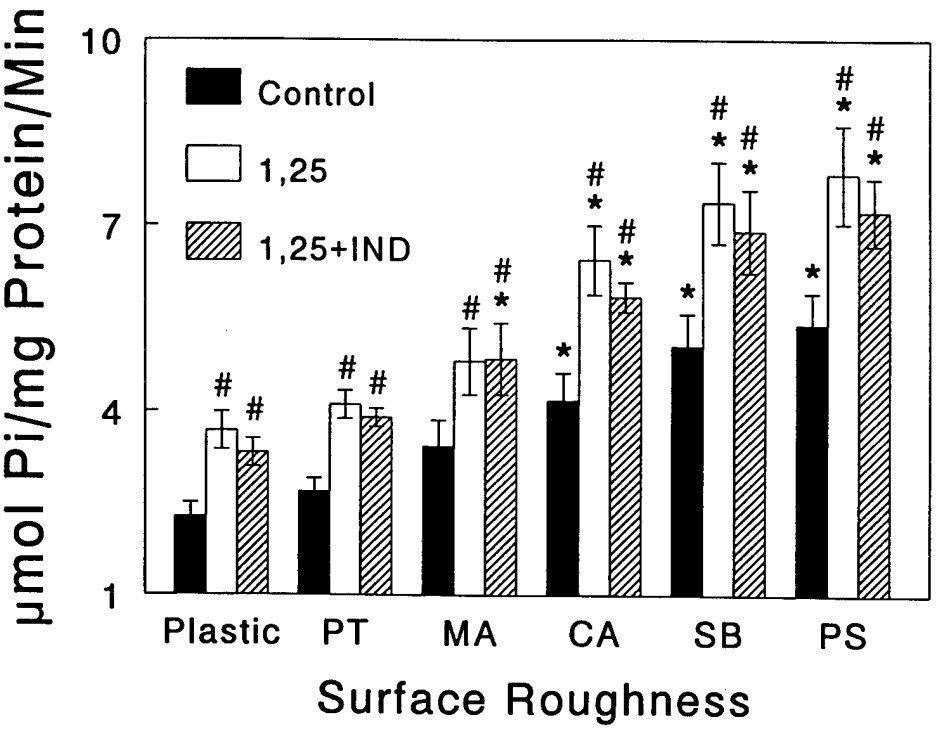

µmol Pi/mg Protein/Min
10
7
4
1
Control
1,25
1,25+IND
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 10.

Alkaline phosphatase specific activity of MG63 cell layers after culture on plastic or Ti disks of varying roughness. Cultures were treated with indomethacin (10^{-7}M) during the entire culture period or for 24 hours after the cells reached confluence. At harvest, the cell layer was released by scraping and then assayed for enzyme activity. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

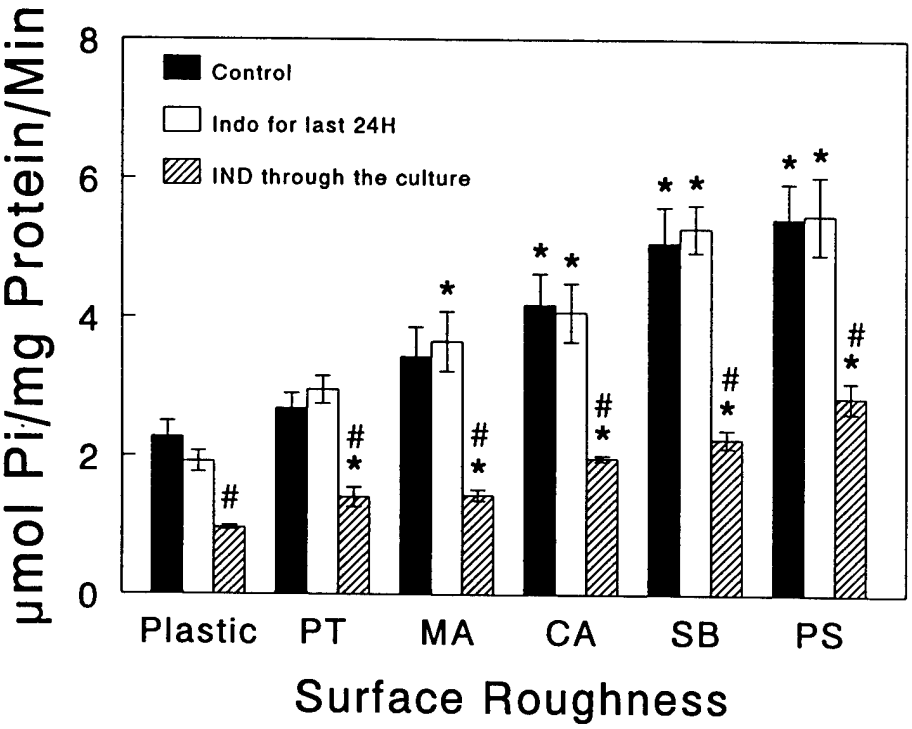

µmol Pi/mg Protein/Min
8
6
4
2
0
Control
Indo for last 24H
IND through the culture
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 11.

Osteocalcin production by MG63 cells during culture on plastic or Ti disks of varying roughness. Confluent cultures were treated for 24 hours with 10^{-7}M 1α,25-$(OH)_2D_3$ or 10^{-7}M 1α,25-$(OH)_2D_3$ plus 10^{-7}M indomethacin (1,25 + IND) and osteocalcin content measured by RIA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *P < 0.05, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

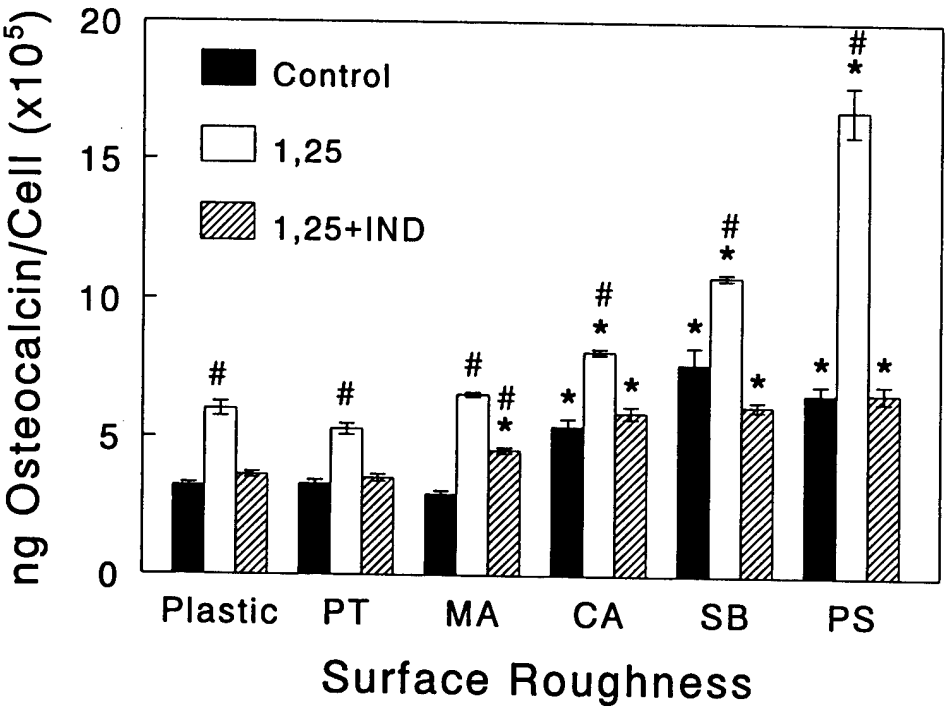

ng Osteocalcin/Cell (x10^5)
20
15
10
5
0
Control
1,25
1,25+IND
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 12.

Osteocalcin production by MG63 cells during culture on plastic or Ti disks of varying roughness. Cultures were treated with indomethacin (10^{-7}M) during the entire culture period or for 24 hours after the cells reached confluence and osteocalcin content measured by RIA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

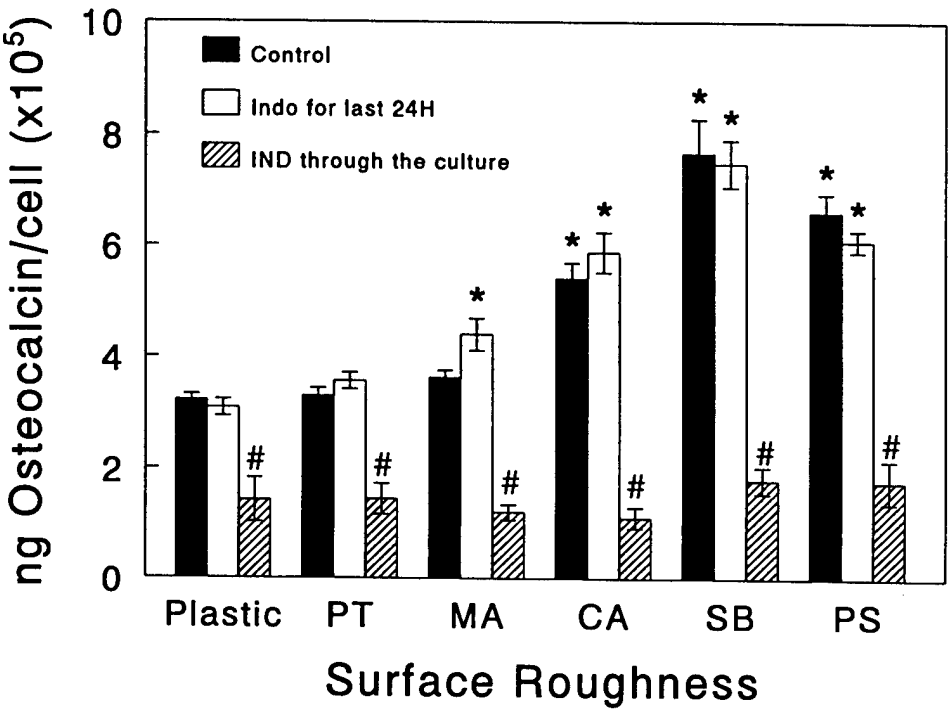

ng Osteocalcin/cell (x10^5)
10
8
6
4
2
0
Control
Indo for last 24H
IND through the culture
*
#
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 13.

Latent TGFβ production by MG63 cells during culture on plastic or Ti disks of varying roughness. Confluent cultures were treated for 24 hours with 10^{-7}M 1α,25-$(OH)_2D_3$ or 10^{-7}M 1α,25-$(OH)_2D_3$ plus 10^{-7}M indomethacin (1,25 + IND) and TGFβ content measured by ELISA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.

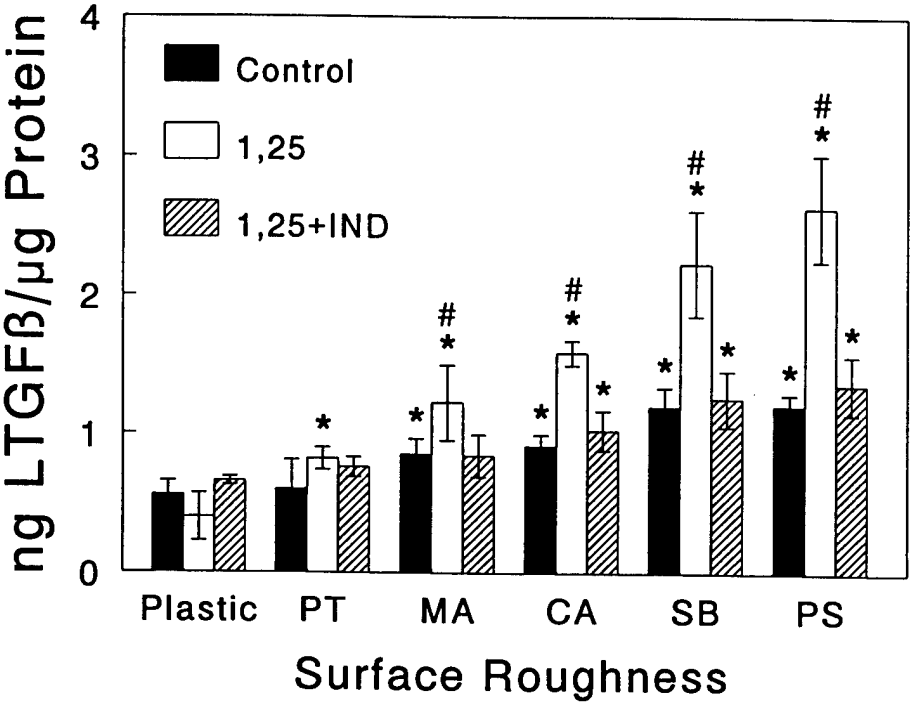
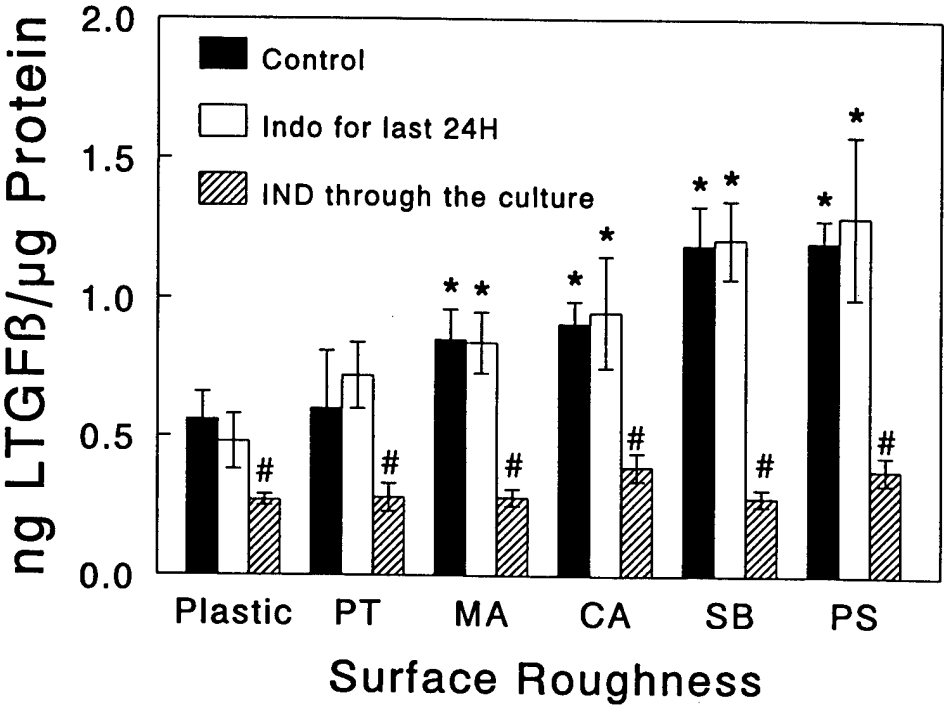

ng LTGFß/µg Protein
4
3
2
1
0
Control
1,25
1,25+IND
Plastic
PT
MA
CA
SB
PS
Surface Roughness

Figure 14.

Latent TGFβ production by MG63 cells during culture on plastic or Ti disks of varying roughness. Cultures were treated with indomethacin (10^{-7}M) during the entire culture period or for 24 hours after the cells reached confluence and TGFβ content measured by ELISA. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments. *$P < 0.05$, Ti disk vs. plastic; $^{\#}p < 0.05$, treated vs. untreated control for a particular surface.


ng LTGFß/µg Protein
2.0
1.5
1.0
0.5
0.0
Control
Indo for last 24H
IND through the culture
Plastic
PT
MA
CA
SB
PS
Surface Roughness

IV. DISCUSSION AND SUMMARY

This study confirmed previous observations that surface roughness can modulate the phenotypic expression of osteoblast-like MG63 cells (Martin *et al.*, 1995; Kieswetter *et al.*, 1996). In general, cells cultured on rougher surfaces tended to exhibit attributes of more differentiated or mature osteoblasts than did those cells cultured on smoother surfaces for comparable periods of time, including reduced cell numbers and increased alkaline phosphatase specific activity. These results are in agreement with the hypothesis of Lian and Stein (1992) regarding osteoblast differentiation. In addition, MG63 cells cultured on the roughest surfaces produced increased levels of osteocalcin, a non-collagenous calcium-binding protein shown to be maximally expressed with mineralization of the extracellular matrix *in vivo* (Hauschka *et al.*, 1989) and *in vitro* (Owen *et al.*, 1990; Aronow *et al.*, 1990) and, therefore, used as a marker of the mature osteoblast.

The enhanced expression of a differentiated phenotype in response to surface roughness has also been demonstrated by Groessner-Schreiber and Tuan (1992), who showed that rough titanium surfaces increased alkaline phosphatase specific activity and calcification in cultures of embryonic chick osteoblasts. This supports the contention that the response of MG63 cells to the materials used in this study is not due to the transformed nature of the osteoblast-like osteosarcoma cell but is a general characteristic of osteoblast cultures. Morphology of the MG63 cells varies considerably as surface roughness increases (Martin *et al.*, 1995) suggesting that changes in the nature of the

focal adhesions used to anchor the cell to its substrate may play a role (Bidanset *et al.*, 1992).

Also, as noted previously (Kieswetter *et al.*, 1996), surface roughness modulated the ability of MG63 cells to synthesize and secrete autocrine and paracrine mediators, such as LTGFβ and PGE_2. As surface roughness increased, production of these factors was stimulated. This indicates that surface roughness has a direct effect on cell activity, and may have an indirect effect mediated through local factor production.

This study demonstrates for the first time that surface roughness-dependent changes in MG63 cells can modify how the cells respond to extrinsic regulatory agents, such as 1,25-$(OH)_2D_3$. When grown under normal culture conditions, MG63 cells respond to 1,25-$(OH)_2D_3$ with decreased cell proliferation (Bonewald *et al.*, 1992a) and increased alkaline phosphatase specific activity (Boyan *et al.*, 1989) and osteocalcin production (Bonewald *et al.*, 1992a). Cells cultured on the cpTi disks also exhibited 1,25-$(OH)_2D_3$-dependent changes typical of osteoblasts but, on the roughest surfaces, the response to the vitamin D metabolite was markedly enhanced; indeed, it was synergistic with the effect of surface roughness alone. One possibility is that growth on the rougher surfaces resulted in a more differentiated cellular phenotype, with a corresponding change in the response to 1,25-$(OH)_2D_3$. In fact, several investigators have shown that the effect of 1,25-$(OH)_2D_3$ on osteoblasts is directly related to the state of differentiation of the cell (Bonewald *et al.*, 1992a; Majeska and Rodan, 1982).

It is also possible that 1,25-$(OH)_2D_3$ altered the response of the MG63 cells to surface roughness by promoting the differentiation of the cells into a more mature osteoblastic phenotype. Treatment with the vitamin D metabolite resulted in increases in

osteocalcin production on all surfaces, showing that osteoblastic differentiation did occur. Studies in our lab using costochondral cartilage cells as a model have shown clear differences in cellular response to surface roughness as a direct consequence of chondrocyte maturation (Schwartz *et al.*, 1996).

Local factor production was also sensitive to the synergistic effects of surface roughness and 1,25-$(OH)_2D_3$ treatment. The production of PGE_2 and LTGFβ were both increased on the rougher surfaces with the greatest effects being observed in cells cultured on the roughest SB and PS disks. It is likely that surface roughness, and not 1,25-$(OH)_2D_3$, was the primary factor sensitizing the cell. Although production of both factors was enhanced by surface roughness alone, increased LTGFβ production was observed on the CA and SB disks only when the highest concentration of hormone was used. Moreover, 1,25-$(OH)_2D_3$ had no effect on PGE_2 production by MG63 cells cultured on plastic or PT surfaces indicating that at least for this aspect of cell function, the hormone alone did not alter phenotypic expression.

It is possible, however, that surface-dependent changes in local factor production played a role in the synergistic response. Under the experimental conditions used in this study the MG63 cells produced LTGFβ rather than the active form of the growth factor, which may account for our failure to observe the TGFβ-dependent inhibition of 1,25-$(OH)_2D_3$ stimulated osteocalcin production previously noted in MG63 cells cultured on plastic (Bonewald *et al.*, 1992a). The interactions between PGE_2 and other hormones have been postulated to play a role in the differentiation of osteoblasts (Raisz and Fall, 1990), and PGE_2 has been proposed to be a second messenger, mediating the effects of other regulatory agents (Schwartz *et al.*, 1992a and b). The autocrine/paracrine actions of

PGE_2 on osteoblasts may stimulate production of cytokines, such as insulin-like growth factor, associated with cellular proliferation and differentiation (McCarthy *et al.*, 1991).

Prostaglandins are known to be among the primary mediators of inflammation. Although its exact mode of action is unknown, indomethacin is known to be a potent inhibitor of prostaglandin synthesis. Indomethacin was used in the present study in various culture situations and at various timepoints to effectively block the production of PGE_2 by MG63 cells. Thus, the effect of PGE_2 produced by MG63 cells on mediating the interactive effect of surface roughness on response of MG63 cells to 1,25-$(OH)_2D_3$ could be assessed. When indomethacin was present throughout the entire culture period, the effect of surface roughness on all parameters was abolished. However, the addition of indomethacin to confluent cultures for the last 24 hours prior to harvest appeared to have little effect. These results suggest that the timing of non-steroidal anti-inflammatory agent administration and the resulting level of PGE_2 production may be important factors in the response of osteoblast-like cells to surface roughness.

The synergistic effect of surface roughness and the hormone 1,25-$(OH)_2D_3$ on cell number and LTGFβ and osteocalcin production was abolished when indomethacin was added to the cultures with 1,25. However, this was not observed for alkaline phosphatase specific activity. These results indicate that the synergistic cell response produced by surface roughness and 1,25-$(OH)_2D_3$ is mediated, at least in part, by PGE_2 produced by the same cells. Furthermore, the synergistic increase in alkaline phosphatase appears to be mediated by a different mechanism. Although high levels of PGE_2 are known to produce significant inflammation and stimulate bone resorption, the current study indicates that a

physiologic amount of PGE_2 may play a crucial role in mediating the beneficial effects of a specific material surface and a healthy endocrine system.

Future studies

While earlier studies in this laboratory used cpTi disks of comparable R_a values to those used here (Martin *et al.*, 1995; Kieswetter *et al.*, 1996), not all of the surface treatments were identical to those used in the present study. Except for those cells cultured on PS surfaces, the nature of the manufacturing technique did not alter the cellular response to surface topology or topography from prior reports. In contrast, the PS disks used here had titanium oxide crystallites on the surface, whereas the Ti plasma-sprayed disks (TPS) used previously did not (see figure 1E, F). The PS surfaces supported increased alkaline phosphatase specific activity while the TPS disks caused a significant inhibition, demonstrating the importance of surface characteristics other than surface roughness alone on phenotypic expression, as has been shown previously (Hambleton *et al.*, 1994). These results indicate that crystallinity may play a role in cell response to a material surface. Hanein *et al.* (1993) reported major differences in the adhesive response of epithelial cells to different crystallographic structures even though these structures were chemically identical. Research correlating crystallinity to cell response is relatively sparse. A recent study by Ong *et al.* (1995) evaluated the cell responses of rat bone marrow cells to well-characterized calcium phosphate coatings and titanium surfaces *in vitro*. No significant differences in protein production were observed. The necessity for further examination of the effect of crystallinity on cell response is apparent.

In the present study, differences in surface roughness were achieved using a variety of techniques, including the use of corundum, potentially resulting in the entrapment of aluminum oxide, and acid etching, potentially resulting in Ti hydride formation. Therefore, it is possible that some of the differences attributed to surface roughness may be due to differences in surface chemistry, further demonstrating the complexity of the issues which may contribute to cell and tissue response to biomaterials. Future study comparing cell response on machined titanium surfaces to response on surfaces which underwent acid-etching, as in the present study, would certainly appear to be beneficial.

Summary

While the precise role of material surface properties in inducing specific cell responses remains to be elucidated, this study suggests that bone cell response to systemic hormones such as 1,25-$(OH)_2D_3$ is modified by surface roughness. Furthermore, it appears that 1,25-$(OH)_2D_3$ increases the responsiveness of MG63 osteoblast-like cells to surface roughness. This effect is mediated, at least in part, by PGE_2 produced by the same cells. It has been demonstrated that PGE_2 directly affects cellular differentiation of MG63 cells on material surfaces. These results suggest that the endocrine system is actively involved in normal healing around implants.

Regardless of the mechanisms involved in the present study, the results clearly demonstrate that titanium surface roughness and the presence of the systemic hormone, 1,25-$(OH)_2D_3$, can affect the production by osteoblast-like cells of local factors involved in bone formation, suggesting that the complement of autocrine and paracrine mediators produced by cells at the bone-biomaterial interface can be directed mechanically and/or

physiologically. The clinical significance of PGE_2 mediation is that perhaps the dosage and/or timing of the non-steroidal anti-inflammatory medications prescribed for patients postoperatively may ultimately contribute to the type of interface that results at the implant site.

Optimal tissue healing and subsequent osseointegration of implants is paramount for the success and long-term function of orthopaedic and dental implants. This research has provided new insight into how surface properties, systemic hormones, and local factors interact to regulate cell behavior during healing. This study, in conjunction with continued research in this area will hopefully aid in the identification and development of an implant surface whose surface properties will maximize the potential for clinical success.

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VITA

Richard C. Batzer was born on July 4, 1958, to Richard Charles and Joan Clark Batzer in Lancaster, Pennsylvania. Following graduation from Manheim Township High School in Lancaster, Pennsylvania, he attended the University of Pittsburgh in Pittsburgh, Pennsylvania where he earned a Bachelor of Science degree in 1980, graduating summa cum laude. He was named a University Scholar in 1979 and 1980. Dr. Batzer received his Doctor of Dental Surgery degree from the University of Maryland in 1984. During dental school, he was elected to the Omicron Kappa Upsilon Honorary Dental Society and graduated magna cum laude. Following his dental training, Dr. Batzer completed a general practice residency at Geisinger Medical Center in Danville, Pennsylvania. From 1985-1990, he was an associate dentist in a private general dentistry practice in Elizabethtown, Pennsylvania. Dr. Batzer was commissioned as a captain in the United States Air Force in 1990 and since has been promoted to the rank of major. After a tour at Plattsburgh Air Force Base in Plattsburgh, New York, he began prosthodontic residency training at Wilford Hall Medical Center at Lackland Air Force Base in San Antonio, Texas. He anticipates a graduation date of June 1998. Dr. Batzer has been married to his wife Kay for 14 years and together they have a daughter Ashley, 11, and two sons, Brenton, 7, and Sean, 6. Upon completion of his training, he anticipates continuing his career and service with the United States Air Force.