ARACHIDONIC ACID AND PROSTAGLANDIN E_2 INFLUENCE HUMAN OSTEOBLAST (MG63) RESPONSE TO TITANIUM SURFACE ROUGHNESS

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Prior studies have shown that implant surface roughness affects osteoblast proliferation, differentiation, matrix synthesis, and local factor production. Further, cell response is modulated by systemic factors, such as $1,25(OH)_2D_3$ and estrogen as well as mechanical forces. Based on the fact that peri-implant bone healing occurs in a site containing elevated amounts of prostaglandin E_2 (PGE_2), the hypothesis of the current study is that PGE_2 and arachidonic acid (AA), the substrate used by cyclooxygenase to form PGE_2, influence osteoblast response to implant surface roughness. To test this hypothesis, 4 different types of commercially pure titanium (cpTi) disks with surfaces of varying roughness (smooth Ti, R_a 0.30 μm; smooth and acid etched Ti [SAE Ti], R_a 0.40 μm; rough Ti, R_a 4.3 μm; rough and acid etched Ti [RAE Ti], R_a 4.15μm) were prepared. MG63 osteoblasts were seeded onto the surfaces, cultured to confluence, and then treated for the last 24 hours of culture with AA (0, 0.1, 1, and 10 nM), PGE_2 (0, 1, 10, 25, and 100 nM), or the general cyclooxygenase inhibitor indomethacin (0 or 100 nM). At harvest, the effect of treatment on cell proliferation was assessed by measuring cell number and [^{3}H]-thymidine incorporation, and the effect on cell differentiation was determined by measuring alkaline phosphatase (ALP) specific activity. The effect of AA and PGE_2 on cell number was somewhat variable but showed a general decrease on plastic and smooth surfaces and an increase on rough surfaces. In contrast, [^{3}H]-thymidine incorporation was uniformly decreased with treatment on all surfaces. ALP demonstrated the most prominent effect of treatment. On smooth surfaces, AA and PGE_2 dose-dependently increased ALP, while on rough surfaces, treatment dose-dependently decreased enzyme specific activity. Indomethacin treatment had either no effect or a slightly inhibitory effect on [^{3}H]-thymidine incorporation on all surfaces. In contrast, indomethacin inhibited ALP on smooth surfaces and stimulated ALP on rough. Taken together, the results indicate that both AA and PGE_2 influence osteoblast response by promoting osteoblast differentiation on smooth surfaces, while inhibiting it on rough surfaces. Because implants with rough surfaces are acknowledged to be superior to those with smooth surfaces, these results suggest that use of nonsterioidal anti-inflammatory drugs to block PGE_2 production and reduce inflammation may be beneficial in the postoperative period after implant placement. They also indicate that manipulation of the AA metabolic pathway may offer a new therapeutic approach for modulating bone healing after implant placement. Because peri-implant healing takes place in a complex cellular environment quite different from the one used in the present study, additional work will be necessary to substantiate these possibilities.

Key Words: arachidonic acid, prostaglandin E_2, titanium, implant surface, osteoblast, cell response

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INTRODUCTION

Commercially pure titanium (cpTi) and its alloys are routinely used in craniofacial and orthopedic implants because of their biocompatibility and favorable mechanical and chemical properties. Texturing of cpTi implant surfaces is an important factor in osseointegration of dental implants.[1,2] While the early Branemark titanium (Ti) dental implants had a smooth surface and required 3 to 6 months of healing before loading,[3] current dental implants rely on acid etching, sandblasting with various media, and chemical modification of the surface to enhance the speed and predictability of osseointegration.[4] A number of in vivo laboratory studies have demonstrated that Ti implants with roughened surfaces achieve improved osseointegration compared with smooth surfaces.[1,5–10] These results have been confirmed in the clinic[11–13] and point to the fact that enhancement of the Ti surface-bone interface is a vital component in early osseointegration.

A large number of in vitro studies have reported the effect of implant surface on cell behavior. Surface characteristics of Ti implants directly influence the composition and conformation of serum components that adsorb onto the surface of the implant and play a role in cell recruitment and attachment.[14,15] In bone, these cells may be mesenchymal stem cells, osteoprogenitor cells, or immature osteoblasts. Once attached, surface roughness plays a role in regulating cell proliferation, differentiation, matrix synthesis, and local factor production.[16–18] For MG63 cells, cell proliferation (cell number and [^{3}H]-thymidine incorporation) is decreased, while differentiation (alkaline phosphatase [ALP] specific activity and osteocalcin production), matrix synthesis ([^{3}H]-proline and [^{35}S]-sulfate incorporation), and local factor production (prostaglandin E_2 [PGE_2] and transforming growth factor-β [TGF-β]) are increased with increasing surface roughness. In addition, osteoblast response to circulating hormones, such as $1,25(OH)_2D_3$ and 17β-estradiol, and shear stress are influenced by surface roughness.[19–21] Most notably, the effects of $1,25(OH)_2D_3$ and, to a lesser extent, 17β-estradiol are synergistic with increasing surface roughness, suggesting that systemic hormones may be important in bone formation around implants.

Implant placement begins a wound healing process similar to that found in fracture repair.[22] At the site of trauma, blood vessels are damaged, blood flows into the wound site, and a hematoma is formed. A fibrin clot seals off the wound site and provides a framework for the infiltrating inflammatory cells, such as macrophages, neutrophils, and mast cells, which are responsible for the production of various cell mediators (IL-1, IL-6, TNF, PDGF, TGF-β). The cell mediators orchestrate the initial phases of bone repair and play a prominent role in stimulating the proliferation and migration of a number of cells, such as mesenchymal stem cells and osteoblasts, into the developing fracture callus. Metabolites of arachidonic acid (AA), such as prostaglandins, are important in this phase of healing and are produced in prodigious amounts during the first 2 weeks after injury.[23,24] Within the fracture callus, cells recruited to the wound synthesize a new mineralized extracellular matrix. Prostaglandins figure prominently in the synthesis of a new matrix, its remodeling, and subsequent rounds of bone resorption and bone formation.

The objective of the present study was to determine whether exogenous AA or PGE_2 regulate osteoblast response to implant surface roughness. The study is based on the fact that systemic hormones have been shown to influence osteoblast response to implant surface roughness and that AA metabolites, such as PGE_2, are involved in the inflammatory phase of bone healing and subsequent bone remodeling. Because many therapeutic modalities for managing preoperative and postoperative pain in patients receiving implants use nonsteroidal anti-inflammatory drugs (NSAIDs) targeting the AA pathway, the results may provide additional insight for optimizing osseointegration and clinical success.

MATERIALS AND METHODS

Titanium disk preparation and characterization

The Ti disks used for this study were prepared from 1 mm thick sheets of grade 2 unalloyed pure Ti (ASTM F67 Unalloyed titanium for surgical implant applications), obtained from Titanium Metals Corporation (Denver, Colo). These disks were fabricated to be 15 mm in diameter and fit into the well of a standard 24-well tissue culture plate. The disks were processed to produce 4 different surfaces of varying roughness.

Smooth disks were prepared by sequentially polishing the surface using 180-, 320-, 400- and 600-grit silicon carbide metallographic paper (Pace Technologies, Tucson, Ariz) until a uniformly smooth surface was achieved. To prepare the rough disks, smooth disks were sandblasted with 60grit (254 μm) white aluminum oxide particles (Duralum Special White, Washington Mills, Niagara Falls, NY). When the surface reached a uniform gray tone, the disks

were washed in sterile deionized, distilled water (dH_2O) and then ultrasonically cleaned in 70% ethanol for 10 minutes.

The surface roughness (R_a) of both smooth and rough disks was measured using a contact profilometer at a high sensitivity setting (Taylor-Hobson Surtronic 3 profilometer, Leicester, UK). Six to ten representative disks from each batch were evaluated and 4 roughness values were obtained for each disk. The smooth disks used in the current study had an R_a of 0.30 $\pm$ 0.02 μm, while the rough disks had an R_a of 4.3 $\pm$ 0.17 μm.

A subset of the smooth and rough disks was acid etched as described by Steinemann and Claes.[25] For the rough disks, this resulted in a surface similar to that found on the ITI SLA dental implant. In brief, disks were placed in a basket constructed of woven Teflon tape, submerged for 1 minute in a boiling acid solution (water, HCl [30%], and H_2SO_4 [60%] in a 10:10:80 ratio), and then immediately rinsed 5 to 6 times in a large volume of distilled water. The disks were then neutralized by immersion in 5% sodium bicarbonate and rinsed with distilled water.

After determining the R_a values (smooth acid etched [SAE] = 0.4 $\pm$ 0.07 μm; rough acid etched [RAE] = 4.1 5 $\pm$ 0.29 μm), the disks were rinsed in water, followed by ultrasonic cleaning with acetone for 10 minutes. The disks were then passivated in 40% nitric acid at room temperature for 30 minutes, rinsed with dH_2O, neutralized in 5% sodium bicarbonate solution, and ultrasonically rinsed in dH_2O for 3 five-minute periods. The disks were sterilized by autoclaving; disks were wrapped in sterile gauze, placed in sterilization pouches, and autoclaved for 30 minutes at 121°C. Before culturing, disks were placed under ultraviolet light for 24 hours. Disks were turned over once to make sure to expose both sides to the ultraviolet light. This process did not alter the morphology of the surface or the thickness of the oxide layer, and it did not introduce contaminates.[16,26] For all experiments, cells were cultured on disks placed in the well of a 24-well plate (Corning, NY). Controls consisted of cells cultured directly on the polystyrene (tissue culture plastic) surface of the 24-well plate.

Cell culture

In this study, MG63 osteoblast-like cells (American Type Culture Collection, Rockville, Md) were used. This cell line was originally isolated from a human osteosarcoma[27] and displays many traits characteristic of immature osteoblasts, including increased production of alkaline phosphatase activity and osteocalcin synthesis in response to 1,25-$(OH)_2D_3$.[27–29] Because MG63 cells exhibit enhanced osteoblastic differentiation when cultured on Ti substrates of increasing roughness,[16] they are excellent for examining the underlying mechanisms involved in the response of osteoblast-like cells to surface topography. Furthermore, MG63 cells exhibit low levels of PGE_2 production when cultured on plastic[30] or smooth Ti surfaces, but they exhibit increased PGE_2 production in response to increasing surface roughness[18,31] and a synergistic increase with 1,25$(OH)_2D_3$ on rough surfaces.[19]

For the current study, cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2 and 100% humidity. Cells were seeded at 9300 cells/cm^2 on both titanium disks and wells of 24-well tissue culture plates (Corning Costar, Cambridge, Mass). After seeding, cultures were maintained and processed as described in the following specific assays.

Determination of cellular proliferation

Changes in cell proliferation were assessed by 2 techniques. Incorporation of [^{3}H]-thymidine was used to measure new DNA synthesis during the last 4 hours of culture, and cell number was used to measure the total number of cells present on the surface after the entire culture period. This latter assay gives a value not only for the effect of treatment during the last 24 hours but also for the effect of the surface during the 2 to 3 days preceding the addition of arachidonic acid or prostaglandin E_2.

DNA synthesis was estimated by measuring [^{3}H]-thymidine incorporation using a modification of a previously described method.[16] At 80% confluence on plastic, the FBS concentration in the media was reduced from 10% to 2%. At confluence, each well received 500 μL of experimental media containing PGE_2 (1, 10, 25, or 100 nM), arachidonic acid (0.1, 1, or 10 nM), or indomethacin (100 nM), and control media with no PGE_2 or arachidonic acid. After adding the experimental and control media, the cultures were harvested 24 hours later. Four hours before harvest, 50 μL [^{3}H]-thymidine (4μ Ci/mL; DuPont NEN Research Products, Boston, Mass) were added to the cultures. At harvest, the media were removed, and the cultures rinsed twice with cold phosphate-buffered saline (PBS) and twice with cold 5% trichloroacetic acid. The cultures were then treated with ice-cold saturated trichloroacetic acid for 30 minutes. The resulting precipitate was dissolved in 0.3 mL of 1% sodium dodecyl sulfate at 20°C and radioactivity was measured by liquid-scintillation counting.

TABLE 1

Osteoblast cell number after culture on titanium (Ti) surfaces of varying roughness and treatment with arachidonic acid (AA) for 24 hours; number of cells ($\times 10^5$) (mean ± SEM)

	Plastic	Smooth Ti	SAE Ti	Rough Ti	RAE Ti
Control	7.10±0.05	6.88±0.21*	4.07±0.12*	1.98±0.12*	3.18±0.37*
0.1 nM AA	7.18±0.14	5.61±0.40**	4.50±0.31	2.12±0.05	3.80±0.24
1.0 nM AA	5.12±0.47**	5.63±0.37**	5.01±0.09**	2.28±0.19	4.19±0.15**
10.0 nM AA	5.56±0.27**	6.48±0.07**	4.36±0.26	2.51±0.34**	3.89±0.22**

*$P < .05$, vs plastic; **$P < .05$, vs untreated control; SAE indicates smooth and acid etched; RAE, rough and acid etched.

To determine cell number, cultures were prepared and treated in an identical manner as described in the [^{3}H]-thymidine incorporation study. At harvest, cells were released from the culture surfaces by adding 0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid in Hank's balanced salts solution for 10 minutes at 37°C, followed by addition of DMEM containing 10% FBS to stop the reaction. This process was repeated one more time and the released cells were pelleted by centrifugation at 500*g* for 10 minutes. Cell pellets were washed with PBS, resuspended in PBS, and counted in a Coulter counter (Hialeah, Fla). Cells harvested in this manner exhibit greater than 95% viability based on trypan blue dye exclusion.

Determination of cellular differentiation

In this study, cellular differentiation was estimated by alkaline phosphatase (ALP) specific activity. The ALP-specific activity was determined on isolated cell layers using the method of Hale et al[32] as described by Martin et al.[16] At confluence, each well received 500 μL of experimental media containing PGE_2 (1, 10, 25, or 100 nM), AA (0.1, 1, or 10 nM), or indomethacin (100 nM), and control media with no PGE_2 or AA. After adding the experimental and control media, the cultures were harvested 24 hours later. At harvest, culture media were decanted, cell layers were 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 0.5 mL deionized water plus 25 μL 1% Triton-X-100.

After lysing the cell layers by means of 3 freeze/thaw cycles in rapid succession using methanol and dry ice, the protein content was determined by a colorimetric assay using a commercially available kit (Micro/Macro BCA; Pierce Chemical Co, Rockford, Ill). The ALP-specific activity (orthophosphoric monoester phosphohydrolase, alkaline [E.C. 3.1.3.1]) was assayed in cell layer lysates by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at a pH of 10.2 as described by Bretaudiere et al.[33]

Statistical interpretation of data

Each data point represents the mean ± standard error of the mean of 4 cultures from an individual experiment. Each experiment was repeated a minimum of three times. The data were analyzed by analysis of variance. Post hoc testing was performed using Bonferroni's modification of the Student *t* test for multiple comparisons. A P value $\leq .05$ was considered significant.

RESULTS

Effect of arachidonic acid

Arachidonic acid treatment altered MG63 cell number, [^{3}H]-thymidine incorporation, and ALP- specific activity in response to Ti surface roughness. Cell number decreased with increasing Ti surface roughness (Table 1); compared with plastic, the cell number on smooth Ti was decreased by 3%, while that on rough Ti was reduced by 72%. The AA treatment dose-dependently reduced cell number on plastic (28% for 1 nM AA) and smooth Ti (18% for 1nM AA), and it increased cell number on SAE (20% for 1nM AA), rough (13% for 1nM AA), and RAE (24% for 1nM AA) Ti.

New DNA synthesis, as measured by [^{3}H]-thymidine incorporation during the last 4 hours in culture, was also decreased with increasing surface roughness (Figure 1); compared with plastic, [^{3}H]-thymidine incorporation on smooth Ti was decreased by 26%, while that on rough Ti was reduced by 50%. In contrast to the cell number results where AA caused a decrease on plastic and smooth Ti and an increase on SAE, rough, and RAE Ti, [^{3}H]-thymidine incorporation was uniformly and dose-dependently decreased on all surfaces with AA treatment. Treatment with 10 nM AA reduced isotope incorporation by 16% on plastic, 41%

on smooth Ti, 46% on SAE Ti, 31% on rough Ti, and 19% on RAE Ti.

The ALP-specific activity was increased with increasing surface roughness (Figure 2). There was a 3-fold increase in enzyme-specific activity between the plastic and RAE Ti surfaces. The AA also influenced osteoblast response to the surfaces. On smooth surfaces, ALP was dose-dependently increased with increasing doses of AA; on plastic the level increased from 0.67 units/mg protein in the controls to 0.89 units/mg protein with 10nM AA treatment. Similarly, on smooth and SAE Ti, enzyme-specific activity increased from 0.81 and 1.21, respectively, in the controls to 1.56 and 2.35, respectively in cultures treated with 10nM AA. In contrast to these results, cultures on rough and RAE Ti showed a significant, dose-dependent decrease in ALP with AA treatment; cultures on rough and RAE Ti contained 1.62 and 2.06 units/mg protein, respectively, in the controls and 0.91 and 0.42 units/mg protein, respectively with 10nM AA treatment.

Effect of prostaglandin E_2

As observed with AA, PGE_2 treatment affected MG63 cell number, [^{3}H]-thymidine incorporation, and ALP-specific activity in response to Ti surface roughness. Cell number generally decreased with increasing Ti surface roughness (Table 2). Except for smooth Ti, which showed an increase in cell number compared with plastic, cell number on SAE Ti, rough Ti, and RAE Ti was reduced by 37%, 58%, and 43%, respectively. The PGE_2 (25nM) treatment reduced cell number on plastic (20%), smooth Ti (19%), and SAE Ti (7%); in contrast, cell number on rough Ti increased 39% and remained virtually unchanged on RAE Ti.

Incorporation of [^{3}H]-thymidine was also decreased with increasing surface roughness (Figure 3). In contrast to the cell number results, where PGE_2 treatment caused a decrease on all surfaces except rough Ti, [^{3}H]-thymidine incorporation by cultures on plastic was virtually unaffected by PGE_2, while cultures on smooth and rough Ti were dose-dependently decreased. Treatment with 25nM PGE_2 reduced isotope incorporation by 7% on plastic, 19% on smooth Ti, and 48% on rough Ti.

The ALP-specific activity increased with increasing surface roughness (Figure 4). There was a 3-fold increase in enzyme-specific activity between the plastic and rough Ti surfaces. The PGE_2 also dose-dependently influenced ALP activity of osteoblasts on the different surfaces: PGE_2 (25 nM) increased alkaline phosphatase by 71% on plastic and 28% on smooth Ti; in contrast, on rough Ti ALP was decreased by 63% and 71% by 25 and 100nM PGE_2, respectively.

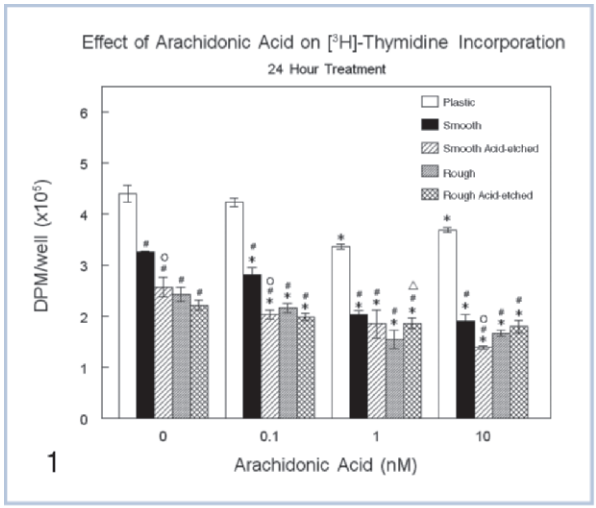
Effect of cyclooxygenase inhibition

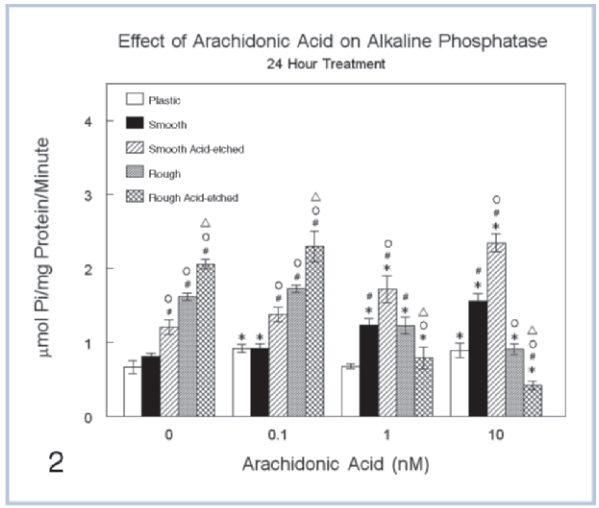
Because AA and PGE_2 were shown in the aforementioned experiments to regulate osteoblast response to implant surface roughness and a number of routinely used analgesic therapies use inhibitors of the AA pathway, the effect of indomethacin, a nonselective cyclooxygenase (COX)-1/COX-2 inhibitor, on osteoblast response to surface roughness was examined (Figures 5 and 6). Incorporation of [^{3}H]-thymidine was decreased with increasing surface roughness (Figure 5). On plastic and RAE Ti, indomethacin was without effect; although isotope incorporation was decreased on RAE Ti, the inhibition never achieved statistical significance. In contrast, on smooth Ti, SAE Ti, and rough Ti, indomethacin decreased [^{3}H]-thymidine incorporation by 10%, 25%, and 18%, respectively. The effect of indomethacin treatment on ALP-specific activity was found to be more robust (Figure 6). There was no significant effect in cultures grown on plastic and SAE Ti; even though enzyme-specific activity was decreased on SAE Ti it never achieved statistical significance. In contrast, on smooth Ti, indomethacin treatment inhibited ALP by 33%. On rough and RAE Ti, enzyme activity was increased by 46% and 62%, respectively.

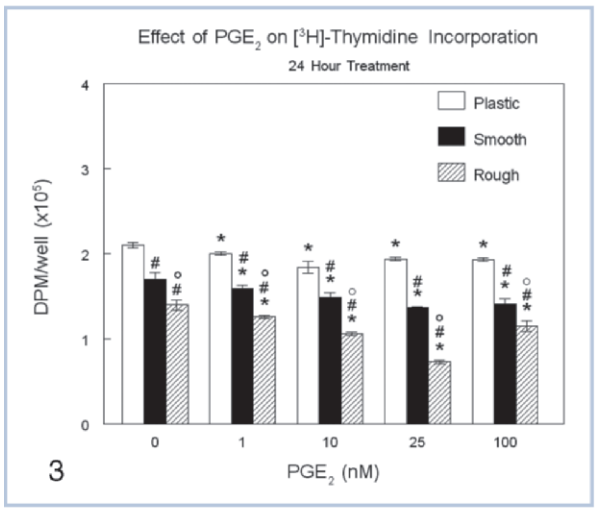
Discussion

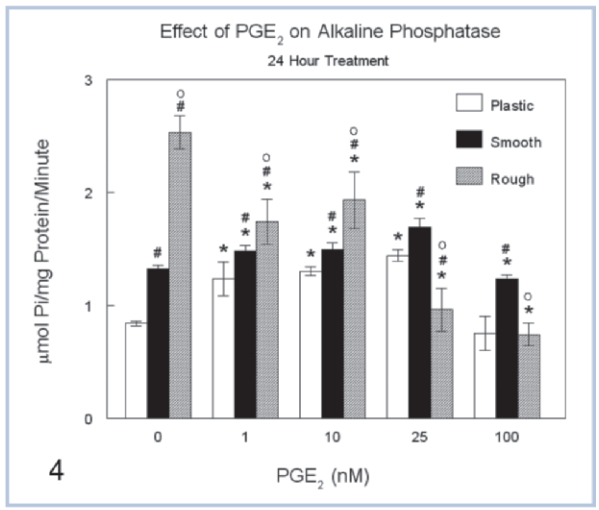
The results of the present study demonstrate that both AA and PGE_2 affect osteoblast response to Ti surface roughness. Using implant surfaces similar to those used in the current study, prior studies have shown that increasing surface roughness decreased osteoblast proliferation and increased differentiation.[16–18] In the present study, cell number on rough Ti was reduced by 60% to 70% compared with plastic, and [^{3}H]-thymidine incorporation was reduced by 40% to 50%; ALP-specific activity of cultures on rough and RAE Ti was increased by 3-fold compared with plastic.

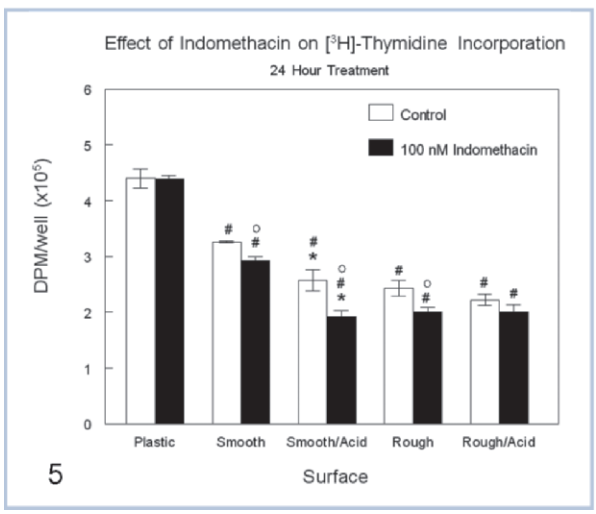
Treatment with AA altered osteoblast response to surface roughness. Cell number on plastic and smooth Ti was reduced with AA treatment and increased as much as 25% on SAE Ti, rough Ti, and RAE Ti surfaces. In contrast, [^{3}H]-thymidine incorporation was decreased on all surfaces with AA treatment. The ALP-specific activity on plastic, smooth Ti, and SAE Ti surfaces was dose-dependently increased with AA treatment, while that on rough Ti and RAE Ti was dose-dependently decreased. Taken together, the results indicate that AA not only modifies the surface

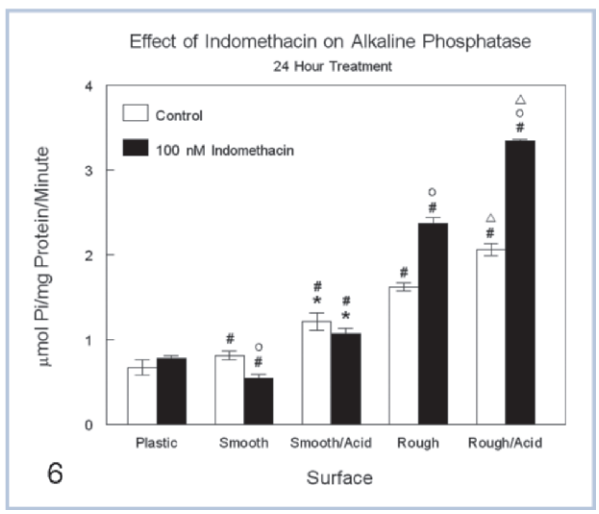












FIGURES 1–6. FIGURE 1. Effect of arachidonic acid (AA) on [^{3}H]-thymidine incorporation by MG63 cells cultured on titanium (Ti) surfaces of varying roughness. The MG63 cells were seeded onto plastic and Ti surfaces and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. At 80% confluence, the FBS concentration was decreased to 2% and the cultures continued to confluence. At confluence, the indicated concentrations of AA were added and the cultures continued for an additional 24

TABLE 2

Osteoblast cell number after culture on titanium (Ti) surfaces of varying roughness and treatment with prostaglandin E_2 (AA) for 24 hours; number of cells ($\times 10^5$) (mean ± SEM)

	Plastic	Smooth Ti	SAE Ti	Rough Ti	RAE Ti
Control	6.09±0.04	6.62±0.28*	3.82±0.21*	2.53±0.10*	3.48±0.23*
0.1 nM PGE_2	5.52±0.12**	6.11±0.16	3.55±0.42	2.30±0.13	3.56±0.29
1 nM PGE_2	4.47±0.13**	5.81±0.35**	3.56±0.41	2.54±0.11	3.02±0.08**
10 nM PGE_2	5.49±0.13**	5.77±0.17**	3.12±0.23**	2.77±0.24	3.22±0.19
25 nM PGE_2	4.75±0.14**	5.35±0.03**	3.54±0.11	3.53±0.33**	3.41±0.17

*$P < .05$, vs plastic; **$P < .05$, vs untreated control; SAE indicates smooth and acid etched; RAE, rough and acid etched.

roughness effect, but it also has the potential to reduce or eliminate it. This was especially obvious with ALP, where enzyme-specific activity was increased in cultures on smooth and SAE Ti and decreased in those on rough Ti and RAE Ti.

The PGE_2 treatment also had an effect on cell response to surface roughness, although it was not as intense as that observed with AA. Cell number on plastic was reduced 20% by 25 nM PGE_2 compared with a 28% decrease with 1 nM AA; similarly, smooth Ti showed a reduction of 19% with 25 nM PGE_2 and an 18% reduction with 1 nM AA. With cells on rough Ti and RAE Ti, the prostaglandin-treated cultures displayed an increase of 39% and no change vs 13% and 24% increase with AA. Similarly, PGE_2 treatment demonstrated less of an effect on [^{3}H]-thymidine incorporation and ALP-specific activity than AA, but the overall effect was still to reverse the effect of surface roughness on cell response. It is unknown why there was a disparity in the effect of AA vs PGE_2, but one possibility is that other metabolites of AA, such as those synthesized by 5- and 12-/15-lipoxygenase, are produced downstream, which may affect bone cells.[34–38]

Prior studies have demonstrated that PGE_2 is produced by bone and has powerful effects on bone metabolism.[39,40] Prostaglandins have an inhibitory effect on osteoclasts, but when given for extended periods of time, they stimulate bone resorption by increasing replication and differentiation of new osteoclasts. In addition to effects on osteoclasts, prostaglandins also have a biphasic effect on bone formation. At relatively low concentrations, the replication and differentiation of osteoblasts is stimulated and bone formation is increased. At high concentrations, PGE_2 inhibits collagen synthesis by osteoblasts and increases the bone-resorbing activity of osteoclasts. These multiple and biphasic effects of PGE_2 on bone has made it difficult to clearly elucidate the mechanism of action of this cytokine. Further, differences in cell-culture systems, species, dosage, and treatment times have further complicated the

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hours. At harvest, [^{3}H]-thymidine incorporation was determined as described in the Materials and Methods. #$P < .05$, vs plastic; *$P < .05$, vs untreated control (no AA); °$P < .05$, vs smooth Ti; $^{\Delta}P < .05$, vs rough Ti. FIGURE 2: Effect of AA acid on alkaline phosphatase (ALP) specific activity of MG63 cells cultured on Ti surfaces of varying roughness. MG63 cells were seeded onto plastic and Ti surfaces and cultured to confluence in DMEM containing 10% FBS and antibiotics. At confluence, the indicated concentrations of AA were added and the cultures continued for an additional 24 hours. At harvest, ALP-specific activity was determined as described in the Materials and Methods. #$P < .05$, vs plastic; *$P < .05$, vs untreated control (no AA); °$P < .05$, vs smooth Ti; $^{\Delta}P < .05$, vs rough Ti. FIGURE 3: Effect of prostaglandin E_2 on [^{3}H]-thymidine incorporation by MG63 cells cultured on Ti surfaces of varying roughness. The MG63 cells were seeded onto plastic and Ti surfaces and cultured in DMEM containing 10% FBS and antibiotics. At 80% confluence, the FBS concentration was decreased to 2% and the cultures continued to confluence. At confluence, the indicated concentrations of prostaglandin E_2 were added and the cultures continued for an additional 24 hours. At harvest, [^{3}H]-thymidine incorporation was determined as described in the Materials and Methods. #$P< .05$, vs plastic; *$P < .05$, vs untreated control (no prostaglandin E_2); °$P < .05$, vs smooth Ti. FIGURE 4: Effect of prostaglandin E_2 on ALP- specific activity of MG63 cells cultured on Ti surfaces of varying roughness. The MG63 cells were seeded onto plastic and Ti surfaces and cultured to confluence in DMEM containing 10% FBS and antibiotics. At confluence, the indicated concentrations of prostaglandin E_2 were added and the cultures continued for an additional 24 hours. At harvest, ALP-specific activity was determined as described in the Materials and Methods. #$P < .05$, vs plastic; *$P < .05$, vs untreated control (no prostaglandin E_2); °$P < .05$, vs smooth Ti. FIGURE 5: Effect of indomethacin on [^{3}H]-thymidine incorporation by MG63 cells cultured on Ti surfaces of varying roughness. The MG63 cells were seeded onto plastic and Ti surfaces and cultured in DMEM containing 10% FBS and antibiotics. At 80% confluence, the FBS concentration was decreased to 2% and the cultures continued to confluence. At confluence, indomethacin (100 nM) or vehicle was added and the cultures continued for an additional 24 hours. At harvest, [^{3}H]-thymidine incorporation was determined as described in the Materials and Methods. #$P < .05$, vs plastic; °$P < .05$, vs no indomethacin treatment; *$P < .05$, vs smooth Ti. FIGURE 6: Effect of indomethacin on ALP-specific activity of MG63 cells cultured on Ti surfaces of varying roughness. The MG63 cells were seeded onto plastic and Ti surfaces and cultured to confluence in DMEM containing 10% FBS and antibiotics. At confluence, indomethacin (100 nM) or vehicle was added and the cultures continued for an additional 24 hours. At harvest, ALP-specific activity was determined as described in the Materials and Methods. #$P < .05$, vs plastic; °$P < .05$, vs no indomethacin; *$P < .05$, vs smooth Ti; $^{\Delta}P < .05$, vs rough Ti.

process of understanding the effect of PGE_2 on bone. In the authors' opinion, one of the major confounding factors affecting knowledge of the effect of PGE_2 on bone is species differences. Much of the work describing the effect of prostanoids and inhibitors of prostanoid production has been performed in rats. As it is well known that physiology of the rat skeleton is different from that of human skeleton, it is possible that much of the reported effects are more relevant to rat than to human bone. Potential differences in NSAID and PGE_2 response between animal studies and humans has recently been noted (for an interesting evidence-based review on NSAIDs, coxibs, smoking, and bone, see http://www.jr2.ox.ac.uk/bandolier/booth/painpag/wisdom/NSAIbone.html).[41] The fact that the present results run somewhat against the present dogma concerning the effect of prostaglandins and NSAIDs on bone healing may be due to the fact that a human cell line was used.

A major rate-limiting step in the synthesis of PGE_2 is the production of free AA from membrane phospholipids by the action of phospholipase A_2. Free AA is metabolized to PGH_2 by COX followed by isomerization by prostaglandin E synthase (PGES).[42] Prior studies using a rat marrow ablation model have demonstrated that Ti implants, but not those of stainless steel, show an increase in matrix vesicle ALP-specific and phospholipase A_2-specific activities in the healing bone[43] suggesting that one possible mechanism accounting for the favorable response of Ti implants lies in the fact that they can induce cells in the wound milieu to synthesize PGE_2 and possibly other factors necessary for new bone formation. Additional studies in the authors' lab have pursued this line of reasoning and further described the role of phospholipase A_2 in the response of osteoblasts to Ti surface roughness and $1,25(OH)_2D_3$,[44,45] while others have reported on the involvement of phospholipase A_2 and COX-2 in augmenting PGE_2 production during mineralization of rat calvarial cells.[46]

Studies have shown that MG63 cells cultured on titanium disks of increasing roughness, similar to those used in the current study, produce increased amounts of PGE_2.[18] On smooth Ti, the cultures were found to produce 50 pg/mL (= 0.7 nM) of PGE_2, while those on rough Ti produced 160 pg/mL (= 4.5 nM). In the present study, the addition of exogenous PGE_2 or substrate in the form of AA would be expected to add to the effective dose seen by the cultures. Because PGE_2 is known to have biphasic effects on osteoblasts, it is possible that this fact may have influenced the data to some extent.

The present study used doses of PGE_2 similar to those reported by others.[47–49] Unlike the present study, where PGE_2 was added for the last 24 hours of culture, other investigators have added PGE_2 for extended periods of time. For example, in Nagata's study,[47] PGE_2 was added to fetal rat calvarial cells during the first 7 days of culture or at days 8 to 14 or 8 to 21 and the effect of PGE_2 treatment was observed. From the results, the authors concluded that PGE_2 has virtually no effect on preconfluent cultures, but stimulates post-confluent cultures to produce increased ALP and bone nodules. Thus, length and time of treatment are important factors in assessing the effect of PGE_2 on bone cells.

Earlier studies in the authors' lab have established a role for PGE_2 in osteoblast response to surface roughness and systemic factors such as $1,25(OH)_2D_3$.[50,51] When indomethacin, a general COX inhibitor of PGE_2 production, was present for the entire culture period, the effect of surface roughness on cell proliferation and osteocalcin and TGF-β production was ablated, while ALP-specific activity was diminished but not blocked. In contrast, when confluent cultures were treated for 24 hours with control media or media containing $1,25(OH)_2D_3$ in the presence or absence of indomethacin, the surface roughness–dependent effects were unaffected by indomethacin treatment, while all of the $1,25(OH)_2D_3$-dependent effects were abolished except for ALP. These results implicate PGE_2 in mediating not only the surface roughness effects in the cultures, but the effect of systemic hormones like $1,25(OH)_2D_3$.

The current study extends these observations by adding exogenous AA or PGE_2 to cultures on Ti surfaces of varying roughness. By adding AA to the cultures, ALP on smooth Ti and SAE Ti was increased, while that on rough Ti and RAE Ti was decreased, indicating that AA has the potential to reduce or eliminate the surface roughness–dependent effect. Further, PGE_2 had a similar but less intense affect on the cultures. Additional insight into the effect of AA metabolites on osteoblast response to surface roughness was gained by adding indomethacin to the cultures. In these experiments, [^{3}H]-thymidine incorporation by cultures on plastic and RAE Ti was unaffected by treatment with indomethacin, while cultures on smooth Ti, SAE Ti, and rough Ti were inhibited 10% to 25%. The effect of indomethacin on ALP activity was more pronounced. Blockade of endogenous PGE_2 production decreased ALP on smooth Ti and SAE Ti, but increased it on rough Ti and RAE Ti. This result is different from that reported by Batzer et al[50] where indomethacin treatment for 24 hours had no effect on enzyme activity and similar to

that reported in Sisk et al[51] where there was an increase in enzyme activity. It is possible that at some point in the growth of MG63 cells (perhaps confluence?) endogenous PGE_2 down regulates ALP. This type of regulation has been as reported for MC3T3-E1 cells.[49,52] The effect of indomethacin in the current study is complementary to the results obtained when exogenous AA or PGE_2 was added to the cultures. For example, increased levels of exogenous AA or PGE_2 were associated with decreased ALP on rough surfaces and increased levels of enzyme-specific activity on smooth surfaces. When indomethacin was added, the effect was the same as that seen with lower levels of PGE_2.

The results of the current study underscore the necessity of carefully evaluating treatment protocols before and immediately after implant placement. Based on the present results, the level of free AA or PGE_2 in a bone wound after surgery has the potential to affect osseointegration. Use of NSAIDS, both general and COX-2 specific, has been controversial and will probably continue to be so.[41,53] Novel protocols using current mainline therapies are viable strategies,[54] but future therapies may include manipulation of the AA pathway [55] or specific agonists of prostanoid receptors to stimulate new bone formation.[56,57]

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