

Effects of Phosphated Titanium and Enamel Matrix Derivatives on Osteoblast Behavior In Vitro

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Purpose: The purpose of this study was to evaluate the effects of phosphated titanium and EMD on osteoblast function. **Materials and Methods:** Primary rat osteoblasts were cultured on disks of either phosphated or nonphosphated titanium. In half of the samples 180 µg of EMD was immediately added. The medium was changed every 2 days for 28 days and then analyzed by transforming growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β) enzyme-linked immunosorbent assays (ELISAs). Scanning electron microscopy and light microscopy were used to evaluate nodule formation and mineralization. **Results:** Microscopic evaluation revealed no differences in osteoblast attachment between the 4 groups. Osteoblast nodule formation was observed in all groups. In the absence of mineralizing media, nodules on the nonphosphated titanium samples showed no evidence of mineralization. All nodules on the phosphated titanium had evidence of mineralization. ELISA analysis revealed no significant differences in IL-1β production between any of the groups. The EMD-treated osteoblasts produced significantly more TGF-β1 than non-EMD-treated cells for up to 8 days, and osteoblasts on phosphated titanium produced significantly more TGF-β1 at 8 days. **Discussion and Conclusion:** Osteoblast attachment appeared unaffected by surface treatment. EMD initiated early TGF-β1 production, but production decreased to control levels within 10 days. Phosphated titanium increased TGF-β1 production at 8 days and induced nodule mineralization even in the absence of mineralizing medium. INT J ORAL MAXILLOFAC IMPLANTS 2007;22:XXX-XXX

Key words: enamel matrix derivatives, interleukin-1β, osteoblasts, phosphate titanium, transforming growth factor-β1

Dental implants have become a widely accepted means of routine tooth replacement. A recent study by Karoussis and associates¹ showed that over

a 10-year period, implants had a success rate of 96.5% in patients who had no history of chronic periodontitis and 90.5% in those with such a history. Implant success rates have proven to be consistently high for nonrisk patients. Research is warranted to develop improved implant surfaces that can increase success rates with high-risk patients. Some examples of high-risk patients are smokers, patients with poorly controlled diabetes, osteoporotic women, and patients with a history of aggressive periodontal disease. Implant failure rates have been shown to be twice as high in smokers compared to nonsmokers,^{2,3} and human studies have shown greater long-term implant failure rates in diabetic patients compared to patients without diabetes.^{4,5}

Many different surface coatings of titanium implant substrates have been tested to improve osseointegration. Any surface that increases successful implant retention in high-risk patients would be beneficial. Some of the surface types that have been studied are smooth, plasma sprayed, and sand-blasted acid-etched (SBAE) surfaces.⁶ These surface treatments were found to influence the growth and

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metabolic activity of cultured osteoblasts, with SBAE surfaces yielding the most favorable results.⁶

Recently, a new titanium surface resulting from electronically coating titanium with phosphate was developed and studied in orthopedic implants (Minevski, personal communication). When titanium is treated by anodic oxidation in phosphoric acid, the phosphate concentration of the surface is increased. The titanium becomes more corrosion resistant, and the surface hardness improves, facilitating biocompatibility.⁷ In a canine study evaluating electrolytic phosphated titanium implants placed in the humerus, the treated titanium had significantly enhanced bone-implant contact and decreased fibrous-tissue interface compared to the nontreated group at 1 month.⁷ Bone formation requires the presence of phosphate and calcium⁸; thus, treating titanium implants with phosphate may accelerate osseointegration.

Interleukin-1 (IL-1) has received attention because of its role as a proinflammatory cytokine. IL-1 β induces bone resorption, an activity reversed by the action of transforming growth factor- β 1 (TGF- β 1).⁹ IL-1 β is the major inflammatory cytokine occurring in gingiva associated with periodontitis,¹⁰ and levels of IL-1 β have been shown to be higher in periodontitis sites compared to healthy sites.^{11,12} Increased levels of IL-1 β have also been shown around implants with peri-implantitis.¹³⁻¹⁵

TGF- β 1 production by osteoblasts is useful in evaluating the early stages of osteoblast response to exposure to new surfaces (eg, dental implants). TGF- β 1 is abundant in bone matrix^{16,17} and has been shown to affect bone metabolism through modulation of both osteoclastic and osteoblastic cell differentiation and activity.¹⁷⁻²¹ It has been suggested that TGF- β 1 may play a role in the pathogenesis and diagnosis of periodontal disease.²² Grafting materials influence the expression of TGF- β 1; Trasatti and associates²³ found that Pepgen P-15 (bovine bone with synthetic P15 protein) caused increased expression of TGF- β 1. Moreover, osteoblasts produce TGF- β , which is embedded in the bone matrix and is activated by bone resorbing osteoclasts.²⁴ Since IL-1 β is a potent proinflammatory cytokine whose activity is suppressed by TGF- β 1,^{9,25} the evaluation of these 2 cytokines together would yield helpful insights regarding the biological response of osteoblasts exposed to a new titanium surface.

More recently porcine-derived enamel matrix derivatives (EMD; eg, Emdogain; Straumann/Biora, Malmo, Sweden), have gained attention because of their ability to induce periodontal regeneration.²⁶ Hammarström and associates²⁶ used preparations of EMD in a monkey model and at 8 weeks found that

EMD proteins significantly regenerated buccal defects, including the reappearance of acellular cementum with inserting collagen fibers (Sharpey's fibers) and the formation of new alveolar bone. Other studies have demonstrated the periodontal regenerative effects of EMD in human models.^{27,28} EMD has recently been shown to increase fibroblast growth factor-2 (FGF-2) expression when added to human osteoblasts.²⁹ The full effects of EMD on the expression of other growth factors are still not known.

Shimizu-Ishiura and associates³⁰ evaluated the use of EMD on trabecular bone induction after the implantation of titanium implants in the rat femur. They noted 30 days postimplantation that the EMD sites had significantly greater trabecular bone formation than sites that did not receive EMD. Casati et al³¹ evaluated guided bone regeneration (GBR) around implants and found that EMD with GBR around titanium implants was superior to GBR alone. Stenport et al³² evaluated EMD placed in implant osteotomies at the time of implantation. However, they found no significant differences between implants with and without EMD.

Since conflicting results were noted, further research appears necessary to further evaluate the influence of EMD on osteoblast behavior in contact with titanium. An *in vitro* osteoblast culture model was used to evaluate the effects of phosphated versus nonphosphated titanium on osteoblast function and to evaluate the added effect of EMD. These *in vitro* studies were designed to critically assess osteoblast responses to the surface phosphate, since phosphate is required for successful matrix mineralization by osteoblasts.

MATERIALS AND METHODS

Cell Culture and Preparation

All procedures were approved by the Baylor College of Dentistry (BCD) Institutional Animal Care and Use Committee. Primary rat osteoblasts were harvested from fetal day 20 rat calvaria as previously described by Williams and associates.³³ Briefly, 4 pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were housed in the BCD animal care facilities and were sacrificed when the fetuses reached 20 days old. The fetuses were removed onto ice and beheaded. The calvaria were removed, and the 2 frontal and parietal bones were dissected from the surrounding sutures. The bones were cut into small pieces and put into phosphate-buffered saline (PBS; Sigma Chemical, St Louis, MO) and stored on ice. The bone pieces were then placed into 2.5 mL digestion solution (calcium [Ca] and magnesium [Mg] contain-

ing PBS and collagenase, 20/mg/mL, Sigma Chemical). After stirring for 20 minutes at 37°C, the supernatant was discarded. This discarded supernatant was considered "fraction 1." The procedure was repeated, and fraction 2 was also discarded, as these 2 fractions were considered to contain mostly fibroblasts.³³ The procedure was repeated another 3 times, and fractions 3, 4, and 5 were retained and stored on ice. These fractions have been shown to be osteoblast-rich.³³ These fractions were combined and centrifuged at 1,000 rpm for 1.5 minutes. The pellet was retained, while the supernatant was discarded. The pellet was resuspended with 3 mL of media and divided evenly into three 3 × 60-mm dishes. Two mL of culture medium—Dulbecco's modified Eagle's medium (DMEM; Gibco BRL/Life Technologies, Rockville, MD); 10% fetal calf serum (FCS; Gibco BRL/Life technologies); an antibiotic mixture of penicillin (76/μg/mL) and streptomycin (76/μg/mL) (Gibco BRL/Life Technologies); 1% insulin, transferrin, and selenium ITS; BD Biosciences, Bedford, MA), and 1% nonessential amino acids (Gibco BRL/Life Technologies) were then added to each dish and placed in a humidified incubator at 37°C with 5% carbon dioxide (CO₂) in air.

The culture medium was changed every 2 days. Cells were monitored by light microscope every 24 hours, and when confluence was reached they were trypsinized (Ca and Mg free Hank's [Author: Ca and Mg-free Hank's balanced salt solution?] plus 0.25 mg/mL trypsin). Subsequently, the cells were divided evenly into 5 new plates. The cells were once again grown to confluence as described. After the third passage, the cells were trypsinized and counted using a hemocytometer.

Culturing Cells on Titanium Disks

Forty-eight 1-cm grade 2 titanium disks (Ti6Al4V) treated by electronic phosphating were supplied by Lynntech (College Station, TX). The disks were degreased in acetone for 10 minutes and then immersed (deoxidized) in 10% tetrafluoroboric acid for 3 minutes. The disks were then washed with deionized water for 5 to 10 seconds and placed in an electrolytic cell. The desired phosphate surface was prepared by the anodic oxidation of titanium samples using 50 volts at room temperature for 30 minutes in 1 mol/L phosphoric acid. These treated disks served as the phosphated titanium group. Forty-eight non-phosphated disks also supplied by the manufacturer served as the nonphosphate control group.

Each disk was placed in 1 well of a 24-well plate (Costar, Corning, NY). Disks were divided into 8 groups. Groups 1 to 4 were phosphate treated, and groups 5 to 8 were not phosphate treated. Groups 1

and 5 had 1 disk each, and were control disks receiving neither cells nor EMD. Groups 2 and 6 had 1 disk each, and were control disks with EMD but not cells. Groups 3 and 7 had 23 disks each and received 10⁶ cells in 400 μL medium. They were allowed to settle in an incubator at 5% CO₂ in air at 37°C for 1 hour. A further 600 μL medium was then added. Groups 4 and 8 had 23 disks each and received 180 μg of EMD immediately prior to the seeding of 10⁶ cells in 400 μL medium. The disks were then allowed to settle in an incubator for 1 hour. Thereafter, 600 μL of culture medium was added to each well, and the cells and disks were cultured at 5% CO₂ in air at 37°C. Ascorbic acid was added daily to each well at a concentration of 100 μg/mL, and the medium was changed at 48-hour intervals.

Preparation of Cells and Disks for Scanning Electron Microscopy

On days 6, 10, and 14, five disks from groups 3, 4, 7, and 8 were removed from the media. From day 14 through day 28, one of the remaining disks in each group was cultured with mineralizing medium (1 mmol/L β-glycerophosphate and 10⁻⁸ mol/L dexamethasone in culture medium) to induce nodule mineralization, and another disk was cultured with regular, nonmineralizing medium as a control. After removal from medium, all of the disks were rinsed with ice-cold PBS, fixed with 4% paraformaldehyde (PFA), and dehydrated. After critical-point drying [Author: hyphenation of this phrase correct?], the samples were sputter coated and viewed using scanning electron microscopy (SEM; JEOL JSM-6300; JEOL USA, Peabody, MA). X-ray energy dispersion analysis (XEDA) was done on the phosphated and nonphosphated surfaces to confirm the presence of mineralized/calcified bone nodules.

Preparation of Cells and Disks for Histologic Analysis of Mineralization

The remaining 6 disks in groups 3, 4, 7, and 8 were cultured in regular medium until day 14. At day 14, 3 disks from each of these groups had the culture medium replaced with mineralizing medium. All groups were continued in their respective media until day 28. At day 28 all disks from groups 1 through 8 were removed from the medium and rinsed with ice-cold PBS and fixed with 4% PFA for 1 hour. They were then washed with deionized H₂O 3 times. Each disk was then embedded in methyl methacrylate, and sections were prepared with an Isomet saw (Buehler, Lake Bluff, IL) and routine polishing procedures. The disks were stained with Stevenel's blue and alizarin red and were evaluated for mineral nodule formation using light microscopy (LM).

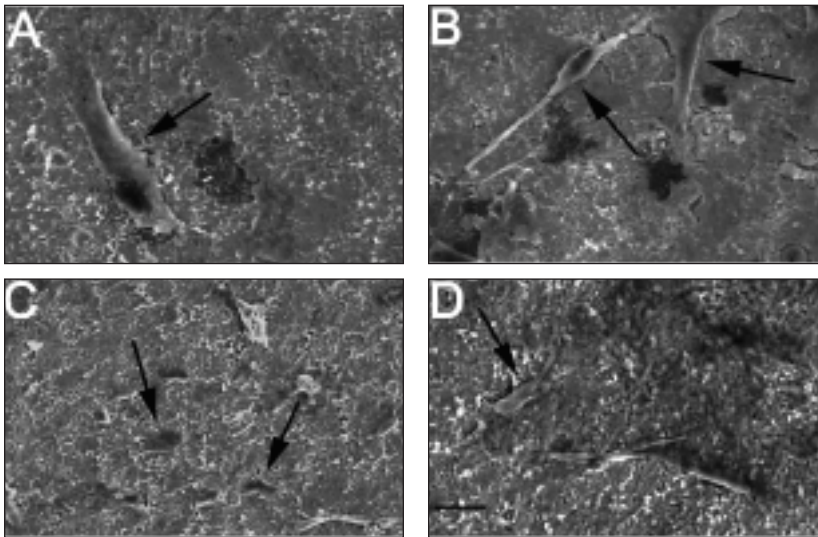


Fig 1 Healthy and flattened osteoblasts (arrows) were present on different titanium surfaces at 6 days. (a) Large osteoblast shown on a control surface. (b) Multiple osteoblasts intertwined in EMD coating on a control surface with EMD. The cytoplasmic processes were easily observed. (c) Smaller but healthy osteoblasts on a phosphated titanium surface. (d) Osteoblasts on a phosphated titanium surface with EMD (original magnification $\times 1,000$; bar = 10 μm).

Preparation of Supernatants for ELISA Assays

Every 2 days, from day 2 to 14, supernatants were collected from all samples in all groups. The Lowry protein assay³⁴ was used to assess total protein content in 10 μL of supernatant, and bovine serum albumin (1 mg/mL) was used to create the standard curve. Samples were read at 750 nm of visible light using Lowry High Sensitivity software on a Beckman DU-64 Spectrophotometer (Beckman Instruments, Fullerton, CA). Active TGF- $\beta 1$ and IL-1 β enzyme-linked immunosorbent assays (ELISAs) were performed on the supernatants using Quantikine immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions in the 96-well microplates provided. Optical densities were read at 450 nm in a microplate reader (Spectra MAX; Molecular Devices, Sunnyvale, CA).

Methods of Analysis

Surface analysis of cell attachment to each disk was done using a variety of magnifications of SEM and LM. Photographs were obtained from predesignated points on each disk to ensure impartiality of data collection between groups. TGF- $\beta 1$ and IL-1 β levels were expressed as pg of growth factor per μg of total protein. Differences between groups were statistically determined using Student *t* test and analysis of variance (ANOVA). The samples treated with EMD were evaluated in the same manner as all the other groups. The EMD samples were compared to the other sample groups to determine whether there were any differences in the early stages of osteoblast attachment and function.

RESULTS

Cells on SEM and LM

SEM revealed cell attachment to the titanium disks in all groups after 6 days in culture (Fig 1). Osteoblasts on all surfaces were flattened and spread out but were not confluent. The surfaces of the disks in the groups treated with EMD had a porous and patchy coating, with osteoblasts intertwined above and below the EMD. While cellularity appeared to increase in all groups over time, no quantitative assessment was done, and no obvious differences were noted between groups after 28 days in culture (Fig 2).

LM confirmed the presence of osteoblasts on all samples in all groups (Fig 3). Blue staining osteoblasts appeared flattened and adhered to the disk surface in all groups, and generally a thickness of 1 to 2 cell layers was observed. Differences in cellularity or cell morphology were noted neither between phosphated and nonphosphated groups nor between EMD treated and non-EMD groups.

Nodule Formation, Nodule Mineralization, and XEDA Analysis

Nodule formation was observed in all groups, in both the presence and absence of mineralizing medium (Fig 4). Counting of nodules on 2 sections from the 2 disks from each group prepared for LM showed increased numbers of nodules when EMD was added but similar numbers of nodules on phosphated versus nonphosphated disks (not shown). Both large and small nodules were noted in all groups. No statistical analysis was done, as numbers of disks and numbers of sections for analysis were too small. All

Fig 2 Osteoblasts (*arrows*) on titanium surfaces on day 28. (*a*) Osteoblasts on a control surface (neither cells nor EMD). (*b*) Osteoblasts on a control surface with EMD. (*c*) Osteoblasts on a phosphated titanium surface. (*d*) Osteoblasts on a phosphated titanium surface with EMD (original magnification $\times 1,000$; bar = 10 μm).

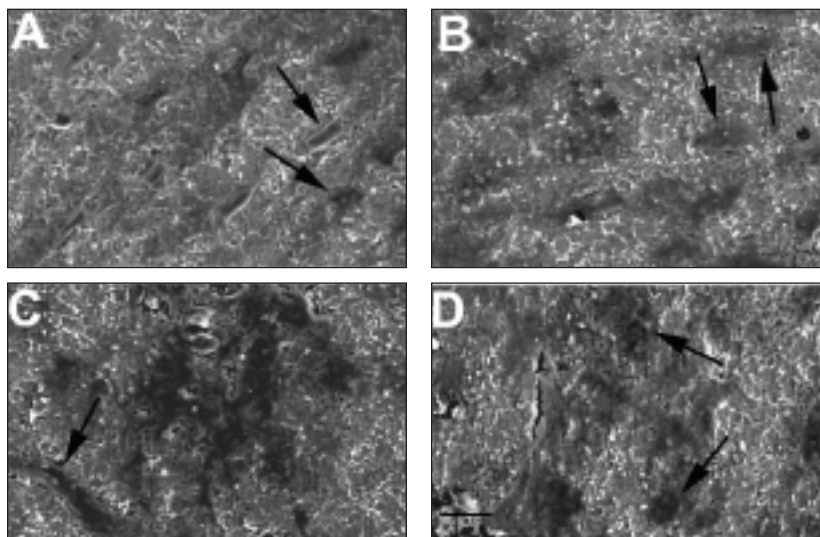


Fig 3 Typical cross-sectional appearance of healthy and flattened blue-stained osteoblasts on a titanium surface at 28 days. (*a*) Osteoblasts on a control surface with EMD. (*b*) Osteoblasts on a phosphated titanium surface with EMD (Stevenel's blue; original magnification, $\times 60$; bar = 50 μm).

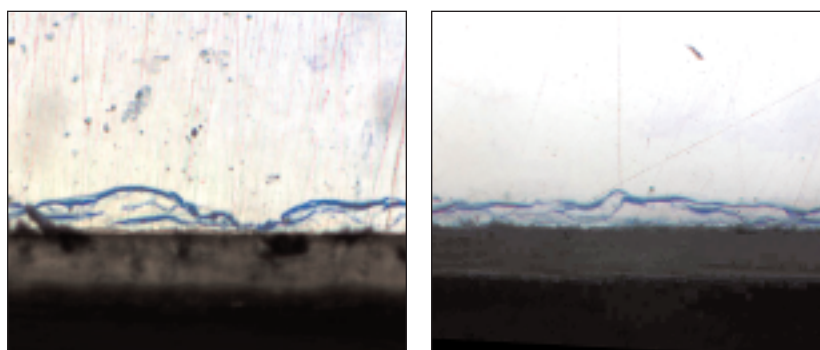
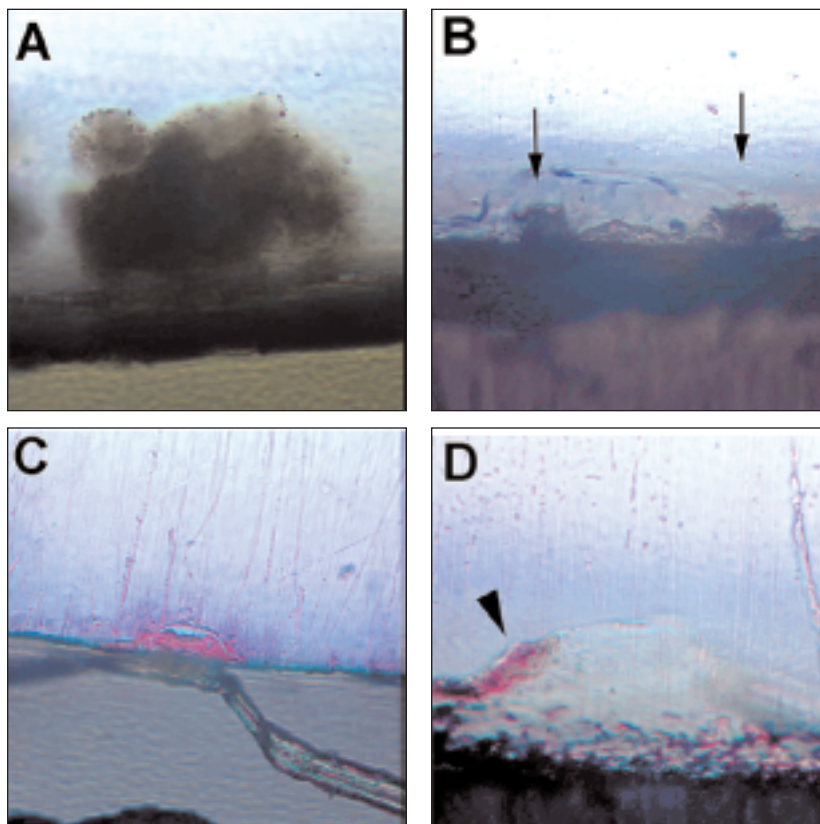


Fig 4 Cross sections of nodules present on different titanium surfaces, none of which were treated with mineralizing medium. Mineralization shows red from alizarin red stain. (*a*) Large nodule present on control surface, note mineralization is not seen. (*b*) Two nodules (*arrows*) present on control surface plus EMD, also with no mineralization seen. (*c*) Nodule present on phosphated titanium surface and mineralization is taking place. (*d*) Large nodule present on phosphated titanium surface with EMD, with mineralization present over the top of the nodule (arrowhead; Stevenel's blue; original magnification $\times 60$; bar = 25 μm).



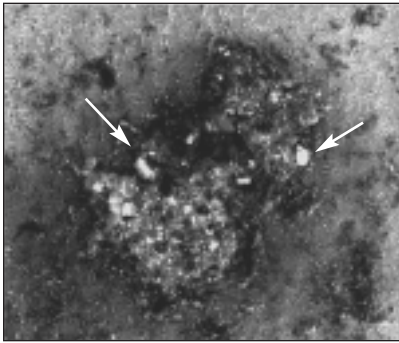


Fig 5 A nodule on a phosphated titanium surface. The nodule is outlined by white dashes. Mineralization took place on the nodule surface (arrows). Bar = 100 μ m.

Table 1 XEDA Analysis of Nodule Mineral Content Compared to Non-nodule Area of Disk

Element	Nodule (%)	PT surface	Fold change
Ca	4.13	0.10	41 \times
P	7.19	0.90	8 \times
Ti	75.41	88.17	No change
Al	8.59	7.32	No change
V	4.68	3.51	No change

Atomic percentages of elements present on forming nodule and on adjacent phosphated titanium (PT) surface where no nodules were present.

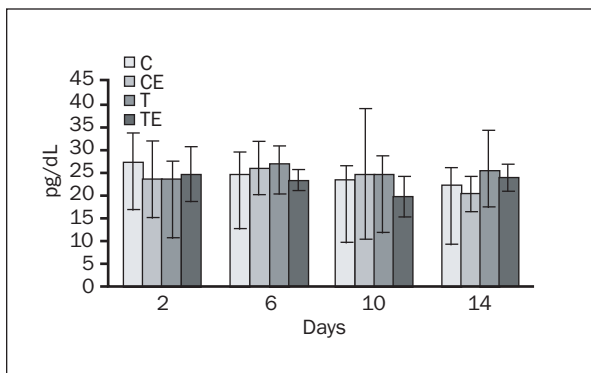


Fig 6 Mean IL-1 β production \pm standard deviation at days 2, 6, 10, and 14. No significant differences were detected between any of the groups. C = nonphosphated titanium; CE = nonphosphated titanium with EMD; T = phosphated titanium; TE = phosphated titanium with EMD.

nodules in all groups showed evidence of calcification when mineralizing medium was added (Fig 4). However, in the absence of mineralizing medium, only nodules on the phosphated titanium disks showed evidence of calcification. Calcification was confirmed by alizarin red positive staining on the nodules.

The presence of mineralized nodules was also noted with SEM (Fig 5) and confirmed with XEDA (Table 1). Roughly 40 times the calcium concentration and 8 times the phosphate concentration were observed in nodules as compared to adjacent titanium surfaces where nodules were not present.

TGF- β 1 and IL-1 β ELISAs

IL-1 β ELISA analysis at days 2, 6, 10, and 14 revealed no significant differences between any of the groups and no significant changes over time (Fig 6). TGF- β 1 ELISA analysis was done at days 2, 4, 6, 8, 10, 12, and 14. On days 2, 4, and 6 significantly ($P < .001$) increased TGF- β 1 production in the EMD treated

phosphate and nonphosphate groups was noted when compared to the non-EMD treated phosphate and nonphosphate groups. In both the phosphate and nonphosphate EMD treated groups, the TGF- β 1 levels were highest at 2 days in culture, and then progressively declined until there was no significant difference at day 10 for all groups (Fig 7).

TGF- β 1 production was not significantly different between the phosphate + EMD and nonphosphate + EMD groups, although the phosphate + EMD group produced more TGF- β 1 at days 2, 4, 8, 10, and 12. In the absence of EMD, the phosphate group produced more TGF- β 1 than the nonphosphate group, except on day 2 [Author: 2 and 14? See Fig 7], but these differences were not significant prior to day 8. At day 8, a significant increase in TGF- β 1 production was noted in the phosphate and phosphate + EMD groups ($P < .001$) compared to the nonphosphate and nonphosphate + EMD groups. No significant differences were detected after 8 days between the phosphated and nonphosphated titanium groups.

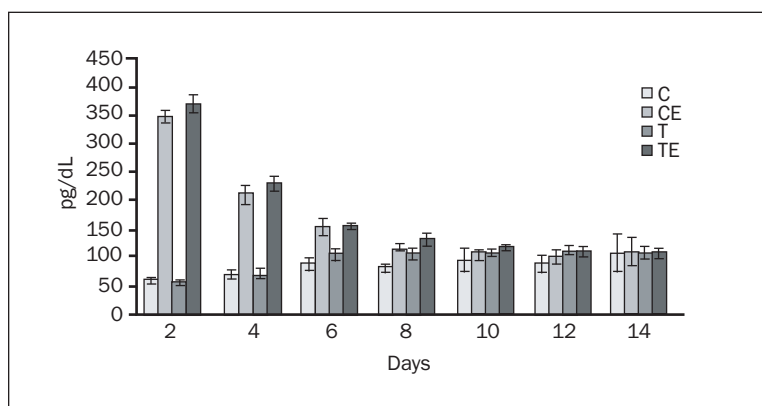
From days 2 to 14, there was a steady decline in TGF- β 1 production by osteoblasts in the EMD groups and a slight but insignificant increase of TGF- β 1 production by osteoblasts in the non-EMD groups. Control wells of phosphated and nonphosphated control titanium disks cultured with EMD and without cells showed no TGF- β 1 expression at any time.

DISCUSSION

Titanium implants are becoming the standard of care in replacing missing teeth and stabilizing dentures. Newer titanium implant surfaces accelerate osseointegration, which shortens the healing phase after surgical implant placement. Interest in phosphated titanium is increasing. Viornery and associates³⁵ evaluated rat osteoblasts grown on phosphoric

Fig 7 Mean TGF- β 1 production \pm standard deviation. Significant differences were detected between the EMD groups and the non-EMD groups up to day 8. After day 8, there were no significant differences between any of the groups. C = nonphosphated titanium; CE = non-phosphated titanium with EMD; T = phosphated titanium; TE = phosphated titanium with EMD.

Au: Figs 6 and 7. It was impossible to tell where the bottom limit on the bars should be in the c and t portions of the original graphs. Please confirm and/or correct.



acid-modified titanium for up to 8 days, with the phosphate covalently bonded to the titanium. They found no differences in the proliferation of osteoblasts on these surfaces compared to controls, showing that the phosphated surfaces were not cytotoxic to the osteoblasts. They also reported that the modified titanium surfaces had significantly more synthesized total protein and collagen type I than the unmodified titanium. The authors concluded that the covalently bonded phosphate might form a scaffold for new bone formation, which ultimately will lead to bonding of the implant to the host tissue.³⁵

A longer-term study was done by Nelson et al,⁷ who looked at electrolytic phosphate-coated implants placed in a dog humeral model for up to 6 months. In their evaluation of the pullout strength and tissue interface, they found no significant difference between phosphate-coated implants and pure titanium implants, but they did find significantly enhanced implant contact with bone and marrow and decreased fibrous tissue interface in the phosphate group. The electrolytic phosphated titanium surface used by Nelson and associates in their study is the same as that used in the present study.

Nodules are created by osteoblasts once they reach confluence in tissue culture. Cells clump together in layers to form the nodules, which then begin forming an osteoid-like matrix, which they then mineralize. Mineralization of nodule matrix usually requires the presence of a mineralizing medium containing an inorganic source of phosphate, such as β -glycerophosphate. Osteoblasts formed nodules of various sizes in all treatment and control groups. The presence of nodules even in the absence of mineralizing media is likely because of the presence of ascorbic acid in the culture medium. The absence of mineralization of nodules noted in the nonphosphate group occurred even in the presence of ascor-

bic acid, indicating that ascorbic acid without a source of phosphate will not result in mineralization.

In the present study mineralization of nodules occurred in the phosphated titanium group when mineralizing medium was not added. However, no mineralization of nodules occurred on the control, nonphosphated surface in the absence of mineralizing medium. This finding suggests that the phosphate treatment of the titanium induced osteoblast differentiation similar to that seen with mineralizing medium. Nodule size and number did not differ among the groups, and EMD did not appear to affect mineralization. The limited number of sections for analysis warrants caution in data interpretation and points to the need for future studies to evaluate nodule formation and subsequent mineralization on the phosphated titanium surface. Such studies are being planned.

The cytokine TGF- β 1 was evaluated in this study because of its role in the regulation of bone growth and healing and its wide recognition by an array of cells in the body. TGF- β 1 is part of the TGF multifunctional polypeptide growth factor family involved in embryogenesis, inflammation, regulation of immune response, angiogenesis, wound healing, and extracellular matrix formation.^{22,36,37} In this study, TGF- β 1 was produced by osteoblasts in all groups, and EMD significantly increased initial TGF- β 1 production by osteoblasts on both phosphated and nonphosphated titanium surfaces. However, TGF- β 1 production declined rapidly over time in these groups. The transient nature of the EMD-initiated TGF- β 1 boost supports evidence for an early role for EMD similar to its role in early wound healing. The fact that EMD stimulated cells to produce TGF- β 1 is consistent with findings from other studies. Okubo and associates³⁸ evaluated the effects of EMD on human periodontal ligament cells and they found that EMD had no appreciable effect on osteoblastic differentiation but

that it did stimulate cell growth and IGF-I and TGF- β 1 production. Lyngstadaas et al³⁹ also evaluated EMD effects on human periodontal ligament cells and found that the cellular interaction with EMD generated an intracellular cyclic adenosine monophosphate (cAMP) signal, after which cells secreted TGF- β 1, IL-6 and platelet-derived growth factor (PDGF).

The present study also addressed the question of whether EMD contained endogenous TGF- β 1 that could be released into the culture media in the absence of cells. No TGF- β 1 was measured in the absence of cells. Therefore, the TGF- β 1 did not come from the EMD; rather, the EMD stimulated TGF- β 1 production by the osteoblasts. Gestrelus et al⁴⁰ reported similar results; in their study, ELISA of EMD showed that GM-CSF [Author: please spell out], calbindin D, EGF, fibronectin, bFGF, gamma-interferon, IL-1 β , 2,3,6, IGF-1, IGF-2, NGF, PDGF, TNF, and TGF- β were not present in culture medium.

The role of EMD in implant osseointegration is still not clear. Schwarz et al⁴¹ evaluated human osteoblasts on SBAE titanium with the addition of varying amounts of EMD. At low concentrations of EMD, the cell viability of groups with EMD was similar to that of groups without it, while high concentrations resulted in statistically significant increases in cell viability compared to the controls.⁴¹ In a dog study, Casati et al³¹ observed periodontal regeneration around implants. They studied animals treated with EMD, GBR, and both EMD and GBR, as well as a control group. They found that EMD alone had no statistically significant effect, but EMD plus GBR was more advantageous than any other treatment. In another study by Shimizu-Ishiyama et al,³⁰ titanium implants were placed into rat femurs with EMD or a control carrier. They found that at 14 and 30 days postimplantation the EMD group had significantly greater trabecular bone areas than the control group. Franke Stenport and associates³² also evaluated titanium implants in rat femurs with the addition of EMD. They found no beneficial effects from the EMD treatment on bone formation around titanium implants. However, they did find that the control group demonstrated significantly higher removal torque and shear force.³² The data reported here do not indicate an added advantage of EMD to the rate of mineralization but show that EMD does provide increased TGF- β 1, which may facilitate the increased cell viability noted by Schwarz et al.⁴¹

IL-1 β was evaluated in this study because of its role in exacerbating chronic inflammation and diseases. IL-1 β is a potent proinflammatory cytokine whose activity is suppressed by TGF- β 1,^{9,25} and its production by osteoblasts could be an indicator of cellular cytotoxicity. The fact that there were no sig-

nificant differences in IL-1 β production between any of the groups and no significant differences at any time periods is important because an increase in IL-1 β would indicate an adverse reaction by the osteoblasts. These data indicate that the treated titanium surfaces are not cytotoxic and are therefore potentially useful for increasing osteoblast function.

In conclusion, results from this study show that electrolytic phosphated titanium is biocompatible with osteoblasts similar to nonphosphated titanium. The increased TGF- β 1 production and nodule calcification caused by the phosphated titanium indicates that the phosphating process has the potential to accelerate implant osseointegration. EMD initiated early TGF- β 1 production but did not accelerate or initiate mineralization without the presence of phosphate, so it is unclear whether the addition of EMD is advantageous to osteoblast function in contact with titanium. Further research is needed to evaluate the long-term effects on bone formation of phosphated titanium surfaces and the role EMD plays in facilitating this process.

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