PROSTAGLANDIN E_2 RECEPTOR EXPRESSION BY OSTEOBLASTS IS MODULATED BY IMPLANT SURFACE ROUGHNESS AND PROSTAGLANDIN E_2

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DEDICATION

This thesis is dedicated to my best friend and wife, You have put more hours into supporting my studies than even I have, and you wouldn't have it any other way. You truly are a gift from God and the inspiration for all I do. I could write a(nother) thesis on that theme alone. To my daughter everything I do is out of love for you and your mother. Secondly, my deepest thanks and love to my parents who somehow managed to raise a stubborn and misbehaved boy into a disciplined and hardworking man. If I had it all to do over again, I promise I would have been less of a hassle! To hanks for your support and love. In the same way that we always have fun together, I truly cannot wait for my kids to get to play with your kids. I guess it will be at about that time that I'll know I've finally achieved adulthood.

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While the predictability of dental implants is ever-increasing, the search for a more rapid and complete healing response, and thus earlier loading, is a much sought-after goal. Of prime importance in early wound healing is the inflammatory process, of which prostaglandins play a major role. Relatively little is known about the cellular receptors for prostaglandins, EP receptors, especially with regard to osteoblast response to implant surface roughness and early events preceding osseointegration. Four EP receptors have been elucidated—EP1, EP2, EP3, and EP4. These receptors play a critical role in both priming and attenuating the inflammatory response via the cAMP-mediated pathway, and thus are likely to be very important in the mechanisms of attachment, proliferation, and differentiation on the implant surfaces. Therefore,

this study sought to further our understanding of the effects of PGE_2 on EP receptor expression by osteoblasts cultured on titanium surfaces of varying roughness. Specifically, the aim of this study was to characterize EP receptor expression as a function of both titanium surface roughness and PGE_2.

Osteoblast-like cells (MG63) were seeded onto tissue culture plastic, and smooth (polished using aluminum oxide grit; $R_a = 0.60 \pm 0.02$) or rough (coarse grit blasted cpTi; ($R_a = 4.0 \pm 0.04$) commercially pure titanium [cpTi] disks and cultured for 24 hrs. After the initial 24-hour incubation, media containing one of four concentrations of PGE_2 were added to each well: 1 nM, 10 nM, 100 nM, or control media lacking PGE_2. Cells were incubated for an additional 3, 6, or 120 hrs to simulate the early response after dental implant placement, after which they were harvested. Total RNA for each sample was extracted, purified, and quantified, and changes in mRNA for EP1, EP2, EP3, and EP4 expression were measured by real-time RT-PCR using 18S-rRNA as the housekeeping gene. Relative expression of each gene was determined by calculating the efficiency and threshold deviation of each unknown sample versus the 18S-rRNA control.

EP1 was not expressed by MG63 cells after 3 hrs on any of the surfaces examined or with any concentration of PGE_2 used. In contrast to EP1, EP2 expression decreased with time in culture on all surfaces. At 3 hrs, EP2 expression was increased on both smooth and rough Ti compared to plastic; however, there was no difference between cultures on smooth or rough Ti. By 6 hrs, EP2 expression on rough Ti was significantly higher than that on smooth Ti and plastic. After 120 hrs, there were no surface-dependent differences in expression; however, expression was significantly decreased compared to that at 3 and 6 hrs. In addition to these surface roughness and time dependent changes, PGE_2 also influenced EP2 expression. At 3 hrs,

EP2 expression was significantly increased by 1 nM PGE2 on smooth, but not rough Ti. Increasingly higher doses of PGE2 decreased EP2 expression on smooth Ti and increased expression on rough Ti. By 100 nM PGE2 there was no difference in EP2 expression on the two surfaces. A similar trend was observed in 6 hr cultures. At 120 hrs, EP2 expression was still significantly higher on smooth vs rough Ti, with 1 nM PGE_2 treatment. Increasing amounts of PGE2 increased EP2 expression on both smooth and rough Ti until, at 100 nM, expression was equivalent on the two surfaces.

Over time, EP3 expression of cultures on plastic increased by up to 5-fold at 120 hrs. On smooth Ti, EP3 expression also increased, but not to the same extent as on plastic. On rough Ti, EP3 expression was increased over plastic and smooth Ti at 3 hrs, but by 6 hrs, EP3 expression was significantly decreased compared to plastic and smooth Ti. By 120 hrs, EP3 expression on rough Ti was greater than seen at 3 hrs. In addition, EP3 expression at 120 hrs was decreased on smooth Ti compared with rough Ti and plastic. EP3 expression was also modulated by PGE_2 treatment. After 3 hrs, EP3 expression of cultures on smooth Ti was increased by approximately 15-fold with 1 nM PGE_2; increasing doses of PGE_2 were without additional effect on EP3 expression. In similar fashion, EP3 expression on rough Ti was increased by up to 4-fold with 10 nM PGE_2 treatment in a dose-dependent manner. At 120 hrs, EP3 expression on smooth Ti was still responsive to PGE_2 treatment and showed a dose-dependent effect. In addition, EP3 expression was increased by 100 nM PGE2 after 120 hrs of culture on rough Ti; lower doses of the prostanoid were without effect.

EP4 expression by osteoblasts also changed with time in culture and surface roughness. Expression on plastic was increased by 2-fold at 6 hrs and then decreased to 1.4-fold at 120 hrs. In contrast, EP4 expression on smooth Ti was elevated by 1.6-fold over plastic at 3 hrs, but then

did not change relative to plastic over time. In contrast, EP4 expression on rough Ti increased with time in culture and was maximal (2-fold) at 120 hrs. After 3 hrs treatment, cultures on rough Ti consistently displayed increased EP4 expression over those on smooth Ti in response to the same PGE2 treatment. Further, at doses of 10 and 100 nM PGE2, EP4 expression on smooth Ti was not different from the untreated control. In contrast, cultures on rough Ti demonstrated increased expression levels in response to all doses of PGE_2 with 1 nM eliciting the greatest increase. A similar trend was observed after 6 hrs of treatment with PGE_2. EP4 expression on both smooth and rough Ti were significantly increased with 1 nM PGE_2 with relatively less expression at higher PGE_2 concentrations. After 120 hrs of treatment with PGE2, EP4 expression was increased on smooth Ti cultures, but the effect was not dose-dependent. In contrast, EP4 expression on rough Ti cultures was dose-dependently increased between 1 and 10 nM PGE2. Further, expression on rough Ti was consistently higher than on smooth Ti for both 10 and 100 nM PGE2.

These results demonstrate that EP2, EP3, and EP4 receptor expression by MG63 cells is significantly affected by changes in implant surface roughness, time of culture, and PGE_2 concentration. It was noted that EP2 and EP4 expression was at least 100 times higher than that of EP3, while EP1 was not expressed at all. Prior studies have shown that EP2 and EP4 receptors mediate increases in intracellular cAMP, while EP3 receptors are involved in opposing effects. Because cAMP serves as an intracellular second messenger in a wide array of biochemical cell-signaling pathways in the osteoblast, it is likely that the effect of PGE_2 on EP receptors plays a role in the inflammatory response and dental implant healing. These results could potentially lead to future clinical applications, including pharmacologic up- or down-

regulation of EP receptor expression or potentially the modification of NSAID use immediately before or after implant placement.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

I. INTRODUCTION AND LITERATURE REVIEW

Implants in the human body, both dental and orthopedic, have revolutionized the standard of care for patients for many years. Endosseous dental implants in particular have become a primary means of restoring an oligodentulous dentition to a functional and esthetic occlusion (Vermylen et al. 2003). Implants have been successful due to osseointegration at the interface between the implant surface and bone, which has been referred to as a functional ankylosis (Schroeder et al. 1981). This ankylosis lacks a periodontal ligament, and is an interface void of fibrous connective tissue or epithelium. Osseointegration was originally defined as "a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant" (Branemark et al. 1977). The current American Academy of Periodontology definition (2001) describes osseointegration as "a direct contact, on the light microscopic level, between living bone tissue and an implant". There is, however, a twenty nanometer glycosaminoglycan layer primarily composed of chondroitin sulfate that exists between the titanium and bone (Listgarten et al. 1991). A key factor in this integration is the biocompatibility of the implant material, typically commercially pure titanium (cpTi) or a titanium alloy (Schmidt et al. 2001, Pohler 2000). It is its high biocompatibility that has made titanium the material of choice for dental implant surfaces. Titanium allows osteoblasts to inhabit and produce bone very close to its surface, allowing for the necessary proximity for integration of dental implants (Branemark et al. 1969).

The early interplay between osteoblasts and the titanium surface is in large part responsible for the eventual success or failure of the implant. The initial trauma caused at implant placement begins the wound healing process that initiates the cascade of acute

inflammation and eventually osteogenesis. Because implant success depends on adequate wound healing, a healthy inflammatory response is vital to the accomplishment of implant stability and survival.

1. The Titanium Surface

Titanium has been modified through the years to create a surface which promotes osseointegration and permanent fixation. Some of these surface modifications include microtopographical changes, such as acid-etching, sandblasting, titanium plasma spraying, and micromachining. Also, there has been much research investigating the addition of coatings such as proteins, enzymes, growth factors, and hydroxyapatite crystals, all in an attempt to create a more biologically acceptable surface environment for optimal osseointegration.

Titanium spontaneously forms a dense passive oxide layer upon contact with oxygen; this oxide is vital to cell adaptation and response to the implant surface, and is an essential component of titanium's biocompatibility and suitability as a restorative metal (Branemark et al. 1977). In titanium-aluminum-vanadium alloy (Ti-6Al-4V), it has been shown that this oxide layer has the ability to protect cells from potentially toxic alloyed elements such as vanadium (Eisenbarth et al. 2002). Similarly, this oxide layer is able to inhibit reactive oxygen species that are often released during the host inflammatory response. Titanium dioxide has the ability to react with superoxide and other free radicals, scavenging potentially harmful electrons and preventing potentially cytotoxic effects (Suzuki et al. 2003).

The two prominent forms of titanium used today, both in research and clinically, are commercially pure titanium and the alloy described above, Ti-6Al-4V. Research has shown that, assuming equal roughness, cpTi surfaces induce osteoblasts to express a more differentiated state

compared to the alloy, as expressed by increased alkaline phosphatase specific activity (Lincks et al. 1998). Johannson and colleagues (1998) demonstrated that the degree of torque required to remove dental implants in rabbit tibiae was higher in cpTi compared to Ti-6Al-4V, and that cpTi had a higher degree of bony contact compared to that of the titanium alloy. Spyrou et al. (2002) recently showed that when comparing cpTi to alloy, Saos-2 osteoblast-like cells on cpTi were more stimulated to release local factors known to activate osteoclasts, such as IL-1 and IL-6. Cells on cpTi also released the lowest levels of osteoprotegerin, a potent inhibitor of bone resorption.

Lincks et al. (1998) and others have suggested that pure titanium with a rough microtopography is likely one of the best surfaces for a dental implant. With regard to dentistry, multiple studies have clearly shown that implants with rough surfaces have higher clinical success rates than smooth surfaces (Cochran 1999). It is also known that interface shear strength of titanium dental implants is affected by surface, since rougher surfaces have a higher removal torque value, and thus improved healing and integration with the bone (Buser et al. 1999).

2. Response of Osteoblasts

The bone-forming cell of the body is the osteoblast, which is responsible for orchestrating osseointegration at the cellular level. Osteoblasts proliferate, differentiate, and begin to synthesize an osteoid matrix, which later goes on to calcify and form bone. Proliferation is measured by an increase in cell number or tritiated thymidine incorporation, while differentiation is typically measured by increases in osteocalcin and alkaline phosphatase activity. Osteoblasts rely on signals that enable them to continue along the osteoblastic phenotype; when these signals

are interrupted or begin to decrease, osteoblasts begin to express the fibroblast phenotype (Shi et al. 1996).

It is known that osteoblasts display increased adhesion in response to increased surface roughness (Bowers et al. 1992). Lincks et al. (1998) and others have found that, in addition to adhering to the rougher surfaces more tightly, cells undergo a morphological change, becoming less well spread and appearing to attach to the surface through cytoplasmic extensions. It has further been shown that titanium surface roughness modulates the ability of osteoblasts to proliferate, differentiate, synthesize their matrix (Martin et al. 1995), and produce local factors and cytokines (Kieswetter et al. 1996). As roughness increases, cell number decreases, while differentiation typically increases. Also, roughness modifies bone cell response to circulating systemic hormones, increasing responsiveness to $1\alpha,25\text{-}(OH)_2D_3$ (Boyan et al. 1998), and this effect may be mediated, in part, by prostaglandins (Batzer et al. 1998).

3. Prostaglandins

Prostaglandins were first isolated from human semen in 1933 by Ulf von Euler of Sweden, who gave them their name because he erroneously believed they were synthesized only in the prostate (von Euler 1939). Since then it has been discovered that prostaglandins are produced and act on virtually every cell in the body. Prostaglandins are a family of lipids whose chemical structure consists of an unsaturated carboxylic acid with a twenty-carbon backbone derived from γ-homolinolenic acid, arachidonic acid, or eicosapentaenoic acid. Of these, arachidonic acid is the most abundant in mammals. Prostaglandins are divided into subclasses based on the structure of the cyclopentane ring, with naturally-occurring subclasses including prostaglandins D, E, F, and I (Bos et al. 2004). The most extensively produced and studied

prostaglandins are those of the E-group, each of which contains either one, two, or three double bonds yielding PGE_1, PGE_2, and PGE_3, respectively (Vane et al. 1995). PGE_2 is the most common of the three due to the fact that the chemical structure of arachidonic acid cannot be converted enzymatically to PGE_1 and PGE_3 (Bos et al. 2004). Prostaglandins are related chemically to the other arachidonic acid metabolites, the eicosanoids and leukotrienes. The enzyme phospholipase A_2 (or diacylglycerol lipase) catalyzes the rate-limiting step in prostaglandin synthesis, liberating phospholipids from the cell membrane, and creating arachidonic acid. This acid is then converted to PGG_2, followed by peroxidization to create PGH_2, both catalyzed by cyclooxygenase (COX), and eventually converted to PGE_2. This COX pathway leads to the production of prostaglandins and eicosanoids, while an alternate pathway catalyzed by the enzyme lipoxygenase leads to the production of the leukotrienes (Kuehl et al. 1980).

The COX enzyme exists in at least two forms in humans, COX-1 and COX-2. COX-1 is expressed constitutively and is important in numerous physiological functions including vision, maintenance of gastric acidity, and platelet aggregation. COX-2, on the other hand, is responsible for the majority of inflammatory effects of prostaglandins (Bos et al. 2004).

PGE_2 induces vasodilation and upregulates a variety of proinflammatory cytokines including interleukin-1 and tumor necrosis factor-α (Williams 1979). It has different effects in different tissues, likely due to the variety of prostanoid receptor subtypes found in the respective tissues, as described in the following section. The plasma half-life of PGE_2 in the circulatory system is approximately thirty seconds and normal plasma levels are 3-12 pg/ml (Hamberg et al. 1971). The half-life *in vitro* is one minute, forty-one seconds (Garrity et al. 1984).

Prostaglandins are among the most important local factors with an autocrine/paracrine role in bone (Suda et al. 1996), and are important mediators for normal differentiation of osteoblasts (Dziak et al. 1983; Nemoto et al. 1997; Sabbieti et al. 1999). Prostaglandin E_2 (PGE_2) works bimodally: at low concentrations, PGE_2 stimulates alkaline phosphatase and osteocalcin activity, while at very high concentrations it causes inflammation and inhibits osteoblast function, while enhancing osteoclastic resorption (Boyan et al. 2001). It has been reported that in MC3T3-E1 osteoblast-like cells, PGE_2 stimulates interleukin-6 synthesis through increases in intracellular calcium ion concentration and cyclic AMP, while negatively regulating interleukin-6 through the protein kinase-C pathway, thus giving one possible mechanism for the bimodal effects of PGE_2 (Kozawa et al. 1998). When MG63 osteoblast-like cells were cultured in media containing the cyclooxygenase inhibitor indomethacin, the effect of surface roughness on cell number, osteocalcin, and latent transforming growth factor-β (TGF-β) production was abolished, responsiveness to $1\alpha,25\text{-}(OH)_2D_3$ was blocked, and alkaline phosphatase activity was reduced (Batzer et al. 1998). These effects were time-dependent. Indomethacin caused a time-dependent decrease in osteocalcin on rough surfaces, eventually eliminating the increase due to surface roughness, but having no effect on smooth surfaces. Indomethacin also decreased TGF-β_1 levels over time (Sisk et al. 2001). Clinically, cyclooxygenase inhibitors like indomethacin are used as analgesic and anti-inflammatory drugs, and have been shown to actually hinder bone formation (Cook et al. 1995; Dimar et al. 1996; Trancik et al. 1989). Thus, it is of vital importance to the field of dental implantology to investigate how prostaglandins mediate their effects at the bone-implant interface. Interestingly, no studies have looked into the effects of PGE_2 on osteoblast response to titanium surfaces. Recently, it has been shown that PGE_2 exerts

an anabolic effect on cementoblastic mineralization through activation of protein kinase C signaling (Camargo et al. 2005).

However, the effect of prostaglandins on bone production has been observed in other studies. For example, one report details that in cyanotic infants with congenital heart defects, PGE_1 was used to keep the ductus arteriosus patent. In these infants, increased cortical bone formation was observed secondary to this prostaglandin treatment (Drvaric et al. 1989). A similar effect was discovered following systemic PGE_2 administration, provoking cortical hyperostosis in an infant with the same heart defect (Jorgensen et al. 1988). In addition to effects on cortical bone, PGE_2 was shown to increase cancellous bone formation in rats after daily systemic injections (Keila et al. 2001). Prostaglandins have also been found to enhance periosteal callous formation (Keller et al. 1992), thus lending further evidence to their role in anabolic bone formation.

Marks and Miller have published a number of studies detailing the effects of PGE_1 on oral and maxillofacial bone formation. At doses of 0.5 to 2.0 mg/week, localized new bone formation occurred with mineralization and a lamellar structure (Marks & Miller 1988). They also determined that higher doses of PGE_1 resulted in mostly woven bone formation while lower doses yielded a more lamellar structure (Miller & Marks 1993). The same group later reported findings of periodontal regeneration adjacent to locally-delivered PGE_1 in beagle dogs with induced periodontal defects. This regeneration included not only increases in bone height and width, but cementum and periodontal ligament as well (Marks & Miller 1994). While dental implant placement clearly does not result in exactly the same wound healing as in periodontal defects, many of the same principles apply with respect to bony adaptation and attachment.

4. E-Type Prostanoid Receptors

Prostaglandins of the E type, such as PGE_2, exert their effects principally through specific G-protein-coupled cell surface proteins known as E-type prostanoid (EP) receptors (Narumiya et al. 1999). There are other less well-characterized receptor subclasses labeled DP, FP, IP, and TP (Kamphuis et al. 2001). Four EP receptor subtypes have been described—EP1, EP2, EP3, and EP4. Each receptor is numbered according to the subclass of PGE with the greatest affinity for the receptor, i.e. PGE_2 has its highest affinity to the EP2 receptor. However, considerable cross-reactivity can be seen between any PGE and the other numbered EP receptors (Breyer et al. 2001). EP1 increases intracellular calcium ion concentration via a phospholipase C-dependent pathway, EP2 and EP4 increase intracellular cyclic AMP levels, and EP3 decreases intracellular cyclic AMP concentration (Coleman et al. 1989). In osteoblasts, PGE_2 upregulates cyclic AMP production and causes an increase in intracellular calcium, indicating the existence of EP1, EP2, and/or EP4 in bone (Yamaguchi et al. 1989; Partridge et al. 1981). Initial characterization of *in vivo* expression of EP1, EP3, and EP4 by *in situ* hybridization showed that, in embryonic and neonatal mice, EP4 is the major form found in bone tissue, especially in preosteoblasts (Ikeda et al. 1995). Expression of EP2 has recently been demonstrated in fetal rat calvariae and long bone, suggesting a role for EP2 in addition to EP4 in the effects of PGE_2 on bone (Nemoto et al. 1997). In addition to bone, EP2 receptors have been shown to have high expression in the ileum, spleen, and liver with lower expression in the kidney, lung, heart, uterus, adrenal gland and skeletal muscle (Guan et al. 2002). In rats, EP3 receptor mRNA was most abundant in liver and kidney, EP2 receptor mRNA was most expressed in spleen, lung, and testis, and EP1 receptor mRNA transcripts were predominantly expressed in the kidney (Boie et al. 1997).

EP4 receptor activation is known to markedly stimulate bone resorption, while EP2 only enhances resorption slightly; EP1 and EP3 activation do not stimulate resorption at all (Suzawa et al. 2000). With regard to the EP1 receptor, it has been shown that, in mouse osteoblasts, PGE_2 autoamplifies its own production via this receptor's upregulation of cyclooxygenase-2. This could be important due to the biological necessity for the typically short-lived PGE_2 to maintain its presence in times of mechanical stress and wound healing, such as in dental implant osseointegration, as well as in pathological bone loss (Suda et al. 1998). Hagel-Bradway et al. (1991) found that a significant increase in calcium uptake was seen in the osteoblast cell line Saos-2 after a 5-minute incubation with 2μM PGE_2. It has also been demonstrated that the EP2 receptor mediates the effects of autocrine PGE_2 on osteocyte gap junctions in response to fluid flow-induced shear stress. The expression of the EP2 receptor, but not EP1, EP3, or EP4, increases in response to fluid flow (Cherian et al. 2003).

5. Purpose of the Current Study

The purpose of the current study was to examine EP receptor expression as a function of PGE_2 dose and time in osteoblast cultures on plastic or titanium surfaces. To do this, we cultured MG63 human osteoblast-like cells on tissue culture plastic and on smooth or rough cpTi disks. Dose and time effects were measured after addition of PGE_2 to the cultures. Messenger ribonucleic acid (mRNA) expression of each EP receptor subtype (EP1, EP2, EP3, and EP4) was measured by real-time reverse transcription-polymerase chain reaction (RT-PCR) after 3, 6, and 120 hours of culture on the different surfaces. The results of this study provide a greater understanding of how PGE_2 and surface roughness influence EP receptor expression and may lead to new pharmacologic strategies to enhance osseointegration and clinical success.

II. MATERIALS AND METHODS

1. Titanium Disk Preparation and Analysis

The titanium disks used for the experiments were manufactured from 1mm-thick sheets of grade two unalloyed pure titanium (ASTM F67 "Unalloyed titanium for surgical implant applications"), obtained from Titanium Metals Corporation (Denver, CO). The disks were fabricated to be fifteen millimeters in diameter and fit into the well of standard 24-well tissue culture plates. The titanium disks were processed to create two different levels of surface roughness.

To apply a finish to the disks, they were first washed and allowed to soak in 70% alcohol overnight. A mark was made with a band saw on one side of each disk so as not to confuse this side with the other side of the disk to be processed to a specified roughness. Using a Bunsen burner to heat a stick of modeling compound, the molten compound was used to affix eight disks to a disk holder. The disks were then polished using 180-grit silicon carbide metallographic paper (Pace Technologies, Tucson, AZ) until the surface was as smooth as possible. Next, sequential 320-, 400- and 600-grit papers were used until a uniformly smooth surface was achieved. Disks were carefully removed without scratching the polished surface. The roughness value was measured on each disk using a diamond stylus contact profilometer at a high sensitivity setting (Taylor-Hobson Surtronic 3 profilometer, Leicester, United Kingdom). The target average surface roughness (R_a) value for the smooth disks was approximately 0.60 μm. If the surface was not within this range, the disks were again polished until the correct R_a was achieved. In order to remove the modeling compound from the back sides of disks, they were

attached to three-inch masking tape, smooth side down and then uniformly sandblasted with air abrasion until all compound was removed.

The rough titanium disks were prepared to uniform smoothness as described above, followed by coarse grit-blasting with 60 grit (254 μm) white aluminum oxide (Duralum® Special White, Washington Mills, Niagara Falls, NY). When the surface reached a uniform gray tone, disks were washed in deionized distilled water, and the roughness determined as described above with profilometry. Because prior studies have shown rough implant surfaces to have R_a values of 3.97 ± 0.04, values of 4.0 ± 0.04 were attained in the current study. R_a values were not determined for the polystyrene tissue culture plastic. The disks were then ultrasonically cleaned in 70% ethanol for ten minutes, making sure that all disks were lying flat and individually without stacking. Disks were rinsed again in water, followed by ultrasonic cleaning with acetone for ten minutes, and then passivated in 40% nitric acid at room temperature for thirty minutes to produce an oxide layer with a thickness of approximately 400Å.

After preparation of the surfaces was complete, the disks were rinsed with distilled deionized water, neutralized in 5% sodium bicarbonate solution, and ultrasonically rinsed in deionized water for three five-minute periods. The disks were wrapped in sterile gauze, placed into a sterilization bag, and then autoclaved at 121°C for 30 minutes at 18 psi. Prior to culture, disks were placed under ultraviolet light for 24-48 hours. Disks were turned over once to make sure to expose both sides to the ultraviolet light. This process did not alter the surface oxide thickness. For all experiments, cells were cultured on disks placed in the wells of 24-well plates (Corning, NY). Controls consisted of cells cultured directly on the polystyrene (tissue culture plastic) surface of the 24-well plate.

2. Cell Model and Culture

MG63 osteoblast-like cells were used for these studies and they were obtained from the American Type Culture Collection (Rockville, MD). MG63 cells were originally isolated from a human osteosarcoma (Franceschi et al. 1985). These cells have been well characterized and exhibit numerous traits characteristic of an immature osteoblast, including increased production of alkaline phosphatase activity and osteocalcin synthesis in response to 1,25-$(OH)_2D_3$ (Franceschi et al. 1985, Boyan et al. 1989, Bonewald et al. 1992). Because MG63 cells exhibit enhanced osteoblastic differentiation when cultured on Ti substrates of increasing roughness (Martin et al. 1995), 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 (Schwartz et al. 1992) or smooth Ti surfaces but exhibit increased PGE_2 production as a function of surface roughness (Kieswetter et al. 1996, Lincks et al. 1998).

For the current study, cells were grown in culture media containing Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (diluted from a stock solution containing 5000 U/ml penicillin, 5000 U/ml streptomycin; GIBCO, Grand Island, NY) at 37°C in an atmosphere of 5% CO_2 in air at 100% humidity. The media were changed every 48 hours until the cells reached confluence on polystyrene.

In the first round of experiments, MG63 cells were seeded at 56,500 cells/cm^2 (≈100,000 cells/well). Cells were plated on smooth or rough titanium disks, and standard tissue culture plastic (Corning Costar, Cambridge, MA) resulting in an *n* of four for each surface. Each well received 500µl of experimental media containing PGE_2 in concentrations of 1, 10, or 100 nM, or

control media with no PGE_2. After plating, assays were performed (as described below) at time points of 3, 6, 24, and 120 hours.

In repeat experiments, the 24-hour time point was eliminated from the study due to the relative lack of an important effect at this time point when compared to the earlier and later time points. Also, cells were plated and then allowed to attach for 24 hrs before adding the experimental media containing PGE_2. Experimental media were prepared by dissolving PGE_2 (Sigma Chemical Co., St. Louis, MO) in absolute ethanol to form a 10mM stock solution, and then diluting the prostanoid in full media to a final concentration of 1, 10, and 100 nM. As above, cells were cultured for 3, 6, and 120 hours. At harvest, mRNA was isolated and EP receptor expression measured as described below.

3. Determination of EP Receptor mRNA Production

For EP1, EP2, EP3, and EP4 receptors, real-time RT-PCR was used to measure the relative ratios of the gene expression as compared to 18S-ribosomal RNA. 18S-rRNA is a housekeeping gene shown to be expressed constitutively and at steady levels regardless of experimental conditions, thus allowing it to serve as a control gene for comparison (Ullmannova et al. 2003, Schmittgen et al. 2000).

Total RNA was extracted with TRIzol reagent, made up of phenol and guanidine isothiocyanate (Gibco-BRL). After aspirating media from the culture wells, 500µl room temperature TRIzol (Invitrogen, Carlsbad, CA) was added to each well and gently agitated and allowed to stand for five minutes at room temperature. All of the TRIzol for each group was transferred to 15-ml tubes. Another 500µl TRIzol was added to the first well of each group, and transferred to the next well in the group, and the next, and so on, thus washing each well a last

time to remove all possible RNA from each well or disk. The tubes of TRIzol were then aliquotted into 1.7-ml microcentrifuge tubes. Next, 0.25ml of chloroform was added to each tube followed by vigorous shaking for fifteen seconds. The tubes were allowed to stand for three minutes, followed by centrifugation at 11,700g for fifteen minutes. The clear top aqueous phase containing the RNA was removed with a pipet and transferred to another 1.7-ml microcentrifuge tube. To the aqueous phase was added 0.625ml isopropanol and gently mixed and incubated at room temperature for ten minutes. Another spin was performed at 11,700g for ten minutes. The supernatant was decanted and the pellet washed with 0.5ml cold 75% ethanol. Finally the tubes were spun again for six minutes. The ethanol was decanted and the tubes turned upside down for two minutes on autoclaved Kim-wipes to allow as much ethanol as possible to drain out. The tubes were turned upright and the pellet allowed to air-dry for at least twenty minutes until most ethanol had evaporated. The pellet was then dissolved in 30µl diethyl pyrocarbonate- (DEPC-) treated water and stored at -80°C until RNA quantification.

All real-time PCR experiments were performed with materials provided by Applied Biosystems Inc. (Foster City, CA), including the Prism 7700 Sequence Detection System, master mix with *Taq*-polymerase, reverse transcriptase, and probe/primers. All EP receptor primers were purchased from Applied Biosystems and the specific catalog numbers and corresponding GenBank sequences were: EP1 (Hs00168752_ml, BC051286); EP2 (Hs00168754_ml, U19487); EP3 (Hs00168755_ml, D86097) and EP4 (Hs00168761_ml, L28175).

For each sample, RNA was quantified by ethidium bromide luminescence. All samples and standards were mixed with NorthernMax 10X MOPS buffer (Ambion, Austin, TX) and ethidium bromide, vortexed, and heated to 70°C for ten minutes. They were then placed on ice for three minutes, followed by loading onto a 1% agarose gel. The gel was electrophoresed at

eight volts/centimeter for thirty minutes. The gel was washed and photographed under ultraviolet light. The pictures were scanned and relative luminosities were quantified using Adobe Photoshop (Adobe Systems International, San Jose, CA). The luminosity values for the standard RNA concentrations (1, 5, 10, 50, and 100 ng human kidney RNA (Ambion, Austin, TX)) were graphed, yielding R^2 values of >0.99. RNA in samples was quantified relative to the standard curve. Fifty nanograms of RNA from each sample was then mixed with DEPC-treated water in order to bring each sample's solution to 10.625µl, and each was aliquoted into 96-well plates. In each well, 12.5µl of master mix was added, along with 1.25µl of each respective primer/probe (EP1, EP2, EP3, or EP4). Finally, 0.625µl of reverse transcriptase was added to each well, bringing the total reaction volume of each well to 25µl.

The plate was then covered, vortexed, and centrifuged for two minutes, followed by real-time PCR as follows: reverse transcription for thirty minutes at 48°C, followed by ten minutes at 95°C to deactivate the reverse transcriptase and activate the polymerase. Finally, 40 PCR cycles were run, consisting of fifteen seconds at 95°C for denaturation and melting, and one minute at 60°C for annealing and extension. The sequence detection system monitored fluorescence of the reference dyes FAM and VIC for EP receptors and 18S-rRNA, respectively. The dyes are contained in the probe/primers, fluorescing when bound to double-stranded DNA, thus allowing for quantification of the accumulation of the amplified product. The passive reference dye used was ROX. Relative quantification of each EP receptor was determined for all samples by the previously described method of Pfaffl (2001). Critical thresholds for PCR amplification were set at the point on the curve where the logarithmic increase began. Real-time PCR efficiencies were calculated for standard quantities of RNA for each EP receptor and 18S-rRNA according to the equation: $E = 10^{[-1/\text{slope}]}$. After determining efficiencies for all genes, the relative expression

ratio of each EP receptor gene was calculated based on the efficiency and threshold deviation of each unknown sample versus the 18S-rRNA control. Thus, relative ratios of each gene as compared to its control were determined.

The experiment was then repeated with some changes in order to increase the number of samples for each experimental time point, surface, and EP receptor. Cells were cultured in 24-well plates as described above. For the three-hour time point, and for each different concentration of PGE_2 in the media, cells were plated in four wells on polystyrene plastic controls, six smooth titanium disks, and six rough titanium disks. The same experimental conditions were used for the six-hour and 120-hour time points. At harvest, all cells from each group of wells were collected together for each time point and PGE_2 concentration. At the ultimate aliquotting into 96-well PCR plates, each sample was analyzed in triplicate.

EP1 receptor mRNA was not assayed in this repeat experiment due to lack of the detection of its expression in the first experiment; real-time PCR showed no expression of EP1. Control experiments were performed with human brain and kidney RNA samples (Ambion, Austin, TX) in which EP1 was detected, thus indicating that the Applied Biosystems EP1 primer was adequate for detecting human EP1 gene expression. As a result, only EP2, EP3, and EP4 were assayed in the repeated experiment.

4. Statistical Interpretation of Data

For cell culture studies, each data point represented the mean ± standard error of the mean of four individual cultures. The data was analyzed by ANOVA. Post hoc testing was performed using Bonferroni's modification of the Student's t test. Statistical significance was determined by comparing each data point to the plastic control. The same methodology was also

used for comparison between two different surface roughness characteristics. P values ≤ 0.05 were considered significant.

III. RESULTS

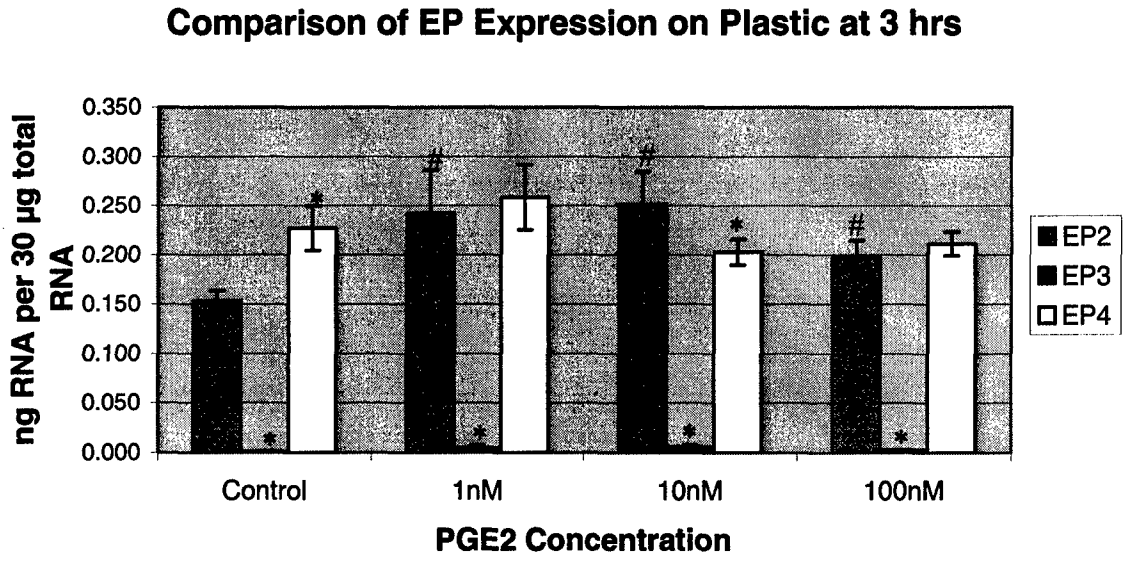
1. EP Receptor mRNA Expression on Plastic

A preliminary experiment was conducted before initiating the detailed studies to investigate EP receptor mRNA expression by MG63 cells cultured for 3 hrs on plastic (Figure 1). It was found that EP1 was not expressed, while EP2 and EP4 were expressed in robust amounts. In contrast, EP3 was expressed at significantly lower levels approaching $1/100^{th}$ that of EP2 and EP4.

These cultures were also treated for 3 hrs with varying doses of PGE_2. EP2 expression was increased by PGE_2, with a maximal effect being observed at 1 and 10 nM PGE_2. No PGE_2-dependent effect on EP3 or EP4 expression was observed.

Figure 1.

EP receptor mRNA expression by MG63 cells cultured on plastic for 3 hrs after addition of PGE_2 to the media. Values are the mean ± SEM of four cultures. EP3 was expressed at levels approaching one hundredth that of EP2 and EP4. *$P<0.05$, significantly different from EP2 within a treatment group. #$P<0.05$, significantly different from untreated (no PGE_2) control.


Comparison of EP Expression on Plastic at 3 hrs
ng RNA per 30 µg total RNA
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Control
1nM
10nM
100nM
PGE2 Concentration
EP2
EP3
EP4

2. EP1 Receptor mRNA Expression

Real-time PCR analysis showed that EP1 was not expressed on plastic or either titanium surface. Experiments demonstrating this were performed twice for verification. Specific human EP1 primers were tested on commercially available RNA from human brain and kidney tissue in order to substantiate the method and the ability of the primers to amplify authentic EP1 mRNA. Indeed, real-time PCR analysis showed significant expression of EP1 receptor in these two tissues.

3. EP2 Receptor mRNA Expression

EP2 receptor mRNA expression was found to vary with length of time in culture, surface roughness, and PGE_2 treatment (Figures 2-8).

EP2 expression varied with time in culture (Figure 2). At 3 hrs, expression was 1.7-fold higher on smooth Ti and 1.45-fold higher on rough Ti, when expression on plastic (3 hrs) was used to normalize the data. By 6 hrs, the relative pattern of expression, compared to that on plastic (3 hrs), was found to have changed. Expression on plastic was increased 1.2-fold, while that on rough Ti was increased by 1.8-fold and expression on smooth Ti was decreased by 10%. By 120 hrs, EP2 expression on all surfaces was signficantly decreased to about 30% of that found at 3 hrs. Over the time of study, EP2 expression on plastic decreased, while that on smooth Ti was relatively high at 3 hrs and then dropped off precipitously over time. In contrast, expression on rough Ti was relatively high at 3 hrs, increased to a maximum by 6 hours, and then dropped off significantly by 120 hrs.

EP2 receptor mRNA expression was sensitive to PGE_2 treatment. On plastic, EP2

expression was upregulated in a dose-dependent manner after as little as 3 hours of treatment (Figure 3). With 1nM PGE_2, expression was increased by 1.5-fold compared to untreated control. Expression was further increased by 2.5-fold by treatment with 100 nM prostanoid. After 6 hours of treatment, EP2 expression was maintained at elevated levels in response to PGE_2, but the effect was not dose-dependent. By 120 hours, expression was much less than that seen at 3 and 6 hours, but the stimulatory effect of PGE_2 was still dose-dependent and significant. EP2 expression by cells cultured on smooth Ti was also affected by PGE_2 treatment (Figure 4). In contrast to the effect seen on plastic at 3 hours, EP2 expression by MG63 cells was significantly upregulated by 3-fold with 1 nM PGE_2 and with increasing doses of prostanoid, EP2 expression decreased. At 6 hours, EP2 expression was still increased by PGE_2 but not to the same extent as seen at 3 hours. In contrast to the effect seen at 3 hours, there was a dose-dependent 5-fold increase in EP2 expression over control at 120 hours that was maximal with 100 nM. Cells on rough Ti also displayed changes in EP2 receptor expression in response to PGE_2 (Figure 5). Cells cultured for 3 hours showed up to a 2-fold increase in EP2 expression that was significant 10 nM; further increase in prostanoid concentration to 100 nM was without additional effect. After 6 hours, there was no striking difference between control and treated cultures, although cells treated with 10 nM displayed a slight decrease in expression. By 120 hours, EP2 expression was significantly reduced in the control cultures compared to expression at 3 and 6 hours. Treatment with PGE_2 dose-dependently increased EP2 expression. At 1 nM the increase was 3-fold over that seen in untreated controls; at 10 nM, the increase was 4-fold and at 100 nM, expression was increased by 8-fold.

In contrast to the above, where the expression data were presented as ratios relative to

expression on a particular surface at 3 hours (Figures 2-5), data were also calculated as ratios relative to expression in untreated controls for a specific treatment time (Figures 6-8). In all cases, the trends were very similar.

Figure 2.

Comparison of EP2 receptor mRNA expression by MG63 cells cultured for 3, 6, and 120 hrs on plastic and smooth and rough titanium surfaces. Changes in EP2 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. Most striking was a time-dependent decrease in EP2 expression on the smooth titanium surface. *$P<0.05$, significantly different from 3 hrs on plastic. #$P<0.05$, significantly different from 3 hrs on smooth Ti. ^$P<0.05$, significantly different from 3 hrs on rough Ti.

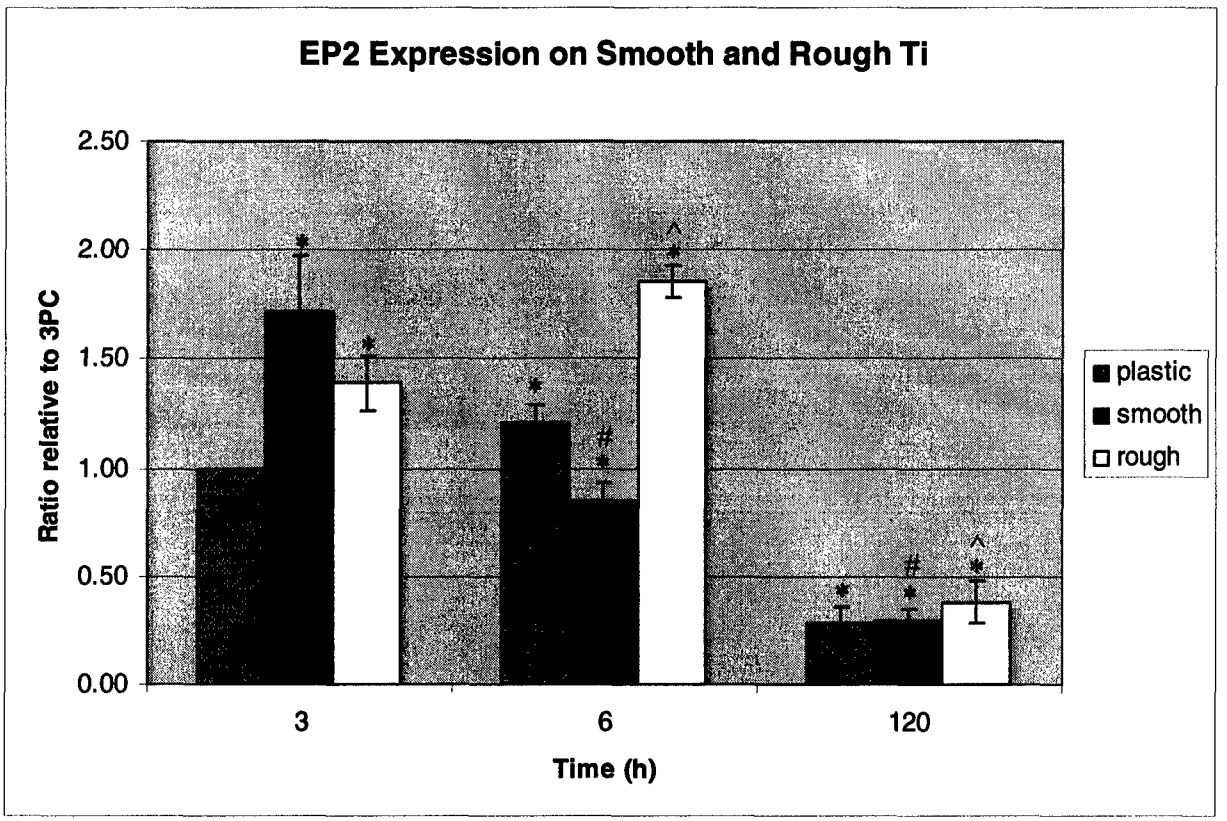

EP2 Expression on Smooth and Rough Ti
Ratio relative to 3PC
2.50
2.00
1.50
1.00
0.50
0.00
3
6
120
Time (h)
plastic
smooth
rough

Figure 3.

EP2 receptor mRNA expression by MG63 cells cultured on plastic for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120-hrs. Changes in EP2 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. There was a trend toward increasing EP2 expression as PGE_2 concentration increased in the media. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2.

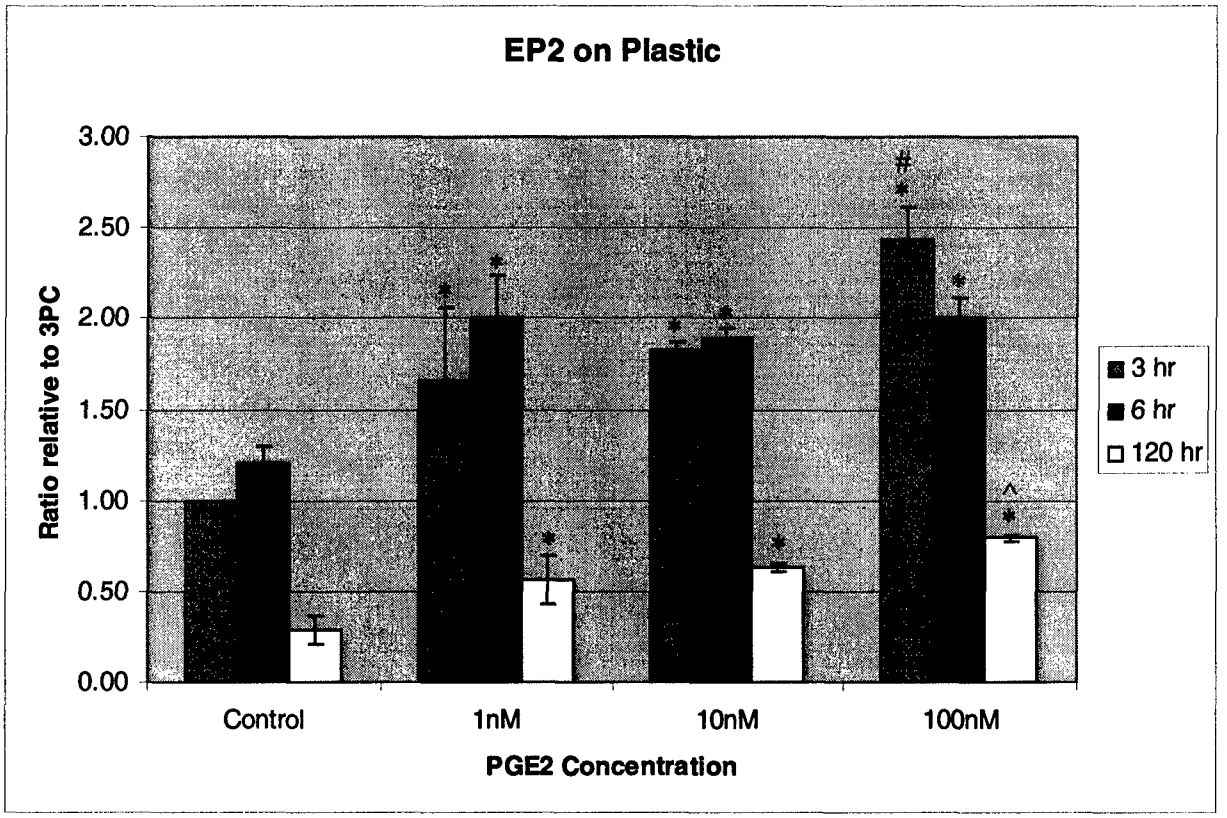

EP2 on Plastic
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ratio relative to 3PC
Control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr
*
#
^

Figure 4.

EP2 receptor mRNA expression by MG63 cells cultured on smooth Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3, 6, and 120 hrs. Changes in EP2 expression are shown as a ratio relative to expression on smooth Ti at 3hrs. Values are the mean ± SEM of four cultures. EP2 was expressed at significantly higher levels at the 3-hour time point, and decreased over time. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2.

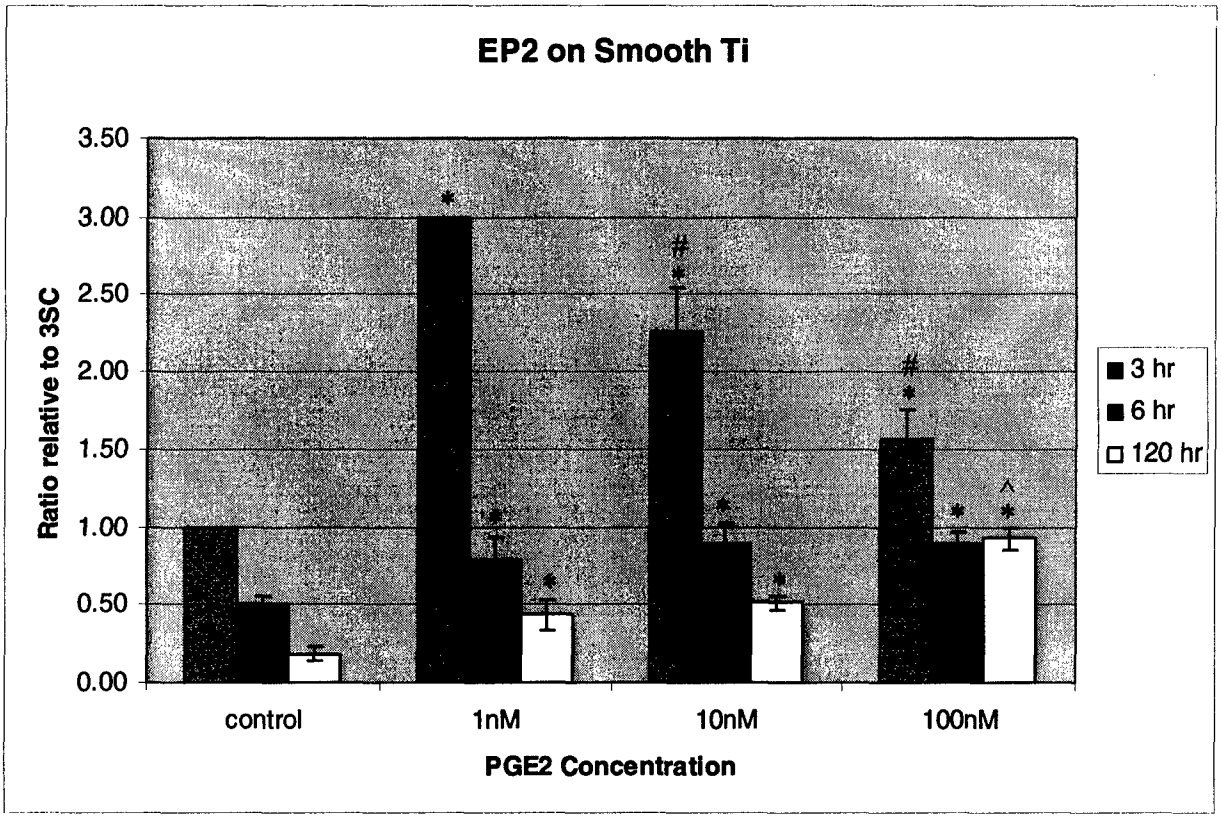

EP2 on Smooth Ti
Ratio relative to 3SC
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 5.

EP2 receptor mRNA expression by MG63 cells cultured on rough Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3, 6, and 120 hrs. Changes in EP2 expression are shown as a ratio relative to expression on rough Ti at 3 hrs. Values are the mean ± SEM of four cultures. EP2 expression was dose-dependently increased by PGE_2 in the 120-hr group. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2.

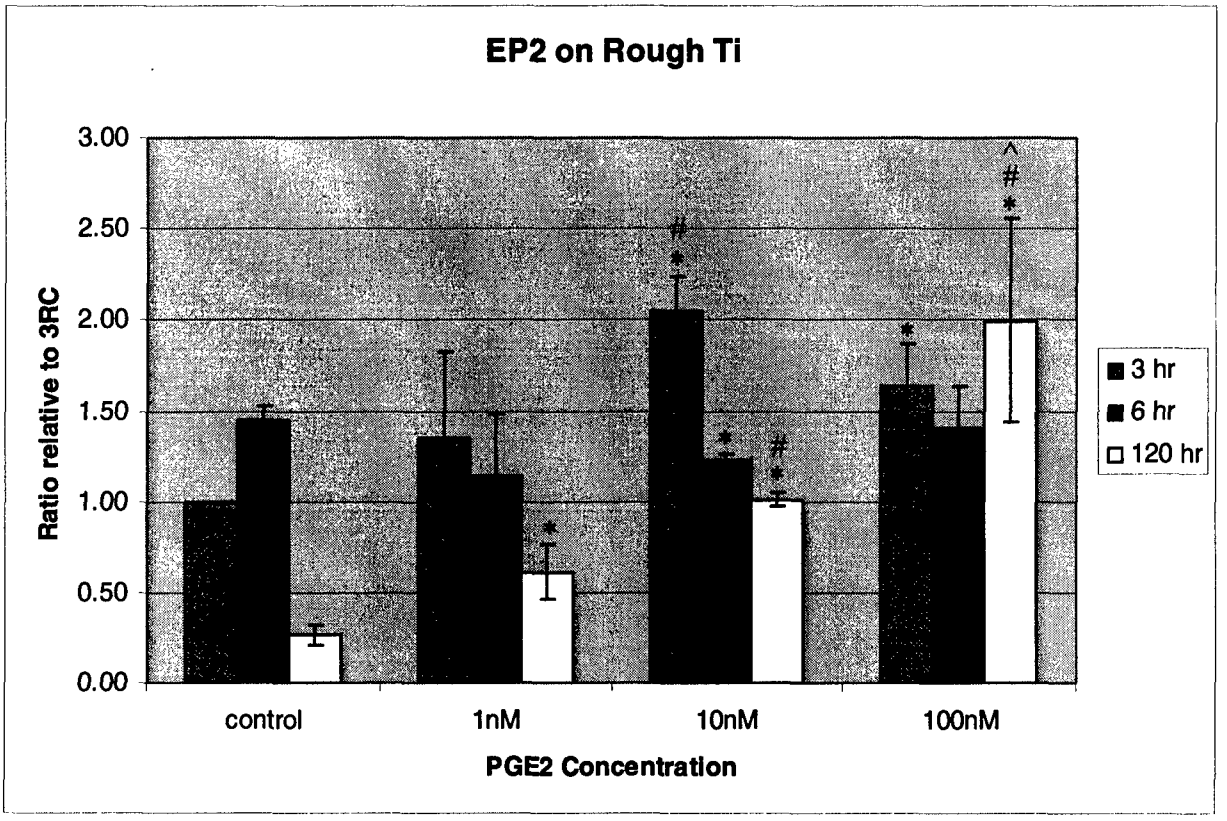

EP2 on Rough Ti
Ratio relative to 3RC
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 6.

EP2 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 3 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3 hrs. Changes in EP2 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP2 expression was maximal in cultures on smooth Ti treated with 1 nM PGE_2 and decreased steadily with increasing concentrations of PGE_2. EP2 expression on rough Ti was significantly increased at 10 nM PGE_2 and remained elevated with 100 nM PGE_2. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2.

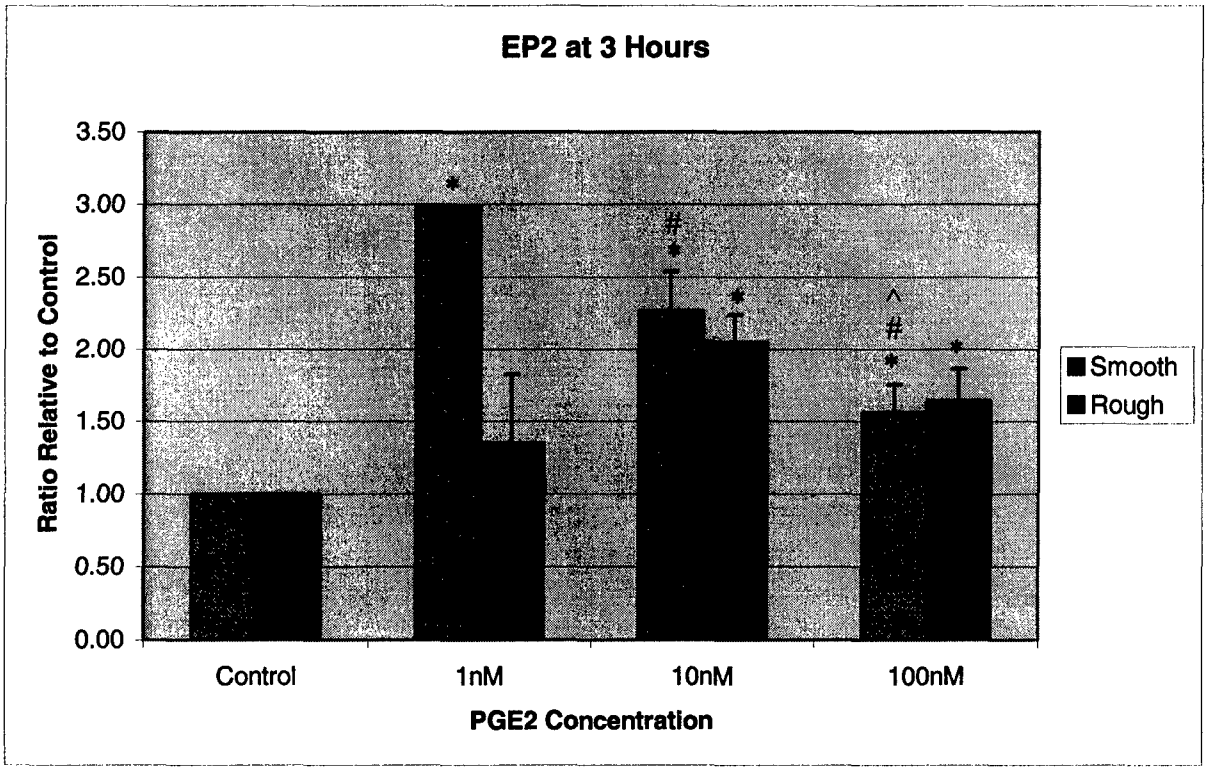

EP2 at 3 Hours
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 7.

EP2 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 6 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 6 hrs. Changes in EP2 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP2 expression was maximal in cultures on smooth Ti treated with 1 nM PGE_2 and decreased slightly with increasing concentrations of PGE_2. EP2 expression on rough Ti was not affected by PGE_2 treatment. $P<0.05$, significantly different from untreated control.

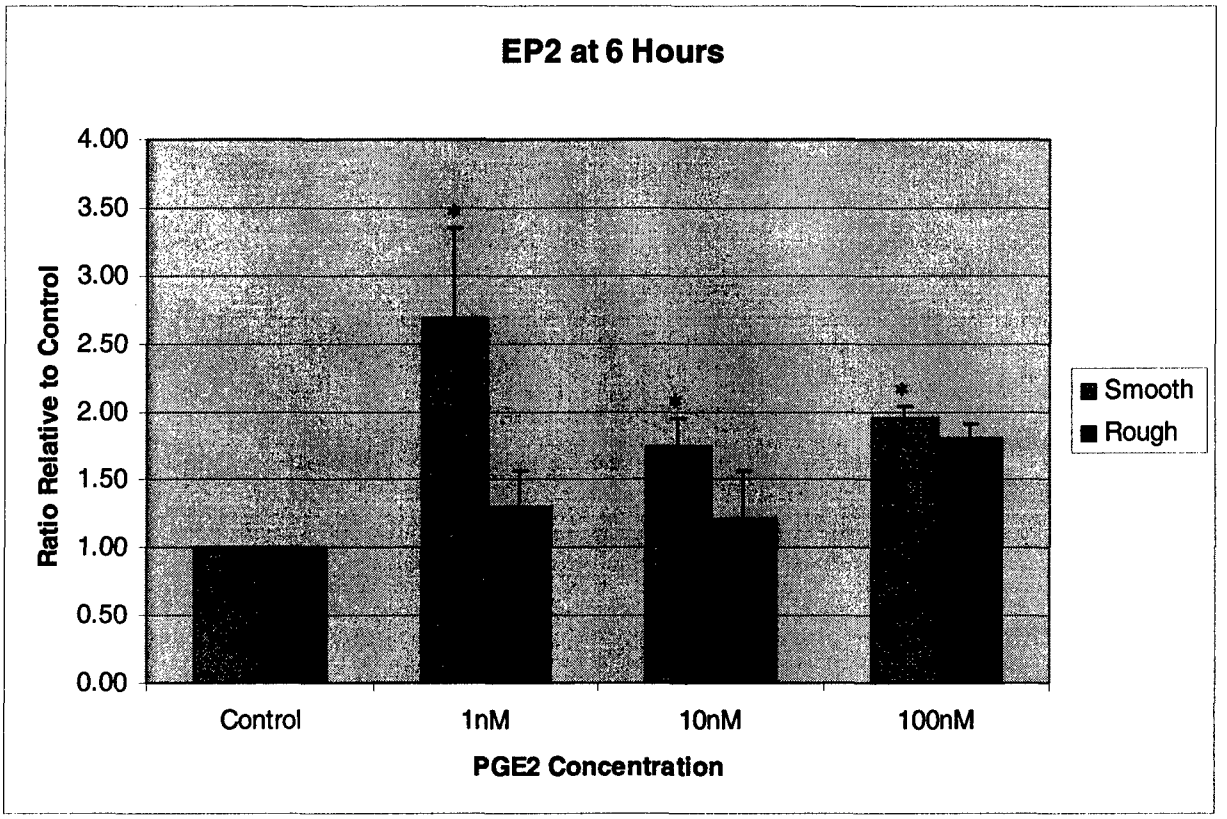
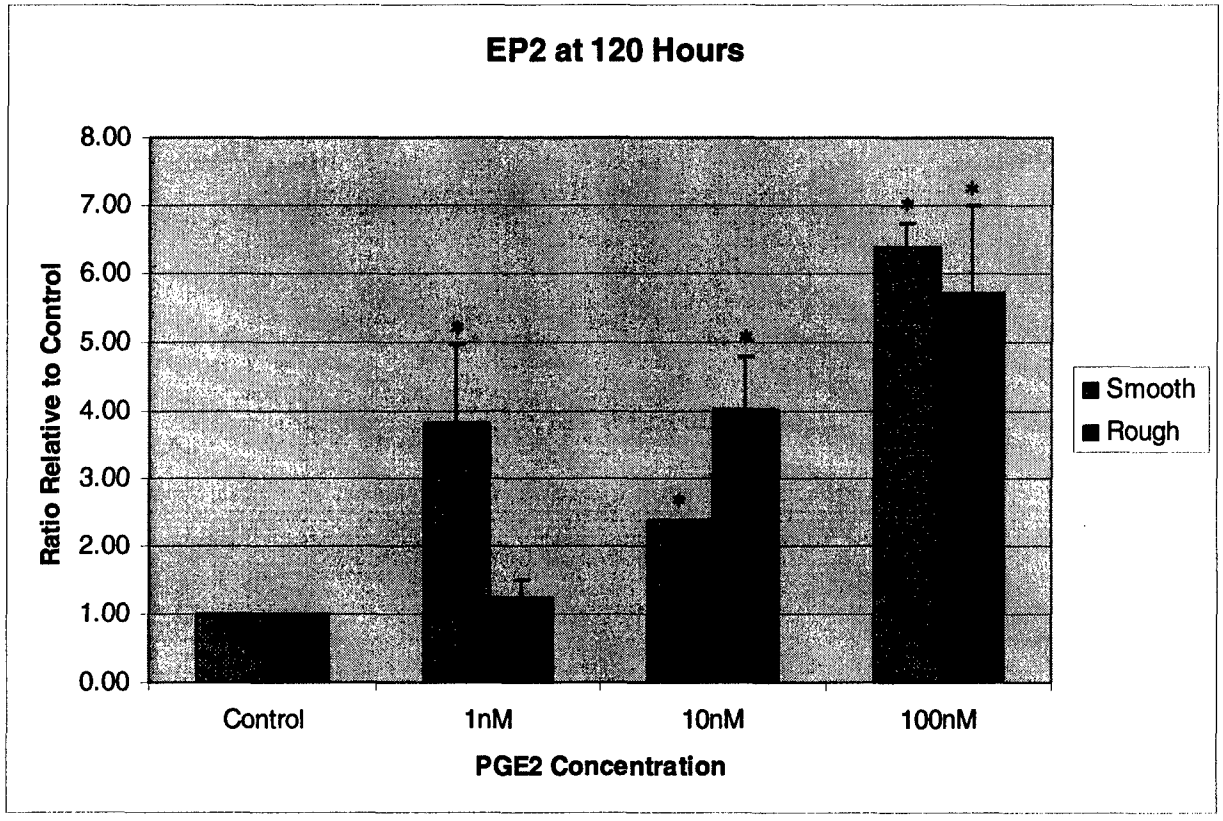

EP2 at 6 Hours
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 8.

EP2 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 120 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 120 hrs. Changes in EP2 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP2 expression was dose-dependently increased in cultures on smooth Ti with PGE_2 treatment. EP2 expression on rough Ti was similarly increased in a dose-dependent manner. $P<0.05$, significantly different from untreated control.


EP2 at 120 Hours
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

4. EP3 Receptor mRNA Expression

As observed for EP2 expression, EP3 receptor mRNA expression was found to vary with length of time in culture, surface roughness, and PGE_2 treatment (Figures 9-15).

EP3 expression varied with time in culture (Figure 9). At 3 hrs, expression was significantly increased by 1.8-fold on rough Ti compared to plastic and smooth Ti. By 6 hrs, the relative pattern had changed and expression on plastic and smooth Ti was significantly increased over that on rough Ti. With continued time in culture, EP3 expression on plastic by 120 hrs was increased compared to smooth Ti and was 5-fold higher than at 3 hrs on the same surface. EP3 expression on smooth Ti after 120 hrs was slightly increased over that at 3 hrs and was unchanged compared to 6 hrs. In contrast, expression on rough Ti at 120 hrs was increased over that on smooth Ti and increased by 50% over expression on rough Ti at 3 hrs.

EP3 receptor mRNA expression was affected by treatment with PGE_2 (Figures 10-15). On plastic, EP3 expression was increased by as much as 7-fold with 10 or 100 nM PGE_2 after 3 hrs (Figure 10). By 6 hrs, expression in the control cultures was increased by 2.3-fold over that found at 3 hrs; in contrast to what was oberved at 3 hrs, expression at 6 hrs was virtually unaffected by PGE_2 treatment, with only a slight decrease found with 10nM. Expression was significantly increased by 5-fold in controls after 120 hrs; treatment with 1nM PGE_2 was without effect, while treatment with 10 nM significantly increased expression by 7-fold, and treatment with 100 nM stimulated expression by 3.8-fold over the 3 hr control. EP3 expression by cells cultured on smooth Ti was also affected by PGE_2 treatment (Figure 11). In contrast to the time-dependent increase in EP3 expression on the plastic control, no time-dependent change in expression was observed on smooth Ti. Treatment with PGE_2 for 3 hrs increased EP3 expression

by up to 15-fold; however, the effect was not dose-dependent. After 6 hrs, EP3 expression was increased by 4.5-fold by 1 nM PGE_2; treatment with 10 and 100 nM PGE_2 did not significantly alter the expression, although it remained significantly higher than observed in the untreated controls. By 120 hrs, EP3 expression was inhibited by 1 nM PGE_2, compared with the untreated control, and unchanged by treatment with 10 or 100 nM PGE_2. Cells on rough Ti also displayed changes in EP3 expression in response to PGE_2 (Figure 12). Osteoblast expression in control cultures was unchanged at 6 hrs compared to that at 3 hrs, but was increased by almost 2-fold after 120 hrs. Treatment with 1 to 100 nM PGE_2 for 3 hrs, increased EP3 expression by 2- to 4-fold, with maximum effect at 10nM. Similarly, treatment with 10 and 100 nM for 6 hrs increased expression by 4-fold, while treatment for 120 hrs was less stimulatory with a 2.8-fold increase seen with 100 nM PGE_2.

In contrast to the above, where the expression data were presented as ratios relative to expression on a particular surface at 3 hrs (Figures 9-12), data were also calculated as ratios relative to expression in untreated controls for a specific treatment time (Figures 13-15). In all cases, the trends were very similar.

Figure 9.

Comparison of EP3 receptor mRNA expression by MG63 cells cultured for 3, 6, and 120 hrs on plastic and smooth and rough Ti surfaces. These values represent the control samples in media lacking PGE_2. Changes in EP3 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. At 3 hrs, EP3 expression was highest on rough Ti, while expression on smooth Ti and plastic were less than that on rough Ti but equivalent. A time-dependent increase in EP3 expression was found on all surfaces, with that on plastic being the greatest. *$P<0.05$, significantly different from 3 hrs on plastic. ^$P<0.05$, significantly different from 3 hrs on rough Ti.

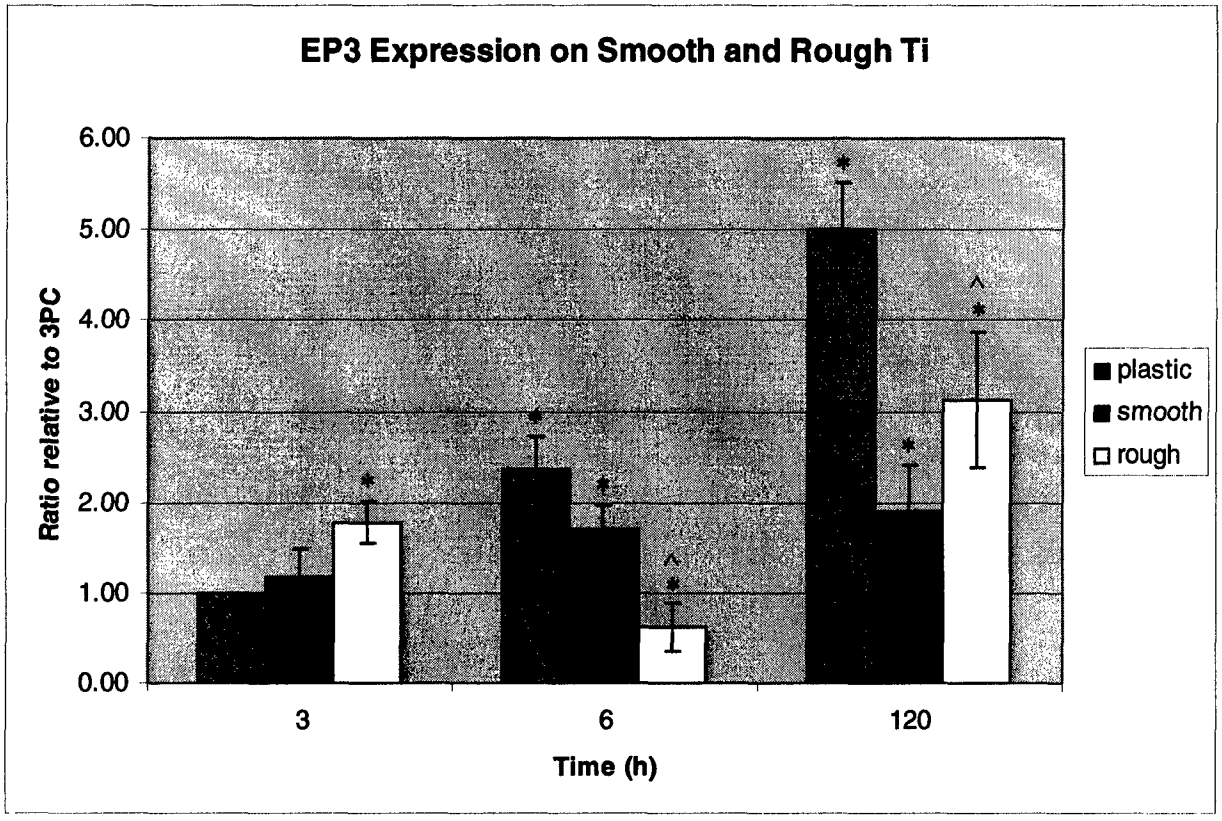

EP3 Expression on Smooth and Rough Ti
Ratio relative to 3PC
6.00
5.00
4.00
3.00
2.00
1.00
0.00
3
6
120
Time (h)
plastic
smooth
rough

Figure 10.

EP3 receptor mRNA expression by MG63 cells cultured on plastic for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP3 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. EP3 expression in the control group increased with time in culture. With 10 and 100 nM PGE_2 treatment EP3 expression was significantly increased at 3 and 120 hrs and decreased with 10 nM PGE_2 at 6 hrs. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2. °$P<0.05$, significantly different from 3 hrs.

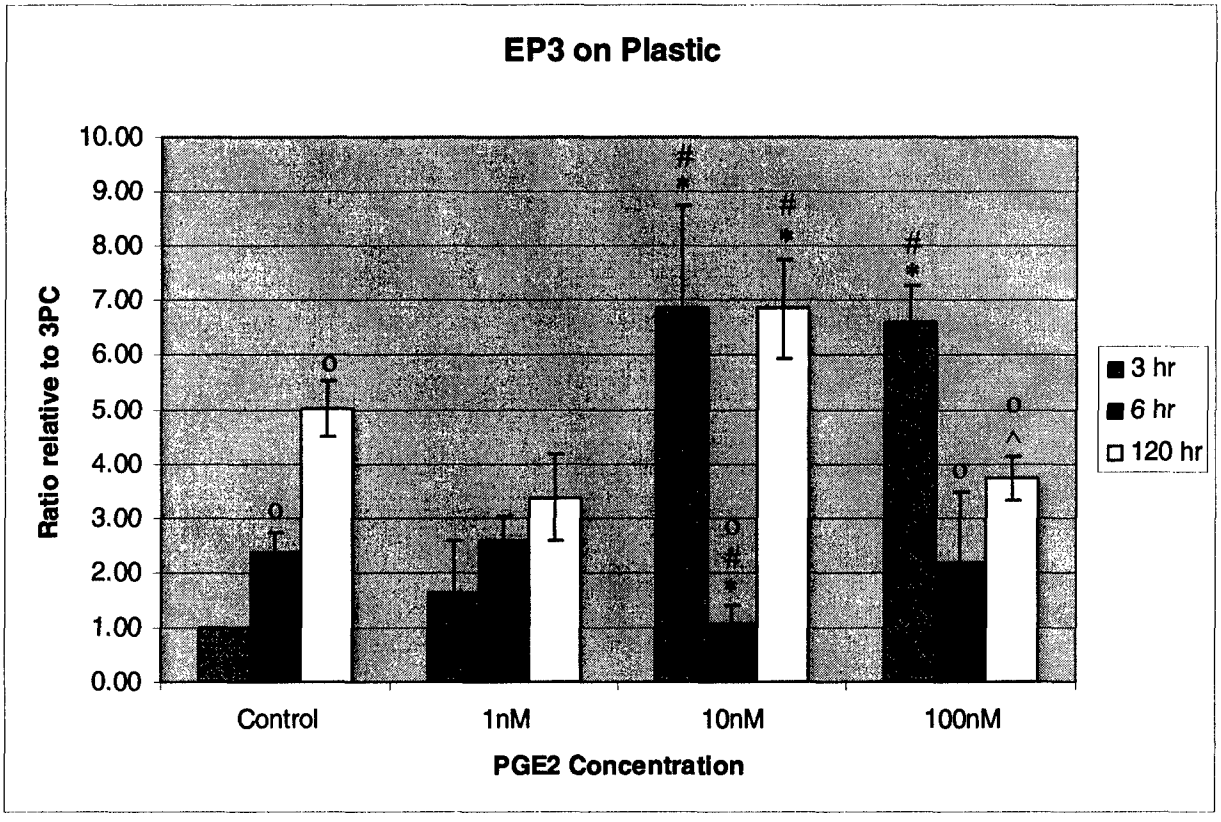

EP3 on Plastic
Ratio relative to 3PC
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 11.

EP3 receptor mRNA expression by MG63 cells cultured on smooth Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP3 expression are shown as a ratio relative to expression on smooth Ti at 3 hrs. Values are the mean ± SEM of four cultures. EP3 expression was significantly increased after 3 hrs of treatment with PGE_2, but the effect was not dose dependent. EP3 expression was also increased at 6 hrs, but not to the extent seen at 3 hrs. *$P<0.05$, significantly different from untreated control. °$P<0.05$, significantly different from 3 hrs.

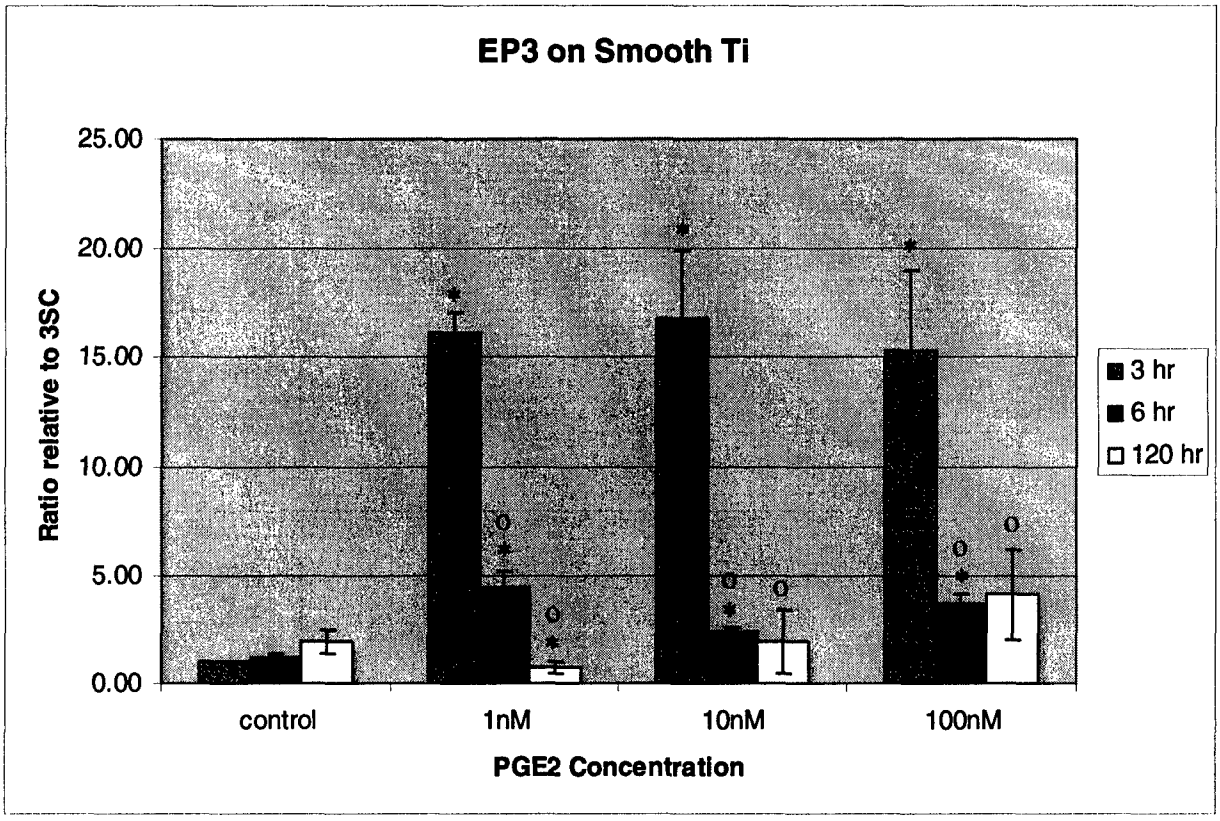

EP3 on Smooth Ti
25.00
20.00
15.00
10.00
5.00
0.00
Ratio relative to 3SC
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 12.

EP3 receptor mRNA expression by MG63 cells cultured on rough Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP3 expression are shown as a ratio relative to expression on rough Ti at 3 hrs. Values are the mean ± SEM of four cultures. EP3 expression in controls was increased at 120 hrs. PGE_2 treatment increased EP3 expression at 3 and 6 hrs, but not 120 hrs. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2. °$P<0.05$, significantly different from 3 hrs.

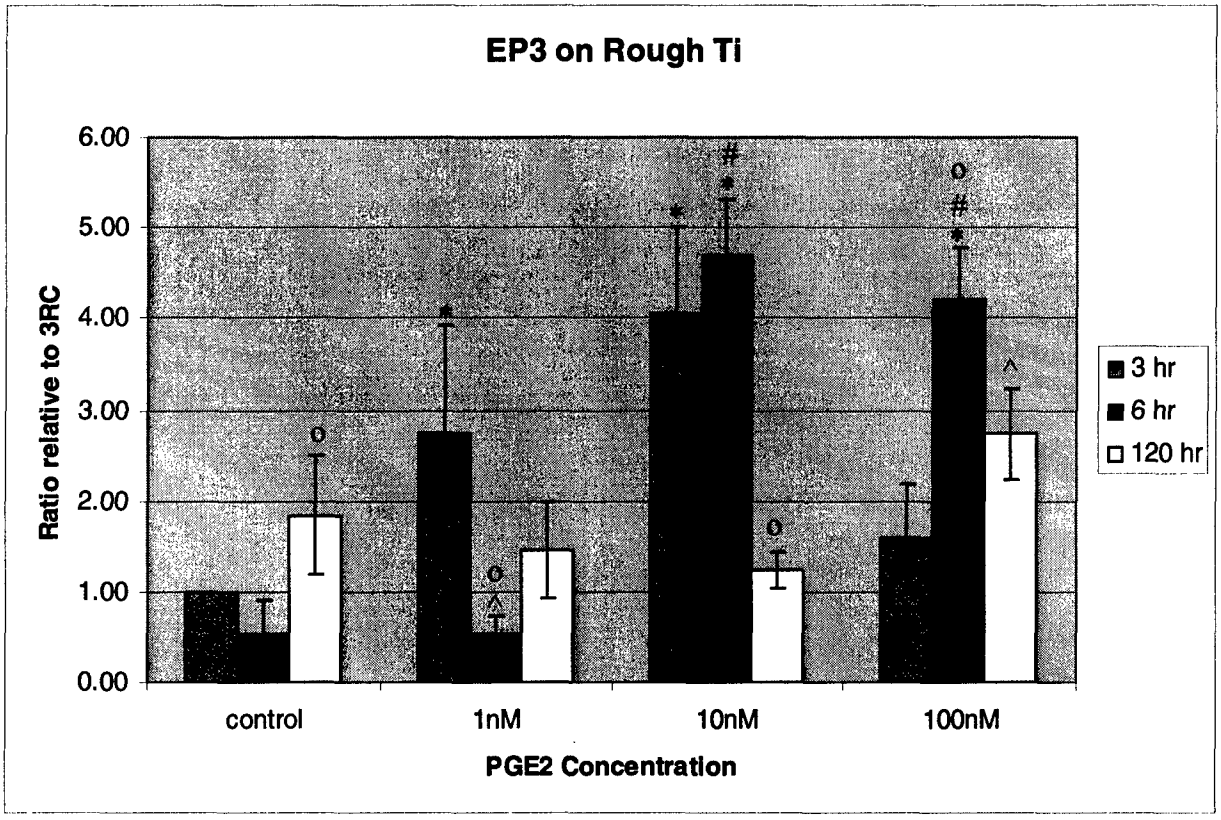

EP3 on Rough Ti
Ratio relative to 3RC
6.00
5.00
4.00
3.00
2.00
1.00
0.00
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 13.

EP3 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 3 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3 hrs. Changes in EP3 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP3 expression on smooth Ti was significantly increased by 16-fold compared to control with 1 nM PGE_2 treatment; no dose-dependent changes were observed with higher concentrations of PGE_2, although 15-fold increased expression was maintained. On rough Ti, expression was dose-dependently increased by up to 5-fold with 1 and 10 nM PGE_2; no significant change compared to control was observed with 100 nM. *$P<0.05$, significantly different from untreated control.

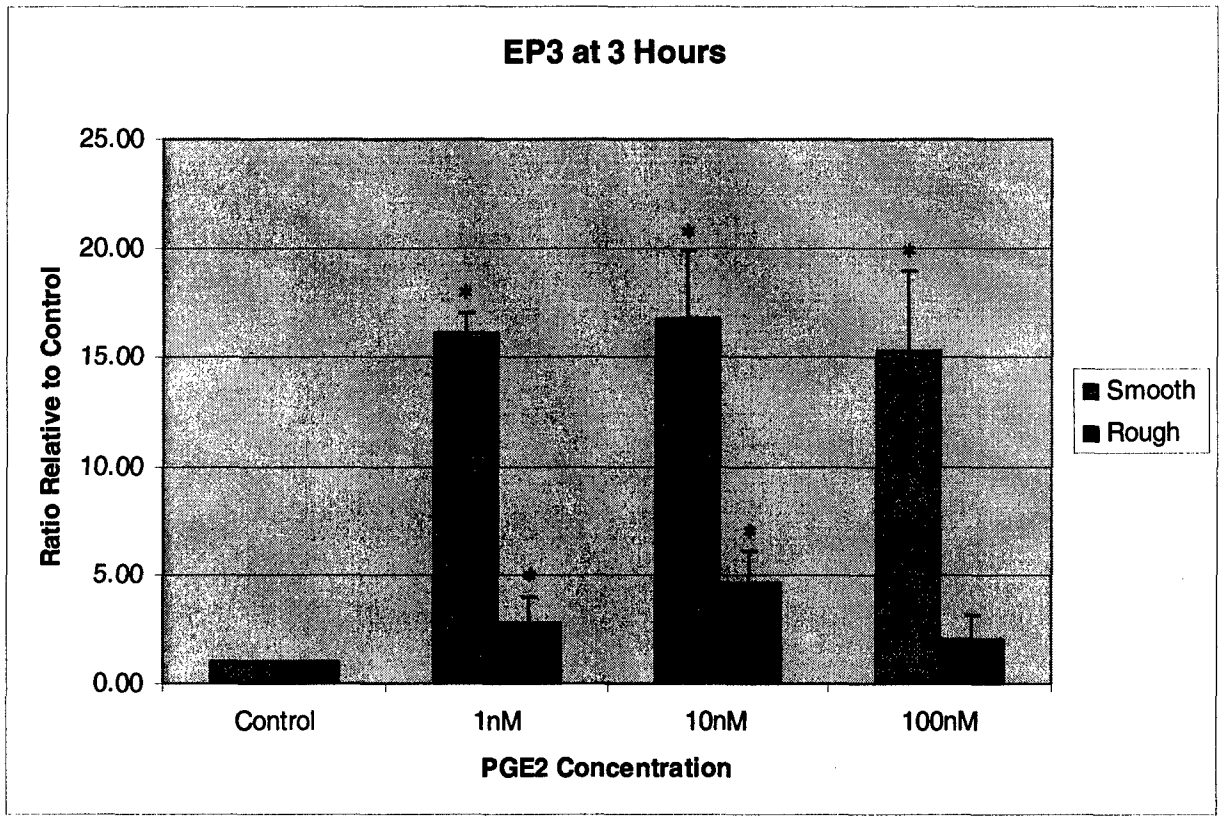

EP3 at 3 Hours
25.00
20.00
15.00
10.00
5.00
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 14.

EP3 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 6 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 6 hrs. Changes in EP3 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP3 expression on both smooth and rough Ti was increased about 3-fold with PGE_2 treatment in all cultures except for those on rough Ti treated with 10 nM PGE_2 which displayed a 16-fold increased in EP3 expression. *$P<0.05$, significantly different from untreated control.

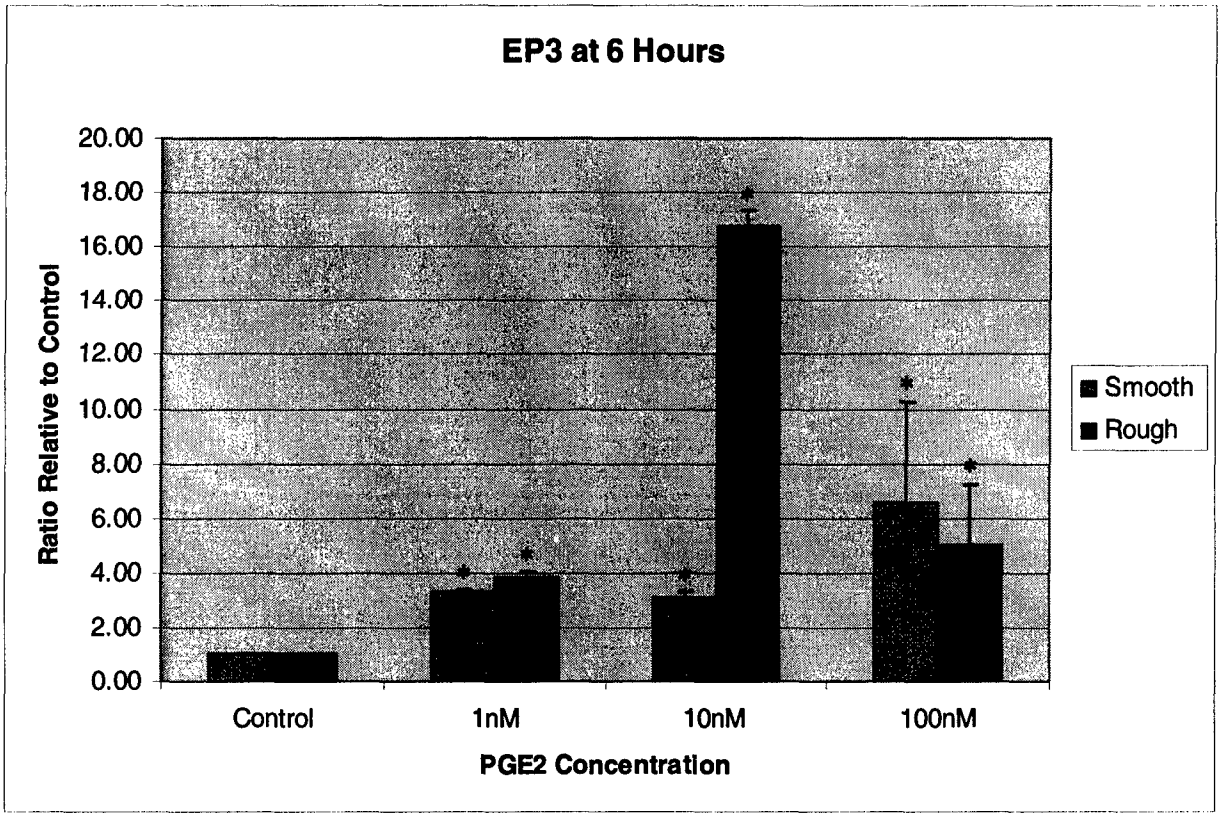
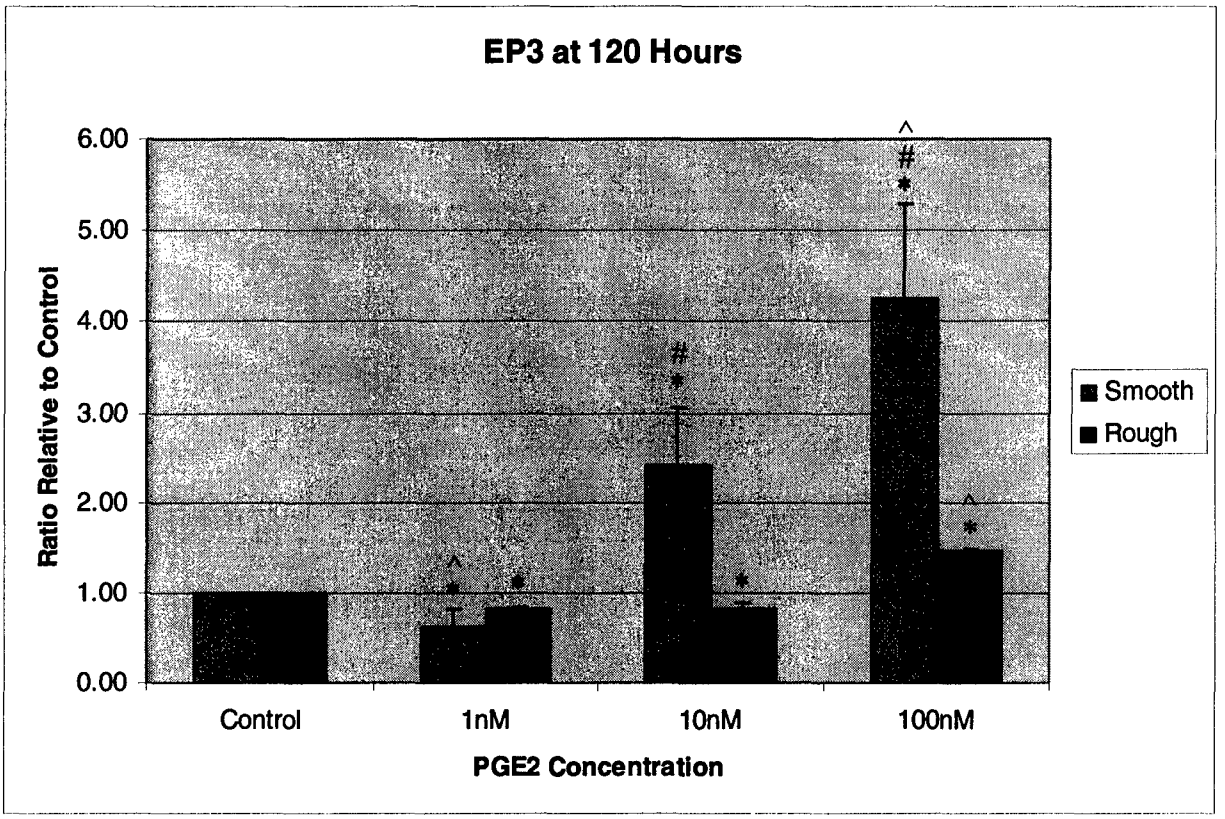

EP3 at 6 Hours
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 15.

EP3 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 120 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 120 hrs. Changes in EP3 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP3 expression on smooth Ti was decreased with 1 nM PGE_2 and dose-dependently increased by 2- and 4-fold with 10 and 100 nM PGE_2, respectively. Expression on rough Ti was inhibited to a small extent with 1 and 10 nM PGE_2 and increased with 100 nM PGE_2. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2.


EP3 at 120 Hours
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

5. EP4 Receptor mRNA Expression

As observed for EP2 and EP3 receptor expression, EP4 receptor mRNA expression was found to vary with length of time in culture, surface roughness, and PGE_2 treatment (Figures 16-22).

At 3 hrs, expression on smooth Ti was significantly higher than that on either plastic or rough Ti (Figure 16). In contrast, by 6 hrs, EP4 receptor expression was higher on plastic and rough Ti than on smooth Ti. Compared to expression at 3 hrs, the expression at 6 hrs was 2-fold higher on plastic, unchanged on smooth Ti, and increased by 1.8-fold on rough Ti. At 120 hrs, EP4 expression on rough Ti was significantly higher than that on plastic; compared to expression on plastic at 3hrs, there was a 2-fold increase on rough Ti. When viewed across the entire time course, EP4 expression increased by 2-fold with time in culture on rough Ti, remained constant on smooth Ti, and displayed a peak level of expression (2-fold) at 6 hrs on plastic.

EP4 expression was affected by PGE_2 treatment (Figures 17-22). On plastic, EP4 mRNA expression was equivalent in all treatment groups after 3 hrs, except for those treated with 100 nM PGE_2 (Figure 17). By 6 hrs, expression in control cultures was increased 2-fold compared to that at 3 hrs; with increasing amounts of PGE_2, expression was increased with 1 nM, but none of the other doses. In contrast to the 3 and 6 hr cultures, PGE_2 treatment dose-dependently increased EP4 receptor expression by up to 3.4-fold (100 nM treatment group) in the 120 hr cultures. PGE_2 treatment of cultures on smooth Ti also affected EP4 receptor expression in a time-dependent manner (Figure 18). At 3 hrs, EP4 expression was increased 1.7-fold by 1 nM PGE_2; none of the other doses of prostanoid affected EP4 expression. By 6 hrs, the effect seen with 1 nM was no longer apparent and there were no differences observed between the different

treatment groups. As seen with cultures on plastic, treatment of MG63 cells for 120 hrs with PGE_2 resulted in a dose-dependent, 2-fold increase in EP4 expression that was maximal at 100nM. Cultures on rough Ti also displayed changes in EP4 expression (Figure 19). At 3 hrs, EP4 expression was significantly increased by PGE_2 treatment; at 1nM, a maximal 2.3-fold increase was observed. A similar pattern was observed with 6 hrs of treatment. In contrast, after 120 hrs of treatment, a dose-dependent increase with PGE_2 was observed; with 10 and 100 nM, 2.8- and 3.1-fold increases, respectively, were observed.

In addition to the above, where changes in expression were calculated in relation to expression on plastic at 3 hrs, the data were also calculated as ratios relative to expression in untreated controls for a specific treatment time (Figures 20-22). In all cases, trends were very similar.

Figure 16.

Comparison of EP4 receptor mRNA expression by MG63 cells cultured for 3, 6, and 120 hrs on plastic and smooth and rough Ti surfaces. These values represent the control samples in media lacking PGE_2. Changes in EP4 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. At 3 hrs, EP4 expression was highest on smooth Ti. At 6 hrs, expression on plastic and rough Ti were increased over that seen at 3 hrs, although there was no change observed with cultures on smooth Ti. By 120 hrs, EP4 expression on rough and smooth Ti was increased over plastic. *$P<0.05$, significantly different from 3 hrs on plastic. ^$P<0.05$, significantly different from 3 hrs on rough Ti. °$P<0.05$, significantly different from 3 hrs on smooth Ti.

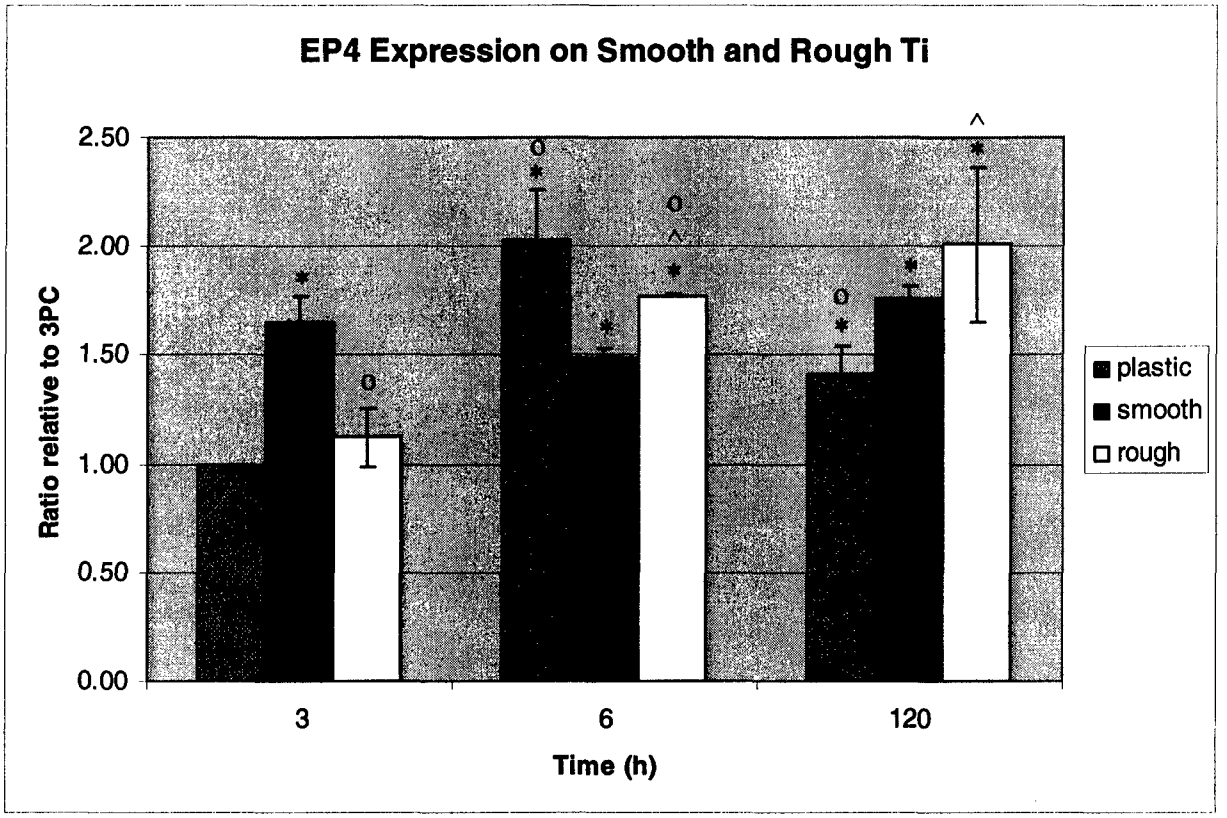

EP4 Expression on Smooth and Rough Ti
Ratio relative to 3PC
2.50
2.00
1.50
1.00
0.50
0.00
3
6
120
Time (h)
plastic
smooth
rough

Figure 17.

EP4 receptor mRNA expression by MG63 cells cultured on plastic for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP4 expression are shown as a ratio relative to expression on plastic surfaces at 3 hrs. Values are the mean ± SEM of four cultures. EP4 expression in the control group was increased by 2-fold after 6 hrs, and 1.5-fold after 120 hrs. PGE_2 dose-dependently increased expression in the 120 hr treatment group. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2. °$P<0.05$, significantly different from 3 hrs.

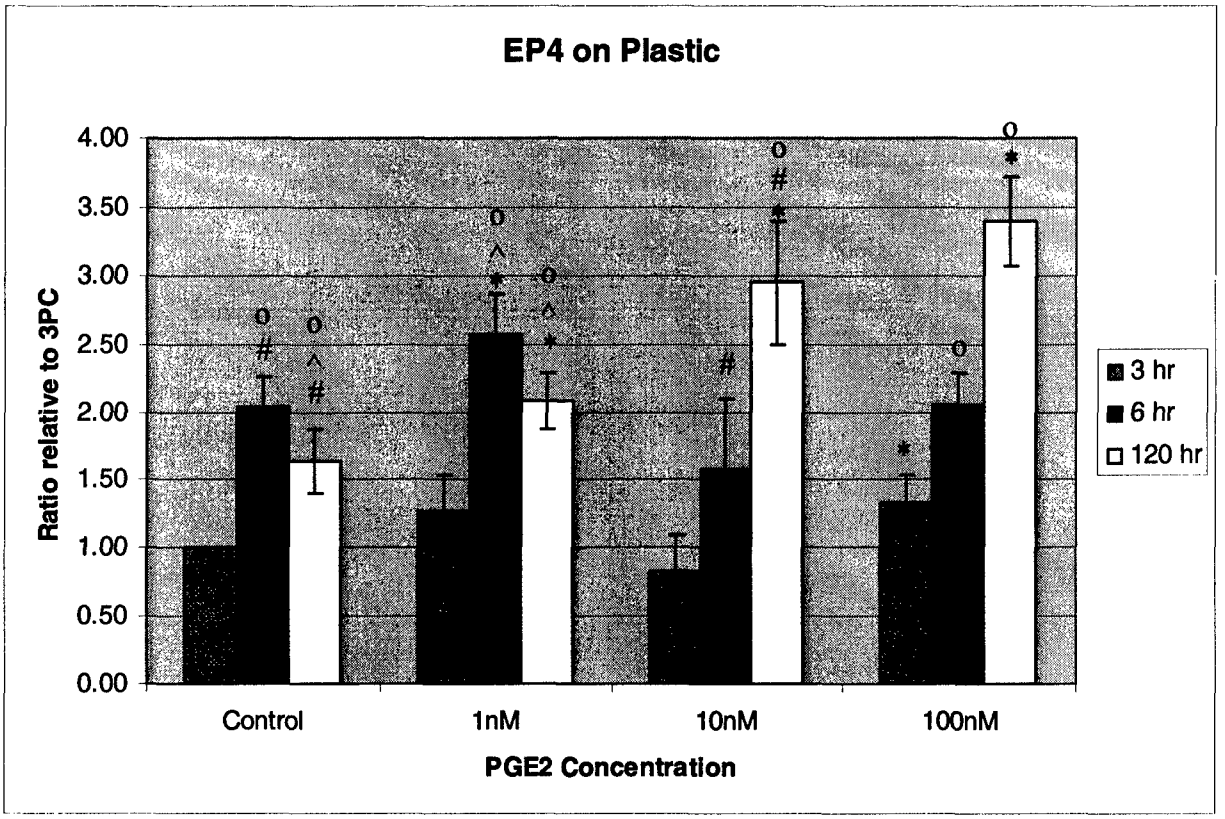

EP4 on Plastic
Ratio relative to 3PC
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 18.

EP4 receptor mRNA expression by MG63 cells cultured on smooth Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP4 expression are shown as a ratio relative to expression on smooth Ti at 3 hrs. Values are the mean ± SEM of four cultures. There were no differences in EP4 expression in the control cultures over time. EP4 expression was affected by PGE_2 treatment; 1 nM PGE_2 but none of the other doses increased expression after 3 hrs, while there was a dose-dependent increased seen after 120 hrs of treatment. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2. °$P<0.05$, significantly different from 3 hrs.

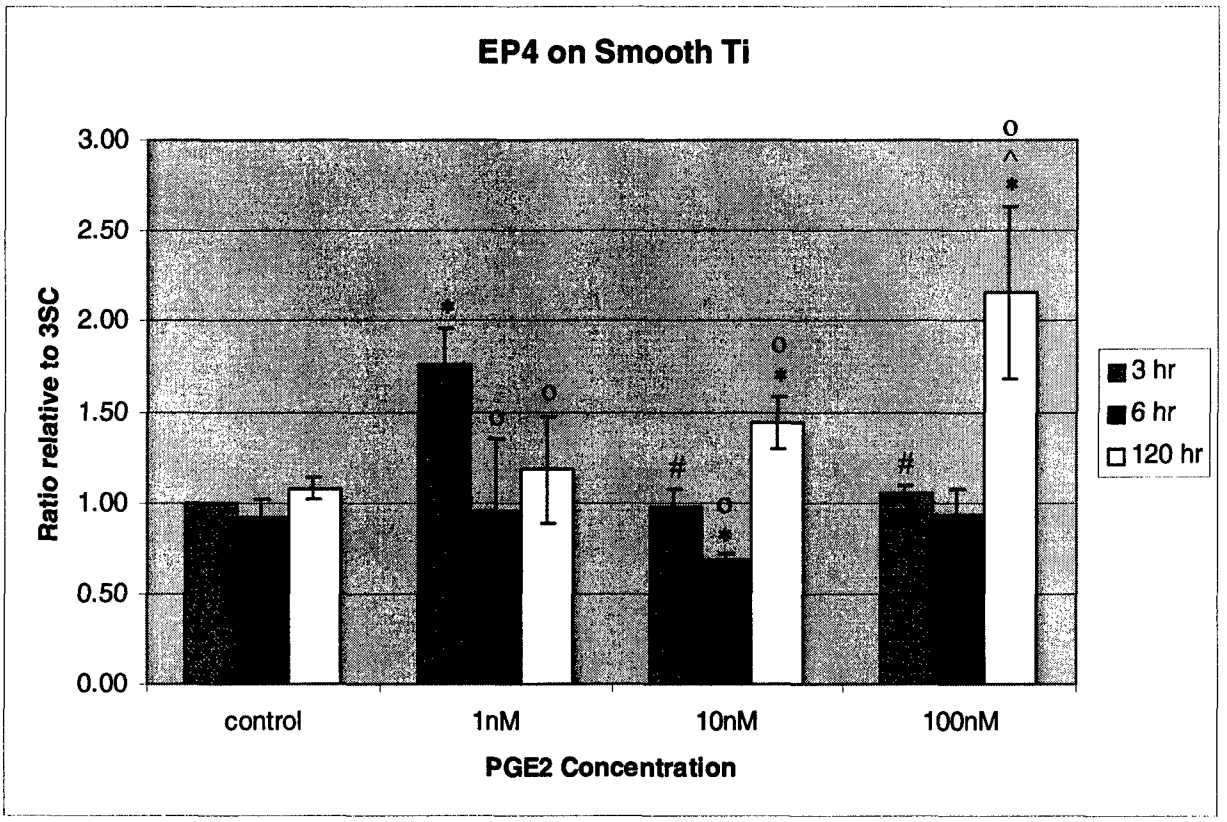

EP4 on Smooth Ti
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ratio relative to 3SC
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 19.

EP4 receptor mRNA expression by MG63 cells cultured on rough Ti for 3, 6, and 120 hrs in the presence and absence of 1, 10, or 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3-, 6-, and 120 hrs. Changes in EP4 expression are shown as a ratio relative to expression on rough Ti at 3 hrs. Values are the mean ± SEM of four cultures. EP4 expression in control cultures increased over time. 1 nM PGE_2 treatment increased EP4 expression at all time points; higher concentrations for 3 or 6 hrs were generally inhibitory, while at 120 hrs, the higher doses of PGE_2 stimulated increased expression. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. ^$P<0.05$, significantly different from 10 nM PGE_2. °$P<0.05$, significantly different from 3 hrs.

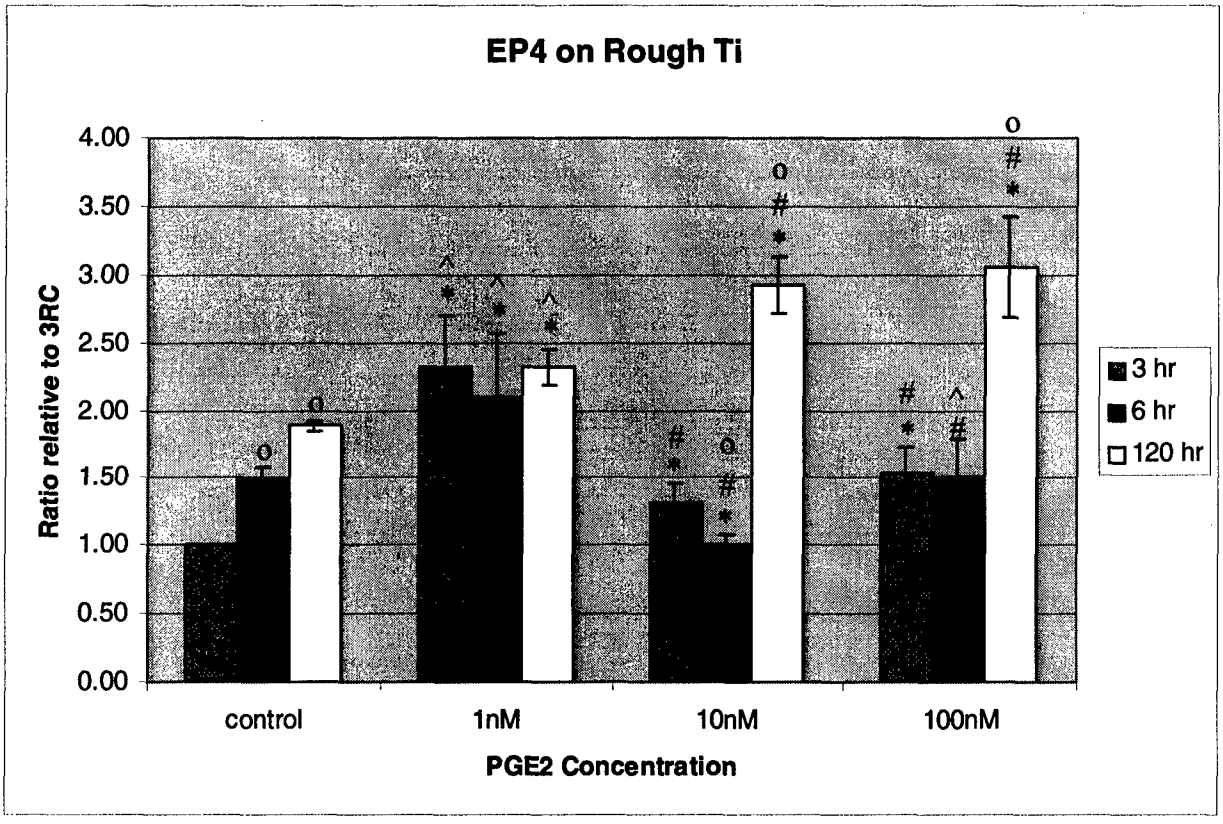

EP4 on Rough Ti
Ratio relative to 3RC
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
control
1nM
10nM
100nM
PGE2 Concentration
3 hr
6 hr
120 hr

Figure 20.

EP4 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 3 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 3 hrs. Changes in EP4 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. EP4 expression was significantly increased by treatment with 1 nM PGE_2; higher doses of PGE_2 reduced expression, although there was a significant difference in expression on smooth and rough Ti at these higher doses. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. Δ$P<0.05$, significantly different from smooth Ti.

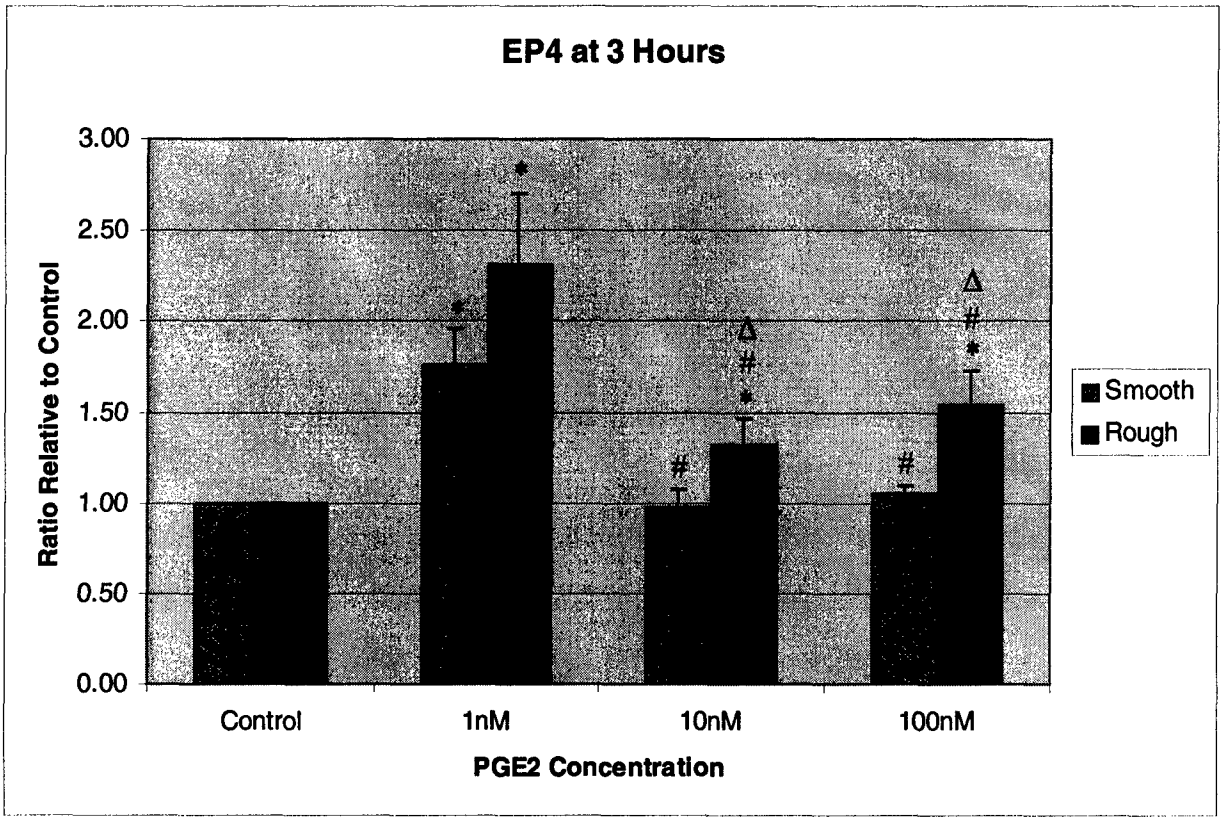

EP4 at 3 Hours
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 21.

EP4 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 6 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 6 hrs. Changes in EP4 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. As seen at 3 hrs, EP4 expression was significantly increased by treatment with 1 nM PGE_2; higher doses of PGE_2 reduced expression. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2.

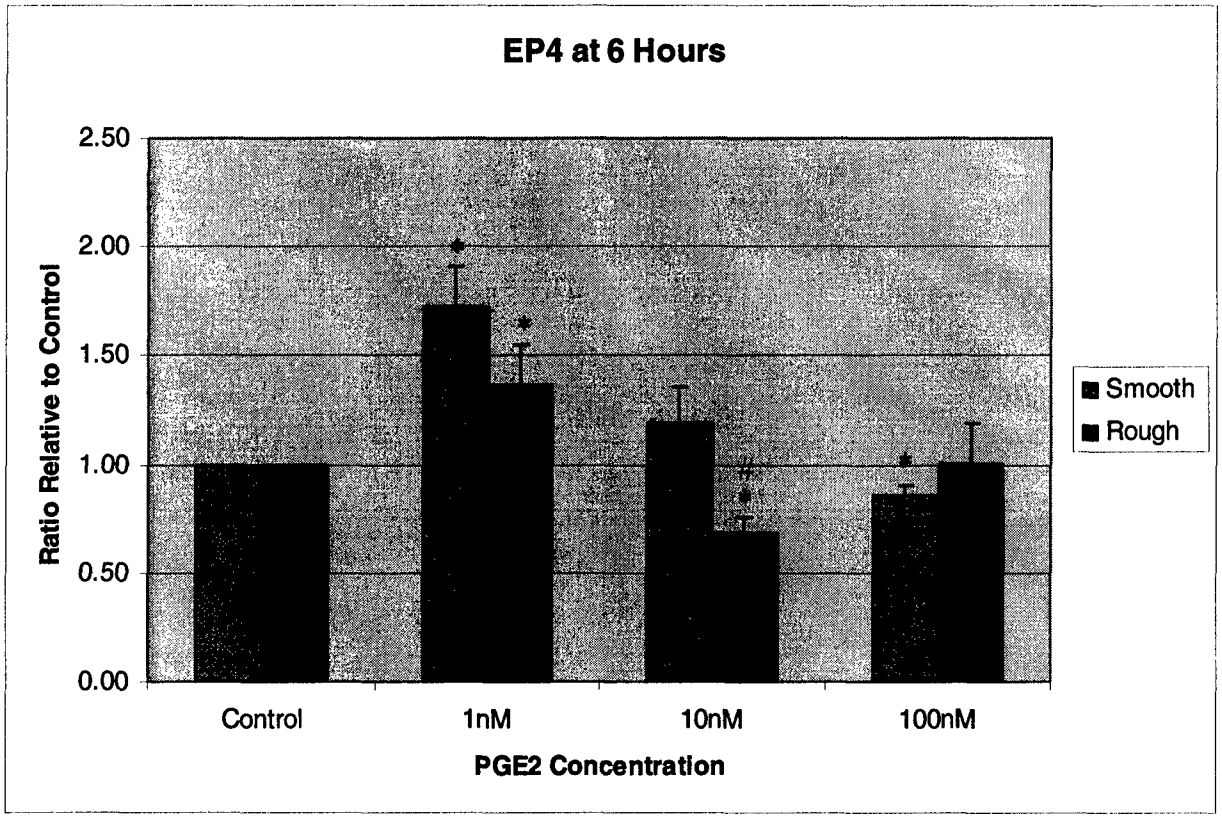
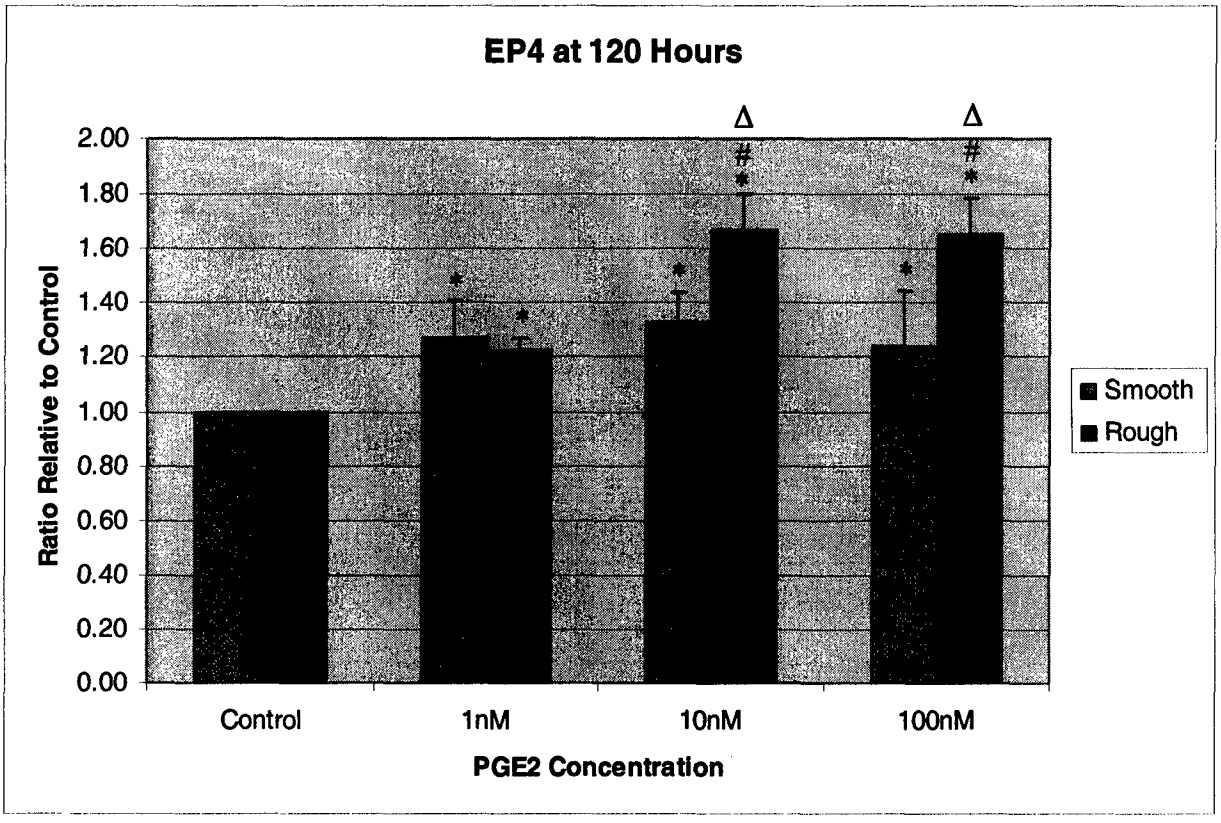

EP4 at 6 Hours
2.50
2.00
1.50
1.00
0.50
0.00
Ratio Relative to Control
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough

Figure 22.

EP4 receptor mRNA expression by MG63 cells cultured on smooth and rough Ti for 120 hrs in the presence and absence of 1, 10, and 100 nM PGE_2. After seeding, the cultures were incubated for 24 hrs to allow for cell attachment. Media containing PGE_2 or vehicle were then added and the incubation continued for 120 hrs. Changes in EP4 expression with PGE_2 treatment are shown as a ratio relative to expression in control cultures. Values are the mean ± SEM of four cultures. *$P<0.05$, significantly different from untreated control. #$P<0.05$, significantly different from 1 nM PGE_2. Δ$P<0.05$, significantly different from smooth Ti.


EP4 at 120 Hours
Ratio Relative to Control
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control
1nM
10nM
100nM
PGE2 Concentration
Smooth
Rough
Δ
Δ
#
#
*
*
*
*
*
*

IV. DISCUSSION

The clinical success of an implant is largely determined by the initial interactions which occur between the implant and the surrounding tissue. Successful osseointegration, therefore, is dependent on the ability of the implant to attract and promote the differentiation of osteogenic cells which will form new bone. Also involved in this complex process are autocrine, paracrine, and endocrine factors that work in concert with implant surface chemistry, energy, topography, and roughness to promote bone formation (Lincks et al. 1998, Schwartz and Boyan 1994).

Our laboratory has focused on examining the role of surface roughness in promoting osteoblast differentiation. Others have demonstrated that *in vivo* rough surfaces demonstrate significantly better bone fixation than smooth surfaces (Buser et al. 1991, Carlsson et al. 1988, Thomas and Cook 1985), suggesting that rough surfaces may have a direct effect on osteoblast attachment, proliferation, and differentiation. Our work and the work of others has supported this suggestion. Osteoblasts exhibit greater initial attachment to rough Ti surfaces (Kasemo and Lausmaa 1988, Lohmann et al. 2000, Michaels et al. 1989). In addition, osteoblast proliferation, differentiation, and local factor production are affected by surface roughness. MG63 cells exhibit decreased proliferation (cell number and [^{3}H]-thymidine incorporation), increased differentiation (alkaline phosphatase specific activity), and increased local factor production (PGE_2 and transforming growth factor-beta, TGF-β) with increasing surface roughness (Martin et al. 1995, Kieswetter et al. 1996, Boyan et al. 2001). Kajii et al. (1999) described how indomethacin increased alkaline phosphatase activity and the accumulation of mineralized tissue MC3T3-E1 osteoblast-like cells by inhibiting the production of PGE_2.

These latter data suggest how an implant may control the osseointegrative process. By regulating the production of local factors involved in bone formation, such as PGE_2 and TGF-β, the implant can participate in bone formation. In addition to the local control just mentioned, bone formation is also influenced by systemic factors. Prior studies have shown that surface roughness modulates osteoblast response to $1,25\text{-}(OH)_2D_3$ (Boyan et al. 1998) and 17β-estradiol (Lohmann et al. 2002). Treatment of MG63 cells with $1,25\text{-}(OH)_2D_3$ causes a synergistic increase in markers of differentiation, such as osteocalcin and alkaline phosphatase specific activity, with surface roughness. Furthermore, production of PGE_2 and TGF-β on plastic and smooth Ti surfaces was unaffected by treatment with the hormone, while that on the rough Ti surface was synergistically increased.

The increased production of PGE_2 by osteoblasts cultured on rough implant surfaces is of great significance because prostaglandins are important mediators of osteoblast differentiation (Dziak et al. 1983, Nemoto et al. 1997, Sabbieti et al. 1999, Suda et al. 1996). Prior studies have demonstrated that osteoblast response to PGE_2 is concentration-dependent. At low concentrations, PGE_2 stimulates alkaline phosphatase activity and osteocalcin production, while at high concentrations it causes inflammation, inhibits osteoblast function, and enhances osteoclastic resorption. For this reason, the synthesis of PGE_2 in the local environment around an implant may be of critical importance to clinical success.

The synthesis of prostaglandins occurs through the action of prostaglandin H_2 (PGH_2) synthase on arachidonic acid which is released from membrane phospholipids by phospholipase A_2. PGH_2 synthase consists of two enzymes, cyclooxygenase (Cox) and peroxidase. Cox is responsible for the conversion of arachidonic acid to PGG_2, which is then metabolized by the

peroxidase to form PGH_2. PGH_2 is then converted to prostaglandins, prostacyclins, and thromboxanes. (Kulmacz 1998, Filizola et al. 1997).

Although cyclooxygenase inhibitors such as indomethacin are widely used to treat postsurgical pain and prevent heterotopic ossification (Knelles et al. 1997, Amstutz et al. 1997), inhibition of prostaglandin production can lead to increased bone formation as well (Bonewald et al. 1997). At the same time, decreased prostaglandin production has also been shown to have a deleterious effect on bone growth. In a rat spinal fusion model, indomethacin significantly decreased the rate of fusion (Dimar et al. 1996) Similarly, indomethacin reduced bone ingrowth around porous coated implants (Trancik et al. 1989). In addition to exerting dose-dependent effects on bone formation, the effects of indomethacin appear to be time-dependent. Exposure to the NSAID for less than three weeks reduced bone formation and pullout strength of transcortical plugs whereas longer exposures were without effect (Cook et al. 1995). Similarly, the effect of exposure of osteoblasts to indomethacin in vitro is time-dependent (Batzer et al. 1998) When MG63 cells were treated with indomethacin throughout the entire culture, the surface roughness and $1,25\text{-}(OH)_2D_3$ effects on osteoblast differentiation were not observed. In contrast, when confluent cultures were treated for the last 24 hours of culture, indomethacin had no effect on osteoblast response to surface roughness, but the effect of $1,25\text{-}(OH)_2D_3$ was not observed. This suggested that the time of exposure to NSAID was critical and may be important immediately before or after implant placement.

PGE_2 begins its cascade by binding to the membrane-bound EP receptor and causing intracellular changes in cAMP and calcium concentrations, which lead to changes in osteoblast proliferation, differentiation, and local factor production. Four of these EP receptor subtypes

have been described by Coleman (1989) and have been found in bone cells (Yamaguchi et al. 1989).

While the above cited studies provide insight into the effect of PGE_2 on bone cells in culture and bone healing in vivo, no prior study has specifically examined how PGE_2 may influence osteoblast response to implant surface roughness. The hypothesis tested in the current study was that PGE_2 mediates its effects on osteoblast response to surface roughness in a dose- and time-dependent manner through changes in EP receptor expression. This study demonstrated that varying the concentration of PGE_2 caused changes in MG63 osteoblast-like cells' expression of EP receptor mRNA *in vitro*.

EP1 was not found to be expressed in any sample, regardless of surface roughness or PGE_2 concentration. This indicates a specific absence of EP1 expression in MG63 cells. One might question whether our primers were capable of detecting EP1 mRNA in human cells. This issue was specifically addressed by demonstrating the presence of EP1 expression in standardized and commercially available RNA preparations of human brain and kidney cells. Thus, we concluded that MG63 cells do not produce EP1 mRNA at detectable levels on plastic or titanium within 120 hours of seeding. This conclusion is somewhat supported by the literature, as EP1 expression has been reported in mouse osteoblasts (Suda et al. 1996) and preosteoblasts/osteoblasts from fetal rat calvarial bone (Kasugai et al. 1995) while MG63 cells are derived from a human osteosarcoma.

In contrast to the absence of EP1 expression by MG63 cells in response to any of the variables tested, expression of EP2, EP3, and EP4 was robust. Changes in receptor expression were observed with surface roughness, time, and PGE_2 treatment.

EP2 expression varied with time in culture on the three different surfaces. In general, EP2 expression on plastic and smooth Ti decreased with time in culture, while that on rough Ti peaked at 6 hrs and then decreased precipitously. EP2 receptor expression was also modulated by PGE_2 treatment. During short treatment times (3 and 6 hrs), 1 nM PGE_2 produced a spike in EP2 expression on smooth Ti that fell off with 10 and 100 nM PGE_2. In contrast, on rough Ti, expression peaked at 10 nM PGE_2 at 3 hrs and 100 nM at 6 hrs. By 120 hrs, EP2 expression dose-dependently increased with increasing doses of PGE_2 on both smooth and rough Ti. Although EP2 has been implicated in bone formation in response to PGE_2, it is difficult to definitively ascribe a role for this receptor in osteoblast response to implant surface roughness. The results in the untreated cultures suggest that, perhaps, EP2 receptor signaling is important in the early hours after dental implant placement, and as time goes on this response wanes. Alternatively, the receptor may be involved in PGE_2-dependent cell proliferation during the early (pre-confluent) phase of the culture. The time-dependent effects of indomethacin treatment on cell response to surface roughness and systemic hormone treatment described above suggests that changes in EP2 receptor expression may occur with varying degrees of confluence.

EP3 receptor expression on all surfaces increased with time in culture. The largest change was observed in cultures on plastic, which demonstrated a 5-fold increase in the 120 hr cultures. EP3 expression was also modulated by PGE_2 treatment. At 3 hrs, PGE_2 treatment resulted in a 15-fold increase in expression on smooth Ti that was not dose-dependent; on rough Ti, the increase was 5-fold and dose-dependent. By 120 hrs, the fold increase in response to PGE_2 was dose-dependent on both smooth and rough Ti, but less robust.

EP3 was expressed at levels approaching 1/100th that of EP2 and EP4. While EP3

expression was minimally expressed when compared to EP2 and EP4, it is important to note that EP3 was perhaps the most responsive of the receptors to changes in PGE_2 concentration. This is true particularly at the early (3 hour) and late (120 hour) time points. The addition of the smallest amount of PGE_2 (1 nM) caused dramatic increases in EP3 receptor expression, and was almost greater on smooth titanium than on rough. This greater expression may or may not be more in line with healthy osteoblast attachment and differentiation. It is important to remember that physiologically, even minor changes in a receptor's gene expression can lead to significant cellular phenotypic changes. While this study did not investigate these potential changes, this is certainly an area in which future studies should examine.

Over time, EP4 receptor gene expression was the most consistent and unchanging of the four receptor subtypes examined. EP4 expression on plastic peaked (2-fold) at 6 hrs and then decreased. In contrast, expression on smooth Ti was consistent and relatively unchanged over time, while that on rough Ti increased with time in culture and showed maximal expression at 120 hrs.

EP4 expression was also affected by PGE_2 treatment. Interestingly, the trends were similar on both smooth and rough Ti, but expression was generally greater in cultures on the rough Ti.. At 3 hrs, EP4 expression was increased by 1 nM PGE_2 on both Ti surfaces, but expression was slightly higher on rough Ti. With amounts of PGE_2 above 1 nM, expression decreased on both surfaces, but in all cases, expression on rough Ti was higher. At 120 hrs, EP4 expression was equivalently increased on both Ti surfaces with 1 nM PGE_2; as the amount of prostanoid was increased to 10 and 100 nM PGE_2, EP4 expression increased in cultures on rough, but not smooth, Ti.

There is mounting evidence in the literature to support an important role for EP4 in PGE_2-induced bone formation. Weinreb et al. (2001) demonstrated that EP4 and not EP2 is expressed in bone tissue of young adult rats. In addition, they also reported that PGE_2 stimulates osteoblastic differentiation in bone marrow stromal cell cultures and that activators of adenylate cyclase and EP4 agonists were able to produce similar effects (Weinreb et al. 1999). It has also been recently discovered that PGE_2, through EP2 and EP4, propagates its own auto-amplification via stimulation of cAMP and eventually the Cox-2 that is responsible for further PGE production (Sakuma et al. 2004). Masuzawa et al. (2005) recently showed that a combination of β-tricalcium phosphate and EP4 agonist yielded greater bone formation around femoral titanium rods than either β-TCP or titanium rod alone. A similar result was found using an EP2 agonist in a study examining the healing of canine long bone fractures (Paralkar et al. 2003).

The results of the present study do not definitively implicate a particular EP receptor in mediating the response of osteoblasts to implant surface roughness or PGE_2. This result is not surprising since it is well known that PGE_2 has variable effects in vitro, depending on the osteoblastic cell type used (Raisz and Koolmans-Beynen 1974, Hakeda et al. 1986, Kaneki et al. 1999). In addition, research in this area is further hampered by a lack of specific reagents or tools for elucidating the relevant pathways. Agonists and antagonists, with known and well characterized specificity and selectivity, are not available (Kozawa et al. 1998, Ono et al.1998). Recent research using animals that have been genetically altered so that specific EP receptors are inactivated were thought to display great promise. In the late 1990's and early 2000, several reports demonstrated that EP4 is involved in PGE_2-induced bone resorption in mice (Ono et al. 1998, Miyaura et al. 2000, Suzawa et al. 2000). Indeed, studies of osteoclast formation in vitro

demonstrated that the induction of bone resorption was dependent on the presence of EP4 in osteoblastic cells. One uncertainty with these models is that the effects of prostanoids in mice are different than in rat and human. Specifically, PGE_2 is a strong stimulator of bone resorption in mice, while in rat and human PGE_2 is a potent stimulator of bone formation. Thus, a number of investigators have argued that more selective agonists/antagonists are needed to dissect out the various effects of PGE_2 in bone metabolism. In short, this important area is fertile ground for discovery.

Another factor which confounds the clear interpretation of our results is the fact that a single ligand, PGE_2, has the potential to act through a multiplicity of receptor subtypes which subserve the same function. This phenomenon has been demonstrated to occur in sensory neurons (Southall and Vasko 2001) and as we unravel this story we expect to observe a similar pattern.

V. SUMMARY

An increase in our knowledge of how PGE_2 mediates its effects on osteoblasts at the bone-implant interface provides us with a greater understanding of the events leading to osseointegration and eventually superior clinical success. The current study demonstrated that EP receptor expression was affected by implant surface roughness. Further, varying the dose and time of exposure to PGE_2 was shown to cause changes in osteoblast EP receptor expression. Future studies will need to explore the role of EP2, EP3, and EP4 in mediating the effect of PGE_2 on osteoblast proliferation, differentiation, and local factor production.

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VITA

Casey McCray Campbell was born on [redacted] Abilene, Texas. Following graduation from Cooper High in 1994, he attended Wake Forest University in Winston-Salem, North Carolina, graduating *magna cum laude* in 1998. Dr. Campbell began dental school in 1999 and was the recipient of the U.S. Air Force Health Professions Scholarship and was commissioned as a 2nd lieutenant. He received his Doctor of Dental Surgery with the distinction *summa cum laude* from the University of Texas Health Science Center at San Antonio in 2003. As a dental student, Dr. Campbell received two AADR Student Research Fellowships as well as the NIDCR fellowship, presented research at the AADR meetings in Chicago and San Antonio, and at the Hatton competition in Memphis. He received many awards including honors in research, basic sciences, and clinical dentistry; the dental student award from the American Association of Oral & Maxillofacial Pathology; the Hanau/Waterpik Prosthodontics Award; and national awards for outstanding achievement in the fields of operative dentistry and TMJ/occlusion. Upon graduation he was inducted into Omicron Kappa Upsilon national dental honor society and was selected to receive the Pierre Fauchard Academy scholarship, given to one graduating senior at each of the nation's dental schools.

Following graduation Dr. Campbell was promoted to the rank of captain in the U.S. Air Force and attended officer training school at Maxwell AFB, Alabama. He was a distinguished graduate and received the Henry H. "Hap" Arnold Award for Leadership Excellence. He then began residency training in periodontics at Wilford Hall Medical Center at Lackland AFB, Texas, where he will graduate in June 2006. Since beginning his residency, he has already won

awards for presentation of research at the Southwest Society of Periodontists annual meeting in Dallas and at the UTHSCSA Dental Science Symposium. He has also presented his results at the AADR meeting in Baltimore. He is currently a member of the ADA, SWSP, and AAP.

Dr. Campbell has been married to his wife for seven years, and they are the parents of a 1-year old daughter. Upon completion of his residency training, he looks forward to continuing his career in the U.S. Air Force, attaining board certification in periodontics, practicing clinical periodontics, and perhaps teaching in a residency program.