Growth Factors Regulate Expression of Mineral Associated Genes in Cementoblasts

N. Esra Saygin,* Yoshihiko Tokiyasu,*† William V. Giannobile,* and Martha J. Somerman*‡

Background: Knowledge of the responsiveness of cells within the periodontal region to specific bioactive agents is important for improving regenerative therapies. The aim of this study was to determine the effect of specific growth factors, insulin-like growth factor-I (IGF-I), platelet-derived growth factor-BB (PDGF-BB), and transforming growth factor-β (TGF-β) on cementoblasts in vitro and ex vivo.

Methods: Osteocalcin (OC) promoter driven SV40 transgenic mice were used to obtain immortalized cementoblasts. Growth factor effects on DNA synthesis were assayed by [^{3}H]-thymidine incorporation. Northern analysis was used to determine the effects of growth factors on gene expression profile. Effects of growth factors on cementoblast induced biomineralization were determined in vitro (von Kossa stain) and ex vivo (re-implantation of cells in immunodeficient (SCID) mice).

Results: All growth factors stimulated DNA synthesis compared to control. Twenty-four hour exposure of cells to PDGF-BB or TGF-β resulted in a decrease in bone sialoprotein (BSP) and osteocalcin (OCN) mRNAs while PDGF-BB also increased osteopontin (OPN) mRNA. Cells exposed to IGF-I for 24 hours exhibited decreased transcripts for OCN and OPN with an upregulation of BSP mRNA noted at 72 hours. In vitro mineralization was inhibited by continuous application of PDGF-BB or TGF-β, while cells exposed to these factors prior to implantation into SCID mice still promoted biomineralization.

Conclusions: These data indicate IGF-I, PDGF-BB, and TGF-β influence mitogenesis, phenotypic gene expression profile, and biomineralization potential of cementoblasts suggesting that such factors alone or in combination with other agents may provide trigger factors required for regenerating periodontal tissues. *J Periodontol 2000;71:1591-1600.*

KEY WORDS

Dental cementum/growth; growth factors, insulin-like; growth factors, platelet-derived; growth factors, transforming.

* Department of Periodontics/Prevention/Geriatrics, University of Michigan, Ann Arbor, MI.
† Department of Pediatric Dentistry, Mekai Universityl Sakado, Saitama, Japan.
‡ Department of Pharmacology, University of Michigan.

Restoring lost periodontium, the ultimate goal of regenerative periodontal therapy, requires functional and anatomical reconstruction of a multitude of tissues including cementum, periodontal ligament, bone, and gingiva. Toward this goal research efforts have focused on determining the cells, factors, and mechanisms fostering periodontal wound healing. Increased understanding of mediators required for promoting periodontal regeneration has led to improved techniques directed toward enhancing healing and regeneration of periodontal tissues lost as a consequence of disease.[1-3] A specific concern for the clinician has been the limited predictability of forming regenerative cementum, which is considered a prerequisite for complete restoration of periodontal tissues.[4] Therefore, understanding the molecular mechanisms and cell dynamics of cementogenesis during development and healing of periodontal tissues is important to improving regenerative techniques. The molecular and cellular events involved in controlling cementogenesis have not been studied widely due to limited tools, including lack of cementoblast cultures.[5,6] Toward this aim our laboratory determined the timed and spatial expression of molecules during tooth root development *in situ*.[7-9] Root lining cells, cementoblasts, were found to express markers associated with cells involved in mineral formation such as bone sialoprotein (BSP) and osteocalcin (OCN). This selective expression profile enabled us to establish cementoblast cell

lines independent from surrounding periodontal ligament (PDL) cells. The availability of cementoblast cell lines allows us to seek answers to many questions that previously have been difficult to address. The accumulating evidence indicating that growth factors, under appropriate conditions, have positive effects on wound healing[10,11] prompted us to evaluate their effects on cementoblasts in vitro.

Growth factors possess diverse activities including the ability to stimulate cell migration, proliferation, differentiation, and matrix synthesis, all activities known to be important in tissue repair.[12] More specifically, increasing evidence has accumulated from both in vitro[13-16] and in vivo[17-21] studies to support a role for growth factors for use in regenerative periodontal therapy. Factors considered to have a positive impact on periodontal regeneration include platelet-derived growth factor (PDGF); insulin-like growth factor (IGF); fibroblast growth factor (FGF); transforming growth factor-β 1, -2, and -3, (TGF-β); and bone morphogenetic proteins (BMPs) belonging to TGF-β superfamily.[3,22] However, specific cells, as well as the response of cells to these factors, have not been completely defined.[4,10] Most notably, limited data exist regarding the effects of growth factors on cementoblasts. Knowledge as to the responsiveness of cementoblasts to growth factors may provide critical information important for designing regenerative therapies.

The aim of this investigation was to determine whether the growth factors IGF-I, PDGF-BB, and TGF-β, known to have an effect on PDL cells and on osteoblasts, alter cementoblast behavior. Cell activities examined included mitogenic activity in vitro, cell differentiation in vitro, and induction of mineralization in vitro and ex vivo.

MATERIALS AND METHODS

Isolation and Culture of Mouse Cementoblast Cell Line

An immortalized cementoblast cell line was used for these studies. As described previously, these cells were obtained using osteocalcin (OC) promoter-driven SV-40 T antigen transgenic mice (OC-TAg) provided by Dr. Jolene Windle.[23,24] The 2.6-kb fragment of rat OC promoter driving the expression of SV-40 TAg ensures that cells expressing OC (cementoblasts) are preferentially immortalized. Therefore when cells from the surrounding tooth are cultured, only cementoblasts, not PDL cells, survive.

Briefly, first mandibular molars of mice day 41 (vaginal plug day = 0) were used, based on previous data *in situ*[8] and in vitro,[7] which indicated that at this time cells along the root surface, cementoblasts, express high levels of mineralized tissue-associated markers BSP and OCN mRNA. Mandibles were dissected and hemisected and molars were removed by carefully cutting into the PDL using a dissecting microscope thereby ensuring that osteoblasts from surrounding alveolar bone were excluded. Collagenase/trypsin digestion was used to isolate cementoblasts from the surface of first mandibular molars. Cultured cells were termed "OC-CM" to indicate cementoblasts (CM) derived from OC-TAg mice. Limiting dilutions were used to obtain clonal cell lines. For this purpose, one cell/well was plated in each of 96-well tissue culture plates and subclones were generated and expanded. OC-CM subclone 30 (OCCM-30), which expresses BSP and OCN mRNAs, indicative of cementoblasts *in situ*, was used for this study. OC-CM-30 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)§ supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µl streptomycin, and incubated in an humidified atmosphere of 5% CO_2 at 37°C. Cells were used between passages 9 to 12.

Growth Factors

The growth factors evaluated in this study included recombinant human insulin-like growth factor-I (rhIGF-I);‖ recombinant human platelet derived growth factor-BB (rhPDGF-BB);‖ and recombinant human transforming growth factor-β1 (rhTGF-β).‖

DNA Synthesis Assay

To measure the mitogenic effects of growth factors on cementoblasts, [^{3}H]-thymidine incorporation assay was used as previously described.[15] Initially a dose-response experiment was carried out with 3 different concentrations for each growth factor to determine cell responsiveness (data not shown). Concentrations used were 50 to 200 ng/ml for IGF-I, 15 to 60 ng/ml for PDGF-BB, and 2 to 8 ng/ml for TGF-β. Concentrations were selected based on previous studies from our laboratory[25] and others where PDL cells and osteoblasts were exposed to growth factors.[15,26,27] Optimal concentrations for growth factors, as determined by ability to enhance proliferation, were 100 ng/ml for IGF-I, 30 ng/ml for PDGF-BB, and 4 ng/ml for TGF-β. Cells were seeded in 24-multiwell plates at a density of 1×10^5 cells/well in triplicate and incubated for 24 hours in DMEM containing 10% FBS and antibiotics. Media were removed, wells were rinsed twice with phosphate buffered saline (PBS), and then serum-free DMEM was added for 24 hours. Next, cells were exposed to growth factors in serum-free DMEM for 20 hours and pulsed with 3 µCi/ml methyl-[^{3}H]-thymidine¶ during the last 4 hours of incubation. Serum-free media were used as a negative, while media with 10% FBS, where FBS is known to contain numerous growth factors, were used as a positive control. Following labeling, cells were rinsed with PBS and incu-

§ Gibco BRL, Gaithersburg, MD.
‖ R&D Systems Inc., Minneapolis, MN.
¶ Amersham, Arlington Heights, IL.

bated with 5% (w/v) trichloroacetic acid (TCA) for one hour. The precipitate was solubilized with 250 µl 1% sodium dodecyl sulfate (SDS) at 55°C for 2 hours and counted in a liquid scintillation counter.# The DNA synthesis assay was repeated 3 times with similar results observed. Results are expressed as CPM ± S.D.

Northern Analysis

OC-CM-30 cementoblasts were seeded at an initial density of 5×10^4 cells/cm^2 in 100 mm cell culture plates using DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µl streptomycin and incubated in an humidified atmosphere of 5% CO_2 at 37°C overnight to allow for cell attachment. On the following day media were changed to DMEM containing 2% FBS with antibiotics, specific growth factors, and mineralization components. In order to promote mineralization 50 µg/ml ascorbic acid (AA) and 10 mM β-glycerophosphate (βGP), previously shown to induce mineralization of cementoblasts and osteoblasts, were added to the cells.[24,28,29] Cells were exposed to these conditions for 24 or 72 hours. Total RNA was isolated using a modified guanidium isothiocyanate, sodium citrate, sodium acetate, phenol, and chloroform extraction. After quantifying by spectroscopy,[30] RNA (10 µg) was electrophoresed on a 6% formaldehyde and 1.2% agarose gel, transferred onto a nylon membrane** and immobilized using an UV irradiation unit.†† Blots were hybridized with random primed ^{32}P radiolabeled probes‡‡ and exposed to Kodak X-OMAT film with intensifying screens at –70°C for 24 to 72 hours.

Probes

Probes used for Northern analysis included: bone sialoprotein: mouse-BSP cDNA in PCR II vector containing a 1 kb PCR product of mouse inserted by TA cloning[31] (a gift from Dr. M. Young, NIDCR/NIH); osteocalcin: mouse-OCN cDNA cloned into pSP65 cloning vector[32] (a gift from Dr. J. Wozney, Genetics Institute); osteopontin: mouse-OP-3 cDNA in PCR II vector containing 1kb PCR product[33] (a gift from Dr. M. Young NIDCR /NIH); and GAPDH: a 1100 bp cDNA cloned into pGEM3.[34] GAPDH a "house keeping gene" which is not affected by cell conditions is used to normalize loading of mRNA.

Mineralization Assay

In vitro: von Kossa. Mineralization assay was performed as previously described.[24,28,29] Concentrations selected were the same as those used for DNA synthesis and gene expression assays. For continuous application groups, cells were plated in 24-multiwell culture dishes at an initial density of 5×10^4 in triplicate and were grown for 8 days in the presence of growth factors, 2% FBS, and AA. For pulse application groups, cells were grown in the presence of growth factors for the first 24 hours, whereas AA and 2% FBS were present during the entire 8-day experimental period. Media were replenished every other day. Control wells received DMEM containing 2% FBS with or without AA, as a positive and negative control, respectively. Three mM inorganic phosphate (monobasic NaH_2PO_4) was added to all wells during the last 48 hours of incubation. At the termination of the experiment the cells were fixed with absolute ethanol and stained by using von Kossa method to detect mineral nodule formation in vitro.[28,29] Black staining is indicative of mineral nodule formation.

In vivo: SCID mice. The influence of growth factors on mineral induction by OC-CM 30 cells ex vivo was determined using SCID mice. Homozygous 7- to 9-week-old male C.B.-17 scid/scid (SCID) mice§§ were used. The ability of OC-CM 30 cells to promote mineralization in this model has been reported previously.[35] A mixture of hydroxyapatite/tricalcium phosphate powder (HA/TCP) and bovine Type I collagen,|||| was used as a delivery system for the cells. OC-CM 30 cells were exposed to DMEM containing 2% FBS and either 30 ng/ml PDGF-BB or 4 ng/ml TGF-β and were maintained in culture for 24 hours or 72 hours. The implants were prepared by using 4 mm × 4 mm sponges and soaking sponges in DMEM for 30 minutes. Sponges were compressed between 2 pieces of sterile Whatman filter paper to remove air prior to adding cells and then were inserted into microcentrifuge tubes containing 2×10^6 cells in 30 µl DMEM. After anesthesia with methoxyflurane¶¶ a mid-longitudinal skin incision of approximately 1 cm in length was made on the dorsal surface. Subcutaneous pouches were created by blunt dissection in 3 different directions in order to enable fascia to separate individual implants.[36] PDGF-BB and TGF-β treated and graft-only implants were engrafted into separate pockets in the same animal and incisions were closed with surgical staples. For each treatment, 3 animals were used. After 6 weeks the mice were sacrificed and samples were retrieved and fixed in neutral buffered formalin (10% v/v) and embedded in paraffin. Five micron thick sections were prepared and stained with hematoxylin and eosin (H&E). All procedures were performed under University of Michigan Unit for Laboratory Animal Medicine (ULAM) approved guidelines for the use of animals in research.

Statistical Analysis

To determine statistical significance one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test were used.

Wallac 1410, Pharamacia, Turku, Finland.
** Duralon-UV, Stratgene Inc., La Jolla, CA.
†† Stratalinker, Stratagene, Inc., La Jolla, CA.
‡‡ Rediprime, Amersham.
§§ Taconic, Germantown, NY.
¶¶ Collagraft, Zimmer Corp., Warsaw, IN.
|||| Metofane, Mallinckrodt Veterinary, Inc., Mundelein, IL.

RESULTS

DNA Synthesis (Fig. 1)

Many growth factors enhance cell proliferation and, therefore, to determine the mitogenic response of OC-CM-30 cells to specific growth factors, a [^{3}H]-thymidine incorporation assay was used. When compared with control (serum-free DMEM), IGF-I, PDGF-BB, and TGF-β increased DNA synthesis in OC-CM 30 cells (P <0.001). [^{3}H]-thymidine uptake for PDGF-BB treated cells was similar to 10% FBS (positive control) and significantly greater than that of TGF-β, IGF-I, and 0% FBS (negative control). TGF-β and IGF-I had comparable effects on DNA synthesis.

Gene Expression (Fig. 2)

In addition to affecting cell proliferation, many growth factors also alter gene expression and subsequently cell differentiation. The effects of the growth factors above on gene expression for OPN, BSP, and OCN, genes associated with the cementoblast phenotype, were determined using Northern analysis. Representative results from one experiment, where similar results were noted on 3 separate occasions are shown in Figure 2. Figures 2A and 2B show the Northern blot data, while in Figure 2C the differences observed have been normalized to GAPDH, to compensate for changes in loading of mRNA. Cells treated with PDGF-BB and TGF-β for 24 hours exhibited a significant down-regulation of BSP and OCN mRNAs, while the PDGF-BB group also upregulated OPN mRNA when compared with 2% FBS plus AA control (2% AA) (Fig. 2A). This effect on OPN mRNA levels was also noted in cells exposed to TGF-β, but only after 72 hours exposure (Fig. 2B). By 72 hours, cells exposed to PDGF-BB and TGF-β had increased mRNAs for both BSP and OCN when compared with mRNAs at 24 hours; however, relative to 2% AA control at 72 hours transcripts for BSP and OCN still remained reduced, with a more noticeable reduction remaining for BSP mRNA (Fig. 2B).

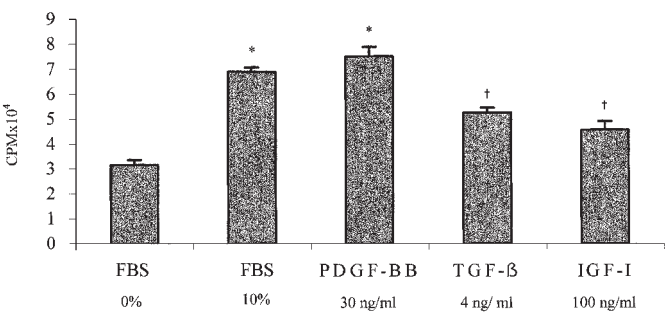

Figure 1.
Effect of growth factors on DNA synthesis measured by [^{3}H]-thymidine incorporation. Each bar represents the mean ± SD. Results from a representative experiment of 3 repetitions are shown.
** P <0.001: 10% FBS and PDGF-BB>TGF-ß and IGF-I and 0% FBS;*
† P <0.001:TGF-ß & IGF-I > 0% FBS.

Effects of IGF-I on these genes were different from those of PDGF-BB and TGF-β. IGF-I inhibited both OPN and OCN gene expression at 24 hours (Fig. 2A). At 72 hours OPN mRNA remained downregulated, whereas OCN mRNA levels were comparable to 2% AA (Fig. 2B). Most interesting was the effect of IGF-I on BSP mRNA, where BSP is considered a key protein in the regulation of mineral formation. At 24 hours IGF-I had no effect on BSP transcripts beyond that of 2% AA control while at 72 hours IGF-I exposed cells expressed higher levels of BSP transcripts when compared to 2% AA control, where this effect was seen in 3 separate experiments (Figs. 2A and 2B).

Mineralization

Since changes in gene expression may reflect changes in other cell activities, the ability of cementoblasts to promote mineralization in vitro and in vivo, after exposure to growth factors, was determined. Results shown in Figure 3 represent one experiment where each agent was examined in triplicate wells. The experiment was repeated 3 times with similar results.

In vitro (Fig. 3). The ability of growth factors to alter cementoblast-induced biomineralization was determined by von Kossa staining. As previously reported,[24] cementoblasts promote mineral nodule formation in the presence of 2% FBS+AA, but not in the absence of AA (Figs. 3A and B, bottom row). Mineral nodule formation was inhibited in cells exposed continuously to PDGF-BB and TGF-β. In contrast, IGF-I did not affect mineralization either in vitro or in vivo when compared to 2% FBS positive control (data not shown). In order to determine whether a short exposure to PDGF-BB or TGF-β, which would promote proliferation, would enhance the ability of cells to induce a mineral matrix, cells were exposed to factors for the first 24 hours of the 8-day experiment. As shown in Figure 3B, under these conditions, cells pulsed with growth factors were able to promote mineral nodule formation but mineralization remained less than the 2% FBS positive control. Furthermore this inhibitory effect was more apparent with TGF-β exposed cells versus PDGF-BB exposed cells.

Ex vivo (Fig. 4). To assess the effect of growth factors on mineral formation induced by OC-CM 30 cells ex vivo, cell/HA/TCP sponges were implanted in SCID mice and prepared for histological analysis 6 weeks post-transplantation. All implants, with or without cells, were encapsulated at the time of retrieval. HA/TCP implant material only, used as the carrier, was shown to be incapable of inducing mineralized matrix at 6 weeks (Fig. 4A). Histological analysis of tissue samples obtained from SCID mice containing OC-CM/HA/TCP implants revealed mineral formation at 6 weeks (positive control, Fig. 4D), but not at 1 week (Fig. 4B) or 3 weeks (Fig. 4C) post-implantation.

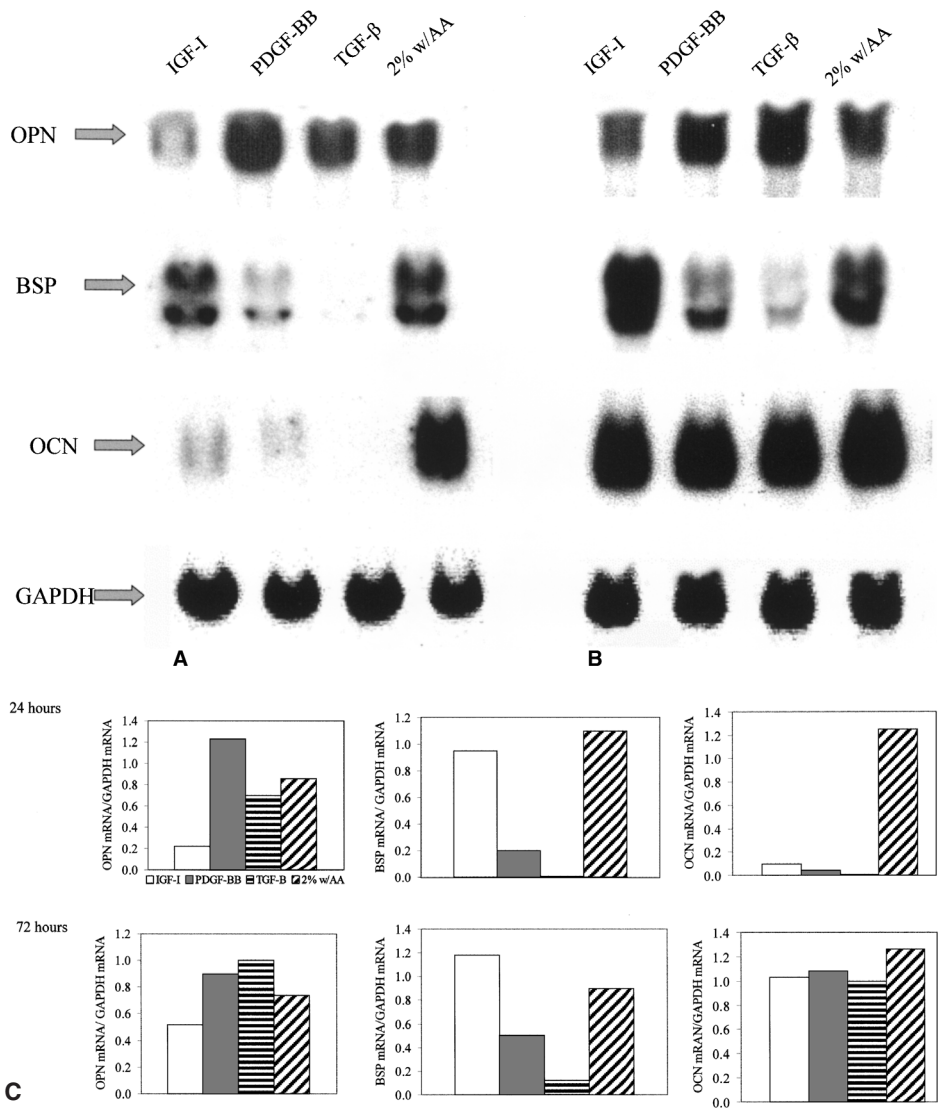

Figure 2.

Gene expression analysis. Total RNA was isolated and subjected to Northern analysis. Genes probed: OPN, osteopontin; BSP, bone sialoprotein; OCN, osteocalcin; and GAPDH, "housekeeping" gene to normalize loading of mRNA. Factors: IGF-I, insulin-like growth factor-I; PDGF-BB, platelet derived growth factor-BB; TGF-ß, transforming growth factor-ß; 2% w/AA: cells incubated with DMEM containing 2% FBS, ascorbic acid and ß-glycerophosphate (positive control). ***A.*** *24-hour exposure to growth factors;* ***B.*** *72-hour exposure to growth factors;* ***C.*** *normalization of OPN, BSP, and OCN mRNA to GAPDH mRNA at 24 and 72 hours post-treatment. Results from a representative experiment of 3 repetitions are shown.*

Hence, 6 weeks of healing was determined as optimal for biomineralization to occur and was used for subsequent studies.

OC-CM 30 cementoblasts, pretreated with either PDGF-BB or TGF-β for 24 or 72 hours in vitro and then seeded onto HA/TCP for implantation in vivo, promoted mineralization (Figs. 4E, F, G, and H). Mineral deposition developed from the surface into the center of the grafts for all specimens that mineralized. Light microscopic evaluation of tissues indicated similar appearance of cells and mineral for PDGF-BB, TGF-β, and positive control. Interestingly, although histomorphometric analysis was not performed, at the macroscopic level PDGF-BB and TGF-β treated specimens appeared to demonstrate enhanced neovascularization at the time of retrieval when compared to positive control (data not shown).

DISCUSSION

Substantial evidence exists suggesting that growth factors can promote periodontal regeneration, although the specific cell interactions have not been established.[2,11,22] Studies here demonstrating that IGF-I, PDGF-BB, and TGF-β influence the activities of cementoblasts, in vitro, further support the use of these factors in regenerative therapies.

A fundamental process for healing and development is cell proliferation. PDGF-BB, TGF-β, and IGF-I all significantly stimulated DNA synthesis in OC-CM 30 cells. PDGF-BB was the most potent at the doses and times studied. This is not surprising since several studies have shown consistently that PDGF promotes proliferation of mesenchymal cells, including human PDL cells,[13,37] human pulp fibroblasts,[37] preosteoblasts and osteoblasts.[26,38,39] Furthermore, as also noted for cementoblasts, Piche and Graves[40] reported that PDGF was more mitogenic than TGF-β, IGF-I, or EGF for human osteoblastic cells. Similarly, results for IGF-I are in line with previous studies, where IGF-I has been shown to be a mitogen for PDL cells and osteoblasts.[15,41] In contrast to the consistent proliferative effects reported for PDGF-BB or IGF-I, existing data indicate that TGF-β has varied effects, including enhancing or inhibiting proliferation and having anabolic or catabolic activity.[42,43] These differences have been attributed to several factors including concentration,[44-46] culture conditions,[47,48] maturity of cells,[49] differences in anatomic origin of cells, and animal species.[47,48,50]

While promotion of cell proliferation is important for bone wound healing, several additional events must occur for periodontal repair to proceed. These include cell differentiation and biomineralization of appropriate tissues.

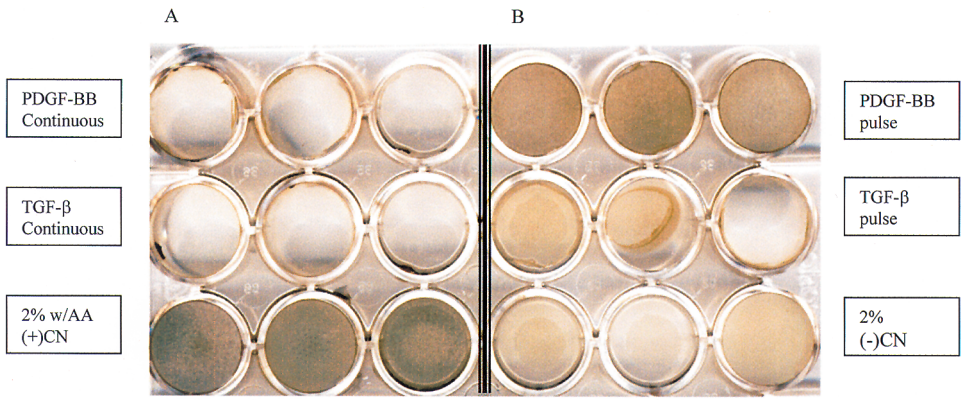

Figure 3.
Eight-day von Kossa mineralization assay. Cells were exposed to DMEM containing 2% FBS plus 50 µg/ml ascorbic acid and PDGF-BB platelet derived growth factor-BB, 30 ng/ml, or TGF-ß transforming growth factor-ß, 4 ng/ml, either in a continuous or pulse fashion. As a positive control (2% w/AA;(+)CN), cells were exposed to DMEM containing 2% FBS plus 50 µg/ml ascorbic acid (AA). Note positive black staining indicative of mineral nodule formation. As a negative control, AA was omitted (2%(-)CN). Note absence of stain. ***A.*** *Continuous treatment: Note that cells exposed to either PDGF or TGF-ß were not capable of promoting mineral nodule formation.* ***B.*** *Pulse application: Note decreased mineral nodule formation. Results from a representative experiment of 3 repetitions are shown, where, in each experiment triplicate wells were used for each growth factor.*

To address these issues with regard to cementoblast function, Northern analyses and mineralization assays were used. Previous studies from our laboratory,[51] as well as others,[52] have shown that cementoblasts express mineral-associated proteins including osteopontin, bone sialoprotein, and osteocalcin; however, factors regulating their expression have not been explored in depth. BSP and OCN are highly specific to mineralized tissues.[53] Evidence to date suggests that BSP is a nucleator of hydroxyapatite crystal formation,[54,55] while osteocalcin appears to play a role in early phases of mineralization and in regulation of crystal growth.[55-57] Osteopontin, which has been identified in several tissues,[58] promotes the migration and attachment of both osteoblasts and osteoclasts and regulates crystal growth.[59,60] Thus, the ability of growth factors to modulate these genes in cementoblasts is likely to have significant effects on cell function.

Northern analysis indicated that cells exposed to PDGF-BB or TGF-β for 24 hours exhibited downregulation of BSP and OCN mRNAs when compared to untreated cells, which were sustained for 72 hours (Figs. 2A and B). Concurrently, OPN mRNA was upregulated at 24 hours in PDGF-BB treated cells, and at 72 hours in TGF-β treated cells. The ability of factors to enhance OPN mRNA levels may prove valuable for use in periodontal regenerative therapies, where elevation of OPN levels in the extracellular matrix, by inhibiting crystal growth, may prevent ankylosis. Effects of PDGF-BB on cementoblast gene expression are consistent with previous data from our laboratory with osteoblast cultures,[25] as well as that of several other laboratories,[61-63] where it has been shown that PDGF-BB promotes cell proliferation, while decreasing the level of genes and gene products associated with cell differentiation. The effect of TGF-β on cementoblast genes is consistent with some previous studies as well. Similar to our findings, rat osteosarcoma cells and non-transformed osteoblast-like cells when exposed to TGF-β, exhibit upregulation of OPN transcripts[64-67] and downregulation of OCN.[27,68]

IGF-I also inhibited OCN mRNA expression; however, unlike PDGF-BB and TGF-β, IGF-I inhibited OPN expression at 24 hours and upregulated BSP at 72 hours. Increased BSP gene expression at 72 hours suggests that IGF-I may have a specific role in mineral formation, since BSP is implicated in promoting crystal growth. Interestingly, PDGF and IGF appear to act synergistically to promote periodontal tissue regeneration.[69] Several reasons for this effect have been proposed including the ability of IGF-I to nullify the collagenolytic effects of PDGF.[15] PDGF also has been reported to activate both the IGF-I receptor gene promoter and corresponding IGF-I receptor mRNA in fibroblasts.[70] Our studies suggest that these effects may be attributed to enhancement of proliferation by PDGF coupled with promotion of mineral formation by IGF (i.e., BSP upregulation). Therefore, factors in combination that can enhance cell proliferation and also promote mineralization may provide the balance needed to establish a functional PDL with intervening bone and cementum formation.

Another requirement of periodontal regeneration is the promotion of biomineralization, thus these experiments were designed to determine whether growth factors influence the ability of cementoblasts to promote mineralization in vitro and ex vivo. Results from the present study indicate that continuous exposure of cells to PDGF-BB or TGF-β inhibited mineral nodule formation in vitro (Fig. 3A) and this correlated with down-regulation of differentiation markers, BSP and OCN (Fig. 2A). When cells were pulsed with PDGF-BB or TGF-β for 24 hours and then factors withdrawn, inhibitory effects on mineralization were reversed to some degree, being more noticeable for PDGF-BB versus TGF-β (Fig. 3B). These findings are supported by other researchers demonstrating that PDGF-BB and TGF-β inhibit mineralization in vitro.[27,63,71] In fact, Hsieh and Graves[63] reported that brief exposure of osteoblasts to PDGF-BB promoted mineralized nodule

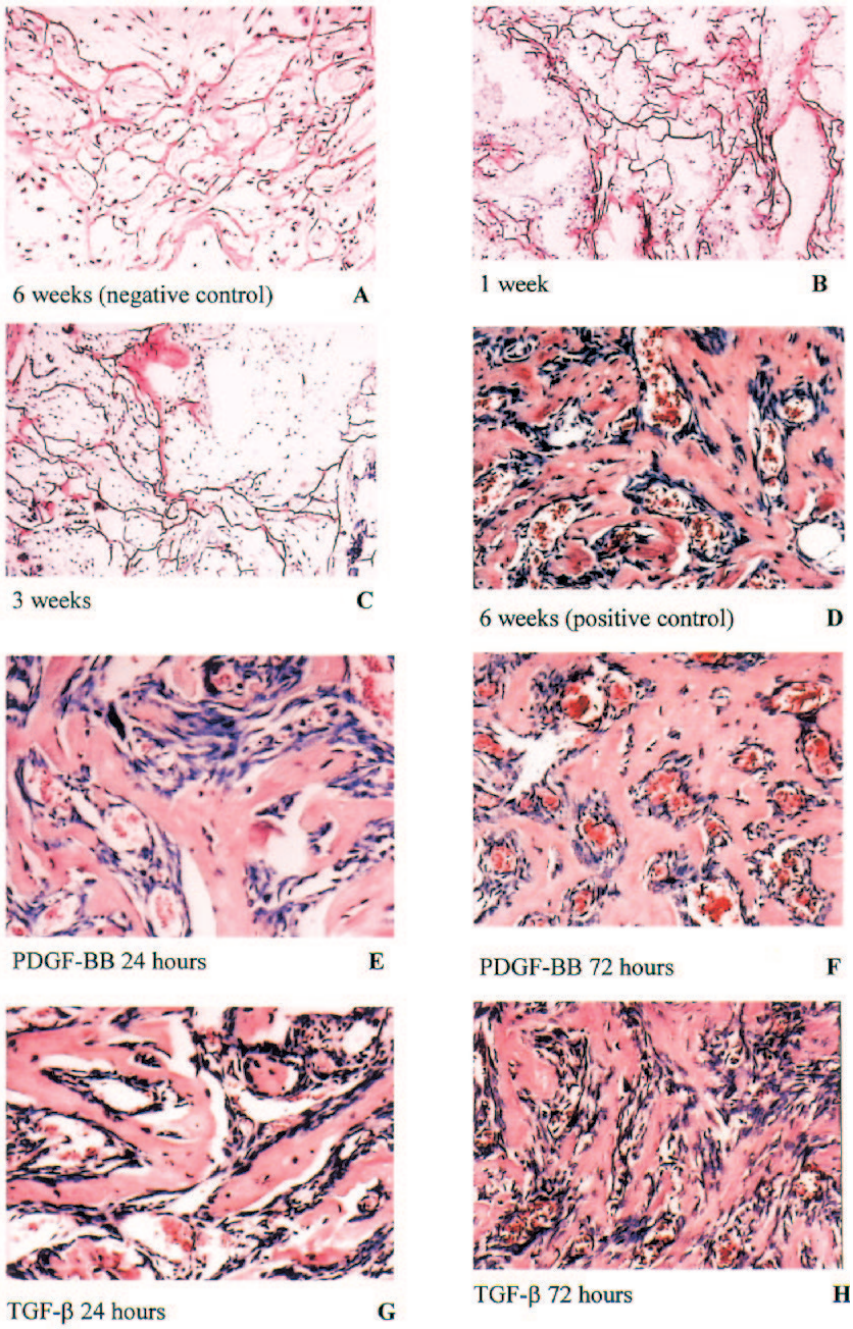

Figure 4.
Biomineralization by cementoblasts ex vivo. Histological sections obtained from implantation of cells and HA/TCP in immunodeficient SCID mice (original magnification, ×10). ***A.*** *material harvested after 6 weeks post-implantation. No mineral formation observed. The scaffold and granular structure of the material remained.* ***B.*** *OC-CM 30 cells grown in DMEM containing 10% FBS and implanted within HA/TCP, harvested 1 week post-implantation. No evidence of mineralization was observed.* ***C.*** *OC-CM 30 cells grown in DMEM containing 10% FBS and seeded in HA/TCP, harvested 3 weeks post-implantation. No evidence of mineralization was observed.* ***D.*** *OC-CM 30 cells grown in DMEM containing 10% FBS and implanted within HA/TCP, harvested 6 weeks post-implantation (positive control). Extensive biomineralization was observed with a woven-type pattern to the mineralized tissue* ***E and F.*** *OC-CM 30 cells treated with PDGF-BB for 24 or 72 hours, implanted within HA/TCP, harvested 6 weeks post-implantation. Biomineralization was observed throughout the graft and particles, with "woven type" mineralization. Note abundance of blood vessels throughout the mineralized matrix, especially in the 72-hour specimen (F).* ***G and H.*** *OC-CM 30 cells treated with TGF-β for 24 or 72 hours and implanted within HA/TCP, harvested 6 weeks post-implantation. Biomineralization was observed throughout the graft and particles, with a pattern similar to OC-CM 30 group in 10% FBS.*

formation, not by promoting cell differentiation, but by enhancing mitogenesis at early stages of bone formation. In contrast to PDGF-BB and TGF-β, IGF-I did not alter mineral formation when compared to controls. This finding correlates with the gene profile for cells exposed to IGF-I; i.e., upregulation of BSP mRNA levels, a molecule associated with mineralization, and down regulation of OCN and OPN mRNA levels; molecules considered to regulate the extent of crystal growth.[57,58,72]

Next, studies were designed to determine if mineralization would be altered under more physiological conditions. Engraftment of OC-CM 30 cells incorporated into a HA/TCP vehicle enabled us to treat these cells with growth factors ex vivo and determine results in an in vivo system (Fig. 4). Pretreatment of cultured cells with PDGF-BB or TGF-β for 24 or 72 hours prior to implantation had no effect on biomineralization. Thus it appears that initial inhibitory effects of these factors on mineralization, in vitro, can be overcome by the interaction of implanted cells with the local environment, in vivo. This finding is supported by others, demonstrating anabolic effects of TGF-β on bone metabolism in vivo.[73-75]

Another interesting finding was the prominent neovascularization noted in implants that had been pretreated with growth factors (Fig. 4). Angiogenesis is a particularly important requirement for regeneration. Thus, use of a combination of factors that promote vascularization, proliferation, and mineralization within a suitable vehicle may prove to be valuable for improving clinical outcomes of periodontal regenerative procedures, especially in challenging areas such as furcations where minimal vascularization may hinder the regenerative potential. Although PDGF-BB and TGF-β do not have a direct impact on proliferation of endothelial cells, TGF-β stimulates endothelial cell growth through a cytokine-related pathway, while PDGF-BB has a direct effect on vascular smooth muscle cells.[76] Therefore, both factors may work in concert to promote neovascularization during periodontal wound healing.

To our knowledge, this study is the first to investigate the effects of growth factors

on cementoblasts in vitro. Results here demonstrate that cementoblasts respond to growth factors and thus may serve as a cell population to target in attempts to regenerate periodontal tissues. Continued studies with these in vitro and ex vivo models will enable us to determine the mechanisms by which growth factors regulate cementoblasts. Information provided from such investigations will assist in designing regenerative therapies based on sound biological principles.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Beverly Sutton for assistance in preparation of this manuscript and Dr. Hongjiao Ouyang, Dr. John D'Errico, Chris Strayhorn, and Jan Berry for development of the cells used for these studies. This work was supported by NIDCR grants DE13047, DE11960, and DE09532.

REFERENCES

1. Wikesjö UM, Sigurdsson TJ, Lee MB, Tatakis DN, Selvig KA. Dynamics of wound healing in periodontal regenerative therapy. *J Calif Dent Assoc* 1995;23:30-35.
2. Amar S. Implications of cellular and molecular biology advances in periodontal regeneration. *Anat Rec* 1996; 245:361-373.
3. Cochran DL, Wozney JM. Biological mediators for periodontal regeneration. *Periodontol 2000* 1999;19:40-58.
4. Saygin NE, Giannobile WV, Somerman MJ. Molecular and cell biology of the cementoblast. *Periodontol 2000* 2000;24:73-98.
5. Arzate H, Alvarez-Perez MA, Aguilar-Mendoza ME, Alvarez-Fregoso O. Human cementum tumor cells have different features from human osteoblastic cells in vitro. *J Periodont Res* 1998;33:249-258.
6. Grzesik WJ, Kuzentsov SA, Uzawa K, Mankani M, Gehron Robey P, Yamauchi M. Normal human cementum-derived cells: Isolation, clonal expansion and in vitro and in vivo characterization. *J Bone Min Res* 1998;13:1547-1554.
7. D'Errico JA, MacNeil RL, Takata T, Berry JE, Strayhorn CL, Somerman MJ. Expression of bone associated markers by tooth root lining cells, in situ and in vitro. *Bone* 1997;20:117-126.
8. MacNeil RL, Berry J, D'Errico J, Strayhorn C, Somerman MJ. Expression of bone sialoprotein mRNA by cells lining the mouse tooth root during cementogenesis. *Arch Oral Biol* 1996;41:827-835.
9. MacNeil RL, Strayhorn C, Berry J, et al. Osteopontin is localized and expressed at specific sites during tooth eruption. In: Davidovitch Z, Norton LA, eds. *Biological Mechanisms of Tooth Movement and Craniofacial Adaptation*. Boston: Harvard Society for the Advancement of Orthodontics; 1996:123-131.
10. Ripamonti U, Reddi AH. Tissue engineering, morphogenesis and regeneration of the periodontal tissues by bone morphogenetic proteins. *Crit Rev Oral Biol Med* 1997;8:154-163.
11. Giannobile WV. Periodontal tissue regeneration by polypeptide growth factors and gene transfer. In: Lynch SE, Genco RJ, Marx RE, eds. *Tissue Engineering*. Carol Stream, IL: Quintessence Books; 1999:231-243.
12. Greenhalgh DG. The role of growth factors in wound healing. *J Trauma* 1996;4:159-167
13. Oates TW, Rouse CA, Cochran DL. Mitogenic effects of growth factors on human periodontal ligament cells in vitro. *J Periodontol* 1993;64:142-148.
14. Mailhot JM, Schuster GS, Garnick JJ, Hanes PJ, Lapp CA, Lewis JB. Human periodontal ligament and gingival fibroblast response to TGF-beta 1 stimulation. *J Clin Periodontol* 1995;22:679-685.
15. Giannobile WV, Whitson SW, Lynch SE. Non-coordinate control of bone formation displayed by growth factor combinations with IGF-I. *J Dent Res* 1997;76:1569-1578.
16. Takayama S, Murakami S, Miki Y, et al. Effects of basic fibroblast growth factor on human periodontal ligament cells. *J Periodont Res* 1997;32:667-675.
17. Lynch SE, Ruiz de Castilla G, Williams RC, et al. The effects of short-term application of a combination of platelet derived growth factor and insulin-like growth factors on periodontal wound healing. *J Periodontol* 1991;62:458-467.
18. Giannobile WV, Finkelman RD, Lynch SE. Comparison of canine and non-human primate animal models for periodontal regenerative therapy: Results following a single administration of PDGF/IFG-I. *J Periodontol* 1994; 65:1158-1168.
19. Wang HL, Pappert TD, Castelli WA, Chiego DJ. The effect of platelet-derived growth factor on the cellular resonse of the periodontium: an autoradiographic study on dogs. *J Periodontol* 1994;65:429-536.
20. Sigurdsson TJ, Lee MB, Kubota K, Turek TJ, Wozney JM, Wikesjö UM. Periodontal repair in dogs: recombinant human bone morphogenetic protein-2 significantly enhances periodontal regeneration. *J Periodontol* 1995; 66:131-138.
21. Howell TH, Fiorellini JP, Paquette DW, Offenbacher S, Giannobile WV, Lynch SE. A phase I/II clinical trial to evaluate a combination of recombinant human platelet-derived growth factor-BB and recombinant human insulin-like growth factor-I in patients with periodontal disease. *J Periodontol* 1997;68:1186-1193.
22. McCauley LK. Somerman MJ. Biologic modifiers in periodontal regeneration. *Dent Clinics N Am* 1998;42:361-387.
23. Chen D, Chen H, Feng JQ, et al. Osteoblastic cell lines derived from a transgenic mouse containing the osteocalcin promoter driving SV40 T-antigen. *Mol Cel Differen* 1995;3:193-212.
24. D'Errico JA, Berry JE, Ouyang H, Strayhorn CL, Windle JJ, Somerman MJ. Employing a transgenic animal model to obtain cementoblasts in vitro. *J Periodontol* 2000;71:63-72.
25. Strayhorn CL, Garrett JS, Dunn RL, Benedict J, Somerman MJ. Growth factors regulate expression of osteoblast associated genes. *J Periodontol* 1999;70: 1345-1354.
26. Matsuda N, Lin WL, Kumar NM, Cho MI, Genco RJ. Mitogenic, chemotactic and synthetic responses of rat periodontal ligament cells to polypeptide growth factors in vitro. *J Periodontol* 1992;63:515-525.
27. Harris SE, Bonewald LF, Harris MA, et al. Effects of transforming growth factor-β on bone nodule formation and expression of BMP-2, osteocalcin, osteopontin, alkaline phosphatase and Type I collagen mRNA in long term cultures of fetal rat calvarial cells. *J Bone Min Res* 1994;9:855-863.
28. Tenenbaum HC. Role of organic phosphate in mineralization of bone in vitro. *J Dent Res* 1981;60(Spec. No. C):1586-1589.
29. Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Min Res* 1994;

9:843-854.
30. Xie WQ, Rothblum LI. Rapid small scale RNA isolation from tissue culture cells. *Biotechniques* 1991;11:324-327.
31. Young MF, Ibaraki K, Kerr JM, Lyu MS, Kozak CA. Murine bone sialoprotein (BSP): cDNA cloning, mRNA expression, and genetic mapping. *Mamm Genome* 1994; 5:108-111.
32. Wozney JM. The bone morphogenetic protein family and osteogenesis. *Molec Reproduct Develop* 1992;32:160-167.
33. Young MF, Kerr JM, Termine JD, et al. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics* 1990;7:491-502.
34. Dugaiczyk A, Haron JA, Stone EM, Dennison OE, Rothblum KN, Schwartz RJ. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. *Biochem* 1983;22:1605-1613.
35. Somerman MJ, Ouyang HJ, Berry JE, et al. Evolution of perodontal regeneration: from the root's point of view. *J Periodont Res* 1999;34:1-5.
36. Kuznetsov SA, Krebsbach PH, Satomura K, et al. Single-colony derived strains of human marrow fibroblasts form bone after transplantation in vivo. *J Bone Min Res* 1997;12:1335-1347.
37. Rutherford RB, Niekrash CE, Kennedy JE, Charette MF. Platelet-derived and insulin-like growth factors stimulate regeneration of periodontal attachment in monkeys. *J Periodont Res* 1992;27:285-290.
38. Canalis E, McCarthy TL, Centerella M. Effects of platelet derived growth factor on bone formation in vitro. *J Cell Physiol* 1989;140:530-537.
39. Hock JM, Canalis E. Platelet derived growth factor enhances bone cell replication but not differentiated function of osteoblasts. *Endocrinol* 1994;14:1423-1428.
40. Piche JE, Graves DT. Study of growth factor requirements of human bone-derived cells: A comparison with human fibroblasts. *Bone* 1989;10:131-138.
41. Canalis E, Lian JB. Effects of bone associated growth factors on DNA, collagen and OC synthesis in cultured fetal rat calvariae. *Bone* 1988;9:243-246.
42. Pfeilschifter J, Oechsner M, Naumann A, Gronwald RG, Minnie HW, Ziegler R. Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth factor, and transforming growth factor beta. *Endocrinol* 1990;127:69-75.
43. Wergedal JE, Mohan S, Lundy M, Baylink DJ. Skeletal growth factor and other growth factors known to be present in bone matrix stimulate proliferation and protein systhesis in human bone cells. *J Bone Min Res* 1990;5: 179-186.
44. Elford PR, Guenther HL, Felix R, Cecchini MG, Fleisch H. Transforming growth factor-beta reduces the phenotypic expression of osteoblastic MC3T3-E1 cells in monolayer culture. *Bone* 1987;8:259-262
45. Noda M, Rodan GA. Type beta transforming growth factor (TGF beta) regulation of alkaline phosphatase expression and other phenotype-related mRNAs in osteoblastic rat osteosarcoma cells. *J Cell Physiol* 1987;133: 426-437.
46. Ibbotson KJ, Orcutt CM, Anglin AM, D'Souza SM. Effects of transforming growth factors beta 1 and beta 2 on a mouse clonal, osteoblastlike cell line MC3T3-E1. *J Bone Min Res* 1989;4:37-45.
47. Centrella M, McCarthy TL, Canalis E. Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *J Biol Chem* 1987;262:2869-2874.
48. Robey PG, Young MF, Flanders KC, et al. Osteoblasts synthesize and respond to transforming growth factor–type β in vitro. *J Cell Biol* 1987;105:457-463.
49. Katagiri T, Lee T, Takeshima H, Suda T, Tanaka H, Omura S. Transforming growth factor-beta modulates proliferation and differentiation of mouse clonal osteoblastic MC3T3-E1 cells depending on their maturation stages. *Bone Min* 1990;11:285-293.
50. Pfeilschifter J, D'Souza SM, Mundy GR. Effects of transforming growth factor beta on osteoblastic osteosarcoma cells. *Endocrinol* 1987;121:212-218.
51. MacNeil RL, Somerman MJ. Development and regeneration of the periodontium: parallels and contrasts. *Periodontol 2000* 1999;19:8-20.
52. Nanci A. Content and distribution of noncollagenous matrix proteins in bone and cementum: relationship to speed of formation and collagen packing density. *J Structur Biol* 1999;126:256-269.
53. Ganns B, Kim RH, Sodek J. Bone siaoloprotein. *Crit Rev Oral Biol Med* 1999;10:79-98.
54. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Nat Acad Sci* (USA) 1993;90:8562-8565.
55. Hunter GK, Hauschka PV, Poole AR, Rosenberg LC, Goldberg HA. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem J* 1996;317:59-64.
56. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 1997;89:747-754.
57. Bronckers AL, Price PA, Schrijvers A, Bervoets TJ, Karsenty G. Studies of osteocalcin function in dentin formation in rodent teeth. *Eur J Oral Scien* 1998;106: 795-807.
58. Butler WT, Ridall AL, McKee MD. Osteopontin. In: Bilezikian JP, Raizs LG, Rodan GA, eds. *Principles of Bone Biology*. San Diego: Academic Press; 1996:167-181.
59. Boskey AL, Maresca M, Ullrich W, Doty SB, Butler WT, Prince CW. Osteopontin-hydroxyapatite interactions in vitro: inhibition of hydroxyapatite formation and growth in a gelatin-gel. *Bone Min* 1993;22:147-159.
60. Hunter GK, Kyle CL, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J* 1994;300:723-728.
61. Centrella M, McCarathy TL, Kusmik WF, Canalis E. Relative binding and biochemical effects of heterodimeric and homodimeric isoforms of platelet-derived growth factor in osteoblast enriched cultures from fetal rat perietal bone. *J Cell Physiol* 1991;147:420-426.
62. Tanaka H, Liang CT. Effect of platelet-derived growth factor on DNA synthesis and gene expression in bone marrow stromal cells derived from adult and old rats. *J Cell Physiol* 1995;164:367-375.
63. Hsieh S, Graves DT. Pulse application of platelet derived growth factor enhances formation of a mineralising matrix while continuous application is inhibitory. *J Cell Biochem* 1998;69:169-180.
64. Noda M, Yoon K, Prince CW, Butler WT, Rodan GA. Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type beta transforming growth factor. *J Biol Chem* 1988;263:13916-13921.

65. Sorensen S, Justesen SJ, Johnsen AH. Identification of a macromolecular crystal growth inhibitor in human urine as osteopontin. *Urol Res* 1995;23:327-334.
66. McCauley LK, Koh AJ, Beecher CA, Cui Y, Decker JD, Franceschi RT. Effects of differentiation and transforming growth factor beta 1 on PTH/PTHrP receptor mRNA levels in MC3T3-E1 cells. *J Bone Min Res* 1995;10: 1243-1255.
67. Takata T, D'Errico JD, Atkins KB, et al. Protein extracts of dentin effect proliferation and differentiation of osteoprogenitor cells in vitro. *J Periodontol* 1998;69:1247-1255.
68. Noda M. Transcriptional regulation of osteocalcin production by transforming growth factor-beta in rat osteoblast-like cells. *Endocrinol* 1989;124:612-617.
69. Giannobile WV. Periodontal tissue engineering by growth factors. *Bone* 1996;19:23S-37S.
70. Rubini M, Werner H, Gandini, Roberts CT Jr, LeRoith D, Baserga R. Platelet-derived growth factor increases the activity of promoter of the insulin-like growth factor-I (IGF-I) receptor gene. *Exp Cell Res* 1994;211:374:3379.
71. Antosz ME, Bellows CG, Aubin JE. Effects of transforming growth factor-β and epidermal growth factor on cell proliferation and the formation of bone nodules in isolated fetal rat calvaria cells. *J Cell Physiol* 1989;140: 386-395.
72. Ducy P, Desbois C, Boyce B, et al. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; 382:448-452.
73. Noda M, Camilliere JJ. In vivo stimulation of bone formation by transforming growth factor-beta. *Endocrinol* 1989;124:2991-2994.
74. Beck LS, Amento EP, Xu Y, et al. TGF-β1 induces bone closure of skull defects: Temporal dynamics of bone formation in defects exposed to rh TGF-β1. *J Bone Min Res* 1993;8:753-761.
75. Erlebacher A, Filvaroff EH, Ye JQ, Derynck R. Osteoblastic responses to TGF-beta during bone remodeling. *Mol Biol Cell* 1998;9:1903-1918.
76. Pusztai L, Lewis CE, Lorenzen J, McGee JO'D. Growth factors: Regulation of normal and neoplastic growth. *J Pathol* 1993;169:191-201.

Send reprint requests to: Dr. Martha Somerman, Department of Periodontics/Prevention/Geriatrics, University of Michigan, School of Dentistry, 1011 North University Avenue, Ann Arbor, MI 48109-1078. Fax: 734/763-5503; e-mail: somerman@umich.edu

Accepted for publication March 27, 2000.