An ultimate goal of periodontal regenerative therapies is to establish treatment modalities that predictably reconstruct periodontal tissues lost as a consequence of disease. This requires formation of bone, cementum, and a functional periodontal ligament (PDL). A key for reengineering these tissues is to first ascertain the necessary factors required for their development. As one approach, the studies described here focus specifically on determining the effects of agents on expression of genes associated with osteoblasts using a well-defined in vitro system. This in vitro model utilizes MC3T3-E1 cells, a mouse osteoblast progenitor cell line. Importantly, when MC3T3-E1 cells in vitro are exposed to ascorbic acid and β-glycerol phosphate, they differentiate along the osteoblast pathway. This differentiation includes expression of osteoblast-associated genes, e.g., alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OCN), and osteopontin (OPN) and secretion of a matrix appropriate for promoting mineral nodule formation. Studies using a rat periodontal regenerative model have shown that these same genes are associated with bone formation in vivo. Thus, these cells provide an excellent system for initial screening of agents to determine whether or not specific factors can influence osteoblast differentiation.
Attractive candidates for promoting osteoblast differentiation include polypeptide growth factors and bone morphogenetic proteins. The studies here focused on determining the role of platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and a partially purified extract of bovine bone (BP) containing a mixture of bone morphogenetic proteins (BMPs), alone and in combination, in regulating proliferation and gene expression in MC3T3-E1 cells. The rationale for selecting these factors was based on several points, including: 1) the known ability of polypeptide growth factors to promote events associated with wound healing including cell migration, cell proliferation, and extracellular matrix production; 2) existing evidence suggesting that PDGF/IGF can act synergistically to promote periodontal regeneration, bone formation, and cell proliferation; 3) the known ability of bone morphogenetic proteins, such as those present in demineralized freeze-dried bone, to induce bone formation and also cementogenesis.

PDGF containing two polypeptide chains as either a homodimer (AA or BB) or a heterodimer (AB) exhibits strong mitogenic activity in many cell types. The ability of PDGF to promote wound healing has been attributed to its proliferative activity, where it may function to increase the number of cells within the local environment at a healing site. IGF-I and -II are anabolic polypeptide growth factors structurally related to insulin. IGFs, produced in bone cells and stored in high concentrations within the bone matrix, are considered to regulate bone cells through both autocrine and paracrine pathways, resulting in an increase in osteoblast maturation. IGF activity and concentration at local sites have been shown to be regulated by several other factors including IGFBPs (IGF binding proteins), PDGF, and bone morphogenetic proteins (BMPs). Interestingly, PDGF appears to decrease expression of IGF mRNA in osteoblasts, while BMPs increase expression of IGF mRNA. Cell receptors for PDGF and IGFs have been identified and, while different from each other, are both tyrosine kinase signaling receptors.

The BMPs, originally isolated from bone, belong to the TGF-β superfamily and have a variety of functions dependent on tissue location and developmental stage. Studies to date provide convincing evidence that BMPs are powerful inducers of osteoblast differentiation in vitro and bone formation in vivo, and thus may have potential for use in clinical situations where new bone formation is required. BMPs act through specific threonine-serine receptor kinases.

Previous in vitro and in vivo studies have demonstrated that PDGF/IGF act synergistically to promote periodontal regeneration, while data from in vitro studies using BMP and IGF in combination suggest that these 2 factors act synergistically to promote osteoblast differentiation. This prompted us to determine whether or not combinations of PDGF/IGF/BP would prove to be useful for promoting periodontal regeneration. As a first step, the studies here focused on examining the effect of PDGF, IGF, and/or BP in various combinations on osteoblast activity in vitro. Factors that can enhance mineral tissue formation in vitro have potential for clinical use to promote both root mineralization and bone formation as required subsequent to loss of periodontal tissues. Results of studies here indicate that both IGF and BP promoted osteoblast differentiation as measured by expression of BSP, OPN, and OCN mRNA in MC3T3-E1 cells, with an additive effect when used in combination. In contrast, PDGF promoted proliferation in MC3T3-E1 cells and blocked differentiation of osteoblasts as measured by decreased expression of OCN mRNA. Furthermore, when added with IGF, PDGF blocked the ability of IGF to induce expression of BSP, OPN, and OCN mRNA.

MATERIALS AND METHODS

Assay for Bovine Bone Protein Activity
BP was obtained as a dry lyophilized powder from a commercial source. The biological activity was assessed using a rat orthotopic calvaria model. BP was prepared by dissolving 35 µg of BP in 35 µl of 10 mM HCl solution. This was added to 5 mg of lyophilized collagen and allowed to soak into the collagen matrix. The resulting BP/collagen mixture was lyophilized and then added to 45 mg of a 50:50 poly (DL-lactide-co-glycolide) polymer (PLG) with an inherent viscosity of 0.17 dl/g.

Adult Sprague Dawley rat calvaria were exposed via a midline incision extending from the nasal bone to the mid-sagittal crest and the skullcap was exposed with blunt dissection. The periosteum was removed from the internal flap surface and the polymer implant containing the lyophilized BP/collagen matrix was placed at the orthotopic site. Incisions were closed with 4-0 silk sutures. The animals were sacrificed at day 28 and their skullcaps surgically removed and processed for histological evaluation. Six micron step serial sections were made through the implant area and slides were analyzed for the overall amount of bone formation within the implant histomorphometrically.

Culturing of MC3T3-E1 Cells
MC3T3-E1 cells originally obtained from Dr. M. Kumegawa (Meikai University, Sakado, Japan) were...
RESULTS
An orthotopic rat calvaria model was used to verify that BP had biological activity. As shown in Figure 1, BP promoted bone formation in this model system, confirming that the BP material had biologic activity. All sites treated with the polymer containing the BP/collagen matrix showed substantial bone formation. No control site treated with collagen/polymer matrix without BP showed bone formation.

Next, to determine the effects of IGF, PDGF, and BP on proliferation of MC3T3-E1 cells, cell counts were done at various time points over an 8-day period. BP at 1 to 20 µg resulted in a decrease in cell number at day 8 when compared with vehicle control (2%) and an increase in cell number when compared with negative control (CN) (Fig. 2A). At day 8, PDGF at 10 ng/ml and 20 ng/ml enhanced cell number when compared to control cells – both vehicle control (2%) and negative control (CN) (Fig. 2B). No significant effect on cell number was noted in cells exposed to 1 ng/ml PDGF. In contrast, IGF at all doses examined here (1 to 20 ng/ml) had no significant effect on cell number beyond that seen for both vehicle control (2%) and negative control (CN) (Fig. 2C).

Existing evidence that PDGF and IGF in combination have a synergistic effect on cell proliferation10,13 prompted us to next examine whether such an effect would be obtained with MC3T3-E1 cells. As shown in Figure 2D, MC3T3-E1 cell number was increased by
PDGF/IGF combination when compared with 2% FBS control cells, but this effect was not greater than that noted for cells exposed to PDGF alone, at comparable doses (Fig. 2B).

As a positive control, cells were exposed to mineralization media plus 10% FBS, and such cells exhibited increased cell numbers when compared with all other conditions. Statistical analyses at day 8 are shown in Table 1.

Northern Analysis
For analysis of genes expressed by MC3T3-E1 cells exposed to the various agents, doses used were as follows: PDGF 10 ng/ml; IGF 10 ng/ml; BP 10 µg/ml; PDGF/IGF 10 ng/ml each; PDGF/BP (10 ng/ml/10 µg/ml); IGF/BP (10 ng/ml/10 µg/ml); and PDGF/IGF/BP (10 ng/ml/10 ng/ml/10 µg/ml). These doses were selected to maintain consistency with the proliferation studies. In most experiments, cells were exposed to agents for the duration of the experiment, 8 days, with media changes every 3 to 4 days as described above.

OCN/BSP
As shown in Figure 3, PDGF blocked expression of OCN, a gene associated with osteoblast differentiation, when compared with vehicle control (2%). Cells exposed to BP and IGF exhibited enhanced expression of BSP compared to vehicle control (2%).

PDGF’s inhibitory effect on OCN expression was noted in cells exposed to PDGF/IGF combination. In contrast, PDGF’s inhibitory effect on OCN was not as prominent in cells exposed to the combination of PDGF/BP or PDGF/IGF/BP (P/I/BP).

OPN
As shown in Figure 4, the effects of these agents on OPN expression were more dramatic. Both IGF and BP promoted OPN mRNA when compared with the vehicle control (2%). Furthermore, the combination of IGF/BP resulted in an even greater promotion of OPN mRNA levels. Interestingly, PDGF blocked IGF-mediated OPN mRNA (PDGF/IGF lane), while PDGF was not able to alter BP-mediated OPN mRNA expression (PDGF/BP lane). In addition, the additive effect of IGF/BP on OPN mRNA expression was blocked by PDGF (P/I/BP lane). Results at day 16 were comparable to those at day 8 (data not shown).

OPN Pulse Experiments
The next series of experiments were designed to determine whether an even greater effect on osteoblast differentiation would be observed if cells were exposed to growth factors early on, with BP added at a later stage (Fig. 4). For these experiments, cells were exposed to IGF (10 ng/ml) or PDGF (10 ng/ml) or PDGF/IGF (10 ng/ml/10 ng/ml) for the total duration of the experiment, 8 days, with the addition of BP (10 µg/ml) the last 2 days. On day 8, RNA was extracted and transcripts for OPN determined by Northern analysis. No differences in gene expression were noted between exposure of cells to IGF/BP for 8 days (IGF/BP lane) versus adding BP on day 6 (I/BP day 6 lane). Similarly, exposing cells to PDGF/BP for 8 days (PDGF/BP lane) versus adding BP on day 6 (P/BP day 6 lane) gave comparable results. PDGF’s inhibitory effect on OPN mRNA expression was noted in PDGF/IGF/BP treated cells (P/I/BP lane), even after addition of BP on day 6 (P/I/BP day 6 lane).

Osf2
Studies by Ducy et al. have identified Osf2/Cbfa1 (osteoblast specific transcription factor/core-binding factor) as an osteoblast-specific transcription factor and more specifically as a regulator of osteoblast differentiation. Thus, as a next step, the effect of exposing cells to PDGF, IGF, and BP on expression of Osf2
was determined. As seen in Figure 5, BP and IGF did not affect expression of Osf2 mRNA, while PDGF inhibited Osf2 expression when compared with the vehicle control cells (2%). Cells exposed to the combination of PDGF and IGF still exhibited decreased levels of Osf2 transcripts, while PDGF was not able to completely block Osf2 mRNA noted in cells exposed to BP. Also, cells exposed to 10% FBS exhibited lower levels of Osf2 mRNA when compared with cells exposed to 2% FBS.

**DISCUSSION**

The studies here were designed to determine the effects of IGF, PDGF, and BP, alone and in combination, on osteoblast differentiation, using an in vitro model system. Furthermore, it was anticipated that the in vitro model could serve as an assay for screening agents for their ability to promote osteoblast differentiation prior to initiating more costly in vivo studies with these agents.

A well-characterized mouse osteoblast progenitor cell line, MC3T3-E1, was used for these studies. Importantly, when these cells are cultured in the presence of ascorbic acid, they are stimulated to differentiate along the osteoblast pathway. With onset of differentiation, there is a cessation of cell replication, secretion of a type I collagen-associated extracellular matrix, and an upregulation of markers of osteoblast differentiation. Thus, in the presence of ascorbic acid, these cells are induced to express mRNA for BSP and OCN, known markers for the osteoblast phenotype. BSP, a glycoprotein selective to mineralized tissues, has been implicated as having a

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**Table 1.**

**Effect of PDGF, IGF and BP on Cell Proliferation; Statistical Analysis at Day 8**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BP 10 µg</th>
<th>PDGF 10 ng</th>
<th>IGF 10 ng</th>
<th>PDGF/IGF 10 ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Vehicle (2%)</td>
<td>*</td>
<td>†</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Negative (CN)</td>
<td>†</td>
<td>†</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NS** = not significant ($P>0.05$).

* $P<0.01$; † $P<0.001$. 

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**Figure 2.**

Effect of PDGF, IGF, and BP on cell proliferation. Cells were plated at a density of 5,000 cells/ml and allowed to adhere overnight. On the following day, mineralization media with appropriate growth factors were added. Media were changed on day 3 and 6 and cells were harvested for cell counts on day 3, 6, and 8. A. BP; B. PDGF; C. IGF; D. PI (PDGF/IGF 10 ng/10 ng).
Increased expression of osteoblast-specific markers, while PDGF inhibits differentiation of MC3T3-E1 cells along the osteoblast pathway. Furthermore, when IGF-stimulated cells also are exposed to PDGF, expression of osteoblast-specific markers induced by IGF is blocked. In contrast, PDGF was not able to block BP-induced expression of osteoblast-specific markers. When BP was added in combination with IGF, the increased expression of OPN, OC and BSP transcripts was still noted, where an additive effect on OPN mRNA expression was detected in these cells when compared with cells treated with either BP or IGF alone. This additive effect on OPN transcripts may prove to have clinical significance. For example, while highly speculative, agents that regulate crystal growth, such as OPN, may provide an appropriate balance between mineral formation and PDL attachment, thus preventing ankylosis, as has been reported with use of various other osteoinductive factors.

Interestingly, at the time we noted this additive effect on OPN mRNA expression, two papers appeared supporting this finding. Yeh et al. demonstrated that BMP-7/OP-1 and IGF-I acted synergistically to promote [3H] thymidine incorporation, alkaline phosphatase activity, PTH-dependent cAMP level, and bone nodule formation, using primary cultures of fetal rat calvaria cells, where maximal synergy was noted when they were added simultaneously. They did not report on the effects of these factors on OCN, BSP, or OPN mRNA levels. In another study, Hayden et al. demonstrated that BMP-7 stimulates IGFBP-3 production in human osteosarcoma cells predominantly by promoting the production of IGFBP-3 nuclear transcripts. The importance of this activity to BMP-7’s ability to promote differentiation remains to be established.

Based on previous reports indicating that PDGF/IGF in combination promote periodontal regeneration, we were surprised that the combination of PDGF/IGF blocked expression of genes associated with osteoblasts. However, the positive effects of PDGF seen at sites of wound healing are attributed to its ability to promote cell migration and proliferation, thus establishing a critical mass of cells at a local site. In fact, several studies report that PDGF, while promoting cell replication, inhibits osteoblast differentiation. Furthermore, Hock and Canalis demonstrated that PDGF decreased IGF mRNA in osteoprogenitor cells and suggested that this effect may, in part, explain PDGF-mediated inhibition of osteoblast differentiation. Thus, our studies support
In summary, the studies here demonstrate that BP is a potent inducer of osteoblast differentiation and may act synergistically with IGF to promote osteoblast differentiation. Further studies, including the use of various doses of BP/IGF and/or PDGF in combination with PDGF, may reveal additional insights into the complex interactions of these growth factors during osteoblast differentiation.

**Figure 5.**
Effect of PDGF, IGF, and BP on Osf2 mRNA in MC3T3-E1 cells. For these experiments, cells were treated as described in Figure 3. GAPDH is shown to indicate relative loading of RNA.
vivo and in vitro, are warranted to determine if there are any benefits in using these factors in combination for periodontal regenerative procedures.

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Dr. Garrett is Senior Vice President and Dr. Dunn is Vice President of Atrix Corporation. Dr. Benedict is Vice President of Sulzer Orthopedics.

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