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# Pulse Application of Platelet-Derived Growth Factor Enhances Formation of a Mineralizing Matrix While Continuous Application Is Inhibitory

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**Abstract** Platelet-derived growth factor (PDGF) stimulates chemotaxis and proliferation of osteoblasts, and induces bone formation in vivo. To determine how PDGF might regulate these cells, the effect of PDGF on long-term mineralizing cultures of fetal rat osteoblastic cells was examined. Although PDGF increased cell proliferation in these cultures, continuous treatment with PDGF caused a dose-dependent decrease in mineralized nodule formation. When cells were treated with multiple, brief (1 day) exposures to PDGF at the osteoblast differentiation stage, there was a significant 50% increase in mineralized nodule area. Based on modulation of alkaline phosphatase activity it appears that longer-term exposure to PDGF reduces mineralized nodule formation largely by inhibiting differentiated osteoblast function, while short-term exposure enhances proliferation without inhibiting the differentiated phenotype. Thus, the ultimate affect of PDGF on bone formation is likely to reflect two processes: a positive effect through enhancing cell number or a negative effect by inhibiting differentiated function. The inhibitory effect of PDGF on formation of a mineralized matrix is unlikely to be simply a result of enhanced proliferation of "fibroblastic" cells since cultures treated with PDGF for 3 days and then transferred to new plastic dishes exhibited a 70% increase in mineralized nodule area compared to controls. These results would predict that multiple, brief exposures to PDGF would enhance bone formation in vivo, while prolonged exposure to PDGF, which is likely to occur in chronic inflammation, would inhibit differentiated osteoblast function and limit bone regeneration. J. Cell. Biochem. 69:169–180, 1998. 

**Key words:** growth factor; bone; osteoblast; inflammation; alkaline phosphatase; differentiation; proliferation; PDGF; mineralized nodules

Platelet-derived growth factor (PDGF) is a member of the polypeptide growth factor family. There are two different PDGF polypeptides that are 56% homologous and encoded by different genes, PDGF-A and PDGF-B. PDGF is present as three dimers: PDGF-AA, PDGF-BB, and PDGF-AB. These three isoforms stimulate mitogenesis and migration in osteoblastic cells [Centrella et al., 1989, 1991, 1992; Piche Graves, 1989; Gilardetti et al., 1991]. In vivo evidence suggests that PDGF is an important factor for bone wound-healing and new bone formation. PDGF A and B genes are induced during fracture repair and expressed by multiple cell types during bone wound healing [Bolander, 1992; Nash et al., 1994; Andrew et al., 1995]; PDGF is produced by osteoblastic cells and is stored in bone [Hauschka et al., 1986; Zhang et al., 1991; Rydziel et al., 1992]. Several studies indicate that PDGF alone, or with other mediators such as IGF-I or dexamethasone, can enhance bone regeneration in both periodontal and orthopedic models of osseous healing [Lynch et al., 1989, 1991; Rutherford et al., 1992, 1993].

The mechanisms by which PDGF may affect osteoblastic cells has also been studied in different in vitro "model" systems including monolayer cultures of human and rat osteoblastic cells, and calvarial organ cultures. Centrella and co-workers reported that PDGF stimulates proliferation of rat calvarial osteoblasts in monolayer cultures, but has little effect on the amount of collagen produced per cell [Centrella et al., 1991]. PDGF also reduces alkaline phosphatase activity in monolayer cultures, a marker of

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osteoblastic cell differentiation. Recent studies demonstrate that PDGF treatment disrupts and inhibits bone matrix formation in the fetal rat calvaria organ cultures [Hock and Canalis, 1994]. These reports suggest that PDGF enhances osteoblast replication, but not differentiated function of osteoblasts. It has also been proposed that PDGF could potentially enhance bone collagen degradation by up-regulating the expression of interstitial collagenase [Varghese et al., 1996]. In addition, PDGF has been reported to induce bone calcium release in mouse calvaria organ cultures [Tashjian et al., 1982; Cochran et al., 1993]. These studies indicate that PDGF may have multiple effects on bone remodeling.

To further investigate the impact of PDGF on osteoblastic cells, we examined mineralized nodule formation in long-term cultures of osteoblast-enriched populations of fetal rat calvarial cells [Bellows et al., 1986]. In this culture system, mineralized "bone-like" nodules are formed following cell proliferation, matrix production, and extracellular matrix mineralization. The formation of mineralized nodules in vitro involves many of the same cellular events and exhibits many of the same morphological, ultrastructural, and biochemical characteristics of woven bone formed in vivo [Bellows et al., 1986; Bhargava et al., 1988; Owen et al., 1990]. Thus long-term cultures of rat calvarial osteoblastic cells have been successfully used as an in vitro model system to gain insight into the effect of growth factors on bone formation [Antosz et al., 1987; Stashenko et al., 1987; Harris et al., 1994]. Despite the wide use of this model, little is known about the effects of the platelet-derived growth factor on mineralized nodule formation.

In studies presented here, we evaluated the effect of continuous and various pulse treatments with PDGF on mineralized nodule formation. The data demonstrate that continuous PDGF treatment inhibits formation of a mineralized matrix. Pulse incubation with PDGF at different stages of osteoblast development showed biphasic effects on mineralized nodule formation. Multiple, brief (1 day) exposures to PDGF at the osteoblast differentiation stage exhibited a statistically significant stimulation of mineralized nodule area. However, if cells were stimulated for long periods of time, PDGF suppressed formation of mineralized nodules. These results suggest that the effect of PDGF on mineralized nodule formation depends on the length of exposure and stage of osteoblast development.

## MATERIALS AND METHODS

### Cell Isolation and Culture

The isolation of fetal rat calvarial cells with osteoblast-like characteristics was performed as described by Bellows and colleagues [Bellows et al., 1986]. Frontal and parietal bones from 20-day fetal rats were cleaned of loose connective tissue and periosteum. The calvaria were minced and subjected to 5 sequential collagenase (2 mg/ml) and trypsin (0.05%) digestions (10, 20, 30, 50, and 70 min of incubation, respectively). Cells from the first two digestions were discarded. Populations from digestion III to V were pooled and re-suspended with a-Minimum Essential Media (α-MEM) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 1% penicillin and streptomycin, and 0.05% Gentamycin, and cultured for 24 hr. Cells were then trypsinized and replated at the density of 5,400 cells/cm$^2$ in 12-well or 24-well plates. Cells were cultured in (α-MEM plus 10% FBS for the first 24 hr, rinsed, and then incubated in mineralizing media (α-MEM, 2.5% fetal bovine serum, 100 μg/ml ascorbic acid, 5 mM β-glycerophosphate, and antibiotics). PDGF-BB was generously provided by Creative Biomolecules (Hopkinton, MA). Media were changed every 3.5 days. All dishes were maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$.

### Staining and Quantifying of Mineralized Nodules

Cultures were fixed with 10% paraformaldehyde and stained for calcium by von Kossa's method [Clark, 1981]. The total area of mineralized bone-like nodules was quantified by projection of the microscopic image onto a computer monitor and quantifying the mineralized area by computer-assisted image analysis.

### DNA Synthesis

DNA synthesis was assessed by measuring the incorporation of [$^3$H] thymidine. Fetal rat calvarial osteoblastic cells were cultured at 5,400 cells/cm$^2$ in 24-well plates and changed to mineralizing media without or with PDGF (2, 20, 50 ng/ml) for 24 hr. [$^3$H] thymidine (2.5 μCi/ml) was added for the last 6 hr. Cells were

then fixed with cold 10% tricholracetic acid (TCA) for 30 min, rinsed with cold 10% TCA, and solubilized in 1% SDS for 30 min. The solubilized cells were transferred to vials with a scintillation cocktail. Incorporated [$^3$H] thymidine was evaluated in a Packard liquid scintillation analyzer (Packard Instrument Co., Downer Grove, IL).

### DNA Quantity

A fluorometric assay was employed to measure DNA quantity as described in Labarca and Paigen [1980]. A standard curve was established with 0 to 5 µg salmon sperm DNA. Cell extract was prepared by adding 1 M perchloric acid to pelleted cells and deoxyribose was liberated by heating at 70°C for 20 min. Fluorescence intensity was measured and compared to a standard curve generated at the same time. Excitation and emission wave lengths were 405 and 520 nm, respectively. The values obtained were used to monitor relative cell number and to normalize alkaline phosphatase activity results.

### Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured by a colorimetric procedure [Lowry et al., 1954]. Enriched populations of fetal rat osteoblastic cells were cultured in 24-well plates and incubated in mineralizing culture media as described above. After 3, 7, 14, 21, 28, or 35 days, cell layers were subjected to three freeze-thaw cycles in 400 µl of 0.01% TritonX-100. After thorough mixing, 50 µl of recovered sample was assayed for enzyme activity in 96-well plates with p-nitrophenylphosphate (Sigma) as a substrate, in a total of 100 µl. After 15 min at 37° C, the reaction was stopped with 100 µl of 1M NaOH, and the absorbance was measured at 410 nm and compared to a standard curve of p-nitrophenol.

### Statistical Analysis

Data were generally expressed as the mean ± standard deviation. Statistical difference between samples was determined by one-way analysis of variance followed by Tukey's multiple comparison test. All experiments were performed at least three times with similar results.

## RESULTS

### Continuous Exposure to PDGF Inhibits Mineralized Nodule Formation

To test the effect of different doses of PDGF-BB on the formation of mineralized bone-like nodules, fetal rat calvarial cells were cultured in mineralizing media with PDGF (0–50 ng/ml), which was changed every 3.5 days for 5 weeks, and then stained by von Kossa's method to quantify the area of mineralized matrix formation. Figure 1 demonstrates that PDGF-BB causes a significant and dose-dependent decrease in the total area of mineralized nodules. It had been reported that unidentified factors in fetal bovine serum (FBS) are indispensable for the formation of a mineralized matrix by osteoblast cell cultures in vitro [Aronow et al., 1990]. We tested whether PDGF-BB had the same effect on inhibiting mineralized matrix formation in 10 or 2.5% FBS. Under both conditions, PDGF at 20 ng/ml inhibited the formation of mineralized matrix by 80–85% (data not shown). In subsequent experiments we used a concentration of 2.5% FBS in mineralizing media.

To examine potential mechanisms by which PDGF could inhibit mineralized nodule formation, we investigated the effect on cell proliferation and alkaline phosphatase activity (Fig. 2). Figure 2A demonstrates that PDGF-BB (0–100 ng/ml) stimulates a dose-dependent increase in [$^3$H] thymidine incorporation, which reaches a plateau of fivefold enhancement at a concentration of 20 ng/ml or higher. Based on these results and those in Figure 1, a concentration of 20 ng/ml PDGF-BB was selected in subsequent experiments. To examine the effect of PDGF on cell proliferation at different stages of long-term mineralizing cultures, total DNA content was measured. DNA content increased over twofold in cells continuously incubated with PDGF compared to the control cells on days 3 and 7 (Fig. 2B). On day 28 and after, there was no significant difference in the amount of DNA per well for PDGF-BB stimulated cells compared to untreated controls, indicating that the major effect of PDGF on cell number occurs early in the first 2 to 3 weeks of the culture system. This is consistent with our previous observations that the effect of PDGF on cell proliferation appears to be down-regulated at the later stages in long-term mineralizing cultures [Xiaohui et al., 1997]. The results also

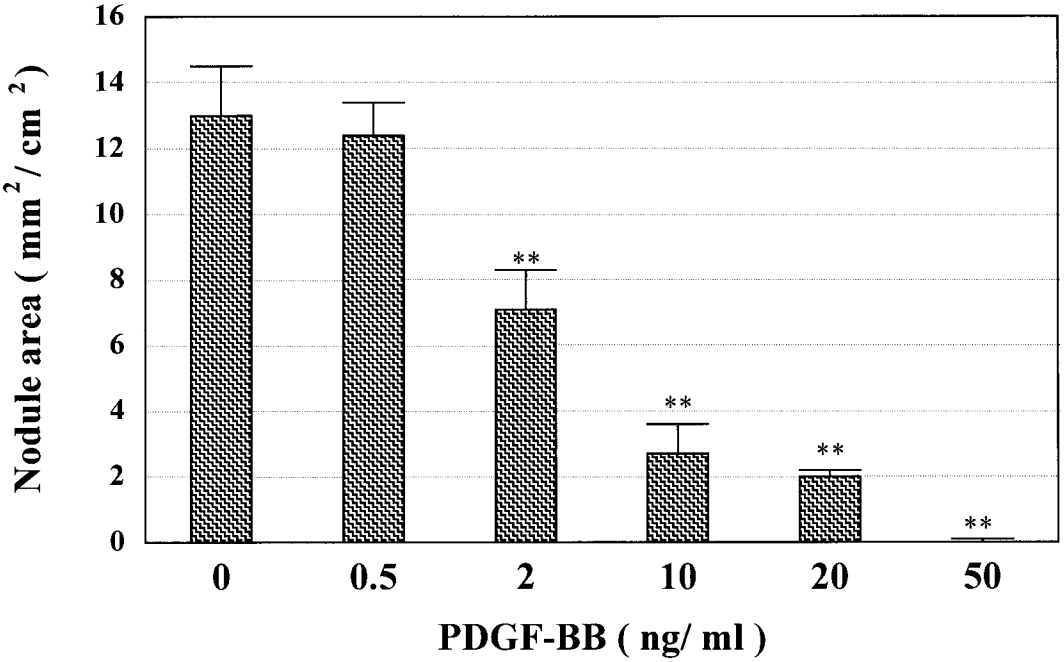


**Fig. 1.** PDGF inhibits formation of a mineralized matrix. Osteoblastic cells were obtained by collagenase digestion of fetal rat calvaria and were cultured in mineralizing culture media containing 2.5% FBS. Each group of cells was continuously treated with different concentrations of PDGF-BB (0–50 ng/ml). Mineralized nodules were stained by von Kossa's method after 5 weeks. The total area of mineralized nodules were quantified using an image analysis system at 2X magnification. Each bar represents the mean ± standard deviation of triplicate wells.**Statistically significant value compared to unstimulated cells: $P \leq 0.01$.

suggest that PDGF causes an initial burst in cell number that reaches a plateau after 14 days. This is not strictly due to contact inhibition, since 100% confluence is reached before 3 days with PDGF and before 7 days in control cultures.

The same cultures were also examined for the effect of PDGF on alkaline phosphatase activity to assess the impact of PDGF on the acquisition of a more mature osteoblastic phenotype. In control cultures, alkaline phosphatase activity was low in the first few days and dramatically increased from day 7 to 14 (Fig. 2C). There was little increase after day 14. Continuous exposure to PDGF consistently reduced alkaline phosphatase activity in the long-term cultures, with the greatest effect occurring before day 21, when there was a 70–80% decrease in the PDGF group compared to controls. Alkaline phosphatase activity increased in the PDGF-treated group during the late stages, but still was less than that of the control group.

### Effect of PDGF on Mineralized Nodule Formation Depends on the Duration of Exposure

To examine whether the duration of incubation with PDGF affects mineralized nodule formation, PDGF-BB pulse treatments of 1 or 7 days were used to stimulate long-term mineralizing cultures at different stages. Figure 3 demonstrates that a 7-day incubation with PDGF from day 0 to day 7, or day 7 to day 14, representing a relatively long-term pulse treatment, suppresses mineralized nodule formation. The min-

**Fig. 2.** PDGF modulates DNA synthesis, proliferation, and alkaline phosphatase activity in long-term mineralizing cultures of fetal rat osteoblastic cells. **A:** Fetal rat osteoblastic cells were cultured in mineralizing media until confluent. Following depletion of mitogenic factors for 24 hr in serum-free media, cells were stimulated with PDGF-BB (0–100 ng/ml) for 24 hr. [3H] thymidine was added for the final 6 hours. Thymidine incorporation was measured as described in Materials and Methods. **B:** To examine the effect of PDGF on cell number, fetal rat osteoblastic cells were cultured in mineralizing media without or with PDGF (20 ng/ml). The relative change in cell number was determined by quantifying the total amount of DNA per well at each time point. **C:** To examine the effect of PDGF on alkaline phosphatase activity, fetal rat osteoblastic cells were cultured in mineralizing media without or with PDGF (20 ng/ml). Cell lysates were collected on days 3, 7, 14, 21, 28, and 35. Alkaline phosphatase activity was expressed as nM p-nitrophenol per microgram of DNA per minute. Each value represents the mean ± standard deviation from quadruplicate wells. **Significantly different from corresponding control: $P \leq 0.01$. *Significantly different from corresponding control: $P \leq 0.05$.

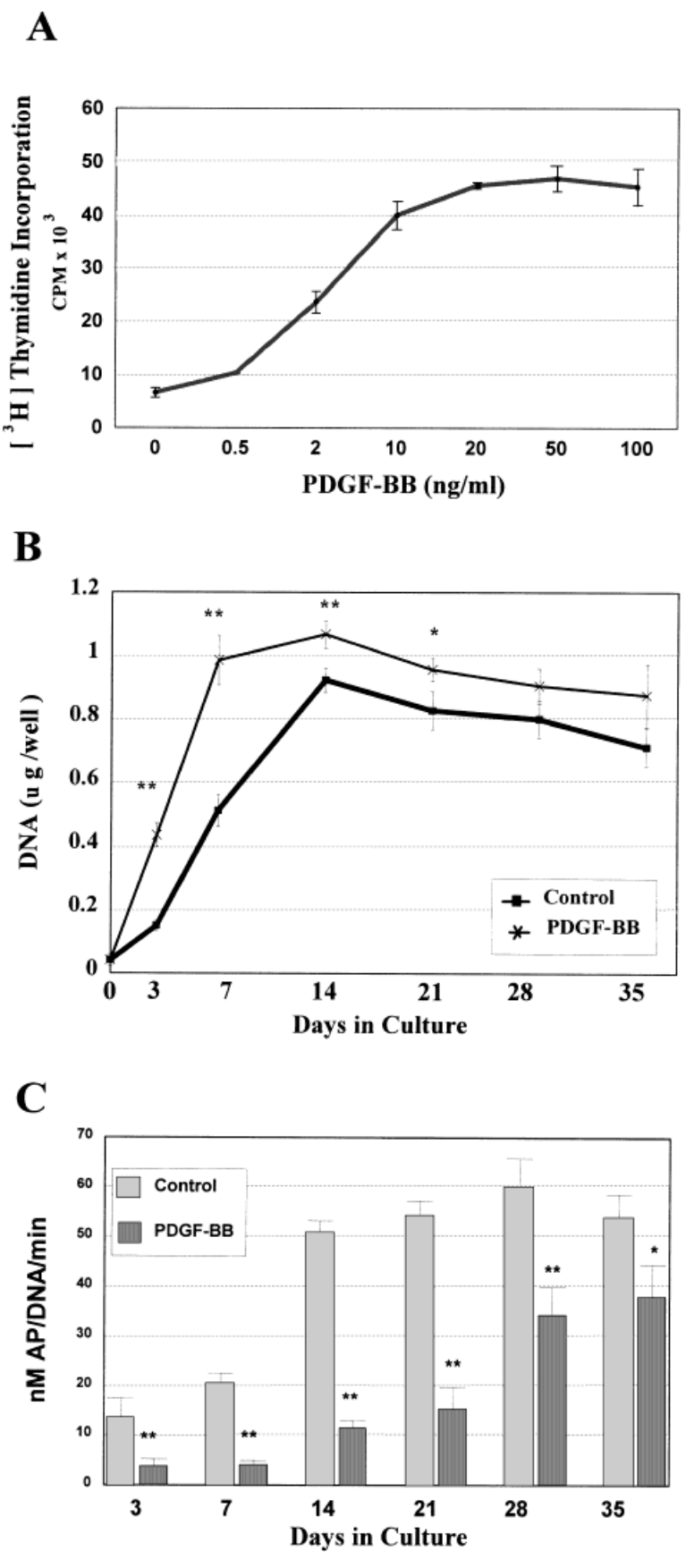
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Figure 2.

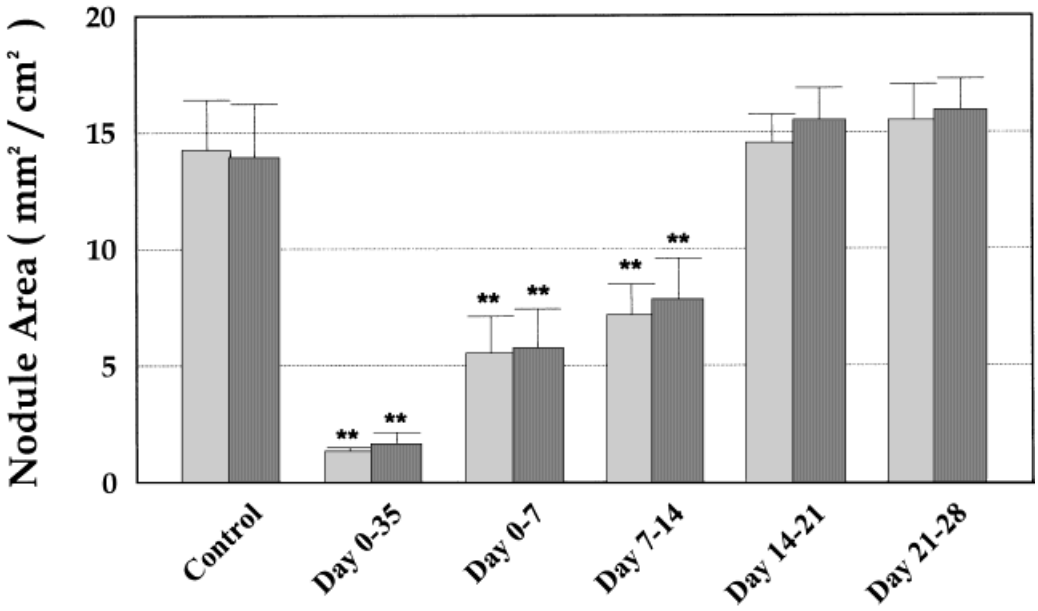


**Fig. 3.** The effect PDGF on formation of a mineralizing matrix is dependent upon the stage of osteoblast maturation. Fetal rat osteoblastic cells were cultured in mineralizing media without or with PDGF (20 ng/ml) either continuously or for periods of 1 week as indicated. The area of von Kossa's stained matrix was measured after 5 weeks (light bars) and 7 weeks (dark bars). Each value represents the mean ± standard deviation for triplicate samples. **Significantly different from corresponding control: $P \leq 0.01$. *Significantly different from corresponding control: $P \leq 0.05$.

eralized area for these groups was only half that of the control, untreated group. In contrast, when PDGF was applied in 7-day pulses at later stages, from days 14–21 or days 21–28, no effect on formation of a mineralized matrix was observed. To establish that the inhibitory effect of the pulse PDGF treatment in the first 2 weeks was not due to a simple delay in mineralized nodule formation, longer time points were also examined, i.e., nodules were fixed and stained after 7 weeks in culture. There was no increase in mineralized nodule area when the length of incubation was extended from 5 to 7 weeks. This indicates that mineralized matrix stops forming after 5 weeks. The results also suggest that the inhibitory effect of a 7-day pulse exposure to PDGF in the early stages is irreversible, since the cultures do not recover and produce mineralized nodules even when PDGF is removed after 1 week.

Table IA demonstrates the effect of 7-day PDGF pulse treatments on alkaline phosphatase activity. We found that a 1-week treatment with PDGF from either days 0–7 or days 7–14 inhibited alkaline phosphatase activity measured before day 28. However, if alkaline phosphatase was measured on day 35, there was no significant difference compared to the control group. PDGF pulse treatment from days 14–21 or days 21–28 had no effect on alkaline phosphatase activity (Table IA). These data indicate that 7-day PDGF-BB pulse exposure inhibits alkaline phosphatase activity if applied at the early stages of cell culture; little effect is observed if PDGF is applied to cultures after day 14. Furthermore, they indicate that if cells are treated with PDGF at the early stages and it is then removed after 1 week, there is a substantial delay in the up-regulation of alkaline phosphatase activity.

Because the above experiments showed that a 7-day pulse exposure to PDGF had the same effect as continuous treatment, shorter pulses were then used. To test when cells would best respond to a short-term stimulation with PDGF-BB, [$^3$H] thymidine incorporation was examined at different stages of the mineralizing culture system (Fig. 4). The rate of DNA synthesis is presented as the amount of [$^3$H] thymidine incorporated per 1,000 cells. A significant mitogenic effect of PDGF-BB was found on day 7 (100% increase), and day 14 (70% increase), but there was no effect on day 21 or later. We then examined the effect of 24-hr pulse treatment vs. long-term treatment on alkaline phosphatase activity (Table I). Continuous or 7-day pulse exposure to PDGF-BB (20 ng/ml) inhibited alkaline phosphatase activity while a short-term

**TABLE IA. Effect of 7-Day Pulse Exposures to PDGF-BB on Alkaline Phosphatase Activity†**

| Alkaline phosphatase activity (nmol/min/μg DNA) | | | | | |
|---|---|---|---|---|---|
| Day cells lysed | Control | PDGF-BB applied for 7 days | | | |
| | | Day 0–7 | Day 7–14 | Day 14–21 | Day 21–28 |
| 3 | 13.4 ± 4.1 | 3.8 ± 2.1* | | | |
| 7 | 20.4 ± 2.2 | 4.4 ± 1.5* | | | |
| 14 | 50.8 ± 2.4 | 19.8 ± 3.3* | 29.8 ± 4.8* | | |
| 21 | 54.1 ± 3.2 | 37.9 ± 4.5* | 39.9 ± 5.3* | 50.1 ± 4.4 | |
| 28 | 60.0 ± 5.9 | 41.2 ± 6.2 | 45.2 ± 4.7 | 57.8 ± 8.6 | 54.4 ± 7.1 |
| 35 | 53.8 ± 4.8 | 45.0 ± 6.8 | 49.2 ± 3.2 | 51.5 ± 7.1 | 53.2 ± 8.4 |

**TABLE IB. Effect of 24-Hr Pulse Exposure to PDGF on Alkaline Phosphatase Activity†**

| Alkaline phosphatase activity (nmol/min/μg DNA) | | | |
|---|---|---|---|
| Day cells lysed | Control | PDGF-BB | |
| | | Day 7 | Day 14 |
| 3 | 15.6 ± 3.2 | | |
| 7 | 21.8 ± 4.7 | | |
| 14 | 49.3 ± 4.1 | 47.8 ± 8.8 | |
| 21 | 53.7 ± 5.3 | 48.7 ± 5.4 | 51.8 ± 4.2 |
| 28 | 55.8 ± 7.8 | 60.2 ± 8.3 | 54.9 ± 7.3 |
| 35 | 51.4 ± 9.2 | 49.2 ± 8.9 | 61.5 ± 8.9 |

†Fetal rat calvarial osteoblastic cells were stimulated with PDGF-BB (20 ng/ml) for discrete periods of time. Cell lysates were tested for alkaline phosphatase activity. Each value represents the mean of triplicate samples ± standard deviation.
*Significantly different from the corresponding control group, $P \leq 0.01$.

incubation did not. Since short-term treatment with PDGF stimulated DNA synthesis without inhibiting differentiated function, we tested the impact of short-term stimulation on mineralized nodule formation (Fig. 5). The results indicate that a single 24-hr incubation with PDGF does not affect mineralized matrix formation. However, when 24-hr pulse treatments were applied at two time points, there was a 50% increase in the area of mineralized matrix compared to the control group (Figs. 5 and 6).

The previous data showed that short-term stimulation with PDGF is mitogenic without interfering with alkaline phosphatase activity, suggesting that it does not inhibit the differentiation process or differentiated function. To investigate this further, osteoblastic cells were pre-treated with or without PDGF-BB for 3 days and then replated to new tissue culture dishes. Cells were then analyzed for formation of a mineralized matrix 32 days following replating. In Figure 7A, two groups were preincubated in mineralizing culture media with (lane E) or without PDGF (lane D) supplementation for 3 days, and then re-plated to new tissue culture wells and incubated under standard mineralizing conditions for 32 days. Several controls were run simultaneously and consisted of similar cells that were not transferred to new plates but were cultured for 35 days in mineralizing media without (Fig. 7A, lane A) or with PDGF for either the first 3 days (Fig. 7A, lane C) or continuously for 35 days (Fig. 7A, lane B). The results indicate that for cells incubated under standard conditions, re-plating had no effect on mineralized matrix area (Fig. 7A, compare lanes A and D). In cells exposed to PDGF and then re-plated in culture media without PDGF (Fig. 7A, lane E) there was a 70% increase on the mineralized nodule area compared to the control cultures (Fig. 7A, lanes A and D). However, when cells were exposed to PDGF-BB for the first 3 days but not transferred (Fig. 7A, lane B), there was no increase in the formation of a mineralized matrix. Thus, transferring PDGF-treated cells to a new surface appears to enhance their capacity to form mineralizing nodules.

We also monitored alkaline phosphatase activity in cells that were transferred to new plates with or without PDGF pre-treatment. Cells pre-treated with PDGF for 3 days (Fig. 7B, lanes C and E) exhibited no significant difference in alkaline phosphatase activity per

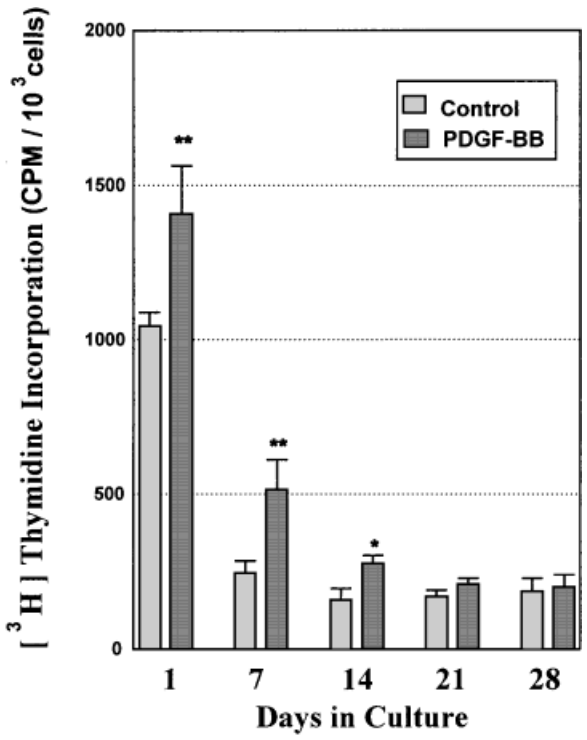


**Fig. 4.** The mitogenic response to PDGF is temporally modulated in long-term mineralizing culture. Osteoblastic cells were cultured in mineralizing culture media. PDGF-BB was added for 24 hr at each indicated time point. DNA synthesis was measured by pulse-labeling with [$^3$H] thymidine for the last 6 hr of culture as described in Materials and Methods. **Significantly different from corresponding control: $P \Leftarrow 0.01$. *Significantly different from corresponding control: $P \leq 0.05$.

cell compared to control cells after 14 days (Fig. 7B, lane A). Thus, a short-term 3-day pretreatment with PDGF-BB had no significant effect on alkaline phosphatase activity per cell, while continuous exposure to PDGF did (Fig. 7B, lane B).

## DISCUSSION

PDGF is an important regulatory molecule for osteoblastic cells. PDGF has been shown to stimulate proliferation of osteoblastic cells in organ cultures [Canalis et al., 1989; Pfeilschifter et al., 1992] and is highly mitogenic for monolayer cultures of osteoblastic cells [Piche and Graves, 1989; Centrella et al., 1989; Kasperk et al., 1990; Abdennagy et al., 1992]. When compared directly, PDGF is more mitogenic for normal human osteoblastic cells than TGF-b, IGF-1, or EGF [Piche and Graves, 1989]. Consistent with these findings, we found that PDGF-BB stimulated DNA synthesis and cell replication in fetal rat calvarial cells prior to day 21 in long-term mineralizing cultures, but not at later time points. In this in vitro model, cells are thought to go through three major stages: (1) cell proliferation, (2) differentiation and formation of an abundant extracellular matrix, and (3) matrix mineralization [Owen et al., 1990]. When cells were continuously incubated with PDGF they proliferated for a longer period of time, suggesting that more cells remained in the cell cycle. That PDGF inhibits upregulation of alkaline phosphatase activity suggests that the inhibitory effect of PDGF on the formation of a mineralized matrix is due to an inhibition of differentiation or differentiated function of osteoblastic cells. While continuous or 1-week incubation with PDGF inhibited alkaline phosphatase activity and mineralized nodule formation, two 24-hr incubations with PDGF increased the mineralized nodule area. It is noteworthy that single or multiple 1-day exposures to PDGF did not inhibit alkaline phosphatase activity in the long-term mineralizing cultures. Brief exposures to PDGF may be effective in enhancing mineralized nodule formation because they have a mitogenic effect without inhibiting differentiation or differentiated function.

Incubation of osteoblastic cells with PDGF from day 0 to day 3 enhanced cell number twofold. Despite this increase in cell number, there was no significant increase in the formation of mineralizing nodules. However, when cells were pre-treated with PDGF for 3 days and then transferred to new dishes, there was a 70% increase in mineralized matrix area. That cells had to be transferred to observe an increase in mineralized nodule area after incubation with PDGF for 3 days suggests that cells are being released from an "inhibitory factor." The simplest interpretation is that PDGF alters the matrix that is produced, which, in turn, limits the formation of mineralizing nodules. Thus, removing cells from matrix produced by PDGF-stimulated cells enables the cells to form a greater amount of mineralized matrix. The inhibitory effect was only noted on cells that had been exposed to PDGF. An alternative interpretation of the inhibitory effect of PDGF on mineralized nodule formation is that PDGF causes selective proliferation of fibroblastic cells, which have limited capacity to form a mineralizing matrix. Although this may occur to some extent, it is unlikely to account for most of the inhibitory effect. If PDGF pre-treatment had selectively stimulated proliferation of fibroblas-

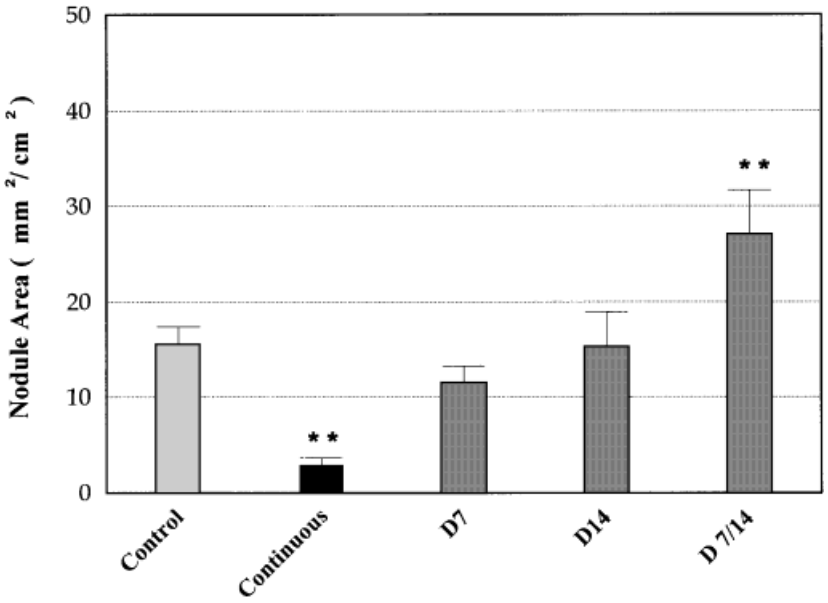


**Fig. 5.** The effect of continuous and pulse exposure to PDGF-BB on mineralized nodule area. Fetal rat osteoblastic cells were cultured in mineralizing media without or with PDGF (20 ng/ml). Each group is represented as follows: Control, mineralizing media without PDGF-BB; Continuous, cells treated with PDGF-BB from day 0 to day 35; D7, pulse exposure to PDGF for 24 hr on day 7; D14, pulse exposure to PDGF for 24 hr on day 14; D7/14, pulse exposure to PDGF-BB for 24 hr on day 7 and again on day 14. Mineralized nodules were identified with von Kossa's stain after 5 weeks and their area was quantified with a computer assisted imaging system. Each bar represents a mean ± standard deviation of triplicate samples. **Significantly different from corresponding control: $P \leq 0.01$. *Significantly different from corresponding control: $P \leq 0.05$.

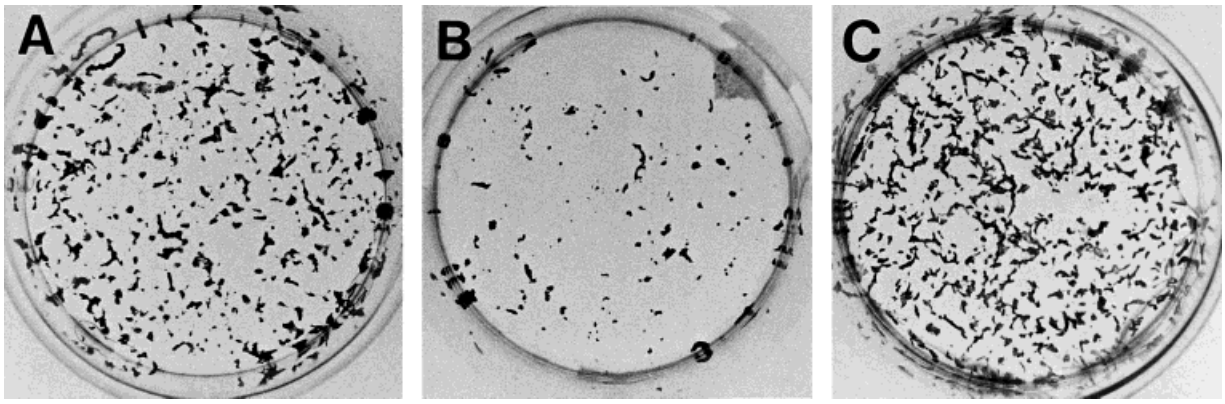


**Fig. 6.** Effect of continuous and pulse exposure to PDGF-BB on mineralized nodule area. Fetal rat osteoblastic cells were cultured in mineralizing media without or with PDGF (20 ng/ml). Mineralized nodules were identified with von Kossa's stain after 5 weeks. **A:** Control osteoblastic cultures in mineralizing media. **B**: Osteoblastic cultures in mineralizing media supplemented with PDGF-BB for 35 days. **C**: Osteoblastic cultures in mineralizing media supplemented with PDGF-BB for 24 hr on day 7 and again on day 14.

tic cells, the increased mineralized nodule formation following PDGF pre-treatment and subsequent transfer to new dishes would have been unlikely.

Numerous studies demonstrate the importance of PDGF in enhancing bone formation in vivo [Lynch et al., 1989, 1991; Rutherford et al., 1992, 1993; Mitlak et al., 1996]. Exogenous application of PDGF has been shown to enhance bone healing in orthopedic models of osseous repair [Nash et al., 1994]. The short-term application of a combination of platelet-derived and insulin-like growth factor-1 enhances new bone formation in a periodontal model of osseous repair [Lynch et al., 1989]. It is noteworthy that these investigators found that the half-life of PDGF at the site of application was only 4 hr. Thus, the exogenous applica-

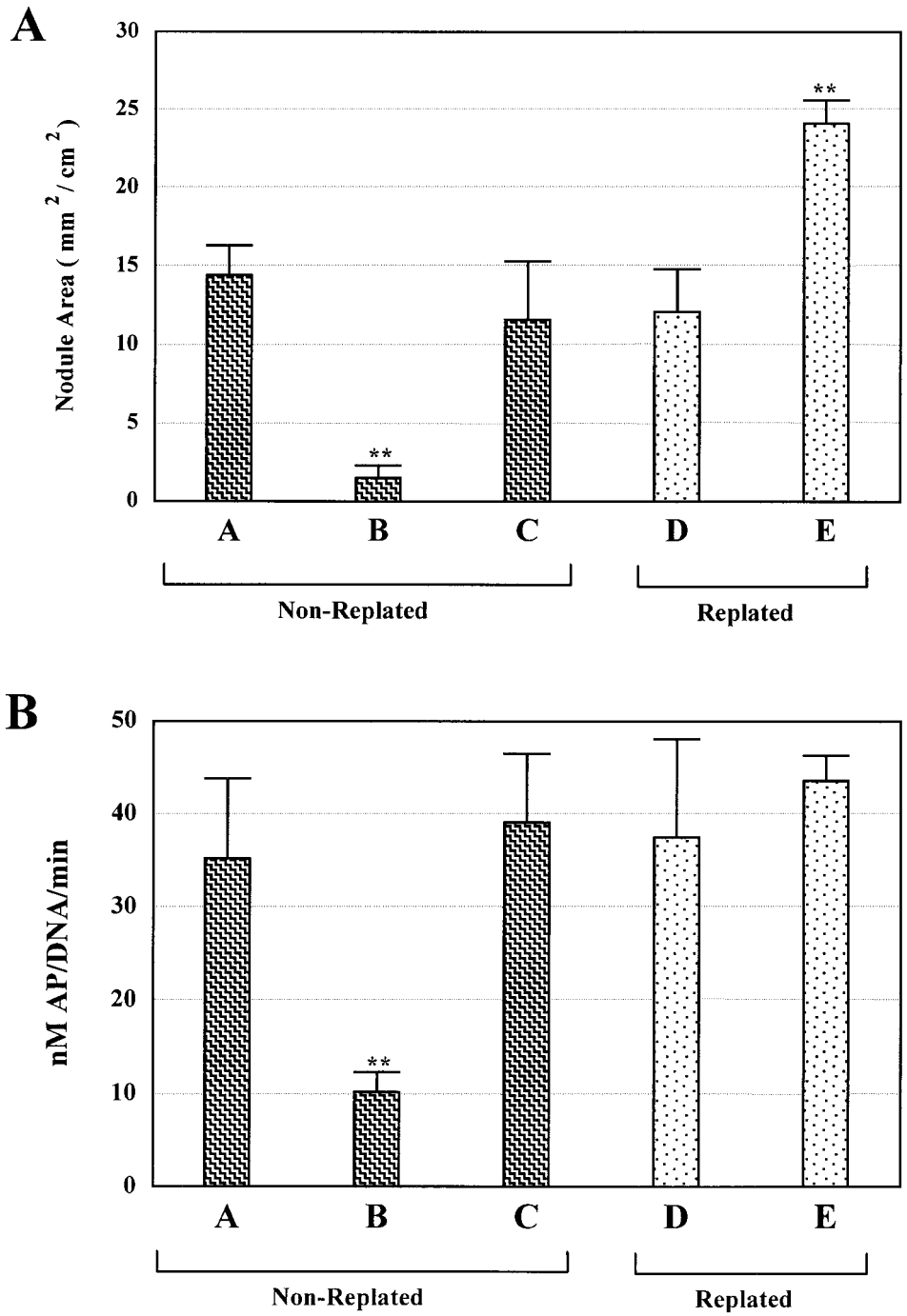


**Fig. 7.** Replating osteoblastic cells following incubation with PDGF enhances mineralized nodule formation. Fetal rat osteoblastic cells obtained by collagenase digestion were cultured in mineralizing media with and without PDGF(20 ng/ml). There were five groups as follows: **Lane A**, control cells incubated in mineralizing media; **Lane B**, cells continuously exposed to PDGF-BB (20 ng/ml) for 35 days; **Lane C,** cells pulse-treated with day PDGF-BB from day 0 to day 3; **Lane D**, cells cultured in mineralizing media from day 0 to day 3, trypsinized and incubated in new tissue culture wells in mineralizing media for 32 days; **Lane E**, cells cultured in mineralizing media with PDGF-BB treatment from day 0 to day 3, trypsinized and incubated in new tissue culture wells in mineralizing medial without PDGF. **A:** Mineralized nodule area. Cells that were not replated were examined for mineralized nodule area after 35 days (zigzag bars). Cells that were replated were examined for mineralized nodule area 32 days after replating (dotted bars). The area of mineralized nodules was determined with computer-assisted image analysis following staining with von Kossa's method. Each bar represents a mean ± standard deviation from triplicate samples. **B:** Alkaline phosphatase activity. Cell lysates were collected on day 14. Alkaline phosphatase activity was expressed as nM p-nitrophenol (released from the substrate p-nitrophenol phosphate) per microgram DNA per minute. Each bar represents a mean ± standard deviation from quadruplicate samples. **Significantly different from corresponding control: $P \leq 0.01$. *Significantly different from corresponding control: $P \leq 0.05$.

tion of PDGF in many in vivo experiments is likely to represent a brief pulse exposure rather than a continuous application. This may be highly significant since we found that a brief exposure to PDGF enhances proliferation of osteoblastic cells without blocking differentiation or differentiated function. This could perhaps explain how exogenous PDGF could enhance osseous healing in vivo despite its dramatic affect on differentiated function.

In chronic inflammation there is substantial recruitment of macrophages and a prolonged up-regulation of growth factor production. It is possible that prolonged exposure to growth factors such as PDGF could inhibit bone formation, and could lead to deficient bone formation in chronic inflammatory diseases such as periodontitis. This hypothesis is supported by findings that chronically high levels of PDGF production are found in failing dental implants, which are characterized by deficient osseointegration and insufficient bone formation [Salcetti et al., 1997].

In summary, the studies presented here demonstrate that multiple brief exposures to PDGF enhance formation of a mineralized matrix while longer-term exposure is inhibitory. Based on this finding, PDGF may have anabolic as well as inhibitory effects on bone formation in vivo, depending upon the length of times that cells are exposed to it. We would predict that multiple, brief exposures to PDGF would enhance bone formation in vivo, while prolonged continuous exposure to PDGF would inhibit differentiated osteoblast function and limit osseous regeneration. Furthermore, prolonged exposure to growth factors such as PDGF that are often generated in chronic inflammation may represent a mechanism whereby bone regeneration is inhibited.

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