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Platelet Derived Growth Factor Stimulates Chondrocyte Proliferation but Prevents Endochondral Maturation

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Platelet-derived growth factor (PDGF) is a cytokine released by platelets at sites of injury to promote mesenchymal cell proliferation. Since many bone wounds heal by endochondral bone formation, we examined the response of chondrocytes in the endochondral lineage to PDGF. Confluent cultures of rat costochondral resting zone cartilage cells were incubated with 0–300 ng/mL PDGF-BB for 24 h to determine whether dose-dependent changes in cell proliferation (cell number and [^{3}H]-thymidine incorporation), alkaline phosphatase specific activity, [^{35}S]-sulfate incorporation, or [^{3}H]-proline incorporation into collagenase-digestible protein (CDP) or noncollagenase-digestible protein (NCP), could be observed. Long-term effects of PDGF were assessed in confluent cultures treated for 1, 2, 4, 6, 8, or 10 d with 37.5 or 150 ng/mL PDGF-BB. To determine whether PDGF-BB could induce resting zone chondrocytes to change maturation state to a growth zone chondrocyte phenotype, confluent resting zone cell cultures were treated for 1, 2, 3, or 5 d with 37.5 or 150 ng/ml PDGF-BB and then challenged for an additional 24 h with 1,25-$(OH)_2D_3$. PDGF-BB caused a dose-dependent increase in cell number and [^{3}H]-thymidine incorporation at 24 h. The proliferative effect of the cytokine decreased with time. PDGF-BB had no effect on alkaline phosphatase at 24 h, but at later times, the cytokine prevented the normal increase in enzyme activity seen in post-confluent cultures. This effect was primarily on the cells and not on the matrix. PDGF-BB stimulated [^{35}S]-sulfate incorporation at all times examined, but had no effect on [^{3}H]-proline incorporation into either the CDP or NCP pools. Thus, percent collagen production was not changed. Treatment of the cells for up to 5 d with PDGF-BB failed to elicit a 1,25-$(OH)_2D_3$ responsive phenotype typical of rat costochondral growth zone cartilage cells. These results show that committed chondrocytes can respond to PDGF-BB with increased proliferation. The effect of the cytokine is to enhance cartilage matrix production, but at the same time to prevent progression of the cells along the endochondral maturation pathway.

Key Words: Platelet-derived growth factor-BB; chondrocytes; proliferation; differentiation; growth plate.

Introduction

Considerable similarities exist between the events that occur in endochondral bone development and those that occur during wound healing in bone. These processes involve the induction of mesenchymal cells into and along the chondrocyte lineage by chondrogenic growth factors like transforming growth factor beta (TGFβ) *(1–3)*, insulin-like growth factor (IGF) *(4–6)*,and basic fibroblast growth factor (bFGF) *(7–9)*.

In bone wound healing in vivo, enhanced proliferation of osteochondroprogenitor cells is an important first step. Platelet-derived growth factor (PDGF), a cytokine that stimulates proliferation of mesenchymal cells in a broad range of tissues *(10–13)*, is released from platelets at wound sites *(14)*. In addition, PDGF is produced by osteoblasts *(15)* and stored in bone *(16)*, further increasing its local concentration, and as a result, increasing the pool of osteochondroprogenitor cells.

PDGF is a disulfide-linked dimer with a molecular weight of approx 25 kDa *(14)*. PDGF-BB is one of three isoforms of PDGF resulting from the dimeric combination of two distinct, but structurally related, polypeptide chains designated as A and B. Fibroblasts, smooth muscle cells, periodontal ligament cells, and osteoblastic cells have all been shown to respond to this cytokine *(17–20)*. In addition to its stimulatory effect on osteoblast proliferation *(17,21,22)*, other aspects of cell metabolism and phenotypic expression are affected as well. Studies examining matrix production and differentiation markers have suggested that exposure of fetal rat calvarial cells to PDGF

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has an inhibitory effect on collagen synthesis and no effect on osteocalcin production *(21)*. Other studies by the same group on cells isolated from fetal rat parietal bone, while showing enhanced rates of increased collagenase-digestible protein (CDP) and noncollagenase-digestible protein (NCP) production, demonstrated no difference in the overall relative collagen synthesis *(17)*. These observations suggest that PDGF has an overall anabolic effect on the cells, but does not promote osteoblastic differentiation.

To date, attention to the effects of PDGF, particularly the BB isoform, on chondrocytes has been relatively limited. In vitro studies by Chen et al. *(23)* appear to indicate that chick limb bud mesodermal chondrogenesis is inhibited by PDGF. Others, however, have observed chondrogenic differentiation when cultures of perichondrial cells were stimulated with PDGF *(24)*. The purpose of this study, therefore, was to examine the effects of PDGF-BB on the proliferation, matrix synthesis, and differentiation of chondrocytes, a cell whose regulation is essential to development of endochondral bone. We used a well-established cell-culture model *(25,26)*, which permits comparison of chondrocytes at two distinct stages of endochondral maturation: the less mature resting zone chondrocyte and the more terminally differentiated growth zone chondrocyte. The first portion of the study examined cellular response of confluent cultures of resting zone cells exposed to PDGF-BB for 24 h. In the second part of the study, we examined the ability of PDGF-BB to promote endochondral bone formation by examining the long-term response of the cells to the cytokine and by determining whether resting zone chondrocytes exposed to the growth factor develop a growth zone chondrocyte phenotype, based on responsiveness to $1,25\text{-}(OH)_2D_3$.

Results

Proliferation

Cultures stimulated for 24 h with concentrations of PDGF-BB ranging from 4.7 to 300 ng/mL had significantly greater cell numbers than their controls (Fig. 1). The relationship between increasing dose and cell number was biphasic, peaking at 75 ng/ml. The results of the addition of PDGF-BB on DNA synthesis, as assessed by [^{3}H]-thymidine incorporation (Fig. 2), showed significantly greater stimulation of thymidine incorporation at all concentrations examined over controls. This increase appeared to be dose-dependent from 2.3 to 37.5 ng/mL, with the effects plateauing at concentrations above 37.5 ng/mL.

Prolonged exposure to PDGF-BB from 1 to 8 d resulted in a significant increase in cell number (Fig. 3). The observed effect was time-dependent, decreasing with time of exposure to the growth factor. In addition, the effect appeared to be dose-dependent since at the first four time points cultures treated with either 37.5 or 150 ng/mL contained significantly more cells than did untreated controls.

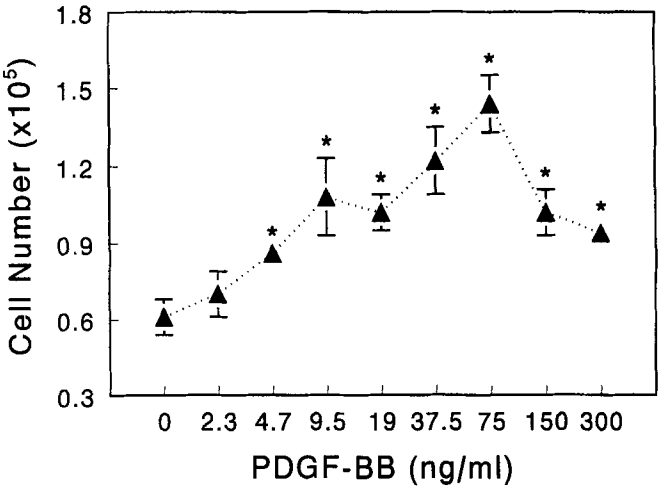

Fig. 1. Number of resting zone chondrocytes after treating confluent cultures for 24 h with 0–300 ng/mL PDGF-BB. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

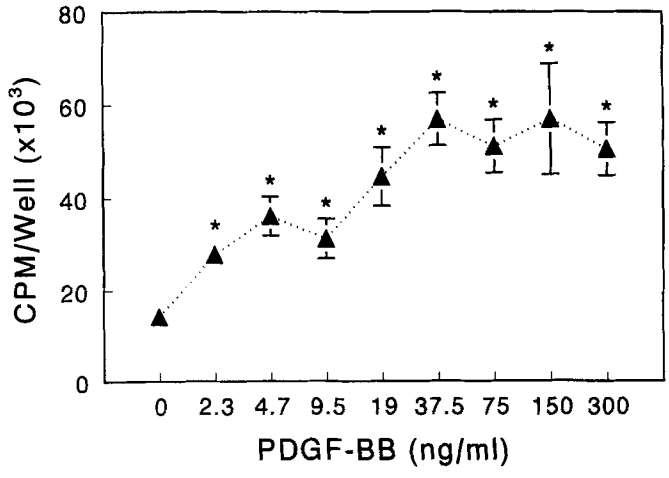

Fig. 2. [^{3}H]-Thymidine incorporation by resting zone chondrocytes after 24 h of treatment with 0–300 ng/mL PDGF-BB. Subconfluent cells were made quiescent by reducing the serum concentration to 1% for 48 h. At that time, the media were changed. Experimental media consisted of PDGF-BB in DMEM containing 1% FBS. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

However, the difference between cultures treated with 37.5 or 150 ng/mL was not statistically significant at any of the time points examined.

Alkaline Phosphatase Specific Activity

There was no evidence of PDGF-BB affecting chondrocyte alkaline phosphatase specific activity after 24 h of treatment (Fig. 4). The enzyme activity of the cell layer, which includes the extracellular matrix and its incorporated matrix vesicles, was not different from that of the controls at any of the various concentrations examined. Similarly,

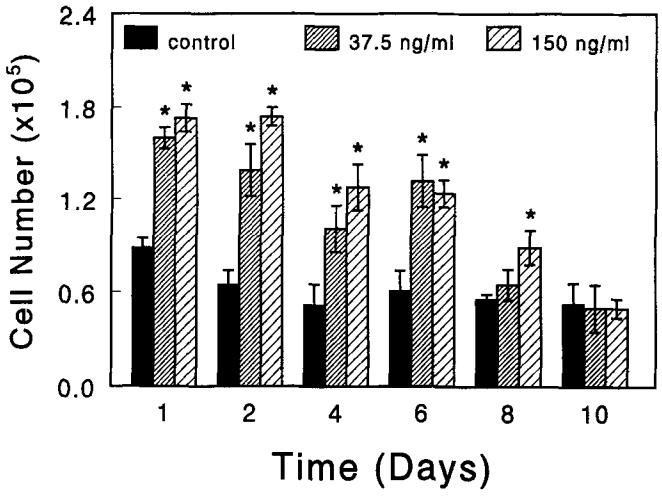

Fig. 3. Number of resting zone chondrocytes after treating confluent cultures with 0, 37.5, or 150 ng/mL PDGF-BB for 1–10 d. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

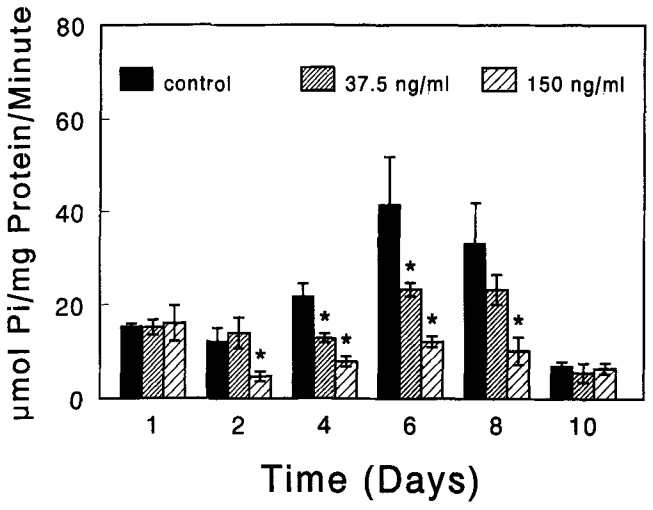

Fig. 5. Alkaline phosphatase specific activity of resting zone chondrocytes isolated from confluent cultures treated with 0, 37.5, or 150 ng/mL PDGF-BB for 1–10 d. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

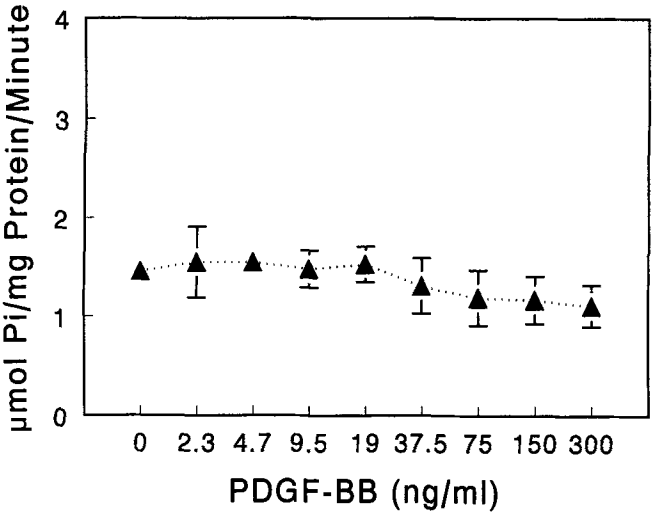

Fig. 4. Alkaline phosphatase specific activity of resting zone chondrocyte cell layers after treatment of confluent cultures with 0–300 ng/mL PDGF-BB for 24 h. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments yielding similar results.

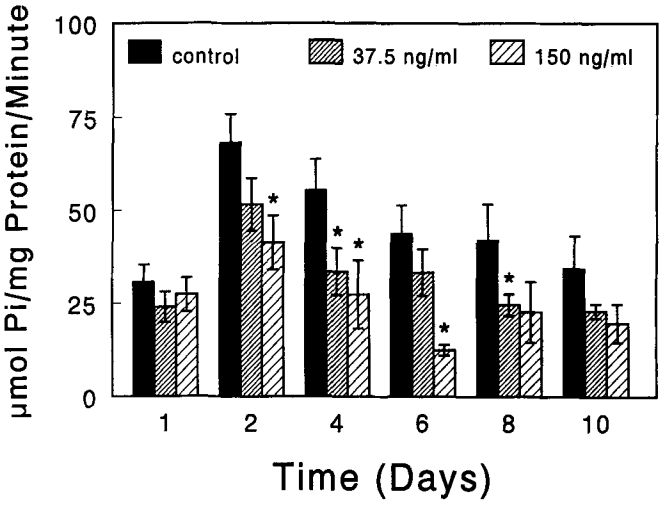

Fig. 6. Alkaline phosphatase specific activity in resting zone chondrocyte cell layers after treatment of confluent cultures with 0, 37.5, or 150 ng/mL PDGF-BB for 1–10 d. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

treatment with PDGF-BB for 24 h had no effect on alkaline phosphatase specific activity of cells isolated from the matrix by trypsinization (data not shown).

Prolonged exposure to PDGF-BB, however, inhibited both isolated cell and cell layer alkaline phosphatase specific activity (Figs. 5 and 6). Significant decreases in cell alkaline phosphatase were noted after cultures were exposed to 150 ng/mL PDGF-BB for 2–8 d (Fig. 5). The inhibitory effects, although less profound, were also noted at d 4 and 6 in cells isolated from cultures exposed to 37.5 ng/mL cytokine (Fig. 5). Cell layer alkaline phosphatase specific activity appeared to be less sensitive to inhibition by PDGF-BB. Although 37.5 ng/mL caused a decrease in enzyme activity in the cell layer, these slightly lower levels were only significantly different from those of the controls at the d 4 and 8 time points (Fig. 6). Treatment with 150 ng/mL for 2–6 d, however, produced a significant decrease in cell-layer alkaline phosphatase specific activity.

[^{35}S]-Sulfate Incorporation

PDGF-BB caused a dose-dependent stimulation of proteoglycan production, as indicated by [^{35}S]-sulfate incorporation. Proteoglycan production by resting zone

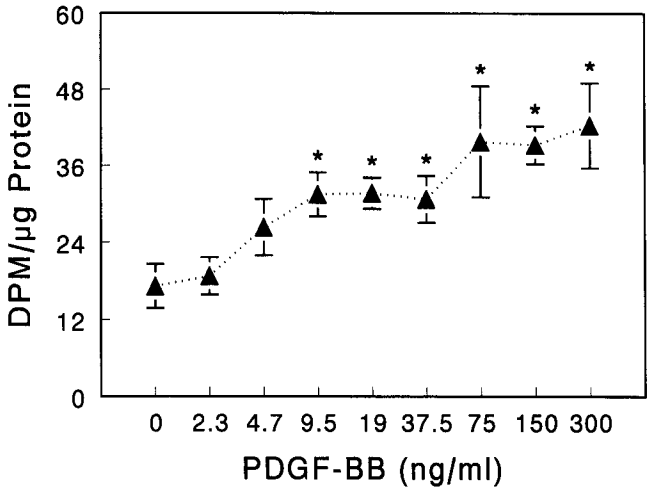

Fig. 7. [^{35}S]-Sulfate incorporation by confluent cultures of resting zone chondrocytes after treatment with 0–300 ng/mL PDGF-BB for 24 h. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control. Data are from one of two replicate experiments yielding similar results.

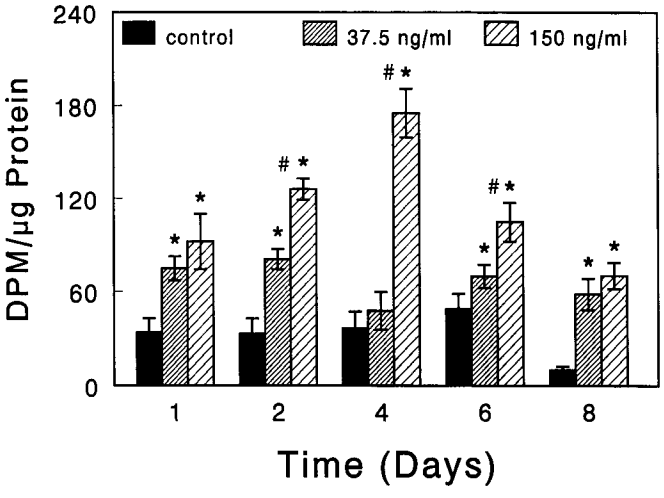

Fig. 8. [^{35}S]-Sulfate incorporation by confluent cultures of resting zone chondrocytes after treatment with 0, 37.5, or 150 ng/mL PDGF-BB for 1–8 d. Values are the mean ± SEM of six cultures. *$P < 0.05$, treatment vs control; $P < 0.05$, 37.5 vs 150 ng/mL. Data are from one of two replicate experiments yielding similar results.

cells was 2.5–3.5 times that of controls (Fig. 7). Dose-dependent increases in proteoglycan production were also noted in cultures treated with PDGF-BB for prolonged time periods (Fig. 8). Production was significantly greater than controls for both concentrations examined at all times examined, except for the 4 d cultures treated with 37.5 ng/mL. Cultures stimulated with 150 ng/ml generally had greater levels of [^{35}S]-sulfate incorporation than did those treated with 37.5 ng/mL. Differences between these two concen-trations, however, were only significant at 2, 4, and 6 d.

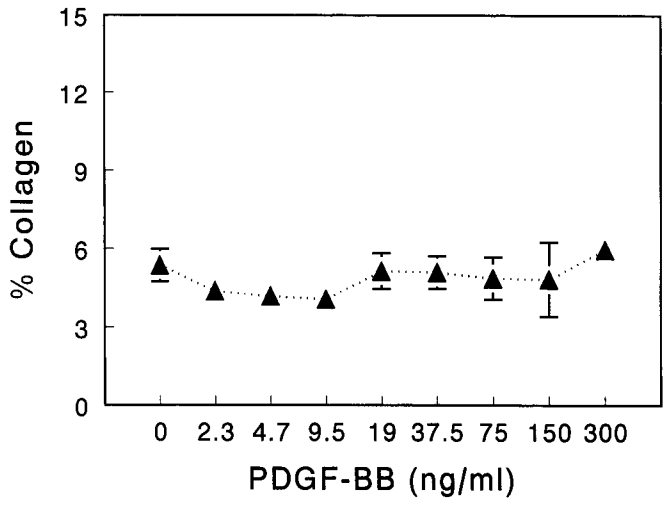

Fig. 9. Percent collagen production by confluent resting zone chondrocytes after 24 h of treatment with 0–300 ng/mL PDGF-BB. Values are the mean ± SEM of six cultures. Data are from one of two replicate experiments yielding similar results.

[^{3}H]-Proline Incorporation

The addition of PDGF-BB to resting zone cultures for 24 h did not alter the amount of either CDP or NCP production (data not shown). Similarly, percent collagen production by cells treated with PDGF-BB was not significantly different from controls (Fig. 9). Furthermore, no effect of PDGF-BB between 1 and 8 d was observed (data not shown).

Effect of PDGF-BB on Resting Zone Chondrocyte Differentiation

Treatment of confluent cultures of resting zone cells with PDGF-BB for 24, 48, 72, or 120 h did not result in a 1,25-$(OH)_2D_3$-responsive phenotype for any of the parameters tested. A typical example of the results obtained is shown in Fig. 10 for alkaline phosphatase activity. Alkaline phosphatase specific activity was inhibited in cultures treated for 48, 72, and 120 h with 150 ng/mL PDGF-BB. When either these cultures, or chondrocytes cultured in control media for comparable periods of time were challenged with 1,25-$(OH)_2D_3$ for an additional 24 h, no change in enzyme activity was noted.

Discussion

Temporally, the release of PDGF from platelets is one of the initial events that occurs in the resolution of a wound. PDGF appears to enhance cartilage and bone formation *(27)*, but it is not known whether this is owing to an increase in the pool of less mature cells, or to a direct effect on the differentiation of those cells. PDGF is well known as a competence growth factor *(10,20)* and has been shown to initiate extensive proliferation of osteoblasts *(17,28)*.

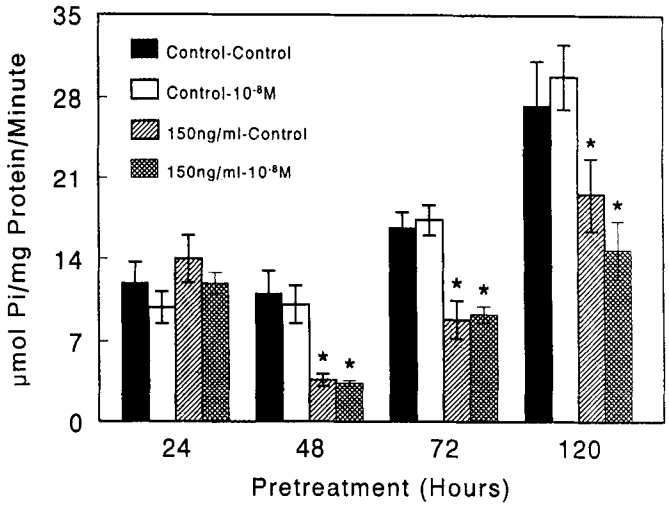

Fig. 10. Alkaline phosphatase specific activity of resting zone chondrocytes after treatment with vehicle (control) or PDGF-BB (150 ng/mL) for 24–120 h. At the end of treatment with vehicle or PDGF-BB, the media were removed and fresh media containing vitamin D vehicle (control) or 1,25-$(OH)_2D_3$ (10^{-8} *M*) added. The cultures were then incubated for an additional 24 h, and the cell layers assayed for alkaline phosphatase specific activity. Values are the mean ± SEM of six cultures. *$P < 0.05$, vs control–control or control-10^{-8} *M* 1,25-$(OH)_2D_3$. Data are from one of two replicate experiments yielding similar results.

Our study demonstrates that PDGF-BB also has a proliferative effect on cells already in the chondrocyte lineage.

Previous studies also have shown that PDGF regulates extracellular matrix synthesis by osteoblasts in addition to its effects on proliferation. The precise effect of PDGF on matrix synthesis is not clear, with some studies reporting an inhibition of collagen production *(21)*, and others reporting no change *(17)*. In the present study, [^{3}H]-proline incorporation was used to examine the effect of PDGF-BB on protein synthesis. Neither the amount of CDP nor the amount of NCP was affected; thus, by this method, there was no change in the amount of collagen synthesized. In contrast, sulfate incorporation was markedly enhanced, suggesting a specific effect of PDGF-BB on expression of the cartilage phenotype. The type of analysis used does not indicate whether there was an increase in synthesis of the proteoglycan core protein. Since no change was noted in the noncollagenase digestible protein produced, it is more likely that the effect of PDGF-BB is on proteins involved in the sulfation of cartilage glycosaminoglycans.

The increase in [^{35}S]-sulfate incorporation in the resting zone cell cultures at 24 h occurred at all concentrations of PDGF used and was evident after 8 d of exposure. Results published by Chen et al. *(23)* using Stage 24 chick limb bud mesodermal cells differed considerably from those presented here. Exposure of these cells to the various isoforms of PDGF for up to 2 d resulted in a large decrease in [^{35}S]-sulfate incorporation. When the chick limb bud cells were exposed to PDGF for 3–4 d, no differences between treatment and control cultures were noted. These differences may be attributed in part to culture conditions, since the studies conducted by Chen et al. were done under serum-deprived conditions. They may also reflect differences in the maturation stage of the cells, with chick limb bud having a greater proportion of pluripotential osteochondral progenitors and the resting zone cell cultures used in the present study being committed chondrocytes.

Although the results of this study indicate that the effect of PDGF-BB on chondrocytes in the early stages of endochondral maturation is to promote proliferation and expression of a matrix typical of a resting zone chondrocyte, they also demonstrate that PDGF-BB inhibits the progression of cells to a more mature growth zone phenotype. Although a 24-h exposure to PDGF-BB had no effect on alkaline phosphatase specific activity in confluent cultures of resting zone cells, by 48 h postconfluence, PDGF-BB decreased alkaline phosphatase specific activity in the cells to levels typical of confluent cultures. Normally, postconfluent cultures of resting zone and growth zone chondrocytes continue to express increased enzyme activity through 24 d in culture *(29)*. This is associated with the formation of nodules, and ultimately, the appearance of von Kossa-positive deposits within the nodules. The effect of PDGF-BB may have been most evident in the isolated cells in comparison with the cell layers at later time-points, because pre-existing matrix vesicle alkaline phosphatase was not affected by the cytokine. Studies using fetal rat calvarial cells demonstrated that PDGF had no effect on osteocalcin production *(17)*. Since an increase in osteocalcin is associated with expression of a differentiated osteoblast phenotype, this observation supports our hypothesis.

Not only was the normal increase in alkaline phosphatase specific activity seen in postconfluent cultures *(30)* inhibited in the present study, but also treatment of the cells with PDGF-BB for up to 5 d failed to elicit a 1,25-$(OH)_2D_3$-responsive phenotype in the resting zone chondrocyte cultures. In contrast, treatment with 24,25-$(OH)_2D_3$ for 36 h *(29)*, with TGFβ for 48 h (unpublished data), or with BMP-2 for 72 h (unpublished data) causes resting zone chondrocytes to respond to 1,25-$(OH)_2D_3$ in a manner identical to a growth zone chondrocyte with respect to stimulation of alkaline phosphatase and regulation of cell proliferation, protein synthesis, and proteoglycan sulfation.

These data show for the first time that PDGF-BB can have a direct effect on chondrocytes in the endochondral lineage. The cytokine stimulates cell proliferation and production of a cartilage-specific matrix based on increased sulfate incorporation. In contrast, PDGF-BB inhibits the increase in alkaline phosphatase specific activity normally seen in post-confluent cultures (30) and does not promote the transition of the cells to a 1,25-$(OH)_2D_3$-responsive growth zone phenotype, suggesting that the cytokine maintains the cells in a less mature phenotype.

Materials And Methods

Cell Culture

Resting zone chondrocytes were isolated from the costochondral cartilage of adult male Sprague-Dawley rats as previously described *(25)*. Cells were released from the cartilage by sequential incubation in 0.25% trypsin (Gibco, Grand Island, NY), and then 0.02% type II collagenase. Following digestion of the matrix, the cells were plated at a density of 10,000 cells/cm^2. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% antibiotics and 50 μg/mL ascorbic acid in an atmosphere of 100% humidity, 5% CO_2, and 37°C. Culture media were changed at 24 h and thereafter at 48-h intervals. At confluence, the cells were subcultured and replated at the initial seeding density. Experiments were conducted using fourth-passage cells plated at a density of 10,000 cells/cm^2. Previous studies have shown that at this passage, the cells retain their cartilage phenotype *(25,26,31)* as well as their responsiveness to hormones *(25,26,29,31,32)* and growth factors (3).

Lyophilized recombinant human PDGF-BB (R & D Systems, Minneapolis, MN) was solubilized in sterile 4 m*M* HCl containing 0.1% bovine serum albumin (BSA) at a concentration of 300 μg/ml. The experimental media were prepared by diluting the PDGF-BB stock solution with DMEM containing 10% FBS, 1% antibiotics, and 50 μg/mL ascorbic acid. All experiments were conducted on confluent cultures. At confluence, the experimental media were added to the wells. Dose-response studies were conducted at concentrations ranging from 0–300 ng/mL. Time course studies were conducted for 1–10 d postconfluence and the cultures exposed to either 37.5 or 150 ng/mL PDGF for the entire culture period. Control cultures were exposed to media containing the vehicle. Media were changed every second day.

Cell Proliferation

Cell Number

At harvest, cells were rinsed with DMEM. They were then released from the polystyrene culture surface by the addition of 0.25% trypsin in Hank's balanced salt solution (HBSS) containing 1 m*M* ethylenediaminetetraacetic acid (EDTA) for 10 min at 37°C. The reaction was terminated by the addition of DMEM containing 10% FBS. Cell suspensions were centrifuged at 500*g* for 10 min and the supernatant decanted. The cell pellet was washed with phosphate-buffered saline (PBS) and resuspended in physiologic saline. Cell viability was assessed by trypan blue exclusion and always found to be > 95%. Cell number was determined using a Coulter Counter (Hialeah, FL).

[^{3}H]-Thymidine Incorporation

At subconfluence (80% confluency), the cells were placed into quiesence by changing the media from DMEM containing 10% FBS to DMEM containing 1% FBS *(26)*. The cells were maintained in the 1% FBS media for 48 hours. At this time, they were confluent, and experimental media containing the cytokine and 1% FBS were added. After 20 h, [^{3}H]-thymidine (1 μCi/ml) in DMEM was added to the wells, resulting in a final isotope concentration of 0.34 μCi/ml. Following the 4-h incubation, the media were removed, and the cells washed twice with PBS, and then twice with 5% trichloroacetic acid (TCA). After the second wash, the cells were treated in TCA for 30 min at 4°C. The wells were air-dried after removal of the TCA and incubated overnight in 1% sodium dodecyl sulfate (SDS). Radioactivity was determined by liquid scintillation spectroscopy.

Alkaline Phosphatase Specific Activity

Since it is well known that the level of alkaline phosphatase activity in growth plate increases as the chondrocytes mature and calcify their matrix, we have used this enzyme as a marker for chondrocyte differentiation *(33–35)*. We have validated this observation in the culture model used in the present studies *(25)*. In culture, alkaline phosphatase activity increases as the cells re-express their in vivo phenotype, typical of their zone of origin within the growth plate. In postconfluent cultures, alkaline phosphatase continues to increase as the chondrocytes form multilamellar nodules *(29)*. The activity of this enzyme is also regulated in a cell maturation-dependent manner *(32)* by hormones known to regulate mineralization.

Alkaline phosphatase (EC 3.1.3.1) specific activity was determined on both the cell layers (cells plus matrix vesicles), prepared as described below, or isolated cells (cells only), prepared as described above for the cell number studies. This enables us to infer the contribution of matrix vesicles to any change in alkaline phosphatase that might occur. Protein content of the wells was determined using commercially available kits (Micro BCA and Macro BCA Protein Assays, Pierce, Rockford, IL). Alkaline phosphatase activity was assessed by the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.25 *(36)*. Specific activity was obtained by normalization of the alkaline phosphatase activity to the amount of protein in the sample.

Cell layers were prepared by the method of Hale et al. *(37)*. At harvest, the cells were washed twice with PBS and removed with a cell scraper. The cells were then centrifuged at 500*g* for 10 min. The supernatant was decanted, and the pellet resuspended in PBS and centrifuged again. Following the second spin, the supernatant was decanted and the pellet resuspended in 0.05% Triton X-100. Isolated cells were obtained via trypsinization, as described above, and resuspended in 0.05% Triton X-100. Enzyme assays were performed on the cell lysates that had been frozen and thawed three times.

[^{35}S]-Sulfate Incorporation

Proteoglycan synthesis was assessed by [^{35}S]-sulfate incorporation according to the method of O'Keefe et al. *(38)*. Four hours prior to harvest, [^{35}S]-sulfate (New England Nuclear, Boston, MA) was added to the media to a final concentration of 9 μCi/ml. At harvest, the wells were washed with 500 μl PBS, the cell matrix collected in two 0.25-mL portions in 0.25*M* NaOH, and protein content determined using commercially available kits (BCA Protein Assays, Pierce, Rockford, IL). In order to determine the extent of [^{35}S]-sulfate incorporation, the sample volume was adjusted to 0.7 mL by the addition of 0.15*M* NaCl. Samples were then transferred into dialysis tubing with a 12,000–14,000 mol wt cut off and dialyzed at 4°C against a buffer containing 0.15M NaCl, 20 m*M* Na_2SO_4, and 20 m*M* Na_2HPO_4 at pH 7.4. The dialysis solution was changed daily until the radioactivity in the dialysate reached background levels and the amount of [^{35}S]-sulfate incorporated into the cell layer determined using a liquid scintillation counter.

[^{3}H]-Proline Incorporation

Matrix protein synthesis was assessed by examining the extent of [^{3}H]-proline incorporation into CDP and NCP by the method of Peterkofsky and Diegelmann *(39)*. At confluence, DMEM containing 10% FBS, vitamin C, antibiotics, 50 mg/mL β-amino proprionitrile (Sigma, St. Louis, MO), and 5 μCi/ml L-[G-^{3}H]-proline (New England Nuclear, Boston, MA), in addition to PDGF-BB, were added to the wells.

The media and cell matrix were retrieved separately at harvest. The cell matrix was collected in two 0.2-mL portions of 0.2*M* NaOH, and proteins in both the media and matrix fractions precipitated with 0.1 mL 100% TCA containing 10% tannic acid and washed with 0.5 ml 10% TCA containing 1% tannic acid. After the initial wash, the two fractions were combined. The combined fractions were washed twice with 0.5 mL 10% TCA containing 1% tannic acid and then twice with 1 mL ice-cold acetone. The final pellets were dissolved in 0.5 mL 0.05*M* NaOH and protein content determined using commercially available kits (Micro BCA and BCA Protein Assays, Pierce, Rockford, IL).

CDP was separated from NCP via a 4-h digestion of the pellet at 37°C with 50 U/mL highly purified clostridial collagenase (Calbiochem, San Diego, CA) in a 0.032*N* HCl solution containing 60 m*M* HEPES (*N*-2-hydroxyethyl piperazine-*NO*-2 ethane sulfonic acid), 1.25 m*M* *N*-ethyl maleimide, and 0.25 m*M* $CaCl_2$. Digestion of the pellet was terminated by the addition of 0.5 mL 10% TCA containing 0.5% tannic acid at 0°C. Samples were then centrifuged for 5 min at 400*g* at 4°C, and the pellet resuspended in 0.5 mL 5% TCA containing 0.25% tannic acid, allowed to sit overnight at 4°C, centrifuged again, and resuspended in 0.5 mL 5% TCA containing 0.25% tannic acid. The supernatants from both spins were retained as the CDP fraction of the sample. The final suspended solution represented the NCP fraction of the sample. Counts in each fraction were determined using a liquid scintillation counter.

NCP synthesis was calculated after multiplying the labeled protein in NCP by 5.4 to correct for its relative abundance in collagen *(40)*. Percent collagen production was calculated by comparing CDP production with total CDP-plus-NCP production (CDP/[CDP + NCP] *100).

Effect of PDGF-BB on Resting Zone Chondrocyte Differentiation

To determine whether PDGF-BB could induce a change in maturation state, we took advantage of the differential responsiveness of resting zone and growth zone chondrocytes to vitamin D metabolites. When resting zone cells are incubated with 10^{-7}M 24,25-$(OH)_2D_3$, cell proliferation is not affected *(26)*; alkaline phosphatase specific activity is stimulated *(31)*, whereas phospholipase A_2 specific activity is *(32)*; [^{35}S]- sulfate incorporation is increased *(29)*; CDP is inhibited, NCP is not affected, and % collagen production is inhibited *(26)*. 1,25-$(OH)_2D_3$ inhibits cell proliferation *(26)* has no effect on alkaline phosphatase or phospholipase A_2 *(32)*; inhibits CDP, has no effect on NCP, and inhibits percent collagen production. In contrast, growth zone chondrocytes respond to 1,25-$(OH)_2D_3$, with inhibition of cell proliferation *(26)*; increased alkaline phosphatase and phospholipase A_2 *(32)*; increased CDP, no effect on NCP, and increased percent collagen production *(26)*.

For these studies, confluent fourth-passage resting zone chondrocyte cultures were incubated with 37.5 or 150 ng/mL PDGF-BB for 24, 48, 72, or 120 h. At the end of each of these times, the media were replaced with experimental media containing 10^{-8}*M* 1α,25-$(OH)_2D_3$ and the cultures incubated for an additional 24 h. At harvest, the cultures were analyzed as described above.

Statistical Analysis

Experiments were conducted at least twice and found to yield reproducible results. The data shown are from one representative experiment. Values given are mean ± standard error of the mean (SEM) of six individual cultures. Data were initially analyzed by analysis of variance (ANOVA), and if differences existed, Student's *t*-tests for multiple comparisons using Bonferroni's correction were used. Differences were considered significant if $p < 0.05$.

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