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Journal of Cellular Biochemistry 107:155–162 (2009)

Journal of Cellular Biochemistry

Inorganic Phosphate Modulates Responsiveness to $24,25(OH)_2D_3$ in Chondrogenic ATDC5 Cells

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ABSTRACT

Chondrogenic ATDC5 cells were used as a model of in vitro endochondral maturation to study the role of inorganic phosphate (Pi) in the regulation of growth plate chondrocytes by vitamin D3 metabolites. ATDC5 cells that were cultured for 10 days post-confluence in differentiation media and then treated for 24 h with Pi produced a type II collagen matrix based on immunohistochemistry and expressed mRNAs for several chondrocytic markers, including aggrecan, collagen types II and X, cartilage oligomeric matrix protein, and SOX9. Pi also caused a decrease in [^{35}S]-sulfate incorporation and stimulated apoptosis, as evidenced by increased DNA fragmentation and caspase-3 activity. In addition, treatment with Pi induced sensitivity to 24,25-dihydroxyvitamin D3 and this effect was both dose-dependent and was blocked by phosphonoformic acid (PFA), a specific inhibitor of sodium dependent type III Pi transporters. Treatment with $24R,25(OH)_2D_3$ reduced cell number and increased alkaline phosphatase specific activity in a dose-dependent manner. Moreover, $24R,25(OH)_2D_3$ reversed the Pi-induced decrease in incorporation of [^{3}H]-thymidine and [^{35}S]-sulfate incorporation, as well as the Pi-induced increase in apoptosis. These results suggest that Pi acts as an early chondrogenic differentiation factor, inducing response to $24R,25(OH)_2D_3$; treatment of committed chondrocytes with Pi induces apoptosis, but $24R,25(OH)_2D_3$ mitigates these effects, indicating a possible inhibitory feedback loop. J. Cell. Biochem. 107: 155–162, 2009.

KEY WORDS: PHOSPHATE; ATDC5 CELLS; CHONDROGENESIS; 24,25-DIHYDROXY VITAMIN D3

The formation of mammalian long bones occurs through the process of endochondral development, which begins with mesenchymal condensation in the embryo to form cartilaginous limb buds. Primary and secondary centers of ossification develop within the cartilage, ultimately becoming bone. The ends of the bones, the epiphyses, are separated from the metaphyses and diaphysis by a region of cartilage called a growth plate, which is spatially organized into zones defined by the differentiation state of chondrocytes resident in that region of the tissue. Nearest to the epiphysis is the reserve or resting zone. Chondrocytes in this region produce an extracellular matrix enriched in type II collagen and proteoglycan aggregates containing sulfated glycosaminoglycans. In embryonic bone, this region is relatively small as cells are rapidly progressing along the endochondral developmental pathway. In contrast, in post-natal growth plates, the resting zone serves as a chondrocyte reservoir and represents a larger component of the tissue. At the base of the resting zone, chondrocytes appear to align in columns to form the proliferative zone, in which they undergo rapid division, providing the major contribution of the growth plate to longitudinal bone growth [Ballock and O'Keefe, 2003]. Following proliferation, the cells undergo a prehypertrophic phase, transitioning into hypertrophy, a period in which the cells remodel their extracellular matrix to accommodate their increase in size and to prepare the matrix for calcification [Pucci et al., 2007]. During this phase, many of the hypertrophic chondrocytes also undergo apoptosis, which causes the growth plate to retain a consistent length despite continued growth of the bone. This process depends upon coordinated mineralization of the matrix. In conditions like vitamin D and phosphate deficient rickets, where the growth plate fails to become calcified, the hypertrophic zone continues to increase in length [Sabbagh et al., 2005].

Previous work examining mouse and rat growth plates has shown that two metabolites of vitamin D, 24,25-dihydroxy vitamin D3 [$24,25(OH)_2D_3$] and 1,25-dihydroxy vitamin D3 [$1,25(OH)_2D_3$], each play a role in regulating the process of endochondral development [Boyan et al., 1992, 2001, 2003]. Chondrocytes from the resting zone

Grant sponsor: NSF; Grant numbers: IGERT-0221600, EEC-9731643; Grant sponsor: NIH Training Grant; Grant number: 1246638; Grant sponsor: Children's Healthcare of Atlanta.

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Received 23 January 2009; Accepted 27 January 2009 • DOI 10.1002/jcb.22111 • 2009 Wiley-Liss, Inc.
Published online 13 March 2009 in Wiley InterScience (www.interscience.wiley.com).

exhibit specific sensitivity to $24,25(OH)_2D_3$, whereas cells in the growth zone no longer exhibit the same responses to $24,25(OH)_2D_3$ but have acquired specific sensitivity to $1,25(OH)_2D_3$. $24R,25(OH)_2D_3$ stimulates extracellular matrix production by resting zone cells, increasing production of sulfated glycosaminoglycans [Schwartz et al., 2001b]. In addition, it causes resting zone chondrocytes to produce extracellular matrix vesicles containing neutral metalloproteinases [Maeda et al., 2001b] and reduces total matrix vesicle metalloproteinase activity in vitro [Dean et al., 1996b] and in vivo [Dean et al., 2001]. In contrast, $1\alpha,25(OH)_2D_3$ inhibits DNA synthesis in prehypertrophic and hypertrophic chondrocytes and reduces synthesis of sulfated proteoglycans [Schwartz et al., 2001b], while increasing production of alkaline phosphatase-enriched matrix vesicles that contain increased metalloproteinase activity [Dean et al., 1996a, 2001]. Moreover, $1\alpha,25(OH)_2D_3$ acts directly on matrix vesicles produced by these cells, activating resident phospholipases, causing loss of membrane integrity and release of matrix processing enzymes [Maeda et al., 2001a]. These observations suggest that $24R,25(OH)_2D_3$ enhances matrix production and maintenance of resting zone cartilage, whereas $1\alpha,25(OH)_2D_3$ modulates the rate and extent of matrix degradation during chondrocyte hypertrophy. Interestingly, $1\alpha,25(OH)_2D_3$ induces production of $24R,25(OH)_2D_3$ by growth zone chondrocytes [Schwartz et al., 2001a], suggesting cross-talk among cells at different maturation states in endochondral development.

Inorganic phosphate (Pi) has also been implicated in the differentiation of the growth plate by acting as a signal affecting the differentiation of mineralization-competent cells [Beck, 2003; Beck et al., 2003; Foster et al., 2006]. The extracellular concentration of Pi is relatively high in the extracellular matrix produced by hypertrophic chondrocytes, in part due to the increased activity of matrix vesicle alkaline phosphatase [Kirsch et al., 1997]. Studies examining the effects of exogenous Pi on chondrocyte phenotype in post-fetal growth plates show that Pi can induce apoptosis [Sabbagh et al., 2005; Teixeira et al., 2007]. This suggests a feed-back loop in which $1\alpha,25(OH)_2D_3$ activates matrix vesicle alkaline phosphatase, releasing Pi into the matrix and Pi then acts back on the chondrocytes to induce apoptosis.

It is less clear how Pi might interact with $24R,25(OH)_2D_3$. To address this question, we took advantage of the embryonic ATDC5 cell model. This prechondrocyte cell line offers a useful culture system for studying the progression of endochondral development. When confluent cultures of ATDC5 cells are grown in high insulin media, they form cartilage nodules that exhibit the differentiation sequence typical of long bone growth plates [Atsumi et al., 1990; Shukunami et al., 1996]. $1\alpha,25(OH)_2D_3$ has been shown to inhibit proliferation and differentiation of ATDC5 cells [Akiyama et al., 1996], but it is not known if these cells are regulated by $24,25(OH)_2D_3$. Interestingly, Pi has been shown to be a regulator of chondrogenic differentiation and apoptosis in these cells, including upregulation of collagen type X, a marker of maturation in the hypertrophic zone of the growth plate [Fujita et al., 2001; Magne et al., 2003]. Pi was also shown to regulate expression of matrix Gla protein (MGP) via ERK1/2 in both ATDC5 cells and primary growth plate organ cultures [Julien et al., 2007]. MGP is an inhibitor of matrix calcification [Murshed et al., 2004], suggesting that Pi may induce production of factors that retard endochondral ossification like $24R,25(OH)_2D_3$, as well as production of factors that stimulate chondrocyte maturation and apoptosis.

The purpose of the present study was to determine if Pi treatment causes ATDC5 cells to become responsive to $1\alpha,25(OH)_2D_3$ or $24R,25(OH)_2D_3$ and if so, what are the consequences to endochondral maturation of the cells. The physiological importance of Pi is supported by the observation that active ion transport through the membrane is required [Guicheux et al., 2000].

MATERIALS AND METHODS

CELL CULTURE

ATDC5 cells were cultured in a maintenance medium consisting of a 1:1 ratio of DMEM/F12 media (Cellgro, Manassas, VA) with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT), 10 μg/ml human transferrin (Sigma Chemical Company, St. Louis, MO), 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA), and 3×10^{-8} M sodium selenite (Sigma). After reaching confluence cells were cultured with differentiation media, which is identical to maintenance media with the addition of 10 μg/ml bovine insulin (Sigma) and 50 μg/ml ascorbic acid (Sigma) [Atsumi et al., 1990; Altaf et al., 2006]. At 10 days post-confluence, cells were cultured for 24 h in differentiation media supplemented with Pi (0–20 mM beyond media basal level) and 10% FBS (10% FBS was used to ensure sufficient serum proteins such as fetuin that help regulate pathologic precipitation of calcium phosphate crystals [Schinke et al., 1996; Price et al., 2003]). To make Pi-supplemented media, a more concentrated volume of Pi was dissolved in warm DMEM/F12 (37°C) using molar ratios of 4 mol of dibasic sodium phosphate Na_2HPO_3 (Sigma) to 1 mole of monobasic sodium phosphate NaH_2PO_3(Sigma) [Beck, 2003]. When the phosphate salts were completely dissolved, the pH was adjusted to 7.4, and the solution was filter sterilized. An appropriate aliquot from the concentrated Pi solution was added to the media preparation to result in the desired final concentration. Control cultures were also treated on day 10 with differentiation media with 10% FBS. Some experiments also included concurrent treatment with the Pi transporter inhibitor phosphonoformic acid (PFA) (sodium phosphonoformate tribasic hexahydrate) (Sigma) to test the effect of phosphate transport inhibition. Cells were returned to differentiation media with 5% FBS on day 11 for treatment with $24R,25(OH)_2D_3$ or $1,25(OH)_2D_3$ or ethanol vehicle (Sigma).

CELL NUMBER

Effects of Pi and $24R,25(OH)_2D_3$ on proliferation were determined by measuring cell number at harvest and also as a function of DNA synthesis (described below). To measure cell number, ATDC5 cells were treated with Pi for 24 h followed by treatment with $24R,25(OH)_2D_3$ for 24 h. At harvest, cells were washed twice with DMEM and trypsinized (Invitrogen) for 10 min. Cells were resuspended in saline, and counted on a Beckman Coulter Z1 particle counter.

ALKALINE PHOSPHATASE ACTIVITY

Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline]-specific activity was used as an indication of chondrocyte differentiation. Harvested cells were suspended in 0.05% Triton-X. After three freeze–thaw cycles to lyse the cells, alkaline phosphatase activity in the cell lysates was determined [Bretaudiere and Spillman, 1984] and normalized to protein content using the Macro BCA Protein Assay Kit (Pierce, Rockford, IL).

[^{35}S]-SULFATE INCORPORATION

To determine the role of Pi in mediating the effects of 24R,25(OH)$_2$D$_3$ on extracellular matrix production, proteoglycan synthesis was assessed by measuring [^{35}S]-sulfate incorporation as described previously [O'Keefe et al., 1988; Schwartz et al., 1995]. Four hours prior to harvest, [^{35}S]-sulfate (Perkin Elmer, Waltham, MA) was added to the cultures. Cell layers were collected and dialyzed to remove any unbound [^{35}S]-sulfate. Radiolabeled incorporated into the cell layer was expressed as disintegration per minute normalized to protein levels for each sample.

HISTOLOGY

To verify that the ATDC5 cells produced a cartilage extracellular matrix, cultures were examined for collagen II protein by immunohistochemistry. Cells were seeded into 4-well chamber slides. Once the cells reached confluence they were cultured with differentiation media and were treated with 20 mM Pi on day 10 and 10^{-7} M 24,25(OH)$_2$D$_3$ on Day 11, respectively, or with the appropriate vehicle. The cells were fixed for 30 min in 4% formalin in PBS, after which the cells were rinsed three times in PBS and stored in 75% absolute ethanol. At time of staining, the fixed cultures were etched with 0.25% pepsin to expose the antigen, followed by PBS washes. Cell monolayers were blocked with 2% horse serum in PBS. Cells were then treated with the mouse anti-collagen II antibody (Hybridoma Bank, University of Iowa). After more PBS washes a biotinylated horse anti-mouse IgG antibody was applied. An alkaline phosphatase ABC kit was used to visualize the biotin. Samples were counterstained with haematoxylin and visualized with light microscopy.

RNA EXTRACTION AND RT-PCR

Cellular RNA was harvested using the Trizol® reagent kit (Invitrogen). RNA samples were converted to cDNA using the Omniscript RT kit (Qiagen, Valencia, CA) and then PCR was performed using HotStar Taq Master Mix Kit (Qiagen). PCR product was visualized using gel electrophoresis in 5% TBE Ready Gels (Biorad, Hercules, CA) and visualized on a Versadoc Model 1000 (Biorad). To better visualize qualitative differences between groups, densities of the visualized bands were measured using Quantity One 4.4.1 Software (Biorad). The gene specific primers (MWG Biotech, Huntsville, AL) used to amplify mRNA were as follows: aggrecan—5′ ATC ACA GCC ACC ACT TCC 3′ (sense) and 5′ CTC CAC TCA CAG ATG TTA TAC C 3′ (anti-sense), collagen type I—5′ GGC TCC TGC TCC TCT TAG 3′ (sense) and 5′ TCT TCT GAG TTT GGT GAT ACG 3′ (anti-sense), collagen type II—5′ GCG GTC CTA ACG GTG TCA G 3′ (sense) and 5′ ACC AGC CTT CTC GTC ATA CC 3′ (anti-sense), collagen type X—5′ GCA CCT ACT GCT GGG TAA GC 3′ (sense) and 5′ GCC AGG TCT CAA TGG TCC TA 3′ (anti-sense), cartilage oligomeric matrix protein (COMP)—5′ CCA CTG ATG ATG ACT ATG C 3′ (sense) and 5′ GAT GTA GCC AAC TTG AGG 3′ (anti-sense), SOX9—5′ GAA CGA GAG CGA GAA GAG ACC 3′ (sense) and 5′ GGC GGA CCC TGA GAT TGC 3′ (anti-sense), and glyceraldehyde phosphate dehydrogenase (GAPDH)—5′ TTC AAC GGC ACA GTC AAG G 3′ (sense) and 5′ TCT CGC TCC TGG AAG ATG G 3′ (anti-sense). The negative control was RNA from mouse liver tissue and the positive control was RNA from mouse cartilage (Zyagen, San Diego, CA).

DNA SYNTHESIS

The effects of Pi and 24R,25(OH)$_2$D$_3$ on DNA synthesis were determined by measuring [^{3}H]-thymidine incorporation into trichloroacetic acid (TCA, Sigma) insoluble cell precipitates as previously described [Schwartz et al., 1989]. ATDC5 cells were treated for 24 h with Pi followed by treatment with 24R,25(OH)$_2$D$_3$. Two hours prior to harvest, [^{3}H]-thymidine (Perkin Elmer) was added. Radioactivity in TCA-precipitable material was measured by liquid scintillation spectroscopy.

ASSAYS FOR APOPTOSIS

DNA fragmentation. Cells were pre-labeled with [^{3}H]-thymidine (Perkin Elmer) for 4 h and then treated with Pi for 24 h followed by 24R,25(OH)$_2$D$_3$ for 24 h, or with Pi followed by vehicle. Cell monolayers were washed with DMEM three times to remove any residual unincorporated [^{3}H]-thymidine and cells were lysed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, 0.2% Triton X-100) for 30 min. Cell lysates were centrifuged at 13,000*g* for 15 min to separate intact DNA from fragmented DNA. The amount of incorporated [^{3}H]-thymidine was determined in each fraction to establish the total amount of [^{3}H]-DNA.

Caspase-3 activity. Caspase-3 activity was assessed using the colorimetric CaspACE™ Assay System (Promega, Madison, WI). Cells were harvested 24 h post-treatment with 200 μl cell lysis buffer followed by two 10 s periods of sonication. After harvest, 2 μl of the caspase-3 selective substrate DEVD-pNA were added to each well containing 100 μl of cell lysate and incubated at 37°C for 4 h. DEVD-pNA cleavage into the colorimetric product pNA was measured at 405 nm. Caspase-3 activity was normalized to protein content as determined by the Pierce Macro BCA Protein Assay Kit.

STATISTICAL ANALYSIS

Data are presented as means ± standard error of the mean (SEM) for six independent cultures for each variable. The results for individual experiments are shown. To ensure validity of the results, all quantitative experiments were repeated at least two or more times. Data were analyzed with ANOVA followed by Bonferroni's modification of Student's *t*-test. Differences in means were considered to be statistically significant if the *P*-value was <0.05.

RESULTS

Pi treatment alone did not affect cell number except at the highest concentration (20 mM) tested (Fig. 1A). 24R,25$(OH)_2D_3$ caused a small but significant decrease in the control cultures and further decreased the effects of 20 mM Pi. The expanded dose–response (Fig. 1B) confirmed the effects of Pi on response to 24R,25$(OH)_2D_3$. Pi reduced ATDC5 cell number at 20 mM. Effects of 24R,25$(OH)_2D_3$ depended on Pi concentration and were dose-dependent from 19.5 to 20.25 mM with the greatest effect at 20 mM.

Pi had a biphasic effect on alkaline phosphatase activity in the ATDC5 cell lysates, with an increase over control levels at 20 mM Pi (Fig. 1C). Effects of 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ on alkaline phosphatase were also sensitive to Pi pretreatment. 1α,25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$ reduced enzyme activity in control cultures. At 5 mM Pi, only 10^{-8} M 1α,25$(OH)_2D_3$ reduced enzyme activity over that seen in Pi treated cells. At 15 mM Pi, both 24R,25$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ stimulated activity but at 20 mM Pi, 24R,25$(OH)_2D_3$ caused a 30% increase in alkaline phosphatase. The stimulatory effect of 20 mM Pi on 24R,25$(OH)_2D_3$-dependent alkaline phosphatase activity was confirmed in the expanded dose–response study (Fig. 1D).

The response of ATDC5 cells to 24R,25$(OH)_2D_3$ was dose-dependent following pretreatment with 20 mM Pi. The reduction in cell number was greatest at 10^{-7} M (Fig. 2A) and the stimulatory effect of alkaline phosphatase was greatest at 10^{-7} M 24R,25$(OH)_2D_3$ (Fig. 2B). The effects of 20 mM Pi were specific based on their inhibition by PFA, which is a specific competitive inhibitor of the type III sodium dependent phosphate transporter Glvr-1. PFA caused a dose-dependent decrease in the Pi-induced reduction in cell number (Fig. 2C) and a dose-dependent decrease in Pi-activated alkaline phosphatase (Fig. 2D).

Treatment with 20 mM Pi on day 10 reduced [^{35}S]-sulfate incorporation at the end of Day 11, but this was restored to control levels when Pi was followed by treatment with 10^{-7} M 24R,25$(OH)_2D_3$ (Fig. 3A). ATDC5 cells produced an extracellular matrix containing type II collagen, regardless of the treatment regimen. Cell layers stained positively with anti-type II collagen antibody whether they were untreated or treated with Pi followed by 24R,25$(OH)_2D_3$ (Fig. 3B). Semiquantitative analysis by RT-PCR showed that mRNA expression of chondrogenic markers was affected (Fig. 4). Pi treatment reduced collagen II mRNA, but dramatically increased collagen X mRNA. During direct exposure to Pi there was an increase in aggrecan and decrease in COMP mRNAs. By itself, 24R,25$(OH)_2D_3$ had minimal effect on any markers, but the

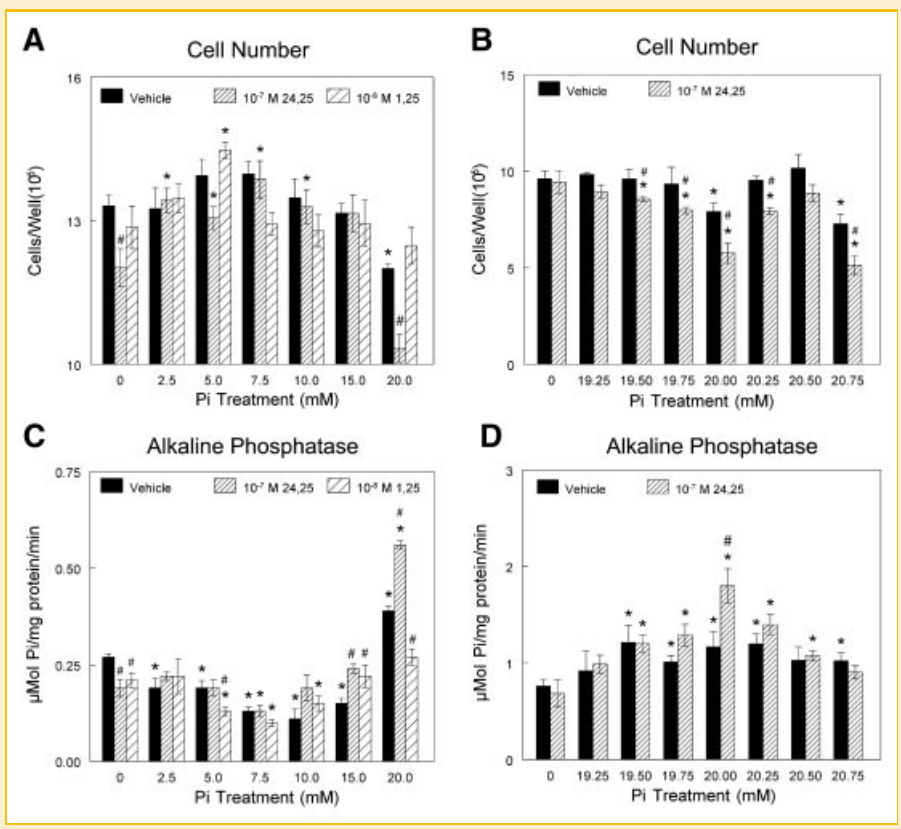

Fig. 1. Pi dose-dependently induces 24R,25$(OH)_2D_3$ sensitivity in ATDC5 cells. Cells were pretreated with control media or media with additional Pi (2.5–20.75 mM) on day 10 followed by vehicle, 10^{-8} M 1,25, or 10^{-7} M 24,25 on Day 11. Cells were harvested at end of day 11 for cell number (A,B) and alkaline phosphatase activity (C,D). #$P < 0.05$ versus vehicle within same Pi-treated group, *$P < 0.05$ versus 0 mM Pi.

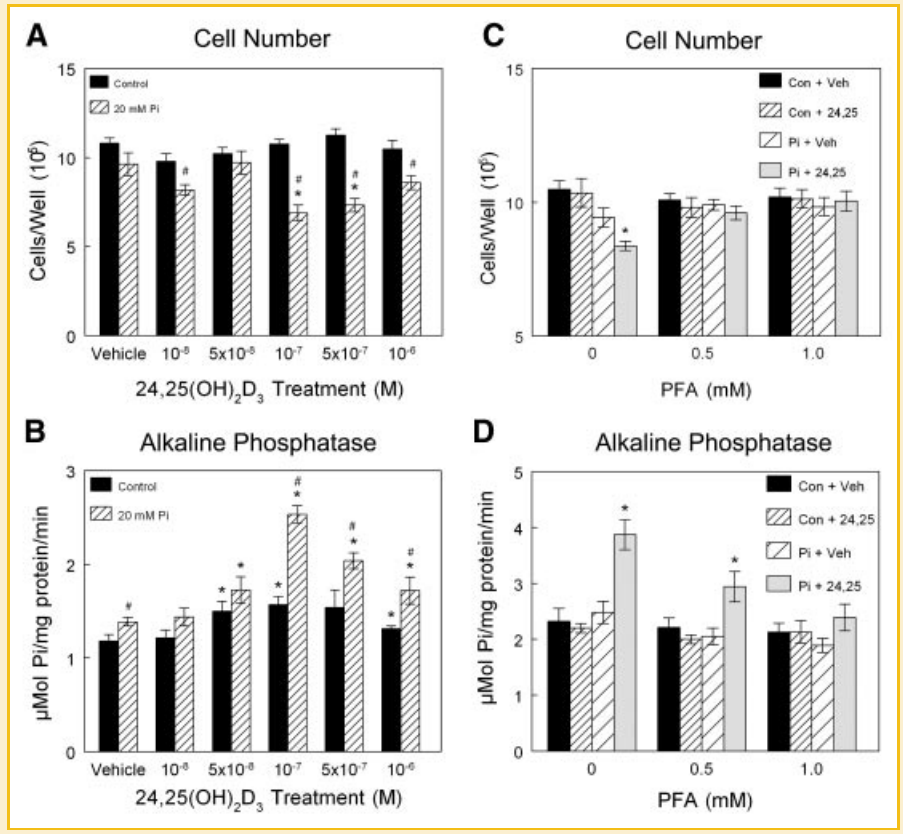

Fig. 2. Response to 24R,25(OH)$_2$D$_3$ is dose-dependent on Pi pretreatment, and Pi transport is required for Pi-induced 24R,25(OH)$_2$D$_3$ sensitivity. A–B: Cells were treated with vehicle or 10^{-8}–10^{-6} M 24R,25(OH)$_2$D$_3$ after pretreatment with 20 mM Pi. Cell number (A) and alkaline phosphatase activity (B) were measured. #$P < 0.05$ versus Control, *$P < 0.05$ versus no 24,25(OH)$_2$D$_3$. C–D: Cells were treated with control or 20 mM Pi on day 10 with 0–1 mM of the Pi transport inhibitor PFA, and then treated on day 11 with vehicle or 10^{-7} M 24R,25(OH)$_2$D$_3$. Cell number (C) and alkaline phosphatase (D) were then measured. *$P < 0.05$ treatment versus control for each PFA concentration.

steroid rescued collagen II mRNA and enhanced collagen X mRNA after pretreatment with Pi.

Pi caused an increase in ATDC5 apoptosis. Pi increased DNA fragmentation (Fig. 5A), increased caspase-3 activity (Fig. 5B), and reduced DNA synthesis (Fig. 5C) by the end of Pi treatment. The stimulatory effect of Pi on apoptosis was reversed by subsequent treatment with 24R,25(OH)$_2$D$_3$. 24R,25(OH)$_2$D$_3$ blocked DNA fragmentation in Pi-treated cells (Fig. 5D), decreased caspase-3 activity (Fig. 5E), and increased DNA synthesis (Fig. 5F).

DISCUSSION

The results presented here demonstrate that exogenous Pi is a potent inducer of endochondral development, not only for hypertrophic cells as has been reported previously [Pucci et al., 2007], but also for prechondrocytes. In response to relatively high levels of Pi, ATDC5 cells exhibited increased levels of mRNA for type II collagen and aggrecan. These cells also exhibited markers of endochondral development, including reduced expression of the early differentiation marker Sox 9, reduced cell numbers and increased alkaline phosphatase specific activity as well as elevated expression of the later-stage marker of hypertrophic chondrocytes, collagen type X. Others have reported a dose-dependent increase in collagen X in ATDC5 cells treated with Pi in the range of 3–30 μm [Fujita et al., 2001], supporting our finding. Moreover, our results confirm that the effects of Pi on endochondral development were specific and were dependent on active transport of the ion because treatment of the cells with the phosphate transporter inhibitor PFA blocked the Pi-induced responses.

Interestingly, the Pi-induced chondrocytes were sensitive to both 1α,25(OH)$_2$D$_3$ and 24R,25(OH)$_2$D$_3$ with respect to reduced cell number and increased alkaline phosphatase at Pi concentrations below 20 mM, but in cultures treated with 20 mM Pi, there was a very specific enhancement of response to the 24R,25(OH)$_2$D$_3$ metabolite of vitamin D3. This was unanticipated since studies using rat [Schwartz et al., 1988, 1995] and mouse [Boyan et al., 2003] costochondral growth plate chondrocytes have shown that resting zone cells are the primary target for 24R,25(OH)$_2$D$_3$, whereas prehypertrophic and hypertrophic chondrocytes are primary targets for 1α,25(OH)$_2$D$_3$. Moreover, 20 mM Pi induced sensitivity of the ATDC5 cells to 10^{-7} M 24R,25(OH)$_2$D$_3$, which is the concentration at which costochondral resting zone cells exhibit maximal responses to the seco-steroid [Boyan et al., 2003] and similar to the level of endogenous 24R,25(OH)$_2$D$_3$ produced by these cells when stimulated in culture [Schwartz et al., 1992].

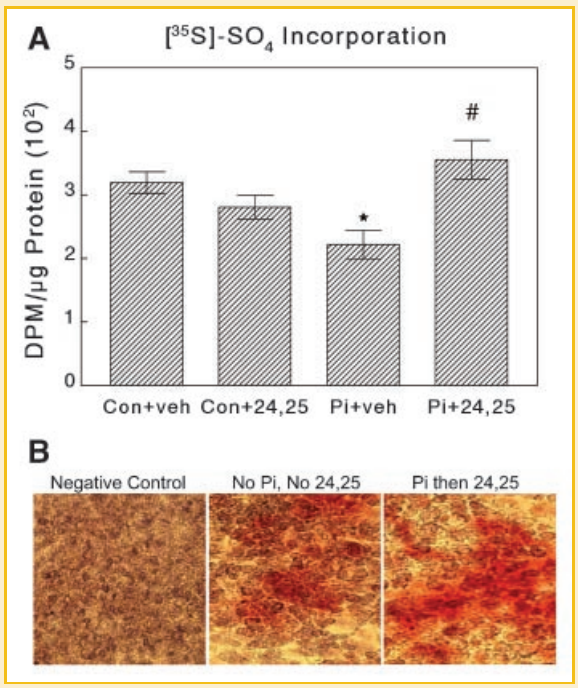

Fig. 3. 24R,25(OH)$_2$D$_3$ recovers Pi-induced reduction of sulfate incorporation, but neither treatment necessary for collagen type II protein expression. A: ATDC5 cells were treated with 20 mM Pi or control media on day 10 and with vehicle or 10^{-7} 24R,25(OH)$_2$D$_3$ on Day 11. Pi on day 10 resulted in lower sulfate incorporation by the end of Day 11, but this effect was not seen for cells that received subsequent 24R,25(OH)$_2$D$_3$ on Day 11. $^*P < 0.05$ versus control, $\#P < 0.05$ versus vehicle. B: ATDC5 cells treated with 20 mM Pi on day 10 and then 10^{-7} M 24R,25(OH)$_2$D$_3$ on day 11 or with neither of these treatments were tested with immunohistochemical staining for collagen type II expression at the end of Day 11. Negative control was performed without primary antibody to test for nonspecific staining.

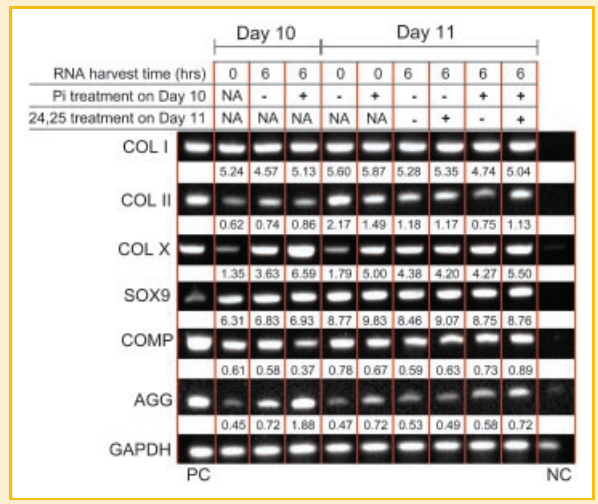

Fig. 4. Chondrocyte marker mRNA expression in ATDC5 cells during Pi and 24R,25(OH)$_2$D$_3$ treatment. ATDC5 cells were treated with ±20 mM Pi on day 10 and $\pm 10^{-7}$ M 24R,25(OH)$_2$D$_3$ on Day 11. RNA was extracted at 0 and 6 h on day 10 and day 11 and RT-PCR was performed to assess mRNA expression of collagen types I, II, and X and also SOX9, COMP, and aggrecan (AGG). GAPDH was also assessed to confirm consistent mRNA levels between samples. The density of each band compared to the density of the respective GAPDH band is shown numerically underneath each sample. Positive control (PC) is mRNA from mouse cartilage tissue and negative control (NC) is mRNA from mouse liver. GAPDH band for negative control is shown to demonstrate RNA present in negative control sample.

These observations suggest that 24R,25(OH)$_2$D$_3$ may serve to protect the early endochondral chondrocytes from premature terminal differentiation due to high levels of exogenous Pi. Our results support this hypothesis. 24R,25(OH)$_2$D$_3$ blocked the inhibitory effect of Pi on [^{35}S]-sulfate incorporation. Moreover, it

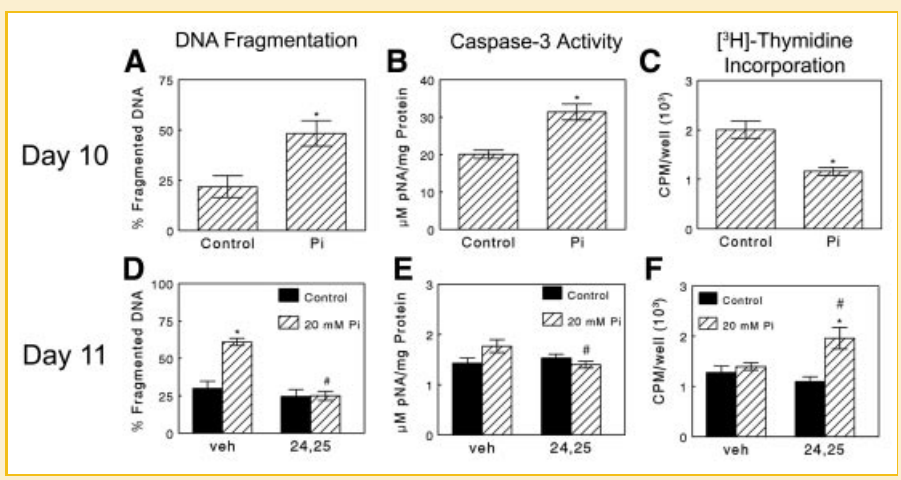

Fig. 5. Time-course effects of Pi and 24R,25(OH)$_2$D$_3$ on apoptosis and [^{3}H]-thymidine incorporation. ATDC5 cells were treated with 20 mM Pi or control on day 10 and then tested after 24 h for DNA fragmentation (A), caspase-3 activity (B), or [^{3}H]-thymidine incorporation(C). At the start of day 11 ATDC5 cells then received vehicle or 10^{-7} M 24R,25(OH)$_2$D$_3$ and were tested for the same assays after 24 h (D–F). 24R,25(OH)$_2$D$_3$ reduced apoptosis and increased proliferation on day 11 when treated with Pi on Day 10. $^*P < 0.05$ versus control. $\#P < 0.05$ versus vehicle.

blocked the stimulatory effects of Pi on apoptosis, based on two different indicators of cell death. 24R,25$(OH)_2D_3$ increased DNA synthesis, reduced DNA fragmentation, and reduced caspase-3 activity in Pi-treated ATDC5 cells.

The fact that the effects of Pi treatment on sensitivity to 24R,25$(OH)_2D_3$ were so narrowly focused in terms of dose may indicate that one or more critical conditions must be met with precision to invoke the need for response to the steroid during endochondral ossification in the embryo. The spatial and temporal sequence of events in embryonic bone formation differs from post-fetal bone growth. Thus, Pi and 24R,25$(OH)_2D_3$ may act together to reduce proliferation and begin the process of hypertrophy, but as alkaline phosphatase increases generating higher levels of exogenous Pi, 24R,25$(OH)_2D_3$ acts as a brake on the apoptotic process induced by the active uptake of Pi.

In summary, our study demonstrates the value of the ATDC5 prechondrocyte model for studying factors that modulate endochondral ossification, as noted by others [Atsumi et al., 1990; Shukunami et al., 1997; Julien et al., 2007]. Our results confirm the importance of exogenous Pi in regulating the differentiation and maturation of chondrocytes in endochondral development. Most importantly, they show that Pi treatment induces sensitivity to vitamin D metabolites 24R,25$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ in a dose-dependent manner and at the higher concentrations of Pi, the cells become specifically responsive to 24R,25$(OH)_2D_3$. This metabolite acts with Pi to reduce cell number and increase endochondral differentiation, but at the same time it blocks the activation of apoptosis, suggesting a role for modulating the rate of terminal differentiation in embryonic bone formation.

ACKNOWLEDGMENTS

The authors thank Tracey Couse for her technical assistance. This research was funded by NSF IGERT-0221600, NSF Grant EEC-9731643, NIH Training Grant 1246638, and Children's Healthcare of Atlanta.

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