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Stathmin Levels in Growth Plate Chondrocytes Are Modulated by Vitamin D_3 Metabolites and Transforming Growth Factor-β1 and Are Associated with Proliferation

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Stathmin is a highly conserved, phosphorylated cytosolic protein that is found at decreased levels in all cells as they become more terminally differentiated, or when they decrease in their rate of proliferation. This study examined the hypothesis that stathmin levels in growth plate chondrocytes decreases as endochondral maturation increases. To test this hypothesis, we used a costochondral growth plate chondrocyte cell culture model. Cells derived from the resting zone (RC) express twice as much stathmin mRNA in culture and have twice as much stathmin protein as cells derived from the post proliferative growth zone ([GC]; prehypertrophic and upper hypertrophic cell zones). Stathmin levels in vivo were assessed by immunohistochemistry. To assess the effects of agents that modulate proliferation and differentiation, RC and GC chondrocytes were cultured in the presence of 10^{-10} to 10^{-8} *M* 1α,25-$(OH)_2D_3$, which regulates proliferation in both cell types but affects differentiation of only GC cells, or 10^{-9} to 10^{-7} *M* 24R,25-$(OH)_2D_3$, which regulates differentiation and maturation of RC cells but decreases proliferation of GC cells. In addition, RC cells were treated with 0.44 or 0.88 ng/mL of recombinant human transforming growth factor β1 (rhTGF-β1), which stimulates proliferation of RC cells and regulates proteoglycan production, but not alkaline phosphatase activity. Stathmin protein levels were determined using quantitative immunoblots, with recombinant human stathmin as a standard. The results show that stathmin levels are associated with proliferation. Proliferating chondrocytes in vivo exhibited higher levels of immunoreactive stathmin than either RC or GC cells in the growth plate. In culture, 1α,25-$(OH)_2D_3$ caused a dose-dependent decrease in stathmin in RC and GC cells within 24 h. 24R,25-$(OH)_2D_3$ also reduced stathmin levels in GC cells within 24 h but only affected RC cells after prolonged exposures (96 h), at which time RC cells express a GC-like phenotype. rhTGF-β1 caused an increase in stathmin levels in RC cells. Stathmin levels are sensitive to protein kinase C (PKC) in other cells. Inhibition of PKC with chelerythrine had no effect on the response of RC cells to 1α,25-$(OH)_2D_3$ but it blocked the effect of rhTGF-β1, indicating that decreases in stathmin by vitamin D_3 metabolites may not be modulated by PKC, whereas increases in stathmin via rhTGF-β1 may be regulated via a PKC-dependent mechanism. These results support the hypothesis that constitutively expressed levels of stathmin are related to cell maturation state and that they are modulated by factors that regulate proliferation.

Key Words: Chondrocyte cultures; stathmin; transforming growth factor-β1; growth plate; 1α,25-$(OH)_2D_3$; 24R,25-$(OH)_2D_3$.

Introduction

Stathmin is a phosphorylated cytosolic protein with expression occurring in a vast number of tissues and species *(1,2)*. It has been described under a variety of names *(3–6)* and is well conserved throughout the evolution of vertebrates *(7, 8)*. This high degree of conservation *(2,7,9,10)* strongly suggests that stathmin serves an important function in mammalian cells, although it is not yet clear what its function may be. It is likely that stathmin plays a role in proliferation, since it has been uniformly observed that stathmin is expressed in actively proliferating cells in culture *(7)*. Recent research focusing on the interaction of stathmin and microtubules indicates that stathmin plays a role in tubulin stability *(6,11,12)*.

In addition to increasing the catastrophe rate of tubulin *(13)*, stathmin is also involved in regulating tubulin dynamics, where it is directly involved in cytokinesis *(14)*. Tubu-

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lin dynamics not only play an integral role in mitotic changes but also modulate signaling pathways and general cell shape *(15–18)*. Both the level of production and the degree of phosphorylation of stathmin have been associated with changes in cell proliferation *(19–22)*, differentiation *(3,23,24)*, and development *(2,25)*. These observations suggest that stathmin levels may be related to differentiation as well as proliferation. This may be important in maturation of mesenchymal cells, such as osteoblasts and chondrocytes, because the onset of differentiation in culture is associated with decreased proliferation *(26,27)*.

Studies using a costochondral growth plate chondrocyte model support this hypothesis *(28)*. In vivo, these cells progress through their lineage cascade in an ordered manner that can be easily visualized under a dissecting microscope owing to the alignment of the cells in adjacent columns. Cells in the resting zone (RC) are less mature with respect to the lineage progression than cells in the prehypertrophic and upper hypertrophic cell zones (growth zone [GC]). In vivo, RC cells enter a period of proliferation before acquiring a GC phenotype. When grown in culture, the less mature RC cells proliferate at a faster rate than GC cells. Both cell types reexpress their in vivo phenotype in culture and respond to regulatory factors in a differential manner *(29)*, making them an ideal model for examining the correlation between chondrocyte maturation and stathmin expression. Using this model, we have shown that RC cell cultures express twice as much stathmin mRNA and have twice as much stathmin protein as GC cell cultures *(28)*, suggesting that stathmin production in vitro is related to maturation state in vivo.

While basal levels of stathmin vary as a function of chondrocyte phenotype, it is not clear whether stathmin is associated with differentiation and proliferation. In vivo, RC cells are preproliferative with respect to endochondral development and GC cells are postproliferative, but both cells proliferate in culture. Moreover, proliferation and differentiation of the cells in culture can be differentially modulated by hormones, growth factors, and cytokines.

In the present study, to test the hypothesis that stathmin production is related to proliferation, we treated RC and GC cells with agents known to regulate proliferation and phenotypic expression *(30,31)*. 1α,25-$(OH)_2D_3$ inhibits proliferation of both RC and GC cells but stimulates differentiation of only GC cells *(32,33)*. 24R,25-$(OH)_2D_3$ inhibits proliferation of GC cells but not of RC cells *(33)*. Moreover, prolonged exposure of RC cells to 24R,25-$(OH)_2D_3$ induces a shift in maturation state, causing RC cells to acquire a GC cell phenotype, including responsiveness to 1α,25-$(OH)_2D_3$ *(34)*. Transforming growth factor-β1 (rhTGF-β1) stimulates proliferation of RC cells in a dose-dependent manner, as shown by [^{3}H]-thymidine incorporation *(35)*. When used at concentrations of 0.44 and 0.88 ng/mL, there are proliferative effects, whereas at lower concentrations, differentiation and maturation are favored over proliferation *(35)*.

Stathmin production has been shown to be regulated via protein kinase C (PKC) in other cell types *(36)*. Many of the effects of 1α,25-$(OH)_2D_3$, 24R,25-$(OH)_2D_3$, and rhTGF-β1 also involve PKC-dependent mechanisms *(33,37–41)*. To test the hypothesis that regulation of stathmin involves a PKC-dependent mechanism in the growth plate chondrocytes, PKC activity was inhibited with chelerythrine.

Results

Immunohistochemistry

Positive identification of stathmin protein was observed in cells throughout costochondral cartilage (data not shown). Visually, a difference between the amount of stathmin present in RC and GC cells was not observed. However, cells in the proliferative zone, the zone separating the RC and GC cell zones, appeared to stain more intensely. In addition, immunoreactive stathmin was reduced in the lower hypertrophic zone cells, which are at their terminal stage of differentiation. Stathmin protein was also detected in the adjacent bone. Sections treated with antibody-blocking peptides were negative, as were sections incubated with preimmune serum (data not shown).

Regulation of Stathmin Protein Levels by 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$

Stathmin protein levels were regulated by 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ in a cell- and metabolite-dependent manner. 1α,25-$(OH)_2D_3$ caused a dose-dependent decrease in the amount of stathmin protein in RC cells (Fig. 1A). This was a consistent observation based on analysis of treatment/control (T/C) ratios for five separate experiments. Unexpectedly, there was no change in stathmin levels in RC cells in response to 24R,25-$(OH)_2D_3$ (Fig. 1B). GC cells responded to both metabolites by decreasing stathmin levels (Fig. 2).

Exposure of RC cells to 24R,25-$(OH)_2D_3$ for 4 d resulted in a dose-dependent decrease in stathmin protein that was significant at both 10^{-8} and 10^{-7} *M* (Fig. 3). In addition, cells that were pretreated for 4 d with 10^{-7} *M* 24R,25-$(OH)_2D_3$ exhibited a dose-dependent decrease in stathmin levels in response to 1α,25-$(OH)_2D_3$ after 24 h (Fig. 4).

Regulation of Stathmin Protein Levels by rhTGF-β1

RC cells exposed to rhTGF-β1 continuously for 72 h exhibited an increase in stathmin levels with respect to the controls (Fig. 5). However, this response was not dependent on concentration.

Role of PKC

Stathmin levels are regulated by PKC-dependent mechanisms (Fig. 6). Inhibition of PKC caused a statistically significant decrease in control levels of stathmin in RC cells (Fig. 6A). However, PKC was not involved in the response of RC cells to 10^{-8} 1α,25-$(OH)_2D_3$ at 24 h. Chelerythrine did not block the effect of 1α,25-$(OH)_2D_3$ but caused a further decrease in stathmin levels in cultures treated with this

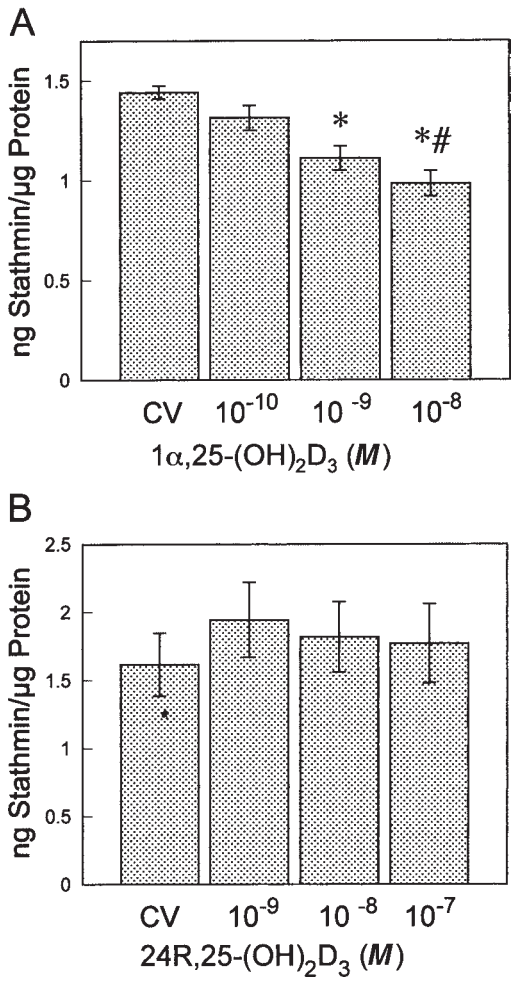

Fig. 1. Effect of 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ on stathmin protein levels in RC chondrocytes. (**A**) Confluent, fourth-passage RC cell cultures were treated with varying concentrations of 1α,25-$(OH)_2D_3$. (**B**) Confluent, fourth-passage GC cell cultures were treated with varying concentrations of 24R,25-$(OH)_2D_3$. Extracts were prepared and then electrophoresed on 15% polyacrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. Values are the mean ± SEM of six cultures of which the data are derived from samples on membranes compared to a membrane with standards. Data are from one of two experiments, each yielding comparable results. (**A**) $*p < 0.05$, vs control plus vehicle (CV); $^{\#}p < 0.05$, vs 10^{-10} *M* 1α,25-$(OH)_2D_3$; and vs 10^{-9} *M* 1α,25-$(OH)_2D_3$. T/C ratios comparing the data from three separate experiments confirmed the observation that 10^{-9} and 10^{-8} M 1α,25-$(OH)_2D_3$ caused a dose-dependent decrease in stathmin in RC cells. (**B**) no significant differences were detected.

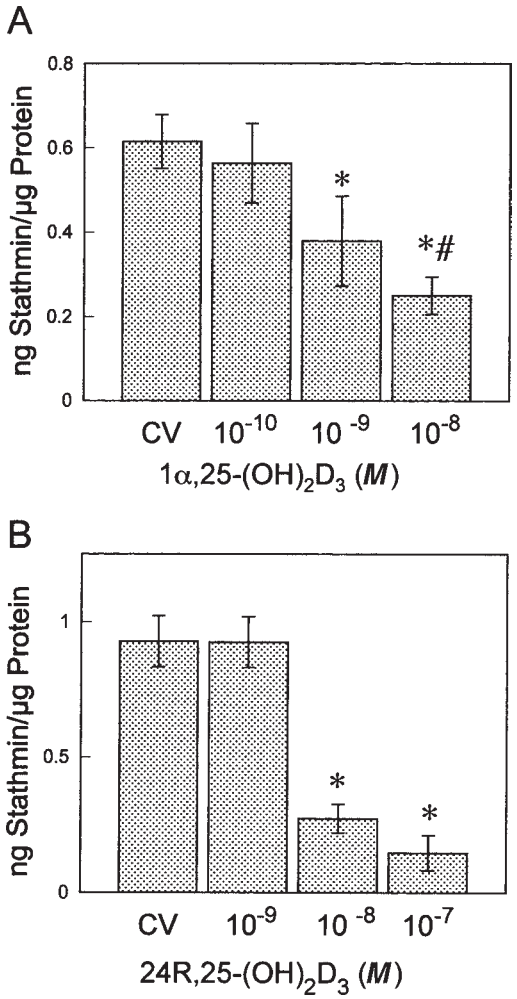

Fig. 2. Effect of 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ on stathmin protein levels in GC chondrocyte cultures. (**A**) Confluent, fourth-passage GC cells were treated with 10^{-10} to 10^{-8} *M* 1α,25-$(OH)_2D_3$ for 24 h. (**B**) GC cells were treated with 10^{-9} to 10^{-7} *M* 24R,25-$(OH)_2D_3$. Stathmin levels were determined by quantitative immunoblots of extracts of culture homogenates. Values are the mean ± SEM of six cultures. Data are from one of two experiments, each yielding comparable results. $*p < 0.05$, vs control plus vehicle (CV); $^{\#}p < 0.05$, vs 10^{-10} *M* 1α,25-$(OH)_2D_3$.

vitamin D_3 metabolite. This was a consistent observation based on analysis of T/C ratios of four separate experiments (Fig. 6B).

By contrast, PKC does mediate the effects of rhTGF-β1 on stathmin levels in RC cells (Fig. 7). Treatment of the cells with 0.88 ng/mL of rhTGF-β1 resulted in a 50% increase in basal levels of stathmin (Fig. 7A). Inhibition of PKC activity by treatment with chelerythrine for 3 d did not alter basal levels of stathmin, but it blocked the rhTGF-β1-dependent increase. This was a consistent observation based on analysis of T/C ratios of four separate experiments (Fig. 7B).

Discussion

This study demonstrates an association of stathmin with proliferation of growth plate chondrocytes and suggests that levels of this protein are regulated as a function of proliferation rather than differentiation. Previously we described the basal expression of stathmin protein in RC and GC cell cultures. In vitro, the less mature RC cells make about twice the amount of mRNA and protein as GC cells. This is similar to the proliferation rate of these cells in vitro and also to their respective states of endochondral maturation in vivo.

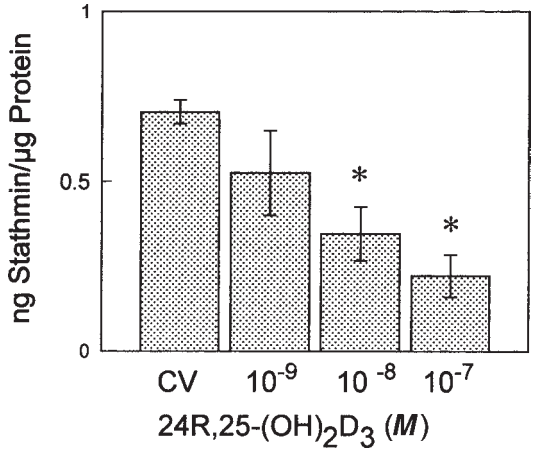

Fig. 3. Effect of treating RC cells for 4 d with 24R,25-$(OH)_2D_3$ on stathmin protein levels. Confluent, fourth-passage RC cells were treated with 10^{-9} to 10^{-7} *M* 24R,25-$(OH)_2D_3$. Extracts were prepared and then electrophoresed on 15% acrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. Values are the mean ± SEM of six cultures of which the data are derived from samples on membranes compared to a membrane with standards. Data are from one of two experiments, each yielding comparable results. *$p < 0.05$, vs control plus vehicle (CV).

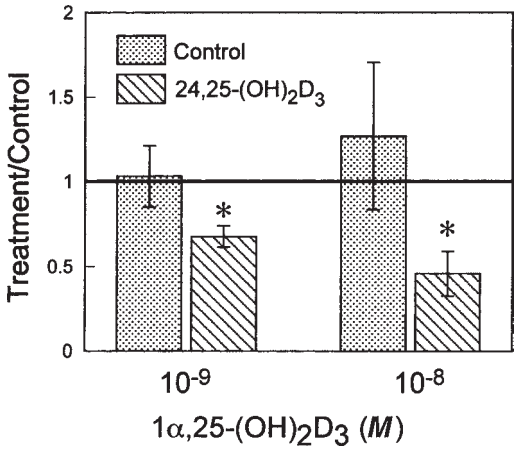

Fig. 4. Effect of treating RC cells with control media plus vehicle (CV) or 10^{-7} *M* 24R,25-$(OH)_2D_3$ for 4 d followed by 1 d of either CV or 10^{-9} or 10^{-8} *M* 1α,25-$(OH)_2D_3$ on stathmin protein levels. Extracts were prepared and then electrophoresed on 15% polyacrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. Values are the mean ± SEM of five the average T/C ratio of separate experiments of which the data for each experiment are derived from samples on membranes compared to a membrane with standards. *$p < 0.05$, vs 1.

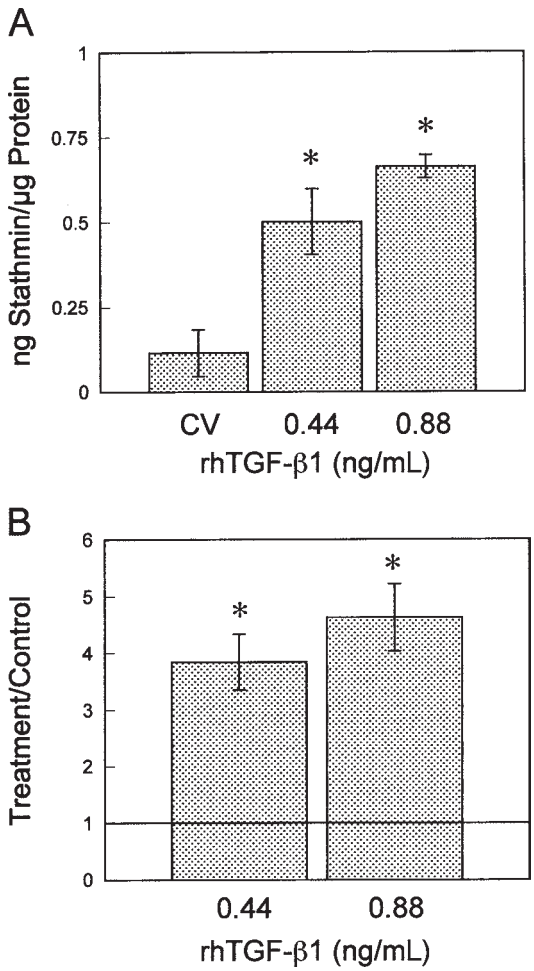

Fig. 5. Effect of treating RC cells with rhTGF-β1 on stathmin protein levels. Confluent, fourth-passage RC cells were treated with 0.44 or 0.88 ng/mL of rhTGF-β1 continuously for 72 h. Extracts were prepared and then electrophoresed on 15% polyacrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. (**A**) Data are from one of three experiments, each yielding comparable results. Values are the mean ± SEM of six cultures of which the data are derived from samples on membranes compared to a membrane with standards. *$p < 0.05$, vs control plus vehicle (CV). (**B**) T/C ratios of the average T/C for three separate experiments. *$p < 0.05$, vs 1.

The immunohistochemical results support the observation that stathmin is present in vivo as well as in vitro. In contrast to our in vitro analysis, however, the RC and GC chondrocytes appear to make the same amount of stathmin in vivo based on the relative visual intensity by immunohistochemistry. This is commensurate with the fact that neither cell population is proliferative in vivo, whereas the intervening cell population, the proliferating cells, exhibits higher levels of the protein.

Cell maturation may also play a role, to some extent, since staining for stathmin in the lower hypertrophic cell region appeared to be reduced. It is possible that the analytical methods used to measure stathmin mRNA and protein in RC and GC cell cultures were more sensitive than the immunohistochemical methods used in the present study *(28)*. It is also possible that the GC cells exhibit a more hypertrophic phenotype in culture than in vivo.

The effects of vitamin D_3 metabolites on stathmin protein levels are associated with their effects on proliferation, rather than differentiation of RC and GC cells. Previous work in our laboratory has demonstrated that exposure of

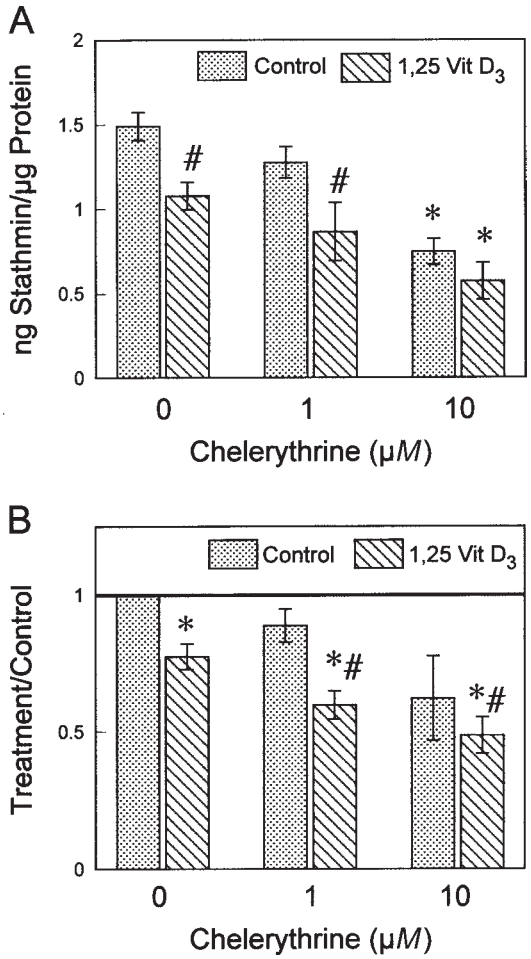

Fig. 6. Effect of treating RC cells with control media plus vehicle (CV) or 10^{-8} *M* 1α,25-$(OH)_2D_3$ (1,25) plus 0, 1, or 10 µ*M* chelerythrine on stathmin protein levels. Extracts were prepared and then electrophoresed on 15% polyacrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. (**A**) Data are from one of three experiments, each yielding comparable results. Values are the mean ± SEM of six cultures of which the data are derived from samples on membranes compared to a membrane with standards. $^*p < 0.05$, vs CV + 0 and CV + 1; $^{\#}p < 0.05$, vs CV + 10. (**B**) T/C ratios from three separate experiments. $^*p < 0.05$, vs 1; $^{\#}p < 0.05$, vs 1,25 + 0.

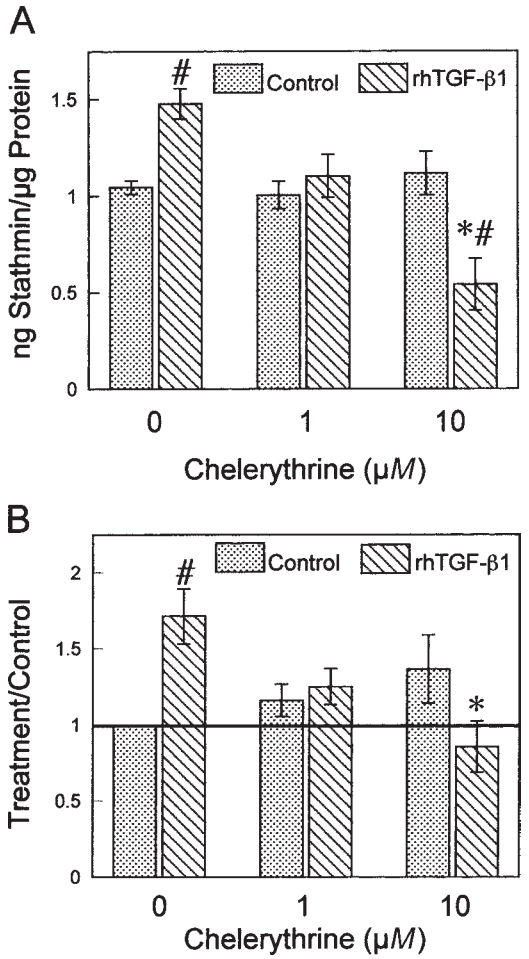

Fig. 7. Effect of treating RC cells with control media plus vehicle (CV) or 0.88 ng/mL of rhTGF-β1 plus 0, 1, or 10 µ*M* chelerythrine on stathmin protein levels. Extracts were prepared and then electrophoresed on 15% polyacrylamide gels. Densitometric analysis of the film produced from the immunoblotted chemiluminescent membrane was performed using NIH Image 1.61. (**A**) Data are from one of three experiments, each yielding comparable results. Values are the mean ± SEM of six cultures of which the data are derived from samples on membranes compared to a membrane with standards. $^*p < 0.05$, vs CV + 0, vs rhTGF-β1 + 0; $^{\#}p < 0.05$, vs CV + 10. (**B**) T/C ratios from three separate experiments. $^*p < 0.05$, vs rhTGF-β1 + 0; $^{\#}p < 0.05$, vs CV + 10.

confluent RC cells to 24R,25-$(OH)_2D_3$ for 24 h has no effect on proliferation, but the cells exhibit a more differentiated phenotype with respect to alkaline phosphatase specific activity *(34)*. By contrast, 1α,25-$(OH)_2D_3$ inhibits proliferation of RC cells but has no effect on alkaline phosphatase specific activity. GC cells respond to 1α,25-$(OH)_2D_3$ but not to 24R,25-$(OH)_2D_3$ with an increase in enzyme activity, whereas both vitamin D_3 metabolites decrease proliferation *(32,33)*. In the present study, RC cells exhibited a decrease in stathmin in response to 1α,25-$(OH)_2D_3$ but not 24R,25-$(OH)_2D_3$. GC cells exhibited decreased stathmin in response to both metabolites. This association of stathmin levels and proliferation of growth plate chondrocytes is further supported by the fact that RC cells treated with 24R,25-$(OH)_2D_3$ for 4 d exhibited a decrease in stathmin to levels similar to those seen in GC cells. Moreover, these cells responded to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ with a decrease in stathmin like that seen in GC cells treated with these metabolites of vitamin D_3. This supports the previous studies showing that prolonged exposure of RC cells to 24R,25-$(OH)_2D_3$ results in a shift to a GC-like phenotype with respect to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$.

In addition to these observations indicating an association of decreased stathmin levels with decreases in proliferation, we observed that increased stathmin levels were associated with increased proliferation. Both RC cell stathmin protein and proliferation were increased by treatment with rhTGF-β1. At the concentrations of rhTGF-β1 used (0.44 and 0.88

ng/mL), RC cells exhibited an increase in [^{35}S]-sulfate incorporation *(42)*, suggesting that matrix synthesis is affected as well. However, endochondral maturation does not occur. There is no effect on alkaline phosphatase specific activity nor is the phenotype of the cells shifted to GC-like *(35)*. Together with the response of the cells to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$, these results indicate that fluctuations in stathmin are associated with proliferation rather than differentiation in growth plate chondrocytes.

Our results support the observations of others that stathmin levels are mediated by PKC-dependent mechanisms. Inhibition of PKC with chelerythrine caused a dose-dependent decrease in control levels of stathmin. As anticipated, inhibition of PKC had no effect on the 1α,25-$(OH)_2D_3$-dependent decrease in stathmin levels. Repeated studies in our laboratory have failed to demonstrate that 1α,25-$(OH)_2D_3$ can stimulate PKC in RC cells, although it does so in GC cells and RC cells have membrane receptors for 1α,25-$(OH)_2D_3$ *(41)*. 1α,25-$(OH)_2D_3$ has also been shown to stimulate PKC in other cell types *(43–45)*. Nevertheless, our results indicate that the 1α,25-$(OH)_2D_3$-dependent decrease in stathmin in RC cells is by another mechanism. By contrast, the increase in stathmin owing to rhTGF-β1 does involve PKC, since inhibition of PKC blocked the stimulatory effect of the growth factor.

Stathmin levels in the RC cultures were time dependent, suggesting that factors present in the media might also play a role in regulating cell response. In confluent cultures treated for 24 h with control media, stathmin levels were maximal at 1.5 ng/μg of protein. This was reduced by 50% in cultures treated with 10 μ*M* chelerythrine. When cultures were treated with control media for 72 h, basal levels of stathmin were reduced to 1 ng/μg of protein and inhibition of PKC had no effect. Moreover, stimulation of the cells with rhTGF-β1 restored stathmin levels to those seen in control cultures at 24 h (1.5 ng/μg of protein). This suggests that depletion of regulatory factors in the conditioned media of the cells over time not only reduces proliferation *(46,47)* but also affects stathmin, again supporting the contention that the two phenomena are interrelated. rhTGF-β1 exerts its stimulatory effect by restoring at least one of the serum-derived regulatory factors.

Materials and Methods

Materials

Reagents were from Sigma (St. Louis, MO) unless otherwise noted. rhTGF-β1 was purchased from R&D Systems (Minneapolis, MN), diluted in phosphate-buffered saline (PBS) to 1 μg/mL, and stored at –20ºC. Chelerythrine was purchased from Calbiochem-Novabiochem (La Jolla, CA), diluted in ethanol to 1 m*M*, and stored at –20ºC. 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ were purchased from BIOMOL (Plymouth Meeting, PA), dissolved in ethanol to 10^{-3} *M* as a stock solution, and stored at –70ºC.

Stathmin Expression In Vivo

Immunohistochemistry was performed on specimens obtained from ribs removed by sharp dissection from male Sprague-Dawley rats (Charles River Breeding Labs, Wilmonton, MA) weighing 120 ± 20 g. Ribs were placed in plastic cassettes and fixed in 10% neutral buffered formalin (Statlab, Lewisville, TX) for at least 24 h. The specimens were washed for 5 min in tap water to remove excess fixative prior to decalcification for 1 wk in 0.5 *M* EDTA, pH 7.2. The decalcified specimens were washed with tap water for 10 min and then processed in an automatic tissue processor (LabTek VIP; Miles Scientific, Naperville, IL) for paraffin embedding. The resultant paraffin blocks were sectioned with a precision microtome (Reichert 2030 Biocut; Cambridge Instruments, Buffalo, NY) into single 4-μm sections, and the sections were placed on clean slides. Sections were dried overnight at room temperature, followed by heating in an oven at 60ºC for 15 min (minimum). After cooling for 1 min, sections were sequentially deparaffinized for 5 min each in xylene (three times), absolute ethyl alcohol (three times), 95% ethyl alcohol, 80% ethyl alcohol, and deionized water. The hydrated slides were placed into a prewarmed water bath at 100ºC containing a citrate buffer target retrieval solution (cat. no. S1699; Dako, Carpenteria, CA) and allowed to incubate for 20 min. These were removed from the water bath, cooled for 20 min, and rinsed three times in 10 m*M* Tris containing 0.9% NaCl and 0.05% Tween-20 (TTBS). Endogenous peroxidases were quenched with 0.1% sodium azide in 3% hydrogen peroxide in 10 m*M* Tris-buffered saline (TBS) for 5 min. These were then rinsed in TTBS two times and blocked with TBS and 10% bovine serum albumin (BSA) for 10 min. Specimens were diluted with TBS containing 10% BSA and 20 mg of sodium azide/mL.

A polyclonal antiserum against human stathmin was used to detect stathmin. This antibody was generated in rabbits to the peptide sequence NH_2-CASGQAFELILSPN-COOH and has been shown to recognize rat stathmin in Western blots *(2,28)*. Specimens were incubated with a 1:300 stathmin antibody solution ([v/v] antibody serum/diluent) overnight at room temperature. The specimens were rinsed, here and for all subsequent rinses between each step, by changing the TTBS and three times letting the specimens stand for 5 min. The specimens were incubated for 30 min in 1:300 (v/v) biotinylated swine antirabbit antibody solution (cat. no. E0353; Dako) and rinsed. These were incubated in a 1:100 (v/v) streptavidin-horseradish peroxidase (HRP) (cat. no. P397; Dako) solution (diluent, cat. no. HX157-5K; Biogenex, San Ramon, CA) for 30 min and then rinsed. The specimens were developed with 3,3' diaminobenzidine tetrahydrochloride (1 mg/mL in TTBS) and 1μL/mL of 30% hydrogen peroxide for 10 min. These specimens were rinsed in PBS, rinsed in deionized water, counterstained in 1% aqueous methyl green for 2 min, and rinsed in water.

The specimens were dehydrated, cleared with xylene, and mounted using Polymount (Poly Scientific, Bayshore, NY). Specimens were also stained with hematoxylin and eosin for comparison.

Cell Culture

Ribs were removed by sharp dissection from Sprague-Dawley rats (Charles River Breeding Labs) weighing 120 ± 20 g and placed in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Rockville, MD) until the costochondral cartilages could be removed. The costochondral resting zone and adjacent growth zone were carefully separated, discarding the intervening proliferating cell zone and the calcified cartilage. This technique takes advantage of the linear architecture of the growth plate and limits contamination with cells at other stages of differentiation/maturation. The tissue was washed two times for 20 min each in Hank's balanced salt solution (HBSS) (Gibco-BRL) containing 1% penicillin-streptomycin. Cartilage was sequentially digested with 1% trypsin (Gibco-BRL) for 1 h, and 0.02% collagenase (type II, Worthington, Freehold, NJ) for 3 h. Both enzymes were dissolved in HBSS (Gibco-BRL). Cells were separated from tissue debris by filtration through a 40-μm nylon cell strainer (Falcon, Franklin Lakes, NJ), followed by centrifugation of the filtrate at 500*g* for 5 min. Cell viability was 95%, as demonstrated by Trypan blue exclusion. Cells were plated in T-75 flasks at 10,000 cells/cm^2 for RC cells and 25,000 cells/cm^2 for GC cells. Incubation was conducted in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 50 μg/mL of sodium ascorbate (complete DMEM) in an atmosphere of 5% CO_2 at 37ºC and 100% humidity for 24 h. At the end of this time, culture media were replaced three times per week. At confluence, cells were subcultured to either T-75 or T-150 flasks using the same plating densities as before and allowed to return to confluence. Both cell populations take 5–8 d (7-d average) to reach confluence in primary culture and in each subculture. Fourth-passage cultures were used for these experiments because a number of studies have demonstrated that these cells retain their differential phenotype up to this number of passages in culture *(31,48,49)*.

For each experiment, confluent cultures of fourth-passage chondrocytes in T-150 or T-75 flasks were treated for the designated times with 20 (T-150) or 10 mL (T-75) of experimental complete DMEM (as already described) plus vehicle as a control or various concentrations of the test agent or agents, as indicated.

Regulation of Stathmin Protein Levels

Effect of 1α,25-(OH)$_2$D$_3$ and 24R,25-(OH)$_2$D$_3$

Previous studies have shown that 1α,25-$(OH)_2D_3$ inhibits proliferation of RC and GC cells, whereas 24R,25-$(OH)_2D_3$ inhibits proliferation of only GC cells *(32,33)*. By contrast, 1α,25-$(OH)_2D_3$ stimulates differentiation of GC cells and 24R,25-$(OH)_2D_3$ stimulates differentiation of RC cells. To assess the effects of these vitamin D metabolites on stathmin production, confluent fourth-passage cultures of RC and GC cells were treated with 10^{-10} to 10^{-8} *M* 1α,25-$(OH)_2D_3$ or 10^{-9} to 10^{-7} *M* 24R,25-$(OH)_2D_3$ for 24 h. Prior to adding the vitamin D_3 metabolites to the cells, the stock solutions were diluted 1:5000 (v/v) with culture medium and appropriate dilutions were made with culture medium. Cells were harvested by scraping, and protein extracts were prepared as described previously *(28)* and briefly described subsequently.

Exposure of RC cells to 24R,25-$(OH)_2D_3$ for periods longer than 36 h results in acquisition of responsiveness to 1α,25-$(OH)_2D_3$ and 24R,25-$(OH)_2D_3$ typical of GC phenotype *(34)*. To determine whether extended exposure to 24R,25-$(OH)_2D_3$ also affects stathmin protein levels, confluent cultures of RC cells were treated with 24R,25-$(OH)_2D_3$ for 1 or 4 d. For the 4-d experiments, culture media were changed at 48 h; thus, they received a second dose of 24R,25-$(OH)_2D_3$ at that time. To determine whether RC cultures become responsive to 1α,25-$(OH)_2D_3$ following an extended exposure to 24R,25-$(OH)_2D_3$, confluent RC cells were treated with 10^{-7} *M* 24R,25-$(OH)_2D_3$ for 4 d (media were changed at 48 h). At that time, the media were replaced with fresh control media or media containing either 10^{-8} *M* 1α,25-$(OH)_2D_3$ or 10^{-7} *M* 24R,25-$(OH)_2D_3$. Cells were harvested 24 h later.

Regulation of Stathmin Levels by rhTGF-β1

RC chondrocytes respond to 0.44 and 0.88 ng/mL of rhTGF-β1 with an increase in proliferation at 72 h *(35)*. These cells also exhibit an increase in proteoglycan production, suggesting that matrix synthesis is regulated by rhTGF-β1 as well. However, alkaline phosphatase activity is not affected by 0.44 or 0.88 ng/mL of rhTGF-β1, indicating that at these concentrations the cells are not stimulated to undergo endochondral maturation. To determine whether stathmin levels are also regulated, confluent fourth-passage RC cells were incubated for 72 h with 0.44 or 0.88 ng/mL of rhTGF-β1. The rhTGF-β1 stock was diluted into culture medium prior to addition to the cultures.

Role of PKC

1α,25-$(OH)_2D_3$ has been shown to exert its effects on costochondral chondrocytes via traditional nuclear receptor–mediated mechanisms as well as via rapid membrane-mediated mechanisms. Membrane-associated receptors for 1α,25-$(OH)_2D_3$ have been identified in both RC and GC cells *(41)*. However, they appear to be active only in GC cells with respect to activation of PKC. Moreover, 1α,25-$(OH)_2D_3$ mediates its effects on proliferation of GC cells via PKC. Similarly, rhTGF-β1 exerts its effects on proliferation of costochondral chondrocytes via a PKC-dependent mechanism. Since PKC plays a role in mediating these proliferation events and may play a role in mediating stathmin production, we examined whether PKC might play a

role in response to 1α,25-$(OH)_2D_3$ or rhTGF-β1. Confluent RC cells were treated for 24 h with control media containing 10^{-8} *M* 1α,25-$(OH)_2D_3$ or for 72 h with rhTGF-β1 in the presence and absence of the PKC inhibitor chelerythrine (1 and 10 μ*M*) as described previously *(50)*.

Stathmin Protein Levels

Cytosolic proteins were isolated from the chondrocyte cultures using a modification of the rapid scheme for purification of stathmin previously described *(28,51)*. This method provides concentrated protein fractions suitable for analysis by polyacrylamide gel electrophoresis (PAGE). After the cells were washed twice with cold PBS, cold homogenization buffer (10 m*M* Tris-HCl, pH 7.4; 0.02% NaN_3; 150 m*M* NaCl; 10 m*M* EDTA; 10 m*M* NaF; 15 m*M* $Na_4P_2O_7$; 25 μg/mL of aprotinin; 10 μg/mL of leupeptin; 10 μ/mL of pepstatin) was added and the cells were homogenized. The homogenate was centrifuged in a Beckman ultracentrifuge (Beckman, Palo Alto, CA) using an SW 50.1 rotor at 100,000*g* for 10 min. The pellet was discarded and the supernatant retained, incubated in a boiling water bath for 10 min, cooled, and centrifuged again at 100,000*g* for 10 min. The pellet was discarded, the supernatant was retained, and the samples were concentrated by dialysis for sodium dodecyl sulfate (SDS) PAGE analysis. The samples were then lyophilized and stored at 4ºC until reconstituted in 50 μL of PBS.

Protein concentrations were determined using BCA protein reagent kit no. 23225 (Pierce, Rockford, IL) with BSA as a standard. Samples were loaded using equal amounts of protein. Recombinant stathmin (RST) (Calbiochem, San Diego, CA) standards, molecular weight markers (SeeBlue; Novex, San Diego, CA), or samples from RC and GC cells (10 or 20 μg of protein) were diluted in 2X sample buffer (0.125 *M* Tris-HCl, pH 6.8; 4.1% SDS; 10% glycerol; 0.02% bromophenol blue; 2% 2-mercaptoethanol). Empty lanes received only 2X buffer. On the day of the experiment, an RST aliquot was thawed and rediluted with PBS to 1 ng/μL. The RST was diluted further in 2X sample buffer to concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, and 25 ng to establish a standard curve for the immunoblots. These samples or standards were heated to 100ºC for 5 min and electrophoresed on precast 15% polyacrylamide mini gels (Bio-Rad, Hercules, CA), according to the procedure of Laemmli *(52)*, at 100 V for 1.6 h on a Ready gel cell (Bio-Rad).

The polyclonal antiserum used to detect stathmin by immunohistochemistry was also used to detect stathmin on immunoblots as described previously *(28)*. Following SDS-PAGE, gels were equilibrated in Towbin transfer buffer (25 m*M* Tris; 192 m*M* glycine; 15% methanol; 0.05% SDS, pH 8.3) for 10 min. Transfer to a 0.2-μm Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) with the transfer buffer in a Mini Trans-Blot electrophoretic transfer cell wet transfer apparatus (Bio-Rad) was accomplished in 1.2 h at 100 V. All incubations or washes were performed on an orbital shaker at low-medium speed. Following transfer, the membranes were equilibrated in 0.05% Tween-20-PBS (T-PBS) for 15 min. Membranes were blocked for nonspecific interaction in membrane blocking solution (Zymed, South San Francisco, CA) for either 1 h at room temperature or overnight at 4ºC. Membranes were washed a minimum of three times for 15 min each in T-PBS. The antistathmin antiserum and goat-antirabbit enhanced chemiluminescence (ECL)-HRP conjugate (Amersham Pharmacia Biotech, Piscataway, NJ) were each diluted in the membrane blocking solution at 1:3000 (v/v). Following transfer, equilibration, and blocking, the membranes were washed a minimum of three times for 15 min each in T-PBS. They were then incubated with the diluted antistathmin antiserum solution for either 1 h at room temperature or overnight at 4ºC. Membranes were again washed a minimum of three times for 15 min each in T-PBS. The membranes were incubated with the secondary diluted goat-antirabbit ECL-HRP antibody solution for 1 h at room temperature. All membranes were washed again as just described. The membranes were then incubated for 1 min with the ECL Western blotting reagents (Amersham Pharmacia Biotech), blotted to remove excess liquid, placed in a protective plastic sleeve, placed in a film cassette with ECL Hyperfilm (Amersham Pharmacia Biotech), and developed in an automatic film processor. The resulting films were scanned in transparency mode to a TIF file and analyzed by NIH Image 1.61 software (NIH, Bethesda, MD) on a Power Macintosh 7200/120 (Apple, Cupertino, CA). Quantitative data from NIH Image and PhosphorImager™ data from ImageQuant™ software were analyzed with StatView 5.0.1 software (SAS, Cary, NC). The amount of immunoreactive protein was calculated based on the RST standard curve, as described previously *(28)*.

Statistical Analyses

The data presented are from a single experiment in which each variable is the mean ± SEM of six independent cultures. Significance between groups was determined by Bonferroni's post hoc analysis using $p < 0.05$ or a student's *t*-test $p < 0.05$. Each experiment was performed two or more times. In some cases, experiments were performed five times, and the results among experiments were compared statistically by determining T/C ratios in each individual experiment. The means ± SEM for the average T/C values from each experiment were then calculated and compared by Bonferroni's post hoc analysis using $p < 0.05$. Results are graphed using Deltagraph 4.05 software (SPSS, Chicago, IL).

Neither proliferation nor PKC activity was assessed directly in this study; thus, direct correlations of either parameter with stathmin levels cannot be made. Measurement of stathmin required use of the entire cell layer. Therefore, it would have been necessary to run parallel cultures for analysis of proliferation or PKC activity. These experiments used primary cultures and not cell lines; for each experi-

ment, 18 rats had to be killed. Because the data showing the effects of 1α,25-$(OH)_2D_3$, 24R,25-$(OH)_2D_3$, and rhTGF-β1 on both proliferation and PKC activity are published as already noted, the use of these additional animals was not adequately justified.

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