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Marrow Stromal Cell Commitment to Mineralization Under the Effect of a Prolyl Hydroxylase Inhibitor

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Abstract Mitochondrial response to the effect of a hydroxylase (PH) inhibitor was tested in marrow stromal cells during stimulation of osteoprogenitor cell (OPC) differentiation. The rationale for this was to explore pathways of regulatory interactions between procollagen synthesis and mitochondrial respiration that could be linked to the commitment of OPCs to mineralization. Stimulated OPCs exposed to the PH inhibitor cis-hydroxyproline (cis-HP), compared to the noninhibiting isomer trans-HP, for 11 days showed a dose-dependent decrease in cell proliferation, the surviving cells showed increased alkaline phosphatase activity. Trans-HP did not influence the cis-HP effect on ALP and on proliferation arrest. Short time exposures, 2–3 days, to cis-HP at different periods suggested that Days 0–3 and 3–5 were critical for the commitment to Day 21 mineralization of OPCs. On Days 0–3 cells were most sensitive to cis-HP, since on Day 11, 8 days after removal of cis-HP, they were too scarce to be counted by the staining method. However, the presence of 5.0 mM cis-HP in the cultures during Days 3–5 has induced on Day 21 close to 24-fold more mineralization/cell than controls, compared to the trans-HP effect, which was only close to 3-fold. The presence of cis-HP in the cultures on Days 0–3 has augmented the mitochondrial Day 3 retention of rhodamine 123 (Rho) in the stromal cells, relative to controls, compared to the presence of trans-HP. However, the presence of cis-HP during Days 3–5 or 3–6 resulted in lower Day 5 Rho retention, relative to controls, which was not significantly different from the retention that resulted from trans-HP. Since Rho retention is related to the final result of aerobic respiration level, these results are interpreted as a cis-HP inhibitory effect on procollagen peptidyl-proline hydroxylation, which may in turn release oxygen surpluses, to be available for mitochondrial consumption. The fall in Rho retention responses to cis-HP between Days 0–3 and 3–5 is suggesting either abrupt decrease in proline hydroxylation or poor mitochondrial sensitivity to oxygen in Day 3–5 cultures. © 1994 Wiley-Liss, Inc.

Key words: cis-hydroxyproline, rhodamine 123, mitochondria, rat bone marrow, dexamethasone, osteoprogenitor cells, differentiation

INTRODUCTION

The growth plate area in the epiphysis of growing bone constitutes a panoramic display of differentiation stages of mineralizing cells. The microenvironment of these skeletal cells becomes temporarily hypoxic in front of the provisional mineralization zone [Brighton and Heppenstall, 1971] and the redox state of these cells is changing accordingly [Shapiro et al., 1982]. In this growth plate hypoxic microenvironment, the chondrocytes rely on glycolysis as a less *efficient* source of energy. This metabolic alteration is characteristic of a differentiation stage in these cells, which seems to occur somewhere between the end of proliferative zone and the hypertrophic zone [Brighton et al., 1969] before the initiation of matrix maturation. Under aerobic conditions, the mitochondria are responsible for the more efficient energy metabolism. In addition, mitochondria participate in controlling the cellular calcium metabolism in both soft and hard tissues. In soft tissues, the mitochondria accumulates calcium by a metabolism dependent process [Carafoli and Sottocasa, 1984], but in skeletal cells there is probably an additional mechanism for calcium binding [Shapiro and Lee, 1975]. Calcium precipitation in the growth plate was found to be preceded by calcium efflux from mitochondria [Brighton and Hunt, 1978]. In addition to the ionized form of calcium, chondrocytic mitochondria accumulates calcium in a precipitated form as well [Lehninger, 1970],

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which is released when cartilage mineralization takes place. Although previous studies have established that calcium is extruded from skeletal cell mitochondria directly into the extracellular matrix, most of the effort has been invested in the study of matrix vesicle mineralization by extracellular calcium rather than cellular events preceding the formation of matrix vesicles [Wuthier, 1993]. The presumed link between transient hypoxia and mitochondrial involvement in the commitment of osteoprogenitor cells (OPCs) to mineralization is based on calcium fluxes across the mitochondrial membrane. Under anaerobic conditions, molecular oxygen is less available in the mitochondria to cooperate with oxidative phosphorylation and extrusion of NADH-derived protons. Accordingly, it has been found that, under anaerobic conditions, in the hypertrophic chondrocytes of the growth plate, the NADH/NAD ratio is elevated [Shapiro et al., 1982]. It was also found that a lower cytosolic pH and accumulation of phosphoenol pyruvate, both connected to the glycolitic anaerobic metabolism, are associated with calcium release from chondrocytic mytochondria [Shapiro and Lee, 1978].

The hypoxic window in the growth plate may be regarded as the result of oxygen deprivation caused by a diffusion barrier, possibly due to a diffusion-resistant extracellular matrix and scarce vascularization. This is the main change in oxygen tension, which presumably brings its intracellular concentration to a new low steady-state level. We surmise that the gross drop in $[O_2]$ makes the mitochondria more sensitive to oxygen consumption by other organelles. We recently found that marrow stromal cell-derived OPCs in culture also undergo a stage with characteristics of anaerobic metabolism during differentiation. Based on this change in energy metaboliosm, OPCs were enriched [Klein et al., 1993a] by inhibiting the mitochondrial enzyme succinate dehydrogenase (SDH), since they were already under unaerobic metabolism and therefore became resistant to malonate. Oxygen availability to the mitochondria, specifically to cytochrome oxidase, is the way by which aerobic respiration is generally regulated [Brown, 1992]. Contrary to the growth plate situation, oxygen supply to mitochondria in the stromal cell culture is less likely to be disturbed by an extracellular matrix (ECM) diffusion barrier and is not dependent on vascularization. Therefore, we assume that an intracellular mechanism is participating in changing oxygen availability to the mitochondria during OPC differentiation. ECM maturation was suggested to regulate osteoblastic proliferation by a negative feedback [Stein et al., 1990; Lian et al., 1991]. ECM is an oxygen consumer, particularly chondroitin sulfate in young cartilage [reviewed by Scott, 1992], but this requires aging of the ECM. More immediate oxygen utilization occurs during collagen synthesis upon cotranslational hydroxylation of prolines. According to Kao et al. [1979], the rate of procollagen secretion from cultured chick tendon fibroblast is 2 nmol/10^9 cells/h; this should use 936 nmol O_2/10^9 cells/60 min, based on 13% of the amino acids of the triple helix that are hydroxylated (of a total 3,600 amino acids). Suppose that the amount of O_2 used/min for procollagen hydroxylation, 15.6 nmol/10^9 cells, is allowed to establish the cellular oxygen concentration, i.e., 15.6 μM, and note that O_2 levels are heterogeneous in various organs, from <1 to 90 μM [Brown, 1992]. This makes it likely that mitochondria will be sensitive to the procollagen hydroxylation in the ER. In this paper we describe the effect of a prolyl hydroxylase inhibitor, cis-HP, on marrow stromal OPC differentiation, and on early commitment to mineralization. We also demonstrate that during an important time point for the comitment to mineralization a change in mitochondrial response to cis-HP occurs.

MATERIALS AND METHODS

Reagents

Cis- and trans-hydroxyproline, ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate and rhodamine 123 were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company, Grand Island, NY.

Stromal Cell Culture

Bone marrow cells obtained from femora and tibiae of female Sabra rats, weighing 60–80 g, were cultured as described by Maniatopoulos at al. [1988], removing the nonadherent hematopoietic cells during the first 10 days of culture. Adherent stromal cells were cultured in DMEM (Dulbacco Modified Eagle's Medium) supplemented with 15% fetal calf serum and antibiotics, in a humid 10% CO_2 atmosphere. For the experimental cultures stromal cells were removed 2 weeks later by trypsinization and were

plated in 96-well microtiter plates, 10,000 cells/ cm^2 and grown in osteoprogenitor cell (OPC) stimulation medium (DEX medium). This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate and 10 mM β glycerophosphate. The medium was changed every 3–4 days.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 or 12 of dexamethasone stimulation was set for ALP assay as described before [Klein et al., 1993b]. Briefly, after medium removal cells were washed twice in situ with 0.2 ml TNC (50 mM Tris, 150 mM NaCl pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TNC, 1.33 mg/ml was dispensed, 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min, and optical density of the hydrolysed pNPP was measured in a multichannel spectrophotometer at 405-nm wavelength. ALP specific activity was expressed either as nmol/time/cell quantity or as an activity index or percentage of control (experimental/control specific ALP ratio).

Rhodamine Retention Measurement

Rhodamine 123 (Rho) stock solution ×20 in distilled water was added to the cultures, 10 μl/well, after an incubation period as indicated; the Rho-containing medium was removed. The cells were washed twice with TNC (50 mM Tris pH 7.6, 150 mM NaCl) and continued their cultivation after the short incubation period until the Rho retention measurement was performed. To measure Rho retention on the following day the growth medium was removed and cells were washed in situ with TNC. The cells were then incubated for 30 min at room temperature with 150 μl of 0.2% Triton-X 100 to dissolve membranes and to release intracellular Rho. The disolved cell sap was transferred to an opaque-white 96 well plate. Rho content was measured in a Perkin Elmer LS-5B luminescence spectrometer with a plate reader, or in cuvettes, by exitation at 505 nm and emission at 540 nm.

Quantitative Cell Staining

After the ALP or the rhodamine retention assay, cells at the lower half of the plates were stained using the methylene blue (MB) staining method [Goldman and Bar-Shavit, 1979]. Cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O and air-dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water and air dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. The O.D. of the eluted MB was measured at 620 nm by a multichannel spectrophotometer; 1.0 unit is equivalent to 5×10^4 stromal cells.

Measurement of In Vitro Precipitated Calcium

After 3 weeks in culture, OPC stimulation medium, as opposed to ordinary medium, induces cell-mediated calcifications in uninhibited cultures [Klein et al., 1993a,b]. To quantitate these precipitates, plates were washed twice with TNC and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations. The precipitates were expressed as g Ca/well and presented as indices of experimental to control ratios.

RESULTS

To examine the differential effect of HP isomers on stromal cell proliferation and on OPC differentiation, isomers were added to stromal cells cultured in OPC stimulation medium.

Dose Effect of HP Isomers on Cell Count and ALP

Figure 1 shows the dose response of Day 11 cell count (Fig. 1A) and specific ALP activity (Fig. 1B) to 4 doses of HP isomers, which were present in culture 11 days. The effect of trans-HP was similar to that of the control OPC stimulation medium alone for both cell count and specific ALP activity at all concentrations. In contrast, cis-HP, above 0.1 mM, decreased the cell count and increased specific ALP activity in a dose-dependent manner. The 1:1 mixture of cis + trans-HP showed a slightly lower but similar effect to that of cis-HP alone. However, it should be noted that in the mixture of cis with trans-HP, each isomer represented one-half the molarity of the corresponding separately administered isomer for each concentration point. Thus, the total molarity of the cis + trans-HP

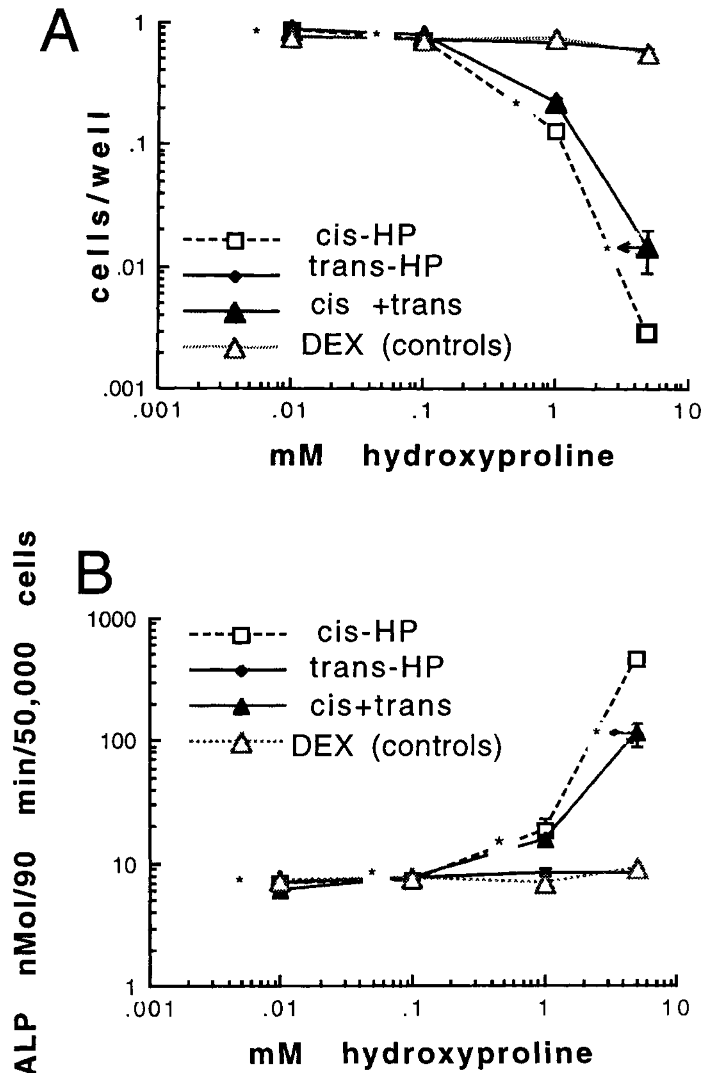

Fig. 1. HP isomers dose effect on stromal cell proliferation and on ALP activity. HP isomers were added, days 0–11, to marrow stromal cells cultures stimulated with DEX medium. On Day 11 cells were counted by MB staining (**A**), and ALP activity was measured in parallel wells (**B**). Each point represents the mean ±SE response to the respective HP concentration, n = 10. Asterisk denotes the hypothetical location of cis + trans-HP if each of the isomers concentration would have been considered separately. Arrows at 5.0 mM marking points of total HP (cis + trans), which ignores separate isomer concentrations, indicate the shift to appropriate concentrations of separate isomers (*), only for this curve (closed triangles). Cells are expressed by O.D. units, 1.0 unit = 50,000 cells.

disregards the existance of the two isomers. Upon shifting the cis + trans-HP points to the left toward the positions that reflect the corresponding separate isomer concentrations (arrow from closed triangle to asterisk, Fig. 1); the new hypothetical curves obtained, represented by the asterisks, coalesce with the cis-HP curve. This demonstrates that the cis + trans-HP effect was similar to that of cis-HP for both cell count and ALP activity. It is thus clear that under these experimental conditions cis-HP preferentially decreases proliferation of non-OPCs in stimulated cultures relatively to controls, whereas trans-HP does not substantially affect, nor does it interfere with, the ability of cis-HP to affect these functions.

The cell count values were inversely related to the specific ALP values, as demonstrated by the logarithmic regression line with a high correlation, r = −0.878. (Fig. 2). This is consistent with the coupling between decreased proliferation rate and onset of differentiation [Stein et al., 1990].

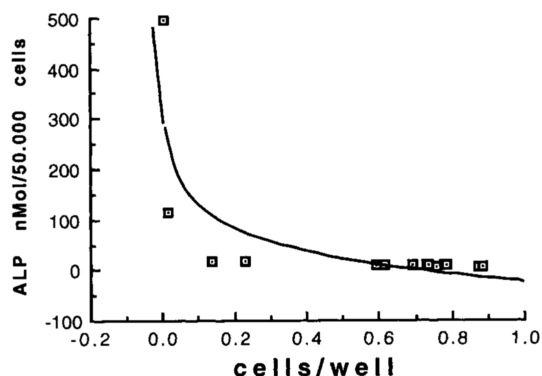

Fig. 2. Correlation between cell counts and specific ALP activities under different HP isomer concentrations. Logarithmic regression computed for mean result pairs (cell count/well versus ALP activity/cell/well), n = 10 for each point, r = −0.878.

Dose Effect of HP Isomers on Day 21 Mineralization

Figure 3 shows the absolute (A) and the relative to control (B) effect of HP isomers on cell mediated mineralization in culture. Regarding the absolute quantity of calcium/well there was no significant difference between effects of cis and trans-HP on Day 21 mineralization, except for a small decrease in calcium under 5.0 mM trans-HP (Fig. 3B). However, if Day 21 calcium quantity were related to Day-11 cell count (taken from Fig. 1B), at 1.0 mM concentration cis-HP should have increased Day 21 mineralization, relative to trans-HP, by one order of magnitude and at 5.0 mM by more then two orders of magnitude. This is based on the assumption that Day 11 cells, committed to mineralize in the cis-HP cultures, were represented 10 days later, among the Day 21 cell populations, similarly to the cells in trans-HP cultures.

The control curve under OPC stimulation medium alone, although persistently lower than the HP-induced curves, followed the trend of these experimental curves (Fig. 3A). For each HP isomer concentration, the three-sample replica of HP and their own DEX controls were cultures in a common plate. Therefore, the shape of the curve indicates a possible effect of the atmosphere of the experimental wells upon the control wells in each plate. The increase in mineralization in the control wells parallel to the experimental wells must have been resulted from a volatile product released under a dose-dependent HP effect on cells in neighboring experimental wells. To determine whether the effect of cis-HP might be related to its inhibitory effect on hydroxylases of collagen, a different prolyl hydroxylase inhibitor, protocatechuate [Kivirikko and Myllyla, 1987], was used.

Dose Effect of Protocatechuate on Stromal Proliferation and OPC Differentiation

Protocatechuate was added to stromal cells cultured in OPC stimulation medium for the duration and concentrations equimolar to those of HP shown in Figure 1. Figure 4 shows Day 11 ALP activity, cell counts, and mineralization responses to protocatechuate doses. The mineralization response extent was similar to that of cis-HP. The pattern of cell proliferation and specific ALP activity were also similar to those caused by cis-HP. Thus, the inhibitory effect on the cotranslational hydroxylation of procollagen amino acids (mainly proline) selectively enriched the stromal cell population with OPCs by decreasing the non-OPCs.

Delineation of Early HP Isomer Effect on Stromal OPC Differentiation

To delineatate the period during stimulated differntiation of stromal cells in which cis-HP affects stromal OPC and non-OPC proliferation, HP isomers were introduced for different periods between Day 0 and 11 into the cultures. Day 11 cell count and ALP activity test were performed. Figure 5 shows differential effects of cis versus trans-HP on Day 11 specific ALP, Day 11 cell counts and also Day 21 cell mediated mineralization (calcium). Among the short exposures, the period of days 3–5 resulted in selected cells with the highest specific ALP, 166% of controls, compared with 99.9% of controls obtained by exposure to trans-HP. The highest effect on mineralization was detected on days 0–3, where it cannot be related to cell number since they were too scarce to count. A substantial effect on mineralization was also seen on days 3–5. This indicates that days 0–5 is the period during which most of the non-OPCs proliferate. Later, from days 6–11, specific ALP activity of the cis-HP cultures came closer to that of the trans-HP cultures and the cell counts remained low relative to the trans cultures. This indicates that at the later stage more of the OPCs were proliferating and synthesizing collagen in the control cultures, in such a way as to become a principal target for cis-HP inhibition between

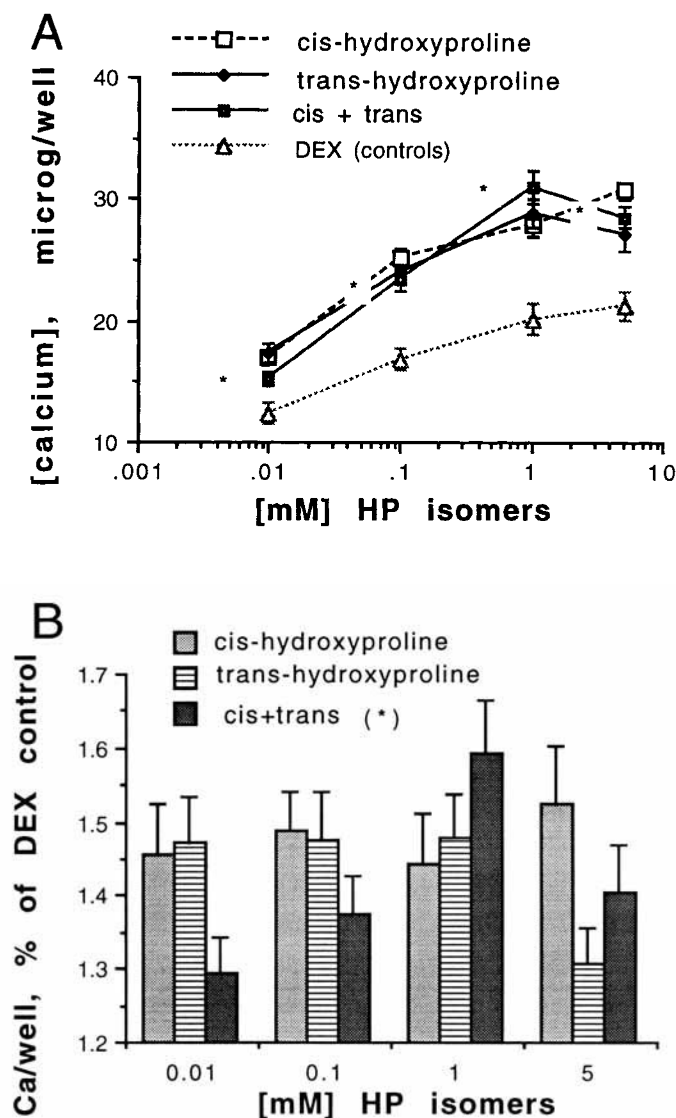

Fig. 3. HP isomers dose effect on stromal OPCs mineralization. Different doses of HP isomers were added to DEX-stimulated marrow stromal cells (as described in Fig. 1). On day 11, HP was removed from all cultures, and cultivation was continued in DEX medium until Day 21, when mineralization was measured. **A:** DEX control curve separated from the HP curves. **B:** Results are presented relative to controls. Asterisk indicates a dual option for assigning concentration to the mixed HP curve, either general HP or separate isomers (as in Fig. 1).

Days 6–11. Contrary to periods between Days 6 to 11, on days 3–5 trans-HP resulted in increased mineralization relative to controls, although less than in the cis cultures. Continuous exposure to cis-HP on days 3–11 resulted in high (416.8% of control) specific ALP activity of a residual cell population, 3.3% of the control. It is clear that exposure to cis-HP during days 3–11 resulted in a most partial OPCs enrichment of the cultures. Only 416.8% increase in specific ALP activity was found in the residual 3.3% cell population, versus an expected increase of 3,000%.

Can cis-HP Augment Differentiated OPCs in the Absence of Dexamethasone?

Table I shows the effect of cis-HP, present in stromal cell cultures during days 3–5, on specific ALP activity and cell proliferation in the abscence of DEX. $DEX^{(-)}$ medium, containing only ascorbate and β-glycerophosphate, was added on Day 0 to the stromal cell cultures. HP iso-

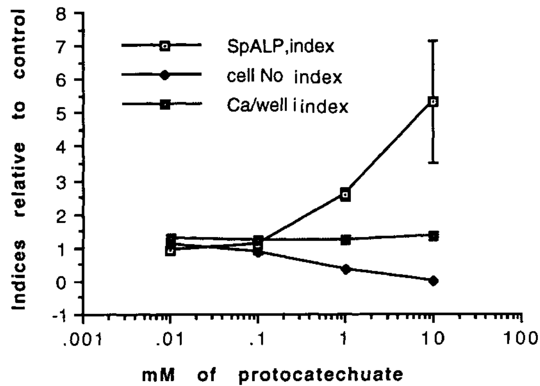

Fig. 4. Protocatechuic acid dose effect on OPC differentiation. Different concentrations of protocatechuate were added to stromal cells on Day 0 of DEX-induced OPC differentiation and maintained up to Day 11. Specific ALP activity (SpALP) and cell counts (cell No) were performed on Day 11 for parallel wells. Similar plates were cultured beyond Day 11 after removal of protocatechuate and maintenance in DEX medium up to Day 21 when mineralization was measured (Ca/well). Mean ±SE for each point (n = 10) is expressed as an index relative to DEX controls.

mers were added to the cultures on Day 3 and removed on Day 5. Under cis-HP Day 11 specific ALP activity augmented to 134.5% ±25.9% above $DEX^{(-)}$ control cultures, whereas under trans-HP they were 93.6% ±13.5% of controls. The cell count under cis-HP was 8.1% of controls compared to 149.3% under trans-HP. The gap between cis and trans-HP induced cell counts, greatly differs from the gap obtained under a similar experimental protocol but with $DEX^{(+)}$ medium (Fig. 5, 3rd and 4th lines). This indicates that DEX is required to expand the initial population of ALP expressing cells and cis-HP causes a relative enrichmant of the ALP expressing cells/well.

Effect of Days 3–5 HP Isomers on Specific Day 21 Mineralization

Stromal cells stimulated with $DEX^{(+)}$ medium were exposed on days 3–5 to HP isomers, Day 21 specific mineralization was 2,543.7% of controls and under trans-HP it was 294.9% (Table II). The cell count under cis-HP was 87% of controls, only about twice in magnitude of the 41.7% from Day 11 (see Fig. 5, 3rd line). However, the specific mineralization, 2543.7% of controls, was substantially high indicating that cis-HP, in addition to arresting cell proliferation, stimulated the commitment to mineralization in the DEX-recruited OPCs.

Effect of HP Isomers on Mitochondrial Respiration

Mitochondrial functioning under aerobic condition can be indirectly estimated according to the level that its inner membrane potential is maintained. This can be measured by mitochondrial retention of rhodamine 123 (Rho) [Chen, 1989]. Since prolonged incubation time of cells with Rho can increase cell proliferation (unpublished), DEX stimulated stromal cells were incubated with 3 Rho concentrations to determine an appropriate incubation period without significant effect on cell proliferation. Figure 6 shows the effect of one long and two short Day 2 Rho incubation periods on Day 3 cell proliferation and specific Rho retention for 3 different Rho concentrations. Due to the minimal effect of 1.0 μg/ml Rho, for 2-h incubation, on the relative cell count and Rho retention under DEX, it was chosen for use under these conditions.

Stromal cells were stimulated with DEX on Day 0, and the cultures were challenged with HP isomers either on Days 0–3 or on Days 3–5, 3–6, and 3–7 all after Day 3. (Day 3 is important for the commitment to mineralization [Klein et al., 1993a].) Cultures were incubated with Rho 1 day prior to cell harvesting, 24 h later, cells were counted and lysed for Rho quantitation. It should be noticed that the above presented Figure 3A suggested a dose-dependent remote influence by the experimental wells upon the controls, transmitted via gas diffusion, presumably oxygen. Therefore the following experiment was performed in two types of tissue culture vessels which differ by their aeration capacity and oxygen diffusion due to differences in the medium depth (Freshney 1988). Figure 7 shows the effect of cis-HP on stromal cell mitochondrial retention of Rho during the first week of OPC differentiation. The highest relative Rho retention (expressed as % of DEX controls) was induced by cis-HP, as compared to trans-HP, during the first 3 days of DEX-induced differentiation. This effect was more prominent in the microtiter wells (close to 1000% of DEX controls, Fig. 7A) than in flat dishes (80% of DEX control, Fig. 7B). These scale differences in the cis-HP effect were probably due to slower gas exchange between the incubator atmosphere and the cytoplasm across the medium-depth in the microtiter wells, which was 3-fold deeper than that of the dishes.

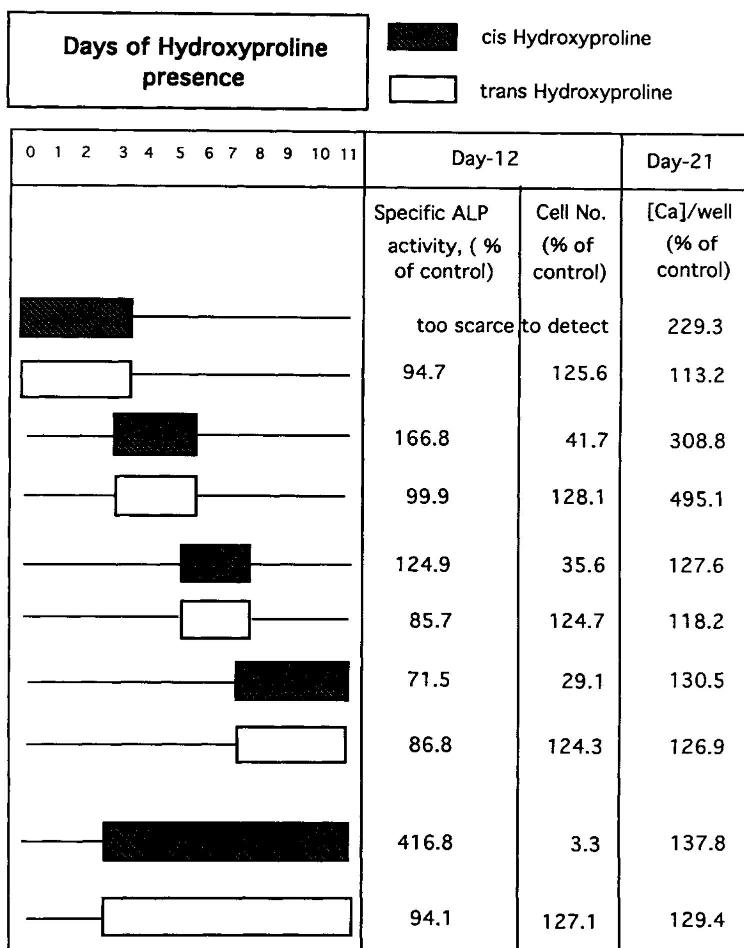

Fig. 5. Dissection of HP isomer effect on OPC differentiation during the first 12 days of DEX stimulation. Stromal cells were stimulated with DEX in multiple sets of microtiter plates. Cis or trans-HP were added to the cultures for restricted time intervals. For each exposure protocol specific ALP and cell counts were performed on Day 12, and a set of plate continued propagation in culture from Day 12 to 21, with DEX medium for quantitation of cell-mediated mineralization. Results are presented as mean % of DEX controls (n = 10).

DISCUSSION

In the present work cis hydroxyproline, unlike trans hydroxyproline, inhibited stromal cell proliferation resulting in less cells with higher ALP activity on Day 11. Cis-HP was shown to arrest proliferation of fibroblasts (Kao and Prockop, 1977) and connective tissue cells but not their transformed counterparts [Liotta et al., 1978] due to inhibition of collagen secretion. Also, coating plastic dishes with collagen was

TABLE I. Effect, on Day 11 ALP, of Days 3–5 HP Isomer Administration to Stromal Cells Cultured in DEX$^{(-)}$ Medium

HP isomer[a]	Specific ALP activity[b]	Specific ALP index[c]	Cell count[d]	Cell count index
Cis	25.926 ± 2.396	1.345 ± 0.259	0.0315 ± 0.0021	0.081 ± 0.013
Trans	21.371 ± 1.968	0.936 ± 0.135	0.482 ± 0.075	1.493 ± 0.275

[a]N = 10 per isomer.
[b]Expressed as nmol/50,000 cells/90 min.
[c]Mean ±SE related to DEX$^{(-)}$ controls.
[d]1.0 units = 50,000 cells.

TABLE II. Effect, on Day 21, of Day 3–5 HP Isomers Administration to Stromal Cells Cultured in DEX Medium

HP isomer[a]	Specific[b] [Ca]	[Ca] index[c]	Cell count[d]	Cell index
Cis	13.154 ± 0.954	24.537 ± 4.372	1.495 ± 0.045	0.870 ± 0.039
Trans	0.934 ± 0.247	2.944 ± 1.017	1.879 ± 0.055	1.122 ± 0.057

[a]N = 10 per isomer.
[b]Expressed as μg/well/50,000 cells.
[c]Mean ±SE related to DEX controls.
[d]1.0 unit = 50,000 cells.

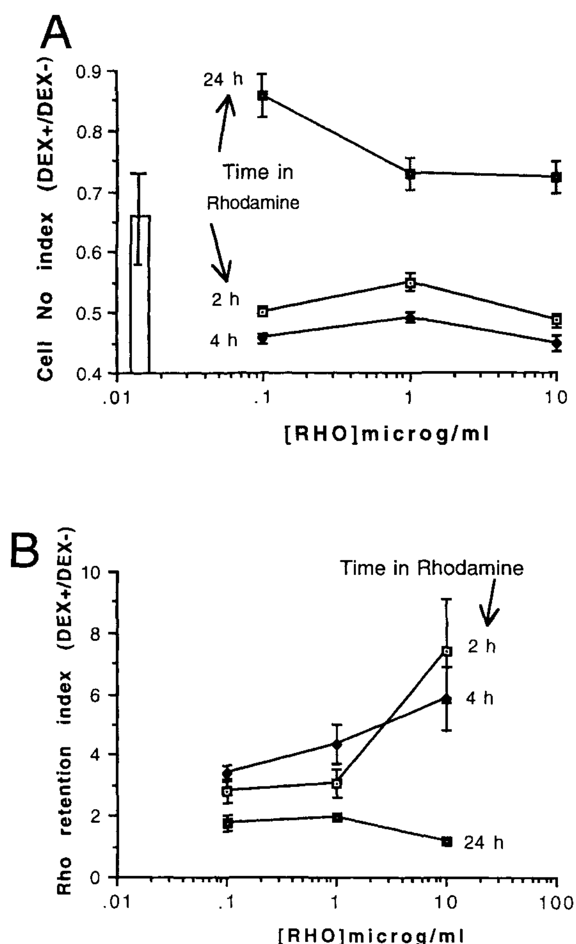

Fig. 6. Incubation-time and dose-effect of rhodamine on stromal cell proliferation and rhodamine retention. DEX stimulated and nonstimulated stromal cells were cultured in microtiter plates. On Day 2 of stimulation, the cells were incubated with different Rho concentrations for different time intervals. On Day 3 the cells were counted by MB staining and cells from parallel wells were extracted for Rho fluorometry. **A:** Cell count dose responses within different incubation time curves (n = 10) expressed as DEX^+/DEX^- index, the bar on the left is the mean ±SE of the proliferation index between DEX and ordinary medium without Rho incubation (n = 20). **B:** Mean ±SE of specific (per cell) Rho retention, expressed as an index between DEX^+ and DEX^- media, n = 10 for each point.

shown to prevent proliferation arrest of cis-HP-treated cells. It is believed that collagen synthesis and secretion can regulate cell proliferation and differentiation, but the precise sequence of events behind the mechanism is not known. However, presence of collagen on the plastic surface seemed inadequate to allow cells to proliferate in our experiments. Note that in the present study, Day 0 was always 3 days after cell seeding attaining subconfluence before collagen synthesis was arrested, yet cis-HP has induced arrest of cell proliferation. Thus, there must be other pathways for cis-HP to stop cell proliferation through hydroxylation inhibition. The specific inhibition of prolyl hydroxylase by cis-HP results in unstable procollagen triple helix [Jimenes and Rosenbloom, 1974], and some of the cis-HP molecules become incorporated into the collagen peptide [Rosenbloom and Prockop, 1971]. Contrarily, trans-HP does not inhibit proline hydroxylation and is not incorporated into the collagen peptide [Rosenbloom, 1971]. In the present studies, trans-HP did not arrest and, in some cases, it has even slightly increased, cell proliferation (Fig. 5). In the cultures under continuous presence of 5.0 mM cis-HP a small fraction of the cells with high ALP activity remained on Day 11 (Fig. 1); therefore, some of the OPCs must be resistant, more than the non-OPCs, to cis-HP. This means that the HP isomers differ by the ability (of cis-HP) to select some ALP expressing cells. Similar ALP and cell count results were obtained (Fig. 4) with 3,4 Dihydroxybenzoate, another prolyl hydroxylase-inhibitor (protocatechuate [Kivirikko and Myllyla, 1987]). Based on these results, inhibition of cotranslational hydroxylation of collagen is an important step in selection of ALP expressing cells. It is possible that all collagen-secreting cis-HP-treated stromal cells undergo proliferation arrest, including ALP-expressing OPCs, but stromal fibroblasts cannot

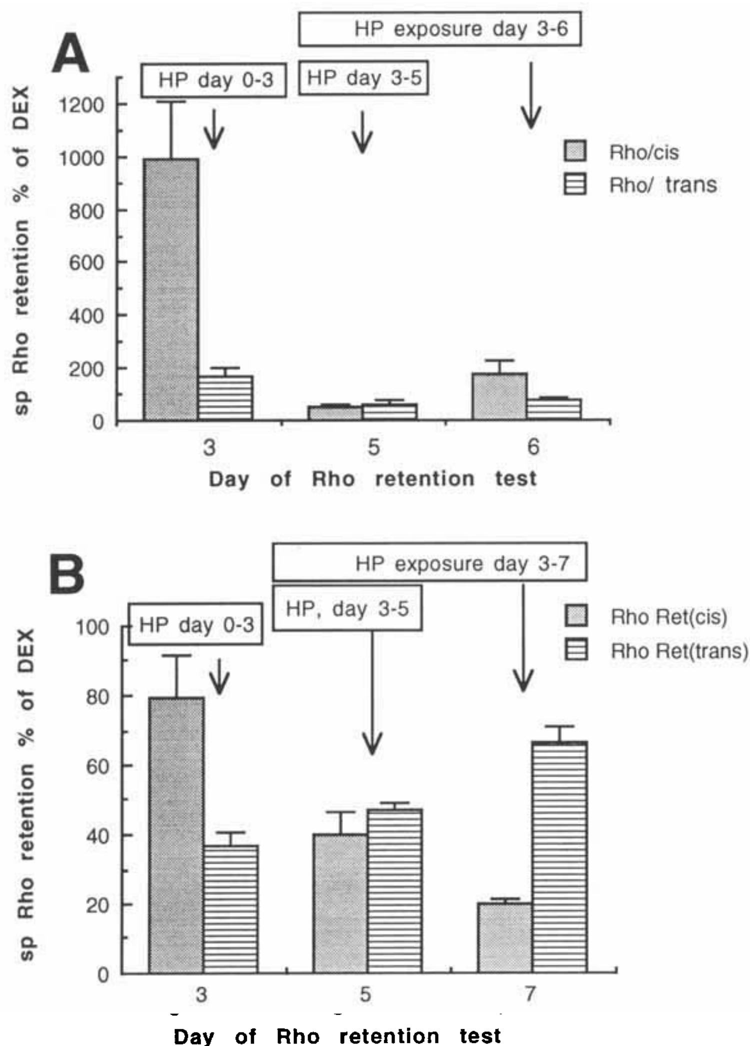

Fig. 7. Differential effect of HP isomers on stromal cell retention of Rho before and after Day 3 of DEX stimulation. Sets of DEX stimulated stromal cell cultures either in microtiter plates (**A**) or in 30-mm plastic dishes (**B**) were propagated with cis or trans-HP during the indicated periods. Rho 1.0 μg/ml was added for 2 hours and 24 h after reintroducing the same medium cells were counted by MB staining (A) or by microscopy (B). Parallel wells were extracted in situ (A) or extracted, after trypsinization and counting (B), with 0.2% Triton X-100. Rho was quantitated as Arbitrary fluorescence units using a fluorometer with plate reader (A) or with plastic cuvettes (B). Specific Rho retention was calculated per cell and expressed as an index relative to DEX-stimulated controls.

survive it, whereas OPCs continue their differentiation under this condition. Advancing differentiation upon proliferation arrest is consistent with the inverted correlation between proliferation and differentiation shown in Figure 2, a mechanism for these two reciprocal activities in the case of bone cells has been recently suggested [Stein et al., 1990; Lian et al., 1991].

The effect of HP isomers appeared less differential on mineralization than on ALP since in Figure 3 cis- and trans-HP showed similar results. However, upon dissection of the differentiation period into shorter intervals (Fig. 5), days 0–5 were discernible as the period of commitment to mineralization, especially days 3–5 where cis-HP seemed more effective than trans-HP. Two additional observations support this as an important time interval. First, malonate selection of OPCs was shown most efficient when started from Day 3 [Klein et al., 1993a]. Second, levamisole, which was shown to inhibit the commitment to mineralization if initiated during the first week in culture [Klein et al., 1993b], has slightly lost its inhibitory capacity, when started on Day 3. In the present study the effect of cis-HP was shown to be superior to that of trans-HP by expressing mineralization/cell rather than on the per well basis (Table II).

Trans-HP has substantially increased mineralization relative to the DEX control, but cis-HP increased it much more. It is thus likely that in addition to induction of OPC differentiation and to relative enrichment of OPCs in culture cis-HP has also positively affected the commitments to mineralization of the induced and selected OPCs. Based on repeated past observations and on the shape of the DEX control curve shown in Figure 3A, OPC differentiation or at least commitment to mineralization must be related and also sensitive to volatile matter. It is known that oxygen tension plays a role in controlling bone growth or at least its mineralization [Brighton and Happenstall, 1971; Shapiro et al., 1982; Deren et al., 1990]. It was therefore interesting to test the effect of cis-HP on an oxygen-dependent function within the stromal cells in two tissue culture vessels that differed by medium depth. The abrupt change in mitochondrial membrane potential between days 3 and 5 induced by cis-HP/control versus trans-HP/control is suggesting that until Day 3 cis-HP had an available enzymatic target reaction to inhibit; this reaction faded by Day 5, or the mitochondria were much less responsive on Day 5 than on Day 3. Based on these results and according to the former possibility, we hypothesize that during days 0–3 prolyl hydroxylase is active in cotranslational hydroxylation of procollagen prolines utilizing a substantial amount of oxygen and α-ketoglutarate (KG) while a molar equivalent of succinate is released [reviewed by Adams, 1980]. Cis- but not trans-HP, inhibited utilization of oxygen, making it available to mitochondrial respiration; this permitted a relatively high Rho retention under cis, compared to Rho retention under trans-HP. However, on Day 5 cis-HP became futile for oxygen diversion from the endoplasmic reticulum (ER) to the mitochondria because proline hydroxylation has diminished due to other cellular regulatory mechanisms. We therefore hypothesize that one of the ways by which collagen synthesis is coupled to cell proliferation in general and to bone cell commitment to mineralization is by a prolyl hydroxylase-dependent "oxygen shuffling." We cannot rule out the possibility of a change in sensitivity in OPCs mitochondria to oxygen between Day 3 and Day 5. The results of this study seem to reveal only the tip of the "iceberg" of a complex biochemical regulatory set of interactions, some of them more general and others perhaps more specific for calcified tissues. One of several interesting questions these results raise is how the β-subunit of PH is reacting to the cis-HP-mediated inhibition of the α-subunit. Such a question should be examined in the light of additional potential catalytic functions of the β-subunit. For example, since it is identical to disulfide isomerase [Pihlajaniemi et al., 1987], it might influence the sulfation of glucosaminoglycans, which participate in the structure of the ECM and thus constitute another regulatory component in the commitment to mineralization.

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