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Polyamine and differentiation: Induction of ornithine decarboxylase by parathyroid hormone is a good marker of differentiated chondrocytes

(glycosaminoglycan biosynthesis/de-differentiation by vitamin A)

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ABSTRACT **The activity of ornithine decarboxylase (ODCase; L-ornithine carboxy-lyase, EC 4.1.1.17) in rabbit costal chondrocytes in culture increased markedly after addition of parathyroid hormone (PTH), reaching a maximum 4 to 5 hr after PTH addition. The increase in ODCase activity was followed by increase in the intracellular concentrations of polyamines, especially putrescine, which increased in 6 hr to about 3-fold that of untreated cultures. The induction of ODCase by PTH was not observed in L, 3T3, HeLa, buffalo rat liver, or BHK cells. Retinyl acetate and retinoic acid both inhibited expression of the differentiated phenotype of chondrocytes by rabbit costal chondrocytes in culture within 3 days after their addition, as judged by morphological change and decrease in sulfate incorporation into glycosaminoglycans but did not inhibit cell proliferation. PTH could not induce an increase in ODCase in de-differentiated cells that had been pretreated with retinyl acetate or retinoic acid for 3 days; but 4 days after removal of the retinoids, these de-differentiated cells regained the ability to synthesize ODCase in response to PTH. These facts suggest that the induction of ODCase and the formation of putrescine by PTH are good markers of the differentiated phenotype of cultured chondrocytes.**

The polyamines putrescine, spermidine, and spermine have been linked to many processes for division, proliferation, and growth of various types of cells. The intracellular contents of polyamines are high in developing embryos and neoplastic tissues and increase in various tissues after appropriate growth stimuli or administration of hormones and drugs (1–7). The polyamine content also increases in cultured mammalian cells stimulated to proliferate and transform (8–10). Ornithine decarboxylase (ODCase; L-ornithine carboxy-lyase, EC 4.1.1.17), the enzyme responsible for putrescine formation and probably the rate-limiting enzyme in polyamine biosynthesis, is rapidly and markedly induced by various growth stimuli and by treatment with hormones and drugs both *in vivo* and in culture (1–7, 11–16). Recently, Conroy *et al.* (17) demonstrated the presence of ODCase and polyamines in cartilage, and Rath and Reddi (18) observed increase in ODCase activity during matrix-induced sequential differentiation of cartilage, bone, and bone marrow *in vivo*. However, although there are many reports of a close relationship between polyamine metabolism and cell proliferation, there have been few studies on the role of polyamine during cell differentiation.

We have shown (19) that growth cartilage cells isolated from the ribs of young rats actively synthesize cartilage matrix and that parathyroid hormone (PTH) stimulates glycosaminoglycan (GAG) synthesis, a differentiated phenotype of chondrocytes. On the other hand, vitamin A is known to inhibit GAG synthesis in chondrocytes from chicken sterna and from rat costal cartilage (20–22). Vitamin A also inhibits differentiation of mesenchymal cells derived from the limb buds of mouse embryos into chondrocytes *in vitro* (23–25).

Recently, we found (11) that PTH induced ODCase in rabbit costal chondrocytes in culture. Recent reports by Boutwell and his coworkers (7, 12) showed that vitamin A analogues inhibited the induction of ODCase by a tumor promoter in mouse epidermis. In the present study, by using vitamin A (retinoids), we investigated the mechanism of induction of ODCase by PTH in rabbit costal chondrocytes in culture and the relationship between the induction of the enzyme and the differentiated phenotype of chondrocytes.

MATERIALS AND METHODS

Chemicals. Bovine PTH-active fragments (synthetic 1–34) were purchased from Beckman Instruments. Retinyl acetate and retinoic acid were purchased from Sigma. DL-[1-^{14}C]-Ornithine-HCl (52.80 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was obtained from New England Nuclear. [^{35}S]Sulfuric acid (carrier-free) was obtained from Japan Atomic Energy Research Institute, Tokyo. Other materials used were commercial products of the highest grade available.

Cell Culture. Chondrocytes were isolated from growth cartilage of ribs of young male New Zealand rabbits weighing 400–700 g by treatment with EDTA, trypsin, and collagenase, as described (19). The isolated chondrocytes were plated at a density of 1 × 10^5 cells per Lux dish (35 mm) and grown in 2 ml of Eagle's minimum essential medium supplemented with 10% inactivated fetal calf serum and 60 μg of kanamycin per ml at 37°C under 5% CO_2 in air. The medium was changed every other day, except in experiments on the effect of vitamin A, when it was changed every day. Mouse L, BALB/c 3T3, and HeLa cells were kindly provided by Y. Mori (Department of Cell Biology, Center for Adult Diseases, Osaka, Japan) and buffalo rat liver cells were supplied by K. Nishikawa (Second Department of Biochemistry, Kanazawa Medical University, Kanazawa, Japan). BHK cells were kindly supplied by N. Maeda (First Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Osaka University, Osaka). These cells were cultured under the conditions described above, except that they were plated at 2.5 × 10^5 cells per Lux dish (60 mm).

Assay of ODCase Activity. For assay of ODCase, the medium was removed and the cell layer was washed five times with ice-cold phosphate-buffered saline. Then the cells were collected with a rubber policeman in 0.8 ml of standard buffer, sonicated at 20 kHz for 60 sec (in two 30-sec periods), and centrifuged at 15,000 × *g* for 15 min at 4°C, as reported (11). The activity of ODCase in the resulting supernatant was de-

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Abbreviations: ODCase, ornithine decarboxylase; PTH, parathyroid hormone; GAG, glycosaminoglycan; IU, international unit.

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termined by measuring the rate of liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine under described conditions (11).

Determination of Intracellular Polyamine Levels. Cultures were rapidly washed five times with ice-cold phosphate-buffered saline and the cells were collected with a rubber policeman in 0.8 ml of 2% chilled perchloric acid. The dishes were washed twice with 0.8 ml of the same acid. The cell suspension and the washings were combined, sonicated at 20 kHz for 60 sec, and centrifuged at 1600 × *g* for 20 min at 4°C. Perchloric acid-insoluble material was used for DNA assay. Polyamines in the supernatant were dansylated and separated by silica gel thin-layer chromatography by the method of Dion and Herbst (26). The fluorescence intensity of dansyl-polyamines extracted from silica gel plates was measured as described (5).

Determination of the Rate of GAG Synthesis. GAG synthesis was monitored by measuring incorporation of [^{35}S]sulfate by a modification of the procedure of Saarni and Tammi (27). The cell layer was washed four times with Hanks' solution containing [^{35}S]sulfate (2 μCi/ml) for 3 hr at 37°C in 5% CO_2 in air. The concentration of sulfate in the medium was 0.794 mM. After labeling, the medium was removed and the cells were washed with 1 ml of chilled Hanks' solution. The medium and the washing solution were combined and chilled. Next, the cells were collected with a rubber policeman in 1 ml of 0.5 M NaOH and the dishes were washed once with 1 ml of 0.5 M NaOH. The suspension of cells was combined with washing solution, and the mixture was solubilized by incubation for 1 hr at 37°C and then neutralized with 1 M HCl. Part of the neutralized material was taken for protein determination. The remainder was combined with an equivalent portion of the cold culture medium described above and used for GAG assay.

Other Assay Methods. Protein was determined by the method of Lowry *et al.* (28) or by the method of Ross and Schatz (29) if it was necessary to avoid interference from sulfhydryl compounds, with bovine serum albumin as standard. DNA was assayed by the modified diphenylamine reaction described by Giles and Myers (30) with calf thymus DNA as standard. All experiments were performed in duplicate.

RESULTS

Induction of ODCase and Changes in Polyamine Levels by PTH. We have already shown that PTH stimulates a differentiated cellular phenotype of chondrocytes (19) and induces ODCase activity (11) in cultured rat or rabbit costal chondrocytes. As shown in Fig. 1, when PTH was added to confluent quiescent cultures of rabbit costal chondrocytes, the activity of ODCase increased rapidly, reaching a maximum of about 5-fold that of untreated cultures 4–5 hr after addition of the hormone. Thereafter the enzyme activity decreased abruptly, returning to nearly the control level after 8 hr. The maximal stimulation was observed at a dose of 2.5 international units (IU) or more of the hormone per ml (11). The increase in enzyme activity was followed by an increase in the cellular concentrations of polyamines (Fig. 1), the concentration of putrescine in particular reaching about 3-fold that of untreated cultures 6 hr after PTH addition.

Effect of PTH on ODCase of Other Cell Lines. Table 1 shows the effect of PTH on ODCase activity in various types of cells. PTH did not increase ODCase activity in L, 3T3, HeLa, buffalo rat liver, or BHK cells. Therefore, these cells are unresponsive under the conditions of dose and time that allow the chondrocytes to respond.

Effects of Retinoids on the Differentiated Phenotype of Chondrocytes. Fig. 2 shows the effects of retinyl acetate and retinoic acid on the morphology of rabbit costal chondrocytes in culture. Chondrocytes grown in control medium had a typical polygonal shape and were surrounded by a refractile matrix

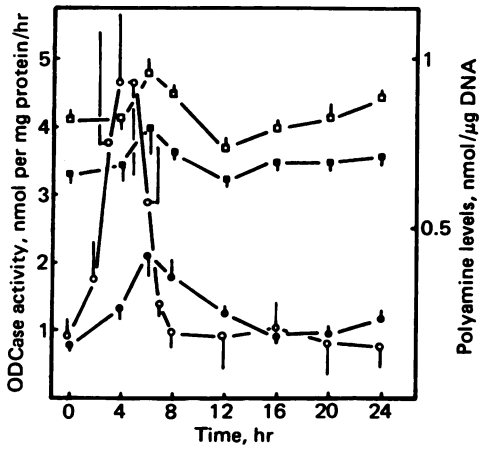

FIG. 1. Changes in ODCase activity and polyamine levels in rabbit costal chondrocytes after addition of PTH. When chondrocytes had reached confluence (day 8), the culture medium was replaced by 2 ml of fresh medium. After 24 hr, PTH (20 μl in phosphate-buffered saline) was added to the culture medium at a final concentration of 2.5 IU/ml, and then cultures were harvested at the times shown. Phosphate-buffered saline had no effect on ODCase activity or on polyamine levels: ○, ODCase; ●, putrescine; □, spermidine; ■, spermine. Points and lines are means ± SEM of results in three to five experiments.

(Fig. 2 *A* and *D*). On the other hand, after 3 days of treatment with retinyl acetate or retinoic acid, the polygonal cells assumed the appearance of fibroblasts and very little matrix was seen (Fig. 2 *B*, *C*, *E*, and *F*). These effects were dose-dependent and the minimum effective concentrations of retinyl acetate and retinoic acid were 0.2 μM and 0.05 μM, respectively (data not shown). The effects were also time-dependent and were apparent after a 2-day treatment with 5 μM retinyl acetate or a 24-hr treatment with 0.2 μM retinoic acid. These retinoids inhibited GAG synthesis in parallel with the morphological changes (Table 2). Retinoic acid was more effective than retinyl acetate: after a 3-day treatment, the inhibition by 0.2 μM retinoic acid was nearly equal to that by 5 μM retinyl acetate.

The effects of retinoids were reversible. Fig. 2 *G* and *H* shows chondrocytes that had been incubated with retinyl acetate and retinoic acid, respectively, for 3 days from day 2 to day 5 of culture and then without retinoids for 4 days from day 5 to day 9. The cells had regained a polygonal shape and refractile matrix. The reversion was observed 2 days after removal of retinoids. The inhibition of incorporation of [^{35}S]sulfate into GAG was also reversed (Table 2). This phenomenon was confirmed by metachromatic staining with toluidine blue.

Effects of Retinoids on Protein and DNA Accumulations.

Table 1. Effect of PTH on ODCase activity in various types of cells

Cell line	ODCase activity, nmol per mg protein/hr − PTH	+ PTH
Chondrocytes	1.02	4.55
L	3.81	3.68
3T3	0.97	0.64
HeLa	3.77	1.43
BRL	0.45	0.31
BHK	0.32	0.32

When the cells reached confluence, the culture medium was replaced by fresh medium. After 24 hr, PTH (2.5 IU/ml) was added to the medium and cells were harvested 4 hr later. Values are the average of duplicate dishes, which varied less than 6% from the mean. Similar results were obtained in repeated experiments. BRL, buffalo rat liver.

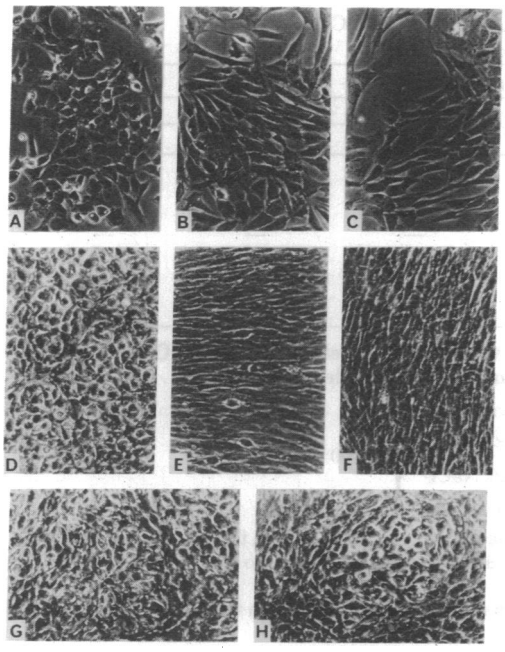

FIG. 2. Effects of retinyl acetate and retinoic acid on the morphology of chondrocytes. Retinyl acetate and retinoic acid in ethanol were added at final concentrations of 5 μM and 0.2 μM, respectively. Ethanol had no effect at the concentration used (0.01%). (*A–C*) and (*D–H*) Photomicrographs of cells on day 5 and day 9, respectively. (*A* and *D*) Ethanol-treated control. The cells were polygonal and surrounded by a refractile matrix. (*B* and *E*) Chondrocytes after 3 days of treatment with medium containing retinyl acetate. The cells became fibroblastic in appearance and secreted very little matrix. (*C* and *F*) Chondrocytes after 3 days of treatment with retinoic acid. The appearance of cells was similar to that of retinyl acetate-treated chondrocytes. (*G* and *H*) Chondrocytes that had been treated with retinyl acetate and retinoic acid, respectively, for 3 days from day 2 to day 5 and then incubated in medium without vitamins until day 9. The appearance of the cells had become similar to that of the controls (*D*).

Neither retinyl acetate nor retinoic acid inhibited protein accumulation under the condition described in Table 2 (Table 3, part A). Table 3, part B shows the effect on cells in the logarithmic phase of proliferation of treatment with retinoids for 3 days. Under these conditions, the protein and DNA contents of control cultures both increased about 3-fold, and the increases were not inhibited by treatment with retinyl acetate or retinoic acid. Thus, the inhibitory effect of these retinoids on the differentiated phenotype was not caused by general cytotoxicity of the retinoids.

Table 2. Effects of retinyl acetate and retinoic acid on [^{35}S]-sulfate incorporation into GAGs in rabbit costal chondrocytes

Pretreatment with vitamin A at days:	^{35}S Incorporation, % of control	
	Retinyl acetate	Retinoic acid
8–9	61.1 ± 5.0	55.6 ± 3.0
7–9	38.4 ± 1.0	—
6–9	32.4 ± 3.8	31.1 ± 3.5
5–9	28.5 ± 5.3	24.6 ± 2.5
2–5*	85.3 ± 9.7	92.3 ± 8.9

Retinyl acetate (5 μM) or retinoic acid (0.2 μM) was present on the days shown. Cultures were labeled with 2 μCi of [^{35}S]sulfate per ml for 3 hr on day 9. The incorporation of [^{35}S]sulfate into control cultures was 114.2 ± 7.0 dpm/μg of protein. Values are means ± SEM for at least four cultures from two separate experiments.

* On day 5, the medium containing vitamin A was removed and the cells were further incubated in the absence of vitamin A until day 9.

Table 3. Effects of retinyl acetate and retinoic acid on accumulations of protein and DNA in rabbit costal chondrocytes in culture

Pretreatment with vitamin A		Protein, μg/dish	DNA, μg/dish
A. Control		617	—
Retinyl acetate	(day 6–9)	702	—
	(day 2–5)*	808	—
Retinoic acid	(day 6–9)	691	—
	(day 2–5)*	679	—
B. Control		353	13.5
Retinyl acetate	(day 4–7)	356	13.3
Retinoic acid	(day 4–7)	380	14.0

Retinyl acetate (5 μM) or retinoic acid (0.2 μM) was present on the days shown. Cultures were harvested on days 9 and 7 in experiments A and B, respectively. Values are averages of duplicate dishes, which varied less than 5% from the mean. Similar results were obtained in repeated experiments.

* On day 5, the medium containing vitamin A was removed and the cells were further incubated in the absence of vitamin A until day 9.

Effects of Retinoids on Induction of ODCase by PTH. Previously, we showed that, when added with PTH, neither retinyl acetate (0.1–10 μM) nor retinoic acid (0.01–10 μM) inhibits the induction of ODCase by PTH in rabbit costal chondrocytes in culture (11). However, when the cells had been pretreated with retinoids for 3 to 4 days, induction of ODCase was suppressed when PTH was added (Fig. 3 and Table 4). The effect was dose-dependent: after 4 days of treatment, ODCase induction by PTH was inhibited about 40% at 0.2 μM retinyl acetate and about 75% at 1 μM retinyl acetate. Retinoic acid was more effective than retinyl acetate: after 3 days of treatment, the inhibition by 0.2 μM retinoic acid was about equal to that by 5 μM retinyl acetate. In addition, the inhibition by retinoic acid was observed earlier than that by retinyl acetate. When chondrocytes that had been pretreated with retinoids were further incubated in medium free of retinoids, the cells regained their ability to synthesize ODCase in response to PTH (Table 4). Additions of retinyl acetate or retinoic acid to the

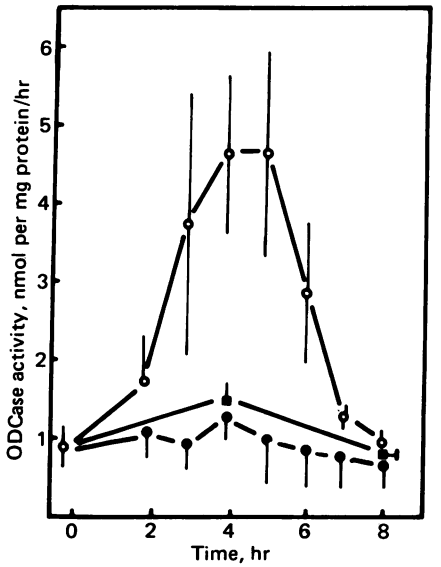

FIG. 3. Effects of pretreatment with retinyl acetate and retinoic acid on induction of ODCase by PTH in rabbit costal chondrocytes. PTH (2.5 IU/ml) was added to cultures that had been pretreated with ethanol (○), 5 μM retinyl acetate (●), or 0.2 μM retinoic acid (■) for 3 days, and then the cultures were harvested at the times shown. Other experimental procedures were as described in the legend to Fig. 1. Points and lines are means ± SEM of results in two or three experiments.

Table 4. Effects of retinyl acetate and retinoic acid on induction of ODCase by PTH in rabbit costal chondrocytes in culture

Pretreatment with vitamin A at days:	Inhibition of induction, %					
	Retinyl acetate			Retinoic acid		
	0.2 μM	1 μM	5 μM	0.02 μM	0.1 μM	0.2 μM
9*	—	−3.5 ± 3.7	−5.8 ± 3.5	—	10.0 ± 7.3	5.8 ± 7.6
8–9	—	29.3 ± 1.4	41.5 ± 12.4	—	—	76.4 ± 9.7
7–9	—	—	56.9 ± 10.2	—	—	—
6–9	—	—	90.5 ± 5.6	52.3 ± 9.2	63.3 ± 1.8	85.3 ± 2.4
5–9	42.0 ± 2.5	74.2 ± 3.4	98.7 ± 5.9	—	71.1 ± 7.8	99.5 ± 1.9
2–5†	—	—	−16.2 ± 5.1	—	11.4 ± 0.5	10.2 ± 6.0

Retinyl acetate or retinoic acid was present on the days shown. PTH was added 4 hr before the assay of ODCase at 2.5 IU/ml. Enzyme activity was assayed on day 9. The percentage inhibition of induction is defined as:

$$\left[1 - \frac{\text{SA in PTH- and vitamin A-treated culture} - \text{SA in vitamin A-treated culture}}{\text{SA in PTH-treated culture} - \text{SA in } P_i/\text{NaCl}\text{-treated culture}}\right] \times 100$$

Values for specific activity (SA) in retinyl acetate- and retinoic acid-treated cultures were almost the same as that in the phosphate-buffered saline (P_i/NaCl)-treated culture. Values are means ± SEM of results of two to five experiments.

* Vitamin A was added with PTH.

† On day 5, the medium containing vitamin A was removed and the cells were further incubated in the absence of vitamin A until day 9.

reaction mixture at a concentration of 10 μM did not alter the enzyme activity (data not shown). These results show that ODCase induction by PTH in chondrocytes is closely correlated with the differentiated phenotype of chondrocytes.

DISCUSSION

Gaillard *et al.* reported that mouse long-bone rudiments treated with relatively low concentrations of PTH induced anabolic reactions in the cartilage by favoring the maturation process, whereas higher concentrations of PTH diminished the number of mature cartilage cells and favored the resorption of bone (31). In our investigations, PTH induced a marked increase in ODCase activity in rabbit costal chondrocytes in culture, followed by increase in the intracellular levels of polyamines and especially putrescine. Previously, we reported (19) that PTH stimulated GAG synthesis and inhibited calcium uptake by these cells. Recently, we showed (11) that activity of ODCase in rabbit costal chondrocytes was not affected by addition of factors associated with calcium metabolism, such as calcitonin, 1,25-dihydroxyvitamin D_3, or vitamin A, when added with or without PTH. Therefore, induction of ODCase by PTH is not related to change in calcium metabolism in the chondrocytes. PTH also induced marked increase in the cyclic AMP level in the cultured chondrocytes 2 min after addition of the hormone (unpublished). Deshmukh *et al.* reported that addition of PTH rapidly increases the cyclic AMP level in rabbit articular cartilage cells in culture (32). Zull *et al.* reported that PTH activated adenyl cyclase in cultured chicken limb bud cells and they suggested that activation of the enzyme is related to the appearance of bone cells (33). In addition, dibutyryl cyclic AMP induced ODCase activity and expression of the differentiated phenotype of chondrocytes in our cell system (unpublished). These facts suggest that PTH induces a series of events—successive increases of ODCase activity, polyamine levels, and expression of differentiated phenotype of chondrocytes—through an increase in cyclic AMP concentration.

Both retinyl acetate and retinoic acid inhibited expression of the differentiated phenotype of chondrocytes, as judged by morphological change and decrease of sulfate incorporation into GAG (Fig. 2 and Table 2). It has already been shown that these retinoids inhibit expression of the differentiated phenotype of chondrocytes from the sterna of chicken embryos and of rats (20–22) and also depress chondrogenesis of mesenchymal cells derived from mouse limb buds (23–25) in culture. Vasan and Lash (21) reported that 10 μM vitamin A inhibited proliferation of chondrocytes from the sterna of chicken embryos. Hassell *et al.* (23) also found that 10 μM retinoic acid inhibited proliferation of prechondrogenic cells plated at high cell density, but not at low cell density. However, Shapiro and Poon (22) reported that a low concentration of retinoic acid (2.2 nM to 2.2 μM) inhibited GAG synthesis without affecting cell proliferation. The concentration of retinyl acetate used in this study was 5 μM and that of retinoic acid was only 0.2 μM, and our results agree with those of Shapiro and Poon, as shown in Table 3.

Verma and Boutwell (12) reported that topical application of retinoic acid or retinyl acetate inhibited phorbol ester-induced ODCase activity in mouse epidermis *in vivo* and that

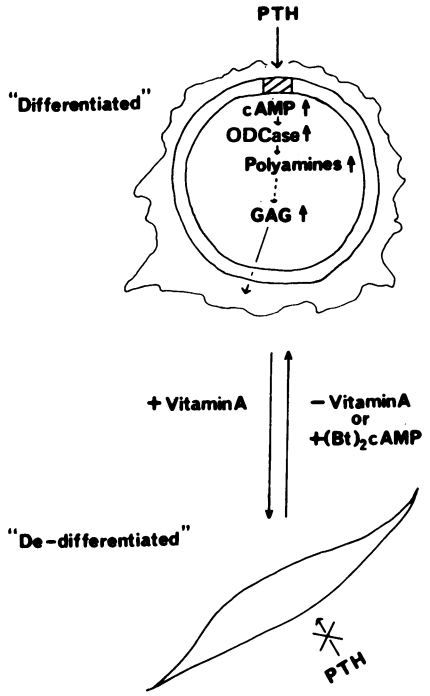

FIG. 4. Possible scheme of the roles of PTH and polyamines in the expression of the differentiated phenotype of chondrocytes. cAMP, cyclic AMP; $(Bt)_2$cAMP, dibutyryl cyclic AMP.

retinoic acid was more effective than retinyl acetate. Table 4 shows that retinoic acid was more effective than retinyl acetate in our system also. Solursh and Meier (20) reported that the inhibitory effect of retinyl acetate (10 μM) was not reversible, but Fig. 2 and Tables 2 and 4 clearly show that the effects of retinoids on the morphology and metabolism of the cells are reversible. This discrepancy requires further investigation. The initial cell density that Solursh and Meier employed was only 1/10th of that in our system. Thus, their chondrocytes may have possessed a lower ability to differentiate, because we have shown that expression of the differentiated phenotype of chondrocytes is greatly influenced by the initial cell density: a high cell density is required for retention of the differentiated phenotype (34). Solursh and Meier also used a higher concentration of retinyl acetate than we did.

In our preliminary experiments, treatment of chondrocytes with retinoids inhibited increase in the intracellular cyclic AMP level by PTH and, when dibutyryl cyclic AMP was added to cultures of de-differentiated chondrocytes that had been pretreated with retinoids, the cells changed from a fibroblastic back to a polygonal shape and regained the ability to exhibit ODCase induction with PTH and GAG synthesis. The details of this phenomenon will be published in another paper.

Recently, Lewis *et al.* reported that addition of vitamin A to prechondrogenic mesenchyme cells resulted in the maintenance of the cell surface features of the mesenchyme cells and hence inhibited chondrogenesis (25). Moreover, as shown in this study, retinoids induced transformation of polygonal cells into fibroblastic cells with loss of responsiveness to PTH. Consequently, vitamin A may affect cell surface proteins and the PTH receptors thought to be present on the surface of chondrocytes.

There are many reports showing a close relationship between polyamine metabolism and cell proliferation and tissue growth. We also reported that polyamines are involved in cell proliferation in isoproterenol-stimulated mouse parotid glands (4) and in the skin of mice after application of ethylphenylpropiolate (5). More recently, we showed that multiplication-stimulating activity, a substance partially purified from serum-free medium conditioned by growth of buffalo rat liver cells, markedly induced not only proliferation of rabbit costal chondrocytes but also their synthesis of GAG in serum-free medium (35). However, PTH had little effect on DNA synthesis of cultured chondrocytes and did not induce any proliferation of the cells (unpublished). Moreover, induction of ODCase by PTH is found only in chondrocytes; other cells tested did not synthesize ODCase in response to PTH (Table 1). Thus, induction of the enzyme by PTH is a useful marker of the differentiated phenotype of chondrocytes.

From this investigation, we postulate, as shown in Fig. 4, that, in our chondrocyte system, accumulation of polyamines triggered by the action of PTH may play an important role in expression of the differentiated phenotype of chondrocytes, in addition to its well-known roles in proliferation of cells. Fig. 4 also indicates the involvement of cyclic AMP in ODCase induction and GAG synthesis by PTH and in the reversible effect of vitamin A.

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