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Synthesis of collagen by chondrocytes in suspension culture: Modulation by calcium, 3′:5′-cyclic AMP, and prostaglandins

(arthritis/protein synthesis *in vitro*)

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ABSTRACT **Rabbit articular chondrocytes synthesize type II collagen [$3\alpha_1$(II)] *in vivo* and type I collagen [$2\alpha_1$(I)·α_2] in monolayer cultures. In suspension culture the nature of phenotype depends on extracellular Ca^{2+}. The relationship of Ca^{2+} and 3′:5′-cyclic AMP (cAMP) in regulation of collagen synthesis has been investigated. In suspension culture, cAMP levels of chondrocytes increase by 2- to 3-fold and then reach basal values regardless of the presence or absence of extracellular Ca^{2+}. The cells, however, synthesize primarily type II collagen in the absence of $CaCl_2$ in the medium and type I collagen in medium containing 1.8 mM $CaCl_2$. If $CaCl_2$ is added when intracellular cAMP levels are low, the phenotype is type I collagen. These observations minimize the role of cAMP as a second messenger in the chondrocyte culture system. Increasing endogenous cAMP with a phosphodiesterase inhibitor or adding exogenous dibutyryl-cAMP leads the cells to synthesize type I collagen, although this effect is significantly less pronounced if the medium contains ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid (EGTA). Increased concentrations of cAMP may mobilize the intracellular calcium pools and activate the cells to switch their phenotypic expression. Prostaglandins E_2 and $F_2\alpha$, thought to be involved in rheumatoid arthritis and bone resorption, have no significant effect on cAMP content of chondrocytes and alter their collagen phenotype to a small extent.**

The cartilage matrix consists of two major macromolecular components, collagen and proteoglycans, which are synthesized by chondrocytes. Normal articular cartilage contains type II collagen, [$3\alpha_1$(II)], and is genetically distinct from type I collagen, [$2\alpha_1$(I)·α_2], of, for example, skin, bone, and tendon (1, 2). The association of type II collagen with proteoglycans imparts unique structural and functional characteristics to this tissue. The cartilage undergoing osteoarthritic degeneration synthesizes type I collagen rather than the tissue-specific type II collagen (3, 4) and also exhibits an abnormality in the formation of proteoglycan aggregates (5). These structural changes lead to a disturbance in the macromolecular assembly and loss of tissue function.

Numerous studies are being done to demonstrate the capability of cells to control their microenvironment and the effect of the extracellular matrix on cell structure and function. Our recent studies have shown that normal rabbit articular chondrocytes synthesize type II collagen *in vivo* or *in vitro*. These cells in culture synthesize type II or type I collagen, depending upon the extracellular conditions. In monolayer cultures they produce type I collagen, while in suspension cultures the phenotypic expression mainly depends upon the presence or absence of Ca^{2+} in the medium. In the absence of Ca^{2+}, the cells synthesize their tissue-specific type II collagen; in the complete medium (containing 1.8 mM $CaCl_2$), the major product is type I collagen (6). This change in the collagen type was observed at a concentration of $CaCl_2$ as low as 0.1 mM. Furthermore, if the cells were treated in monolayer cultures in complete medium with the divalent cation ionophore A23187, an increased influx of calcium into the cells was induced, which was followed by an increase in intracellular adenosine 3′:5′-cyclic monophosphate (cAMP). These pretreated cells synthesized mainly type I collagen in suspension cultures in the absence of extracellular $CaCl_2$ (7). Calcitonin and parathyroid hormone, the hormones involved in bone metabolism, also cause changes in the type of collagen synthesized, by modulation of the rates of influx or efflux of Ca^{2+} and in intracellular cAMP of chondrocytes (8).

The interrelated involvement of Ca^{2+} and cAMP in cell excitation and response has been reported in a number of biological mechanisms, such as insulin production, various exocrine and endocrine secretions, action of some peptide hormones, and neuromuscular transmission (9). Borle has described in detail the cellular calcium homeostasis, transcellular calcium transport, stimulation of these processes by cAMP, and the role of mitochondria as their controller in various cell systems (10, 11). The present communication describes the relationship between extracellular Ca^{2+} and intracellular cAMP and their effect on the phenotypic expression of chondrocytes in suspension culture.

It has been shown that prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) are commonly found in synovial fluid of inflammatory arthritic joints, and PGE_2 has been reported to be a potent stimulator of bone resorption (12–14). We have investigated the effect of these two prostaglandins on chondrocyte metabolism in culture systems.

MATERIALS AND METHODS

Cell Culture. The chondrocytes were isolated from articular cartilage of knee and hip joints of 2- to 3-month-old rabbits by treatment with hyaluronidase, trypsin, and clostridiopeptidase (15). The cells were plated at 1×10^6 cells per Falcon flask (75 cm^2) and were grown in monolayer culture in 10 ml of Ham's F-12 nutrient mixture, containing 10% fetal calf serum (vol/vol) and antibiotics and in an atmosphere of 5% CO_2 in air until they reached confluency (7–8 days). The cells were fed twice with fresh medium during this time. The chondrocytes were removed from monolayer culture flasks by trypsinization and transferred to Bellco suspension culture flasks in Dulbecco's modified Eagle's medium containing 4.5 g of glucose per liter, no $CaCl_2$, 10% fetal calf serum (vol/vol), and antibiotics. In

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Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; isoBuMeXan, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N′*-tetraacetic acid; PGE_2, prostaglandin E_2; $PGF_2\alpha$, prostaglandin $F_2\alpha$; CM-cellulose, carboxymethylcellulose; CNBr, cyanogen bromide.

some experiments, Dulbecco's complete medium (same medium containing 1.8 mM $CaCl_2$) was used.

The cAMP-related effect on the collagen phenotype was followed by conducting different sets of experiments. In all the experiments, chondrocytes from monolayer primary cultures were directly transferred to suspension culture flasks with medium containing the additives. (*A*) The cells were maintained in suspension medium containing a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (isoBuMeXan) (Aldrich) (50 μM) and without $CaCl_2$. (*B*) A similar experiment was done in the presence of isoBuMeXan (50 μM) and ethylene glycol bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA; 50 μM) and again without $CaCl_2$. (*C*) The cells were incubated in suspension culture medium without $CaCl_2$ for 6 hr, when the intracellular cAMP content reached the minimum level (see *Results*). $CaCl_2$ was added subsequently to adjust the final concentration to 1.8 mM and the incubation was continued further. (*D*) The cells were maintained in medium devoid of $CaCl_2$, containing 1 mM dibutyryl-cAMP (Sigma). (*E*) A similar experiment was carried out in the presence of dibutyryl-cAMP (1 mM) and EGTA (50 μM).

The effect of PGE_2 and $PGF_{2\alpha}$ (Upjohn) on the type of collagen produced by these cells was studied by addition of these compounds at various concentrations to the medium containing no $CaCl_2$.

After a 24-hr incubation under various conditions, ascorbic acid (25 μg/ml), β-aminopropionitrile (Aldrich) (50 μg/ml), and [2,3-^{3}H]proline (10 μCi/ml, specific activity 24.5 Ci/mmol) were added to the medium and the incubation was continued further for 24 hr (6).

Isolation and Characterization of Collagen. The medium from each flask was centrifuged and the cell pellet was extracted with 0.5 M acetic acid at 4° overnight. The medium and the cell extract were mixed together, dialyzed exhaustively against water, lyophilized, and dissolved in 0.5M acetic acid. The mixture was treated with pepsin (100 μg/ml) for 48 hr at 4° and the enzyme was then inactivated by increasing the pH to 8–8.5. Rat skin acid-soluble collagen (6–8 mg) was added as a carrier, the mixture was dialyzed against 1 M NaCl in 50 mM Tris:HCl, pH 7.5, and the labeled collagen together with the carrier collagen was precipitated out with 20% NaCl (wt/vol). The precipitate was dissolved in 0.5 M acetic acid, dialyzed against 60 mM Na acetate buffer (pH 4.8) containing 1 M urea, and chromatographed on a 0.9 × 10-cm column of carboxymethyl (CM)-cellulose at 42° (6). Collagen subunits were eluted by developing a linear gradient between 0 and 100 mM NaCl (80 ml each). The effluent was monitored at 230 nm with a Gilford spectrophotometer, and the aliquots of the effluent fractions were used for radioactivity measurements. [^{3}H]-Hydroxyproline was estimated with the fractions containing radioactivity to confirm the nature of products as collagen subunits (6).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed on a portion of pepsin-treated and purified labeled collagen before and after reduction with mercaptoethanol (16).

Chromatography of CNBr Peptides. The labeled collagen subunits were further identified from their characteristic CNBr peptides. The fractions corresponding to α_1- and α_2-chains from a CM-cellulose chromatogram were pooled, dialyzed, and treated with CNBr to achieve cleavage at methionyl residues, as described in a previous communication (17). The CNBr peptides were lyophilized, dissolved in 20 mM Na citrate buffer (pH 3.6) containing 20 mM NaCl, and separated by chromatography on a CM-cellulose column (0.9 × 10 cm) at 42°, using a linear gradient between 20 and 140 mM NaCl over a total volume of 200 ml. The effluent was monitored at 234 nm and the radioactivity of the aliquots of the fractions was determined.

Table 1. Change in cAMP content of chondrocytes in suspension culture

Incubation period, min	cAMP, pmol/10^6 cells*	
	Medium without $CaCl_2$	Complete medium
0	12.7 ± 1.1	12.5 ± 1.2
5	19.8 ± 1.2	20.2 ± 1.0
15	29.8 ± 1.7	30.4 ± 1.1
30	17.3 ± 1.8	18.8 ± 0.9
60	18.0 ± 0.8	15.7 ± 1.2
120	15.0 ± 1.0	12.9 ± 0.6
240	8.7 ± 0.5	8.5 ± 0.7
360	7.0 ± 0.6	7.1 ± 0.7

* Mean ± SD; $n = 4$.

Estimation of Intracellular cAMP. The chondrocytes from confluent monolayer cultures were trypsinized and incubated in suspension culture flasks in Dulbecco's medium without $CaCl_2$ or the complete medium containing 10% fetal calf serum and antibiotics, for various time periods. In some experiments, isoBuMeXan, PGE_2, or $PGF_{2\alpha}$ was added at various concentrations to the medium devoid of $CaCl_2$. As described earlier, in one set of experiments, the cells were maintained in the medium with no $CaCl_2$ for 6 hr, $CaCl_2$ was then added to adjust the concentration to 1.8 mM, and the incubation was continued further. At the end of each time period, the cell suspension was centrifuged, 2 ml of 0.3 M perchloric acid containing 0.05 pmol of [^{3}H]cAMP (specific activity 34.7 Ci/mmol) was added to the cell pellet, and the mixture was sonicated and frozen immediately. cAMP was estimated by the method of Gilman (18) using commercial protein kinase (Sigma).

RESULTS

Change in Intracellular cAMP Content. After the transfer of cells from monolayer to suspension culture flasks, the intracellular cAMP levels increased rapidly during the first 15 min of incubation and then decreased steadily, reaching a minimum level within 4–6 hr (Table 1). The cells were activated by treatment with trypsin because the value for cAMP at zero time in suspension culture was higher than that previously reported for chondrocytes in resting monolayer cultures (7). The change in cAMP with time was of the same magnitude for the cells maintained in medium without $CaCl_2$ and those in complete medium. In other words, the extracellular Ca^{2+} concentration had no apparent effect on the intracellular cAMP levels.

The addition of a phosphodiesterase inhibitor, isoBuMeXan, to the medium without $CaCl_2$ led to a 8- to 9-fold increase in cAMP during the initial incubation period. There was a steady fall in these amounts, although they never reached the basal level (Table 2). The presence of PGE_2 or $PGF_{2\alpha}$ in medium containing no $CaCl_2$ did not significantly alter the normal pattern of change in cAMP content of chondrocytes (Tables 1 and 2). In one set of experiments, the cells were preincubated in medium without $CaCl_2$ for 6 hr. At this time, the cellular cAMP content had reached the basal levels. Addition of $CaCl_2$ to these cells caused no significant change in the cAMP content (Table 3).

Collagen Synthesis. Table 4 shows the α_1:α_2 chain ratios of collagen synthesized by the representative cell populations after various treatments. The chain ratios were calculated from the

Table 2. Effect of isoBuMeXan, PGE_2, and $PGF_{2\alpha}$ on cAMP content of chondrocytes in suspension culture*

Incubation period, min	cAMP, pmol/10^6 cells†		
	isoBuMeXan, 50 μM	PGE_2, 100 ng/ml	$PGF_{2\alpha}$, 100 ng/ml
0	13.0 ± 0.9	12.7 ± 1.8	12.8 ± 1.5
5	68.6 ± 4.5	20.5 ± 0.9	20.9 ± 1.3
15	111.5 ± 12.1	30.0 ± 3.1	32.7 ± 2.0
30	76.0 ± 8.3	16.2 ± 2.7	17.5 ± 2.2
60	51.2 ± 2.8	15.8 ± 1.3	16.2 ± 1.9
120	32.0 ± 2.3	12.9 ± 1.7	11.4 ± 0.7

* isoBuMeXan, PGE_2, and $PGF_{2\alpha}$ were added to the medium containing no $CaCl_2$.
† Mean ± SD; $n = 4$.

amount of radioactivity coincident with the areas of elution of α_1 and α_2 chains of unlabeled carrier collagen on the CM-cellulose chromatogram. In all the experiments, less than 5% of the total radioactivity eluted in the area of the chromatogram where β_{12} chains appear. The labeled α chains were further characterized from the elution pattern of their CNBr peptides on a CM-cellulose column and by comparison with those of the peptides of α_1(I) and α_2 chains of rat skin acid-soluble collagen or α_1(II) chains of rabbit cartilage collagen.

In complete suspension culture medium, chondrocytes synthesized type I collagen. The radioactivity eluted in the areas of α_1 and α_2 chains on the chromatogram (Fig. 1*a*), and the α_1:α_2 chain ratio was close to 2.0 (Table 4). Furthermore, the elution patterns of CNBr peptides of these chains coincided with those of α_1(I) and α_2 chains of rat skin acid-soluble collagen (Fig. 2 *a* and *b*), confirming the nature of collagen as type I collagen. On the other hand, the cells maintained in suspension medium without $CaCl_2$ produced collagen that eluted primarily as α_1 chains (Fig. 1*b*). The elution pattern of their major CNBr peptides did not coincide with that of α_1(I) chains (Fig. 2*c*) and was similar to the peptides of α_1(II) chains of rabbit cartilage collagen. These results confirm our previous findings (6).

From the data presented so far, it is apparent that the change in cAMP content of chondrocytes in suspension culture occurs irrespective of the presence or absence of Ca^{2+} in the medium. However, the extracellular calcium concentration plays a great role in the phenotypic expression of the cells. To test further the role of cAMP in the switch of the type of collagen synthesized by chondrocytes, we increased their cAMP content significantly beyond normal levels by addition of isoBuMeXan to the medium containing no $CaCl_2$. Under these conditions, the cells were capable of synthesis of type I collagen. The presence of EGTA in the medium reduced this effect significantly (Table 4). In our previous studies, we have observed that addition of dibutyryl-cAMP to the suspension culture medium allowed the cells to produce type I collagen in the absence of extracellular calcium (7). This switch in collagen type did not occur to the same extent if the medium contained EGTA (Table 4). When the cells were maintained in medium without $CaCl_2$ for 6 hr (at which time the intracellular cAMP had reached the minimum levels) and $CaCl_2$ was then added to make the concentration equivalent to that of complete medium, these chondrocytes synthesized type I collagen, although no change in cAMP occurred after the addition of $CaCl_2$.

Table 3. Effect of addition of $CaCl_2$ after 6 hr of incubation on the cAMP content of chondrocytes

Incubation period, min*	cAMP, pmol/10^6 cells†
0	6.9 ± 0.5
15	7.8 ± 0.9
30	7.2 ± 1.0
60	5.9 ± 0.8

* The cells were incubated in the medium without $CaCl_2$ for 6 hr. $CaCl_2$ was then added to adjust the concentration to 1.8 mM and the incubation was continued. cAMP content was estimated at various time intervals after the addition of $CaCl_2$.
† Mean ± SD; $n = 4$.

Table 4. Synthesis of collagen by chondrocytes in suspension culture after various treatments

Treatment	α_1:α_2 chain ratio*
No $CaCl_2$	>30.0
$CaCl_2$ (1.8 mM)	2.0 ± 0.2
isoBuMeXan (50 μM)	2.3 ± 0.3
isoBuMeXan (50 μM) + EGTA (50 μM)	8.0 ± 1.0
Dibutyryl-cAMP (1 mM)	2.8 ± 0.2
Dibutyryl-cAMP (1 mM) + EGTA (50 μM)	8.6 ± 0.4
$CaCl_2$ (1.8 mM) after 6 hr	2.2 ± 0.1
PGE_2 (100 ng/ml)	9.1 ± 1.1
$PGF_{2\alpha}$ (100 ng/ml)	10.5 ± 1.2

* Mean ± SD. Each value (except the one with no $CaCl_2$) represents the mean of three estimations.

PGE_2 and $PGF_{2\alpha}$ had no effect on the cAMP levels of chondrocytes; in their presence in the medium without $CaCl_2$, the cells synthesized approximately 70% or more of type II collagen.

In all these experiments, where α_1:α_2 chain ratio was higher than 2.0, the presence of type II collagen was apparent from its typical CNBr peptides. We have not encountered type III collagen or type I trimer under these conditions. Type III collagen was not detected on sodium dodecyl sulfate/polyacrylamide gel electrophoretograms of purified labeled collagen before or after reduction with mercaptoethanol.

DISCUSSION

The studies reported in this communication were initiated to investigate the interrelated role of Ca^{2+} and cAMP in the stimulation of chondrocytes in suspension culture and modu-

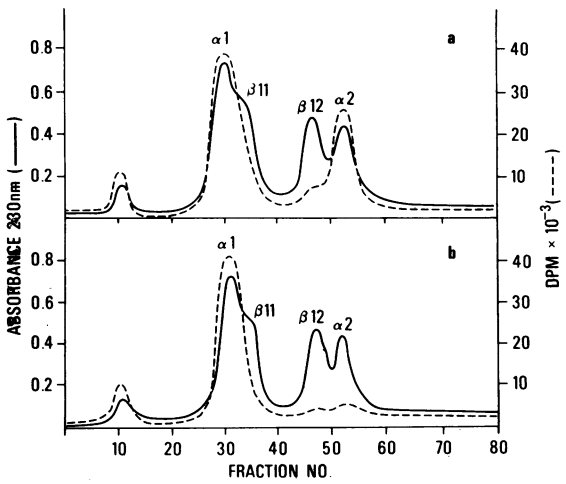

FIG. 1. CM-cellulose chromatogram of collagen. (—) Rat skin acid-soluble collagen. (*a*) (---) Type I collagen synthesized by chondrocytes under various conditions. (*b*) (---) Type II collagen synthesized by chondrocytes in the absence of extracellular $CaCl_2$.

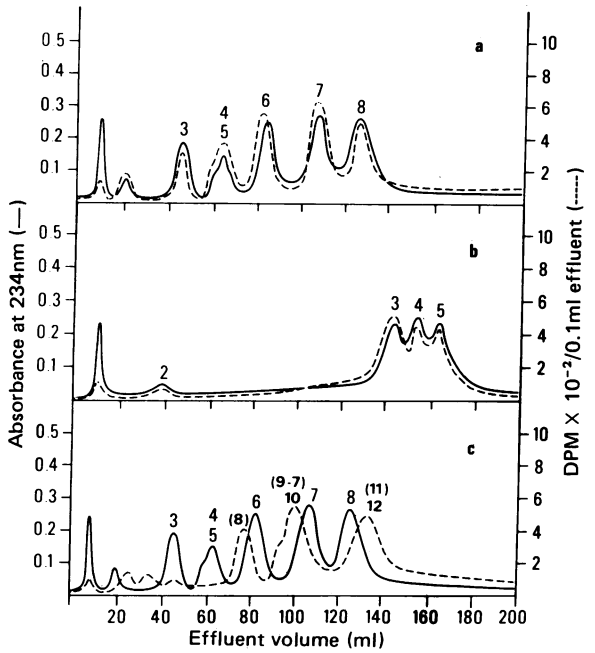

FIG. 2. Elution pattern of CNBr peptides of: (*a*) (—) α_1(I) chains of rat skin acid-soluble collagen; (---) α_1(I) chains synthesized by chondrocytes in culture. (*b*) (—) α_2 chains of rat skin acid-soluble collagen; (---) α_2 chains synthesized by chondrocytes in culture. (*c*) (—) α_1(I) chains of rat skin acid-soluble collagen; (---) α_1(II) chains synthesized by chondrocytes in the absence of extracellular $CaCl_2$.

lation of the type of collagen synthesized by these cells. Our previous data suggest that the chondrocytes switch their synthesis of type II collagen to type I collagen when isolated from cartilage and grown in monolayer cultures. Upon transfer from monolayer to suspension cultures, the nature of the phenotype depends on the presence or absence of calcium in the extracellular medium (6). The cells synthesized primarily type I collagen if the concentration of $CaCl_2$ in the medium was 100 μM or higher (7). Dialyzed or nondialyzed fetal calf serum at 10% concentration did not contribute significant amounts of free calcium ions since when it was present in the medium with no $CaCl_2$, the major phenotype was type II collagen. In addition, we have also observed that the presence of dibutyryl-cAMP in the suspension culture medium containing no $CaCl_2$ led the cells to synthesize type I collagen (7). In other words, in the absence of extracellular Ca^{2+}, exogenous dibutyryl-cAMP could induce the cells to switch their phenotype to type I collagen. The addition of butyric acid did not cause any change in the phenotype (results not shown). In many tissues and cell systems there is apparently a widespread association between Ca^{2+} and cAMP in cell activation processes (9, 19), but this association is not universal (20). In the case of exocrine pancreas or in adrenal medulla, the activation of enzyme secretion or the release of epinephrine by acetylcholine requires extracellular Ca^{2+}. The exogenous cAMP or dibutyryl-cAMP, however, can induce such secretion in the absence of extracellular calcium. In tissues in which cAMP is not a second messenger in the normal activation process, exogenous cAMP can mobilize calcium from an intracellular pool and thereby activate a calcium-dependent process (20). With chondrocytes in suspension cultures, an increase in cAMP followed by a decrease to the basal level was observed in the presence or absence of extracellular calcium, while the change in phenotypic expression of the cells was dependent on calcium in the medium. Furthermore, addition of $CaCl_2$ to the medium when cellular cAMP levels were low caused a change in the type of collagen produced without any significant increase in cAMP. These data minimize the role of cAMP as a second messenger in this system. The addition of isoBuMeXan to the medium to increase intracellular cAMP levels or the presence of exogenous dibutyryl-cAMP allowed the cells to synthesize type I collagen. It is possible that the increase in endogenous or exogenous cAMP may lead to the mobilization of the intracellular calcium pool of chondrocytes and produce an effect similar to that of extracellular calcium. The presence of EGTA in the medium under such conditions inhibited the switching of collagen phenotype to some extent and allowed the cells to synthesize significant amounts of type II collagen. The presence of EGTA in the extracellular medium, however, does not affect the regulation of cytoplasmic calcium activity by mitochondria. Similar effects of EGTA have been observed with, for example, isolated renal tubules, adipose tissues, and liver, and are mainly dependent upon the rates of calcium fluxes across the cell membrane (9). A recent report indicates that exogenous dibutyryl-cAMP also stimulates the release of proteoglycans from cultured rabbit ear cartilage (21). Theophylline alone did not significantly stimulate such release, but potentiated the action of dibutyryl-cAMP.

The role of naturally occurring prostaglandins in inflammation was been established. PGE_2 and $PGF_{2\alpha}$ are the major prostaglandins that are synthesized by rheumatoid synovia in culture (22, 23). It has been suggested that these prostaglandins participate in the pathogenesis of inflammatory rheumatic diseases by acting as the mediators of inflammation and that they also promote bone resorption (12, 13, 24). These two prostaglandins had no effect on the normal change in cAMP of chondrocytes in suspension culture. Furthermore, the cells synthesized approximately 70–80% of their normal phenotype, i.e., type II collagen, in the presence of these prostaglandins.

The phenomenon of switching of collagen synthesis by chondrocytes dependent upon the extracellular conditions is of great significance. As mentioned earlier, the cartilage involved in degenerative arthritis produces type I collagen in addition to the normal type II collagen (3, 4). The degenerative conditions are generally associated with, for example, remodeling of bone, ectopic calcification, and osteophyte formation. All these changes lead to the alterations in cell–matrix interactions. Such altered interactions can be achieved *in vitro*, which result in the change in collagen phenotype. Examples of these situations are the treatment of cartilage by lysosomal enzymes *in vitro* (25) or the prolonged treatment of chondrocytes with testicular hyaluronidase (26). The genetic changes involved in switching of the phenotype by chondrocytes remain to be studied. It could be argued that there are two different cell populations responsible for the synthesis of type I or type II collagen, and that their synthetic process is selectively turned on or off depending upon the extracellular conditions. The recent reports by Mayne *et al.* (27, 28), however, show that the clones of chick embryonic chondrocytes switch their synthesis of collagen from type II to type I collagen and type I trimer in the presence of 5-bromodeoxyuridine or as a result of aging in culture.

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