ROLE OF CALCIUM IN THE PHENOTYPIC EXPRESSION OF RABBIT ARTICULAR CHONDROCYTES IN CULTURE

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1. Introduction

Articular cartilage contains Type II collagen that is comprised of 3α(II) chains and is genetically distinct from the more ubiquitous Type I collagen [$2\alpha_1$(I)·α_2] from skin, tendon, bone and dentine [1,2]. The osteoarthritic human cartilage synthesizes Type I collagen in organ culture in addition to its tissue specific Type II collagen [3]. Our more recent studies on normal rabbit articular chondrocytes in culture have shown that these cells produce Type I collagen in monolayer cultures. The same cells are capable of synthesing of Type II collagen in suspension cultures in the medium with no added calcium and Type I collagen in the medium containing 1.8 mM $CaCl_2$ [4]. These data suggest the dependence of phenotypic expression of cells on the extracellular conditions. Extracellular levels of Ca^{2+} play a great role in cell division and cell activation in various systems [5,6]. To understand the mechanism underlying the changes in the osteoarthritic cartilage, it is essential to study the behavior of chondrocytes in various defined conditions. In this paper, we demonstrate a direct effect of availability of calcium to the cells on their phenotypic expression. We have also studied the cell behavior after treatment with ionophore A23187, which is known to stimulate the increase in the transmembranous flux of Ca^{2+} into the cells [7–9]; and in the presence of cyclic nucleotides.

* Dulbecco's complete medium - Dulbecco's Modified Eagle's medium containing 4500 mg glucose/liter.

** Dulbecco's special medium - Same medium without $CaCl_2$.

2. Materials and methods

2.1. *Cell cultures*

The chondrocytes were obtained from the articular cartilage of knee and hip joints of 2–3 month old rabbits [10] and were grown to confluency in monolayer cultures with Ham's F-12 nutrient mixture containing 10% fetal calf serum, antibiotics, and in the atmosphere of 5% CO_2 in air. The confluent cells were trypsinized and transferred to the suspension cultures with Dulbecco's complete medium* or special medium** containing 10% fetal calf serum and antibiotics. After 24-48 h, the medium was replaced with the fresh medium which contained, in addition, ascorbic acid (25 μg/ml), β-aminopropinitrile (50 μg/ml) and 100 μCi of [2,3-^{3}H]proline. The incubation was continued for 24 h.

In the control experiments, the chondrocytes were maintained in the suspension culture with special medium. The effect of calcium on the cells was studied by transferring them from monolayer to suspension culture with the complete medium; or adding various amounts of $CaCl_2$ to the special medium. The effect of cyclic nucleotides was observed by the addition of dibutyryl cAMP or dibutyryl cGMP (Sigma) directly to the special medium. The calcium ionophore A23187 (Eli Lilly & Co.) was used as an artificial stimulus to increase the uptake of calcium by the cells. F-12 medium of monolayer cultures was replaced by Dulbecco's complete medium containing A23187 (10^{-5} M–10^{-7} M) and the incubation was carried out for 1 h at 37°C. The cells were trypsinized for

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5 min and transferred to the suspension culture flasks with special medium.

2.2. *Isolation and characterization of collagen*

The culture medium was centrifuged and the cell pellet was extracted with 0.5 M acetic acid. The medium and the cell extract were treated with pepsin (100 μg/ml) for 24 h at 4°C. Rabbit skin acid-soluble collagen was added as a carrier and the mixed collagen was precipitated by the addition of 20% NaCl (w/v) at pH 7.5. The precipitate was dissolved in 0.5 M acetic acid; dialyzed against 0.06 M Na-acetate buffer, pH 4.8, containing 1 M urea and chromatographed on a CM-cellulose column [11].

The labelled collagen synthesized by chondrocytes was mixed with either rabbit skin or rabbit cartilage acid-soluble collagen and was treated with cyanogen bromide [12]. The peptides were characterized by CM-cellulose chromatography.

2.3. *Uptake of ^{45}Ca by chondrocytes and the intracellular levels of cAMP*

The influx of calcium in presence and absence of A23184 was studied by estimating the uptake of ^{45}Ca by the cells in monolayer cultures in the complete medium with 10 μCi of ^{45}Ca. The levels of intracellular cAMP were estimated by the method of Gilman [13].

Table 1
Effect of A23187 on chondrocytes

Treatment	Ca Uptake (cpm/10^6 cells)	cAMP (pmoles/10^6 cells)
None	151 ± 11	3.8 ± 0.9
A23187		
10^{-5} M	506 ± 18	9.3 ± 1.1
10^{-6} M	287 ± 9	7.2 ± 0.8

The data represent mean ± s.e.m. of triplicate samples from two separate experiments.

3. Results and discussion

The types of collagen synthesized by chondrocytes in the suspension cultures in various conditions were assessed from the elution pattern of [^{3}H]-labelled collagen on CM-cellulose column (fig.1A and 1B). The analysis of CNBr peptides confirmed the characterization of collagen type. When the ratio of α_1 :α_2 chains was 2.0, the CNBr peptides revealed the presence of α_1(I) and α_2 chains only (fig:2A). When α_1 :α_2 chain

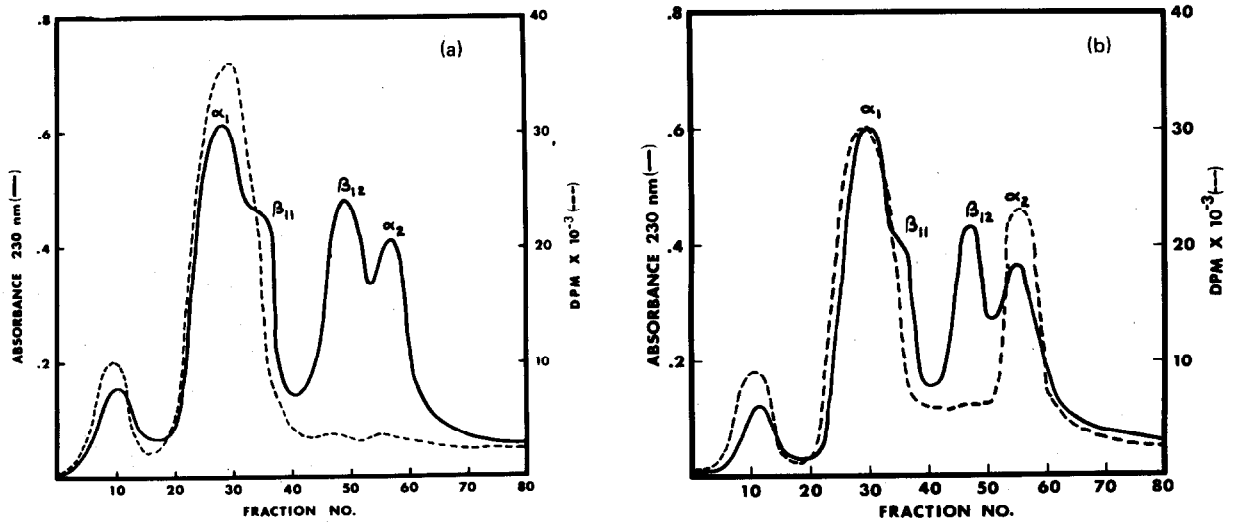

Fig.1. CM-cellulose elution pattern of collagen. (——) Rabbit skin acid soluble collagen. (– – – –) Collagen synthesized by chondrocytes in (A) special medium (no added calcium) and (B) complete medium.

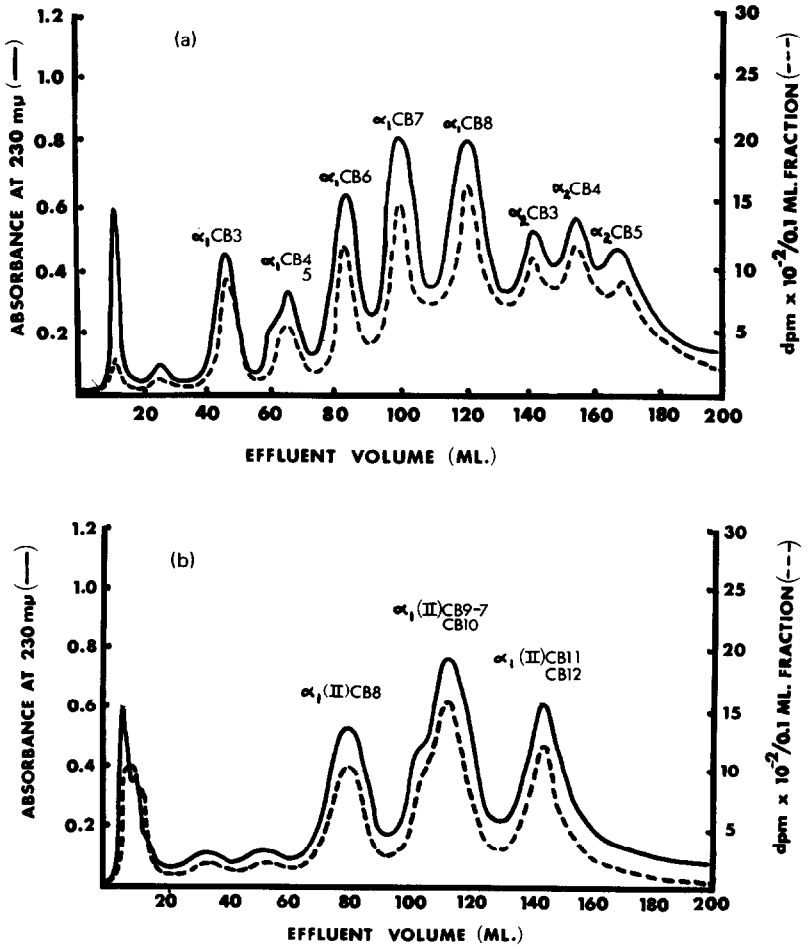

Fig.2. Elution pattern of CNBr peptides. (A) (——) Rabbit skin acid soluble collagen. (– – – –) Chondrocyte collagen with α_1(I) and α_2 chains. (B) (——) Rabbit articular cartilage collagen. (– – – –) Chondrocyte collagen with α_1(II) chains.

ratio was greater than 15 or 20, the peptides were predominantly derived from α_1(II) chains (fig.2B).

Table 2 illustrates the relative proportions of Type I and Type II collagen synthesized under various conditions. The chondrocytes produced Type II collagen in special medium. They changed their phenotype almost exclusively to Type I collagen in complete medium [4]. The cells also responded to the lower extracellular calcium levels, although at 10^{-5} M concentration of $CaCl_2$ this effect was minimal. Calcium present in the serum had no apparent effect on the cells. Incubation of chondrocytes in monolayer cultures with A23187 in the complete medium increased the uptake of calcium by about 2–3-fold at 10^{-6} M and 10^{-5} M concentrations of the ionophore (table 1). The intracellular levels of cAMP were also elevated, When the treated cells were transferred to suspension cultures with special medium, they exhibited a significant change in the synthesis of collagen type. Similar effects were seen when chondrocytes were maintained in the special medium in presence of dibutyryl cAMP. Dibutyryl cGMP had no apparent effect. Neither of these cyclic nucleotides interferred with the change produced by calcium.

The results therefore indicate that extracellular calcium has a definite role in stimulation of these

Table 2
Relative proportions of Type I and Type II collagen synthesized by chondrocytes under various conditions

Treatment		Type I (%)	Type II (%)
Control		0	100
$CaCl_2$	10^{-3} M	91	9
	10^{-4} M	86	14
	10^{-5} M	20	80
A23187	10^{-5} M	61	39
	10^{-6} M	59	41
	10^{-7} M	20	80
db-cAMP	10^{-3} M	76	24
	10^{-4} M	59	41
db-cGMP	10^{-3} M	10	90
	10^{-4} M	0	100
db-cAMP + $CaCl_2$	10^{-3} M, 10^{-4} M	88	12
db-cGMP + $CaCl_2$	10^{-3} M, 10^{-4} M	93	7

The values are the mean of three experiments.

cells to synthesize Type I collagen instead of Type II. Such stimulation also occurs as a pretreatment of the cells with A23187. The reports in the literature suggest that in many cases the primary stimulant may not be calcium itself, but needs calcium for its proper effect [5,6]. Intracellular cAMP then serves as a second messenger. In the absence of external calcium, the intracellular levels of cAMP may be raised, but no subsequent physiological response is observed. Large concentrations of cAMP in the extracellular fluids can mimic the effect of the first stimulant [6]. Whether extracellular cAMP influences the cell by the same mechanism as that of intracellular cAMP is not yet fully understood. In the case of chondrocyte cultures, addition of dibutyryl cAMP to the special medium mimics to some extent the effect of extracellular calcium on the phenotype.

The change in the synthesis of collagen by normal chondrocytes from Type II to Type I in presence of calcium is of great significance. Osteoarthritic joints show the signs of remodelling of subchondral bone, ectopic calcification, focal penetration of blood vessels into the cartilage and osteophyte formation [14]. The elevation of alkaline phosphatase, inorganic pyrophosphate concentrations and the deposition of calcium pyrophosphate in the synovial fluid and the cartilage from osteoarthritic joints have been reported [15–17]. As a result of the elevated level of calcium around the chondrocytes, the cells may switch to the synthesis of Type I collagen [3].

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