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Expression of Adenylyl Cyclase Isoforms in Articular Chondrocytes of Rat

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Abstract.- Chondrocytes exhibit a life cycle of proliferation, differentiation, maturation, and apoptosis; rate of each of these processes is dependent on the expression of temporal and spatial signals. Identification of these signals is essential to understand the molecular basis of chondrocyte physiology. Ca^{2+} and cAMP are second messengers involved in signal transduction cascade. Adenylyl cyclase (AC) is an integral membrane protein that catalyzes the synthesis of cAMP from ATP. In this study we have employed immunohistochemical technique to study expression of AC isoforms in articular chondrocytes. Our results show that AC isoforms I, II, III, IV and V/VI are differentially expressed in articular chondrocytes of the shoulder, elbow, hip, and knee joints. The significance of these findings and the role of expressed isoforms in physiology of articular chondrocytes are discussed.

Keywords: Adenylyl cyclase, second messenger system, articular chondrocytes.

INTRODUCTION

Adenylyl cyclase (AC) is an integral membrane protein that catalyzes the synthesis of 3' 5'-cyclic AMP (cAMP) from ATP. To date, 10 isoforms of AC have been cloned in mammals, each with a distinct set of regulatory elements and varying degrees of amino acid homology. Nine of these are membrane-bound proteins with 12 hydrophobic membrane-spanning domains (Guillou *et al.,* 1999). A soluble AC isoform with a molecular mass of 48 kDa has recently been isolated from rat testis (Buck *et al.,* 1999). All AC isoforms are activated by Gαs subunit of heterotrimeric G protein (Yan *et al.,* 1998). These isoforms are involved in critical signal transduction pathways where they regulate gene transcription, metabolism, and ion channel activity (Krupinski *et al.,* 1992; Tang *et al.,* 1991; Guillou *et al.,* 1999). On the basis of regulatory properties, particularly Ca^{2+}/ calmodu1in sensitivity and amino acid homology, AC isoforms have been classified into five subgroups (Ishikawa and Homcy, 1997). AC isoforms I and VIII form a neuronal subgroup and are mainly expressed in the brain. Both the isoforms are stimulated by Ca^{2+}/calmodulin. Gßγ subunits of heterotrimeric G protein inhibit the catalytic activity of type I; however, it has no effect on isoforms VIII catalytic activity (Cali *et al.,* 1996). AC isoforms II, IV, and VII form a ubiquitous subgroup, which is expressed in multiple tissues. These isoforms are insensitive to Ca^{2+}/calmodulin. In contrast to the neuronal isoforms, the ubiquitous isoforms are stimulated by Gßγ subunits (Volkel *et al.,* 1996). AC isoform III, originally isolated from olfactory tissue, is Ca^{2+}/calmodulin sensitive (Bakalyar *et al.,* 1990; Choi *et al.,* 1992). AC isoforms V and VI are the most closely related isoforms within the mammalian adenyly1 cyclase family. Ca^{2+}/ca1modu1in regulate AC isoforms V/VI in negative manner (Scholich *et al.,* 1997; Wayman *et al.,* 1995; Yoshimura and Copper, 1992). These constitute the cardiac subgroup and are abundantly found in heart muscle. These isoforms are also expressed in several tissues including liver and neurons. AC isoform IX, isolated from a pituitary tumor cell line, shows a unique interaction with calcineurin, a calcium-sensitive serine threonine phosphatase widely expressed in mammalian cells. (Paterson *et al.,* 1995; Antoni *et al.,* 1996)

Articular chondrocytes are essential to maintain a healthy, friction-less articulating surface in synovial joints. Articular cartilage being an

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avascular tissue, articular chondrocytes are dependent on the synovial fluid for nutrients. In such avascular tissues second messenger system may play an essential role in maintaining the healthy state of individual cells. cAMP is an important 2nd messenger of G protein-mediated signal transduction cascade. It is known to regulate intermediary metabolism, cellular proliferation and neuronal signaling (Taussig and Gilman, 1995; Sunahara *et al*., 1996; Montminy, 1997). AC being the effector enzyme in this cascade, it is important to know about the expression of AC isoforms in articular chondrocytes. AC isoforms are known to exhibit tissue-specific expression (Iyengar, 1993). To our knowledge, expression of AC isoforms in articular chondrocytes is not established. In this study we have attempted to localize Ca^{2+}/calmodulin-activated (AC I and AC III), Ca^{2+}/calmodulin-independent (AC II and AC IV) and Ca^{2+}-inhibited (AC V/VI) isoforms in articular chondrocytes in the hip, knee, shoulder and elbow joints of rats using immunohistochemical technique.

MATERIALS AND METHODS

Reagents

Purified rabbit polyclonal antibodies against AC I, II, III, IV, V/VI and their corresponding blocking peptides were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Diaminobenzidine (DAB) substrate kit and Vectastain *Elite* ABC kit were purchased from Vector laboratories, Burlingame, CA, USA. All other reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Animals

Sprague-Dawley rats were housed in the Aga Khan University animal house. Research proposal was approved by the University Ethics Committee for Research on Animals.

Dissection and tissue processing

For this investigation, 5-10 weeks old Sprague-Dawley rats of either sex were anesthetized with ether and perfused transcardially with the fixative consisting of 2% paraformaldehyde 0.1% glutaraldehyde in 100mM phosphate buffer, pH 7.4 at 4°C. Fixative was injected into shoulder, elbow, hip and knee joint cavities to obtain adequate preservation of articular cartilage. Subsequently the limbs were detached under dissecting microscope. Surrounding muscles and other structures were removed and joint capsules were opened. The articulating bones were cut distal to the epiphyses and immediately transferred to cold fixative for additional 3-4 hours. Specimens were washed overnight in 100mM phosphate buffer, and transferred to 3% EDTA at 4°C for decalcification of the attached bone. EDTA solution was changed daily for 10 days. Following decalcification tissues were washed in buffer, dehydrated in graded ethanol series and embedded in paraffin. Tissue sections were cut at 5 µm on rotary microtome and used for immunohistochemical localization of AC isoforms in articular chondrocytes.

Immunostaining

Sections were deparaffinized and rehydrated in graded ethanol series and washed with 100 mM phosphate buffered saline (PBS), pH 7.4 at room temperature. The sections were incubated in 0.1 % sodium borohydride and 50 mM glycine in PBS and 3% hydrogen peroxide in water to quench free aldehyde groups and block endogenous peroxidase activity, respectively. The sections were then incubated with normal serum for one hour to block nonspecific binding. Sections were incubated with the primary antibody solutions at the dilutions that varied from 1:25 to 1:500 for 12-16 hours at 4°C. All subsequent incubations were done at room temperature. Sections were washed with PBS and then incubated with biotinylated secondary antibody for 30 minutes. After washing with PBS sections were incubated with the ABC reagent for 30 minutes. Sections were washed with PBS and incubated with the peroxidase substrate. Sections were washed in tap water and one section on each slide was counter-stained with hematoxylin and mounted with glycerol. Sections incubated with primary antibody preabsorbed with the control peptide served as negative controls. Tissues known to express AC isoforms (cerebrum, cerebellum, hippocampus and olfactory epithelium) were used as positive controls (results not shown). Sections were photographed on Olympus Vanox microscope using Kodak Pro Image 100 film.

RESULTS

In the present work we report the presence of AC isoforms in articular chondrocytes. We have employed immunohistochemistry technique to study cellular localization and distribution of six AC isoforms in articular chondrocytes of rat hip, knee, shoulder and elbow joints. Antibodies were diluted at 1:25, 1:100, 1:200, 1:300 and 1:500. Minimal concentration of the antibody that gave positive results in control tissues was used to detect the AC isoforms in articular chondrocytes.

In all the joints it was noted that articular chondrocytes near epiphyses (deeply placed in the articular cartilage) were larger in size. These cells showed vacuolization of the cytoplasm and empty lacunae were often present in this region of the articular cartilage. The middle zone of articular cartilage exhibited good chondrocyte morphology and few empty lacunae were present. The chondrocytes near the free surface of articular cartilage were invariably smaller in size and empty lacunae were seldom present. Though chondrocytes in all three regions of the articular cartilage exhibited positive immunoreactivity, intensity of staining varied from zone to zone.

Immunoreactivity for AC isoforms I, II, III, IV and V/VI was observed in articular cartilages of scapula, humerus, radius, ulna, hip bone, femur and tibia (Figs. 1, 3, 5, 7 and 9). No immunoreactivity was observed in the articular cartilages upon incubation with preabsorbed antibody solutions (Figs. 2, 4, 6, 8 and 10). For positive controls we used the following tissues: cerebellum for AC isoforms I, IV and V/VI; hippocampus for AC isoforms I and II; olfactory epithelium for AC isoform III (results not shown for positive controls).

Differential immunoreactivity was noted in articular cartilages of all the joints examined. Intensity of immunostaining was found to vary among the AC isoforms as well (Figs. 1, 3, 5, 7 and 9). AC I and AC III immunoreactivity was determined to be intense (Figs. 1 and 5). Chondrocytes in the middle zone were immunoreactive whereas cells in the deeper zone were not. Surface chondrocytes were weakly reactive (Fig.1). AC III immunoreactive chondrocytes were present in the deeper and middle zones. Chondrocytes near the free surface of the articular cartilage lacked immunoreactivity (Fig.5). Moderate immunoreactivity was observed in articular cartilage incubated with antibodies for AC isoforms II and IV (Figs. 3, 5 and 7). For AC II uniform staining was seen in the deep and middle zones. Chondrocytes near the surface were weakly immunoreactive (Fig. 3). For AC IV isoform, moderate immunoreactivity was observed in all three zones of the articular cartilage (Fig. 7). AC V/VI immunoreactivity was considered to be weak, though all chondrocytes were immunoreactive (Fig.9).

DISCUSSION

In the present investigation specific patterns of AC isoform immunoreactivity have been observed in articular chondrocytes of hip, knee, shoulder, and elbow joints in the rat. External stimuli are presumed to bind with specific receptors upstream in the cAMP cascade that couple to specific G proteins, which in turn stimulate or inhibit membrane-bound AC enzymatic activity affecting cAMP levels (Taussig and Gilman, 1995). Since AC isoforms exhibit tissue-specific distribution, AC enzymatic activity at a given membrane site may reflect expression of one or more isoforms defining a unique pharmacology (Tang and Gilman, 1995; Hurley, 1999; Antoni, 2000; Onda *et al.,* 2001). Modulation of the enzymatic activity of individual isoforms with specific pharmacology at discrete cellular sites is key to the transduction by the cAMP-second messenger cascade of signal derived from external stimulus upstream to effect a biological response downstream (Drescher *et al.,* 2000). cAMP activates protein kinase A (PKA) to modulate physiological functions including transcription (Scott, 1991; Taussig *et al.,* 1994; Iwami *et al.,* 1995).

The distinct properties of the individual isoforms allow them to play important roles in signal transduction. The integration of the multiple signals by AC is a dynamic process and that the ability of the different AC types to respond to activated Gαs, Gαi, Gßγ, Ca^{2+}, and phosphokinases places the enzyme at a central position for cross-talk between different signaling pathways (Nicole *et al.,* 2000). Depending on the properties and the relative

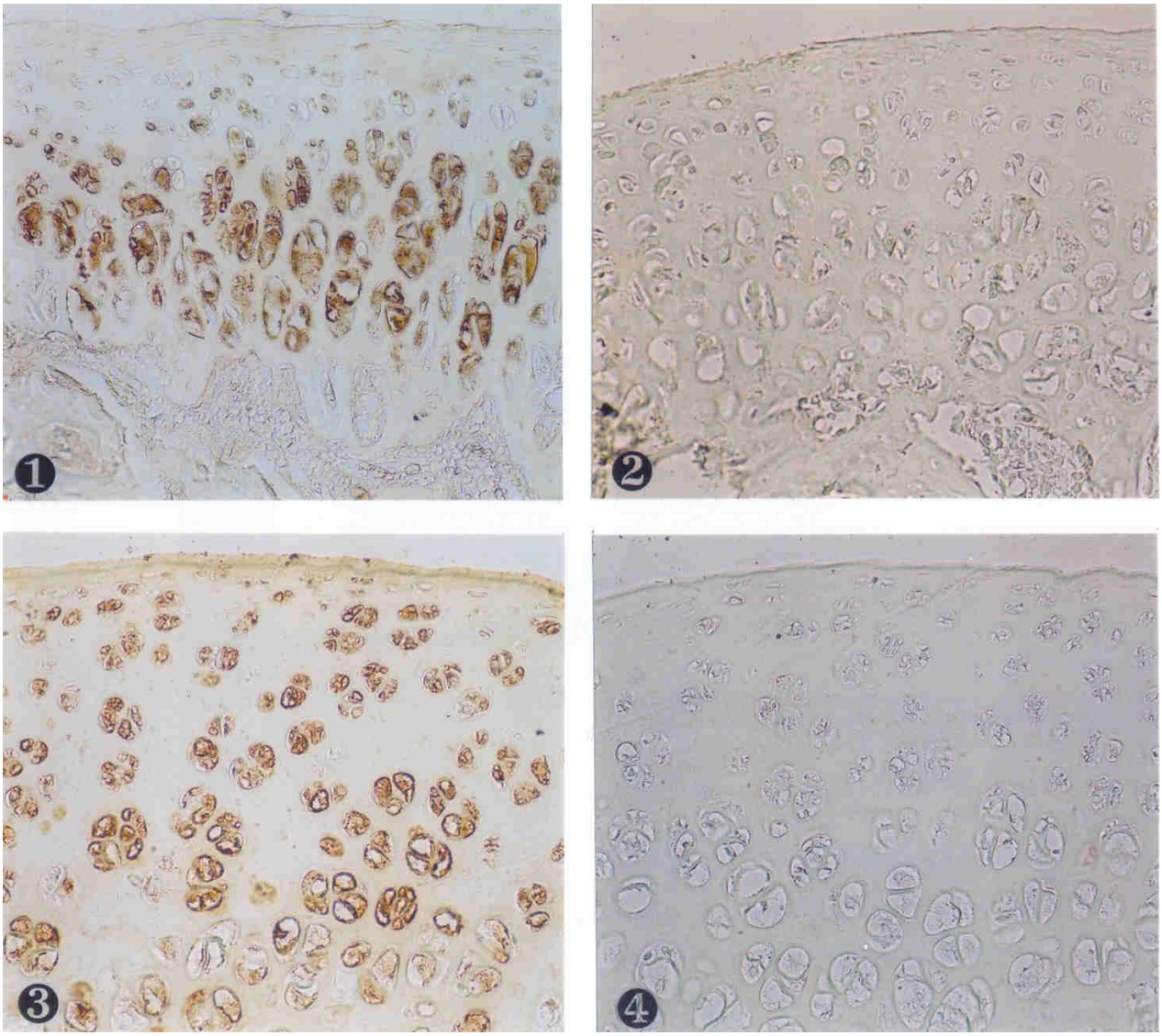

Fig. 1. Immunoreactivity to AC I (1:100 dilution) in articular chondrocytes in the head of femur. Articular chondrocytes in middle zone of cartilage show intense staining. Near the free surface of the articular cartilage chondrocytes are smaller with weak staining. Chondrocytes in the deeper zone do not exhibit immunoreactivity.

Fig. 2. No immunoreactivity is seen in articular chondrocytes in the section of head of femur incubated with AC I antibody preabsorbed with the control peptide.

Fig. 3. Immunoreactivity to AC II (1:500 dilution) in articular chondrocytes in the condyle of femur. Chondrocytes in the middle and deeper zones exhibit moderate staining. Surface chondrocytes show weak immunoreactivity.

Fig. 4. No immunoreactivity is seen in articular chondrocytes in the section of condyle of femur incubated with AC II antibody preabsorbed with the control peptide.

Sections were photographed with 20 x objective lens and negatives were magnified 4.5 x. Final magnification of all figures is 90 x.

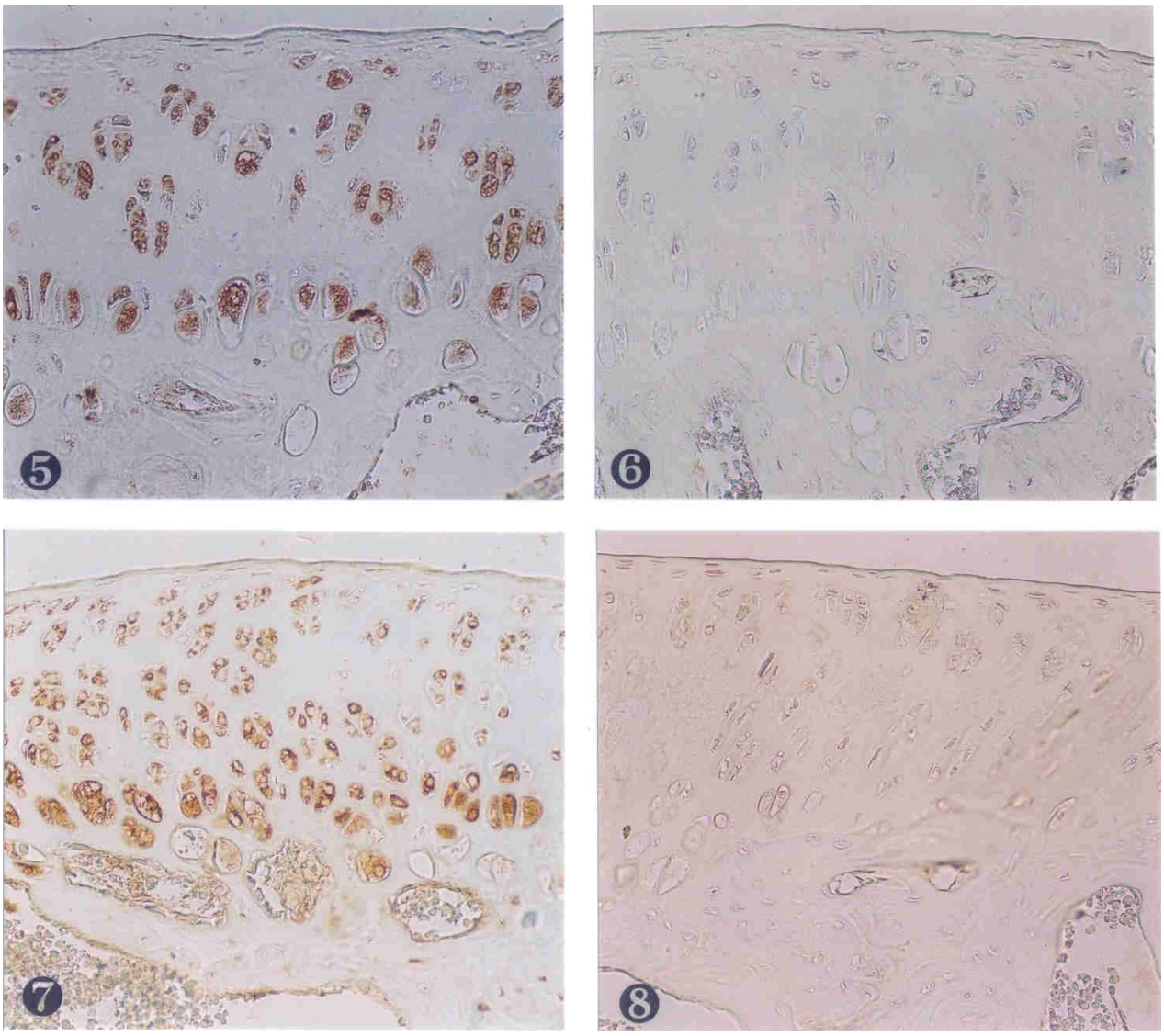

Fig. 5. Immunoreactivity to AC III (1 :500 dilution) in articular chondrocytes in the condyle of tibia. Articular chondrocytes in the deeper and middle zones exhibit intense staining. Surface chondrocytes do not show staining.

Fig. 6. No immunoreactivity is seen in articular chondrocytes in the section of condyle of tibia incubated with AC III antibody preabsorbed with the control peptide.

Fig. 7. Articular chondrocytes in the head of femur show uniformly distributed moderate immunoreactivity to AC IV (1 :500 dilution).

Fig. 8. No immunoreactivity is seen in articular chondrocytes in the section of head of femur incubated with AC IV antibody preabsorbed with the control peptide.

Sections were photographed with 20 x objective lens and negatives were magnified 4.5 x. Final magnification of all figures is 90 x.

levels of the AC isoforms expressed in a cell, extracellular signals can be differently integrated. In our study the presence of AC isoforms in articular chondrocytes suggest a role for cAMP in signaling cascade. Though immunohistochemistry is a qualitative technique, differences in intensity of immunoreactivity for various AC isoforms noted in articular cartilage of all the joints examined may be

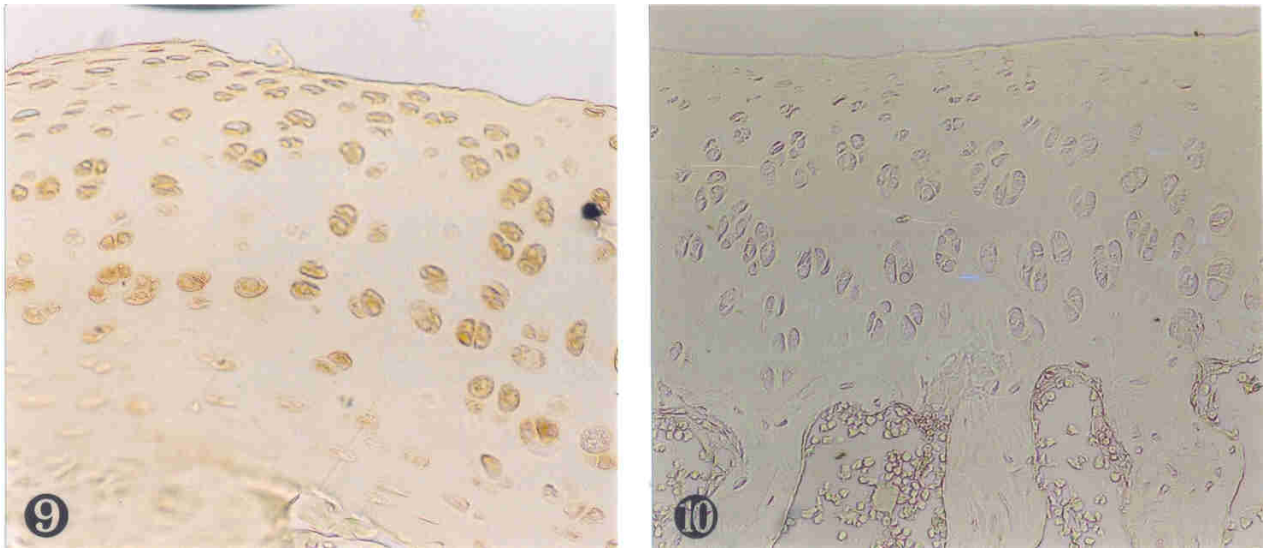

Fig. 9. Note uniformly distributed weak immunoreactivity to AC V/VI (1:500 dilution) in articular chondrocytes in the head of femur.

Fig. 10. No immunoreactivity is seen in articular chondrocytes in the section of head of femur incubated with AC V/VI antibody preabsorbed with the control peptide.

Sections were photographed with 20 x objective lens and negatives were magnified 4.5 x. Final magnification of all figures is 90 x.

an indication of variations in cAMP-dependent signal transduction cascade in articular chondrocytes. There is evidence that suggests that qualitative and quantitative expression of AC isoforms provide a biochemical signature of tissue-specific cAMP generation (Takeshi *at al.,* 2001). It is thus tempting to speculate that expression of Ca^{2+}/calmodulin-dependent and Ca^{2+}/calmodulin-independent AC isoforms in articular chondrocytes maintain cartilage-specific level of cAMP.

Articular cartilage is a unique tissue; it is avascular and unlike cartilage present elsewhere in the body, it lacks perichondrium. Synovial fluid is the major, if not the only, source of nutrients for articular chondrocytes. These cells have to maintain a differentiated state in order to synthesize and secrete cartilage-specific matrix components. Differentiated state of chondrocytes is regulated by complex protein kinase signaling pathways, among which protein kinase C (PKC) has an integral role (Yoon *et al.,* 2002; Lee *et al.,* 2004). PKC is known to stimulate AC I, II and III isoforms (Jacobowitz *et al.,* 1993; Lustig *et al.,* 1993). We have noted moderate to intense immunoreactivity to AC II, I and III isoforms, respectively (Figs. 1, 3 and 5). Presence of PKC and AC isoforms in middle and deeper zones of articular cartilage suggest an intricate balance between these enzymes and their importance in maintaining the differentiated state of articular chondrocytes.

Chondrocytes in middle zone of the articular cartilage are important for maintaining the cartilaginous matrix. In order to support the matrix, chondrocytes have to maintain their differentiated state, as well as these cells have to increase their number by interstitial growth. In order to carry out these diverse functions, chondrocytes would require cross-talk between signal transduction pathways. AC I and III isoforms are members of Ca^{2+}/calmodulin-dependent AC subfamily. Intense AC I and AC III immunoreactivity in these chondrocytes suggest intricate balance in the availability of free cytosolic calcium in articular chondrocytes. The increase in free cytosolic calcium in articular chondrocytes in response to insulin-growth factors and 1 and 2 (IGF-1 and IGF-2) have been shown (Poiraudeau *et al.,* 1997). These observations indicate that two possibilities exist for cross-talk between signal transduction pathways in articular chondrocytes of deeper and middle zones.

Firstly, IGF-l and IGF-2 induce increase in intracellular Ca^{2+} causing activation of AC I and AC III isoforms resulting in cAMP production. cAMP in turn would activate protein kinase A (PKA). PKA would activate phospholipase C resulting in downstream activation of protein kinase C through inositol phosphate and diacylglycerol. Once activated, PKC would maintain the differentiated state of chondrocytes (Lee *et al.,* 2004). The other possibility is that cAMP-activated PKA would enter the nucleus and phosphorylate cAMP-response element binding protein (CREB) gene. CREB gene is known to be involved in proliferation of hepatocytes (Della Fazia *et al.,* 1997). It is likely that chondrocyte proliferation, as seen during interstitial growth of cartilage, is also modulated by cAMP-activated PKA and downstream phosphorylation of CREB gene.

Importance of cAMP as a second messenger is established almost ubiquitously. Immuno-histochemical localization of AC isoforms in articular chondrocytes suggests that cAMP is involved in signaling cascades. Analysis of expression of more than one AC isoforms in articular chondrocytes is a challenging task. Further studies are necessary to understand the complexity of signaling pathways. We intend to characterize other components of cAMP regulated pathways in articular chondrocytes.

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