Histol Histopathol (1999) 14: 1011-1022

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Sodium transport systems in human chondrocytes I. Morphological and functional expression of the Na^+,K^+-ATPase α and ß subunit isoforms in healthy and arthritic chondrocytes

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Summary. The chondrocyte is the cell responsible for the maintenance of the articular cartilage matrix. The negative charges of proteoglycans of the matrix draw cations, principally Na^+, into the matrix to balance the negative charge distribution. The Na^+,K^+-ATPase is the plasma membrane enzyme that maintains the intracellular Na^+ and K^+ concentrations. The enzyme is composed of an α and a ß subunit, so far, 4 α and 3 ß isoforms have been identified in mammals. Chondrocytes are sensitive to their ionic and osmotic environment and are capable of adaptive responses to ionic environmental perturbations particularly changes to extracellular [Na^+]. In this article we show that human fetal and adult chondrocytes express three α (α1, α2 and the neural form of α3) and the three ß isoforms (ß1, ß2 and ß3) of the Na^+,K^+-ATPase. The presence of multiple Na^+,K^+-ATPase isoforms in the plasma membrane of chondrocytes suggests a variety of kinetic properties that reflects a cartilage specific and very fine specialization in order to maintain the Na^+/K^+ gradients. Changes in the ionic and osmotic environment of chondrocytes occur in osteoarthritis and rheumatoid arthritis as result of tissue hydration and proteoglycan loss leading to a fall in tissue Na^+ and K^+ content. Although the expression levels and cellular distribution of the proteins tested do not vary, we detect changes in *p*-nitrophenylphosphatase activity "in situ" between control and pathological samples. This change in the sodium pump enzymatic activity suggests that the chondrocyte responds to these cationic environmental changes with a variation of the active isozyme types present in the plasma membrane.

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Key words: Na^+,K^+-ATPase, Osteoarthritis, Cartilage, Rheumatoid arthritis

Introduction

Chondrocytes are the cells of the cartilage and form groups of one to five cells, which are called chondrons. The concentration of free cations in the extracellular environment of the cartilage is unusually high (350-450 mM Na^+, 8-15 mM K^+) (Maroudas, 1979). This very high saline concentration is due to the fixed negative charges of glycosaminoglycans, which are constituents of extracellular matrix proteoglycans. Glycosaminoglycans control the ionic composition (particularly Na^+ concentration) of the extracellular matrix of cartilage (Maroudas, 1979; Mobasheri et al., 1998). The extracellular Na^+ concentration ([Na^+]) is directly related to the concentration of the fixed negative charges, and hence to the local concentration of glycosaminoglycans. In addition, the extracellular [Na^+] in the vicinity of chondrocytes will vary during static joint loading, as fluid expression from cartilage will increase the local glycosaminoglycan concentrations, simultaneously increasing the extracellular [Na^+] (Maroudas, 1979). The intracellular [Na^+] will depend on chondrocyte permeability and extracellular [Na^+]; although the permeability of cations such as Na^+ and K^+ across the chondrocyte plasma membrane is low, they tend to move down their electrochemical gradient.

The Na^+,K^+-ATPase (EC. 3.6.1.37) is the membrane-bound protein responsible for maintaining the K^+ and Na^+ intracellular concentrations characteristic of most animal cells. The functional enzyme consists of two subunits: the catalytic α subunit (112 kDa) and a smaller ß subunit (a 45kDa glycoprotein) (Sweadner, 1989; Fambrough et al., 1994). So far, four different

isoform variants have been described for the α subunit (α1-4) (Shamraj and Lingrel, 1994). Of the four known α isoform genes, α1 appears to be ubiquitously expressed in all cells and tissues tested so far (Fambrough et al., 1994; Shamraj and Lingrel, 1994; Sweadner, 1995) whereas the α2 and α3 isoforms are expressed primarily in excitable tissues; α2 has been found mainly in the brain, heart and skeletal muscle (Sweadner, 1989); α3 is the most tissue-restricted in its expression, being found primarily in neurons in the brain (Sweadner, 1995) and in human and primate myocardium (Sweadner et al., 1994); α4 expressed only in testis (Shamraj and Lingrel, 1994).

The ß subunit is a highly glycosylated protein that interacts with the α-subunit and is involved in ion recognition (Jaisser et al., 1992). Three different isoforms of the ß subunit (ß1-3) have been described in mammals (Martin-Vasallo et al., 1989; Malik et al, 1996). The ß2 isoform is an adhesion molecule found on glial cells (AMOG), specifically involved in neuron-astrocyte adhesion (Gloor et al., 1990). No adhesion function has been reported for the ß1 or ß3 isoform. A mutual dependency of the α and ß subunits for the adoption of a correct conformation necessary for their transport out of the endoplasmic reticulum has been demonstrated (Geering, 1991).

The chondrocyte has a high number of Na^+,K^+-ATPase copies per cell (~1.5 x 10^5) (Mobasheri et al., 1994). If extracellular [Na^+] is experimentally elevated, Na^+ ions gain entry to the cytosol in chondrocytes via several proteins abundantly expressed in the plasma membrane. Increased intracellular [Na^+] is a stimulus for Na^+,K^+-ATPase upregulation (Brodie and Sampson, 1989). Therefore, since Na^+,K^+-ATPase abundance is regulated by intracellular [Na^+], the expression and plasma membrane density of the pump is likely to vary with extracellular [Na^+] and play an important role in the adaptation of the chondrocyte to changes in its extracellular environment.

Immunofluorescence microscopy and "in situ" hybridization were used to examine the expression of the α and ß subunit isoforms of the Na^+,K^+-ATPase and localize the distribution of these subunits in human fetal and adult normal chondrocytes and in chondrocytes of patients affected of rheumatoid arthritis (RA) and osteoarthritis (OA). The Na^+,K^+-ATPase enzymatic activity was detected as K^+-dependent *p*-nitrophenylphosphatase (*p*-NPPase) activity "in situ" in cartilage sections.

RA is a chronic multisystem disease of unknown etiology that affects from 0.3 to 2.1% of the population and courses with marked joint deformity and progressive disability. The characteristic feature of RA is a persistent synovial inflammation that causes cartilage destruction and bone erosion and subsequently joint deformities. OA, also called degenerative joint disease, is the failure of the diarthrodal joint. OA can be primary (unknown cause) or secondary to an attributable underlying cause. Load-bearing areas of articular cartilage are the most affected in OA. In the early stages the cartilage is thicker and chondrocytes replicate forming clusters. As the disease progresses, the cartilage becomes thinner, softer and the surface breached develops vertical clefts; chondrocytes disappear little by little, and the cartilage becomes hypocellular. A common feature of the cartilage in both diseases is the destructuration of glycosaminoglycans that lead to changes in the ionic strength (Maroudas, 1979, 1980) and might subsequently regulate the expression or redistribution of ionic transporters of the chondrocyte plasma membrane. Since the Na^+,K^+-ATPase is a principal regulator of cellular ion homeostasis, it was likely to be involved in situations where the ionic environment of chondrocytes is drastically altered, as in RA and OA, where tissue hydration and proteoglycan loss occurs leading to a fall in tissue Na^+ and K^+ content.

The results of the present study demonstrate that the chondrocyte expresses the α1, α2 and α3 isoforms of the catalytic subunit and the ß1, ß2 and ß3 isoforms of the regulatory subunit of the plasma membrane Na^+,K^+-ATPase. This expression pattern does not change in the chondrocyte of the cartilage affected by RA or OA, although variations in *p*-NPPase activity suggest a change in the isoforms protomeric association.

Materials and methods

Tissue sampling

Samples of articular cartilage from human fetus were taken from metacarpal phalangeal and tibia epiphysis of 4 fetus ranging from 22 to 25 weeks. Pathological samples of human hip (29 samples, 20 OA and 9 RA) and knee (14 samples, 8 OA and 6 RA) joints were obtained following orthopedic replacements, hip (5 samples) and knee (4 samples) from traumatic incidents and necropsies (5 samples) were obtained fresh. All the samples were obtained from the Pathology Department Bank of the Hospital Universitario de Canarias following the regulations of the Helsinki Convention and the Ethical Committee of the Hospital. Pieces of 0.5 to 1.0 cm length by 0.25 cm wide and 0.20 to 0.25 cm thick of hyaline cartilage were dissected, rinsed in saline solution (NaCl 9 g/L) and placed in the same solution until processing for histology. For studies on the ß3 isoform, joints from legs of 2 month old Sprague-Dawley rats were obtained from La Laguna University Animal House. The samples were embedded in Tissue Tek (Miles Inc., Elkhart, IN), frozen and sectioned with a cryostat.

Antibodies used

Information and references about the specific antibodies used in this study to detect isoforms of the Na^+,K^+-ATPase subunits are shown in Table 1.

Immunofluorescence microscopy

Cryostat 5 μm sections were fixed in 4% paraformaldehyde in phosphate buffer 0.1M pH 7.4 for 15

Table 1. Antibodies used in this study to detect isoforms of the Na,K-ATPase subunits

ANTIBODY	ISOFORM	WORKING DILUTION	SOURCE	REFERENCE
McK1	α1	1:5	K.J. Sweadner	Peng et al., 1997
McB2	α2	1:5	K.J. Sweadner	Peng et al., 1997
McBX3	α3	1:5	K.J. Sweadner	Arystarkhova and Sweadner, 1996
XVIF9G10	α3	1:5	K.J. Sweadner	Arystarkhova and Sweadner, 1996
SpETß1	ß1	1:1500	P. Martin-Vasallo	Gonzalez-Martinez et al., 1994
SpETß2	ß2	1:2000	P. Martin-Vasallo	Gonzalez-Martinez et al., 1994
RTN ß3	ß3	1:2000	K.J. Sweadner	Arystarkhova and Sweadner, 1997

minutes at room temperature, except the sections probed with McK1, McB2 and McBX3, that were fixed with periodate-lysine-paraformaldehyde (McLean and Nakane, 1974). Subsequent steps were common for all the antibodies. The sections were washed twice and permeabilized in 0.3% Triton X-100, 0.1% bovine serum albumin (BSA) in phosphate buffer 0.1M pH 7.4 for 10 minutes at room temperature. This buffer was also used for all the washing steps. Non specific staining was prevented by treatment with 2% BSA, 0.3% Triton X-100, phosphate buffer 0.1M pH 7.4 for 30 minutes at room temperature. The same solution was also used for dilution of all antibodies. The primary antibodies were diluted to their working concentration (Table 1) and sections were incubated in a humid chamber either 2 hours at room temperature or overnight at 4 °C. The unbound antibody was removed by treatment with washing buffer three times, 10 minutes each, at room temperature. Fluorescent secondary antibodies were obtained from Sigma Chemical Company (St. Louis, MO). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used with polyclonal primary antibodies at 1:250 and tetramethylrhodamine-conjugated goat anti-mouse IgG was used with monoclonal primary antibodies at 1:50. These antibodies were applied to the sections and incubated for 1 hour at room temperature in a humid chamber. The slides were then washed 3 times for 10 minutes and mounted in glycerol:phosphate buffer saline (PBS) 9:1. Slides were examined and photographed with an Olympus BX-50 fluorescence microscope. Control slides were incubated without either primary or secondary antibody. In order to test further the specificity of the staining competing experiments were carried out. For this purpose, binding of the antibodies was competed including a specific antigen in the incubation mix. For ß1 and ß2 the antigens were 0.8 ng/μL of the truncated proteins expressed in *E. coli* (Gonzalez-Martinez et al., 1994). For ß3 the competitor was 40 ng/μL of a semipurified preparation of Na,K-ATPase from plasma membranes of C6 cell line, which has been shown to express only ß3 isoform (Arystarkhova and Sweadner, 1997), kindly provided by K.J. Sweadner (Massachusetts General Hospital). The rest of the procedure was as described above.

Immunohistochemistry of the α1, α2 and α3 isoforms in fetal cartilage was performed with the avidin-biotin system of Vector Laboratories (Burlingame, CA). Sections were first incubated with 1% hydrogen peroxide in 100% methanol for 20 min to quench and thus eliminate endogenous peroxidase staining. Following this treatment, the sections were washed twice for 20 minutes each in 0.1M phosphate buffer with 0.1% BSA and 0.2% Triton X-100. This buffer was used in the following washes and incubations. Sections were then incubated overnight at 4 °C with specific antibodies. The sections were then washed twice in buffer, incubated for 2 hours at 22 °C with the secondary antibody (biotinylated anti-mouse IgG, Vector, diluted 1:250) and again washed 3 times with buffer. Peroxidase activity was revealed with 0.01% hydrogen peroxide, using 3,3'-diaminobenzidine (DAB) as the chromogen. Immunostaining was absent when incubation with the primary antibody was omitted.

"In situ" hybridization

Sense and antisense digoxigenin (DIG)-labeled cRNA probes were generated by in vitro transcription. T7 or T3 RNA polymerases were used in the presence of 10 mM ATP, CTP, GTP and DIG-11-UTP and 1 μg of linearized plasmid containing inserts of either α2 (229 base pairs), α3 (157) or ß3 (634). This inserts were cloned from polymerase chain reaction (PCR) products obtained with the following isoform-specific primers:
Fα2: GGAATTCGAAGAACCAGGCATGGAA
Rα2: CGGGATCCAAAGGATCTGGTCGGGG
Fα3: GGAATTCGGCGCTGCAGAGGCTCC
Rα3: CGGGATCCGCGCTCCTTGCCCTTGT
Fß3: CGGGATCCCGTGACCAGATTCCTAG
Rß3: GCCCTAGGCAACACAACATTTACTCT

The amplified regions show low homology with other Na^+,K^+-ATPase isoforms.

Samples of normal articular cartilage from human femoral head from traumatic incidents were rinsed in diethyl pirocarbonate (DEPC) treated PBS and fixed in 4% formaldehyde before freezing them. Cryostat 5 μm sections were spread in gelatine-coated slides, dried in a 50 °C oven and stored at -80 °C.

"In situ" hybridization and detection of hybridized DIG-probes was performed essentially as described (Deleersnijder et al., 1996). Briefly, sections were rinsed in DEPC-treated PBS and the same buffer plus 100 mM glycine, permeabilized with 0.3% Triton X-100, washed again in PBS and deproteinized for 30 minutes at 37 °C

with 1 μg/mL proteinase K in 100 mM Tris-HCl, 50 mM ethylene diamine tetraacetic acid (EDTA), pH 8. Sections were postfixed for 5 minutes at 4 °C with 4% formaldehyde in PBS, rinsed again in PBS and acetylated for 10 minutes with 0.25% acetic anhydride in 0.1M triethanolamine buffer. Then, sections were prehybridized for 10 minutes at 37 °C with 4xSSC (1xSSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.2) containing 50% formamide. Hybridization was performed in 40% formamide, 10% dextran sulfate, 1x Denhart's solution (0.02% ficoll, 0.02% polyvinyl-pyrrolidone, 10 mg/mL RNase-free BSA), 4xSSC, 1mg/mL yeast t-RNA and 1mg/mL denatured and sheared salmon sperm DNA. Sections were overlaid with 100 μL of hybridization solution containing 10 ng of DIG-labeled cRNA probe, incubated at 42 °C for 16 hours and washed twice with 2x SSC and twice with 1xSSC at 37 °C 15 minutes each time. To digest unbound single stranded probes sections were incubated for 30 minutes at 37 °C with 20 μg/ml RNase A in 500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8 and washed twice with 0.1xSSC at 37 ºC 30 minutes each time. Immunological detection was performed with the Boehringer Mannheim (Mannheim, Germany) immuno-detection kit following manufacturer instructions. The alkaline phosphatase-conjugated anti-DIG antibody was used at 1:1000 dilution. The color reaction was developed for 16 hours in the presence of 4-nitro blue tetrazolium chloride and 5-bromo 4-chloro 3-indolyl phosphate and stopped with 10 mM tris-HCl, 1 mM EDTA pH 8. Sections were rinsed with distilled water, mounted in PBS:glicerol 1:9 and visualized under a Olympus BX50 microscope. Tissues were not counter-stained. Negative controls consisted in treating sections with RNase A prior to hybridization, omitting the probe or the anti-DIG antibody. All of them resulted in the absence of signal.

Cytochemical localization of K⁺-dependent p-nitrophenylphosphatase activity

Cartilage human samples from femoral head (3 from traumatic incidents and 6 from orthopedic replacements, 3 OA and 3 RA) were rinsed in saline and frozen. Cryostat 5 microns sections were stored at –20 °C until use. The following procedure was essentially as described (Zemanová and Pácha, 1996) with minor changes. Sections were fixed for 1 minute at 4 °C with 2% formaldehyde in 100 mM cacodylate buffer. They were then incubated for 30 minutes at room temperature in 50 mM Tricine, 8 mM levamisole and 40 mM L-phenylalanine, pH 7.5. Levamisole and phenylalanine were added to inhibit alkaline phosphatase activity. Nonspecific staining was established as the background signal when the incubation medium included 10 mM ouabain. Sections were then incubated for 75 minutes at 37 ºC in the reaction medium, which consisted in 10 mM $MgCl_2$, 50 mM KCl, 2 mM Mg-p-nitropheylphosphate (p-NPP), 2 mM $CeCl_3$, 40 mM L-phenylalanine, 8 mM levamisole, 50 mM Tricine, pH 7.5. In controls sections, NaCl substituted KCl, p-NPP was omitted or 10 mM ouabain was added. Sections were rinsed three times at room temperature with 50 mM Tricine, pH 7.5 and the visualization medium (1.4 mM DAB, 0.002% H_2O_2, 100 mM sodium azide, 12 mM nickel hexammonium sulfate, 50 mM Tris-HCl, pH 7.6) (Halbhuber et al., 1988), was added for 15 minutes at 60 °C. Sodium azide was added to inhibit endogenous peroxidase activity. Sections were rinsed in distilled water and mounted in PBS:glycerol 1:9 for visualization under light microscopy.

Results

The expression of the different isoforms of the plasma membrane Na⁺,K⁺-ATPase α and ß subunits have been studied in the chondrocyte of the human articular cartilage. These studies were done in fetal cartilage, in healthy adult cartilage and in cartilage of patients afflicted with OA and RA. We have also studied the "in situ" enzymatic activity of Na⁺,K⁺-ATPase.

The fetal chondrocytes appear like immature mesenchymal cells that tend to have a star-like profile with some slender lengthening. All the cells show immunoreactivity for the α1, α2, α3, ß1 and ß2 isoforms of Na⁺,K⁺-ATPase (Fig. 1). The α4 isoform was not tested because of two reasons: it is considered to be a testes specific isoform and there are no specific antibodies available.

Figure 2 shows the immunolocalization of the a subunit isoforms of Na⁺,K⁺-ATPase of the normal and pathologic adult cartilage. Chondrocytes of the normal adult cartilage (Fig. 2A-C) are spheroid and generally are present as single cells, whereas some times occur in groups of two or three per chondron. Chondrocytes show positive immunoreactivity for all of the three (α1, α2, and α3) isoform antibodies. α3 isoform was also detected with XVIF9-G10 antibody (data not shown). No spurious labeling is seen inside the chondrons when primary antibody is omitted, although there is an intense, diffuse background in the intercellular matrix and in the perichondral region. This signal was present in every sample, even when the secondary antibody was omitted (data not shown). Thus this background is due to intrinsic tissue fluorescence. This signal persisted with other blocking strategies, but it does not interfere with the specific staining seen inside the chondrons. This effect is more evident in cartilage from adult than in cartilage from fetus. Previous studies in cartilaginous tissues have shown that collagen and elastin fibres of connective tissues results in a high degree of tissue autofluorescence (Swatland, 1988). Other studies have demonstrated that this intrinsic fluorescence increases with aging as a direct result of collagen cross-linking by lysyl oxidase in various connective tissues such as the cornea (Sady et al., 1995) and rabbit articular cartilage (Pokharna et al., 1995).

In the cartilage of patients affected of OA (Fig. 2D-F), chondrons appear swollen and distended with

proliferation of the chondrocytes within the chondron, in groups of three to five cells per chondron. Osteoarthritic chondrocytes express the three α subunit isoforms. Not detectable variation in the relative expression levels of any isoform with respect to the control was observed. The cartilage of the patient affected of RA (Fig. 2G-I) shows its chondrocytes as regressive cells with irregular surface and degenerated-like look. The pericellular lacunar spaces are not evident. Chondrocytes of RA express the α1, α2, and α3 subunit isoforms. Neither detectable variation in the expression levels of this isoforms are seen. Just the morphological changes described previously can be found among the three groups (normal, OA and RA).

Figure 3 shows the immunolocalization of the ß subunit isoforms of the Na^+,K^+-ATPase in the groups reported before. Only the ß1 and ß2 isoforms were studied in humans because there are not human anti-ß3 antibodies available. Chondrocytes in figure 3A to G show immunoreactivity like for ß1 and ß2 isoforms. Again the expression pattern does not vary between the healthy (Fig. 3A,B) and the arthritic (Fig. 3C-E and G) articular cartilage. To test further the specificity of the staining, we performed competition experiments. In such experiments along with the antibody we incubated specific antigen, that is, the recombinant proteins used as

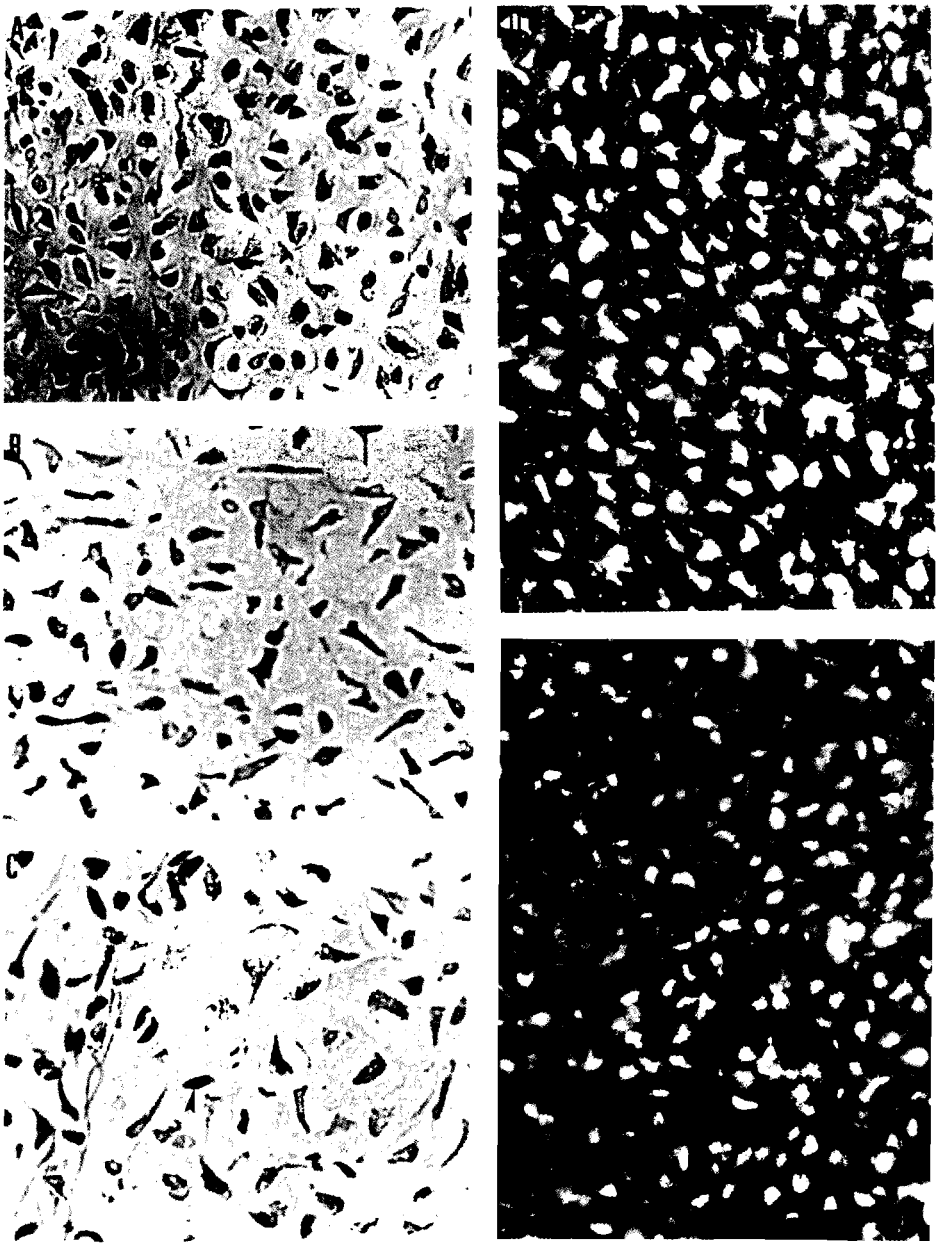

Fig. 1. Immunocytochemical localization of the Na^+,K^+-ATPase in articular cartilage of human fetus. **A, B and C.** Localization by avidin-biotin-peroxidase of α1, α2 and α3 isoforms in 5 μm sections of metacarpal phalangeal cartilage of a 23-week human fetus. **D and E.** Localization by indirect immunofluorescence of ß1 and ß2 isoforms in sections of tibia epiphysis of a 22-week human fetus. x 500

antigens for obtaining the antibodies (Gonzalez-Martinez et al., 1994). Figure 3 shows chondrocytes immuno-reactive for the ß2 isoform (Fig. 3G) and a negative staining for the same antibody incubated along with the recombinant ß2 isoform protein during the procedure (Fig. 3H).

Table 2 compiles the data of number and clustering of cells per chondron and the relative amount of expression of the isoforms studied.

In order to better understand the expression of the regulatory subunit distribution of the ß3 isoform in articular cartilage we used a polyclonal rat-specific antibody (Arystarkhova and Sweadner, 1997). As shown in figure 4A, the chondrocyte shows a bright positive immunoreactivity for this antibody. To confirm the presence of ß3 isoform in human cartilage, we performed "in situ" hybridization to detect ß3 mRNA in normal cryostat sections of the adult cartilage. Figure 4B

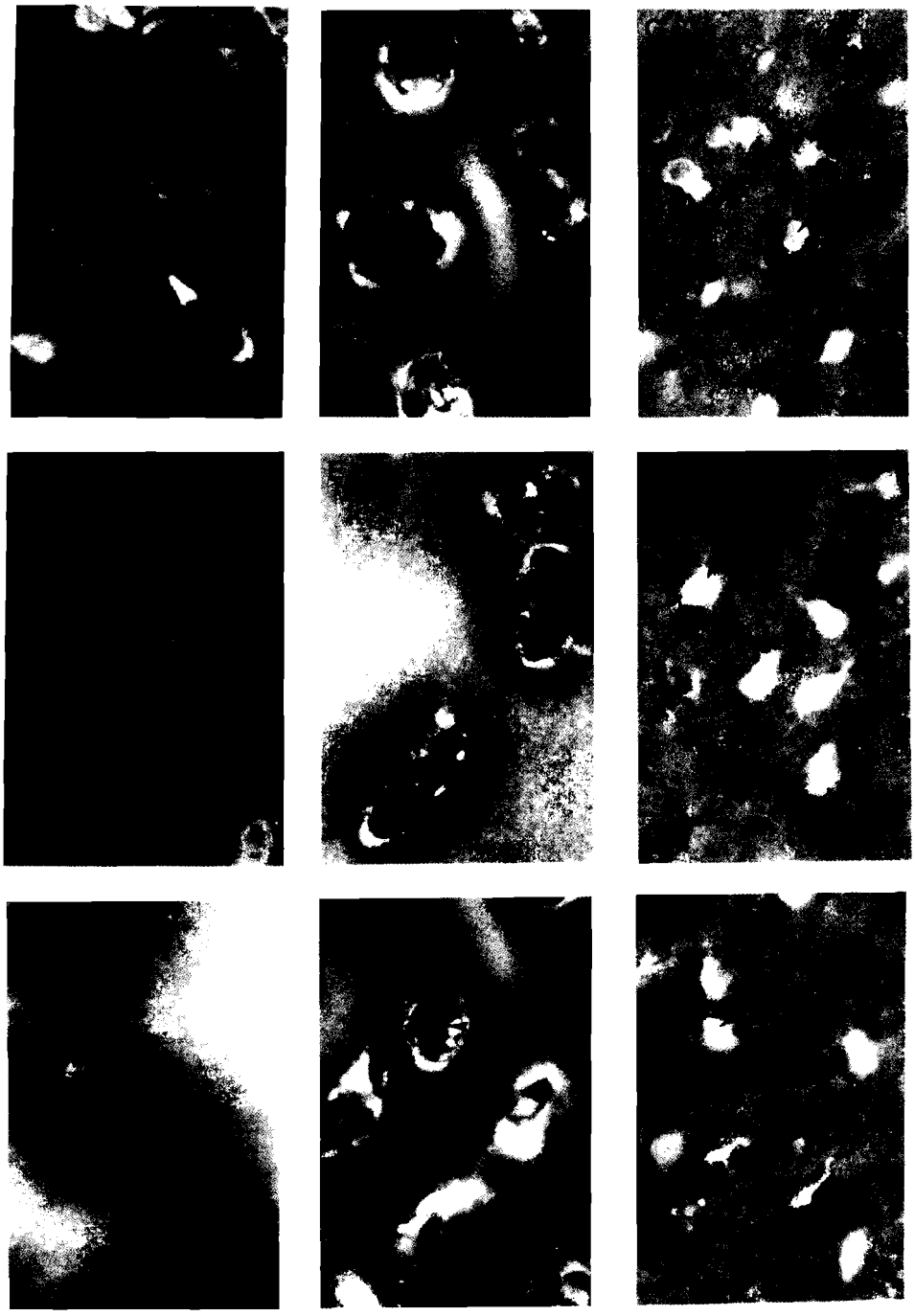

Fig. 2. Immunolocalization by indirect immunofluorescence of the Na^+,K^+-ATPase α subunit isoforms in 5 μm cryostat sections of the adult cartilage. Specific bound antibodies were visualized with rhodamine-conjugated goat anti-mouse Ig G. **A, B and C.** Localization of α1, α2, and α3 isoforms in the adult normal cartilage of a femoral head of a 53 year old female. **D, E and F.** Same localization of α1, α2, and α3 isoforms in cartilage of a patient affected of gonarthrosis (lateral femoral condyle), 67-year-old female. **G, H and I.** Localization of α1, α2, and α3 isoforms in cartilage of the femoral head of a 48-year-old female affected of RA. The α3 isoform was detected with McBX3. Arrowheads pointing inside the chondrons show plasma membrane specific staining. Arrows in panels A and C point the diffuse background in the perichondron region due to intrinsic tissue fluorescence. x 570

shows a positive hybridization signal when antisense cRNA probe to ß3 isoform is used. As a negative control, we used a sense cRNA probe that results in the absence of signal (Fig. 4C). "In situ" hybridization was also used to further demonstrate the presence of α2 isoform in human cartilage, as our immunohistochemical data were opposite to a previous report from another group (Mobasheri et al., 1997b). We are able to detect mRNA coding for α2 isoform. This demonstrates the presence of this isoform in human cartilage (data not shown).

To gain insight into Na^+,K^+-ATPase functionality in human cartilage, we performed a cytochemical method to detect sodium pump enzymatic activity "in situ" (Zemanová and Pachá, 1996). K^+-dependent *p*-NPPase activity is demonstrated in the surface of adult healthy human chondrocytes (Fig. 5A). The reaction product occupies the whole of the cells. The amount of the reaction product is increased in pathological cartilage. The p-NPPase activity is also increased in RA cartilage

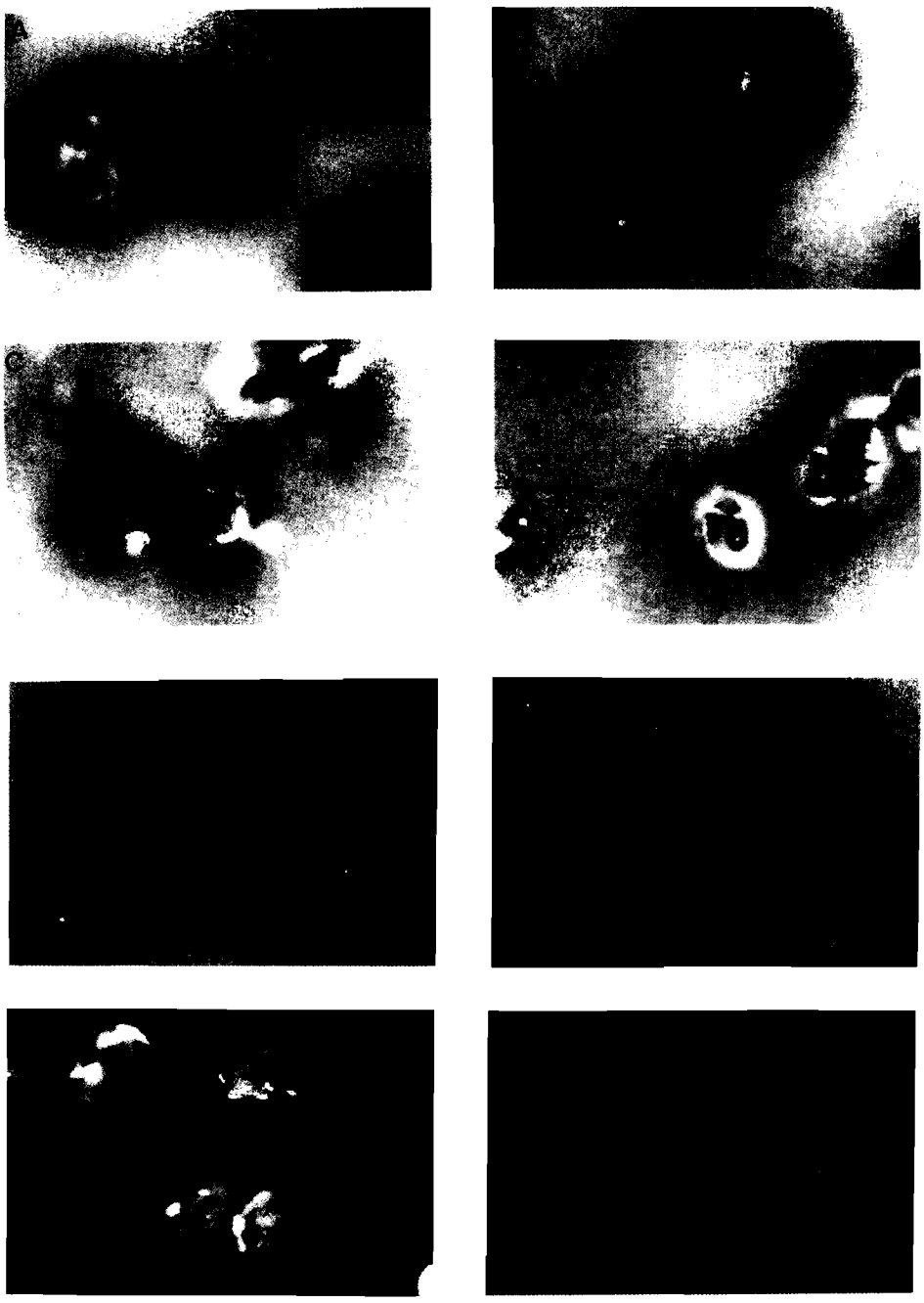

Fig. 3. Immunolocalization by indirect immunofluorescence of ß1 (A, C and E) and ß2 (panels **B, D, F, G and H**) isoforms of the Na^+,K^+-ATPase in adult normal cartilage (A and B) and in cartilage of patients affected with OA (C, D, G and H) and RA (E and F). Visualized with fluorescein-conjugated goat anti-rabbit Ig G. **A and B.** Medial femoral condyle 32 year-old normal male. **C and D.** Femoral head 68 year-old female affected of OA. **E and D.** Femoral head of a 51 year old female affected of RA. **G and H.** Competition experiment; G: staining with SpETß2 and H: staining with SpETb2 antiserum and ß2 recombinant protein together. x 570

when compared with OA cartilage (Fig. 5B and C show representative pictures). The reaction is nearly abolished by incubations with 10 mM ouabain (Fig. 5D). When incubation is carried out in the absence of K^+, no staining of tissues is detected (Fig. 5E).

Table 2. Parameters of cellularity of the normal and pathological cartilage showing number and clustering of cells per chondron and the relative amount of expression of the isoforms of both subunits of Na^+, K^+-ATPase.

	NORMAL	ARTHROSIS	ARTHRITIS
Cells/field	18±1	27±2	34±1
Chondrons/field	16±2	18±2	34±1
m.f.cells/chondron	1-2	3±1	1
α1	+++	+++	+++
α2	+	+	+
α3	++	++	++
ß1	+++	+++	+++
ß2	++	++	++

The number of cells was obtained counting through the 25X lens. m.f.cells/condron: most frequent number of cells per chondron.

Discussion

The human articular chondrocytes express three α (α1, α2, α3) and three ß subunit isoforms (ß1, ß2, ß3). This means that the chondrocyte Na^+,K^+-ATPase must exist in multiple molecular forms. Nine different isozymes could be formed, that is, α1ß1, α1ß2 α1ß3, α2ß1, α2ß2... and so on. These varieties of isozymes play different roles in the physiology of normal cartilage chondrocytes. The Na^+,K^+-ATPase is the principal regulator of intracellular [Na^+] and [K^+]. The α1 and the ß1 isoforms serves as the 'housekeeping' isoforms due to its abundance and ubiquitous cellular distribution. The remaining isoforms exhibit a more restricted tissue specific and developmental pattern of expression; the α2 isoform is expressed most abundantly in cardiac muscle, skeletal muscle and adipose tissue (Sweadner et al., 1994) and its expression is insulin sensitive (Russo and Sweadner, 1993). Previous report in an animal model

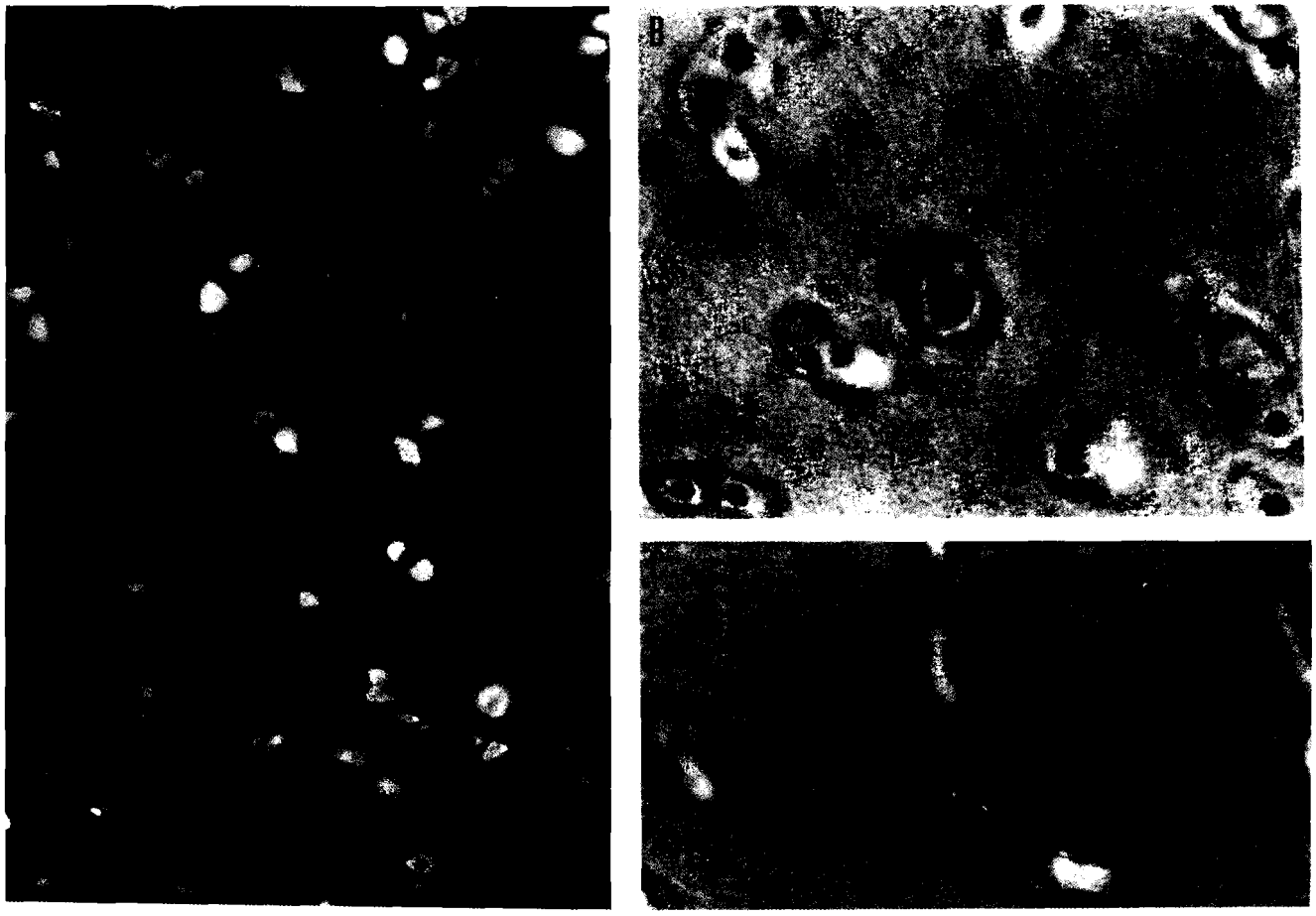

Fig. 4. Localization of the ß3 isoform in articular cartilage. **A.** Immunocytochemical localization by indirect fluorescence of ß3 isoform in adult rat knee articular cartilage. **B.** "In situ" hybridization of antisense cRNA probe to ß3 isoform in normal cryostat sections of the adult cartilage. **C.** "in situ" hybridization of sense cRNA probe to ß3 isoform in normal cryostat sections of the adult cartilage. Note the lack of specific label. x 570

indicated that the chondrocytes do not express the α2 isoform (Mobasheri et al., 1997b) as seen by immunofluorescence and RT-PCR. In this study we use a different fixation method that gives better results with McB2 antibody (K.J. Sweadner, personal communication). The presence of α2 isoform in human cartilage has been further confirmed by "in situ" hybridization. The failure of RT-PCR on detecting α2 (Mobasheri, 1998) can be explain because there is no information about the sequence of this isoform in bovine tissues, so no specific primers are available.

The α3 and ß2 isoforms are found in high concentrations in neurons in the central nervous system (Sweadner, 1995; Peng et al., 1997). The α4 isoform appears to be specific to the testis (Shamraj and Lingrel, 1994) and ß3 is expressed mainly in the lung, liver, testis and skeletal muscle, with small amounts found in kidney, heart and brain (Malik et al., 1996; Arystarkhova and Sweadner, 1997).

There is evidence that α3 is post-translationally modified at the site of binding of McBX3 antibody in a tissue-specific manner, being present in the heart of human, rat, dog and macaque and in the kidney of some species, but absent in brain and retina (Arystarkhova and Sweadner, 1996). This modification abolishes the binding of McBX3 to its epitope. The α3 isoform present in human cartilage does not present this modification, as McBX3 is able to recognize the protein. The expression of α3 has been further demonstrated using the XVIF9-G10 antibody that is not affected by tissue-specific variations (Arystarkhova and Sweadner, 1996). This clearly demonstrates that the α3 isoform of the cartilage is the brain- or neural-like α3 isoform. The catalytic α isoforms have been shown to exhibit significantly different kinetic properties; the affinity of the α3 isoform for intracellular Na^+ is orders of magnitude lower than that of the α1 isoform (Jewell and Lingrel, 1991). Thus, the expression of the α3 isoform may be related to the high extracellular concentration of Na^+ in cartilage and the substantial transmembrane concentration gradient for Na^+ that exists in chondrocytes. The α3 isoform with its low affinity for Na^+ working in parallel with the α1 isoform may allow chondrocytes to respond more effectively to the physiological changes in intracellular [Na^+] that occur frequently *in vivo*. In addition the affinity of the α3

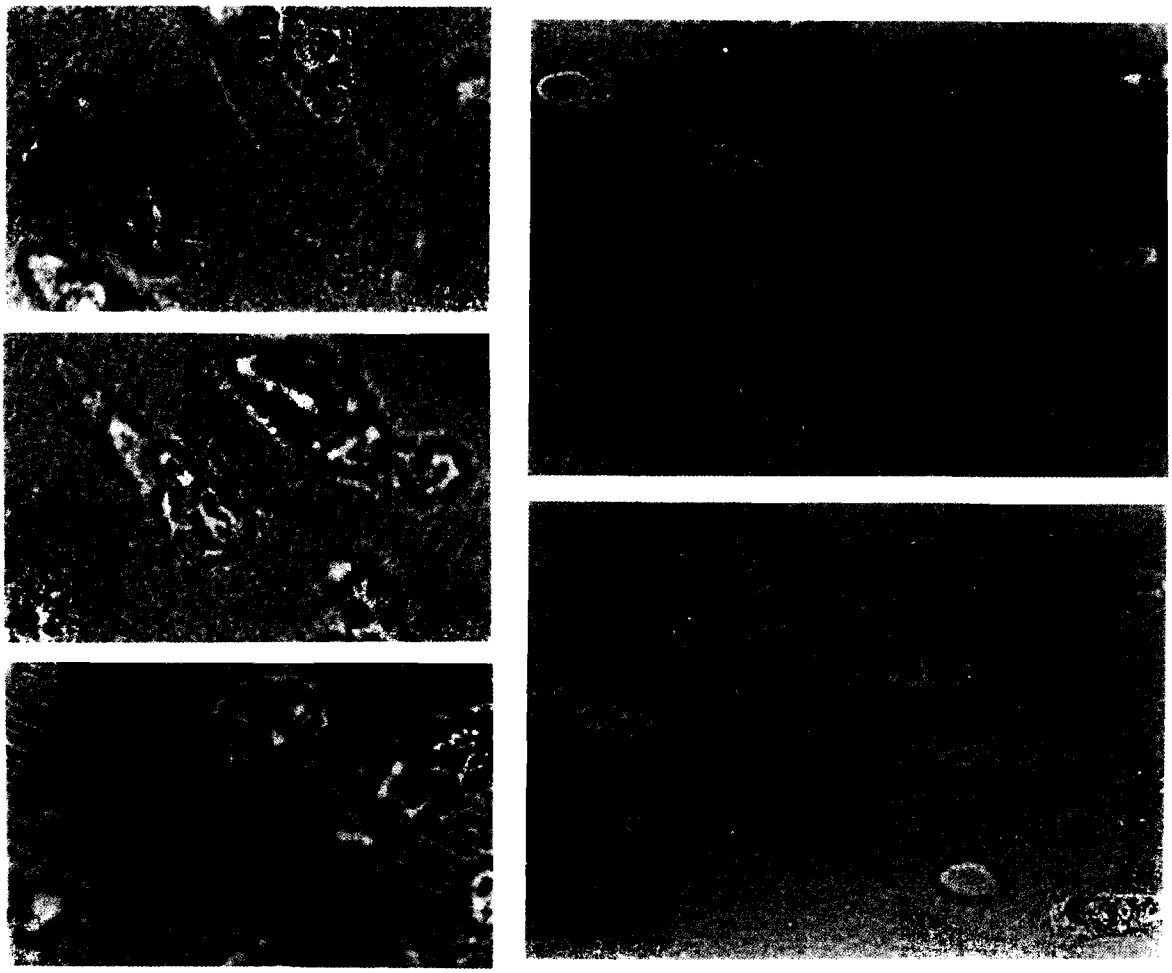

Fig. 5. Cytochemical localization of K^+-dependent p-NPPase activity in adult human articular cartilage. **A.** Normal cartilage. **B.** Osteoarthritis cartilage. **C.** Rheumatoid arthritis cartilage. **D.** Parallel section incubated with 10 mM ouabain. **E.** Parallel section incubated in the absence of K^+.

isoform for the cardiac glycoside ouabain is higher than α1 raising the possibility of independent regulation of Na^+,K^+-ATPase units containing these different isoforms at the tissue level by endogenous inhibitors or endo-ouabain-like compounds (Mobasheri et al., 1998).

It is important to comment that in RA cartilage (Fig. 2G- I) it appears that the α3 subunit isoforms show a higher level of fluorescence compared to the normal and osteoarthritic specimens. However, these qualitative observations need to be substantiated by quantitative studies. Our preliminary densitometric analysis showed no significant differences.

The variability of isozymes may be related to other pathophysiological states; for instance, the synthesis, secretion and general turnover of pro-inflammatory cytokines in mononuclear cells depends on the activity of the Na^+,K^+-ATPase (Foey et al., 1997). Furthermore, the inhibition of the Na^+,K^+-ATPase by ouabain treatment upregulates catabolic cytokines including tumor necrosis factor α and interleukin-1ß. The effect of these cytokines have been extensively studied in clinical and experimental models of arthritis and the current consensus is that they promote cartilage matrix destruction (Borden and Heller, 1997).

The influence of tissue fixed charge density on the ion and water content of the matrix may have important biochemical cellular and patho-physiological consequences. For example, the synthesis of cartilage matrix by chondrocytes is sensitive to extracellular $[Na^+]$ (Urban and Bayliss, 1989). Chondrocytes sense and respond adaptively to short-term changes in their ionic and osmotic environment. In the short-term they regulate the intracellular concentration of ions in an attempt to return the ion concentrations to their physiological levels. In the long-term such changes may affect the equilibrium between matrix synthesis and degradation. This may compromise the mechanical properties of the tissue and result in greater variation in the ionic and osmotic environment, thus playing a significant role in early cartilage pathology. Autoradiographic studies using ^3H-ouabain suggest that Na^+,K^+-ATPase density is sensitive to the ionic and osmotic environment of the chondrocyte and long-term increases in extracellular $[Na^+]$ result in the upregulation of the Na^+,K^+-ATPase in cartilage explants cells (Mobasheri et al., 1997a) and isolated cells (Mobasheri et al., 1997b). It has also been shown that the "in vivo" Na^+/K^+ pump density correlates with $[Na^+]$ and [glycosaminoglycan] (Mobasheri, 1998). The observation that Na^+,K^+-ATPase density depends on the ionic and osmotic environment suggests that chondrocytes are sensitive to their extracellular environment.

The expression pattern of both subunit isoforms of the sodium pump does not seem to be altered in the cartilage pathologies studied in this work. However, there is an increase in p-NPPase enzymatic activity in arthritic chondrocytes when compared to healthy ones. This activity is mainly due to the Na^+,K^+-ATPase because it is almost abolished when the incubation medium includes ouabain. The higher enzymatic activity of sodium pump in arthritic chondrocytes could be explained in two ways, a higher amount of active pumps or a different association between protomers that form a new proportion of isozymes. The variability in the association of isoforms to form different isozymes has been previously reported in rat oxidative-fibers skeletal muscle. This tissue shows an insulin-dependent upregulation of Na^+,K^+-ATPase activity based on a specific translocation of α2 and ß1 isoforms from intracellular to plasma membranes (Lavoie et al., 1996).

The role of the Na^+,K^+-ATPase in pathologies of cartilage is unknown at the present time. Since the Na^+,K^+-ATPase is a principal regulator of cellular ion homeostasis, it is likely to be involved in situations where the ionic environment of chondrocytes is drastically altered. Changes in the ionic and osmotic environment of chondrocytes occur during the initial stages of human OA and RA where tissue hydration and proteoglycan loss occurs leading to a fall in tissue $[Na^+]$ and $[K^+]$ content (Maroudas, 1979, 1980). If the chondrocyte Na^+, K^+-ATPase does not maintain the optimal Na^+/K^+ ratio within prerequisite physiological limits, there may be deleterious alterations to extracellular matrix synthesis rates in the short-term (Urban et al., 1993) and possibly also in the long-term. The intracellular $[K^+]$ is particularly significant because of the requirements of intracellular enzymes (Kernan, 1980), these include those responsible for the synthesis of collagen and glycans. During the later stages of OA and RA large quantities of proteoglycans are lost from the matrix of affected areas of articular cartilage and this further exacerbates the ionic and osmotic changes to the environment of chondrocytes.

Spite of the unusual ionic environment of chondrocytes and the changes that this medium undergoes during the development of arthritic pathologies in cartilage, the number of studies about this topic is scarce. This is the first of a series of articles in which we study ionic transport systems and their alterations in human normal and arthritic cartilage.

Acknowledgements. We thank Dr. Kathleen J. Sweadner (Massachusetts General Hospital) for her kind gift of antibodies. We thank Dr. Zemanová (Institute of Physiology, Czech Academy of Sciences) for technical advice in the "in situ" p-NPPase activity. This work was supported by grants 93/831 and 96/0453 from FIS, Spain, to P.M.-V. and the Department of Biomedical Sciences, School of Biosciences, University of Westminster (grant number V001173) to A.M. Elisa Trujillo and Diego Alvarez de la Rosa contributed equally to this paper, and thus both are first authors. E.T. is recipient of a fellowship from Unidad de Investigación of Hospital Universitario de Canarias.

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Accepted February 10, 1999