

Biological Variation of Rabbit Chondrocyte 5'Nucleotidase (41655)

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Abstract. The activity of 5'nucleotidase was investigated in rabbit chondrocytes. Studies of chondrocytes in organ culture and intact cells revealed that ecto-5'nucleotidase activity varies with the age of the animal. Specific activities of 0.29, 0.62, 1.97, and 1.39 $\mu\text{mole/hr}/10^6$ cells were observed in intact cells from 3-week-, 3-month-, 6-month-, and 2-year-old animals. Specific activities of 15.5, 33.1, 80.3, and 39.5 $\mu\text{mole/hr/mg}$ protein, respectively, were observed when the enzyme protein was solubilized from disrupted cells with the detergent octyl- β -D-glucopyranoside. Membrane-bound nonspecific neutral phosphatase activity did not vary with age. Activity of 5'nucleotidase was observed on all chondrocytes using a histochemical staining technique. Kinetic properties of the enzyme from animals of different ages were investigated. No differences were noted in apparent K_m value for AMP (23 μM), pH optima (7.3), substrate competition studies, and effects of MgCl_2 or CaCl_2 . These data demonstrate a biological variation in rabbit chondrocyte 5'nucleotidase activity with age. The variation of activity represents an apparent increase in the amount of enzyme protein rather than an alteration in kinetic properties.

Recent evidence suggests an important role for purine catabolism in pathological conditions involving cartilaginous tissues. Variation in the activity of 5'nucleotidase (EC 3.1.3.5) has been observed in extracts of articular cartilage from normals and patients with osteoarthritis and calcium pyrophosphate dihydrate (CPPD) deposition disease with elevated activity implicated in the pathogenesis of the latter condition (1). Increased 5'nucleotidase activity could lead to increased inorganic pyrophosphate (PP_i) accumulation by removing AMP, the coproduct of PP_i generation from the degradation of ATP by nucleoside triphosphate pyrophosphohydrolase (Fig. 1). The accumulated PP_i would be available for formation of CPPD crystals. Additionally, chondro-osseous dysplasia has been observed in children with adenosine deaminase deficiency (2), a disease in which variation of 5'nucleotidase activity may in part determine tissue-selective cytotoxicity (3, 4).

The enzyme 5'nucleotidase catalyzes the dephosphorylation of nucleoside monophosphates in the reaction: nucleoside monophosphate + $\text{H}_2\text{O} \rightarrow$ nucleoside + P_i . Despite occupying a critical position in the scheme of purine nucleotide catabolism, the biological significance of this enzyme is uncertain. Although 5'nucleotidase has been investigated in many tissues (5-7) including biochemical

studies in homogenates of chick embryo epiphyseal cartilage (8) and histochemical studies in bovine cartilage (9), little is known concerning this enzyme in mammalian chondrocytes. We have undertaken the study of 5'nucleotidase activity of rabbit chondrocytes to determine whether changes in activity occur with aging, as the prevalence of human CPPD deposition clearly increases with age (10).

Materials and Methods. Bovine serum albumin, 5'AMP, 2'3'AMP, cacodylic acid, glutaraldehyde, hyaluronidase and trypsin were purchased from the Sigma Chemical Company, St. Louis, Missouri. From Fisher Scientific Company, Pittsburgh, Pennsylvania, were purchased lead nitrate, ammonium sulfide, neutral red, and magnesium chloride. Magnesium nitrate and maleic acid were purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey. Hanks balanced salt solution (HBSS) (calcium-magnesium-free) and Eagle's minimal essential media were purchased from GIBCO Laboratories, Grand Island, New York. Collagenase was purchased from Millipore, Bedford, Massachusetts. Octyl- β -D-glucopyranoside was purchased from Calbiochem-Behring Corporation, LaJolla, California, and α,β -methylene adenosine 5'diphosphate (AOPCP) from Miles Laboratory, Elkhart, Indiana. From Amersham Cor-

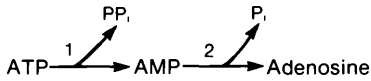


FIG. 1. Increased activity of 5'nucleotidase (enzyme 2) may lead to increased pyrophosphate (PP_i) generation by removing AMP, the coproduct of ATP degradation by nucleoside triphosphate pyrophosphohydrolase (enzyme 1) driving the reaction to the right.

poration, Arlington Heights, Illinois, was purchased [8-¹⁴C]AMP. All other reagents were of the highest quality commercially available.

Assays. The activity of 5'nucleotidase was quantitated by measuring the conversion of radioisotopic AMP to radiolabeled nucleoside products (4). Intact, viable chondrocytes were obtained from rabbits of various ages (3 weeks to 2 years) by hyaluronidase, trypsin, and collagenase digestion of the articular cartilage as previously described (11) and suspended in HBSS (calcium-magnesium-free) to 0.5–2.0 × 10⁶ cells/ml for 3-week- and 3-month-old rabbits and 0.05–0.15 × 10⁶ cells/ml for 6-month- and 1-year-old rabbits. Greater than 90% viability was present in cell chondrocyte preparations as determined by trypan blue exclusions. Aliquots (25 μl) of cell suspension were incubated for 15 min in a total volume of 100 μl with 40 mM Tris-HCl, pH 7.4, 5.0 mM magnesium chloride, 0.2 mM [8-¹⁴C]AMP, and 0 or 0.025 mM AOPCP (a specific inhibitor of 5'nucleotidase activity). In all assays, AOPCP caused greater than 95% inhibition of the reaction. Results were expressed per 10⁶ cells. The assay was linear for 30 min and with respect to cell number within the ranges employed.

Activity of 5'nucleotidase was also determined in protein solubilized by the addition of 40 mM octyl-β-D-glucopyranoside to cell suspensions, which caused immediate disruption of all cells. The resultant solution was centrifuged at 100,000g for 2 hr. The supernatant was passed through a 0.45-μm filter and dialyzed in 10 mM octyl-β-D-glucopyranoside, 1.0 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA for 2–6 hours. The 5'nucleotidase activity was measured by the above method and expressed per milligram protein. Protein concentrations were determined by the method of Markwell (12).

Freshly harvested rabbit articular cartilage was minced into approximately 5 mg pieces

and wet weight determined. Specimens (less than 200 mg) were cultured overnight in 1.5 cm³ of Eagle's essential media and 5% fetal calf serum in 3.5-cm petri dishes. After washing three times with 10 mM Tris-HCl, pH 7.4, in 150 mM NaCl, activity of 5'nucleotidase was assayed as described by Edwards *et al.* (13). Reaction mixture (2 ml) containing 50 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 150 mM NaCl, and 1.0 mM AMP or 2'3'AMP was added to the organ culture and incubated at 37°C. One hundred microliters of supernatant was removed at time 0, 10, 20, 30, and 40 min and assayed for inorganic phosphate content by the method of Chen *et al.* (14). Cell number was estimated using the method of Cheung and Ryan (11). The nucleotidase activity was linear with time to 40 min when less than 60 mg of cartilage was employed.

Nonspecific ecto-neutral phosphatase activity was determined on intact chondrocytes released from the cartilage matrix as described above, using *p*-nitrophenylphosphate as the substrate at pH 7.4 (15).

Histochemical staining for ecto-5'nucleotidase activity was performed by the method of Silber *et al.* (16) with modifications described by Recker *et al.* (17). Released chondrocytes were washed twice in HBSS (calcium-magnesium-free) and resuspended in Hanks buffer to a final concentration of 1–1.5 × 10⁷ cells/ml. Smears were prepared on clear glass coverslips and allowed to dry for 20 min. The slides were incubated for 20 hr at 37°C in 0.25 M Tris-maleate, pH 8.0, 10 mM Mg(NO₃)₂, 2 mM Pb(NO₃)₂, and 3 mM 5'AMP. To assay background nonspecific phosphatase activity which could hydrolyze 5'AMP, 2'3'AMP (3 mM) was substituted for 5'AMP. Slides were fixed in a mixture of 0.4% glutaraldehyde, 1% sucrose, and 0.16% cacodylic acid at 4°C. After rinsing twice in water, slides were immersed in 0.5% ammonium sulfide for 30 sec, rinsed twice more in water, counterstained for 1 min with 1% neutral red, and rinsed again with water. The presence of 5'nucleotidase activity was evidenced by a black precipitate over the cells. Generally less than 2% of the cells were positive in control plates in which 2'3'AMP was the substrate.

Kinetic studies. The following studies were performed on intact chondrocytes and di-

alyzed protein solubilized from the cells as described above using the radiochemical assay. Kinetic studies were performed with [8-¹⁴C]AMP 3.3 to 20 μ M. The values of the apparent Michaelis constants were estimated from Lineweaver-Burk plots. Activity of the protein over a pH range was assessed by assaying samples in the presence of 50 mM sodium acetate, pH 4.5 to 6.0, or 50 mM Tris-HCl, pH 6.5 to 10.0.

Substrate competition studies were performed using the radiochemical assay described above in the presence or absence of 1 mM 5'AMP, 5'GMP, 5'IMP, 5'deoxyAMP, 5'deoxyGMP, 5'deoxyIMP, 2'3'AMP, cyclic AMP, and *p*-nitrophenylphosphate. The effect of calcium and magnesium was determined by assaying 5'nucleotidase activity with 0-25 mM MgCl₂ or CaCl₂.

Statistical analysis. The results of enzyme specific activities were tested for simultaneous equality with one-way analysis of variance. Pairwise testing of means was performed by Student's *t* test.

Results. Rabbit chondrocyte ecto-5'nucleotidase activity varies significantly with the age of the animal (Table I, Fig. 2). Mean specific activities of 0.29, 0.62, 1.97, and 1.39 μ mole/hr/10⁶ cells were obtained from animals of 3 weeks, 3 months, 6 months, and 2 years, respectively. Ecto-neutral nonspecific phosphatase activity, on the other hand, did

not vary with the age of the animal and gave mean values of 23.2 nmole/hr/10⁶ cells. To ensure that the variation in enzyme activity was not attributable to an artifact during release of the cells, 5'nucleotidase was assayed in organ culture as described above for 3-week- and 6-month-old rabbits. A similar and significant ($P < 0.01$) variation with age was observed (Table I), although activities measured in organ culture were 3- to 3.6-fold lower than those determined for released cells (a variation most likely attributable to differences in accessibility to substrate and diffusion).

The 5'nucleotidase activity was assayed on cell protein solubilized with the detergent octyl- β -D-glucopyranoside to determine whether the increased activity in the old animals was due to the presence of more enzyme protein or an increased enzyme-substrate accessibility. Again an age-related change was noted with mean specific activities of 15.5, 33.1, 80.3, and 39.5 μ mole/hr/mg protein observed (Table I).

A difference in 5'nucleotidase activity has been observed in human lymphoid cells and attributed to the proportion of 5'nucleotidase-positive and -negative subpopulations (16). Histochemical staining procedures demonstrated 5'nucleotidase activity on virtually all chondrocytes from rabbits of each age indicating that the increased activity could not be explained by a difference in subpopulations

TABLE I. SPECIFIC ACTIVITY OF RABBIT CHONDROCYTE 5'-NUCLEOTIDASE: VARIATION WITH AGE

Assay method	3 Week (N) mean	3 Month (N) mean	6 Month (N) mean	2 Year (N) mean
Released cells ^a (μ mole/hr/10 ⁶ cells)	(18) 0.29	(6) 0.62	(18) 1.97	(11) 1.39
Organ culture ^b (μ mole/hr/10 ⁶ cells)	(4) 0.099	ND	(4) 0.549	ND
Solubilized cell ^c protein (μ mole/hr/mg protein)	(8) 15.5	(4) 33	(6) 80.3	(5) 39.5

^a Intact, viable chondrocytes were obtained by hyaluronidase, trypsin, and collagenase digestion of articular cartilages and specific activity was measured by the conversion of radioisotopic AMP to radiolabeled products. One-way analysis of variance indicates a significant difference among the set of four means ($P < 0.001$). Comparisons among pairs gave $P < 0.02$, $P < 0.001$, and $P < 0.001$ for comparisons of 3 weeks activity with 3 months, 6 months, and 2 years, respectively. The difference between activities for 6 month and 2 year chondrocytes was not significant.

^b Enzyme activity was determined in organ culture by measuring the accumulation of inorganic phosphate in media with 5'-AMP as the substrate. The difference in activities for 3-week and 6-month rabbit cartilage is significant ($P < 0.01$). Activities for 3-month and 2-year-old animals were not determined (ND).

^c Activities of 5'-nucleotidase determined by the radiochemical method in protein solubilized by adding 40 mM octyl- β -D-glucopyranoside to cell suspensions were different for chondrocytes 3 weeks through 6 months of age ($P < 0.01$) and 6 months to 2 years ($P < 0.05$).

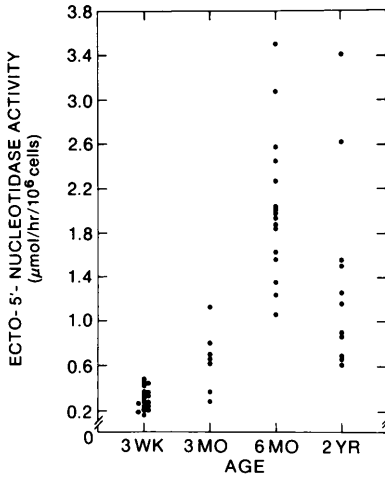


FIG. 2. Chondrocyte ecto-5'nucleotidase activity varied with the age of the rabbit. Each dot represents the mean of duplicate determinations of intact chondrocyte ecto-5'nucleotidase activity from one rabbit. Assays were performed on multiple occasions with activities for rabbits of more than one age determined each time.

and was more likely due to an increased amount of enzyme per cell.

The enzyme protein was investigated to determine whether the variation in enzyme activity was due to altered kinetic or physical properties. The following results were similar in experiments employing both intact viable chondrocytes as well as cell proteins solubilized with detergent. Initial velocity studies revealed an apparent Michaelis constant value for AMP of 23 μM for animals of all four ages studied (Fig. 3). The enzyme from the animals of each age were compared by examining their activity from pH 4.5 to 10.0. The enzyme activity from each age rabbit was maximal at pH 7.4, with a plateau of near optimal activity extending into the alkaline pH range (Fig. 4).

Substrate competition studies failed to show any difference in either substrate competition or inhibition for the 5'nucleotidase activities from cells of 3-week- and 6-month-old rabbits (Table II). Similarly, there were no differences in the effects of varying concentrations of MgCl_2 or CaCl_2 (0–25 μM) on the enzyme activity with neither divalent cation causing marked enhancement or loss of activity (Table III).

Discussion. These data demonstrate a variation in rabbit chondrocyte 5'nucleotidase ac-

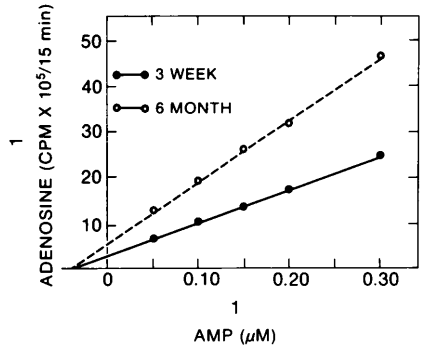


FIG. 3. Lineweaver-Burk plots provided an apparent K_m value of 23 μM for AMP for ecto-5'nucleotidase activity for chondrocytes from 3-week- and 6-month-old rabbits. Results (not shown) were similar for 3-month- and 2-year-old rabbits. In the experiment displayed chondrocytes from a 3-week- (0.62×10^6 cells/ml) and a 6-month-old rabbit (0.09×10^6 cells/ml) were incubated for 15 min with 3.3 to 20 μM [8-¹⁴C]AMP. Chondrocytes from these rabbits had ecto-5'nucleotidase specific activities of 0.322 (3 week) and 1.33 $\mu\text{mole/hr}/10^6$ cells (6 month).

tivity with age. This observation is in keeping with previous observations of variability of this enzyme in cartilage and other tissues. Rodan *et al.* have observed a pronounced increase in 5'nucleotidase activity during chick epiphyseal cartilage maturation (8) and Otte reported 5'nucleotidase activity localized to cells of the superficial zone in immature bovine cartilage compared to activity in all zones in the adult (9). An inverse correlation between 5'nucleotidase activity and cell proliferation has been reported in hepatic cells *in vivo* (18,

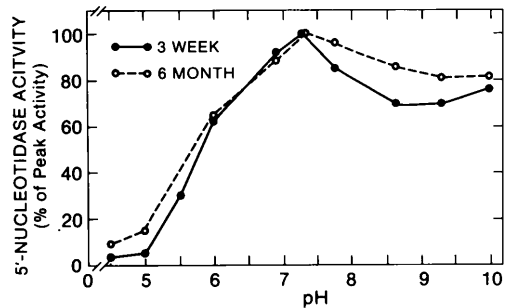


FIG. 4. Rabbit chondrocyte ecto-5'nucleotidase activity over a pH range of 4.5 to 10. The pH maximum of 7.4 with a plateau of near maximum activity in the alkaline range was noted for the enzyme activity was observed for chondrocytes from rabbits of ages 3 weeks and 6 months.

TABLE II. SUBSTRATE COMPETITION^a

Compound added (1.0 mM)	Percentage of control	
	3 Week	6 Month
Normal saline	100	100
5'-AMP	20	20
5'-IMP	30	31
5'-dGMP	63	62
5'-dIMP	88	84
5'-dAMP	88	91
2',3'-AMP	97	95
p-nitrophenyl-P	96	102
Cyclic AMP	103	103

^a Substrate specificity for chondrocyte ecto-5'nucleotidase activity was estimated by substrate competition experiments for 3-week- and 6-month-old rabbits. The amounts of radiolabeled adenosine formed from incubation of intact chondrocytes with 200 μ M [8-¹⁴C]AMP were compared in the absence and presence of 1.0 mM nonradiolabeled compounds.

19). In cell culture, cell lines that have unlimited lifespans have very low 5'nucleotidase activities, whereas in cells with limited lifespans 5'nucleotidase activity increases as cells become senescent (20). Ecto-5'nucleotidase activity increases as T lymphocytes differentiate from lymphoblasts to post-thymic circulating cells (21) and the activity declines on circulating T cells with age (22).

Although variation in 5'nucleotidase activity with age has been observed, no studies to date allow determination of whether the differences are due to differences in enzyme amount or the effect of activity-modifying factors. The

TABLE III. EFFECT OF DIVALENT CATIONS^a

	mM	Percentage of Control	
		3 Week	6 Month
MgCl ₂	1.25	115	121
	5.0	123	129
	25.0	122	122
CaCl ₂	1.25	113	117
	5.0	100	100
	25.0	81	63

^a The effect of divalent cations on chondrocyte ecto-5'nucleotidase activity was compared for rabbits of 3 weeks and 6 months of age. Results are expressed as percentage of control when enzyme activity was assayed in the absence of divalent cation.

results of these studies suggest the former. The fact that the age variation was maintained in solubilized proteins suggests that the increase is due to an increase in the amount of enzyme per cell rather than the inaccessibility of enzyme to substrate in younger cells. The histochemical demonstration of 5'nucleotidase activity in all chondrocytes rules out the possibility of enzyme-positive and -negative subpopulations. The similarity in apparent K_m values, pH optima, effects of divalent cations, and results of substrate competition studies for the enzyme from cells from different age animals further suggests that the older animals have more 5'nucleotidase activity due to more enzyme present rather than having developed a more active molecule.

The variation of 5'nucleotidase activity with age suggests an important regulatory role for the enzyme in the processes of differentiation and aging. However, the exact physiological significance of this enzyme is not understood. Whether the change in 5'nucleotidase activity could be related to variations in the generation of adenosine or regulation of nucleotide levels is purely speculative. Tenenbaum *et al.* have observed a threefold increase of 5'nucleotidase activity in extracts of cartilage from patients with calcium pyrophosphate deposition disease compared to extracts from patients with osteoarthritic or normal cartilage (1). In these same extracts PP_i generation from ATP could be demonstrated, perhaps by a nucleoside triphosphate pyrophosphohydrolase. Thus, by degrading AMP, a coproduct with PP_i , 5'nucleotidase could push the pyrophosphohydrolase reaction to the right, generating more PP_i . This observation suggests a critical role for 5'nucleotidase in CPPD deposition disease. The demonstration of variation of 5'nucleotidase activity in rabbit chondrocytes provides a model for the investigation of the role of this enzyme in disease states of cartilage and may in part explain the increased prevalence of CPPD deposition with aging.

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