Hexosaminidase and alkaline phosphatase activities in articular chondrocytes and relationship to cell culture conditions

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Abstract. Hexosaminidase and alkaline phosphatase activities in rabbit articular chondrocytes have been studied under different cell culture conditions. Chondrocytes were cultured in monolayer primary culture, monolayer subcultured to the fifth passage (in vitro aging) and cultured within a collagen gel; enzymatically released cartilage cells were used as control. Under these conditions, the two enzymes behave quite differently in relationship to alteration of the chondrocyte phenotype in culture. Increased lysosomal hexosaminidase activity could be considered to be a marker of the dedifferentiated phenotype in monolayer subculture; membrane alkaline phosphatase activity could be used as a marker of non-proliferating cells.

Key words. Hexosaminidase; alkaline phosphatase; rabbit; articular chondrocytes; cell culture conditions.

The etiology of osteoarthritis in most cases is unknown; however, the progression of this disease is probably attributable to the secretion of degradative enzymes by chondrocytes. Thus in osteoarthritic cartilage, increased production of alkaline phosphatase and lysosomal enzymes has been reported [1–4], as well as the appearance of type I collagen, which is a sign of chondrocyte dedifferentiation [5]. This suggests that the production of these enzymes by chondrocytes could be linked to the dedifferentiation of these cells. To investigate this hypothesis we compared the activities of alkaline phosphatase and hexosaminidase from rabbit articular chondrocytes grown under a variety of cell culture conditions, allowing progressive modulation of their differentiated phenotype. Hexosaminidase (HEX) and alkaline phosphatase (AP) were previously shown to be present in rabbit articular chondrocytes in monolayer cultures [6, 7]. In the present study, HEX and AP activities were determined in freshly isolated rabbit articular chondrocytes cultured in monolayer (primary culture and subcultures) and subcultured within a collagen gel. It is well established that, when liberated from their matrix and cultivated in monolayer for several passages, articular chondrocytes progressively lose their differentiation phenotype [5, 8, 9]. In contrast, 'three-dimensional' cell culture techniques, such as collagen gel cultures, have been employed with success with the aim of maintaining the differentiated phenotype [9, 10]. The present results provide evidence that AP and HEX activities are independently regulated during the modulation of the chondrocyte phenotype in culture.

Materials and methods

Cell culture

Isolation and primary culture of articular chondrocyte. Articular chondrocytes were enzymatically released from knee and shoulder joints of 1- to 3-month-old 'Fauve de Bourgogne' rabbits, according to the experimental procedure of Green [11] with minor modifications [12]. When cellular confluency was reached, the cells were harvested by mild trypsinization, and were then subcultured in monolayer or in collagen gels.

Monolayer subcultures. In vitro aging was performed by weekly subculture of cells with a constant seeding density of 2×10^4 cells/cm^2 in plastic flasks. Chondrocytes were considered to be young at the first passage and senescent at the fifth passage with respect to cartilage cells (enzymatically released) as a control. Chondrocytes are considered to be senescent at the fifth passage, as they showed a finite lifespan of 7–9 subcultures [13].

Three-dimensional subculture. Chondrocytes were embedded within Bovine Skin Collagen (BIOETICA, France) according to Bell's procedure [14] with minor modifications [10]. Briefly, the cell suspension (primary culture) was diluted to a final density of 2×10^5 cell/ml in concentrated HAM's F12 solution containing fetal calf serum (IBF, France) up to 10% v/v. Bovine skin collagen was then added at a final concentration of 0.15 mg/ml. Aliquots of 2 ml were pipetted into 35-mm diameter Petri dishes and left to polymerise at 37 °C for one hour.

Preparation of cells for enzyme assays

Monolayer cultured cells were harvested by brief trypsinization and counted in a hemocytometer. Cells were harvested from monolayer by exposure to a mixture of trypsin (0.1% w/v) (CHOAY France, 45 IU/mg) and EDTA (0.02% w/v) in PBS for 30–45 s. Collagen gels were transferred from the dishes to a 0.1% w/v *Clostridium* collagenase solution in PBS for 20 min at 37 °C with magnetic stirring. After digestion of the gel the cells were counted. The cells were washed twice with cold 150 mM NaCl, centrifuged ($2600 \times g$, 5 min) and the cell pellets suspended in 0.5 ml 150 mM NaCl, pH 7. The cells were then sonicated three times for 15 s. (Sonicator MSE

PGE100, England). Lysates were used as the enzyme source.

Enzyme assays

AP and HEX activities were determined as previously described, using the fluorometric substrates 4-methylumbelliferylphosphate and 4-methylumbelliferyl-β-D-glucosaminide (Sigma), respectively[7]. Each assay was performed in triplicate and with parallel blanks of both cell lysate and subtrate. The results are expressed as nanomoles of 4-methylumbelliferone released per h, per million cells and per mg of protein. Protein concentration was measured by the method of Lowry[15], using serum albumin as standard. It must be pointed out that independent experiments correspond to different donors; this explains the substantial variations observed. For statistical analysis, therefore, the non-parametric Mann-Whitney U-test was used, with a significance level of $p < 0.01$.

Results and discussion

Tables 1 and 2 relate specific HEX and AP activities to protein content and to number of cells. HEX and AP activities in rabbit articular chondrocytes differ in their response to transfer from cartilage to culture conditions. In primary culture and gel collagen culture (fully differentiated chondrocytes), HEX activity in relation to protein content tended to be in the same range as that of cartilage cells (table 1): the differences are not significant. Identical results were obtained for HEX activity with respect to the number of cells for collagen gel-cultured chondrocytes compared with cells released from cartilage. Modulation of the differentiated chondrocyte phenotype by monolayer subculture induced a dramatic increase in HEX activity (× 4). It is noteworthy that the subsequent phenotypic changes associated with senescence of chondrocytes during aging in vitro does not change HEX activity in relation to protein content. By contrast, a marked change in HEX activity from first to fifth subculture was apparent in relation to number of cells. This alteration may be explicable in terms of the enlargement of the chondrocytes during aging[16]. These data suggest that, in chondrocytes, intracellular HEX activity may be linked to dedifferentiation in monolayer subculture.

In contrast to HEX, AP showed a remarkable dependency upon the 'age' of the cells in culture and upon the culture conditions (table 2). AP activity was depressed when articular chondrocytes were isolated from the surrounding matrix and placed in primary monolayer cultures. AP activity subsequently became undetectable in early subcultures, but increased abruptly at the fifth subculture. Identical results were obtained with respect to the number of cells or to protein content. These data are in agreement with earlier observations of other authors. In chondrocytes isolated from fetal bovine growth-plate cartilage, marked membrane-immunoreaction was observed with monoclonal antibodies directed against AP, when cells were cultured for three days or longer, no AP could be detected on their plasma membrane[17]. In pelleted cultures of rabbit growth-plate chondrocytes, AP activity started to increase at the cessation of cell division[18]. In a previous study, we have established a correlation between growth and AP activity induced by retinoic acid treatment of cultured rabbit articular chondrocytes[19]. The present study provides further evidence that AP may have a possible role as a marker of non-proliferating cells. With an increasing number of monolayer subcultures the proliferative capacity of chondrocytes fell markedly[13]; chondrocytes subcultured within collagen gels divided, but at a very reduced proliferation rate when compared with the monolayer culture[10]. Thus, the chondrocytes at the fifth monolayer subculture or cultured in collagen gels showed AP activity.

In conclusion, the present study provides evidence that HEX and AP are independently regulated in chondrocytes. HEX can be considered as a marker of the dedifferentiated chondrocyte phenotype in monolayer subcultures. AP appears as a possible marker of non-proliferating cells. The results presented here suggest separate control of growth and differentiation and show that cell culture conditions can bring about independent changes in these two processes in chondrocytes.

Table 1. Hexosaminidase activity of articular chondrocytes in different culture conditions. Results are the mean of several independent experiments, each in triplicate ± standard deviation. Mann-Whitney U-test: [a] not significantly different from chondrocytes released from cartilage; [b] $p < 0.01$.

Chondrocytes	Hexosaminidase activity in nmol/h Versus protein (mg)	n	Versus 10^6 cells
Released from cartilage	702 ± 105	9	252 ± 37
Primary culture	773 ± 40[a]	6	142 ± 13[b]
Monolayer subcultures			
first subculture	3074 ± 172	9	674 ± 75
fifth subculture	3259 ± 304	9	1687 ± 509
Collagen gel culture	869 ± 163[a]	12	258 ± 30[a]

Table 2. Alkaline phosphatase activity of articular chondrocytes in different culture conditions. Results are the mean of several independent experiments, each in triplicate ± standard deviation. nd = not determined.

Chondrocytes	Alkaline phosphatase activity in nmol/h Versus protein (mg)	n	Versus 10^6 cells
Released from cartilage	421 ± 153	9	252 ± 65
Primary culture	85 ± 6	6	15 ± 2
Monolayer subcultures			
first subculture	nd		nd
fifth subculture	173 ± 24	9	101 ± 24
Collagen gel culture	130 ± 19	12	39 ± 6

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Evidence for the extranuclear localization of thymosins in thymus

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Abstract. A new radioimmunoassay has been developed for thymosin β_4 by generating rabbit polyclonal antibodies against the synthetic N-terminal peptide fragment 1–15 coupled to KLH. The synthetic analogue [Tyr^{12}]-thymosin β_4 (1–15) was used as tracer. This radioimmunoassay, with a useful range of 10–1000 pmoles, showed cross-reactivity with the second homologous β-thymosin of man and rat (thymosin β_{10}) but not of calf (thymosin β_9). This radioimmunoassay, together with an improved radioimmunoassay for the N-terminus of parathymosin α, was employed for the measurement of the levels of thymosin β_4 and parathymosin α in nuclear and extranuclear extracts of calf thymus. The bulk of these polypeptides was found in the extranuclear material whereas only traces were observed in the nuclear environment, which indicates the extranuclear localisation of α- and β-thymosins.
Key words. Thymosin β_4; parathymosin α; thymosin; thymus.

Thymosins, polypeptides initially isolated from thymus, belong to α- and β-homologous families. The α-family contains prothymosin α^1 and parathymosin α^2, which have approximately 100 residues. The β-family contains thymosin β_4[3] and thymosin β_9, in calf[4], or thymosin β_{10} in human[5] and rat[6] tissues, with approximately 40 residues. Thymosins are widely distributed in mammalian tissues[7,8], including blood plasma[9,10]. They can be identified by chromatographic analysis and specific radioimmunoassays[11–16]. Despite the wide distribution of thymosins, little is known about their physiological role. Reports have indicated the involvement of thymosins in cell-mediated immunity phenomena[17–20] and cell proliferation[21–24].

Conflicting results exist as to the intracellular location of prothymosin α, suggesting its presence inside the nucleus[25,26], outside the nucleus[27–30] and in both compartments[31]. We present here results showing the extranuclear location of two other thymosins, i.e. thymosin β_4 and parathymosin α, by cellular fractionation and measurement of the cross-reactive materials by radioimmunoassay.

Materials and methods

Materials. Thymus was collected from a 14-month-old calf. Fractionation of nuclear and extranuclear material and whole tissue extraction was carried out as previously reported[30]. Bovine lung thymosin β_4 and thymosin β_9[32], rat thymus thymosin β_{10}[6], human thymus prothymosin α[33] and rat liver parathymosin α[17] were isolated by established procedures.

Radioimmunoassay for thymosin β_4. Peptide fragment thymosin β_4 (1–15) and the analog [Tyr^{12}]-thymosin β_4 (1–15) were prepared by solid phase synthesis on an automatic Synostat-peptide synthesizer (Biotronik, Maintal, Germany) using 4-(hydroxymethyl)-phenoxymethyl-copoly-(styrene-1% divinyl-benzene)-resin preloaded with FMOC-Ser (tBu)-OH (for each synthesis 0.33 g = 0.26 mmol). Both syntheses were performed by the BOP/HOBt coupling procedure using FMOC amino acids with tert.-butyl side chain protecting groups. All amino acids were coupled twice in DMF in threefold excess in the presence of 0.35 g BOP/0.11 g HOBt and 1.6 ml diisopropylethylamine (25% in DMF). For the synthesis of [Tyr^{12}]-thymosin β_4 (1–15) FMOC-Phe-OH