Biochemical Pharmacology, Vol. 37, No. 10, pp. 1939–1945, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

EFFECTS OF PEPTIDIC GLYCOSAMINOGLYCANS COMPLEX ON HUMAN CHONDROCYTES CULTIVATED IN THREE DIMENSIONS*

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(*Received* 27 *July* 1987; *accepted* 13 *November* 1987)

Abstract—Human chondrocytes from the pelvic joint were cultivated in suspension; under these conditions, after a few days, cells aggregated. These chondrocytes were morphologically differentiated (round shape, situated inside cavities and surrounded by a matrix synthesized during cultivation) and biosynthetically differentiated (synthesis of type II collagen and cartilage proteoglycans (PG) (Bassleer *et al. In vitro* **22**, 115–120, 1986).

In this work, we present the metabolic and cellular effects of a peptidic-glycosaminoglycan (P-GAG) complex isolated from calf cartilage and bone marrow. We analyzed the effects of P-GAG on DNA synthesis (appreciated by ^{3}H-thymidine incorporation into DNA), on type II collagen and on PG synthesis analyzed by specific radioimmunoassays.

According to its final concentration in culture medium, P-GAG was able to stimulate proliferation or to favor the production of specific components of cartilage matrix, type II collagen and PG.

It is well known that chondrocytes are characterized by their ability to synthesize an extracellular matrix. This matrix is essentially composed of type II collagen [1, 2] and cartilage proteoglycans (PG) [3]. Metabolic functions of chondrocytes have mainly been studied by cultivating chick embryo chondrocytes [4], mammalian articular chondrocytes [5, 6] or human chondrocytes [7] in monolayer. Under these conditions, chondrocytes in culture usually had an unstable phenotype and tended to dedifferentiate [8, 9]. In order to avoid this difficulty, chondrocytes were cultivated in agarose gel [10], in organ culture [11] or in tridimensional culture [12]. Human chondrocytes from macroscopically normal parts of human femoral head cartilage were cultivated in suspension. After a few days under these conditions, cells formed a cluster. These chondrocytes were morphologically differentiated (round shape, situated inside cavities and surrounded by a new matrix) and biosynthetically differentiated: they synthesized type II collagen and specific cartilage PG [12].

A peptidic-glycosaminoglycan (P-GAG) complex was isolated from cartilage and bone marrow. This complex was recommended for treatment of osteoarthritis. Some double blind studies strongly favored its clinical efficacy [13–17].

In order to define the metabolic and cellular effects of this GAG-complex on human cartilage, the preparation was tested on human articular chondrocytes in tridimensional culture. Cell multiplication, type II collagen and PG synthesis were analyzed.

* Supported by grant 3.4517.86 from the FRSM.

MATERIALS AND METHODS

1. *Compound to be tested*

The active component was isolated from Rumalon® [13] (Robapharm, Bâle). The commercial preparation was ultrafiltered on Amicon filter (cut-off of 100,000 d). The active component consisted in a glycosaminoglycan-peptide (P-GAG) complex with molecular weight ranging from 10^5 to 2.10^6 d., as determined by filtration on Sepharose 4B (Pharmacia) (personal communication). This complex was lyophilized and dissolved in sterile tridistilled water to obtain 2.3 mg/ml, a concentration equivalent to that of the active commercial complex in Rumalon® for pharmaceutical use and corresponding to 2.3 10^{-5}–1.15 10^{-6} M concentration.

2. *Human chondrocytes in culture*

Human chondrocytes were cultivated in *Ham's* F 12 medium (Flow Labs) supplemented with Ultroser G (2%) (IBF, LKB, France). Morphologically normal cartilage was taken from human femoral heads immediately after surgical operations. This hyaline cartilage was cut into small fragments which were digested by clostridial collagenase (Boehringer Mannheim, GmbH; 1 mg/ml) in a carbonate–bicarbonate buffer (pH 7.4, $CaCl_2$ 1 mM), for 24 hr. After six successive washings and centrifugations, cells were suspended (10^6 cells per flask) in 2 ml culture medium and placed on a gyratory shaker (100 rpm). Cultures were maintained at 37°, in an air atmosphere with 5% CO_2 [12]. After treatment, human chondrocytes were incubated with the active component (P-GAG) to be tested: the culture medium (2 ml total volume per flask) contained 0, 2.3, 23,

230 μg/ml of the peptidic glycosaminoglycan complex. Human chondrocytes were incubated with the compound for 5, 8, 11 and 14 days, or between the 15th and the 30th day of culture. For each concentration of P-GAG to be tested and for the corresponding controls, three flasks were used, each flask contained one chondrocyte cluster obtained from 10^6 isolated chondrocytes.

3. *Proteoglycans (PG) radioimmunoassay*

PG released into culture medium and PG present inside chondrocyte clusters were assayed according to the radioimmunoassay method previously described [18].

Culture media were directly assayed for proteoglycans. Chondrocyte clusters were washed three times with PBS (phosphate buffer saline), then homogenized in PBS by ultrasonic dissociation at 4° (1 min; power: 200 W/cm^2). The extraction allowed to recover 70–80% of all the HCl–guanidine extractable proteoglycans. Various concentrations of calf P-GAG (2.3, 23, 230 μg/ml) had no significant effect on the binding of labeled human PG to their antibodies.

4. *Type II collagen radioimmunoassay*

(a) *Preparation of type II collagen.* Type II collagen was extracted from human articular cartilage by the method described by Herbage *et al.* [19]. The purity of type II collagen was assessed by SDS. Polyacrylamide gel electrophoresis at 7.5%, according to Laemmli [20], slightly modified by the omission of urea in the electrophoresis buffer.

(b) *Antisera.* Antisera against type II collagen were raised in guinea-pigs using the Vaitukaitis *et al.* method [21] consisting in multiple intradermal injections of the antigen in suspension in incomplete Freund adjuvant.

Iodination: Type II collagen was labeled with ^{125}iodine according to the iodogen (R) method [22].

Radioimmunoassay: The radioimmunoassay was performed classically in 400 μl containing respectively 100 μl of the tracer (diluted to obtain 20,000 cpm); 100 μl of the incubation buffer; 100 μl of the reference solution of type II collagen (1–1000 ng) or of culture medium, or of cluster extracts to be measured, and finally, 100 μl of a 1/1000 dilution of the antiserum. After a 48 hr incubation at 4°, the collagen antibody complexes were separated from the free labeled collagen by the double antibody system [23].

Various concentrations of P-GAG (2.3, 23, 230 μg/ml) had no significant effect on the binding of labeled type II collagen to its antibodies. Cartilage proteoglycans, fibronectin, laminin, link protein, as well as type I and III collagen did not cross-react with type II collagen in the radioimmunoassay.

5. *Analysis of cell multiplication*

After different culture durations in the presence of P-GAG (4, 7, 10, 13 and 27 days), chondrocyte clusters were further incubated in culture medium supplemented with P-GAG and (^{3}H)-methyl thymidine (2 μCi/ml; 5 Ci/mmole, Amersham) for 24 hr. After this period of incubation, chondrocyte clusters were then washed with phosphate buffer saline (PBS) and ultrasonicated. Incorporated radioactivity was counted with a beta counter and chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [24].

6. *Calculation and statistical analysis*

Results were expressed as rates of production per 24 hr and per μg of DNA (daily production released into culture medium), and as cumulative production. The total production of PG and type II collagen was also calculated by adding the amount found in culture media and inside corresponding clusters.

Means and standard deviations of variables (^{3}H-thymidine incorporation, type II collagen and cartilage PG synthesis) were calculated for each concentration of P-GAG studied here. To assess a possible dose-effect on the variables, a one-way analysis of variance was performed (*F*-test). Wherever a significant result ($P < 0.05$) was obtained, simultaneous confidence intervals for mean contrast were calculated in order to identify the differing dose. We compared response curves for the four dose levels (0, 2.3, 23 and 230 μg/ml culture medium) by applying Zerbe's method [25]. This method allows comparison of response curves not only for each culture day, but also over any time interval, thus providing a global assessment of dose differences.

RESULTS

1. *Effects of P-GAG on ^{3}H-thymidine incorporation*

Table 1 shows that in the controls, ^{3}H-thymidine incorporation into DNA increased during the first days of culture. The incorporation was maximal after 8 days of culture; then it decreased as a function of culture duration. After 5, 8, 11 and 14 days of

Table 1. ^{3}H Thymidine incorporation into DNA (cpm/μg DNA)

Culture duration (days)	Control cultures (cpm/μg DNA)	2.3 μg P-GAG/ml (cpm/μg DNA)	23 μg P-GAG/ml (cpm/μg DNA)	230 μg P-GAG/ml (cpm/μg DNA)
5	1381 ± 265	2467 ± 386*	1775 ± 190 (NS)	1327 ± 196 (NS)
8	2970 ± 315	4247 ± 532*	3248 ± 260 (NS)	2991 ± 229 (NS)
11	1500 ± 301	2655 ± 102*	2015 ± 241 (NS)	1700 ± 141 (NS)
14	481 ± 40	784 ± 97*	580 ± 88 (NS)	483 ± 39 (NS)
28	363 ± 82	357 ± 48	502 ± 78 (NS)	473 ± 65 (NS)

NS non significative value (U-test Mann–Whitney).
* $P < 0.05$ (U-test Mann–Whitney) compared to control.

Table 2. Daily cartilage PG released into culture medium (μg PG/μg DNA—24 hr Mean ± SD)

Period of incubation		Control cultures	2.3 μg P-GAG/ml	23 μg P-GAG/ml	230 μg P-GAG/ml
A	1–4	1.24 ± 0.15	1.20 ± 0.16 (NS)	1.60 ± 0.20*	2.16 ± 0.36*
	4–7	0.96 ± 0.21	1.19 ± 0.10 (NS)	1.59 ± 0.12*	1.75 ± 0.21*
	7–10	0.81 ± 0.10	0.71 ± 0.10 (NS)	1.34 ± 0.11*	1.63 ± 0.15*
	10–13	0.50 ± 0.06	0.47 ± 0.07 (NS)	0.90 ± 0.20*	1.23 ± 0.20*
B	15–17	0.51 ± 0.07	0.73 ± 0.06*	0.71 ± 0.06*	0.91 ± 0.09*
	17–20	0.30 ± 0.02	0.49 ± 0.03*	0.55 ± 0.06*	0.57 ± 0.06*
	20–22	0.32 ± 0.03	0.50 ± 0.04*	0.54 ± 0.07*	0.70 ± 0.07*
	22–24	0.35 ± 0.03	0.50 ± 0.06*	0.63 ± 0.05*	0.67 ± 0.07*
	24–27	0.24 ± 0.03	0.38 ± 0.06*	0.42 ± 0.06*	0.49 ± 0.04*

* $P < 0.05$ (compared to control).
NS non-significant value.

incubation, 2.3 μg P-GAG per ml of culture medium significantly stimulated ^{3}H-thymidine incorporation ($P < 0.05$), while 23 and 230 μg/ml had no effect on this metabolic parameter (Table 1). When chondrocytes were incubated between the 14th and the 28th day of culture, P-GAG had no effect on DNA synthesis in chondrocyte clusters on the 28th day of culture (Table 1).

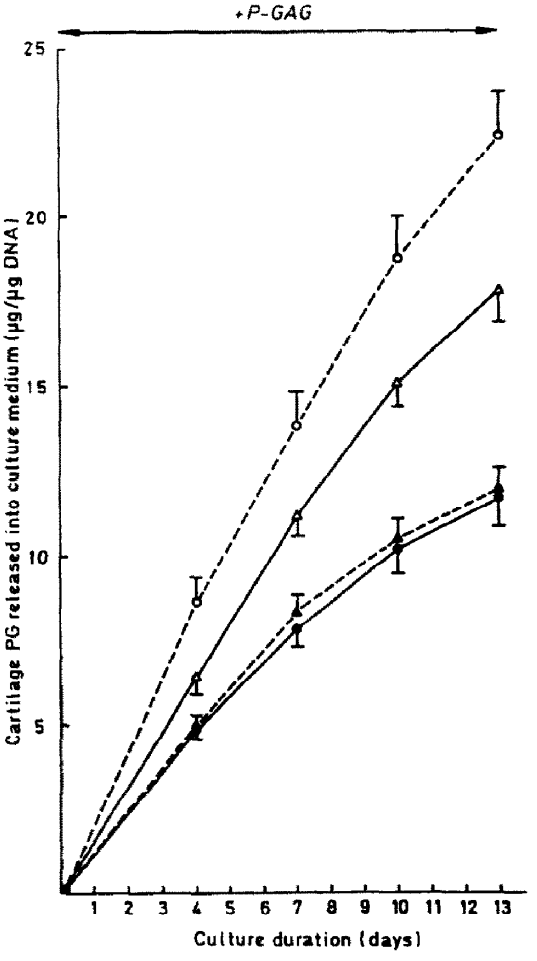

Fig. 1. Effects of P-GAG on human cartilage proteoglycans released into culture medium as a function of culture duration (cumulative values; mean ± SEM): ●—●, 0 μg P-GAG/ml; ▲—▲, 2.3 μg P-GAG/ml; △—△, 23 μg P-GAG/ml; ○—○, 230 μg P-GAG/ml.

2. *Effects of P-GAG on PG synthesis*

(a) *PG released into culture medium.* Table 2A shows that a progressive decrease of the daily release of PG into culture medium took place as a function of culture duration (0–13 days): there was a highly significant negative correlation ($r = -0.91$) between the PG released into culture medium and the culture duration, for each concentration of P-GAG studied.

When human chondrocytes were treated between the 1st and the 14th day of culture, 2.3 μg P-GAG per ml of culture medium had no effect on the PG released daily into culture medium per μg of DNA, while 23 or 230 μg P-GAG/ml significantly stimulated the release of cartilage PG into culture medium (Table 2).

The analysis of variances showed a significant dose-effect on the PG released daily into culture medium ($P < 0.05$).

When chondrocytes were incubated, between the 14th and 28th day of culture, 2.3, 23 and 230 μg P-GAG per ml culture medium significantly stimulated the PG release, in a dose-dependent way (Table 2B). Analysis of variances showed a significant dose-effect of the PG released daily into culture medium ($P < 0.05$). There was a highly significant negative correlation between the PG released into culture medium and the culture duration for each concentration of P-GAG studied ($r = -0.73$).

The cumulative PG production released into culture medium increased as a function of culture duration until the 13th day of culture (Fig. 1). Randomization analysis of the response curves of cumulative PG released into culture medium showed a significant difference ($P < 0.01$) between the four P-GAG doses for the first 14 days of culture. In contrast there was no significant difference between the cumulative PG production curves for the last 14 days of culture, whatever the dose of P-GAG complex studied.

(b) *Cartilage PG content inside chondrocyte clusters.* After 8 and 11 days of incubation, P-GAG (2.3, 23 and 230 μg/ml) was found to have no significant effect on the amount of PG present inside the chon-

Table 3A. Cartilage PG content inside chondrocyte clusters (μg/μg DNA)

Culture duration	Control cultures	2.3 μg P-GAG/ml	23 μg P-GAG/ml	230 μg P-GAG/ml
8	1.60 ± 0.20	1.48 ± 0.08 (NS)	1.64 ± 0.06 (NS)	1.46 ± 0.14 (NS)
11	1.56 ± 0.18	1.42 ± 0.14 (NS)	1.54 ± 0.22 (NS)	1.38 ± 0.10 (NS)
14	1.88 ± 0.18	2.02 ± 0.15 (NS)	3.28 ± 0.17*	3.00 ± 0.26*
28	1.35 ± 0.06	1.31 ± 0.13 (NS)	1.38 ± 0.14 (NS)	1.37 ± 0.15 (NS)

* $P < 0.025$ compared to the control.
NS non-significant value compared to the control.

Table 3B. Total PG production (μg PG/μg DNA)

Culture duration (days)	Controls	2.3 μg P-GAG/ml	23 μg P-GAG/ml	230 μg P-GAG/ml
0–8	10.4 ± 1.66	11.04 ± 1.12	14.4 ± 1.34	17.10 ± 2.42
0–11	12.79 ± 1.82	13.11 ± 1.48	13.32 ± 1.83	21.91 ± 2.83
0–14	14.61 ± 2.09	15.12 ± 1.7	22.76 ± 2.38	27.22 ± 3.59
14–28	6.11 ± 0.52	8.53 ± 0.86	9.17 ± 1.03	10.51 ± 0.97

drocyte clusters. After 14 days of incubation, P-GAG (23 and 230 μg/ml) induced a significant ($P < 0.025$) increase of the amount of PG inside the chondrocyte clusters (Table 3A). There was no dose–effect relation. When chondrocytes were treated between the 14th and the 28th days of culture, P-GAG had no effect on the PG level inside the chondrocyte clusters (Table 3A).

(c) *Total production.* By adding the amounts of PG released into culture medium (μg/μg DNA) and the amount of PG present in the clusters (μg/μg DNA), it was clear that for each period of incubation the mean PG production increased in a dose-dependent manner when chondrocytes were incubated with P-GAG (Table 3B).

3. *Effects of P-GAG on type II collagen synthesis*

(a) *Type II collagen released into culture medium.* Table 4A shows a progressive decrease of the daily production of type II collagen released into culture medium, as a function of culture duration (0–13 days). For each dose of P-GAG studied, $r = -0.9$, there was a highly significant negative correlation between the type II collagen released daily into culture medium and the culture duration.

P-GAG (23 and 230 μg/ml) significantly increased the daily production of type II collagen in culture medium, while 2.3 μg P-GAG/ml had no effect on this parameter (Table 4A). The analysis of variance showed a significant dose-effect on type II collagen released into culture medium ($P < 0.05$).

When chondrocytes were incubated with P-GAG between the 14th and the 28th day, no modification of the daily production of type II collagen was observed, whatever the dose of P-GAG studied. Randomization analysis of cumulative response curves of type II collagen released into culture medium (Fig. 2) showed a significant difference ($P < 0.01$) between the four P-GAG doses (respectively: 0, 2.3, 23 and 230 μg/ml). In contrast, there

Table 4A. Daily type II collagen released into culture medium (μg/μg DNA/24 hr)

Period of incubation (days)	Control cultures	2.3 μg P-GAG/ml	23 μg P-GAG/ml	230 μg P-GAG/ml
4	1.25 ± 0.10	1.27 ± 0.13 (NS)	1.60 ± 0.17*	1.75 ± 0.18*
7	0.60 ± 0.07	0.63 ± 0.06 (NS)	0.84 ± 0.07*	0.86 ± 0.08*
10	0.40 ± 0.03	0.40 ± 0.035 (NS)	0.60 ± 0.075*	0.62 ± 0.08*
13	0.33 ± 0.03	0.37 ± 0.004 (NS)	0.52 ± 0.055*	0.62 ± 0.065*

* $P < 0.05$ compared to control.
NS not significantly different.

Table 4B. Total type II collagen production (μg/μg DNA)

Culture duration (days)	Controls	2.3 μg P-GAG/ml	23 μg P-GAG/ml	230 μg P-GAG/ml
0–8	7.80 ± 0.70	7.98 ± 0.78	10.13 ± 0.98	10.84 ± 1.05
0–11	9.00 ± 0.79	9.23 ± 0.90	11.98 ± 1.20	12.72 ± 1.33
0–14	10.10 ± 0.94	10.43 ± 0.93	13.63 ± 1.40	14.69 ± 1.54
14–28	1.22 ± 0.13	1.25 ± 0.10	1.44 ± 0.09	1.45 ± 0.08

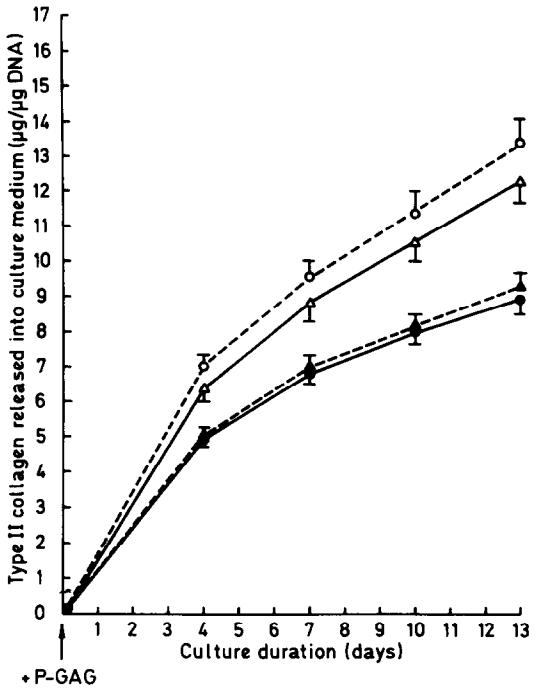

Fig. 2. Effects of P-GAG on type II collagen released into culture medium as a function of culture duration (cumulative values; mean ± SEM); ●—●, 0 µg P-GAG/ml; ▲—▲, 2.3 µg P-GAG/ml; △—△, 23 µg P-GAG/ml; ○—○, 230 µg P-GAG/ml.

was no significant increase when P-GAG was added from the 14th to the 28th day of culture whatever the dose of P-GAG studied.

b. *Type II collagen content inside chondrocyte clusters*. There was no apparent effect of P-GAG (2.3, 23 and 230 µg/ml) on the amount of type II collagen in chondrocyte clusters.

c. *Total production*. By adding the amount of type II collagen released into culture medium (µg/µg DNA) and the amount of type II collagen present in chondrocyte clusters (µg/µg DNA), it was apparent that for each period of incubation, the production of type II collagen was dose-dependently increased when human chondrocytes were incubated with P-GAG (Table 4B).

DISCUSSION

In our previously reported studies [12], chondrocytes were shown to differentiate morphologically as well as biosynthetically, and to synthesize a new cartilage matrix composed among other components of type II collagen and cartilage proteoglycans [18, 26]. This new PG and collagen production was accompanied by an increase of the net weight of the chondrocyte clusters and by an important development of the matrix, as demonstrated by biochemical and histological analysis (electron and optic microscope observations and immunofluorescence analysis) [12, 26]. In this work, we used this model for testing the effects of a peptidic-glycosaminoglycan (P-GAG) complex on anabolic cellular parameters (DNA, type II collagen and cartilage PG synthesis).

This work confirmed previous personal results concerning the production of specific substances by differentiated human chondrocytes cultivated under these conditions. Synthesis of PG and type II collagen (expressed as amounts released into culture medium per 24 hr and per µg DNA) and cell multiplication were active but decreased as a function of culture duration [12].

When human chondrocytes were cultivated according to the same method, but in presence of P-GAG, synthesis of PG and type II collagen were significantly stimulated. Thus, PG and type II collagen released into culture medium, cumulative and total production were increased in a dose-dependent way when chondrocytes were incubated with P-GAG. These clearcut effects were in keeping with those of Vacha *et al.* [27], who have shown that Arteparon®, a glycosaminoglycan polysulfate (GAG-PS) stimulated the chondrocyte synthesis of RNA, of collagen and of the components. Moreover, our results can be related to studies of the action of GAG and PG on cartilage. Nevo and Dorfman [28] observed that the synthesis of chondroitin sulfate-PG (CS-PG) by suspension cultured chondrocytes was stimulated by the addition of a variety of polyanions, including chondroitin sulfate (200 µg/ml). This effect was confirmed by Huang [29]. In contrast, high concentrations of exogenous PG (10 ng/ml) can selectively depress both collagen and PG synthesis by cultured chondrocytes [30]. Similar concentrations of GAG only had a slight inhibitory effect [31]. CS-PG can apparently act by both positive and negative feedback mechanisms on matrix synthesis. PG is rapidly deposited in the pericellular matrix of freshly dissociated cartilage cells [32].

The effects of P-GAG on DNA synthesis depended on its concentration in culture medium: the concentrations of P-GAG which stimulated DNA synthesis had no effect on the PG and the type II collagen synthesis, and reciprocally. So, according to the dose, P-GAG can stimulate either cell multiplication, or PG and type II collagen synthesis. Such a pattern is similar to that of other substances such as FGF (fibroblast growth factor) [33]. A few substances can simultaneously stimulate chondrocyte multiplication and synthesis of their specific products as, for example, ascorbic acid [34] or cartilage derived factor (CDF; [35, 36]). This was not the case for the P-GAG complex. These biological effects were obtained with molar concentrations situated between 2.10^{-9} M and 2.10^{-7} M, which are active concentrations, not only for many pharmaceutical drugs, but also for many hormones which are present in these concentrations in biological fluids (sex steroids in follicular fluid [37], cortisol in plasma [38].

When results were expressed as percentages, it was clear that the effects of P-GAG were stronger on the PG production than on the production of type II collagen. This quantitative difference may be related to the fact that in normal cartilage, PG turnover is more active than that of type II collagen [39, 40].

When considering the results obtained in absolute values, the effects of P-GAG when chondrocytes

were treated during the first 14 days of culture were also stronger than when the treatment was applied between the 14th and the 28th day. This suggested that the effects can be different when the substances acted at the beginning of cultivation or after a relatively long period of culture: at the beginning of culture, chondrocytes constituted a new intercellular matrix (high metabolic activity). Between the 14th and the 28th day of culture, the clusters were constituted: the cluster's fresh weight was stable [12] and metabolic activity was decreasing.

The lack of obvious effect of P-GAG on PG and type II collagen content of clusters may be related to the fact that a large part of PG and type II collagen were released into culture medium and were not integrated inside chondrocyte clusters. This interpretation was supported by comparing the daily PG production in culture medium with the cluster's PG content. For example, after 8 days of culture, 80% of the total PG production was released into culture medium, while 20% total of the PG production was integrated in chondrocyte clusters. Therefore significant variations of PG content inside chondrocyte clusters were not easily detected. Another explanation could be that although P-GAG had a stimulatory effect on the production of PG and type II collagen, a defect in chondrocyte metabolism prevented adequate production of hyaluronic acid, link protein or chondronectin and thus a correct integration inside the clusters.

The stimulatory effects of P-GAG on the human chondrocyte metabolism observed in our model *in vitro* could explain the clinically significant results obtained with this substance in a disease characterized by a decrease of the function of chondrocytes, as in osteoarthritic cartilage [13–17]. In clinically defined osteoarthritis, chondrocyte function is depressed after a compensatory period [41–43], leading to cytologic alteration, decrease of PG content [44, 45] and cell death [46, 47]. P-GAG could be an alternative to compensate the osteoarthritic related decrease of chondroformation.

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