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Effects of Glucosamine, Dibutyryl Cyclic AMP and Lymphocytes on Retrobulbar Fibroblast Synthesis of Hyaluronic Acid (39695)[1]

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The interaction of cultured human retrobulbar fibroblasts with lymphocytes has been used as an *in vitro* analogy to Graves' ophthalmopathy (1-3). Previously we have demonstrated that cyclic 3′,5′-adenosine monophosphate (cAMP) mediates stimulation of hyaluronic acid (HA) synthesis in the fibroblasts induced by lymphocytes. We now show that glucosamine (GlcN), a precursor of HA, enters the fibroblasts and is incorporated into HA. The use of GlcN on the cultures facilitated investigation of loci in the biosynthetic process at which the cAMP could act. Studies here reported show that the intracellular concentration of GlcN is rate limiting in fibroblast synthesis of HA, and that lymphocytes and dibutyryl cAMP (DbcAMP) appear to stimulate HA synthesis at a point which is dependent upon preformed GlcN.

Materials and methods. Cultures of fibroblasts from normal human retrobulbar connective tissue were initiated and perpetuated as described previously (2). Lymphocytes from normal subjects were obtained and frozen-thawed before use by a method noted in a prior publication (1). D-Glucosamine-HCl, D-galactosamine-HCl (GalN), and glucosamine-6-PO_4 as the sodium salt (GlcN-6-PO_4) were purchased from the Sigma Chemical Company (St. Louis, Mo.); N_6-2′-*O*-dibutyryl adenosine 3′,5′-cyclic phosphate (DbcAMP) was obtained from Schwarz/Mann (Orangeburg, N.Y.), D-[1-^{14}C]glucosamine-HCl ([^{14}C]GlcN), 9.6 mCi/nmole, was purchased from New England Nuclear Corporation (Boston, Mass.). The above materials were dissolved in CMRL 1066 medium and sterilized by filtration.

Medium composition was as recorded before (2), and experimental design, except where noted, was as previously described (4). Methods for analyzing media glucose, media glycosaminoglycans (assumed to be HA from prior studies), and fibroblast protein have been reported (2).

[^{14}C]GlcN was added to cultures in the quantity of 0.9 μCi and 92 nmole per flask. The [^{14}C]HA was determined by dissolving the cetylpyridinium-HA precipitate (obtained in the extraction GAG from media for uronic acid analysis) in methanol and counting the ^{14}C in a liquid scintillation counter. The ^{14}C background counts which relate to contaminating [^{14}C]GlcN were obtained by adding the 0.9 μCi of [^{14}C]GlcN to flasks in each experimental group at the end of the culture period; this background activity (5% or less of the total ^{14}C count rate) was subtracted to give the net [^{14}C]HA activity. The net [^{14}C]HA was divided by the total [^{14}C]GlcN activity added to give the fraction of medium GlcN converted to HA. The total HA derived from medium GlcN was then calculated by multiplying the fraction converted by the nanomoles of GlcN present in the medium.

Results. Additions of GlcN, 5×10^{-4}-5×10^{-3} *M*, to the media of fibroblast cultures resulted in dose-related increases in HA synthesis. However, no additional HA production was observed when GlcN concentrations were increased above 5×10^{-3} *M*; indeed, the quantities of HA recovered in these circumstances were often less (Tables I and II). Glucose utilization was inconsistently affected, and fibroblast protein of each flask was unaltered by the presence of GlcN.

Glucosamine-6-PO_4, the intracellular form of GlcN entering the HA biosynthetic

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pathway, was no more effective and, at times, less effective than GlcN in enhancing HA synthesis. Additions of GalN, 10^{-4}–10^{-2} M, did not alter HA production. Glucose enrichment of media up to 5×10^{-3} M resulted in little or no increases in HA production.

Stimulations of fibroblasts by DbcAMP in the presence of GlcN, 5×10^{-3} M, produced quantities of HA which were greater than those calculated by summing the HA responses observed when DbcAMP and GlcN were added independently to cultures (Table II). Concentrations of GlcN down to 10^{-4} M potentiated the DbcAMP effect. However, increasing the concentrations of GlcN to 10^{-2} M in association with DbcAMP resulted in no additional synthesis of HA. Comparable effects of GlcN on the synthesis of HA were seen in experiments where lymphocytes replaced DbcAMP (Table II).

To evaluate the importance of intracellular GlcN in the response to DbcAMP control medium and medium containing GlcN were removed from the cultures after 2 days and just before the addition of the cyclic nucleotide derivative (Table III). In Group B (Table III), GlcN was introduced into the cultures for only a few minutes before DbcAMP to assess the effect of residual GlcN in the medium, i.e., the GlcN which could not be removed in the change of media. Residual GlcN enhanced the stimulation of HA synthesis by DbcAMP (Group B compared with Group A). However, when the fibroblasts were exposed to medium enriched with GlcN for 2 days before the addition of DbcAMP, the production of HA was significantly greater than that obtained when GlcN had been present only briefly (Group C compared with Group B in Student's t test).

By following the fate of radiolabeled GlcN, the GlcN in medium was observed to be readily incorporated into HA (Table IV). In the presence of medium enriched with GlcN (Group B in Table IV), most, if not all, of the HA was composed of GlcN which

TABLE I. EFFECTS OF GLUCOSAMINE ON RETROBULBAR FIBROBLAST SYNTHESIS OF HA.

Concentration of GlcN ($\times 10^{-3}$ M)	No. of flasks	HA in media[a] (nmole of uronic acid/mg fibroblast protein)[b]	(E/C)[c]
0.0	(4)	71 ± 4	
0.5	(4)	124 ± 10	1.75
1.0	(4)	149 ± 7	2.10
5.0	(4)	180 ± 17	2.54

[a] Media collected over Days 2–4 of culture.
[b] Mean ± SEM.
[c] Experimental group mean ÷ control group mean.

TABLE II. EFFECTS OF GLUCOSAMINE ON THE STIMULATION OF RETROBULBAR FIBROBLAST PRODUCTION OF GAG BY DIBUTYRYL CYCLIC AMP AND LYMPHOCYTES.

Additives and concentrations		No. of flasks	HA in media[a] (nmole of uronic acid/mg of fibroblast protein)[b]	(increments above basal concentration)
Experiment No. 1				
None		(4)	151 ± 5	
DbcAMP	2×10^{-3} M	(3)	587 ± 15	436
GlcN	5×10^{-3} M	(4)	264 ± 4	113
GlcN	10^{-2} M	(4)	245 ± 7	94
DbcAMP plus GlcN	2×10^{-3} M + 5×10^{-3} M	(4)	1034 ± 26	883
DbcAMP plus GlcN	2×10^{-3} M + 10^{-2} M	(3)	995 ± 63	844
Experiment No. 2				
None		(4)	61 ± 5	
Lymphocytes	5×10^5/ml	(4)	167 ± 6	106
GlcN	5×10^{-3} M	(4)	240 ± 14	179
GlcN	10^{-2} M	(4)	231 ± 11	170
Lymphocytes plus GlcN	5×10^5/ml + 5×10^{-3} M	(4)	479 ± 23	418
Lymphocytes plus GlcN	5×10^5/ml + 10^{-2} M	(4)	389 ± 21	328

[a] Media collected over Days 2–4 of culture.
[b] Mean ± SEM.

originated external to the cells. Medium GlcN, at 5×10^{-3} *M*, also served as the sole source of this precursor when HA synthesis was stimulated by DbcAMP (Group D in Table IV). The HA calculated (by ^{14}C counting) to be derived from medium GlcN in Group D (269 nmole) seems to exceed the total HA measured as uronic acid (231 nmole), but the two values are not significantly different by Student's *t* test.

Discussion. In the presence of media enriched with GlcN, there is enhanced synthesis of HA production in retrobulbar fibroblast cultures stimulated by lymphocytes and by DbcAMP (which is considered to act as does the intracellular mediator, cAMP, of the lymphocytes' action). Potentiation could have related to the effect of cAMP on an expanded intracellular pool of GlcN or to cellular uptake of GlcN which was accelerated by cAMP. In Table III, greater potentiation was seen when the fibroblasts were exposed to GlcN for 2 days before the introduction of DbcAMP in new media (Group C) than when GlcN was presented to the cells for only a few minutes (Group B): these data indicate that at least some of the potentiation of the DbcAMP stimulation resulted from GlcN which had already entered the fibroblasts.

There was some potentiation of the response to DbcAMP in Group B in Table III which must have been related to residual GlcN not removed from the culture flasks when the media were changed. This residual GlcN could have been taken up by the fibro-

TABLE III. EFFECTS ON RETROBULBAR FIBROBLAST PRODUCTION OF GAG WHEN GLUCOSAMINE IS ADDED TO AND REMOVED FROM MEDIA.

Groups	Additives and concentrations: Days 2-3[a]	Additives and concentrations: Days 3-5	No. of flasks	HA in media (nmole of uronic acid/mg of fibroblast protein)[b]	(increment above respective basal concentrations[c])
A. (1)	None	None	(5)	43 ± 1	356
(2)	None	DbcAMP, 2×10^{-3} *M*	(5)	399 ± 11	
B. (1)	None	GlcN, 5×10^{-3} *M* briefly, then none	(5)	47 ± 2	416
(2)	None	GlcN, 5×10^{-3} *M* briefly, then DbcAMP, 2×10^{-3} *M*	(5)	463 ± 33	
C. (1)	GlcN, 5×10^{-3} *M*	None	(5)	78 ± 3	499
(2)	GlcN, 5×10^{-3} *M*	DbcAMP, 2×10^{-3} *M*	(5)	577 ± 28	
D.	GlcN, 5×10^{-3} *M*	GlcN, 5×10^{-3} *M*	(5)	135 ± 4	

[a] After Day 3, media were removed and flasks were washed with 1 ml of respective medium to be used on Days 3-5, then medium for Days 3-5 added. In group B, GlcN, 5×10^{-3} *M*, 2.5 ml, was added before wash and immediately removed.

[b] Mean ± SEM.

[c] In each group, mean of Subgroup (1) subtracted from mean of Subgroup (2).

TABLE IV. INFLUENCE AND FATE OF MEDIA GLUCOSAMINE IN THE SYNTHESIS OF HYALURONIC ACID BY RETROBULBAR FIBROBLASTS.

Additives to media	Hyaluronic acid synthesized[a] (nmole of uronic acid/flask): No.[c]	(nmole of uronic acid/flask): Mean ± SEM	(nmole derived from GlcN in media)[b]: No.[c]	(nmole derived from GlcN in media)[b]: Mean ± SEM
A. None	(4)	26 ± 2	(4)	0.62 ± 0.06
B. GlcN, 5×10^{-3} *M*	(4)	55 ± 6	(4)	43 ± 12
C. DbcAMP, 10^{-3} *M*	(4)	87 ± 2	(4)	3.5 ± 0.5
D. GlcN, 5×10^{-3} *M* plus DbcAMP 10^{-3} *M*	(4)	231 ± 3	(4)	269 ± 31

[a] Media collected over Days 2-4 of culture.

[b] Calculated by recording the percent of [^{14}C]GlcN incorporated into HA and relating this value to the total GlcN in initial media: Groups A and C had 92 nmole of GlcN/flask (from the commercial [^{14}C]GlcN), and Groups B and D had 12,592 nmole of GlcN/flask.

[c] Media obtained from parallel flasks for each assay.

blasts through a cAMP-dependent or a cAMP-independent process. Thus, although DbcAMP, and presumably cAMP, can be shown to act on intracellular GlcN or on a site dependent upon intracellular GlcN, the possibility that the cyclic nucleotide also enhances cellular entry of GlcN cannot be excluded.

In HA synthesis, preformed GlcN appears to be preferentially utilized over GlcN converted from glucose by the fibroblasts (Table IV). Thus, the availability of GlcN seems to be one rate-limiting step in the synthesis of HA. However, since medium concentrations of GlcN greater than 5×10^{-3} *M* induce no additional effect, but rates of HA synthesis can be further enhanced by DbcAMP, there also appears to be a second, cAMP-dependent, rate-limiting step. This second step is dependent upon, but subsequent to, the availability of intracellular GlcN.

There are alternative explanations of the observed data. It is possible that the system which transports GlcN into the cell becomes saturated when medium concentrations of GlcN reach 5×10^{-3} *M*. Although at least some effects of DbcAMP relate to the intracellular GlcN, an action of the cyclic nucleotide derivative which enhances the entry of GlcN into fibroblasts, even after the normal transport system has been saturated, is possible. In this case, no rate-limiting step subsequent to the formation of GlcN and dependent upon cAMP needs to be postulated.

Another mechanism may contribute to the results of the experiments. Glucosamine, at concentrations above 2×10^{-3} *M*, has been shown to inhibit glycolytic enzymes and to reduce the concentration of ATP in retinas (5). Therefore, the failure of concentrations of GlcN above 5×10^{-3} *M* to increase HA synthesis by retrobulbar fibroblasts may relate to toxic effects attributable to GlcN. The toxic effects may also have limited the synthesis of endogenous cAMP, a deficiency remedied by the added DbcAMP. This action of GlcN eliminates the need to invoke a cAMP-dependent, rate-limiting step in physiological HA synthesis. In this explanation, the effect of DbcAMP, which is potentiated by concentrations of GlcN less than 5×10^{-3} *M*, may derive from enhanced cellular uptake of medium GlcN and a replenishment of intracellular cAMP stores which are reduced by the presence of abnormally large quantities of GlcN.

Summary. When added to the media of cultures of retrobulbar fibroblasts, GlcN, a precursor of HA, increased the synthesis of, and was readily incorporated into, HA. The formation of GlcN by the fibroblasts appears to be rate limiting in the biosynthetic pathway of HA.

Media enriched with GlcN potentiated the stimulations of HA production induced by lymphocytes and DbcAMP. The potentiation related to an expanded intracellular quantity of GlcN. Increasing the GlcN available to the fibroblasts resulted in a maximum HA synthesis which could be exceeded by the simultaneous presence of DbcAMP. There may be a cAMP-dependent step in HA synthesis subsequent to the formation of glucosamine.

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