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Mechanisms by which Retrobulbar Fibroblasts are Stimulated by Lymphocytes: Role of Cyclic Nucleotide (39677)

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Accumulations of glycosaminoglycans (GAG) and aggregations of lymphocytes in orbital tissues are characteristic features of active Graves' eye disease (1, 2). Cultures of fibroblasts derived from human retrobulbar connective tissue produce a specific GAG, hyaluronic acid (HA), whose synthetic activity is stimulated by lymphocytes and lymphocyte products (3-5). The increased synthesis of HA by cultured cells from the human orbit may provide an analogy to Graves' ophthalmopathy. It is of interest, therefore, to investigate the intracellular processes by which stimulation takes place.

Cyclic 3',5'-adenosine monophosphate (cAMP) is a mediator of many intracellular changes including the stimulation of thyroid cells by thyrotropin (6) and the enhanced HA synthesis by synovial fibroblasts (7). The studies reported in this communication demonstrate in retrobulbar fibroblasts enhanced synthesis of HA in response to dibutyryl cyclic AMP (DbcAMP) and to theophylline and a rise in intracellular cAMP after exposure to lymphocytes. These findings support the concept that cAMP mediates the lymphocyte stimulation of HA synthesis in the cultured cells. Hydrocortisone, which inhibits the lymphocyte stimulation of fibroblasts (4, 5), appears to do so by interfering with the intracellular cAMP response to the stimulus.

Materials and methods. Cultures of fibroblasts derived from the normal human retrobulbar connective tissue of two patients were initiated and perpetuated as described in an earlier publication (4). For experiments, T-15 flasks were plated with 3.5-4.5 $\times$ 10^5 fibroblasts in 2 ml of stock medium (4). After allowing 1 day to initiate growth, stock media were replaced by 2.5 ml of nutrient solutions containing test materials but no human serum. Except in the first experiment, where cells from two donors were studied, fibroblasts from a single patient were used. Lymphocytes were obtained from the peripheral blood of normal donors by a method previously reported (3) and contained less than 1% granulocytes and 5% monocytes. The lymphocytes were incubated, 5 $\times$ 10^6/ml, in 199 medium (Difco, Detroit, Mich.) supplemented with 10% fetal calf serum at 37° for 24 hr after which they were frozen in 6.0% dimethyl sulfoxide and stored at −78° until use. When needed, lymphocytes were melted and centrifuged at 150*g* after which the supernatant was discarded and the cells frozen-thawed three times in sterile distilled water, 5 $\times$ 10^6 cells/0.1 ml. Freeze-thawing was utilized to help ensure more uniform preparations for stimulation; no evidence of HA production or glucose utilization was found in flasks containing frozen-thawed lymphocytes and media. The lymphocyte preparations were added to cultures in the experimental media at a final concentration equivalent to 5 $\times$ 10^5/ml, a quantity found to stimulate fibroblast production of HA regularly to a significant degree (3).

Hydrocortisone (Sigma Chemical Co., St. Louis, Mo.) was prepared as recorded in a prior publication (4). Theophylline was purchased from Sigma. N_6-2'-*O*-dibutyryl adenosine 3',5'-cyclic phosphate was obtained from Schwarz/Mann (Orangeburg, N.Y.). The above materials were dissolved in CMRL 1066 medium and sterilized by filtration.

Experiments were carried out for 3 days (Days 2-4 of the culture) at 37° after which the medium was harvested for determina-

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tions of residual glucose and of GAG which was expressed as uronic acid (3). The fibroblast sheets were washed three times with buffered saline and analyzed for protein (4). Appropriate blank cultures without fibroblasts were included to ascertain initial glucose and GAG values in media.

Intracellular cAMP was extracted from the washed fibroblast sheet in the flasks with 6% trichloroacetic acid (TCA). Ethyl ether saturated with water was then used to extract the TCA from the aqueous phase containing the cAMP; after evaporation, the cAMP residues were taken up in 0.05 *M* sodium phosphate buffer, pH 6.2, and the concentrations measured by radioimmunoassay using kits purchased from Schwarz/Mann. Recovery of cAMP was monitored by counting [^{3}H]adenosine, previously added to the TCA, in the final solutions, as suggested by Sato *et al.* (8).

The nature of the GAG after extraction from media was determined as described earlier (3). Intrinsic viscosities of HA in unextracted media were determined by relating viscometer flow rates observed before and after the addition of hyaluronidase, a method described by Castor and Prince (9). The results are relatively specific for the viscosity of HA and were used to calculate molecular weights of the HA produced by some cultures.

Statistical comparisons were by Student's *t* test.

Results. Evidence for cyclic AMP as a mediator in the stimulation of HA synthesis. Addition of DbcAMP to the cultures of retrobulbar fibroblasts produced dose-dependent increases in the media GAG (Table I). Fibroblast protein was unchanged except at the highest doses of DbcAMP where small increases or decreases in the mean cellular protein were observed. Glucose utilization by the fibroblasts was stimulated less than GAG by DbcAMP; not until DbcAMP concentrations reached 2×10^{-3} *M*, or higher, was the glucose utilization increased by 20% or more. The increases in glucose utilization following additions of DbcAMP were also less than those observed after the additions of lymphocytes which gave comparable stimulation in GAG synthesis (Table II).

Theophylline, at 10^{-4} *M* concentration, induced a modest increase in media GAG but potentiated the GAG responses to lymphocytes (Table II). In separate experiments, additions of dimethyl sulfoxide in quantities that contaminate the lymphocyte preparation had no effect on any measurement made on the cultured fibroblasts.

Two hours after the addition of frozen-thawed lymphocytes, the concentration of intracellular cAMP in the fibroblasts was significantly increased (Table III). Shorter periods of incubation gave inconsistent stimulation of cAMP.

Inhibitory action of hydrocortisone. Hydrocortisone reduced the concentration of the cyclic nucleotide within the cultured cells to an insignificant degree (Table III). But this steroid significantly inhibited the

TABLE I. EFFECTS OF DIBUTYRYL CYCLIC AMP ON HUMAN RETROBULBAR FIBROBLASTS.[a]

DbcAMP concentration ($\times 10^{-3}$ *M*)	No. of flasks	GAG in media (nmole of uronic acid/mg of fibroblast protein)[b]	(E/C)[c]	Glucose utilized (μmole/mg of fibroblast protein)[b]	(E/C)[c]
Experiment No. 1, fibroblasts from patient A					
0.0	(5)	122 ± 3		11.9 ± 0.1	
0.2	(5)	155 ± 4	1.28	11.6 ± 0.3	0.97
0.5	(5)	198 ± 6	1.63	11.9 ± 0.3	1.00
1.0	(5)	275 ± 7	2.26	12.2 ± 0.3	1.02
1.5	(5)	422 ± 9	3.48	12.0 ± 0.1	1.01
Experiment No. 2, fibroblasts from patient B					
0.0	(4)	54 ± 2		11.6 ± 0.8	
2.0	(3)	489 ± 20	9.0	12.9 ± 0.7	1.11
5.0	(3)	912 ± 24	16.8	17.3 ± 0.4	1.49
10.0	(4)	1330 ± 69	24.5	18.0 ± 0.8	1.55

[a] Media collected over Days 2–4 of culture; fibroblast protein determined on Day 4.
[b] Mean ± SEM.
[c] Experimental group mean ÷ control group mean.

TABLE II. THEOPHYLLINE POTENTIATION OF LYMPHOCYTE STIMULATION OF GAG PRODUCTION BY RETROBULBAR FIBROBLASTS.

Concentrations of additives	No. of flasks	GAG in media[a] (nmole of uronic acid/mg of fibroblast protein)[b]	GAG in media[a] (increments above basal concentrations)
None	(5)	44 ± 2	
Theophylline, 10^{-4} *M*	(3)	65 ± 6	21
Lymphocytes, 10^5/ml	(3)	76 ± 4	32
Lymphocytes, 5×10^5/ml	(3)	145 ± 4	101
Lymphocytes, 10^5/ml, plus theophylline, 10^{-4} *M*	(3)	186 ± 3	142
Lymphocytes, 5×10^5/ml, plus theophylline, 10^{-4} *M*	(3)	298 ± 5	254

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

TABLE III. EFFECTS OF LYMPHOCYTES AND HYDROCORTISONE ON INTRACELLULAR cAMP IN RETROBULBAR FIBROBLASTS

Additions to media[a]	No. of flasks	Intracellular cAMP (pmole/mg of fibroblast protein, mean ± SEM)
None	(4)	33.5 ± 3.9
Lymphocytes,[b] 5×10^5	(4)	70.0 ± 5.8[c]
Hydrocortisone, 1 μg/ml	(4)	29.0 ± 2.4
Lymphocytes,[b] 5×10^5, plus hydrocortisone, 1 μg/ml	(4)	37.6 ± 4.0

[a] Incubation for 2 hr
[b] Lymphocytes, frozen-thawed, contained less than 25% of cAMP found in unstimulated fibroblasts; much of this added cAMP would have been discarded with removal of the media.
[c] Differed from each group, $P < 0.01$.

lymphocyte stimulation of intracellular cAMP within the fibroblasts (Table III). However, hydrocortisone did not reduce the synthesis of GAG induced by DbcAMP (2×10^{-3} *M*): media GAG following fibroblast exposure to DbcAMP alone was, in nanomoles of uronic acid per milligram of fibroblast protein, 445 ± 28 (over six times the control level) and after exposure to both DbcAMP and hydrocortisone was 565 ± 25. Indeed, there was a mild potentiation of the DbcAMP effect when hydrocortisone was added.

Differences in fibroblast synthesis of GAG in response to DbcAMP and lymphocytes. By paper chromatography and by differential solubility in Na_2SO_4-CPC, the GAG produced by the DbcAMP-stimulated fibroblasts appeared as HA (Table IV). When the steps of extraction of culture media are taken into account, the reproducibility of measurements of uronic acid are within 10%, and hexosamine within 15%. These variabilities in results deviate the hexosamine/hexuronic ratios in Table IV from the theoretical value of 1 for HA, but, within the error of assays, the ratios are consistent with the concept of HA as the only GAG present in the media. The absence of galactosamine in the GAG weighs strongly against appreciable quantities of chondroitin sulfates and dermatan sulfate as constituents of the GAG, an observation which points to HA as the major, if not the only, component.

Measurements of intrinsic viscosity on unextracted media indicated differences in the molecular sizes of the HA synthesized after exposures to lymphocytes and to DbcAMP (Table IV). The intrinsic viscosity of the HA produced by fibroblasts after lymphocyte stimulation was lower than basal values while that of the HA synthesized after additions of DbcAMP was higher; the molecular weights of HA, calculated from the respective intrinsic viscosities, were accordingly different: 2×10^5 and 5×10^6 daltons. In separate experiments, incubation of authentic HA in media with lymphocytes or DbcAMP, added in concentrations similar to those used in the above experiments, resulted in no change in the intrinsic viscosity after 3 days.

Discussion. In this communication, changes in concentrations of GAG (shown to be HA) in the media of fibroblast cultures was attributed to alterations in synthesis of the polysaccharide. There is no evidence

TABLE IV. NATURE OF GAG IN MEDIA AFTER RETROBULBAR FIBROBLAST STIMULATION BY LYMPHOCYTES AND DIBUTYRYL CYCLIC AMP.

	Experiment No. 1 Additives to Cultures		Experiment No. 2 Additives to Cultures	
	None	Lymphocytes, 5×10^5/ml	None	DbcAMP, 2×10^{-3} *M*
A. Fraction of extracted GAG soluble in 0.2 *M* Na_2SO_4-CPC solution[a,b]	0.73	0.80	0.82	1.00
B. Type of GAG on paper chromatography[b]				
1. Ammonium-methanol-formate solvent[c]	HA	HA	HA	HA
2. $MgCl_2$, 0.1 *M* solvent [c]	HA	HA	HA	HA
C. Hexosamine/hexuronic acid ratio[b]	0.77	0.90	0.87	1.06
D. Types of hexosamine on paper chromatography[b]	Only glucosamine	Only glucosamine	Only glucosamine	Only glucosamine
E. Intrinsic viscosity[b]				
Deciliters/gram of HA	11.2	4.3	13.6	61.1
Calculated molecular weight of HA (millions)	0.6	0.2	0.8	5.0

[a] HA is soluble in this solution.

[b] Methods recorded in Ref. (3) and (9).

[c] The majority, but not the total, stainable GAG was in the HA fractions in all cases.

that HA is degraded by the fibroblasts, and the intracellular content of GAG was found to be low compared to that in media (10); therefore, it is unlikely that the observed increases in media GAG were derived from diminished degradation or from release from intracellular stores. Further, in unpublished studies, it was found that [^{14}C]glucosamine was incorporated into media HA in patterns consistent with the concept that experimental manipulations affect only synthesis rates.

That cAMP mediates the lymphocyte stimulation of HA synthesis in retrobulbar fibroblasts is indicated by the observations that (a) DbcAMP produced the same type of HA response; (b) theophylline, a phosphodiesterase inhibitor, potentiated the lymphocyte action; and (c) exposure of preparations to lymphocytes induced an increase in the cAMP concentration in the fibroblasts within 2 hr. Although a previous publication from this laboratory reported that cAMP added to retrobulbar fibroblast cultures produced no effect (5), it seems likely that the cAMP then used was either rapidly degraded or did not penetrate cells as well as the dibutyryl derivative of the nucleotide.

However, lymphocytes affected two responses in the cultured cells that differed from those observed after additions of DbcAMP. Relative to the effects on HA synthesis, DbcAMP consistently increased glucose utilization less than did the lymphocytes. HA was the only type of GAG found in culture media after the addition of either stimulating agent, but the intrinsic viscosity (and, therefore, the molecular weight) of HA decreased as a consequence of lymphocyte action, and increased as a result of the presence of DbcAMP. It is likely that the stimulation produced by lymphocytes resulted in changes in glucose metabolism separate from those in the adenyl cyclase system; the former effects may play a role in modifying the polymer size of HA.

Hydrocortisone was previously reported to inhibit the lymphocyte stimulation of HA synthesis in the fibroblast cultures (4, 5). Hydrocortisone also markedly reduced the intracellular cAMP response to lymphocytes. Thus, the inhibitory effects of the steroid hormone occur at an early stage in the events consequent to the interaction of lymphocytes and fibroblasts. Possibly hydrocortisone prevents binding of the active factor within lymphocytes to a fibroblast receptor.

In contrast, hydrocortisone did not interfere with but rather potentiated the DbcAMP effects on HA synthesis in the cultures. The potentiation was possibly related to unmeasured intracellular actions of hydrocortisone on retrobulbar fibroblast metabolism.

Summary. It was shown that lymphocytes and lymphocyte products stimulated hyaluronic acid (HA) synthesis in cultures of human retrobulbar fibroblasts. This stimulation was simulated by dibutyryl cyclic AMP (DbcAMP), 2×10^{-4}–10^{-2} *M*, and potentiated by theophylline, 10^{-4} *M*. Lymphocytes induced a significant increase in the

cAMP concentration within the fibroblasts. These data support the concept that the lymphocyte stimulation of HA synthesis is mediated by cAMP.

However, lymphocytes induced greater glucose utilization by the cultures than DbcAMP. Also, the intrinsic viscosity of the HA was decreased by lymphocytes but increased by DbcAMP. Lymphocytes probably produce more effects in the fibroblasts than those mediated by cAMP.

Hydrocortisone inhibited both the cAMP and the HA responses to lymphocytes but potentiated the stimulation of HA synthesis by DbcAMP. Hydrocortisone thus appears to interfere with the earliest effects of lymphocytes, possibly inhibiting the binding of the active factor in lymphocytes to retrobulbar fibroblasts.

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