Exp. Eye Res. (1971) **12**, 285–292

Stimulation of Glucose Utilization and Glycosaminoglycans Production by Fibroblasts Derived from Retrobulbar Tissue*

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(Received 28 October 1970, and in revised form 17 June 1971, Boston)

Cultures of fibroblasts derived from human retrobulbar tissue responded to the presence of living lymphocytes with increased release of glycosaminoglycans to the media and accelerated glucose utilization. At times the fibroblast proliferation rate was enhanced by the lymphocytes, but the metabolic changes were out of proportion to the changes in fibroblast number.

Normal ranges of responses in glycosaminoglycan production and glucose utilization by the cultures were defined for the addition of lymphocytes, at two dose levels, from 19 normal subjects.

Unpurified leukocytes were more potent stimulators of these cultures than were lymphocytes, but the latter were obtained in almost pure form and clearly contained the stimulating properties under investigation. Erythrocytes and platelets were devoid of activity in this system. Freeze-thawed lymphocytes contained activity, but this was found only after 24 hr of preincubation and quantitatively was much less predictable than that in the living lymphocytes. Hyaluronic acid comprised nearly all, if not all, the media glycosaminoglycans in both stimulated and nonstimulated cultures. No metachromasia was detected in the fibroblasts after they were stained with toluidine blue-O, which suggests that the stimulating mechanism(s) did not augment the intracellular glycosaminoglycans of the cultures.

1. Introduction

Monolayer cultures of fibroblasts derived from various human tissues release glycosaminoglycans (GAG) into the culture media. This function has been stimulated by extracts of various human cells, including leukocytes (Yaron and Castor, 1969; Castor and Yaron, 1969); such stimulation may play an important role in the metabolic changes of acute and chronic inflammation.

The biochemical nature of the increased retrobulbar volume seen in some patients with Graves' disease has not been fully identified. The orbital fat content of sialic acid was abnormally high in one case of Graves' ophthalmopathy (Haddad, 1967); and increased concentrations of glycoproteins, but not GAG, were found in the retrobulbar tissues of carp in which experimental exophthalmos was induced (Prame, 1968). However, because the retrobulbar tissues in individuals with Graves' disease may be infiltrated with GAG (Smelser, 1937; Wegelius, Asboe-Hanson and Lamberg, 1957), and because this process may have an autoimmune basis (Hall, Doniach, Kirkham and El Kabir, 1970) possibly related to lymphocyte infiltration (Smelser, 1937; Wegelius et al., 1957), it is of interest to evaluate these factors in the metabolism of orbital connective tissue. We have previously demonstrated that monolayer fibroblasts can be grown from explants of human retrobulbar tissue, and that GAG in culture medium and the utilization of glucose by the cells are parameters readily and reproducibly measured (Sisson, Spaugh and Vanderburg, 1970). This is a report of the metabolic responses of retrobulbar fibroblasts following exposure to various cellular

* This investigation was supported by PHS grants (AM-08495 and EY-00106) from National Institute of Arthritis and Metabolic Diseases and the National Eye Institute, U.S. Public Health Service.

preparations. The results consequent to the introduction of human lymphocytes into the cultures are emphasized because of the possible relevance of fibroblast-lymphocyte interaction to human disease.

2. Materials and Methods

Monolayer cultures of human fibroblasts were derived from the explanted, normal retrobulbar tissues of three patients who underwent enucleation for intraocular disease. These separate strains of fibroblasts were initiated and perpetuated as previously described (Sisson et al., 1970).

Blood donors were healthy and between 21 and 41 years of age. Freshly drawn blood, usually 30–60 ml, was mixed (20:1 v/v) with Hank's balanced salt solution containing 12% dextran (molecular weight 100,000–200,000) and heparin 300 I.U./ml, and erythrocytes were permitted to sediment over 60–90 min. Unpurified leukocytes were recovered in the supernatant. Nongranular leukocytes were obtained by passing the supernatant twice through 2·5–3·5 ml of tightly packed cotton in a sterile plastic 5-ml syringe barrel. Eight milliters of 199 medium (Difco: Detroit, Michigan, U.S.A.) were then passed through the cotton. The eluates were centrifuged in 10–12 ml volumes for 12 min at 150 **g** and the cells pooled in the known volume of 199 medium containing 10% fetal calf serum (199-FCS). The nongranular leukocytes (hereafter referred to as lymphocytes) were recovered (30–50% of the original number) in the pooled eluates; granular leukocytes comprised less than 1% and monocytes less than 5% of the leukocytes in the eluates. Both erythrocytes, in numbers equal to or greater than the lymphocytes, and platelets were found in the eluates. Leukocytes were counted in a hemocytometer after mixing with 0·5% acetic acid to remove the erythrocytes.

Concentrated platelets were obtained by a standard method (Chappell, 1966). Erythrocytes used in the experiments were aspirated from the sediment of the original blood specimen.

Except where noted, the following protocol was adhered to for all experiments. Fibroblasts were plated at $0{\cdot}8 \times 10^6$ in T-30 flasks with 5 ml of medium. After 1 day, 4·5 ml of fresh medium [CMRL 1066 (Grand Island Biological Co., Grand Island, N.Y., U.S.A.) supplemented with 10% fetal calf serum, 100 I.U./ml penicillin g, 0·1 mg/ml streptomycin sulfate, and 2×10^{-3} L-glutamine] replaced the original. Lymphocytes or other cells were then added in a volume of 0·5 ml. Control flasks received 0·5 ml of 199-FCS. Media from all cultures were harvested 3 days after the addition of the experimental material (days 2–4 of the culture) and the fibroblasts were trypsinized and counted as described before (Sisson et al., 1970).

The media were analyzed for residual glucose using Glucostat (Worthington Biochemical, Freehold, N.J., U.S.A.) reagents. GAG were extracted from media using the method described by Castor, Wright and Buckingham (1968b) in which 2 ml of medium was mixed with 8 ml of absolute ethanol, and the GAG-protein complex allowed to precipitate, with the aid of several mixings, for 1 hr at room temperature. The mixture was centrifuged at 17,300 **g** (room temperature) for 10 min and the supernatant discarded. Lipids were extracted by mixing the precipitate with 3 ml of acetone at intervals during 40 min, still at room temperature. The acetone supernatant was discarded after a second centrifugation, and the precipitate allowed to dry overnight in the open tube. The precipitate was dissolved as the protein contained in it was digested by 1 mg of pronase (California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A.) in 2 ml of 0·05 M Tris buffer, pH 7·9, during a 1-hr period at 37°C. The mixture was then diluted with 5 ml of water, and the GAG were reprecipitated by the addition of 0·2 ml of 5% cetylpyridinium chloride (CPC) and 0·2 ml of 0·2 M Na_2SO_4, and incubation continued for 1 hr at 37°C. The flocculated GAG were recovered by centrifugation (27,000 **g** for 30 min). After discarding the supernatant, the GAG were dissolved in 1·2 ml of 2·0 M sodium acetate at

37°C for 1 hr, and 1 ml was used to determine hexuronic acid by a borate carbazole procedure (Bitter and Muir, 1962). The GAG extracted from media were recorded in terms of the hexuronic acid content.

For identification of hyaluronic acid (HA), GAG were extracted from larger quantities of media (8–16 ml) in an analogous manner, except that after the precipitation with CPC-Na_2SO_4, the GAG were dissolved in 2 ml of methanol and reprecipitated with 0·2 ml of 10% sodium acetate in methanol. Again the supernatant was discarded after centrifugation; the precipitate was washed twice with 2 ml of methanol and dried. This GAG precipitate was dissolved in water before the various assays were carried out.

Recoveries of the media GAG were estimated in each experiment using commercial HA dissolved in fresh medium and were 88–90% complete.

Special controls in which the lymphocytes or other added cell preparations were incubated in flasks, but without fibroblasts, were included for each experimental group. Glucose utilization was determined by subtracting the residual concentration in each fibroblast flask from that found in the special control for the respective group. No significant glucose utilization by living lymphocytes or unpurified leukocytes was recorded until 5×10^6 or more were added. The GAG extracted from the media of these special flasks gave small reactions in the analysis of hexuronic acid, and these were subtracted from the values for each flask in the respective group. The incubated lymphocytes and unpurified leukocytes, in quantities up to 10^7, did not appreciably contribute to the hexuronic acid in the extracted GAG; the basal hexuronic acid level was inherent in the media used.

Although mean values for control flasks (containing fibroblasts to which only 199-FCS was added) did not vary greatly, it was found that uniformity of results among experiments was best achieved by expressing glucose utilization and GAG extracted from media as the mean of experimental flasks (receiving leukocytes) per mean of control flasks. There were always at least four flasks in a control group for each experiment. After 3–5 months of continuous subculturing the metabolic parameters of a fibroblast strain declined and the cultures were discarded. Previously frozen fibroblasts derived from the same explant could then be taken from storage and used (Sisson et al., 1970). Because data obtained in stimulated cultures could be derived from different portions of dose-response curves in different experiments, it was deemed necessary to express results for each experiment from at least two doses of stimulating material.

For estimation of changes in intracellular GAG, fibroblasts ($0{\cdot}5 \times 10^6$) were plated in glass Petri dishes (19 cm^2) containing cover glasses and 3·5 ml of medium. Incubation was carried out in a sealed chamber with an atmosphere of 5% CO_2 in air at 37°C. The protocol used for the experiments described above for T-30 flasks was followed, and fresh living lymphocytes were added to these cultures. Residual media glucose and GAG extracted from the media were determined as before, and cover glasses were removed, dried, fixed and stained with toluidine blue-O according to the method described by Matalon and Dorfman (1969). A search was then made for metachromatic changes within the fibroblasts.

3. Results

Stimulating activity of lymphocytes

Dose-response curves to fresh living lymphocytes are shown in Fig. 1 for the glucose utilization and production of media GAG by retrobulbar fibroblast culture. From this experiment and from similar results in another dose-response experiment, it appeared that additions of 10^6 and $2{\cdot}5 \times 10^6$ lymphocytes to the cultures would elicit changes that could be most readily reproduced.

Glucose utilization and GAG production by the fibroblasts were more stimulated, per cell added, by unpurified leukocytes, presumably through the granular leukocyte component, than by lymphocytes. However, the granular leukocytes were too few

(less than 1%) to contribute appreciably to the effects observed in experiments using lymphocytes.

Under identical circumstances erythrocytes (10^7/flask) and platelets (6×10^8/flask) were devoid of stimulating activity. Clearly, the responses in fibroblast cultures that followed the addition of lymphocyte preparations were produced by the lymphocytes.

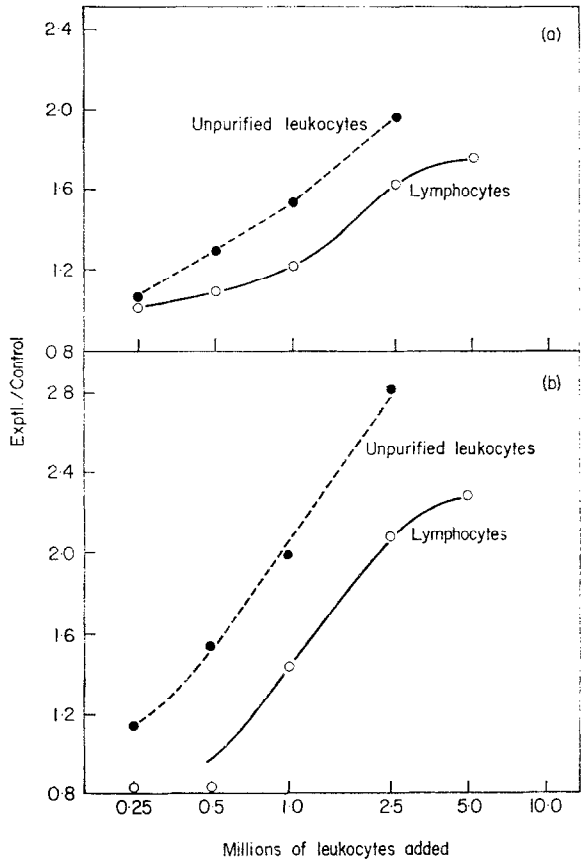

FIG. 1. Dose responses by fibroblast cultures to fresh living leukocytes (consisting of 62% granular leukocytes including 59% neutrophils, 37% lymphocytes and 1% monocytes) and lymphocytes from the same donor. Responses are in terms of mean results in two experimental flasks ÷ mean of four control flask values after 3 days of culture: (a) glucose utilization; and (b) hexuronic acid in GAG extracted from media. These figures were derived from a single experiment but repetition of dose responses gave similar results.

The magnitude and statistical variability of the changes induced by lymphocytes from two normal subjects are recorded in Table I. As a rule, increases in glucose utilization and GAG production which exceeded experimental and control values of 1·25 and 1·30, respectively, were statistically significant when analyzed by the "Student" t-test ($P<0{\cdot}05$), if at least four flasks were included in both control and experimental groups. In general, the proliferation of fibroblasts was acclerated by the presence of lymphocytes (which were easily distinguished by size in the counting of cells), but these changes were rarely statistically significant and, in most instances, could account for only a portion of the increments observed in glucose utilization and GAG production.

TABLE I

Effects of fresh living lymphocytes from 2 subjects on retrobulbar fibroblasts

No. lymphocytes added (millions)	No. flasks	Glucose utilized (mg/flask)	Glucose utilized (Exptl./Control)	GAG extracted from media (μmoles $\times 10^3$ hexuronic acid/flask)	GAG extracted from media (Exptl./Control)	Fibroblasts (millions per flask)
JQ (Subject 7)						
2·5	(2)	2·52	1·49	205	1·83	1·25
1·0	(4)†	2·11±0·06	1·25	152±6	1·36	1·34±0·14
TD (Subject 8)						
2·5	(2)	2·94	1·74	254	2·27	1·18
1·0	(4)†	2·35±0·08	1·39	214±9	1·91	1·16±0·05
(None control)	(4)†	1·69±0·06		112±7		0·98±0·14

* Lymphocytes were added before day 2 of cultures and media were collected during days 2–4. Fibroblasts initially were plated at $0{\cdot}8 \times 10^6$/flask, and were counted after day 4.

† Where 4 flasks were included in a group, the mean ± S.E. is given.

TABLE II

*Responses of a single strain of retrobulbar fibroblasts to the addition of lymphocytes from 19 normal subjects**

	Millions of lymphocytes added 1·0	Millions of lymphocytes added 2·5
Glucose utilization (Exptl./Control)		
Mean ± S.D.	1·30±0·095	1·60±0·162
Range for ±2 S.D.	1·11–1·49	1·28–1·92
GAG extracted from media (Exptl./Control)		
Mean ±S.D.	1·66±0·329	2·27±0·358
Range for ±2 S.D.	1·00–2·32	1·55–2·99
Number of fibroblasts (Exptl./Control)		
Mean ±S.D.	1·11±0·18	1·11±0·16
Range for ±2 S.D.	0·75–1·47	0·79–1·43

In the 8 experiments from which the above data were derived, the mean ±1 S.D. for the experimental control groups was: for glucose utilized/flask, 1·61±0·28 mg; for GAG extracted from medium/flask, $107 \pm 26 \times 10^3$ μmole uronic acid; for fibroblast/flask, $1{\cdot}08 \pm 0{\cdot}16 \times 10^6$ cells.

* 14 Men, 5 women.

Fibroblast cultures derived from one patient responded to two dose levels of lymphocytes from 19 normal individuals (Table II). The proportional increases in glucose utilization were of smaller magnitude but with a narrower range of variability than those obtained for GAG production after lymphocyte stimulation.

From experiment to experiment, some variability was recorded in the responses of lymphocytes from the same subject (maximum differences observed: glucose utilization 20%; GAG in media 70%), but the discrepancies appeared to be no less in the experiments that utilized fibroblasts from a single source than in those in which the cultures originated from retrobulbar tissues from different patients.

The stimulating activity was also present in preparations of dead lymphocytes (freeze-thawed three times in 0·04 ml H_2O for each 10^6 lymphocytes, then reconstituted to the desired volume with 199-FCS). This activity could be detected only after 24 hr of incubation in 199-FCS at 37°C, and none was found in lymphocytes killed within 2 hr of harvest from the donor.

Although waning with time, increased production of GAG hexuronic acid persisted for at least 11 days, and through two subcultures after washing the fibroblast cultures free of lymphocytic material. The augmented glucose utilization rate, however, returned to normal before 11 days had passed.

The nature of the GAG extracted from culture media

A number of systems were used to establish the presence of HA as the major constituent of the GAG extracted from media. Each of the results represent, as a minimum, close agreement between determinations on duplicate samples.

The hexuronic acid values obtained for dermatan sulfate and for heparan sulfate by the carbazole method (Dische, 1947) differ considerably from those observed with the orcinol reagent (Volkin and Cohn, 1954; Hoffman, Linker and Meyer, 1956; Castor, Greene, Prince and Hazelton, 1968a). These are in contrast to a ratio of carbazole/orcinol (C/O) hexuronic acid values near unity expected for HA. In the experiments described above, the C/O ratios were 1·17 and 1·04, respectively, for the GAG extracted from media of control and lymphocyte-stimulated fibroblast cultures.

Molar ratios of hexosamine (determined by the method described by Roseman and Daffner, 1956) to hexuronic acids (Bitter and Muir, 1962) measured in the extracted GAG were 0·77 and 0·90, respectively, for the media from control and stimulated cultures. Although an equal number of moles of hexosamines and hexuronic acids would be anticipated for HA and the chondroitin sulfates, the above-recorded ratios suggested that the major portion of GAG consisted of these polymers. The added extraction step required for the hexosamine analyses beyond that for hexuronic acid measurement may contribute to some loss of the former, and, in fact, the ratios of the two components may be closer to unity than was observed.

Identification on paper chromatography (Heyworth, Perkins and Walker, 1961) of glucosamine, but not galactosamine, in the GAG hexosamines extracted from both control and stimulated cultures excludes the presence of appreciable quantities of chondroitin 4- and 6-sulfates, and points to HA as the major, if not the only, constituent of the GAG.

Solubility in 0·2 M Na_2SO_4 in the presence of CPC characterizes HA (Castor et al., 1968a), and 73% (control) and 80% (stimulated) of the GAG (measured as hexuronic acid) extracted from media were dissolved under this condition. All but small quantities (0·2–0·45 of the total GAG by weight) of protein (Lowry, Rosebrough, Farr and Randall, 1951), which might alter GAG solubilities, were removed before this

procedure. Although HA would, in this system, again appear to be the major component of the GAG, the presence of other polymers as small fractions of the total could not be excluded.

Paper chromatographic separation (Castor and Dorstewitz, 1967) of the various types of GAG demonstrated patterns of movement—remaining at the origin in ammonium formate-methanol and traveling to the front in 0·1 M $MgCl_2$ solvents—consistent with those expected for HA in the GAG extracted from both the control and stimulated culture media.

The viscosity of the media obtained from both control and stimulated fibroblast cultures dropped after treatment with bovine testicular hyaluronidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). 300 U.S.P. units of hyaluronidase was added to 4 ml of media and the viscosity was measured in an Ostwald viscometer (Castor and Prince, 1964). This enzyme treatment caused a decrease in viscosity of about 30% in both control and stimulated fibroblast media. This indicates that some of the viscosity of the media is due to the presence of hyaluronic acid.

As in the experiments carried out in flasks, increases in media GAG were seen after exposure of fibroblasts in Petri dish cultures to lymphocytes. Although changes in the extracellular GAG were distinct (ratio between experimental and control: 3:1), no definite alteration in morphology and no intracellular metachromasia after staining with toluidine blue-0 was observed within the fibroblasts, even when there was intimate contact with lymphocytes.

4. Discussion

Human lymphocytes, in a predictable way, can influence the metabolism of cells derived from retrobulbar connective tissue cells in a culture system. The increases in glucose utilization and media GAG of these fibroblast cultures by lymphocytes from 19 healthy individuals provide normal ranges of these parameters for reference in future studies of pathologic properties of lymphocytes. The responses of fibroblasts derived from different subjects to fresh living lymphocytes obtained from a single donor were sufficiently uniform so that detection of unusual potency in future lymphocyte specimens should not be dependent upon the availability of a single fibroblast source.

Glucose utilization was nearly always enhanced out of proportion to the requirements for increased GAG synthesis; this suggests that either the locus of actions of the stimulator(s) was at some relatively fundamental level in GAG production, or that several loci are involved. In keeping with this concept Castor and Yaron (1969) have shown that lactate production also is enhanced following stimulation in similar fibroblast cultures.

Castor and co-workers (Yaron and Castor, 1969; Castor and Yaron, 1969) have shown that stimulator(s) of fibroblast cultures are contained in a wide variety of human cells, but the mechanism(s) of stimulation have not been completely elucidated. Very likely, fibroblasts derived from different areas of the body will retain in culture some of the specialized characteristics basic to their respective functions in the human organism, and will therefore differ in responses to various stimuli.

Although preparations of unpurified leukocytes were, per cell, more potent stimulators than were lymphocytes in this in vitro system, the latter were obtained in almost pure form and clearly contained the properties under investigation. This may have some relevance to disease processes in vivo since lymphocytic infiltration

of retrobulbar tissues (Smelser, 1937; Wegelius et al., 1957), and a possible immunologic basis, were reported for Graves' ophthalmopathy (Hall et al., 1970).

Manifestations of Graves' eye disease have been reported to include accumulations of GAG in and about the orbit (Smelser, 1937; Wegelius et al., 1957), but the biochemical changes of this disorder have not been completely elucidated. In fact, increases in orbital GAG are absent in the experimental exophthalmos of carp (Prame, 1968). Although GAG have been identified in human orbital tissues (Prame, Gardell and Antonopoulos, 1966), increased concentration of sialic acid appeared as an important component, at least of the orbital fat, in a case of Graves' ophthalmopathy (Haddad, 1967). Unusual quantities of sulfated GAG, but not HA, are reported to be excreted in the urine of patients exhibiting active exophthalmos (Winand, 1968). The various studies of pathogenesis of the ophthalmopathy must remain, at least for now, somewhat removed from the active process, and, for this reason, the literature data may appear more conflicting than is actually true. In the model used for the above investigations, a single GAG polymer, HA, would seem to be reasonably established as an important product of the lymphocyte-stimulated fibroblast cultures. The precise relation of HA to the changes in Graves' eye disease, of course, remains uncertain.

ACKNOWLEDGMENT

Mr Joel Vanderburg and Miss Beatrice Spaugh provided invaluable technical help in this work. I am indebted to Dr C. W. Castor for many suggestions and criticisms and to Dr J. Matovinovic for his continuing support.

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