INHIBITION BY 5-BROMO-2'-DEOXYURIDINE OF DIFFERENTIATION-DEPENDENT CHANGES IN GLYCOSAMINOGLYCANS OF THE RETINA

John E. Morris[1] and Albert Dorfman

Departments of Pediatrics and Biochemistry
Joseph P. Kennedy, Jr. Mental Retardation Research Center
Pritzker School of Medicine
University of Chicago
Chicago, Illinois 60637

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SUMMARY

Embryonic chick neural retinas incorporated radio-labeled precursors into glycosaminoglycans in the same relative amounts whether cultured as intact tissues, cell aggregates, or monolayers. Incubation with 5-bromo-2'-deoxyuridine inhibited histogenesis and caused the pattern of synthesis to remain more like that in undifferentiated tissue, when compared with controls without this nucleoside analog. This was determined by the level of incorporation and the ratios of chondroitin sulfate to heparan sulfate and chondroitin-4-sulfate to chondroitin-6-sulfate incorporation. Incubation with 4-methylumbelliferyl-β-D-xylopyranoside stimulated synthesis and release of chondroitin sulfate and heparan sulfate into the medium. The results taken together imply that the production of specific glycosaminoglycans during the course of differentiation in the retina is regulated at the gene level in parallel with histogenesis in this tissue.

The inducibility by corticosteroids of glutamine synthetase in the embryonic chick retina depends upon the cells being organized into specific multicellular associations (1). Furthermore, the differentiation of both the specific multicellular associations and glutamine synthetase inducibility appears to be controlled at the gene level, as suggested by their tandem inhibition by the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) (2). Because the pattern of glycosaminoglycan (GAG) synthesis progressively changes in the retina in stages that correlated with histogenesis (3), we have sought to deter mine whether this pattern of synthesis also is influenced by cell association and BrdU.

1 This work was done while J.E.M. was on sabbatical leave from Oregon State University. Current address: Department of Zoology, Oregon State University, Corvallis, Oregon 97331.

Abbreviations: glycosaminoglycan (GAG), 5-bromo-2'-deoxyuridine (BrdU), chondroitin sulfate (CS), heparan sulfate (HS), hyaluronic acid (HA), cetylpyridinium chloride (CPC), 2-amino-2-deoxy-D-glucose (GlcN).

MATERIALS AND METHODS

Neural retinas were dissected from chick embryos and cultured in 3 to 5 ml of Eagle's minimum essential medium supplemented with 10% (v/v) fetal calf serum and antibiotics (1,2). Cell aggregates and cell monolayers were prepared by trypsin dissociation of retinas from 9-day embryos (1) and were cultured 24 hr before adding radio-labeled precursors in fresh medium for a second 24 hr. For the BrdU experiments, retinas from 7-day embryos were cultured for 2 days in medium with or without 5×10^{-5} M of the inhibitor. The tissues were transferred to fresh medium without BrdU but with 10^{-4} M thymidine for an additional 6 days. The medium was changed every 2 days, and labeled precursors were included during the final 24 hr. For the experiments with 4-methylumbelliferyl-β-D-xylopyranoside the tissues were incubated in regular medium for 1 hr before adding fresh medium with 4×10^{-4} M xyloside plus labeled precursors.

The details for preparing the polysaccharides will be described elsewhere (3). Essentially, this preparation involved digestion for 2 or 3 days in the cold with 0.5 M NaOH, after which time a fraction of the digest was assayed for protein (Lowry method), and the balance was neutralized with acetic acid and dialyzed against water. The contents of the dialysis bag were mixed with carrier GAG, adjusted to 0.02 M NaCl, and precipitated with 4 volumes of ethanol. The precipitates were dried with ether, dissolved in 0.1 M tris-HCl, pH 7.9, and sequentially digested in the presence of toluene at 37° with DNase (2 hr), trypsin (1 day) and Pronase (2 days). Any undigested proteins were removed by centrifugation after their precipitation with trichloroacetic acid. The supernatant fraction was dialyzed for 3 to 4 days against cold running water and lyophilized. This crude polysaccharide fraction was redissolved in 2 ml of 0.2 M pyridine-acetate buffer and filtered on a column (1 x 200 cm) of Sephadex G-50 to separate the GAG from the lower molecular weight components. In some cases 10% cetylpyridinium chloride (CPC) was added dropwise in the presence of 0.02 M NaCl to precipitate the GAG as an additional purification step prior to Sephadex chromatography (4). The CPC precipitates were allowed to form for 1 hr at 37°, after which time they were sedimented by centrifugation, dissolved in a 2 M NaCl, and precipitated twice with ethanol.

The GAG were characterized by digestion with a) streptococcal hyaluronidase (Varidase, Lederle Labs.; 1,500 units/3 ml of 0.15 N NaCl in 0.01 M NaH_2PO_4, pH 6.0, overnight at 37°) to degrade hyaluronic acid (HA) and chondroitin (4,5); b) chondroitinase AC or ABC (Miles Labs.; 0.625 units/ 3 ml of 0.1 or 0.01 M tris-HCl, pH 7.9, 4 to 6 hr at 37°) to degrade chondroitin sulfate (CS) plus HA and chondroitin (4,6); and c) nitrous acid (1 ml each of water, 5% $NaNO_2$, and 33% acetic acid, 2 hr at room temperature) to degrade heparan sulfate (HS) and heparin (7). The degration products were characterized and isolated by Sephadex G-50 chromatography. The products of hyaluronidase were identified by descending paper chromatography in 1 M ammonium acetate (pH 5.0): ethanol (35:65) for 15 hr (8), and the products of chondroitinase were chromatographed in a 2 N ammonia: isobutyric acid (3:5) for 40 hr (9). The heterogeneity of the nitrous acid degradation products seen after chromatography on Sephadex C-50 indicated that most, if not all, of the products were HS rather than heparin.

RESULTS

Lack of effect of tissue dissociation on the differentiation of GAG pattern. Retinas were cultured intact or were dissociated with trypsin and cultured either on plates as cell monolayers or suspended in flasks on a gyratory shaker

TABLE 1

Synthesis of GAG by Cultured Retina Tissue and Cell Monolayers [a]

	Intact Tissue				Cell Monolayers			
	[^{3}H] GlcN		$^{35}SO_4$		[^{3}H] GlcN		$^{35}SO_4$	
	dpm/mg	%	dpm/mg	%	dpm/mg	%	dpm/mg	%
Initial Total GAG [b]								
Cells	11,436		15,516		16,531		14,909	
Medium	1,463		1,974		16,542		18,450	
Aliquots [c]								
Hyaluronic acid	456	12 [d]	76	2	78	2 [d]	21	1
Chondroitin sulfate	1,476	55	2,604	60	2,233	50	2,292	59
Heparan sulfate	3,536	58	3,474	57	4,777	47	3,720	53

[a] Retinas from 9-day embryos were cultured as intact tissues or cell monolayers (quadruplicate cultures, one retina/culture) with 100 μCi/ml of $H_2{}^{35}SO_4$ (carrier-free) and 1 μCi/ml [^{3}H]GlcN (3 Ci/mmole). [b] Initial total GAG were isolated from the crude polysaccharides by CPC precipitation and chromatography on Sephadex G-50. The data are presented as dpm/mg of tissue or cell protein and are shown for a single experiment. A duplicate experiment yielded comparable results. [c] Initial total fractions were divided into three aliquots for separate determinations. One aliquot was digested with streptococcal hyaluronidase, another with chondroitinase and the third with nitrous acid. The percentages are for single aliquots, not the total GAG; thus, they do not total 100%. [d] The small number of counts in HA made these percents uncertain. In other experiments (3) the HA incorporation was found to vary between 1 and 2% of the total GAG.

to permit reassociation as cell aggregates. The amount of incorporation of [^{3}H]glucosamine ([^{3}H]GlcN) and $^{35}SO_4$ into GAG was similar in the intact tissue controls and cell monolayers (Table 1). Data for cell aggregates are omitted from Table 1 but were in all aspects similar to the data for intact tissues. In contrast to the intact tissue, the monolayers released the major proportion of their labeled material into the medium. However, the conditions of culture appeared to have no effect on the types of GAG synthesized.

Inhibition of GAG differentiation by BrdU. During normal differentiation of the retina there is a decrease in GAG synthesis per cell and an increase in sulfation and in both the CS/HS and chondroitin-4-SO_4/chondroitin-6-SO_4

(C-4-S/C-6-S) ratios (3). Because BrdU will inhibit in the retina certain aspects of histogenesis in culture (2), it was of interest whether the pattern of GAG synthesis also would be inhibited when 7-day retinas were cultured for 7 days (i.e., to attain a chronological age comparable to a 14-day retina). After 2 days of culture in the presence of BrdU the incorporation of labeled precursors into GAG 5 days later was higher than the controls (Table 2). The cultures treated with BrdU showed on the average a somewhat lower than control incorporation into CS and a correspondingly higher incorporation into HS. The ratio of C-4-S to C-6-S was lower than in the controls. For comparison, during normal differentiation of the retina the C-4-S/C-6-S ($^{35}SO_4$) ratio increases from 1.8 to 7.0 between 7 and 14 days of age (3).

Stimulation of CS synthesis by β-D-xyloside. Certain xylosides initiate the synthesis and release into the medium of free CS chains, presumably by substituting for the xylosyl-serine link to the core protein (10). To determine whether CS synthesis in the retina might also be controlled by the availability of active core protein, 14-day retinas were cultured with 4-methylumbelliferyl-β-D-xylopyranoside and $H_2{}^{35}SO_4$. During 24 hr of culture the xyloside was found to stimulate the incorporation of $^{35}SO_4$ into not only CS (8X control level) but into HS (3X control level) (Table 3).

DISCUSSION

These data indicate that unlike glutamine synthetase induction (1) the pattern of GAG synthesis in the 9-day neural retina was not influenced by specific cell association, at least during the 2 days of culture studied. On the other hand, the progressive change with differentiation in GAG synthesis did appear to be inhibited by BrdU.

During normal differentiation in the chick neural retina, the predominant GAG synthesized at 5 days of age is HS (89% of the [^{3}H]GlcN in GAG); when examined at 7, 10, and 14 days, the trend in synthesis switches to CS (CS increases 4X to 40%, while HS declines to 59%) (3). During this period there is a 3.5X increase in $^{35}SO_4$ relative to [^{3}H]GlcN in both CS and HS and a 5.8X increase

TABLE 2

Changes in the Pattern of GAG Synthesis with BrdU Treatment [a]

	[^{3}H] GlcN and [^{3}H] acetate		$H_2{}^{35}SO_4$	
	- BrdU	+ BrdU	- BrdU	+ BrdU
Initial Total GAG (dpm/mg protein) [b]				
Expt. 1 ([^{3}H]-acetate)				
Tissue	15,110	21,174	6,720	13,060
Medium	6,330	17,126	3,057	2,528
Expt. 2 ([^{3}H]-GlcN)				
Tissue	31,267	41,110	22,288	35,946
Medium	7,264	10,887	4,465	6,862
Percent Composition of GAG [c]				
Hyaluronic acid				
Tissue	1	2	0.03	0.08
Medium	3	17	1	0.04
Chondroitin sulfate				
Tissue	32	23	34	32
Medium	75	48	60	68
Heparan sulfate				
Tissue	56	68	64	68
Medium	22	34	39	32
Ratio of C-4-S/C-6-S [d]				
Tissue	6.6	2.6	5.0	2.6
Medium	7.2	3.2	4.5	2.6

[a] Intact 7-day retinas were cultured for 2 days in medium with or without BrdU (triplicate cultures, two retinas/culture) and in fresh medium without BrdU for 5 more days. For an additional day the medium contained $^{35}SO_4$ plus [^{3}H]GlcN (as in Table 1) or [^{3}H]acetate (55 µCi/ml, 300 mCi/mmole). [b] The initial total GAG fractions were prepared as for Table 1, except that CPC precipitation was omitted, and are expressed as dpm/mg of tissue protein. [c] Percent composition was determined by the sequential digestion of the initial total GAG with hyaluronidase, followed by chondroitinase, and finally nitrous acid. After each digestion the products were isolated on Sephadex G-50 and characterized by paper chromatography. The void volume from the Sephadex column was lyophilized and reconstituted for the next digestion in the sequence. The data represent the average of determinations from experiments 1 and 2 from the top of this table. [d] Ratios of C-4-S to C-6-S are averages for the two experiments calculated on the basis of counts obtained from paper chromatographs of chondroitinase digests.

TABLE 3

Effect of 4-Methylumbelliferyl-β-D-Xylopyranoside on GAG Synthesis [a]

	Initial GAG [b] (dpm/mg protein)	% Composition [c]		% Stimulation	
		CS	HS	CS	HS
Control:					
Tissue:	10,759	62	36		
Medium	7,094	73	24		
Xyloside:					
Tissue	6,388	56	43	52	72
Medium	49,717	87	11	839	303

[a] Intact 14-day retinas were incubated 24 hr in fresh medium containing β-D-xyloside and 200 μCi/ml $H_2{}^{35}SO_4$ (triplicate cultures, one retina/culture). [b] The GAG were isolated as for Table 1, except that CPC precipitation was used alone (i.e., no Sephadex chromatography). [c] The GAG were digested with chondroitinase ABC, and portions of these digests were sampled for counting to calculate the baseline for the percent CS. The undigested GAG were precipitated with CPC in the presence of freshly added carrier GAG, and the supernatant fractions were counted to determine the CS. The precipitates were dissolved in 2M NaCl, precipitated with ethanol, reconstituted in water, and digested with nitrous acid. The digest was lyophilized and sampled for counting to provide a baseline for calculating the percent HS. The undigested GAG was precipitated with CPC in the presence of freshly added carrier GAG, and the supernatant fraction was counted to calculate the HS.

in the C-4-S/C-6-S ratio for $^{35}SO_4$ (3). At no time during this period did HA synthesis comprise more than about 2% of the total GAG (3). Because of the irreversible inhibitory effect of BrdU on histogenesis in the retina (2) it is of interest that 2 days of culture with BrdU were found to inhibit at least some aspects of differentiation in the pattern of GAG synthesis in this tissue, when tested after 5 days of subsequent culture in BrdU-free medium containing thymidine. Most notable was an inhibition of the increase in C-4-S relative to C-6-S and a relatively higher total incorporation.

Work with immature chondrocytes (11) has suggested that BrdU interferes with the normal expression of genes controlling the synthesis of core protein for CS proteoglycans. In the retina the site of action of BrdU is also likely

the DNA (2), but it is not yet clear whether the inhibitor affects histogenesis, glutamine synthetase inducibility, and GAG synthesis independently or whether it affects a different level of control for all three characteristics. The increase in incorporation into retina GAG after exposure to the inhibitor could be explained by changes in the properties of the core protein and/or a decrease in degradative processes. However, at present other possibilities, such as inhibition of some process that reduces incorporation during culture or increase in permeability to labeled precursors, cannot be ruled out. The increased incorporation with BrdU cannot simply be explained by a reduction in protein content of the cells because this remained essentially the same in treated and control cultures.

The β-D-xylosides by-pass the need in chondrocytes, limb bud mesenchyme, rat glial tumor, mouse neuroblastoma, rat hepatoma (10), and cartilage slices (12) for core protein and the first enzyme in CS chain initiation (xylosyltransferase), presumably by providing an alternative substrate for chain formation. The present observation adds to this list yet another cell type in which all the enzymes for CS chain elongation appear to be present but are limited in their function by the availability of core protein and/or xylosyltransferase. When considered together with the inhibition of changes in the pattern of GAG synthesis by BrdU and the failure of cell dissociation to alter the pattern, we suggest that the pattern of change in GAG synthesis in the retina most likely is controlled at the gene level. The close parallel between these changes in synthesis and other aspects of retina differentiation (1-3) raises the important question now under investigation whether GAG have a functional role in retina histogenesis.

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