

# Reversible Effects of Retinoic Acid on Glycosaminoglycan Synthesis during Differentiation of HL-60 Leukemia Cells<sup>1</sup>

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## ABSTRACT

Glycosaminoglycans (GAGs) play an important role in cell-cell and cell-substratum interactions, and undergo specific changes during neutrophil development. Previous studies (Luikart, S. D., Maniglia, C. A., and Sartorelli, A. C. *Cancer Res.*, 44: 2907-2912, 1984) have shown that both dimethyl sulfoxide and 4- $\beta$ -phorbol-12- $\beta$ -myristate-13- $\alpha$ -acetate decreased GAG production by a hypoxanthine-guanine phosphoribosyl transferase-deficient clone of HL-60 promyelocytic leukemia cells prior to the appearance of a mature myeloid or monocytoid phenotype. To expand these investigations further, GAGs were analyzed by cetylpyridinium chloride precipitation and DEAE-Sephacel ion-exchange chromatography after labeling of parental HL-60 cultures with [<sup>35</sup>S]sulfate and D-[<sup>3</sup>H]glucosamine for 6 h, following treatment with 1  $\mu$ M all-*trans* retinoic acid (RA). Chondroitin sulfate represented the major GAG species produced, although endo- $\beta$ -galactosidase-sensitive undersulfated macromolecules which possibly might be keratan sulfate, were also identified. GAG production decreased over a time period of 144 h in culture. RA treatment reduced the amount of radiolabeled cell-associated GAGs by 50% after 48, 96, and 144 h of exposure. In contrast, commitment to myelocytic maturation of the majority (*i.e.*, approximately 60%) of the cells occurred between 72 and 96 h of RA treatment. Concurrently with the appearance of mature granulocytic cells, two-thirds of the radiolabeled GAGs were recovered from the medium, compared to one-third in untreated cultures, a phenomenon that resulted in an overall alteration in the distribution of GAGs. When RA was removed by washing after either 48 h (*i.e.*, precommitment to differentiation) or 96 h (*i.e.*, postcommitment to differentiation), a 1.5- to 3.5-fold increase in GAG production was noted 48 h later; this increase was unrelated to the medium change or to alterations in cell cycle distribution. The amounts of endo- $\beta$ -galactosidase-sensitive macromolecules were unaltered. Thus, although 1  $\mu$ M RA inhibited the synthesis of chondroitin sulfate by HL-60 leukemia cells, this inhibition was reversible by removal of the drug and appeared to be unrelated to the commitment to myelocytic maturation.

## INTRODUCTION

GAGs<sup>3</sup> are highly charged, linear, high-molecular-weight poly-

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<sup>3</sup> The abbreviations used are: GAG, glycosaminoglycan; RA, all-*trans*-retinoic acid; TPA, 4- $\beta$ -phorbol-12- $\beta$ -myristate-13- $\alpha$ -acetate; CPC, cetylpyridinium chloride; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline, in g/liter, [KH<sub>2</sub>PO<sub>4</sub>, 0.2; Na<sub>2</sub>HPO<sub>4</sub>, 1.5; NaCl, 8.0 (pH 7.4)]; DMSO, dimethyl sulfoxide.

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saccharide chains, most often covalently bound to a core protein on the cell surface (18). These molecules are composed of repeating disaccharide units which have a strong negative charge, due largely to varying degrees of sulfation (18). The biological functions of these molecules are probably related to these physicochemical properties, and they play an important role in cell-cell and cell-substratum interactions (30). Changes in GAG synthesis have been correlated with discrete stages of development in embryonic systems (35), and the normal maturation of granulocytes and monocytes also appears to be accompanied by specific changes in GAG profiles (20, 28). Our laboratory (21) has recently reported that GAG synthesis by HL-60/HGPRT<sup>-</sup> human promyelocytic leukemia cells was decreased by approximately 75% during exposure to DMSO or TPA, and that this effect preceded the appearance of the mature phenotype induced by these drugs. Treatment with a phorbol ester which did not cause differentiation resulted in a much smaller decrease in GAG production.

To further explore the role of GAGs in the maturation of HL-60 cells, we have extended these studies to another class of differentiating agents. These investigations measured the effects of RA on the production of GAGs by parental HL-60 cells at 48-h intervals during the induction of differentiation. RA has been reported to alter GAG synthesis in various normal epithelial (19) and mesenchymal (17, 32) tissues, as well as in neoplastic cells (22). Moreover, RA is one of the most potent known inducers of granulocytic maturation in HL-60 cells (1, 15), and 13-*cis*-retinoic acid is currently under clinical investigation for the treatment of preleukemic syndromes (12). Although the effects of retinoids on cell differentiation are believed to result from alterations in gene transcription (33), it has also been suggested that these agents are directly involved in glycosyltransfer reactions (6) and, therefore, they might well affect GAG synthesis in this manner. Thus, studies with RA might provide further information on whether the observed changes in GAG production were indeed directly linked to the development of a mature phenotype by HL-60 cells, or were a result of the effects of the drug on GAG processing itself.

In this report, we provide evidence that the production of chondroitin sulfate, the major species of GAG found on HL-60 cells, is inhibited by RA, but that this effect can be reversed when the drug is removed, even after commitment to a program of terminal differentiation has taken place. In addition, our findings indicate that HL-60 cells produce a second class of large endo- $\beta$ -galactosidase-sensitive glycoconjugates, the production of which is not affected by removal of RA. Thus, our findings suggest that RA might regulate the production of specific glycoconjugates by HL-60 cells independently of its effects on cell differentiation.

## MATERIALS AND METHODS

**Materials.** All-*trans*- $\beta$ -retinoic acid (Sigma Chemical Co., St. Louis, MO) was freshly dissolved in 100% ethanol for each experiment. All manipulations involving RA were performed in subdued light. NBT, TPA, DNase I, chondroitin ABC lyase, and CPC were obtained from the Sigma Chemical Co., and *Streptomyces hyaluronidase*, as well as *Pseudomonas endo- $\beta$ -galactosidase* (keratanase), and chondroitin AC lyase were from Seikagaku Kogyo, Tokyo, Japan. Proteinase K was purchased from E. M. Biochemicals, Cincinnati, OH, and Pronase and neuraminidase were from Calbiochem-Behring Corp., La Jolla, CA. Chondroitin 6-sulfate (Sigma Chemical Co.), dissolved in PBS at a concentration of 1 mg/ml, was used as a carrier. Alcian blue (phthalocyanine dye) was obtained from Matheson, Coleman & Bell, Norwood, OH.

**Cell Culture.** HL-60 cell passages 40 through 50 were used for these experiments (10). The cells were maintained in RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal bovine serum (Grand Island Biological Company, Grand Island, NY), and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. For all experiments, cells were seeded at a density of 1.0 to 1.5  $\times 10^5$  cells/ml. Stock cultures were maintained in exponential growth by subculturing every 4 to 5 days. RA dissolved in 100% ethanol was added to the cultures to give a final concentration of 1  $\mu$ M; the final concentration of ethanol never exceeded 0.1%. Cell numbers were determined using a Model ZBI Coulter particle counter.

**Measurement of Cellular Differentiation.** The differentiation of HL-60 cells was quantitated by determining the fraction of the cell population which acquired the ability to reduce NBT, a reflection of the attainment of phagocytic activity (10). Cell suspensions were mixed with an equal volume of 0.2% NBT (w/v) dissolved in PBS, to which TPA was added to a final concentration of 3  $\mu$ M. After incubation of this mixture for 20 min at 37°C, the cells were collected by centrifugation at 100  $\times g$  for 5 min, and the supernatant was discarded. The cell pellet was resuspended in 50  $\mu$ l of fetal bovine serum, and smeared onto slides which were stained with Wright's stain (Fisher Scientific Co., Pittsburgh, PA). The slides were examined under a microscope and the number of cells containing dark blue-black formazan granules were scored. A total of 200 cells were examined per sample.

**Radiolabeling and Analysis of GAGs.** Cultures were labeled with either 40  $\mu$ l of sodium [<sup>35</sup>S]sulfate/ml (1 Ci/mmol; New England Nuclear, Boston, MA) only, or simultaneously with 20  $\mu$ l/ml of D-[<sup>3</sup>H]glucosamine-HCl (39.4 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 6 h at 37°C. The medium and 2 PBS washes were collected and combined as the medium fraction. The cell pellets were lysed by 3 cycles of alternate freezing and thawing. The lysate was treated with an equal volume of 3 mM MgCl<sub>2</sub> containing 0.08 mg of DNase/ml for 12 h at 37°C. Both cellular and medium samples were then treated with Pronase (1 mg/sample) for 24 h at 37°C, and with proteinase K (1 mg/sample) for another 24 h at 37°C. Samples were adjusted to pH 10.0 with 1.0 N NaOH to remove possible peptide residues from the linkage region, and were neutralized with 1.0 N HCl. GAGs in the medium samples were precipitated with 4 volumes of 100% ethanol at -20°C overnight, in the presence of carrier chondroitin 6-sulfate; the precipitates were collected by centrifugation at 10,000  $\times g$  for 20 min at 0°C, and resolubilized in a small volume of PBS. Relative acid-soluble pool sizes of radiolabeled precursors were determined by precipitation with 1% phosphotungstic acid in 0.5 M HCl, followed by 2 extractions with 10% trichloroacetic acid, and were found to be equal by 6 h for both [<sup>35</sup>S]sulfate and [<sup>3</sup>H]glucosamine. Radioactive GAGs were quantitated by a modification of the method of Glimelius *et al.* (11). After the addition of 200  $\mu$ g of carrier chondroitin 6-sulfate, 200- $\mu$ l samples were treated with 1 ml of 2% (w/v) CPC in 40 mM Na<sub>2</sub>SO<sub>4</sub> at 37°C for 1 h. Precipitates were collected on 0.45- $\mu$  HA Millipore filters (Millipore Corp., Bedford, MA) and rinsed with 15 ml of 1% (w/v) CPC in 40 mM Na<sub>2</sub>SO<sub>4</sub>. The filters were air dried, placed in glass scintillation vials containing 10 ml of Biofluor (New England Nuclear), and radioactivity was determined using a Beckman Model LS7500 scintillation spectrometer. To determine the species of GAG

produced, cultures were labeled with [<sup>35</sup>S]sulfate and D-[<sup>3</sup>H]glucosamine HCl simultaneously for 6 h. GAGs were isolated and purified as described above, and samples were applied to DEAE-Sephacel (Pharmacia, Uppsala, Sweden) columns equilibrated with 0.15 M LiCl in 0.04 M sodium acetate (pH 4.0), and eluted with a linear gradient of 0.15 to 1.5 M LiCl in 0.04 M sodium acetate (pH 4.0) at 20°C. The ion-exchange profiles were compared to chromatograms obtained from samples pretreated with either chondroitin ABC lyase (1 unit/ml) for 12 h at 37°C (31), *Streptomyces hyaluronidase* (20 turbidity-reducing units/ml in 0.02 M sodium acetate, pH 6.0) for 1 h at 60°C (25), endo- $\beta$ -galactosidase (250 milliunits/ml in 0.1 M Tris-HCl, pH 7.4) for 12 h at 37°C, chondroitin AC lyase (1 unit/ml in 0.02 M sodium acetate, pH 6.0) for 12 h at 37°C, or nitrous acid (4). Total cell-associated sulfated GAGs were determined spectrophotometrically, as described by Gold (13).

## RESULTS

The growth rate of HL-60 cells exposed to 1  $\mu$ M RA was not significantly different from that of untreated control cells during the exponential phase of growth (Chart 1). The fraction of functionally mature cells, as ascertained by their ability to generate reactive oxygen species that reduce NBT following activation by TPA, increased gradually during continuous exposure to RA. After 6 days of drug treatment, approximately 95% of the population was composed of cells with a mature phenotype. These results are in general agreement with previous reports on the effects of 1  $\mu$ M RA on the growth and differentiation of HL-60 leukemia cells (1, 15).

To determine whether the previously reported changes in GAG production that accompanied the induction of maturation of HL-60 cells by TPA and DMSO (21) also occurred with RA treatment, exponentially growing cells were cultured in the presence of 1  $\mu$ M

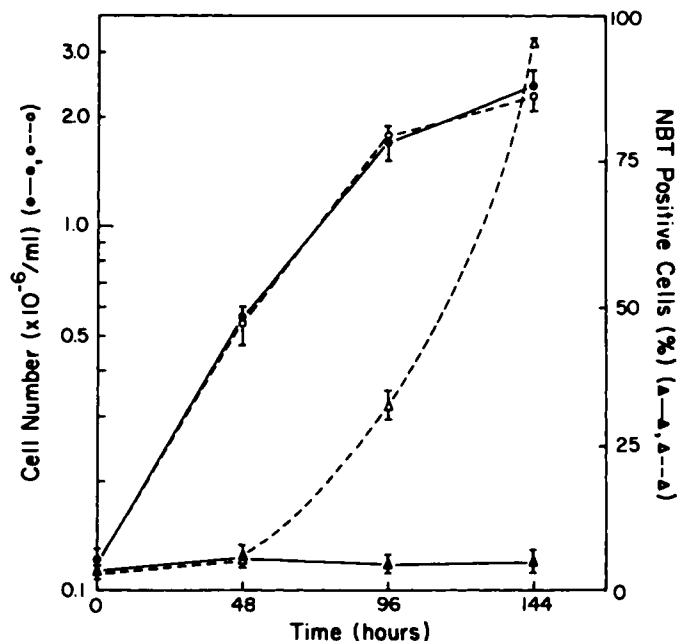


Chart 1. Growth and functional maturation of HL-60 cells during exposure to RA. Exponentially growing cells were seeded at a density of 1.0 to 1.5  $\times 10^5$  cells/ml in duplicate flasks in the presence of 1  $\mu$ M all-*trans* RA (○), or 0.1% (v/v) ethanol only (●). At various times thereafter, cell numbers were determined using a Model ZBI Coulter particle counter, and the percentage of NBT-positive cells exposed to RA (△) was compared to control cells receiving ethanol-containing vehicle alone (▲). The results are the means and SE of 5 independent experiments, with duplicate flasks being used in each experiment.

RA, and after 1, 48, 96, or 144 h of exposure, cultures were labeled with sodium [<sup>35</sup>S]sulfate for 6 h, and GAGs associated with the cells (cellular fraction), as well as those released into the medium (medium fraction), were analyzed. The amounts of CPC-precipitable radiolabeled GAGs recovered at various times are shown in Table 1. The production of GAGs decreased with increasing time in culture in both control and RA-treated cultures. This phenomenon appeared to be related to the phase of growth, since GAG production by exponentially growing cells (*i.e.*, cells at 1 and 48 h in culture) was approximately 5-fold higher than that of cells entering plateau phase (96 and 144 h in culture). Exposure to RA for 48, 96, or 144 h resulted in a further decrease in cell-associated radiolabeled GAGs of approximately 50%, compared to untreated cultures. It is of interest that even after 1 h of exposure to RA, the incorporation of [<sup>35</sup>S]sulfate into CPC-precipitable GAGs by HL-60 cells was already decreased by 10 to 25%, even though relative acid-soluble pool sizes of radiolabeled precursors were equal in control and treated cells.

Table 2 shows the compartmental distribution of the radiolabeled GAGs recovered from RA-treated and untreated cultures. In untreated cultures, approximately 65 to 75% of the GAGs were found in the cellular fraction for up to 144 h in culture. Exposure of HL-60 cells to RA increased the amount of radiolabeled GAGs recovered from the medium relative to that present in the cellular fraction. Thus, the amount of material present in the medium accounted for approximately 65% of all the GAGs in cultures treated with RA for 96 to 144 h (Table 2). Whether this change in GAG deposition caused by RA resulted from a relative increase in the amount of GAG released into the medium, or from a faster rate of degradation of cellular GAGs, remains to be established.

It appeared important to establish whether a linkage exists between the changes in GAG production that have been observed, and the commitment of HL-60 cells to terminal differentiation. The early inhibition of GAG synthesis by RA (Table 1) suggested that the 2 processes might not be related. To accomplish these measurements, cells were exposed to RA for various periods of time; the drug was then removed by washing, and

Table 1

*Biosynthesis of sulfated GAGs by HL-60 leukemia cells during exposure to RA*

Exponentially growing cells were seeded at 1.0 to 1.5 × 10<sup>6</sup> cells/ml in the presence of 1 μM RA or the solvent 0.1% ethanol. At the indicated times, duplicate flasks were labeled with 40 μCi of [<sup>35</sup>S]sulfate/ml for 6 h. Radiolabeled GAGs were isolated and purified from the cellular and medium fractions, and precipitated with 2% (w/v) CPC and 40 mM Na<sub>2</sub>SO<sub>4</sub>. A 2-tailed Student's *t* test for paired observations was applied to evaluate differences.

Treatment	Time (h)	Radiolabeled [ <sup>35</sup> S]GAGs (dpm/10 <sup>6</sup> cells × 10 <sup>-4</sup> )		
		Total	Cellular	Medium
None	1	13.31 ± 1.69 <sup>a, b</sup>	7.77 ± 0.75 <sup>b</sup>	5.54 ± 1.80 <sup>b</sup>
		9.70 ± 3.49	5.17 ± 1.39	4.53 ± 1.20
RA	48	8.85 ± 0.39 <sup>b</sup>	6.53 ± 0.85 <sup>c</sup>	2.32 ± 0.65 <sup>b</sup>
		6.78 ± 0.45	3.81 ± 0.64	2.97 ± 1.07
None	96	2.45 ± 0.30 <sup>b</sup>	1.38 ± 0.31 <sup>d</sup>	1.08 ± 0.15 <sup>b</sup>
		1.87 ± 0.29	0.76 ± 0.22	1.10 ± 0.22
RA	144	1.11 ± 0.12 <sup>b</sup>	0.62 ± 0.07 <sup>d</sup>	0.49 ± 0.13 <sup>b</sup>
		0.90 ± 0.19	0.32 ± 0.09	0.56 ± 0.16

<sup>a</sup> Mean ± SE of 4 independent experiments.  
<sup>b</sup> Not significant.  
<sup>c</sup> P < 0.02.  
<sup>d</sup> P < 0.05.

Table 2

*Distribution of radiolabeled GAGs in cultures of HL-60 cells*

Exponentially growing cells were treated with 1 μM RA, and exposed to [<sup>35</sup>S]sulfate to label GAGs as described in Table 1. Radiolabeled, CPC-precipitated GAGs associated with the cellular and the medium fractions are expressed as a percentage of the total GAGs recovered from the cultures, and are compared to non-RA treated controls. The results are presented as the range of 4 independent experiments. The difference in distribution of GAGs between RA-treated and control cultures was significant at 96 and 144 h (P < 0.05) as assessed by Student's *t* test for paired observations.

Treatment	Time (h)	% of distribution	
		Cells	Medium
None	1	66–76 (71) <sup>a</sup>	24–34 (29)
		54–80 (66)	20–46 (34)
RA	48	74–87 (82)	13–26 (18)
		63–80 (72)	20–27 (28)
None	96	50–70 (61)	30–50 (39)
		23–49 (33)	51–77 (67)
RA	144	62–78 (68)	22–38 (32)
		26–42 (34)	58–74 (66)

<sup>a</sup> Numbers in parentheses, mean of 4 independent experiments.

Table 3

*Reversibility of the effects of RA on GAG biosynthesis in relation to the commitment of HL-60 cells to a differentiation pathway*

Exponentially growing cells were seeded in the presence of 1 μM RA as described in Table 1. At the indicated times, cells were washed twice and resuspended in fresh medium. The percentage of cells capable of reducing NBT was determined on Day 6, to assess the fraction of committed cells at the time of removal of RA. At 48 h after the removal of RA, cultures were labeled with [<sup>35</sup>S]sulfate and GAGs were analyzed as described in Table 1. A 2-tailed Student's *t* test was used for statistical analysis. Total cell-associated sulfated GAGs, determined spectrophotometrically (13) on Day 6, 48 h after removal of RA, were 4.44 and 9.11 pg/cell for untreated and RA treated cells, respectively.

Time of exposure to RA (h)	Committed cells (% of total)	Radiolabeled [ <sup>35</sup> S]GAGs (dpm/10 <sup>6</sup> cells × 10 <sup>-4</sup> )	
		Control	RA treated
24	3–6		
48	3–4	2.53 ± 0.65 <sup>a</sup>	3.98 ± 0.24 <sup>b</sup>
72	13–26		
96	74–81	0.91 ± 0.31	2.79 ± 0.54 <sup>c</sup>
120	88–94		

<sup>a</sup> Mean ± SE of 4 independent experiments.  
<sup>b</sup> P < 0.05.  
<sup>c</sup> P < 0.025.

cells were resuspended in fresh medium in the absence of inducer. At the end of 6 days in culture, the fraction of mature (NBT-reducing) cells was determined. Table 3 shows that the majority of cells enter a state of irreversible commitment to differentiation within a relatively short period of exposure to RA (*i.e.*, between 72 and 96 h following addition of drug).

The effects of removal of RA on GAG production were investigated at 2 time points, one prior to commitment, and one after most of the population was committed to a terminal program of maturation. Cells were exposed to RA for either 48 or 96 h, followed by resuspension in fresh medium. Two days later, GAG production was measured by radiolabeling the cultures with sodium [<sup>35</sup>S]sulfate for 6 h. Control cultures were treated in an identical manner, to ensure that the condition of the medium and cell densities were comparable at the end of the experiment. As in previous experiments, total GAG production in untreated cultures decreased as a function of cell density (Table 3). Thus, GAG production was lower in 6-day-old (plateau phase) cultures than in 4-day-old (exponentially growing) cultures. The effects of removing RA from cultures that had been treated with this

inducer for 48 or 96 h were marked, in that GAG production by cells exposed to RA for 2 days, followed by 2 days in fresh culture medium, was about 1.5-fold higher than that of untreated control cells. Cells exposed to RA for 4 days (approximately 80% of these cells were committed to a differentiation pathway) had an even greater increase in GAG production over control values assayed 48 h later. GAG production by these cells was 3-fold higher than that of corresponding untreated cells. Thus, although treatment of HL-60 cells with RA for 48 or 96 h resulted in a significant decrease in precursor incorporation into cellular GAGs (Table 1), resuspension in fresh medium without drug for an additional 48 h increased the production of radiolabeled GAGs 1.5- to 3-fold. This rebound phenomenon was independent of the induction of a committed state. The total amount of cell-associated sulfated GAGs in RA-treated cultures determined spectrophotometrically on Day 6 (48 h after drug removal) was 9.11 pg/cell, compared to 4.44 pg/cell in untreated cultures. Thus, the rebound effect observed in radioactive precursor incorporation was reflected by an equivalent increase in the absolute amount of cell-associated GAGs. The distribution of radiolabeled GAGs between the cellular and medium fractions 48 h after removal of RA was similar to that in untreated control cultures (data not shown). Thus, the effects of 1  $\mu$ M RA on both GAG synthesis and distribution were reversible after removal of the drug.

To assess the GAG species affected by RA treatment, a similar set of washout experiments was performed, in which cultures were labeled with both [ $^{35}$ S]sulfate and [ $^3$ H]glucosamine; GAGs were then isolated and purified, and analyzed by ion-exchange chromatography. The results of these experiments presented in Chart 2 show that highly sulfated GAGs eluted as a single sharp peak at 0.45 M LiCl. This material was almost entirely (95%) degradable by chondroitin ABC lyase, as well as by chondroitin AC lyase, indicating that it consisted of chondroitin sulfate species, both in control cultures and in cultures pretreated with 1  $\mu$ M RA for 96 h, and that no detectable amount of dermatan sulfate was produced. It is clear that the amount of  $^{35}$ S-labeled GAGs recovered from RA-pretreated cells was much larger (80% in the sample shown) than from control cells, largely due to the appearance of a shoulder which eluted prior to the main peak. The observed increase confirmed the data from CPC precipitation experiments (Table 3). Whether this material represents a single subspecies of chondroitin sulfate or shorter chain lengths remains to be established. A second peak of undersulfated, chondroitin ABC lyase-resistant material which eluted at 0.30 to 0.35 M LiCl, remained both in RA-pretreated and control preparations following chondroitin ABC lyase treatment. These large and/or highly charged molecules were not digested by *Streptomyces* hyaluronidase, and were resistant to heparitinase and nitrous acid treatment as well. This material apparently was not sialated, since neuraminidase treatment did not alter the profile. However, complete degradation of this material could be achieved with endo- $\beta$ -galactosidase from *Pseudomonas*. This enzyme preferentially cleaves the  $\beta$ -D-galactosyl(1  $\rightarrow$  4)-2-acetamido-2-deoxy-6-O-sulfo-D-glucose linkages present in keratan sulfates; sulfation at the galactose residue inhibits the action of the enzyme(s). Thus, the material which eluted at 0.30 to 0.35 M LiCl may well represent undersulfated keratan sulfate. It is of interest that this material was not detectable in the culture medium; thus, it appeared to be retained by the cells, which

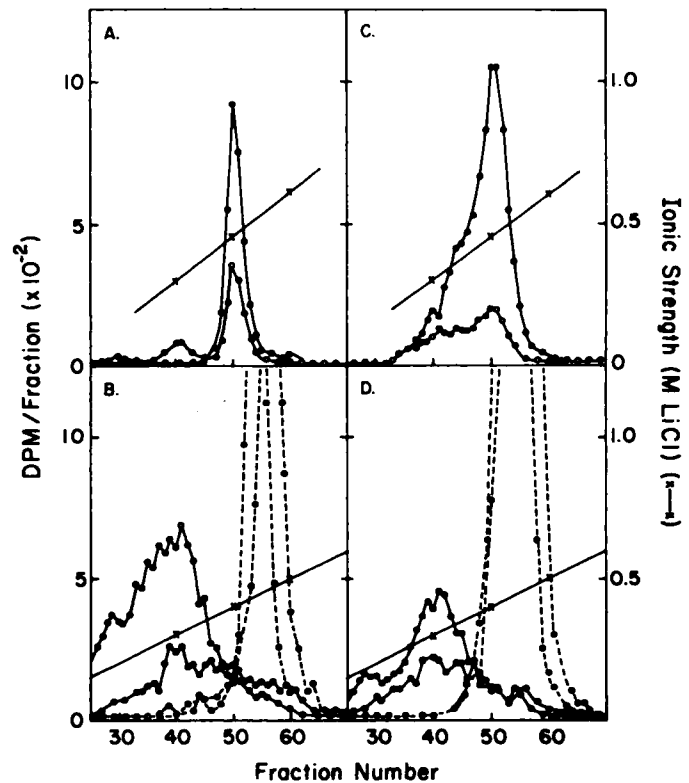


Chart 2. Ion-exchange chromatography of GAGs synthesized by HL-60 cells treated with RA. Exponentially growing cells were exposed to 1  $\mu$ M RA for 96 h, followed by resuspension in fresh medium for an additional 48 h before labeling of GAGs. Purified [ $^{35}$ S]sulfate- and [ $^3$ H]glucosamine-labeled GAG preparations were chromatographed on DEAE-Sephacel, and eluted with a gradient of 0.15 to 1.5 M LiCl. A, elution profile of GAGs from vehicle-treated cells (O—O,  $^3$ H; ●—●,  $^{35}$ S). B, same preparation as in A, pretreated with either chondroitin ABC lyase (O—O,  $^3$ H; ●—●,  $^{35}$ S); or endo- $\beta$ -galactosidase (O—O,  $^3$ H; ●—●,  $^{35}$ S). C, elution profile of cellular GAGs from RA-treated cultures (O—O,  $^3$ H; ●—●,  $^{35}$ S). D, same preparation as in C, pretreated with either chondroitin ABC lyase (O—O,  $^3$ H; ●—●,  $^{35}$ S); or endo- $\beta$ -galactosidase (O—O,  $^3$ H; ●—●,  $^{35}$ S). The results are expressed as dpm/fraction corrected for differences in cell numbers between control and RA-treated cultures. The experiment was performed in duplicate, and chromatography was repeated at least twice for each condition, of which one representative example is shown.

contrasts with chondroitin sulfate which was released into the medium. As is apparent in Chart 2, the relative amount of keratan sulfate synthesized by control cells, as well as by cells pretreated with RA, appears to be equivalent. This observation contrasts with the rebound increase in the synthesis of chondroitin sulfate seen following removal of RA, which was described above. Thus, the synthesis and distribution of 2 different species of sulfated glycoconjugates appeared to be affected independently by treatment with RA and its subsequent removal.

## DISCUSSION

RA is a potent inducer of myeloid differentiation of HL-60 promyelocytic leukemia cells, with the optimal concentration being 1  $\mu$ M (1, 15). Our findings are consistent with these results, in that exposure of exponentially growing HL-60 cells to 1  $\mu$ M RA for 144 h produced a population of 80% or more of mature myeloid cells (Chart 1; Table 3). Maturation of normal neutrophilic granulocytes is accompanied by a number of changes in GAG production and deposition (5, 26, 28, 36, 37). Payne and Ackerman (28) showed that [ $^{35}$ S]sulfate is incorporated into primary

granules during the promyelocyte stage of development, as well as into the tertiary granules of fully mature neutrophils. Furthermore, detachment of granulocytes from bone marrow stromal cells appears to be accompanied by the *de novo* appearance of chondroitin sulfate on the cell surface (5, 36), whereas no membrane-associated GAGs are found in immature precursor cells, or on leukemic myeloblasts (37). In addition, adhesion of peripheral neutrophils appears to be correlated with the release of chondroitin sulfate by the cells, and the appearance of heparan sulfate and hyaluronic acid on the cell surface (36). The experiments described in this report indicate that chondroitin sulfate is the major species of GAG synthesized by HL-60 cells, as had been shown previously by others (14, 21). No dermatan sulfate, heparin/heparan sulfate, or hyaluronic acid were detected. In contrast, Parmley *et al.* (27) and Del Rosso *et al.* (5) have identified small amounts of heparan sulfate-like material and dermatan sulfate in mature neutrophils, as well as in myeloid precursors, and Murata (23) has detected heparan sulfate and hyaluronic acid in leukemic myeloblasts. It is possible that HL-60 cells do not synthesize any GAG species other than chondroitin sulfate, and that this represents a characteristic of the abnormal phenotype of these cells. Alternatively, the relatively short labeling period of 6 h may not have been sufficient to detect very low levels of synthesis of other GAG species.

Luikart *et al.* (21) have previously shown that both DMSO and TPA decreased cell-associated GAG production by HL-60/HGPRT<sup>-</sup> cells by 75%, at a time point which preceded the appearance of a mature phenotype. This observation suggested that changes in GAG production might represent early events in the process of HL-60 cell differentiation. However, the finding that 4 $\alpha$ -phorbol-12,13-didecanoate also decreased GAG production by 40% shed some doubt on this assumption, as this phorbol ester does not induce maturation of HL-60 cells (21). We have extended these observations further by using RA, one of the most potent inducers of myeloid differentiation in HL-60 cells, by measuring GAG production at various intervals of drug exposure. The reduction of GAG production as a function of increasing cell density which we observed was not unexpected, since this phenomenon has been noted by others previously (2). The synthesis of chondroitin sulfate was moderately reduced in HL-60 cultures within 7 h of exposure to 1  $\mu$ M RA. Compared to untreated cells, the incorporation of radiolabel into cellular GAGs in RA-treated cultures was significantly decreased by 50% at 48 h, and this difference remained constant throughout the remaining 4 days of exposure used in these experiments. RA treatment also altered the deposition of radiolabeled GAGs (Table 2); thus, at 96 and 144 h of exposure to this inducer, the fraction of cell-associated radiolabeled material was significantly less than in untreated cultures. This finding suggested that a change in the processing of newly synthesized GAGs occurred, possibly related to alterations in lysosomal enzyme release by retinoids described previously (7). In the earlier experiments performed by Luikart *et al.* (21) with DMSO and TPA, a similar shift in the distribution of radiolabeled GAGs was noted; however, only 40% of the total material was found to be associated with the cells in untreated cultures, whereas in the present experiments, 60% of the GAGs were cell associated at 96 h in culture. This difference could possibly be accounted for by the fact that trypsin-released material was included with the medium fraction in the previous report (21). This technical difference may also be responsible for

the greater decrease in cell-associated GAG production observed with TPA and DMSO (21) than that seen in the present findings with RA treatment. Alternatively, differences in mechanisms of action or relative potencies of the drugs used may be involved, in a manner analogous to the different quantitative changes in activities of various glycosyltransferases induced by RA and TPA, as noted by others (8).

The effects of retinoids on the growth and differentiation of both normal and neoplastic cells have recently been reviewed (16, 33). Variable effects are observed *in vitro*, depending upon the compound tested and the level of inducer used, the cell types used (normal versus neoplastic, epithelial, or mesenchymal), and the culture conditions (1, 15, 16, 22, 34). It has been proposed that these complex and variable actions of the retinoids reflect alterations in genetic expression (33). For example, it has recently been shown that exposure of HL-60 cells to RA results in a reduced expression of the *c-myc* gene, which appears to play a role in the control of growth and differentiation (38). Similarly, the activity of various glycosyltransferases is altered in a pattern specific for myeloid differentiation, independently of the inducer used (8). However, the suppression of GAG synthesis by HL-60 cells caused by exposure to RA does not appear to be part of the program of terminal differentiation induced by the drug for 2 reasons. First, a beginning inhibition of GAG synthesis is observed within the first 7 h of drug treatment; and secondly, it is reversible after removal of RA, even beyond the time that most cells have reached the committed state. If these changes in GAG synthesis are the result of alterations in gene expression, one must assume that the activity of enzymes involved in GAG synthesis is suppressed, or degradative enzymes are induced. Moreover, these changes would selectively affect the processing of chondroitin sulfate only (e.g., at the level of chain initiation by xylosyltransferase), since the synthesis of endo- $\beta$ -galactosidase-sensitive (O-linked) molecules is not altered. Alternatively, a direct role for retinoids in mannosyl- and galactosyltransfer reactions has been suggested (5, 29). However, RA is not metabolized to retinol, and there is no convincing evidence for the existence of a metabolite of RA which could assume such a sugar transfer function (33). Moreover, a selective effect on only one class of glycoconjugates as we have observed would be hard to explain by this pathway (9). Alternatively, it might be speculated that RA could induce core proteins as part of the program of terminal differentiation, but suppress the expression of enzymes involved in GAG chain initiation and/or elongation. Removal of the drug after the maturation program is initiated would then result in an increase in GAG synthesis on the core proteins which had become expressed in the interim.

The precise identity of the endo- $\beta$ -galactosidase-sensitive undersulfated glycoconjugates identified by ion-exchange chromatography (Chart 2) remains to be established. It is likely, however, that this material corresponds to a class of high-molecular-weight glycopeptides identified by Cossu *et al.* (3) on HL-60 cells. In both instances, the material was (a) labeled with glucosamine; (b) sensitive to endo- $\beta$ -galactosidase; and (c) resistant to neuraminidase treatment. Considering both its elution position at fairly high ionic strength, and the fact that this material was sulfated to some degree, it can tentatively be classified as keratan sulfate (24).

Since retinoids and other inducers of differentiation are entering clinical trial for the treatment of myelodysplastic and leukemic

syndromes (12), the identification of biochemical processes affected by these drugs becomes of considerable interest. It is clear that the effects of RA in HL-60 cells are complex and in part dependent upon the presence of the drug. As clinical parameters for the activity of inducing agents are sought, caution should be exercised that the measurements selected truly reflect the maturation of malignant cells.

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