Altered Glycosaminoglycan Production by HL-60 Cells Treated With 4-Methylumbelliferyl-β-D-Xyloside

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Glycosaminoglycans, mainly chondroitin 4-sulfate, are located in the primary granules of human myeloid cells. These polyanionic carbohydrates are believed to play an important role in leukocyte maturation and function. To study the effect of altered chondroitin sulfate metabolism on human promyelocytic leukemia cells, we have treated HL-60 cells with 4-methylumbelliferyl-β-D-xyloside. β-D-Xylosides initiate the synthesis of free chondroitin sulfate chains. Cytotoxic studies of treated cells demonstrated a marked increase in cytoplasmic granules stained with cationic dyes. This was confirmed by radiolabeled precursor incorporation studies that demonstrated a 344% increase in 35S-sulfate uptake into glycosaminoglycans associated with the cells and a 39% increase in incorporation into glycosaminoglycans released into the media.

Chromatographic analyses of these glycosaminoglycans from treated cells demonstrated that the newly formed chondroitin sulfate chains were not attached to protein core and were of shorter length, but of greater charge density than chondroitin sulfate produced by control cells. Thus, β-D-xyloside appears to alter the protein linkage, chain length, and sulfation of chondroitin sulfate produced by HL-60 cells, and these changes are morphologically evident. These biochemically altered cells may provide important information concerning the role of these macromolecules in myeloid development.

MATERIALS AND METHODS

Materials. 35S-sulfate was obtained from ICN Chemical and Radioisotope Division (Irvine, Calif.). DNase, chondroitin ABC lyase, 4-methylumbelliferyl-β-D-xyloside, disopropylfluorophosphate, Triton X-100, Sephadex G25, and Sepharose CL-2B were purchased from Sigma Chemical Co (St Louis). DEAE-sephacel was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Chondrosulfatasases were provided by Miles Laboratories (Elk hart, Ind.). Proteinase K was obtained from Boehringer Mannheim (Indi-
anapolis). Alcian blue was purchased from Matheson, Coleman and Bell Manufacturing Chemists (Norwood, Ohio). N-dodecyl-$N$-$N$-dimethyl-3-ammonia-1-propane-sulfonate (Zwittergent 3-12) was obtained from Calbiochem-Behring Corp (San Diego). Spectrum No. 3 dialysis tubing was obtained from Fisher Scientific (Pittsburgh).

**Cell culture.** HL-60 cells were obtained from Dr R.C. Gallo (National Cancer Institute, Bethesda, Md) and maintained in RPMI 1640 supplemented with 50 μg/mL of gentamicin and 15% heat-inactivated fetal calf serum.

For measurement of the rates of cellular proliferation, HL-60 cells were plated at a density of $1 \times 10^5$ cells per milliliter; cells were untreated or treated with various concentrations of 4-methylumbelliferyl-$\beta$-D-xyloside for four days. The cells were centrifuged at 300 g for four minutes, fixed in 2.7% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35, for one hour at 4°C, and rinsed three times in 0.1 mol/L cacodylate buffer, pH 7.35. High iron diamine (HID) staining was performed as previously described.23 Control samples were incubated for 18 hours at 4°C, and rinsed twice with 0.1 mol/L cacodylate buffer, pH 7.35. HID staining was enhanced by staining after Alcian blue staining, and after myeloperoxidase staining.22

**Electron microscopy.** HL-60 cells were grown in the absence or presence of 0.3 mmol/L 4-methylumbelliferyl-$\beta$-D-xyloside for four days and then incubated with 40 μCi/mL of 35S-sulfate (1 Ci/mmol) for six hours; the cells were centrifuged at 300 g for four minutes, fixed in 2.7% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35, for one hour at 4°C, and rinsed three times in 0.1 mol/L cacodylate buffer, pH 7.35. High iron diamine (HID) staining was performed as previously described.23 Control samples were incubated for 18 hours at 22°C with a MgCl2 solution (1.4 mL MgCl2 to 50 mL H2O), pH 1.4. All pellets were rinsed, dehydrated, and embedded in Spurr low-viscosity medium. HID staining was enhanced by staining thin sections with thiocarbohydrazide and silver proteinate (TCH-SP) by the technique of Sannes et al.24

**Radiolabeling and analysis of glycosaminoglycans.** Cells were seeded at $1 \times 10^4$/mL in culture with or without 0.3 mmol/L 4-methylumbelliferyl-$\beta$-D-xyloside for four days and then incubated with 40 μCi/mL of 35S-sulfate (1 Ci/mmol) for six hours; the medium and two phosphate-buffered saline (KH2PO4, 0.2 g/L; Na2HPO4, 1.15 g/L; KCl, 0.2 g/L; NaCl, 8.0 g/L; pH 7.4) washes were combined as the medium fraction and were treated with 4 vol of 100% ethanol at −20°C to precipitate glycosaminoglycans. The cells were counted and lysed by alternate freezing and thawing three times. The lysate was then treated with an equal volume of 3 mmol/L MgCl2 containing 0.08 mg DNase per milliliter for 12 hours at 37°C.25 After centrifugation of the medium fraction at 10,000 g for 20 minutes, the medium and cellular fractions were treated twice with 1 mg of proteinase K for 24 hours each at 37°C, adjusted to pH 12 with 1.0 N NaOH to remove possible peptide residues from the cell membrane, cultures were labeled with 40 μCi/mL 35S-sulfate, as previously described. The medium fractions were discarded, and the cell proteoglycans were extracted by a modification of the method of Kimura et al.25 Briefly, 2 mL of 8% (wt/vol) Zwittergent 3-12 in a buffer of 0.1 mol/L 6-aminohexanoic acid, 10 mmol/L Na2 EDTA, 5 mmol/L benzamidine HCl, 1 mmol/L sodium iodoacetamide, and 0.1 mol/L sodium acetate, pH 5.8, was added to each culture for 30 minutes at 4°C. Then, an equal volume of 8 mol/L guanidine HCl in the same inhibitor-buffer was added for an additional 30 minutes. The extracts were removed, dialyzed against 7 mol/L urea in the inhibitor-buffer at 4°C overnight, applied to a DEAE-sephacel column (6 mL bed volume), and washed with 0.15 mol/L LiCl in 7 mol/L urea, 0.2% Triton X-100, and the inhibitor-buffer and eluted with 1.5 mol/L LiCl and the same other buffer components. Recovery was greater than 95%, and each column was used only once. Aliquots of the radiolabeled material were then applied to a Sepharose CL-2B column (0.6 × 115.0 cm) and eluted in the presence of 4 mol/L guanidine HCl, 0.1 mol/L Tris HCl, 0.1 mol/L NaSO4, and the protease inhibitors, pH 7.5. Flow rate was 1.3 mL/h. Fractions of 0.3 mL were collected and counted in a scintillation spectrometer (Beckman, Fullerton, Calif). Protein linkage was determined by susceptibility to alkaline hydrolysis.3

**RESULTS**

The effects of 4-methylumbelliferyl-$\beta$-D-xyloside on the rate of proliferation of HL-60 cells in culture were measured (Fig 1). The rate of replication of the HL-60 cells was not significantly altered by the presence of 4-methylumbelliferyl-$\beta$-D-xyloside in the lower concentrations tested. At 0.5 mmol/L concentration, cellular proliferation was somewhat inhibited after prolonged exposure. We therefore elected to study the effects of 0.3 mmol/L 4-methylumbelliferyl-$\beta$-D-xyloside on glycosaminoglycan metabolism. There was a dramatic change in cellular morphology. As seen in Fig 2, there was a marked increase in azurophilic granules. These changes were more evident by light microscopy after 48 hours of exposure. Alcian blue staining also demonstrated a marked increase in cytoplasmic granules, suggesting that intracellular polyanionic carbohydrates were increased (data not shown). However, peroxidase staining of all primary granules revealed similar number and size of granules in control and xyloside-treated cells. At the ultrastructural level, those cells treated with 0.3 mmol/L 4-methylumbelliferyl-$\beta$-D-xyloside exhibited numerous granules that were stained with HID–TCH-SP, as seen in Fig 3. This stain specifically identifies sulfated glycoconjugates.23 The granules contained an electron-dense center and a lucent rim, which is characteristic of immature primary granules.
Fig 1. The effect of 4-methylumbelliferyl-β-D-xyloside on the replication of HL-60 cells. Cells were seeded at a density of $1 \times 10^5$ per 10 ml in a 25 cm² flask in the absence (●) or presence of 0.1 mmol/L β-D-xyloside (◇), 0.3 mmol/L β-D-xyloside (▲), or 0.5 mmol/L β-D-xyloside (□). Cell numbers were determined with a model ZF Coulter particle counter. Each point represents the mean of duplicate flasks, with less than 10% deviation between flasks.

Similar but less numerous granules were seen in control cells. Staining control specimens of both xyloside-treated and control cells, which were exposed to an acid MgCl₂ solution instead of HID, lacked significant TCH-SP staining (not shown).

$^{35}$S-sulfate incorporation into glycosaminoglycans was measured in control and β-D-xyloside-treated cultures of similar cell density. As shown in Fig 4, $^{35}$S-sulfate-labeled glycosaminoglycans were increased from $1.8 \times 10^2$ disintegrations per minute (DPM) per control cell to $8.0 \times 10^2$ DPM per xyloside-treated cell, an increment of 344% in the cell-associated material, and from $2.6 \times 10^2$ DPM per control cell to $3.6 \times 10^2$ DPM per xyloside-treated cell, a 39% increase in material released into the media. Thus, total cetylpyridinium chloride-precipitable glycosaminoglycans were increased 164% in treated cultures, and the distribution of glycosaminoglycans between the cellular and medium compartments was altered by exposure to β-D-xyloside. In control cultures, 40% of the radiolabeled glycosaminoglycans were found in the cellular compartment, compared with 67% in cultures exposed to β-D-xyloside.

Because of previous reports of qualitative changes in glycosaminoglycan chains produced by β-D-xyloside-treated cells, aliquots of the $^{35}$S-sulfate–labeled material were subjected to ion-exchange chromatography by using a LiCl linear gradient to define changes in the degree of sulfation of the glycosaminoglycan molecules. As shown in Fig 5, the elution profile of glycosaminoglycans from both media and cell-associated compartments of treated cultures demonstrated a small component similar to control cultures and a second peak eluting at a higher ionic strength, suggesting an increase in charge density.

To compare the size of glycosaminoglycan molecules produced by cells grown in the presence of β-D-xyloside with those produced by control cells, each peak from the ion-exchange elution profile was collected, precipitated with ethanol, and resuspended for application to a high-performance gel chromatography system. As seen in Fig 6, the higher ionic strength material from xyloside-treated cells eluted later than material from control cells. This shift toward higher $K_{AV}$ values was especially evident in the cell-associated material (control $K_{AV} < .16$, high ionic
Fig 4. Incorporation of $^{35}$S-sulfate into glycosaminoglycans of HL-60 cells during a six-hour labeling period. Each number is calculated from the mean determination of three control flasks and three xyloside-treated cultures, representing one of two experiments with similar results. Standard deviations were less than 10%.

Fig 5. TSK 545 DEAE chromatography of glycosaminoglycans present in control HL-60 (C) and xyloside-treated (E) cells. $^{35}$S-sulfate–labeled glycosaminoglycans were applied to a TSK 545 DEAE column, equilibrated with 0.04 mol/L sodium acetate (pH 4.0) and 0.15 mol/L LiCl, and eluted with a gradient of 0.15 to 1.5 mol/L LiCl. (A) Media. (B) Cell-associated compartment.

Fig 6. Gel filtration chromatography of glycosaminoglycans. The peaks eluting from the DEAE column chromatography (Fig 5) were individually precipitated with ethanol, resuspended in chondroitin ABC lyase buffer, and subjected to gel filtration chromatography. The void volume and total volume of the TSK-G 3000 column were 10.5 and 26.5 mL, respectively. (A) Media from control cells (C); (B) first peak (low ionic strength region) from media of xyloside-treated cells (E); (C) second peak from media of xyloside-treated cells (E); (D) cell-associated material from control cells (C); (E) first peak from cell-associated material of xyloside-treated cells (E); (F) second peak from cell-associated material of xyloside-treated cells (E).

strength material from xyloside-treated cells $K_{av} = 0.47$ and suggests that these chains from xyloside-treated cells are shorter.

All of the $^{35}$S-sulfate–labeled material in each of the cultures was sensitive to chondroitin ABC lyase when analyzed by gel chromatography. We have previously reported that the major sulfated glycosaminoglycan produced by HL-60 cells is chondroitin 4-sulfate. To further examine the more highly charged chondroitin sulfate molecule identified by ion-exchange chromatography, degradation products were incubated with chondro-4-sulfatase, chondro-6-sulfatase, or a mixture of the two enzymes and applied to a gel filtration chromatography column (Sephadex G-25). All of the material was sensitive to chondro-4-sulfatase, liberating free $^{35}$S-sulfate from the disaccharide (Fig 7). No chondroitin 6-sulfate was detected.

To evaluate the protein linkage of glycosaminoglycans, $^{35}$S-sulfate–labeled intracellular proteoglycans from HL-60 cells cultured with and without $\beta$-d-xyloside were applied to a Sepharose CL-2B gel filtration column. As seen in Fig 8, material from control cultures eluted in a broad area, including a proteoglycan-enriched region (susceptible to alkaline hydrolysis), whereas material from $\beta$-d-xyloside–treated cells eluted to a lesser extent before fraction 92, with most material eluting in fractions 92 through 104, similar to free chains released by alkaline hydrolysis. In addition, material from control cultures eluting later than fraction 92 suggests that some free glycosaminoglycan chains may be...
DISCUSSION

In the present experiments, HL-60 cells cultured in the presence of 4-methylumbelliferyl-β-D-xyloside demonstrated an increased incorporation of 35S-sulfate into chondroitin sulfate glycosaminoglycans. Such increased precursor incorporation has been previously reported for neutrophils treated with β-D-xylosides. The newly formed glycosaminoglycans isolated from β-D-xyloside–treated cells appear to be linked to the β-D-xyloside rather than to protein core. In addition, gel filtration chromatography of labeled glycosaminoglycans suggested a decrease in chain length of chondroitin sulfate molecules from β-D-xyloside–treated cultures compared with controls. This increase in free glycosaminoglycan chain production and decrease in mol wt has been previously observed during β-D-xyloside treatment of chondrocytes and led other investigators to suggest that the length of glycosaminoglycan chains may be related to the availability of xylosyl acceptor, ie, with excess acceptor, more chains of shorter length are produced.

As was seen with mast cells, chondroitin sulfate of untreated and β-D-xyloside–treated HL-60 cells are distributed in the intracellular granules. This distribution is in contrast to chondrocytes, where lower mol wt chondroitin sulfate chains produced in the presence of β-D-xyloside are found exclusively in the media compartment, and it suggests that differences in glycosaminoglycan secretion exist in the different cell types. We did not see changes in the total number of primary granules, as determined by peroxidase staining. Thus, the altered glycosaminoglycan metabolism does not appear to perturb granule production, although staining with cationic dyes is altered.

Another difference between xyloside-induced glycosaminoglycan alterations in chondrocytes and in our system was the observation by Gibson et al that in chondrocytes, xyloside-initiated short chains had an inferior ability to accept sulfate. However, in the HL-60 cells treated with β-D-xyloside, the short chains showed a higher charge density. The relationship between chain extension and sulfation is complex. It has been suggested that sulfation can occur during polymerization or subsequent to polysaccharide chain formation. Our data, taken with observations of Gibson et al, suggest that molecular size is not a major determinant of the ability to accept sulfate and is in agreement with observations of Meezan and Davidson and Derge and Davidson. However, the altered degree of sulfation after β-D-xyloside chain initiation seen here and previously reported, as well as the increased ability to accept sulfate
observed after pronase treatment,\textsuperscript{37} suggest that the protein core is an important determinant of sulfate incorporation.

It has been proposed by previous observers that the rate of chondroitin sulfate synthesis in the presence of \( \beta\)-D-xyloside is a measure of the cell's total capacity for chondroitin sulfate synthesis.\textsuperscript{23,42} We have observed an increase in \( ^{35}S \)-sulfate incorporation into chondroitin sulfate produced by HL-60 cells in the presence of \( \beta\)-D-xylosides. Our results suggest that the native chondroitin sulfate chains produced by HL-60 cells are not maximally sulfated, in contrast to the all-or-nothing sulfation observed in the chick embryo cartilage system.\textsuperscript{29} Despite the increased acceptance of sulfate by these newly formed chains, no chondroitin 4,6-sulfate was produced. Because these cells have the potential to differentiate into macrophage-like cells,\textsuperscript{43} which have been associated with chondroitin 4,6-sulfate production, it appears that the 6-sulphotransferase is not present at this stage of development. This again suggests the stage-specificity of glycosaminoglycan production in leukocytes.\textsuperscript{8,14} Alternatively, the HL-60 cell line or our in vitro conditions may be responsible for our inability to detect production of glycosaminoglycans other than chondroitin 4-sulfate. Previous reports have noted the lack of other normal leukocyte functions, such as bacterial ingestion, complement secretion, hexas monophosphate shunt activity, and superoxide generation, after phorbol-induced macrophage maturation of the cells in vitro.\textsuperscript{44} Because glycosaminoglycans have been implicated in many facets of leukocyte behavior,\textsuperscript{5,12,13} and may play an important role in the regulation of hematopoiesis,\textsuperscript{45} we are currently studying effects of these specific xyloside-induced alterations in chondroitin sulfate production by HL-60 cells on subsequent maturation and function.

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