Hyposialylation of Differentiation-Inducer–Resistant HL-60 Cells

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The total sialic acid content of retinoic acid (RA)-resistant or 6-thioguanine (6TG)-resistant HL-60 cells was more than tenfold lower and of dimethylsulfoxide (DMSO)-resistant HL-60 cells was approximately twofold lower than that of parental, wild-type (wt) HL-60 cells. Neuraminidase-inaccessible, ie residual cell–associated sialic acid after neuraminidase treatment, was four- to twelvefold lower in the three differentiation-inducer–resistant sublines than in the parent line. Neuraminidase treatment of 125I-labeled surface membrane glycoproteins (SMGs) from wt HL-60 cells converted the two-dimensional gel electrophoretic pattern to one having features in common with RA- and 6TG-resistant cells. However, neuraminidase treatment did not alter the sensitivity of wt HL-60 cells to differentiation induction by RA, hypoxanthine (purine base), or DMSO. These results indicate that differences in peripheral, neuraminidase-accessible sialic acids are important determinants of the gel electrophoretic mobility of the SMGs of the HL-60 line and sublines but are not likely related to the differentiation-resistance mechanism. Further studies are required to determine if hyposialylation of cryptic, neuraminidase-inaccessible sites has functional significance.

MATERIALS AND METHODS

Cell culture lines and methods. The RA-resistant, 6TG-resistant and DMSO-resistant HL-60 sublines were derived and maintained as previously described.11,12 Differentiation induction assays with 10⁻⁴ mol/L RA for five days, and 169 mmol/L DMSO or 6 mmol/L hypoxanthine for seven days, including the monitoring of granulocytic differentiation by cytologic examination of Wright stained smears and by the nitroblue tetrazolium (NBT) dye reduction test, were as previously described.11,12 Short-term neuraminidase treatment (with Vibrio cholerae, Berhringwerke Marburg, FRG) of wt HL-60 cells prior to differentiation induction experiments (pretreatment) was performed using phosphate buffered saline (PBS)-washed cells, as described below. Continuous neuraminidase treatment involved adding 0.05 IU/mL neuraminidase to the complete tissue culture medium immediately after reestablishment in culture following neuraminidase pretreatment and replenishing with medium containing fresh neuraminidase on days 3 and 7. All biochemical procedures were performed on cells harvested from the mid-to-late-log phase of growth, when cell viability exceeded 90% by trypan blue dye exclusion.

Determination of total and neuraminidase-releasable sialic acid. Cell suspensions of approximately 10⁶ wt or variant HL-60 cells were washed three times in 5 mL PBS with 1 mg Ca²⁺ per liter (PBS-Ca²⁺; GIBCO, Grand Island, NY). For determination of total sialic acid, the washed cells were suspended in 0.5 mL of 0.1N H₂SO₄ at 80 °C for one hour to hydrolyse glycosidically-linked sialic acids and release sialic acid from intracellular sites, followed by the removal of cell debris by centrifugation at 1,000 × g for five minutes. For determination of neuraminidase-releasable sialic acid, cells were suspended in 0.5 mL of PBS-Ca²⁺, pH 7.4, containing 0.05 IU

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Vibrio cholerae neuraminidase (0.1 IU/mL) and incubated at 37 °C for 15 minutes, followed by clarification by centrifugation at 200 x g for seven minutes. Kinetic experiments demonstrated that under these conditions neuraminidase was present in considerable excess, since with the use of 0.02 IU/mL the release of sialic acid was complete in 15 minutes. The sialic acid content of the hydrolysates was directly determined by the thiobarbituric acid colorimetric assay, as modified by Roboz et al to eliminate 2-deoxyribose interference, using sialic acid from sheep submaxillary glands (Sigma, St Louis) as a standard. Digests of 2 x 10^7 cells were assayed for protein content according to the method of Bradford, using bovine serum albumin as a protein standard. Each sialic acid measurement represents the average of three independent tests. The neuraminidase-releasable sialic acid was calculated as the difference between that measured in the presence and absence of the enzyme. The statistical significance of the variations of the sialic acid determinations in the variant v wt HL-60 cells was calculated by Students t test.

SMG gel electrophoretic procedures. Two x 10^6 viable cells were surface-labeled with 1-125 by the Iodo-Gen (Pierce Chemical, Rockford, IL) method using 0.5 mCi sodium-125I (New England Nuclear, Boston) as previously detailed. The labeled and washed cells were treated with diisopropylfluorophosphate and extracted with ice-cold 2% Triton X-100 (Sigma) in PBS with Ca^2+ containing 2 mmol/L phenylmethylsulfonylfluoride (Sigma). Neuraminidase treatment was performed on the labeled, washed cells (Fig 1) or on

![Fig 1. Autoradiographs of surface proteins after 1-125 labeling and two-dimensional polyacrylamide gel electrophoresis. The first (horizontal) dimension was isoelectric focusing with an acidic pH of 4.5 on the extreme left and an alkaline pH of 8.0 on the extreme right. The second (vertical) dimension was SDS-polyacrylamide gel electrophoresis from top to bottom in which the indicated molecular weight markers correspond to 200, 116, 93, 69, and 45 kD. The nomenclature was derived from graphic displays of SMG spots from multiple radioautographic exposures as described in ref 13. Cell sources: (A), wt HL-60; (B), RA-resistant HL-60; (C), neuraminidase-treated wt HL-60.](image-url)
the Triton X-100 protein extracts at 0.1 IU/mL under conditions described in the previous section, which produced a similar, complete reaction from five to >30 minutes of treatment. The 1-125-labeled extracts were subjected to isoelectric focusing followed by polyacrylamide gel electrophoresis as described previously,\(^{16}\) calibrating the second dimension with mixed high molecular weight standard proteins (myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 92,000; bovine serum albumin, 69,000; and ovalbumin, 45,000) (Bio-Rad, Richmond, CA).

RESULTS

Quantitation of total and neuraminidase-releasable sialic acid. Both the RA- and 6TG-resistant sublines had less than 10% of the total amount of sialic acid present in wt HL-60 cells (Table 1). The amount of sialic acid in the DMSO-resistant cells was also significantly lower at approximately 45% of the wt cellular level. Qualitatively similar results were obtained by the periodate-resorcinol method,\(^{16}\) but we were unable to obtain quantitatively highly reproducible results due to the presence of variable amounts of interfering substances. After neuraminidase treatment, approximately 50% of the sialic acid residues were releasable from the wt HL-60 cells (Table 1). Relatively more sialic acid (73%) was releasable by neuraminidase-treatment of the DMSO-resistant cells. Thus, a four- to twofold higher residual amount of neuraminidase-inaccessible sialic acid was present in wt HL-60 cells than in all three variant sublines (Table 1, column 3).

Effect of neuraminidase treatment on SMGs. After 125I-labeling, extraction, and two-dimensional gel electrophoresis, the surface-labeled proteins of HL-60 cells appeared as groups of multiple-charged polypeptides and/or as fused horizontal smears of proteins, as typical of SMGs.\(^{20}\) From studies of radioautographs after different exposure times, up to 12 distinguishable SMGs could be identified in wt HL-60 cells, while only nine SMGs were distinguishable in RA-resistant cells (ref 13 and Figs 1A and B). The SMG gel electrophoretic pattern of the DMSO-resistant cells closely resembled that of wt cells, while the SMG pattern of the 6TG-resistant cells was very similar to that of the RA-resistant cells.\(^{13}\) Furthermore, four SMGs present in the RA- or 6TG-resistant cells were not observed in the wt or DMSO-resistant cells (proteins A through D, Fig 1B). Most notably, the wt or DMSO-resistant cells manifested three closely associated, prominent acidic SMGs in the 120- to 160-kD range (proteins 4, 5, and 6, Fig 1A), while the RA-/6TG-resistant cells showed a single predominant acidic SMG of >200 kD (protein A, Fig 1B). Treatment of 125I-labeled wild-type cells with Vibrio cholerae neuraminidase, converted the SMG gel electrophoretic pattern to one with features resembling that of the RA- or 6TG-resistant cells (Fig 1C). This included the following notable changes: (1) the loss or shift of the three acidic 120- to 160-kD proteins (proteins 4, 5, and 6); (2) the appearance of a prominent higher apparent molecular weight acidic SMG (170- to 120-kD), which resembles protein A but was of significantly less apparent molecular mass; (3) the appearance of a prominent 130- to 150-kD SMG with intermediate isoelectric mobility (protein D); (4) a marked increase in intensity of a 115- to 125-kD, pI 6.0-7.0 SMG (protein 7); and (5) a basic shift of the prominent 85- to 95-kD, pI 6-7 SMG, previously identified as the transferrin receptor in both wt and RA-resistant HL-60 cells (protein 8, Ref 13). Similar results were obtained whether neuraminidase treatment was performed before or after detergent extraction of the SMGs.

Tests of neuraminidase treatment for effects on growth and differentiation of wt HL-60 cells. The above results raised the possibilities that the hyposialylation and/or altered SMGs of the RA-resistant and 6TG-resistant cells could be mechanistically related to their differentiation resistance and that neuraminidase treatment of the wt HL-60 cells might reduce their sensitivity to differentiation stimulators. We demonstrated by direct sialic acid assay or by the binding of the lectin peanut agglutinin, which binds only to hyposialylated HL-60 cells\(^{21}\) (and Gallagher et al, manuscript in preparation), that wt HL-60 cells continuously treated with neuraminidase (see MATERIALS and METHODS) were persistently hyposialylated, the lowest recorded level being 6.1 μmol/g protein (compare with neuraminidase-inaccessible values in Table 1). Such treatment did not affect cell growth rate or the overall level of spontaneous differentiation (data not shown). In several dose-response experiments comparing untreated and acutely or continuously neuraminidase-treated cells, no loss of granulocytic differentiation responsiveness to RA (representatively shown in Fig 2), hypoxanthine, or DMSO was detected, as determined by cytologic evaluation of Wright stains or by the NBT test. The cytologic appearance of the induced HL-60 neutrophilic granulocytes was not discernibly different in the untreated and neuraminidase-treated cells.

DISCUSSION

The principle finding of this study is that differentiation-inducer resistant HL-60 cells are hyposialylated compared to wt HL-60 cells. This finding is relevant to issues which we previously raised about these cultured cells.\(^{13}\) First, it can at least partly account for the differences in SMG gel electrophoretic profiles between the RA-resistant and 6TG-resistant HL-60 cells compared to wt cells, since neuraminidase treatment of wt cells produced a SMG gel profile with features of the variant SMG pattern (Fig 1). In this context,
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Fig 2. Lack of effect of neuraminidase treatment on the growth and granulocytic differentiation of wt HL-60 cells in the absence and presence of various concentrations of RA. Cells were grown for 4 weeks in the continuous presence of neuraminidase added freshly two times per week to a final concentration of 0.05 IU/mL and at the initiation of the five-day induction period with retinoic acid. (A) Percent of control cell count, ie percent of growth of RA-treated cells to growth of control cells in the absence of RA treatment; (B) percentage of cells positive by the NBT test; (C) percentage of mature neutrophils without neuraminidase treatment; (X) with neuraminidase treatment.

Panel A: Percentage mature neutrophils without neuraminidase treatment; (x) with neuraminidase treatment.

Panel C: O, percentage mature neutrophils without neuraminidase treatment; x, percentage of mature neutrophils with neuraminidase treatment.

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