Treatment of HL-60 cells with dimethyl sulphoxide inhibits the formation of β -N-acetylhexosaminidase S

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β -N-Acetylhexosaminidase of HL-60 cells was separated into two main forms, A and S, by chromatography on DEAE-cellulose. Analysis of developmental changes in the isoenzyme pattern was complicated by the fact that the specific activity of β -N-acetylhexosaminidase underwent a 6-fold change during the normal growth cycle. Two other lysosomal enzymes, β -galactosidase and α -mannosidase, behaved similarly. Induction of differentiation of HL-60 cells with dimethyl sulphoxide at a low cell density (3×10^5 cells/ml) had a greater effect on the abundance of α -subunits of β -N-acetylhexosaminidase, measured with 4-methylumbelliferyl- β -N-acetylglucosaminide 6-sulphate, than of β -subunits, measured with 4-methylumbelliferyl- β -N-acetylglucosamine, and resulted in an isoenzyme profile in which A and B were the major forms, with the levels of form S greatly decreased.

INTRODUCTION

The products of the *HEX A* and *HEX B* genes associate to form β -N-acetylhexosaminidase A, an $\alpha\beta$ dimer. Normal human tissues also contain the $\beta\beta$ dimer, β -N-acetylhexosaminidase B, but the $\alpha\alpha$ dimer (β -N-acetylhexosaminidase S) is present in very small amounts (see Neufeld, 1989, for a review). The proportions of these forms are due at least in part to the relative levels of production of the two subunits. This can be seen in extreme forms in Tay–Sachs disease, where loss of α -subunits results in the formation of elevated amounts of β -N-acetylhexosaminidase B, and in Sandhoff's disease, where mutations of *HEX B* result in the increased formation of β -N-acetylhexosaminidase S.

In their survey of glycosidases in lymphoblastoid cells, Dreyfus *et al.* (1984) demonstrated that an unexpectedly high proportion of cell types had severely decreased β -N-acetylhexosaminidase activities. The β -N-acetylhexosaminidase isoenzyme pattern in four out of 25 cell lines resembled that of the residual activity in Sandhoff's disease, in that the major isoenzyme form detected on Cellophor electrophoresis appeared to be β -N-acetylhexosaminidase S. Similar observations were made by Swallow *et al.* (1977). Dreyfus *et al.* (1984) were of the opinion that such a high frequency of the Sandhoff's disease phenotype was unlikely to be due to mutations of the gene for the β -N-acetylhexosaminidase β -subunit because they occur so rarely in the population, and these authors suggested that the expression of the *HEX B* gene may be down-regulated in these cells. A number of lymphoblastoid cell lines had less severely altered proportions of β -N-acetylhexosaminidase isoenzymes, and in these too the pattern of isoenzyme forms was consistent with down-regulation of *HEX B* and the consequent formation of the dimer of α -subunits, β -N-acetylhexosaminidase S.

HL-60 is a promyelocytic cell line that is able to proliferate *in vitro* without changes in its state of differentiation (Collins *et al.*, 1977). Differentiation along the pathway towards granulocytes can be induced by treating the cells in culture with dimethyl sulphoxide (DMSO), which causes a number of morphological changes characteristic of mature granulocytes to take place and

the cells acquire the capacity for physiological phagocytosis (Collins *et al.*, 1978). We have shown that HL-60 cells contain very little β -N-acetylhexosaminidase B and that, in addition to β -N-acetylhexosaminidase A, a high proportion of the total activity is β -N-acetylhexosaminidase S (Emiliani *et al.*, 1990a).

In this paper we describe the changes of β -N-acetylhexosaminidase activity during the growth cycle of HL-60 cells and examine the effects of DMSO on the isoenzyme profiles. A decrease in the abundance of β -N-acetylhexosaminidase S and the increase in β -N-acetylhexosaminidase B are likely to result from lowered production of α -subunits rather than increased expression of β -subunits.

EXPERIMENTAL

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium and fetal calf serum were obtained from Gibco Europe, Breda, Netherlands; Miranol H2M was from Miranol Chemical Co., Irvington, NJ, U.S.A.; 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside, 4-methylumbelliferyl α -D-mannoside and 4-methylumbelliferyl β -D-galactoside were from Sigma Chemical Co., St. Louis, MO, U.S.A., and 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside 6-sulphate was from the HSC Research Corporation, Toronto, Canada. The Bio-Rad protein assay and bovine γ -globulin were from Bio-Rad Laboratories, Richmond, CA, U.S.A.; DEAE-cellulose was from Whatman Biochemicals, Maidstone, Kent, U.K. All other reagents were of analytical grade.

Cell culture

The HL-60 cell line was cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum in 75 cm² culture flasks in a humidified atmosphere containing 5% CO₂. Treatment of cells with DMSO was performed as follows. Cells were sub-cultured in fresh medium (2×10^5 cells/ml) and after 24 h, when the cell concentration was 3×10^5 cells/ml, DMSO

Abbreviations used: 4-MUGlcNAc, 4-methylumbelliferyl- β -N-acetylglucosamine; 4-MUGlcNAc-6-SO₄, 4-methylumbelliferyl- β -N-acetylglucosaminide 6-sulphate; DMSO, dimethyl sulphoxide.

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was added to give a final concentration of 1.25% (v/v). Cells were harvested after 72 h of exposure to DMSO and control cells were collected at 24 h and 96 h after sub-culture. The viability of cells was estimated by examining their ability to exclude Trypan Blue (0.1% in 0.9% NaCl).

Preparation of cell lysates and enzyme extraction

Cell samples (2×10^7) were washed three times with 2 ml of 0.9% NaCl (1000 rev./min, r_{av} 10.5 cm, for 10 min in an MSE benchtop centrifuge) and suspended in 1 ml of 10 mM-sodium phosphate buffer, pH 6.0, and 0.1% (v/v) Miranol H2M. They were then sonicated using a 100 W MSE ultrasonic disintegrator equipped with a 'microtip' at 12 μ m wave amplitude (four sonications; 15 s each) followed by centrifugation at 36000 g (r_{av} 8.19 cm) for 20 min. All procedures were carried out at 4 °C. The supernatants were used as cell lysates.

Enzyme assays

β -N-Acetylhexosaminidase activity was measured as described by Beccari *et al.* (1987) using 3 mM-4-methylumbelliferyl- β -N-acetylglucosamine (4-MUGlcNAc) or 0.3 mM-4-methylumbelliferyl- β -N-acetylglucosaminide 6-sulphate (4-MUGlcNAc-6-SO₄) in 0.1 M-citrate/0.2 M-phosphate buffer, pH 4.5. α -Mannosidase and β -galactosidase were both assayed as above with 3 mM solutions of the appropriate 4-methylumbelliferyl glycosides, except that α -mannosidase was assayed at pH 4.0. Fluorescence of the liberated 4-methylumbelliferone was measured on a Perkin-Elmer LS-3 fluorimeter (excitation 360 nm, emission 446 nm).

One unit is the amount of enzyme that hydrolyses 1 μ mol of substrate/min at 37 °C. Lactate dehydrogenase was assayed using reagents and methods supplied by Eurochima div. Elvi Spa, Milano, Italy. Protein was measured by the method of Bradford (1976) using crystalline bovine γ -globulin as a standard. Specific activity was expressed as units/mg of protein.

DEAE-cellulose chromatography

DEAE-cellulose chromatography was performed according to Robinson & Stirling (1968) using a 1 ml column equilibrated with 10 mM-sodium phosphate buffer, pH 6.0. Enzyme activity retained by the column was eluted in a linear gradient of NaCl that reached a concentration of 0.5 M in 50 ml of buffer. Fractions (0.5 ml) were collected and assayed for activity towards the substrates 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄.

RESULTS

Changes in β -N-acetylhexosaminidase and other glycosidase activities during the growth of HL-60 cells in culture

During the growth of HL-60 cells (Fig. 1a) the total intracellular activity of β -N-acetylhexosaminidase showed a number of remarkable changes. These were detected with both 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄, although the changes were more exaggerated with the latter substrate. When HL-60 cells, harvested at a density of 2×10^6 /ml, were resuspended in fresh medium at a density of 2.5×10^5 /ml, there was a rapid and sustained decrease in the activity per cell which reached its lowest point after 6–8 h. Specific activities of β -N-acetylhexosaminidase decreased in this time from 16.6 munits/mg of protein to 2.5 munits/mg of protein when assayed with 4-MUGlcNAc. In marked contrast to this behaviour, HL-60 cells resuspended in fresh medium at the same density as when they were harvested (2×10^6 cells/ml) showed no decrease in β -N-acetylhexosaminidase activity.

After 12 h the cells began to grow exponentially and there was a gradual increase in the activity per cell that was sustained until

Fig. 1. Intracellular content of lysosomal enzymes in HL-60 cells during growth

(a) Cells were sub-cultured in fresh culture medium at a density of 2.5×10^5 cells/ml. Samples of growth medium containing HL-60 cells were harvested at the times indicated and counted; extracts were assayed for β -N-acetylhexosaminidase activity towards 4-MUGlcNAc (●) and 4-MUGlcNAc-6-SO₄ (○). (b) Intracellular contents of β -N-acetylhexosaminidase (○), α -mannosidase (●), β -galactosidase (■) and lactate dehydrogenase (▲). The activity of each enzyme in HL-60 cell extracts is expressed as a percentage of the activity found in the cells at 0 h.

72 h. Thereafter the rate of accumulation of β -N-acetylhexosaminidase increased and maximal activities per cell were reached after 120 h. At this stage of growth the specific activities were 15 and 2 munits/mg of protein for the activities measured with 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄ respectively. On entering the stationary phase there was a decline in the β -N-acetylhexosaminidase activity measured with both substrates. More than 99% of cells were viable throughout growth. At 216 h cell viability was 98%, but it had declined to only 30% by 264 h.

β -N-Acetylhexosaminidase activity accumulated in the medium during growth, and the rate of accumulation was greatest between 96 and 120 h. It was noticeable that the initial rapid loss of intracellular activity following sub-culture was not accompanied by a corresponding increase in the extracellular activity. After 144 h the activity in the medium declined steadily (results not shown).

β -N-Acetylhexosaminidase was not the only lysosomal glycosidase to show changes in specific activity during the growth of HL-60 cells. Results for α -mannosidase and β -galactosidase are

Fig. 2. Separation of β -N-acetylhexosaminidase from HL-60 cells by DEAE-cellulose chromatography

HL-60 cells were harvested after 24 h in culture when the cell density was 3×10^5 cells/ml. An extract was made and applied to a DEAE-cellulose column equilibrated with 10 mM-sodium phosphate buffer, pH 6.0. Enzyme activity retained by the column was eluted in a linear gradient of NaCl that reached a concentration of 0.5 M in 50 ml of buffer. Fractions (0.5 ml) were assayed for activity towards 4-MUGlcNAc (●) and 4-MUGlcNAc-6-SO₄ (○).

shown in Fig. 1(b) and closely follow the gross changes observed with β -N-acetylhexosaminidase. There was a considerable difference between the behaviour of these lysosomal enzymes and that of the cytosolic enzyme lactate dehydrogenase, which after an initial increase showed virtually no change in specific activity over 144 h.

Separation of β -N-acetylhexosaminidases from HL-60 cells by DEAE-cellulose chromatography

When extracts of HL-60 cells (harvested after 24 h in culture when the cell density was 3×10^5 /ml) were applied to columns of DEAE-cellulose and the fractions assayed with 4-MUGlcNAc, there was a small unretained peak of β -N-acetylhexosaminidase B which accounted for less than 1% of applied activity. Three further peaks of activity were eluted by the salt gradient (Fig. 2). The first of these, β -N-acetylhexosaminidase I, represented 13%, the second, β -N-acetylhexosaminidase A, represented 50% and the third, β -N-acetylhexosaminidase S, represented 25% of the applied activity. Assay with 4-MUGlcNAc-6-SO₄ detected only the peaks in the salt gradient, β -N-acetylhexosaminidase A representing 17% and β -N-acetylhexosaminidase S 35% of the applied activity. There was an overall recovery of 88% when β -N-acetylhexosaminidase was assayed with 4-MUGlcNAc and 56% with 4-MUGlcNAc-6-SO₄.

Effect of DMSO on the growth rate of HL-60 cells

Inclusion of 1.25% DMSO in the culture medium of HL-60 cells had little effect on their growth rate, and the cell concentrations were 1.65×10^6 and 1.42×10^6 cells/ml for the control and DMSO-treated cells respectively after 72 h of treatment.

Effect of DMSO on the β -N-acetylhexosaminidases of HL-60 cells during growth

β -N-Acetylhexosaminidase isoenzyme profiles from HL-60 cells grown in the absence of DMSO and in the presence of 1.25% DMSO are shown in Figs. 3(a) and 3(b) respectively. Two main effects of DMSO treatment were observed. The β -N-acetylhexosaminidase B peak was very greatly increased, to 17%

Fig. 3. β -N-Acetylhexosaminidase in HL-60 cells grown in the absence (a) and presence (b) of DMSO

Cells were sub-cultured in fresh medium (2.5×10^5 cells/ml) for 24 h and either were left to grow without any additions to the medium, or had DMSO added to a final concentration of 1.25% (v/v). Treated and untreated cells were harvested after a further 72 h. Cell extracts were analysed for β -N-acetylhexosaminidase isoenzymes using DEAE-cellulose chromatography described in the legend to Fig. 2: ●, activity towards 4-MUGlcNAc; ○, activity towards 4-MUGlcNAc-6-SO₄.

of the total activity measured with 4-MUGlcNAc, and the β -N-acetylhexosaminidase S peak was decreased to less than 25% of its abundance in the untreated control (Fig. 3a). The effect of DMSO treatment on β -N-acetylhexosaminidase S was most noticeable when assayed with the sulphated substrate (Fig. 3b). A small peak of β -N-acetylhexosaminidase I, which was present after 24 h of growth (Fig. 2), persisted in the DMSO-treated cells (Fig. 3b), but not in the controls (Fig. 3a).

Effect of DMSO on β -N-acetylhexosaminidases of HL-60 cells approaching the stationary phase

HL-60 cells at high density (1.5×10^6 cells/ml) when treated with DMSO for a period of 72 h showed no alterations to the β -N-acetylhexosaminidase profiles.

Specific activity of β -N-acetylhexosaminidase in HL-60 cells treated with DMSO

Comparison of the specific activities of β -N-acetylhexosaminidase of HL-60 cells treated with DMSO during growth and

Table 1. Effect of DMSO treatment on β -N-acetylhexosaminidase activities of HL-60 cells

Activities were measured with 3 mM-4-MUGlcNAc and 0.3 mM-4-MUGlcNAc-6-SO₄ as described in the Experimental section. Values are means $\pm$ S.D. of the numbers of experiments in parentheses.

Time in culture (h)	Activity (munits/10 ⁶ cells)		
	4-MUGlcNAc (a)	4-MUGlcNAc-6-SO ₄ (b)	(b)/(a)
24 (4)	0.65 $\pm$ 0.31	0.052 $\pm$ 0.037	0.08
96 (-DMSO) (4)	1.53 $\pm$ 0.22	0.190 $\pm$ 0.027	0.12
96 (+DMSO) (3)	1.29 $\pm$ 0.25	0.100 $\pm$ 0.019	0.07

their controls was made using 4-MUGlcNAc and 4-MUGlcNAc-6-SO₄ as substrates (Table 1). There was little difference between these activities when assayed with the non-sulphated substrate, but the specific activity of DMSO-treated cells was approximately half that of the controls when measured with 4-MUGlcNAc-6-SO₄ as substrate. Stationary-phase cells exposed to DMSO for 72 h did not show this effect, thereby eliminating the possibility that the presence of DMSO *per se* caused the decrease of activity towards 4-MUGlcNAc-6-SO₄.

DISCUSSION

Much of the interest in the lysosomal enzymes of human cells in culture has derived from their importance in the analysis of genetic diseases. Study of the behaviour of these enzymes in fibroblasts and amniotic fluid cells during growth has been necessary to establish reliable methods for diagnosis of lysosomal storage diseases (Galjaard *et al.*, 1974; Young *et al.*, 1975).

In lower eukaryotes, however, glycosidases and particularly β -N-acetylhexosaminidases have been studied in the context of differentiation, with substantial increases in specific activity accompanying the transition from growth to differentiation in *Dictyostelium discoideum* (Loomis, 1975) and *Physarum polycephalum* (Cook *et al.*, 1983). HL-60 is a human cell line widely used for analysing cellular and molecular events during the induction of myeloid differentiation (Collins, 1987), and we were interested to find that it expressed an 'extra' form of β -N-acetylhexosaminidase which is lacking in normal cells and is present in only small amounts in a variety of leukaemic cell lines, although it is the major form in some (Dreyfus *et al.*, 1984). We wondered whether this 'extra' form, which we now know to be β -N-acetylhexosaminidase S (Emiliani *et al.*, 1990a,b), was expressed throughout growth and persisted in the developmental end-products of myeloid cell differentiation.

Sub-culturing of HL-60 cells caused a rapid decrease in the specific activities of β -N-acetylhexosaminidase and two other lysosomal enzymes. This was not due to losses incurred during centrifugation or washing prior to suspension in fresh medium, and did not seem to be due to release of activity into the medium. The decrease was a progressive one that took place over 6–8 h and contrasted with the behaviour of lactate dehydrogenase, which maintained its specific activity throughout growth. Myeloperoxidase (Olsson & Olofsson, 1981; Hur *et al.*, 1989), non-specific granulocyte collagenase, lysozyme and chymotrypsin-like cationic protein behave similarly to lactate dehydrogenase in this respect (Olsson & Olofsson, 1981).

During normal growth between 24 and 96 h (Figs. 2 and 3) there were proportionate increases in both β -N-acetylhexosaminidases A and S, but in the presence of DMSO there were two changes in the DEAE-cellulose elution profile of β -N-

acetylhexosaminidase when assayed with 4-MUGlcNAc. There was a substantial increase in the proportion of the total activity contributed by β -N-acetylhexosaminidase B, and a decrease in that contributed by the S form. The increase in β -N-acetylhexosaminidase B might be due to an increase in the rate of synthesis of β -subunits relative to α -subunits, or conversely to a decreased rate of synthesis of α -subunits relative to β -subunits. 4-MUGlcNAc is hydrolysed by both α - and β -subunits, but the contribution of the α -subunit must be very small in comparison with that of the β -subunit as the specific activity of β -N-acetylhexosaminidase B ($\beta\beta$) is nearly twice that of β -N-acetylhexosaminidase A ($\alpha\beta$) (Lee & Yoshida, 1976; Sandhoff *et al.*, 1977). Total activities of β -N-acetylhexosaminidase measured with 4-MUGlcNAc largely reflect the activities of β -subunits, and because these activities are not altered appreciably by DMSO treatment, there is no reason to suppose that the rates of β -subunit production are affected.

4-MUGlcNAc-6-SO₄ is hydrolysed by α -subunits, and there is practically no hydrolysis of this substrate by β -subunits (Kytzia & Sandhoff, 1985). Treatment of HL-60 cells with DMSO had a profound effect on 4-MUGlcNAc-6-SO₄ hydrolysis, and this is consistent with a decreased rate of production of α -subunits. There would be two consequences of this for the isoenzyme profile. Availability of α -subunit would become limiting, so that the $\alpha\alpha$ dimer (β -N-acetylhexosaminidase S) would no longer be formed and the β -subunits, now being produced in excess, would form the $\beta\beta$ dimer (β -N-acetylhexosaminidase B), just as they do in normal tissues. Residual β -N-acetylhexosaminidase S in the cells after 72 h of treatment with DMSO was decreased by about 4-fold relative to the activity of this form in the control cells after this time, and this is the proportion expected from dilution of the cells in the inoculum with growing cells not producing β -N-acetylhexosaminidase S. Such dilution can only take place in a growing culture and it is therefore not surprising that DMSO treatment had no effect on the β -N-acetylhexosaminidase profiles of cells at high density in which the growth rate is very low.

In our characterization of the 'extra' form of β -N-acetylhexosaminidase in HL-60 cells, we concluded that it has properties very similar to those of β -N-acetylhexosaminidase S. The two enzymes differ, however, in an important respect. While β -N-acetylhexosaminidase S is abundant in HL-60 cells, in the more extreme case of β -subunit limitation, i.e. Sandhoff's disease, the residual β -N-acetylhexosaminidase S, at least in fibroblasts, does not seem to be stable. It may be that this reflects differences in the proteolytic capacities of the two cell types, but the possibility remains that post-translational modification of α -subunits in HL-60 cells may render them less susceptible to proteolytic attack.

DMSO is known to affect a variety of biochemical functions in HL-60 cells, including increases in protein kinase C and CMP-N-acetylneuraminic acid: lactosylceramide sialyltransferase (Xia *et al.*, 1989), but its effects on development are best documented in mouse erythroleukaemia cells. Here, treatment with DMSO results in an increased rate of transcription of the β -globin gene and down-regulation of the expression of *c-myc* (Lachman & Skoultschi, 1984; Ganguly *et al.*, 1986; Coppola *et al.*, 1989). It remains to be established whether the effect of DMSO on β -N-acetylhexosaminidases of HL-60 cells is due to down-regulation of the expression of α -subunits or to a decrease in their stability resulting from an alteration of post-translational modification. Experiments reported here do not address the mechanism of the effect we describe, which will require the use of α -chain- and β -chain-specific antisera and DNA probes. It is, nevertheless, very clear that induction of differentiation of HL-60 cells with DMSO restores the β -N-acetylhexosaminidase isoenzyme profile to one that more closely resembles that of normal differentiated cells.

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