Regulation of $O$-acetylation of sialic acids by sialate-$O$-acytetyltransferase and sialate-$O$-acetylesterase activities in childhood acute lymphoblastic leukemia

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Enhanced expression of 9-$O$-acytetylated sialoglycoproteins (Neu5,9Ac$_2$GPs) and 9-$O$-acytetylated disialoganglioside (9-OAcGD3) was observed on lymphoblasts of childhood acute lymphoblastic leukemia (ALL). Sialate-$O$-acytetyltransferase (SOAT) and sialate-$O$-acetylesterase (SIAE) are the two main enzymes responsible for the quantity of the $O$-acytety ester groups on sialic acids (Sias). We have earlier shown an enhanced level of SOAT activity, capable of transferring acetyl groups to Sias of glycoconjugates in the microsomes of lymphoblasts of these children. We further observed a decreased SIAE activity in both lysosomal and cytosolic fractions of ALL cell lines and primary cells from bone marrow of patients compared with peripheral blood mononuclear cells from healthy donors, which preferentially hydrolyze $O$-acytetyl groups at C-9 of Sia. The level of $O$-acytetylated Sias in the cytosolic and the lysosomal fractions showed a good correlation with SIAE activity in the corresponding fractions. The apparent $K_M$ values for SIAE in the lysosomal and the cytosolic fractions from lymphoblasts of ALL patients are 0.38 and 0.39 mM, respectively. These studies demonstrate that both SIAE and SOAT activities seem to be responsible for the enhanced level of Neu5,9Ac$_2$ in lymphoblasts, which is a hallmark in ALL. This was subsequently confirmed by using an enzyme-linked immunosorbent assay that also demonstrated a steady decline in SOAT activities even in cell lysates of lymphoblasts during successful chemotheraphy, like radioactive methods have shown earlier.

Keywords: acute lymphoblastic leukemia (ALL) / lymphoblast / 9-$O$-acytetylated sialic acid / sialate-$O$-acetylesterase / sialate-$O$-acytetyltransferase

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant transformation of lymphoblasts and commonly observed in children. Existing chemotherapy regimens are able to cure ~80% of the patients in Western countries. In contrast, the survival is far from being satisfactory in many underdeveloped countries including India (Pui et al. 2008). The relapse rate is also high due to the presence of existing, yet undetectable, lymphoblasts often referred as minimal residual disease (MRD) or to cancer stem cells. The mandatory technical expertise needed for successful search of MRD, in clinical set up, is restricted. Therefore, the identification of new biomarkers for consistent monitoring individual chemotherapeutic response and predicting impending relapse is an urgent need (Marshall et al. 2003).

The $O$-acytetylation of sialic acid (Sia) side chain, at C-7, C-8 and C-9, is found frequently on glycoproteins or glycolipids in all higher vertebrates and in a few bacteria. Enhanced expression of $O$-acytetylated Sias was reported in childhood ALL (Sinha et al. 1999; Mandal et al. 2000), skin melanoma (Ravindranaths et al. 1988; Ritter et al. 1990), basaloma (Fahr and Schauer 2001) and human breast cancer (Marquina et al. 1996). Alternatively, decreased $O$-acytetylation of Sias in human colon carcinoma was observed (Corfield et al. 1999). The expression of $O$-acytetylated Sias on glycoproteins or glycolipids is cell type-specific, developmentally regulated and plays a key role in modulating immune reactions, cell signaling, growth, differentiation, cell–cell adhesion, virus–cell adhesion, apoptosis and malignancy (Kelin and Roussel 1998; Schauer 2000, 2004; Schauer et al. 2011; Angata and Varki 2002; Malisan et al. 2002; Erdmann et al. 2006; Mukherjee et al. 2007, 2011; Wipfiter et al. 2011).

The key enzyme, responsible for such modifications, is the specific sialate-$O$-acytetyltransferase (SOAT) generally located in the Golgi apparatus (Schauer 2004). The ester groups are primarily incorporated into the C-7 of Sia from where they migrate to C-9. The presence of $O$-acytetylated Sias in the bovine submandibular gland (Vandamme-Feldhaus and Schauer 1998; Lrhorfi et al. 2007), livers of rat (Higa et al. 1987; Butor et al.

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Sialate-Ö-acetylesterases (SIAEs) are another important enzymes in Sia metabolism. They hydrolyze 4- or 9-Ö-acetyl groups of either glycosidically linked or free Sias released from glycoconjugates by sialidases (Schauer 2000, 2004; Angata and Varki 2002). Two forms of 9-Ö-acetyl-SIAE, one in the cytoplasm and the other in the lysosomal compartment (i.e. membrane-bound), were reported in mammals (Higa et al. 1987). While lysosomal SIAE is thought to be responsible for the removal of 9-Ö-acetyl groups of the Sias of sialoglycoconjugates, the cytosolic SIAE may be engaged in de-Ö-acetylation of cytosolic 9-Ö-acetylated Sias. Nevertheless, the main function of this cytosolic SIAE has yet to be investigated (Higa et al. 1987; Shen, Kohla, et al. 2004; Schauer and Shukla 2008). SIAE activity was documented in bacterial strains and fecal extracts from normal individuals and patients with human colorectal carcinoma (Shen, Kohla, et al. 2004). This enzyme negatively regulates B lymphocyte antigen receptor signaling which is required for the maintenance of immunological tolerance in mice (Surolia et al. 2010). To the best of our knowledge, SIAE activity has not been reported in childhood ALL. Considering the enhanced presence of 9-Ö-acetylated Sias on both glycolipids (Mukherjee et al. 2008) and glycoproteins (Sinha et al. 1999; Mandal et al. 2000; Pal, Ghosh, Bandhyopadhyay, et al. 2004; Pal, Ghosh, Mandal, et al. 2004) on lymphoblasts of ALL and their role in survival (Ghosh et al. 2007; Mukherjee et al. 2008), understanding of the regulation of this enzyme is important.

We have demonstrated enhanced SOAT activity in ALL, which is possibly responsible for the increased expression of Neu5,9Ac2-GPs (Mandal et al. 2009). Therefore, it may be envisaged that, in disease condition, the relative activities of the complementary enzymes SOAT and SIAE play the crucial role for the enhanced expression of Neu5,9Ac2-GPs. Accordingly, we have measured SIAE activity both in lysosomal and cytosolic fractions of these lymphoblasts, which preferentially hydrolyze the 9-Ö-acetyl group on Sia in ALL. Subsequently, the level of 9-Ö-acetylated Sias in the cytosolic and lysosomal fractions was determined, which showed a good correlation with SIAE activity in the corresponding fractions. Additionally, we also monitored the SOAT activity using a non-radioactive enzyme-linked immunosorbent assay (ELISA). This assay represents a potent tool for such measurements even in the cell lysate of lymphoblasts and therefore is useful for the monitoring the disease progression in ALL. This study demonstrates the contribution of both SIAE and SOAT activities for the enhanced level of Neu5,9Ac2 in lymphoblasts.

Results
Reduced 9-Ö-acetylesterase activity in lysosomal and cytosolic fractions of ALL cells compared with peripheral blood mononuclear cell from normal healthy individuals

The 9-Ö-acetylesterases activity in the cytosolic fractions of representative B-ALL (REH) and T-ALL (CEMC7, MOLT3 and MOLT4) cells were 266 ± 15, 231 ± 18, 287 ± 21 and 234 ± 17 pmol/min × mg protein, respectively, in contrast to normal human peripheral blood mononuclear cell (PBMCN; 754 ± 52) using 4-methylumbelliferyl acetate (MUAc) as a substrate (Figure 1A). Reduced 9-Ö-acetylesterase activity were also observed in the lysosomal fractions of REH (404 ± 35), CEMC7 (385 ± 23), MOLT3 (390 ± 28) and MOLT4 (427 ± 33) pmol/min × mg proteins compared with PBMCN, being 698 ± 45 pmol/min × mg protein.

Similarly, the 9-Ö-acetylesterase activity in the cytosolic fraction of lymphoblasts from ALL patients, at the presentation of disease, was 0.33-fold lower, being 247 ± 22 pmol/min × mg protein than in the cytosol from PBMCN. The 9-Ö-acetylesterase activity lysosomal fractions of these lymphoblasts were also 0.53-fold decreased being 403 ± 26 pmol/min × mg protein when compared with PBMCN (Figure 1A). These results suggest that at least two 9-Ö-acetylesterases exist according to the localization, in the lymphoblasts and PBMCN, respectively, i.e. one soluble form localized in the cytosol and another membrane-associated form in the lysosomes.

Genetic expression of lysosomal and cytosolic SIAEs

This enzyme which removes 9-Ö-acetylation from Sias is encoded by a gene localized in chromosome 11q24 (Guimaraes et al. 1996). Multiple transcript variants of this gene encode different isoforms found either in the lysosome or in the cytosol. Variant 1 represents the shorter transcript but encodes the longer protein (isoform 1), referred as the lysosomal form of the protein. Variant 2 differs in the 5′-UTR and coding region compared with transcript variant 1. The resulting protein (isoform 2) has a distinct N-terminus and also referred as the cytosolic form of the protein. Accordingly, we have checked the status of these two isoforms of the SIAE gene of lysosomal and cytosolic localization. The genetic expression of lysosomal and cytosolic forms of SIAE was significantly down-regulated in both lymphoblasts of ALL patients and ALL cell line in comparison to PBMCN as analyzed by semi-quantitative RT-PCR using specific primers for both SIAE genes (Figure 1B). Real-time PCR analysis revealed ~75-fold down-regulation of lysosomal SIAE gene expression in lymphoblast of patients compared with PBMCN (Figure 1C). Similar analysis revealed ~5000-fold down-regulation of the cytosolic SIAE of lymphoblasts compared with PBMCN.

The $V_{max}$ of 9-Ö-acetylesterase reaction is low in lymphoblasts of ALL compared with PBMCN

Hydrolysis of the acetyl group by lysosomal fractions from CEMC7 cells showed an apparent $K_M$ of 55.42 ± 1.4 μM with a $V_{max}$ of 705 ± 38 pmol/min × mg protein using MUAc as a substrate. Lysosomal fractions from lymphoblasts of ALL patients demonstrated an apparent $K_M$ of 62.67 ± 2.3 μM with a $V_{max}$ of 770 ± 35 pmol/min × mg protein (Figure 2A and B). In contrast, an apparent $K_M$ of 46.3 ± 1.3 μM with $V_{max}$ being 923 ± 50 pmol/min × mg protein was observed with the lysosomal fraction from PBMCN.

In parallel, the hydrolysis of acetyl groups by the cytosolic fraction from the CEMC7 cells and lymphoblasts from ALL
patients showed an apparent $K_M$ of $59.4 \pm 1.6 \mu M$ with a $V_{\text{max}}$ of $514 \pm 29 \text{ pmol/min} \times \text{mg protein}$ and $64.42 \pm 2.0 \mu M (K_M)$ with $590 \pm 30 \text{ pmol/min} \times \text{mg protein} (V_{\text{max}})$, respectively (Figure 2C and D). The corresponding values for PBMCN were $43.8 \pm 1.1 \mu M (K_M)$ and $1035 \pm 49 \text{ pmol/min} \times \text{mg protein} (V_{\text{max}})$.

Reduced hydrolysis of the O-acetyl group from Neu5,9Ac$_2$ by lysosomal and cytosolic SIAEs of lymphoblasts compared with PBMCN

Equal amounts of the Neu5,9Ac$_2$-enriched fraction were incubated with the lysosomal fraction of CEMC7 cells (Figure 3A and B) and PBMCN (Figure 3C and D). The peak corresponding to Neu5,9Ac$_2$ was decreased with a corresponding increase in the N-acetylneuraminic acid (Neu5Ac) peak in both cases. The kinetics of hydrolysis of O-acetyl groups by SIAE present in this fraction of lymphoblasts of patients revealed an apparent $K_M$ of $0.38 \text{ mM}$ (Figure 3E). However, the percent of hydrolysis of O-acetyl groups from Neu5,9Ac$_2$ was higher with PBMCN ($70 \pm 6\%$) compared with that from primary cells from patients ($46 \pm 4\%$) and CEMC7 ($40 \pm 3\%$) cells (Figure 3F).

A similar trend was observed for cytosolic enzyme in CEMC7 cells (Figure 4A and B) and PBMCN (Figure 4C and D). The percent of hydrolysis of Neu5,9Ac$_2$ by cytosolic fractions from PBMCN ($85 \pm 6\%$) was also more when compared with lymphoblasts of ALL patients and CEMC7, being $39 \pm 5$ and $38 \pm 3\%$, respectively (Figure 4F). The apparent $K_M$ for the hydrolysis of acetyl groups by this fraction of lymphoblasts of patients was $0.39 \text{ mM}$ (Figure 4E).

The relative increase in the O-acetylated Sia level in CEMC7 compared with PBMCN

The predominant types of glycoconjugate-bound Sias were Neu5Ac and Neu5,9Ac$_2$ in the lysosomal and cytosolic fractions of CEMC7 cells and PBMCN as determined by fluorimetric high-performance liquid chromatography (HPLC). A slight reduction in Neu5Ac and increased Neu5,9Ac$_2$ was observed in both the fractions of CEMC7 and lymphoblasts compared with PBMCN (Figure 5B–G). The data were normalized by the equal amount of protein. We used bovine submandibular gland mucin (BSM) as a source of standard Sias for the confirmation of the nature of Sia derivatives (Figure 5A).
Reduced Neu5Ac content (μg/mg protein) was also observed in both lysosomal (68 ± 4) and cytosolic (56 ± 5) fractions in CEMC7 compared with corresponding fractions of PBMCN, being 84 ± 5 and 92 ± 5 μg/mg protein, respectively, as measured by the fluorimetric acetyl-acetone method (Figure 5H). In contrast, the expression of Neu5,9Ac2 was increased in the lysosomal (23 ± 3%) and the cytosolic (17 ± 3%) fractions of CEMC7 compared with PBMCN lysosomal (8 ± 1.5%) and cytosolic (7.5 ± 1.5%) fractions (Figure 5I).

Enhanced SOAT activity in ALL lymphoblasts compared with PBMCN

To establish the underlying mechanism responsible for the alteration of O-acetylated Sias in ALL, we have also monitored the SOAT activity to determine the extent of transfer of acetyl groups to Sias by a non-radiometric ELISA even by using cell lysates of ALL lymphoblasts.

Typically, cell lysates were incubated on immobilized de-O-acetylated BSM (de-OAcBSM) in the presence of acetyl-Coenzyme A (AcCoA), and the reaction product was quantified using Achatinin-H. The SOAT activity was linear in the range of 15–30 ng Sia content of the immobilized acceptor (Figure 6A); hence, this was found to be suitable for this assay. Negligible absorbance in the absence of acceptor served as a control. The dependence of O-acetylation activity increased between 2.5 and 10 nM of donor AcCoA; hence, any concentration in this range can be used for this assay (Figure 6B).

The SOAT activity of cell lysates from ALL lymphoblasts linearly increased with increasing amount of protein (Figure 6C), whereas only a slight increase in SOAT activity was observed with cell lysate from PBMCN (Figure 6C). A minute difference between the absorbance in the absence of AcCoA and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) control suggested negligible amounts of endogenous AcCoA in PBMCN. In parallel, microsomal fractions of lymphoblasts were used for comparison.

As the collection of peripheral blood (PB) is simpler than that of bone marrow (BM), we compared the SOAT activity of lymphoblasts in both PB and BM, at presentation of the disease. No significant difference in the SOAT activity between PB and BM was observed (Figure 7A). Approximately 3-fold increase in SOAT activity was observed in lymphoblasts of ALL patients (n = 25); the mean ± SD (standard deviation) of SOAT activity was 762 ± 39 ng OAcSias/mg protein × min (Figure 7B) when compared with PBMCN (242 ± 22 ng OAcSias/mg protein × min) (Figure 7C). In contrast, the lymphocytes from PB of patients in clinical remission led to an absorbance like PBMCN, SOAT being 267 ± 5 ng OAcSias/mg protein × min. However, the patients being in clinical relapse showed SOAT activity 770 ± 24 ng OAcSias/mg protein × min.

Distinct differences in the SOAT activity of lymphoblasts from patients at different weeks of treatment were observed. In patients, being in clinical remission, the absorbance rapidly decreased. As the weeks of treatments progressed, activity declined to almost normal values at the 148th week. The
activity was slightly higher even after 44 weeks in a few patients who were in clinical remission, although at this stage there was no clinical symptom of relapse. Interestingly, the clinical symptoms of relapse of these patients were found to be appearing after 48 weeks and the activity was higher than during presentation of the disease (Figure 7B).

CEMC7, MOLT3, MOLT4 and REH cells showed 3.14-, 3.17-, 2.9- and 3.17-fold increase in SOAT activity being 759 ± 27, 767 ± 42, 705 ± 32 and 766 ± 40 ng OAcSias/mg protein × min, respectively, compared with PBMCN (Figure 7C).

All five acceptors containing α2-6-linked Sias showed comparable activity; the SOAT activity mean ± SD being 729.04 ± 32 ng OAcSias/mg protein × min using cell lysate from a representative B-ALL patient as an enzyme source (Table I). Negligible absorbances were observed with immobilized de-sialylated BSM and asialofetuin, confirming the specificity of transfer to Sia. In the absence of cell lysate, only the acceptor served as a control.

We have observed a good positive correlation for SOAT activity for the same patients between the radiometric assay and the ELISA (Figure 7D), the correlation coefficient value being $r = 0.9911$. Accordingly, the ELISA may be considered as an equally useful method for the measurement of SOAT activity.

At diagnosis, the SOAT activities in cell lysate were high but the O-acetylerase activities of cytosolic fractions were low in comparison with PBMCN (Figure 7E). A similar comparison was observed with the lysosomal fraction. Thus, these two enzymes (SOAT and O-acetylerases) are negatively correlated and the correlation coefficients ($r$) being −0.9875 and −0.9906 for both lysosomal and cytosolic O-acetylerase activities, respectively.

**Discussion**

Previous studies have shown an increase in Neu5,9Ac$_2$-GPs and enhanced SOAT activity in lymphoblasts of children with ALL (Sinha et al. 1999; Mandal et al. 2000, 2009). However, very little progress has been made in examining the balance between the activities of SOAT and SIAE which regulate the expression of Neu5,9Ac$_2$-GPs, a hallmark in ALL. The major achievement of the current investigation is the demonstration...
of reduced SIAE activity in the lysosomal and cytosolic fractions of these lymphoblasts. Furthermore, SOAT activity can be detected even in a small aliquot of cell lysate by a non-radioactive ELISA for successful monitoring of ALL patients at different stages of treatment. The ELISA shows good positive correlation with the established radiometric SOAT assay.

O-Acetylerase activities with different localizations have previously been reported to occur in a variety of mammalian tissues (Schauer et al. 1989; Schauer and Shukla 2008; Shen, Kohla, et al. 2004). In accordance with this, two distinct SIAE activities, one in the lysosomal and another in the cytoplasm compartment, were also found in ALL lymphoblasts. SIAE of the cytosolic fraction is known to regulate the level of free Neu5,9Ac2 (Shen, Kohla, et al. 2004). It is generally accepted that Neu5,9Ac2, probably resulting from the action of a lysosomal sialidase, is transported into the cytoplasm. In contrast, lysosomal SIAE specifically hydrolyzes Neu5,9Ac2 bound to glycoprotein. Thus, the expression of Neu5,9Ac2-GPs seems to be controlled by the relative activity of lysosomal SIAE.

Two forms of cellular SIAE genes were present in mammals, one in the lysosomal (transcript variant 1) and the other in the cytoplasmic (transcript variant 2) compartment. Transcript variant 2 differs in the 5′-UTR and coding region compared with transcript variant 1. Cytosolic SIAE contains a cleavable N-terminal signal sequence and is secreted from

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**Fig. 4.** The hydrolysis of the O-acetyl group from Neu5,9Ac2 by cytosolic SIAE (A–D). Cytosolic fractions from CEMC7 cells (A and B) and PBMCN (C and D) were incubated with Neu5,9Ac2 (0.5 mM)-enriched Sia samples at 37°C for 60 min. The hydrolysis of Neu5,9Ac2 from 0 to 60 min was monitored by fluorimetric HPLC. The peaks 1 and 2 represent Neu5Ac and Neu5,9Ac2, respectively. The decrease in the peak (2) area was pointed out by an arrow. (E) Cytosolic fractions from lymphoblasts of a representative B-ALL patient were incubated with different concentrations of Neu5,9Ac2 (0.00–1.00 mM)-enriched Sia sample and processed as described in (A)–(D). (F) The percent of hydrolysis of Neu5,9Ac2 (0.5 mM) to form Neu5Ac by the cytosolic protein from lymphoblasts of ALL patients (n = 3), CEMC7 cells and PBMCN was estimated from corresponding peak areas.

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**Fig. 5.** Fluorimetric-HPLC analysis of Sias. Sias, isolated from equal amounts of lysosomal and cytosolic protein of a representative ALL cell line (CEMC7), lymphoblasts from a representative ALL patient and PBMCN were analyzed by fluorimetric HPLC. This is the representation of at least three different experiments. (A) Sias from BSM shown as (1) Neu5Gc, (2) Neu5Ac, (3) Neu5,7Ac2, (4) Neu5Ga9Ac, (5) Neu5,8Ac2 and (6) Neu5,9Ac2 were used as standards. (B–D) Sias from the lysosomal fractions of CEMC7, lymphoblasts from a representative ALL patient and PBMCN. The peak (6) corresponding to Neu5,9Ac2 was pointed out by an arrow. (E–G) Cytosolic Sias from CEMC7, lymphoblasts from a representative ALL patient and PBMCN. The numbering of the identified peaks corresponds to that in (A). (H and I) Quantification of O-acetylated Sias by the fluorimetric acetyl-acetone method. (H) Neu5Ac content (mg/mg protein) in the lysosomal fraction from CEMC7 (CEMC7 lyso) and PBMCN (normal lyso) and cytosolic fraction from CEMC7 (CEMC7 cyto) and PBMCN (normal cyto) was quantified. (I) The presence of O-acetylated Sia (%) in the lysosomal fraction from CEMC7 (CEMC7 lyso) and PBMCN (normal lyso) and cytosolic fraction from CEMC7 (CEMC7 cyto) and PBMCN (normal cyto) was also quantified.
COS cells as a glycoprotein with an apparent molecular mass of 62 kDa (Guimaraes et al. 1996), whereas in rat liver it is found predominantly as a heterodimer composed of small (28 kDa) and large (38 kDa) subunits connected by disulfide bridges.

Significantly lower O-acetylerase, in both lysosomal and cytosolic fractions of ALL cells and primary cells of ALL patients compared with normal lymphocytes, was reflected in the decreased $V_{\text{max}}$ value. This lower $V_{\text{max}}$ may be due to the less availability of these enzymes in their corresponding fractions as it is genetically down-regulated in ALL lymphoblast compared with PBMC-N.

The SIAE present in both lysosomal and cytosolic proteins of the ALL cell lines and primary cells of patients is capable of hydrolyzing Neu5,9Ac2 as demonstrated by the decreased peak area corresponding to Neu5,9Ac2 with the increase in the Neu5Ac peak specifying the substrate specificity of the enzyme. The percentage of hydrolysis of Neu5,9Ac2 by the SIAE was lower in ALL, which is possibly responsible for less removal of the O-acetyl group from OAcsias. This may be one of the reasons for higher cell surface expression of OAcsias as previously established on ALL lymphoblasts. Therefore, the presence of glycoside-bound higher Neu5,9Ac2 content in both the fractions of lymphoblasts and CEMC7 cells was also corroborated by lower SIAE activity. This is the first report of the subcellular distribution of different Sias in ALL cells.

Therefore, enhanced SOAT activity in microsomal fractions (Mandal et al. 2009) and higher quantity of O-acetylated Sias on the cell surface of ALL lymphoblasts is not only due to the lower SIAE activity but also to the higher SOAT activity. Accordingly, SOAT may be considered as a possible biomarker for monitoring ALL patients. Thus, we have developed a simple, non-radioactive ELISA for quantification of SOAT activity which demonstrated significant enzyme activity even using minute amounts of cell lysates of lymphoblasts as an enzyme source. The marked increase in detectable absorbance suggesting enhanced SOAT activity at diagnosis and again with clinical relapse is also in good agreement with the higher expression of Neu5,9Ac2-GP on the lymphoblast, signifying that the cell lysate may be suitable for examining the disease status by ELISA.

This ELISA was further validated successfully for long-term monitoring of patients at different stages of treatment and found to be a very sensitive, specific and safe method. A sharp decline in SOAT activity as determined by ELISA in patients who underwent successful chemotherapy was corroborated by their clinical remission. This suggests that measurement of SOAT activity even with cell lysate can be effectively used for screening these children. The positive association observed between the SOAT activity from ELISA and radioactivity of the radiometric method suggests that ELISA is as sensitive as the radiometric method for this purpose.

The acceptor substrate, donor substrate and enzyme concentrations needed are very low. Another advantage of ELISA over established the HPLC-based technique (Shen et al. 2004) is that there is no need to release and purify Sias before analysis, which is less accurate and a lengthy experiment. The similarity of enzyme activities of PB and BM cells suggests that instead of using cell lysates from BM we might use PB for diagnosis of ALL patients; and this definitely is an additional benefit.

Sequencing of purified eukaryotic SOAT protein and identification of corresponding encoding gene(s) is not yet available. Therefore, investigation of SOAT expression clone is continuing. The discovery of O-acetyl ganglioside synthase clone showed considerable homology to an unrelated protein (Ogura et al. 1996). Although a clone encoding truncated form of vitamin D binding protein and another cDNA clone encoding a chimeric protein showed increased O-acetylation, possibly they are not directly involved in transfer of O-acetyl groups (Shi et al. 1998). A new gene (CASD1) was reported to encode an enzyme which specifically increase the O-acetylation of GD3 (Arming et al. 2011). However, whether CASD1 is directly capable to O-acetylate the sialoglycopeptides is still an open question. Therefore, it may be envisaged that pattern recognition receptors possibly identify expressed mRNA or protein which is directly or indirectly responsible for O-acetylation of Sias (Mandal et al. 2011).

It may be concluded that the level of Sia O-acetylation depends on the relative activities of SOAT: O-acetylerase as corroborated by a good negative correlation between the cytosolic and lysosomal O-acetylerase and the SOAT activities for the same set of patients. The high cell surface expression of Neu5,9Ac2-GPs may be due to the high SOAT activity and low SIAE activity of the patients which is in contrast to normal individuals.

The information reported here adds considerably to our understanding of the O-acetylation of Sias in general and not only in leukemia. It may be envisaged that reducing the level of O-acetylation of Sia by stimulation of the SIAE activity or by inhibition of the SOAT activity may lead to the development of new drugs in childhood ALL.

Materials and methods

Chemicals

Reversed-phase columns (RP18, LiChrophor 100, particle size 5 mm) and HPLC solvents (gradient grade) were obtained from Merck (Darmstadt, Germany). Dowex 2X8 (200–400 mesh) was purchased from Pharmacia Biosystems (Freiburg, Germany). Protease inhibitor cocktail and ABTS were from Roche Molecular Biochemicals (Mannheim, Germany). DMB (1,2-diamino-4,5-methylenedioxybenzene), FCS (fetal calf serum), transferrin (Debray et al. 1981), $\alpha$-acid glycoprotein (Schultz et al. 1958), fetuin (Baenziger et al. 1979), SYBR Green Jump Start Ready mix, 4-methylumbelliferon (MU), MUAc, AcCoA and Neu5Ac were from Sigma-Aldrich (St Louis). All PE (phycoerythrin)- and Cy5 (cyanin 5)-conjugated lineage-specific monoclonal antibodies (Mabs) were from BD Pharmingen (San Diego).

The mucins were purified from both BSM (Murphy and Gottschalk 1961) and ovine submundibular gland mucin (OSM) (Bertolini and Pigman 1970). About 38% Neu5,9Ac2 in BSM is reported to be in $\alpha$2-$\epsilon$-linkage to subterminal $N$-acetylgalactosamine (GalNAc) as estimated fluorimetrically (Shukla and Schauer 1982). de-OAcBSM was prepared by
saponifying BSM with NaOH (0.2 M) for 30 min at 25°C followed by neutralization with HCl (0.2 M), and this substance was used as an O-acetyl acceptor of SOA T enzyme (Mandal et al. 2009).

Achatinin-H, a snail lectin, was purified from the hemolymph of Achatina fulica by affinity chromatography using BSM as an affinity matrix (Sen and Mandal 1995). The presence of Neu5,9Ac2-GP on lymphoblasts of children with ALL was demonstrated by utilizing the preferential affinity of Achatinin-H toward Neu5,9Ac2α2-6GalNAc. However, the presence of 7-O- or/and 8-O-acetylated Sias on these glycoproteins also should be taken into consideration because a possible affinity of this lectin toward these Sias may exist.

Cell line

Human B-ALL (REH) and T-ALL (CEMC7, MOLT3 and MOLT4) cell lines from the American Type Culture Collection (Maryland) were cultured in RPMI-1640 medium supplemented with heat-inactivated FCS [10% (v/v)], L-glutamine (0.002 M), antibiotics and antimycotics.

Clinical samples

Either BM or PB or both (1–2 mL) was collected from clinically confirmed ALL patients (20 males and 5 females, median age: 6 years, range: 0.8–15 years, median white blood cell count: 12 × 10⁹/L, range: 0.4–1000 × 10⁹/L) from the Kothari Medical Centre (Kolkata) and sent to the Indian Institute of Chemical Biology (Kolkata). The diagnosis was established by cytological examination of BM smears according to the FAB group recommendations, belonging to L1 or L2. Lymphoblasts from patients (40–65% leukemia blasts as per morphological estimation) were separated by density gradient centrifugation (Ficoll-Hypaque, Amersham Pharmacia, Uppsala, Sweden). The diagnosis of these patients was further confirmed by immunophenotyping using flow cytometry (FACSCalibur, Becton Dickinson, San Jose). The PE- and PECy5-conjugated lineage-specific Mabs (BD Pharmingen) were used for immunologic subgroups. The patients included were from both B (CD19⁺, CD10⁺, cyt-μ⁺, Slg⁻, n = 10) and T (CD3⁺, CD7⁺, n = 5) lineage ALL. Normal human PB mononuclear cells (PBMCN) separated from healthy donors were used for comparison. The data were analyzed with the CellQuestPro software (Becton Dickinson). Lymphoblasts (70–95%) of these children always showed enhanced ALL-associated antigens (Neu5,9Ac2-GPs) on their cell surface using FITC-Achatinin-H by flow cytometry (Pal, Ghosh, Bandhyopadhyay, et al. 2004; Pal, Ghosh, Mandal, et al. 2004; Chowdhury et al. 2008). The sera of these patients were accompanied by high levels of antibodies against Neu5,9Ac2-GPs, A₄₀₅nm being 0.95–1.12 when compared with 0.04–0.20 observed in normal sera by an ELISA using...
BSM as a coating antigen developed in our laboratory (Pal, Bandhyopadhyay, et al. 2004; Bandhyopadhyay et al. 2005).

Children were treated as per MCP841 protocol (Chowdhury et al. 2008). Clinical samples were also assayed during clinical remission and relapse. Children ($n = 25$) were longitudinally monitored using cell lysates (200 ng protein) of lymphoblasts from ALL patients of B- and T-lineages. Transfer of acetyl groups from AcCoA into de-OAcBSM was monitored by ELISA. Twenty patients remained in clinical remission (open squares). Other five children showed relapse (filled squares) after >30 weeks. (C) The assay was also repeated with cell lysate (200 ng) from REH, CEMC7, MOLT3 and MOLT4 cells and PBMC$_N$. (D) Detection of SOAT activity by both the radiochemical technique (Mandal et al. 2009) and ELISA. The correlation ($r = 0.9911$) between these two methods was done with lymphoblasts from ALL patients at presentation (filled squares) and normal PBMC (open squares) using cell lysate. (E) Correlation between O-acetyltransferase and SOAT activity. The correlation between O-acetyltransferase activity obtained fluorimetrically from lysosomal (filled circles, $r = 0.9875$) and cytosolic fractions (filled squares, $r = 0.9906$) of ALL patients during presentation; lysosomal (open circles, $r = 0.9875$) and cytosolic fraction (open squares, $r = 0.9906$) from PBMC$_N$ with their SOAT activity based on ELISA.

**Fig. 7.** SOAT activity in lymphoblast of ALL patients, ALL cell lines and normal PBMCs. (A) The ELISA was performed with cell lysate (200 ng) from PB and BM of ALL patients at the presentation of disease with immobilized de-OAcBSM (20 ng) and AcCoA (5 nM) by incubating at 37°C for 1 h. (B) SOAT activity in the course of leukemic treatment and relapse. Children ($n = 25$) were longitudinally monitored using cell lysates (200 ng protein) of lymphoblasts from ALL patients of B- and T-lineages. Transfer of acetyl groups from AcCoA into de-OAcBSM was monitored by ELISA. Twenty patients remained in clinical remission (open squares). Other five children showed relapse (filled squares) after >30 weeks. (C) The assay was also repeated with cell lysate (200 ng) from REH, CEMC7, MOLT3 and MOLT4 cells and PBMC$_N$. (D) Detection of SOAT activity by both the radiochemical technique (Mandal et al. 2009) and ELISA. The correlation ($r = 0.9911$) between these two methods was done with lymphoblasts from ALL patients at presentation (filled squares) and normal PBMC (open squares) using cell lysate. (E) Correlation between O-acetyltransferase and SOAT activity. The correlation between O-acetyltransferase activity obtained fluorimetrically from lysosomal (filled circles, $r = 0.9875$) and cytosolic fractions (filled squares, $r = 0.9906$) of ALL patients during presentation; lysosomal (open circles, $r = 0.9875$) and cytosolic fraction (open squares, $r = 0.9906$) from PBMC$_N$ with their SOAT activity based on ELISA.

Separation of cytosol, lysosomal and microsomal fractions

Lymphoblasts from BM or PB from individual patients and PBMC$_N$ were washed with cold phosphate-buffered saline (PBS, containing 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, 137 mM NaCl, pH 7.4), resuspended in cold PBS with protease inhibitors (1 μg/mL pepstatin A, 10 μg/mL aprotinin, 10 μg/mL leupeptin) and lysed by sonication (3 pulse for 8 s and giving break for 1 min). The supernatant obtained after centrifugation at 800 × g for 10 min at 4°C was subsequently centrifuged at 100,000 × g for 30 min at 4°C and the supernatant used as a cytosol. The pellet containing the membrane fraction was dissolved in ice-cold PBS.
Table 1. SOAT transfers acetyl groups to α2-6-linked sialoglycoproteins

<table>
<thead>
<tr>
<th>Acceptors*</th>
<th>Type of linkages</th>
<th>Sia (%)b</th>
<th>Transfer of the acetyl group by SOAT detected by</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-0AcBSM</td>
<td>Neu5Acα2-6-β-D-GalNAc</td>
<td>18.5</td>
<td>ELISA (ng OAcSias/mg protein × min) 2951 ± 161</td>
</tr>
<tr>
<td>Ship submaxillary mucin</td>
<td>Neu5Acα2-6-β-D-GalNAc</td>
<td>20</td>
<td>Radiometric assay (cpm) 2922 ± 110</td>
</tr>
<tr>
<td>Fetuin</td>
<td>Neu5Acα2-6-β-D-GalNAc, Neu5Acα2-3-β-D-Gal</td>
<td>6.5</td>
<td>721 ± 33 2843 ± 137</td>
</tr>
<tr>
<td>α1-Acid glycoprotein</td>
<td>Neu5Ac α2-6-β-D-Gal, Neu5Ac α2-3-β-D-Gal</td>
<td>4.8</td>
<td>705 ± 30 2935 ± 120</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Neu5Ac α2-6-β-D-Gal</td>
<td>6.4</td>
<td>728 ± 31 2839 ± 145</td>
</tr>
<tr>
<td>de-sialylated BSMc</td>
<td>—</td>
<td>160 ± 8</td>
<td>ND</td>
</tr>
<tr>
<td>Asialofetuin4</td>
<td>—</td>
<td>147 ± 7</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

a Equal amount of acceptors (20 ng) have been used based on their Sia content in ELISA, whereas 5 μg was used for radiometric assay.

b Sia was estimated according to the method describe in Pal, Ghosh, Bandhyopadhyay, et al. (2004).

c Using [3H]AcCoA as a donor.

d The amount BSM (0.11 μg) containing 20 ng of Sias was used for de-sialylated BSM (0.11 μg). Similarly, the amount fetuin (0.335 μg) containing 20 ng of Sias was used for asialofetuin (0.335 μg).

For the preparation of lysosomal membranes (Butor et al. 1993), cells (1 × 10⁵) were homogenized in 0.25 M sucrose in 20 mM Tris–HCl (pH 7.2) in a glass homogenizer and spun at 800 × g for 10 min at 4°C. The pellet was suspended in 0.25 M sucrose in 0.4 M KCl (pH 7.0) and spun at 11,000 × g for 20 min at 4°C. The pellet was resuspended in 0.025 M sucrose, incubated for 30 min on ice and spun again at 11,000 × g for 20 min at 4°C. Finally, the supernatant was spun at 100,000 × g for 1 h and the pellet was suspended in PBS (200 μL). β-Galactosidase activity of this fraction was ~2-fold higher than in the crude homogenate, and negligible activity was observed in the cytosolic fraction indicating that lysosomes had not been disrupted. In this lysosomal preparation, acid phosphatase activity was enriched ~71- and 86-fold in the comparison with cytosolic fractions and crude homogenate, respectively, implying no apparent contamination between these two fractions.

We routinely took only the lysosomal fraction from one set of cell lysate and discarded the cytosolic and other fractions. Similarly for the collection of cytosolic fraction, we used another new set of cell lysate from the same patient.

The microsomal fractions were obtained by differential sucrose density gradient centrifugation and immediately used for SOAT assays (Leelavathi et al. 1970; Butor et al. 1993; Shen et al. 2004; Mandal et al. 2009; Carey and Hirschberg 1981). Protein was estimated by the method of Lowry et al. (1951).

O-Acetylgalactosaminidase

O-Acetylgalactosaminidase activity in lysosomal and cytosolic fractions prepared from ALL patients and PBMC were initially assayed using MUAc as a substrate. Briefly, protein (50 μg) was incubated with MUAc (60 μM) in Tris–HCl buffer (40 μL, 50 mM pH 7.8) with a total volume of 100 μL for 1 h at 37°C. The reaction was stopped by the addition of ethanol (100 μL) and water (800 μL). The fluorescence intensity was measured at the excitation at 365 nm and emission at 450 nm (Shen, Kohla et al. 2004; Schauer and Shukla 2008).

The kinetics (K_M and V_max) of O-acetylgalactosaminidase were studied by varying the concentration of MUAc (0–150 μM) using 50 μg of cytosolic and lysosomal fractions prepared from lymphoblasts of patients and PBMC in Tris–HCl buffer (50 mM, pH 7.8) at 37°C for 1 h. K_M and V_max values were determined from plots of [F] vs [S], where [F] is the initial velocity at substrate concentration [S].

Genetic expression of lysosomal and cytosolic SIAEs

Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) and treated with an RNase-free DNase I (Invitrogen, Carlsbad, CA 92080, USA) following the manufacturer’s instruction. First-strand cDNA was synthesized by ImProm-II Reverse transcription system (Promega, Madison, WI) according to the manufacturer’s protocol. Semi-quantitative RT–PCR was performed on PTC-100 (MJ Research, Gaithersburg, MD) using PCR Kit (Invitrogen). Expression of β-actin house-keeping gene was used to determine the cDNA yield and to normalize PCR product. The PCR were cycled 40 times after initial denaturation (95°C, 10 min) with the following parameters: denaturation at 94°C, 30 s; annealing at 52°C, 45 s and extension at 72°C, 30 s; followed by 72°C, 4 min. The primers used for this analysis were

lysozymal SIAE—Fw: 5′AC CCACTTCCCAGTCTCA3′ and Rev: 5′TGAGCTTCTCACCTGGTACTT3′—and cytolytic SIAE—Fw: 5′TACCCTCCACGCTTATGTCC3′ and Rev: 5′GGAATCTTTCTGAA GGGCTGAC3′.

Quantitative analysis was performed by a real-time PCR using a LightCycler rapid thermal cycler system (Bio-Rad, Richmond, Richmond, CA) with SYBR Green Jump Start Ready mix, following the manufacturer’s instruction. The specificity of the reactions was confirmed by melting curve analysis. β-Actin was used as an internal control.

SIAE activity assay

Sias were purified from BSM, after liberation by 4 h hydrolysis in 2 M propionic acid at 80°C. After the removal of precipitated proteins by centrifugation at 14,000 × g for 30 min in the cold, the supernatant was lyophilized. Liberated Sias were purified on a column of Dowex 2X8 (200–400 mesh), derivatized with DMB reagent and separated by fluorimetric HPLC (Hara et al. 1989).
The hydrolysis of O-acetyl groups from Neu5,9Ac2 by esterase was determined by separately incubating lysosomal and cytosolic proteins (50 μg) in Tris–HCl buffer (50 mM, pH 7.8) with Neu5,9Ac2-enriched (0.5 mM) Sias, purified from BSM and incubated at 37°C for 1 h. The reaction products were identified and quantified by fluorimetric HPLC (Hara et al. 1989; Shen, Kohla et al. 2004; Schauer and Shukla 2008; Mandal et al. 2009).

Analysis of Sias in cytosolic and lysosomal fractions
Sias present in both the lysosomal and the cytosomal fractions were quantified fluorimetrically using the acetyl-acetone method (Shukla and Schauer 1982). Sias were also liberated by propionic acid from the lysosomal and the cytosolic proteins and detected by fluorimetric HPLC.

Monitoring SOAT activity using a newly developed non-radioactive ELISA
In order to immobilize appropriate amounts of Sias as acceptors, determined by fluorimetric measurement, 96-well flat-bottomed polystyrene microtitre plates (Nunc-Immuno plate, Denmark) were coated with increasing concentrations of de-OAcBSM (0–30 ng according to Sia content) in 0.02 M phosphate buffer, pH 7.2/100 μL/well. Based on a calibration curve, de-OAcBSM (20 ng/100 μL/well) was immobilized by incubating overnight at 4°C. The wells were washed thrice with PBS and the non-specific binding sites were blocked with bovine serum albumin (BSA, 2%) for 2 h at 4°C with Tris-buffered saline (TBS) containing Tris (0.05 M, pH 7.2) and NaCl (0.150 M). The reaction mixture (100 μL) containing either cell lysate (200 ng) or microsomal fraction (50 ng) of an ALL patient, as a source of SOAT and AcCoA (5 nM) in TKM buffer (Tris–HCl 10 mM, KCl 150 mM, MgCl2 1 mM, pH 7.2) was added and incubated for 60 min at 37°C. Subsequently, the wells were washed thrice with TBS and the reaction product was quantified by incubating the wells with Achatinin-H (0.50 μg) in TBS–BSA (2%) containing 30 mM CaCl2 overnight at 4°C. The extent of specifically bound lectin was detected with a rabbit anti-Achatinin-H antibody. The bound complex was estimated using horse radish peroxidase (HRP)-goat anti-rabbit immunoglobulin G (1:5000, Sigma-Aldrich) and the chromogenic substrate, ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); AcCoA, acetyl-Coenzyme A; ALL, acute lymphoblastic leukaemia; BM, bone marrow; BSA, bovine serum albumin; BSM, bovine submandibular gland mucin; cyt-μ, cytoplasmic μ; Cy5, cyanin 5; de-OAcBSM, de-O-acetylated BSM; DMB, 1,2-diamino-4,5-methylenedioxybenzene; ELISA, enzyme-linked immunosorbent assay; FAB, French–American–British; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GalNAc, N-acetylgalactosamine; GD3, di-sialo ganglioside; HPLC, high-performance liquid chromatography; HRP, horse radish peroxidase; Mabs, monoclonal antibodies; MRD, minimal residual disease; MU, 4-methylumbelliferon; MUAc, 4-methylumbelliferyl acetate; Neu5Ac, N-acetylneuraminic acid; Neu5,7Ac2, N-acetyl-7-O-acetylneuraminic acid; Neu5,8Ac2, N-acetyl-8-O-acetylneuraminic acid; Neu5,9Ac2, N-acetyl-9-O-acetylneuraminic acid; Neu5,9Ac2-GPs, O-acetylated sialoglycoproteins; OSM, ovine submandibular gland mucin; PB, peripheral blood; PBMCN, normal human peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PE, phycoerythrin; RT-PCR, Reverse transcription polymerase chain reaction; SD, standard deviation; Sia, sialic acid; SIAE, sialate-esterase; Slg,
surface membrane Ig; SOAT, sialate-O-acetyltransferase; TBS, Tris-buffered saline; 5'-UTR, five prime untranslated region.
Enzymatic regulation of O-acetylated sialic acids in ALL


