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O-Acetylation of GD3 Prevents its Apoptotic Effect and Promotes Survival of Lymphoblasts in Childhood Acute Lymphoblastic Leukaemia

Kankana Mukherjee,[1] Anil Kumar Chava,[1] Chandan Mandal,[1] Sailendra Nath Dey,[1] Bernhard Kniep,[2] Sarmila Chandra,[3] and Chitra Mandal[1*]

[1]*Infectious Diseases and Immunology Division, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Kolkata 700032, India*

[2]*Institute of Immunology, Technical University of Dresden, Fetscherstr. 74 D-01307, Germany*

[3]*Kothari Medical Centre 8/3, Alipore Road, Kolkata 700027, India*

ABSTRACT

We have previously demonstrated induction of *O*-acetylated sialoglycoproteins on lymphoblasts of childhood acute lymphoblastic leukaemia (ALL). These molecules promote survival of lymphoblasts by preventing apoptosis. Although *O*-acetylated sialoglycoproteins are over expressed, the status of *O*-acetylation of gangliosides and their role in lymphoblasts survival remains to be explored in ALL patients. Here, we have observed enhanced levels of 9-*O*-acetylated GD3 (9-*O*-AcGD3) in the lymphoblasts of patients and leukaemic cell line versus disialoganglioside GD3 in comparison to the normal cells. Localization of GD3 and 9-*O*-AcGD3 on mitochondria of patient's lymphoblasts has been demonstrated by immuno-electron microscopy. The exogenous administration of GD3-induced apoptosis in lymphoblasts as evident from the nuclear fragmentation and sub G0/G1 apoptotic peak. In contrast, 9-*O*-AcGD3 failed to induce such apoptosis. We further explored the mitochondria-dependent pathway triggered during GD3-induced apoptosis in lymphoblasts. GD3 caused a time-dependent depolarization of mitochondrial membrane potential, release of cytochrome *c* and 7.4- and 8-fold increased in caspase 9 and caspase 3 activity respectively. However, under identical conditions, an equimolar concentration of 9-*O*-AcGD3 failed to induce similar effects. Interestingly, 9-*O*-AcGD3 protected the lymphoblasts from GD3-induced apoptosis when administered in equimolar concentrations simultaneously. In situ de-*O*-acetylation of 9-*O*-AcGD3 with sodium salicylate restores the GD3-responsiveness to apoptotic signals. Although both GD3 and 9-*O*-acetyl GD3 localize to mitochondria, these two structurally related molecules may play different roles in ALL-disease biology. Taken together, our results suggest that *O*-acetylation of GD3, like that of *O*-acetylated sialoglycoproteins, might be a general strategy adopted by leukaemic blasts towards survival in ALL. J. Cell. Biochem. 105: 724–734, 2008.

KEY WORDS: ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL); GD3; 9-*O*-ACETYL GD3 (9-*O*-AcGD3); MITOCHONDRIAL MEMBRANE POTENTIAL; CYTOCHROME *c*; CASPASE 9; APOPTOSIS

Acute lymphoblastic leukaemia (ALL) is a malignant transformation of lymphoblasts and represents the single most common type of cancer in the paediatric population. Although increase of 9-*O*-acetylated sialoglycoproteins have been convincingly demonstrated as an important determinant on lymphoblasts [Sinha et al., 1999; Pal et al., 2004a,b], the status of *O*-acetylated sialic acids on the glycosphingolipids and their role in the disease biology of ALL has not yet been explored.

Gangliosides are prominent members of glycosphingolipids (GSLs) that are distinguished by the presence of one or more sialic

Abbreviations used: 9-*O*-AcGD3, 9-*O*-acetyl GD3; cyt *c*, cytochrome *c*; ALL, lymphoblasts from childhood acute lymphoblastic leukaemia patients.

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Anil Kumar Chava's present address is Department of Biological Sciences, Northern Kentucky University, Kentucky 41099.

*Correspondence to: Chitra Mandal, Deputy Director, Infectious Diseases and Immunology Division, Indian Institute of Chemical Biology, Raja S.C. Mullick Road, Kolkata 700 032, India.
E-mail: cmandal@iicb.res.in; chitra_mandal@yahoo.com

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acid (SA) residues and are commonly present on the outer leaflet of the plasma membrane [Kolter et al., 2002]. In addition to these functions, an emerging role of GSLs, particularly the ganglioside GD3, implicated in fundamental cell processes such as growth, differentiation, recognition and adhesion and their role as regulators of apoptosis, is increasingly recognized [Hakomori and Igarashi, 1995]. Addition of GD3 to intact cells induces apoptosis in breast carcinoma cells and oligodendrocytes [Simon et al., 2002; Ma et al., 2004]. GD3 added to isolated mitochondria or intact cells caused loss of mitochondrial transmembrane potential and membrane permeabilization. This event is followed by generation of reactive oxygen species (ROS), release of apoptogenic factors, such as cytochrome *c*, apoptosis-inducing factor (AIF) [Susin et al., 1999; Garcia-Ruiz et al., 2000] and mitochondrial caspases [Crompton, 1999; Saleh et al., 1999; Ferri and Kroemer, 2000].

Accumulation of GD3 has been reported in a variety of tumours [Portoukalian et al., 1976; Hakomori, 1984, 1996; Merritt et al., 1987, 1988; Reaman et al., 1990]. The concomitant presence of an acetylated modification of GD3, 9-*O*-acetyl GD3 (9-*O*-AcGD3), was observed in some tumours such as melanoma [Cheresh et al., 1984a,b] and breast cancer [Marquina et al., 1996; Gocht et al., 1998] as well as in tumour cell lines like MOLT-4 [Fox et al., 1989] and SKMel28 [Kawashima et al., 1993]. Recently, differential expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 during apoptosis of human mature erythrocytes [Mukherjee et al., 2007] and lymphocytes [Erdmann et al., 2006] has been reported. *O*-acetylation of GD3 has been reported to suppress its apoptotic potential [Malisan et al., 2002; Kniep et al., 2006]. Here, we explored the role of 9-*O*-acetyl GD3 on survival of lymphoblasts in patients suffering from ALL as well as ALL cell line and it did show a role distinct from GD3.

Recent studies using immunoelectron and laser confocal microscopy showed the physical interaction and accumulation of GD3 in mitochondria from human lymphoblastoid CEM cell line [Rippo et al., 2000], intact hepatocytes [Garcia-Ruiz et al., 2002], or human colon HT-29 cells [Colell et al., 2002] exposed to C2-ceramide or tumour necrosis factor. It is however, unclear if 9-*O*-AcGD3 is also relocalized to mitochondria from plasma membrane during stress but fails to induce apoptotic effects like GD3 due to *O*-acetylation of its outermost sialic acid.

The present investigation (i) depicts enhanced expression of 9-*O*-acetyl GD3 on ALL lymphoblasts as compared to GD3 versus normal cells, (ii) reports a mitochondria dependent apoptotic pathway induced by GD3 in lymphoblasts from ALL patients at presentation of the disease, (iii) explores the role of 9-*O*-acetyl GD3 on survival of these lymphoblasts. (iv) This study also visualizes the localization of both 9-*O*-AcGD3 and GD3 in lymphoblasts from ALL patients on mitochondria by immunoelectron microscopy. Taken together, this report proposes *O*-acetylation as a general strategy adopted by the lymphoblasts for survival in ALL.

MATERIALS AND METHODS

CELL CULTURE

Human leukaemic T-(MOLT-4) ALL cell line (American Type Culture Collection, VA) and human melanoma cell lines (Mel 28) were cultured at 37°C in 5% CO_2 in RPMI-1640 medium (Sigma, St. Louis) with L-glutamine (0.002 M), antibiotics, antimycotics (Medium A) and 10% (v/v) heat inactivated fetal calf serum (FCS).

STUDY SUBJECT

The children with ALL were selected based on their cytomorphological, immunophenotypic and clinical parameters (Table I). Venous blood (2–4 ml) were collected at Kothari Medical Sciences and then sent to the Indian Institute of Chemical Biology. Peripheral blood mononuclear cells (PBMC) from individual patient (n = 7) with B-, T- and mixed lineages ALL and age matched healthy volunteers (n = 7) were isolated separately by Ficoll-hypaque density centrifugation and immunophenotyped by using PE-conjugated anti-CD10, CD19 and CD7 monoclonal antibodies. Additionally, ALL-associated enhanced expression of 9-*O*-acetylated sialoglycoproteins on lymphoblasts were assessed by using FITC-conjugated 9-*O*-acetylated sialic binding lectin, Achatinin-H as described by Pal et al. [2004a]. PBMC from each patient were used individually for all the assays as described below. Informed consent was obtained from donors, patients and parents or guardians. The Institutional Human Ethical Committee as per the protocol of Indian Council of Medical Research, New Delhi approved the study.

GANGLIOSIDES AND ANTIBODIES

9-*O*-AcGD3 was purified from bovine buttermilk and identified by mass spectrometry [Kniep et al., 1992, 1995]. Bovine GD3, GM1, GM2, GM3 (Calbiochem, La Jolla, CA), purified mouse anti-GD3 monoclonal antibodies (mAb, R24, IgG3) [Pukel et al., 1982] and protein-G purified anti-9-*O*-AcGD3 mAb (IgM) from the M-T6004

TABLE I. Immunophenotype of ALL Patients by FACS Analysis

Sample	Morphological estimation of blasts (%)	CD10 (%)[a]	CD19 (%)[a]	CD7 (%)[a]	9-*O*-acetylated sialoglycoprotein (%)[b]
B-ALL (n = 3)	40–50	55–57	44–46	50–53	55–70
T-ALL (n = 2)	70–80	5.5–8	7.5–9	95–97	90–95
Mixed lineage ALL (n = 2)	38–45	80–83	10–12	93–95	75–77
MOLT-4	NA	ND	ND	93–95	85–93
Normal PBMC (n = 7)	NA	6–9	8–10	60–70	5–10

NA, not applicable; ND, not done.
[a]Enhanced expression of CD10, CD19, CD7 on ALL-lymphoblasts was detected by using PE-conjugated respective monoclonal antibodies.
[b]Enhanced expression of 9-*O*-acetylated sialoglycoproteins on ALL-lymphoblasts was assessed by using FITC-conjugated 9-*O*-acetylated sialic binding lectin, Achatinin-H as described in Pal et al. [2004a].

hybridoma culture supernatants [Kniep et al., 2006] were used for all the experiments as described below.

GANGLIOSIDE FEEDING STUDY

Cells (1×10^6/ml) were grown in Medium A with heat inactivated FCS (10%, v/v) in six-well plates. Dried GD3 and 9-*O*-AcGD3 were resuspended in water, briefly sonicated in a water bath sonicator and filtered through a 0.2 μm sterile filter. Initially cells were cultured in the presence or absence of GD3 (0–200 μM) for 24 and 48 h. The exogenous administration of GD3 was optimised at concentration of 100 μM for 24 h. An equimolar concentration of 9-*O*-AcGD3 (i.e., 100 μM) or a combination of GD3 (50 μM) and 9-*O*-AcGD3 (50 μM) was used under similar culture conditions. Etoposide (1.0 μM), an established inducer of apoptosis, served as positive control for apoptosis studies.

ANALYSIS OF GD3 AND 9-*O*-AcGD3 LEVELS

Gangliosides were extracted from equal number of (1×10^8) MOLT-4 cells, PBMC from individual patients (n = 7) and healthy normal donors (n = 7) and Mel 28 (1×10^5) cells separately [Malisan et al., 2002]. Cells were broken by three cycles of freezing and thawing and aqueous pellet was extracted with chloroform/methanol (1:2) followed by 30 s sonication at 10 W. After 15 min vortex at 25°C and 5 min centrifugation at 14,000*g*, water was added to the supernatant to obtain a final ratio of chloroform/methanol/water (4:8:5,v/v/v). Upper phase was recovered and two volumes of methanol were added and evaporated to dryness under nitrogen gas. Gangliosides were dissolved in water and passed through LC 18, VISIPREP (1.0 ml, Suplco) as per manufacturer's instruction. Bound gangliosides were eluted with methanol and subsequently with chloroform/methanol (2:1) and dried under nitrogen gas. Gangliosides were spotted on HPTLC Si 60 plates (Merck, NJ) in chloroform/methanol (1:2) and chromatographed in chloroform/methanol/$CaCl_2$ (0.2% w/v; 2:1:0.2, v/v/v) along with authentic standards (GM1, GM2, GM3, GD3 and 9-*O*-AcGD3).

For checking the ganglioside profile, the plate was sprayed with 0.3% α-napthol (Sigma) in 95% ethanol. Dried plate was placed in 50% sulphuric acid and heated at 100°C for 5 min. Additionally, GD3 and 9-*O*-AcGD3 were immuno-detected by incubating plates for 1 h at 25°C with murine anti-GD3 and anti-9-*O*-AcGD3 mAb separately, washed twice with PBS-Tween-20 followed by further incubation for 1 h at 25°C with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibodies (Cappel, Irvine, CA). Bands were detected by enhanced chemiluminescence system (Amersham Biosciences, NJ). The results shown are means of three independent experiments. The densitometric scanning of the TLC plates was done using Image Master Total Lab V 1.11 software (Amersham Pharmacia Biotech).

NUCLEAR CONDENSATION BY CONFOCAL MICROSCOPY

Apoptotic cells were evaluated by nuclear condensation of chromatin as identified microscopically using the fluorescent nuclear stain Hoechst 33342 (Ex_{max} 350 nm, Em_{max} 461 nm; Molecular Probes, CA). Lymphoblasts from ALL patients (1×10^6/ml) were exposed to either GD3 (100 μM) or 9-*O*-AcGD3 (100 μM) or etoposide (1.0 μM) separately for 24 h. Cells were then washed and loaded with Hoechst 33342, final concentration being 10 μg/ml in Medium A for 45 min at 25°C. They were centrifuged, resuspended in cold PBS, attached on glass slides, mounted in glycerol: PBS (1:1) and examined under a confocal microscope (Leica, Germany). Each experiment was performed in duplicate cover slips and more than 300 cells were examined. Each image was obtained at 40× magnification of the confocal microscope.

DETECTION OF APOPTOTIC SUB G0/G1 PEAK BY FACS

Fragmented, apoptotic nuclei were recognized by their sub-diploid DNA content. The cultured cells exposed to either GD3 or 9-*O*-AcGD3 or equimolar mixture of both the gangliosides for 24 h were washed with PBS, fixed with ice-cold methanol for 3 min at 25°C and kept at 4°C for 1 h. Fixed cells were treated with RNase (500 μg/ml, Sigma) for 30 min at 37°C and stained with 50 μg/ml of propidium iodide for 30 min at 25°C. The percentage of cells in the sub G0/G1 phase was assessed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) using the Cell Quest Pro software.

MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL

To measure dissipation of mitochondrial membrane potential ($\Delta\psi_m$), the cells (1×10^6/ml), after being exposed to gangliosides for 0–4 h were centrifuged, resuspended in Medium A containing JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, 10 μg/ml; Molecular Probes) and incubated at 37°C for 30 min, washed with PBS, resuspended in PBS and analysed. Forward scattering (FSC) and side scattering (SSC) were used to eliminate debris and aggregates. Changes of ψ_m were obtained by measuring successive samples of 10,000 living cells and expressed as the ratio between red (590 nm) and green fluorescence (530 nm).

DETECTION OF CYTOCHROME *c* BY WESTERN BLOT ANALYSIS

The ganglioside exposed cells were washed with ice-cold PBS, incubated on ice for 30 min in an extraction buffer (100 μl), containing mannitol (220 mM), sucrose (68 mM), HEPES-KOH (50 mM, pH 7.4), KCl (50 mM), EGTA (5 mM), $MgCl_2$ (2 mM), dithiothreitol (DTT, 1 mM) and protease inhibitor cocktail (Calbiochem) and homogenized. After centrifugation at 12,000*g* for 1 h, the protein concentrations of the supernatants were measured and stored at −70°C. The protein (50 μg/lane) was resolved on SDS–PAGE (12%) and electrophoretically transferred to nitrocellulose membrane at 100 V on transfer buffer (20 mM Tris, 150 mM glycine and 20% methanol) at 4°C. Cytochrome *c* was detected by incubating the membrane with anti-cytochrome *c* mAb (clone 7H8.2C12, 1:250, Pharmingen, San Diego, CA). Detection was performed using HRP-conjugated goat anti-mouse secondary antibody (1:1,000, Cappel) and an enhanced chemiluminescence system.

DETECTION OF CASPASE 9 AND 3 ACTIVATION

The enzymatic activities of caspase 9 and caspase 3 were assayed spectrofluorimetrically using BD ApoAlert Caspase assay plates (Clontech Laboratories, Inc., Mountain View, CA). The cultured cells were centrifuged and resuspended in lysis buffer and centrifuged at 14,000 rpm for 5 min at 4°C. The cytosolic fraction (50 μl, equivalent to 2×10^5 cells/well) was transferred into each well of caspase

profiling assay plate and then incubated at 37°C for 2 h. After protease activation, the activated caspases recognize their respective substrates covalently linked to the fluorogenic dye, 7-amino-4-methyl coumarin (DEVD-AMC for caspase 3 and LEHD-AMC for caspase 9). Upon cleavage by the respective caspase, the free dye can be detected using a spectrofluorometer (Perkin Elmer, Wellesley, MD) with the excitation and emission wavelengths of 380 and 460 nm, respectively. The increased fluorescence showed increase in caspase activity as folds increase relative to un-induced cells. The specific caspase inhibitors provided with the kit were used as controls.

IN SITU DE-*O*-ACETYLATION OF 9-*O*-AcGD3 AND APOPTOTIC ASSAY

To de-*O*-acetylate the sialoglycoconjugates on lymphoblasts, drug treatment was done according to Kniep et al. [2006]. Briefly, lymphoblasts (1×10^6/ml) were cultured in the presence of 5 mM sodium salicylate (neutralized with 1.0 M aqueous HEPES; 0–8 μl/2×10^6 cells) or with an equimolar aqueous solution of butyric acid (Sigma) for 24 h. Accumulation of GD3 in the cells pre-treated with salicylate and butyric acid was detected by TLC immunostaining using specific antibody against GD3.

De-*O*-acetylated lymphoblasts were then cultured in serum-deprived condition (2% FCS) for another 24 h. Apoptosis was detected by staining cultured cells with annexin V and propidium iodide. Briefly, cells were washed twice with cold PBS, and resuspended in annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were then incubated in dark for 15 min at 25°C with FITC-annexin-V (0.10 μg) and propidium iodide (50 μg/ml) and analysed by flow cytometry. Untreated lymphoblasts kept under similar condition served as control.

IMMUNOELECTRON MICROSCOPY

ALL blasts were washed with 0.25 M PBS and resuspended in 3% glutaraldehyde in PBS for 4 h at 4°C. Cells were again washed with PBS, resuspended in 6% glutaraldehyde for 3 h at 4°C and post fixed with 2% osmium tetraoxide (OsO_4) overnight at room temperature. Cells were washed, pelleted and solid blocks were prepared using 2% agarose. Sample was dehydrated with increasing concentration of ethanol (50–100%). Spur infiltration was done and polymerized at 70°C for 48 h. Ultra thin sections (golden coloured) were cut using an ultra-microtome (Lieca Ultracut UC6) and collected in nickel grids. Grids were incubated for 10 min with 10% H_2O_2, rinsed in distilled water, and treated with 2% bovine serum albumin in 0.1 M PBS for 30 min to minimize nonspecific staining. Sections were incubated for 1 h with GD3 (R24) or 9-*O*-AcGD3 (MT-6004) monoclonal antibodies followed by anti-mouse polyclonal antibodies (rabbit IgG, Cappel). Sections were thoroughly washed and incubated with 10-nm gold-conjugated protein-A (Sigma). Sections were counter-stained with 2% uranyl acetate (5 min) and lead citrate (3 min) and then analyzed and photographed using transmission electron microscope (Tecnai G2 Spirit Bio Twin, FEI, Netherlands) and images were taken using SIS ProTEM software. At least six grids for each condition were examined, counting 10–20 sections. When magnification was 60,000, three to five mitochondria per field were observed. Controls with secondary antibodies and gold-labelled protein-A were performed, and no signal was obtained (not shown).

STATISTICAL ANALYSIS

Results are reported as mean ± SD. All statistical analyses were done using graph pad prism software (San Diego, CA).

RESULTS

EXPRESSION OF ENHANCED 9-*O*-AcGD3 IN LYMPHOBLASTS

Equal number of cells (1×10^8) from individual patients with B-, T- and mixed lineage ALL at presentation of the disease, normal PBMC and MOLT-4 cells were tested for the expression of GD3 and 9-*O*-acetyl GD3 by ImmunoTLC. A representative profile of GD3 (Fig. 1A) and 9-*O*-acetyl GD3 (Fig. 1B) shows that ALL lymphoblasts and MOLT-4 cells express both GD3 and 9-*O*-acetyl GD3. Densitometry of the stained TLC plates allowed an approximation of the level of GD3 in lipid extracts of ALL lymphoblasts (0.8-fold), MOLT-4 cells (2.1-fold) as compared to that in normal PBMC (Fig. 1C). The expression of 9-*O*-AcGD3 in ALL lymphoblasts was 5.3-fold and that in MOLT-4 cells was 14.3-fold more than that of normal PBMC (Fig. 1D). Therefore, 9-*O*-acetyl GD3 but not GD3, is upregulated in ALL lymphoblasts.

For direct comparison of GD3 and 9-*O*-acetyl GD3 levels, cellular lipid extracted from equal number of ALL blasts and normal PBMC were passed through a LC 18, VISIPREP column. Bound gangliosides were eluted, separated on HPTLC Si 60 plates and stained with α-napthol (Fig. 1E). The predominance of 9-*O*-AcGD3 expressions was observed in ALL lymphoblasts. In contrast, the GD3 expression can be barely seen in these lymphoblasts in comparison to normal cells.

9-*O*-AcGD3 FAILS TO INDUCE APOPTOSIS OF LYMPHOBLASTS UNLIKE GD3 AND SUPPRESSES GD3-INDUCED APOPTOSIS

GD3 is well known as a lipid mediator of the apoptotic pathway [De Maria et al., 1997]. To investigate whether GD3 can induce similar effects in ALL lymphoblasts, we administered exogenous GD3 to lymphoblasts cultured as described before. As shown in Figure 2A, lymphoblasts exposed to GD3 showed a similar pattern of nuclear condensation with Hoechst staining as cells treated with a known inducer of apoptosis, etoposide. Blebbing of the lymphoblast nucleus was observed in GD3 treated cells whereas in untreated cells, the nuclei appeared as discrete blue spots. Therefore, GD3 induces apoptosis in ALL lymphoblasts. However, lymphoblasts exposed to equimolar concentration of 9-*O*-acetyl GD3 under similar experimental conditions, lacked apoptotic nuclei.

Furthermore, degree of apoptosis in lymphoblasts treated with equimolar concentrations of GD3 or 9-*O*-AcGD3 was determined from the sub G0/G1 peak by staining DNA with propidium iodide. Propidium iodide binds to both DNA and RNA by intercalating between the bases with little or no sequence preference and generally used to stain DNA for cell cycle studies. On exposure to GD3 percentage of lymphoblasts in the sub G0/G1 phase was 10.4 ± 2.3 as opposed to 1.4 ± 0.67 in untreated cells. The percentage of DNA in the apoptotic region was reduced to 3.8 ± 1.4, when cells were exposed to an equimolar concentration

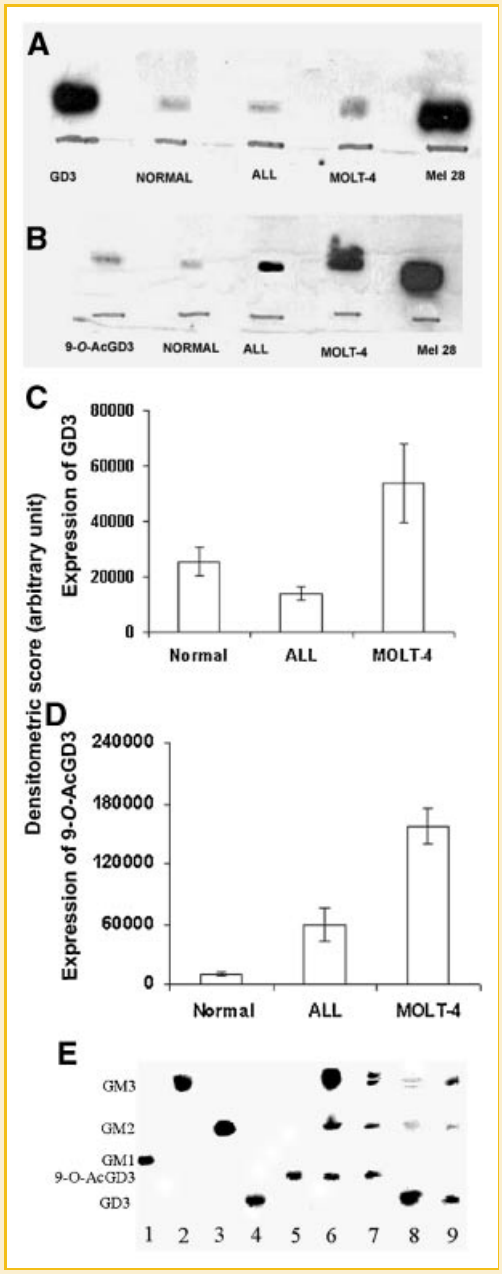

Fig. 1. Expression of GD3 and 9-*O*-AcGD3 in normal PBMC, cell lines and ALL lymphoblasts A,B: Gangliosides were extracted separately from equal number (1×10^8) of lymphoblasts (n = 7), normal PBMC (n = 7) and MOLT-4 cells, separated on HPTLC Si 60 plates in chloroform/methanol/$CaCl_2$ (0.2% w/v; 2:1:0.2, v/v/v) and analyzed by immuno TLC as described in Materials and Methods Section. Standards GD3 and 9-*O*-AcGD3 along with gangliosides extracted similarly from Mel 28 cells (5×10^5) were run in parallel. A representative profile of immunoTLC with anti-GD3 mAb (A) and anti-9-*O*-AcGD3 mAb (B) is shown. C,D: Densitometric quantitation of GD3 (C) and 9-*O*-AcGD3 (D) in lipid extracts from normal PBMC, MOLT-4 and ALL lymphoblasts obtained from immuno TLC. Data are mean ± SD of three independent experiments. E: Lipid extracted from equal number of ALL lymphoblasts and normal cells (1×10^8) were further purified by using column, as described in Materials and Methods, separated on HPTLC plates followed by staining with α-napthol. Lanes 1–5 are standards for GM1, GM3, GM2, GD3, and 9-*O*-AcGD3 respectively. Lanes 6 and 7 are lymphoblasts from two representative ALL patients Lanes 8 and 9 are PBMC from two representative normal donors.

of 9-*O*-acetyl GD3. Additionally, when cells were treated with a combination of both GD3 and 9-*O*-AcGD3 (50 μM each), the apoptotic peak was further decreased (2.6 ± 1.8). Therefore, 9-*O*-acetyl GD3 not only fails to induce apoptosis of ALL lymphoblasts unlike GD3 but also suppresses apoptosis induced by GD3 (Fig. 2B).

9-*O*-AcGD3 IS UNABLE TO INDUCE THE MITOCHONDRIAL APOPTOTIC MACHINERY IN LYMPHOBLASTS

To investigate the mechanism of apoptosis caused by GD3 in intact lymphoblasts, we studied the loss of the mitochondrial membrane potential (ψ_m) that is an early event in cellular death. In the healthy cells with high ψ_m, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In unhealthy or apoptotic cells, with low ψ_m, JC-1 remains in the monomeric form, which shows only green fluorescence. The ratio of red and green fluorescence intensity, that is, 585/530 represents J-aggregates within the mitochondria versus monomer in the cytosol and is positively correlated with the $\Delta\psi_m$ [Malisan et al., 2002].

When the cells were exposed to exogenous GD3, the ψ_m dropped within 30 min and a complete collapse after 2 h of incubation was observed as compared to the untreated cells (Fig. 3A). On the other hand, exogenous addition of 9-*O*-acetyl GD3 failed to cause a drop in the mitochondrial membrane potential when monitored up to 4 h. Taken together; the data indicates that although GD3 caused drastic depolarization of mitochondria even after 30 min, 9-*O*-acetyl GD3 had no effect.

We further examined the apoptogenic factors involved in GD3 induced cell death. As observed by Western blot analysis, exposure of GD3, disruption of the outer mitochondrial membrane by apoptotic stimuli resulted in the release of cytochrome *c* from mitochondria of the lymphoblasts (Fig. 3B) as compared to untreated lymphoblasts. The release was comparable with cells exposed to etoposide that served as positive control. In contrast, 9-*O*-AcGD3 exposures failed to release detectable amounts of cytochrome *c* in the cytosol of the lymphoblasts (Fig. 3B). Once released in the cytosol, cytochrome *c* and ATP serve as cofactors for the apaf-1-mediated activation of caspase 9 [Saleh et al., 1999].

Caspase 9 and caspase 3 activity was measured using BD ApoAlert Caspase assay Plates (Fig. 3C). In GD3-exposed cells, caspase 9 activity increased 7.4-fold as compared to the untreated cells. GD3 had no effect on caspase 9 in the presence of caspase 9 inhibitor. In contrast, in 9-*O*-AcGD3 treated cells caspase 9 activity was negligible and comparable to untreated cells. Similarly, caspase 3 activity was markedly higher (eightfold) in cells exposed to GD3 than in 9-*O*-AcGD3 treated cells (Fig. 3D). In presence of specific inhibitors for caspases, activity was minimal. Therefore, 9-*O*-AcGD3 does not trigger the apoptotic pathway induced by GD3, involving the mitochondria in ALL lymphoblasts. In other words, 9-*O*-AcGD3 fails to induce the mitochondrial apoptotic machinery in these lymphoblasts.

IN SITU DEACETYLATION OF ENDOGENOUS 9-*O*-AcGD3 RESTORES EFFECT OF GD3 IN ALL LYMPHOBLASTS

To show the effect of de-*O*-acetylation on the role of 9-*O*-AcGD3, we first cultured ALL lymphoblasts with 5 mM sodium salicylate or an equimolar concentration of butyric acid for 24 h. Figure 4A shows

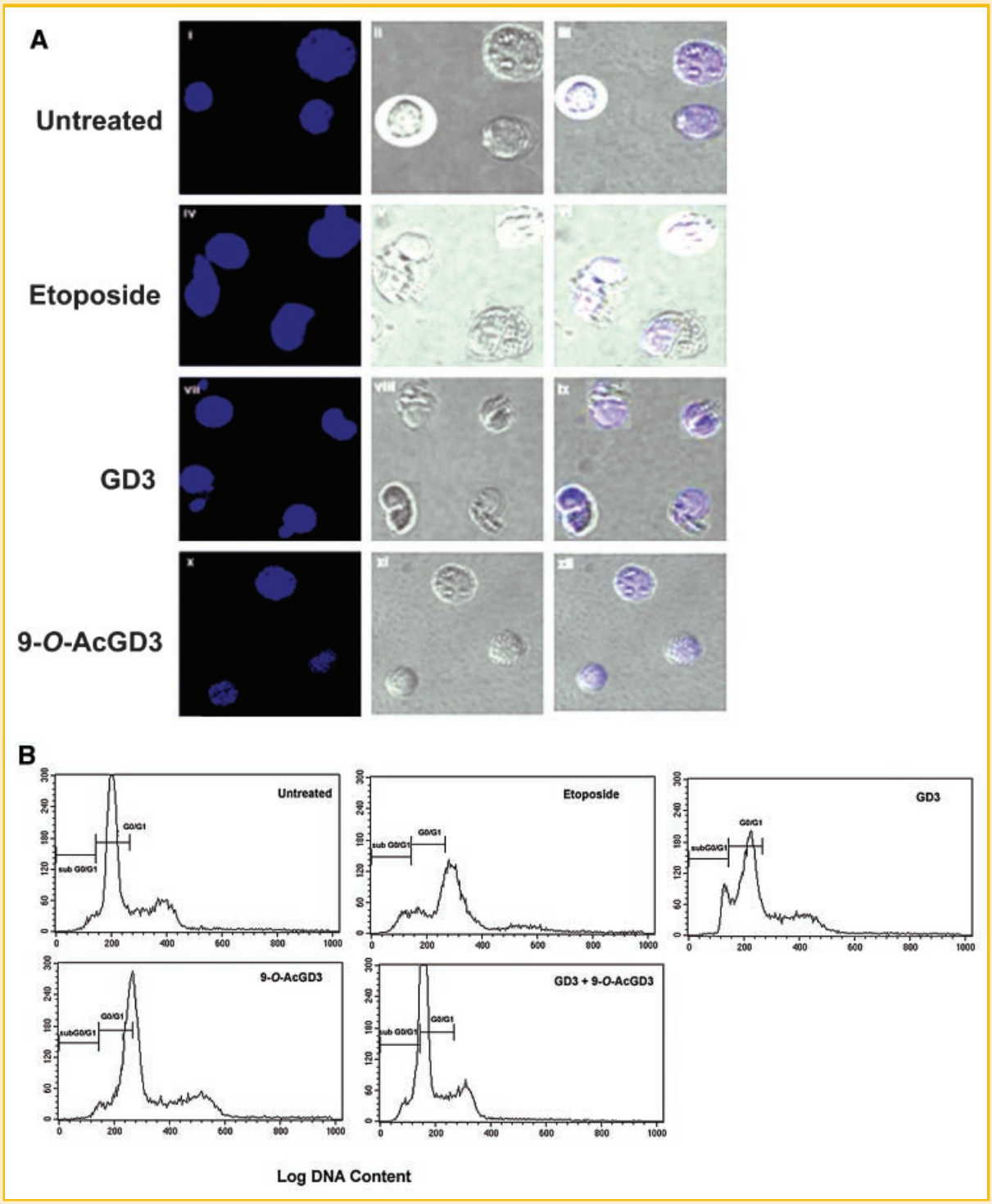

Fig. 2. 9-*O*-AcGD3 fails to induce apoptosis unlike GD3 in intact lymphoblasts (A): Lymphoblasts from ALL patients (1×10^6/ml) were incubated with GD3 (100 μM), 9-*O*-AcGD3 (100 μM) or etoposide (1.0 μM) separately for 24 h, washed and loaded with Hoechst 33342 for 45 min at 25°C as described in Materials and Methods Section and nuclear morphology was analyzed by confocal microscopy. Fluorescence, phase contrast and overlay of the previous two images of the untreated cells (i–iii), etoposide treated cells (iv–vi), GD3 treated cells (vii–ix) and 9-*O*-Ac-GD3 treated cells (x–xii) are shown. Data are representative of three independent experiments. B: Lymphoblasts (1×10^6/ml) were exposed to equimolar concentrations (100 μM) of GD3 and 9-*O*-AcGD3 separately or a combination of GD3 and 9-*O*-AcGD3 (50 μM of each) for 24 h. DNA fragmentation was studied by propidium iodide staining. A representative histogram of lymphoblasts (untreated), exposed to etoposide, GD3, 9-*O*-AcGD3 and combination of GD3 and 9-*O*-AcGD3 (GD3 + 9-*O*-AcGD3) are shown. Data was analyzed using the Cell Quest Pro software. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

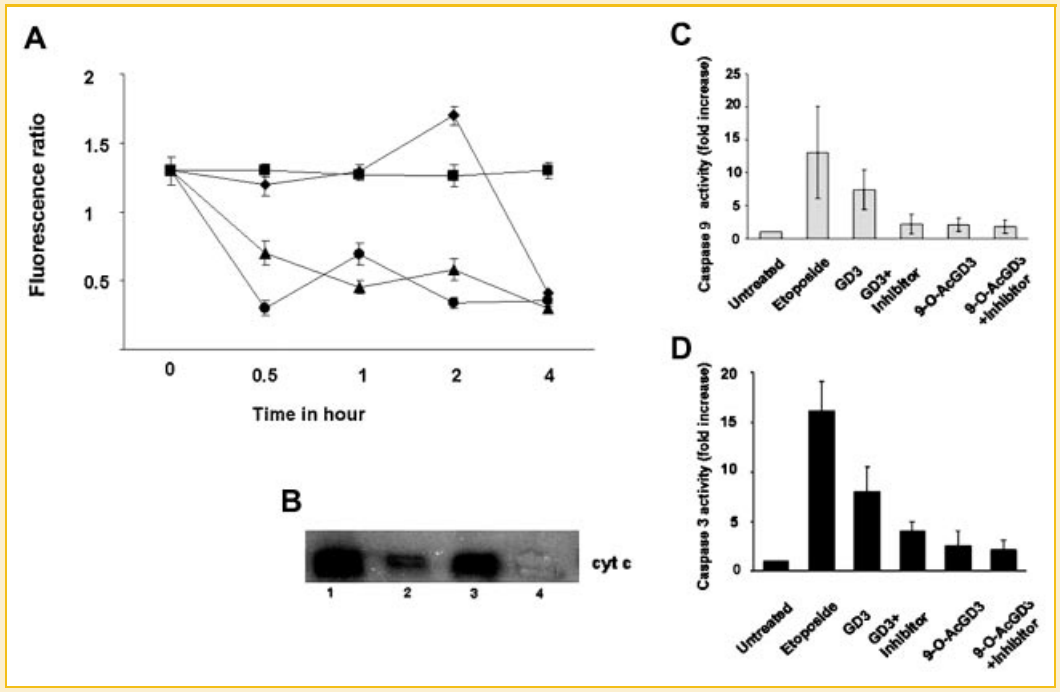

Fig. 3. 9-*O*-AcGD3 is unable to induce mitochondrial changes in lymphoblasts unlike GD3. A: Lymphoblasts (1×10^6/ml) were cultured in absence or presence of equimolar concentrations (100 μM) of GD3 (▲) or 9-*O*-AcGD3 (◆) separately for 0–4 h, washed and probed with JC-1 (10 μg/ml) for 30 min at 37°C and analyzed by a flow cytometer as described in Materials and Methods Section. The ratio of 585/530 nm, that is, J-aggregates in the mitochondria versus monomers in the cytosol represents mitochondrial membrane potential ($\Delta\psi_m$). In parallel, untreated lymphoblasts (■) were exposed to etoposide (1.0 μM; ●) and processed similarly that served as negative and positive controls respectively. B: Lymphoblasts were exposed without or with equimolar concentrations (100 μM) of GD3 and 9-*O*-AcGD3 separately for 30 min. Cytosolic fractions with equal protein content (50 μg) were electrophoresed and immunoblotted for cytochrome *c* with anti-cytochrome *c* mAb as described in the Materials and Methods Section. Lanes 3 and 4 shows a representative profile of the status of cytochrome *c* in lymphoblasts exposed with GD3 and 9-*O*-acetyl GD3 respectively. Lane 2 is the unexposed cells. In parallel, etoposide (1.0 μM) exposed cells were analysed similarly that served as positive control (lane 1). C,D: Lymphoblasts were exposed with the equal concentration of either GD3 or 9-*O*-AcGD3 (100 μM) and assayed for activation of caspase 9 (C) and caspase 3 (D) by using the BD ApoAlert Caspase assay Plates according to the manufacturer's instructions. The activated caspases was measured as increase in fluorescence using a spectrofluorometer. Data are mean ± SD obtained from three independent experiments. To check the specificity, the experiment was repeated in presence of specific caspase inhibitors (provided in the kit). In parallel, etoposide (1.0 μM) exposed cells were checked similarly that served as positive control.

accumulation of GD3 in salicylate treated cells (lane c, d) against the control drug, butyric acid at two different concentration (Fig. 4A, lane a, b). Sodium salicylate has sufficient nucleophilic activity and therefore able to de-*O*-acetylate *O*-AcGD3 to GD3; hence accumulation of GD3 is detected by immunoTLC with GD3 specific antibody (Fig. 4A). Decrease in the 9-*O*-AcGD3 expressions in de-*O*-acetylated cells was further confirmed by flow cytometry by staining cells with anti-9-*O*-AcGD3 specific antibody (Fig. 4B).

Furthermore, to show whether de-*O*-acetylation has any effect on the viability of the cells, the sodium salicylate-treated cells were kept in serum-deprived condition (2% FCS) for 24 h and checked for apoptosis by annexin V and propidium iodide staining. The apoptotic index in untreated cells, kept in serum-deprived condition, was only 3.18 ± 2.6%. Under identical condition, a dramatic increase in annexin V and propidium iodide positivity (44.08 ± 10.5%) was observed in salicylate treated cells (Fig. 4C).

BOTH GD3 and 9-*O*-AcGD3 LOCALIZE TO MITOCHONDRIA IN ALL LYMPHOBLASTS

Recent reports show physical interaction and accumulation of GD3 in mitochondria in intact cells when exposed to various apoptotic stimuli [Colell et al., 2002; Garcia-Ruiz et al., 2002]. To investigate whether 9-*O*-AcGD3 shares its localization with GD3 in the mitochondria in lymphoblasts, immunoelectron microscopy was used. Localization of GD3 and 9-*O*-AcGD3 in the mitochondria was observed by staining sections with anti-GD3 mAb, and anti-9-*O*-AcGD3 mAb respectively followed by secondary anti-murine antibodies and detected by labelling with protein-A gold (Fig. 5A,B). After careful screening of the immuno-gold labelled sections, the clustered pattern of grains of gold conjugated to protein-A appeared to be distributed on transverse and longitudinal sections of mitochondria in the ALL lymphoblasts. Thus both GD3 and 9-*O*-AcGD3 localize in the mitochondria in ALL lymphoblasts.

DISCUSSION

Acetylation is a covalent modification of biological molecules, which may profoundly affect their stability, specificity and function. Although enhanced expression of 9-*O*-acetylated sialoglycoproteins on lymphoblasts has been shown to play an important role in the survival of lymphoblasts [Ghosh et al., 2005a,b], little is known

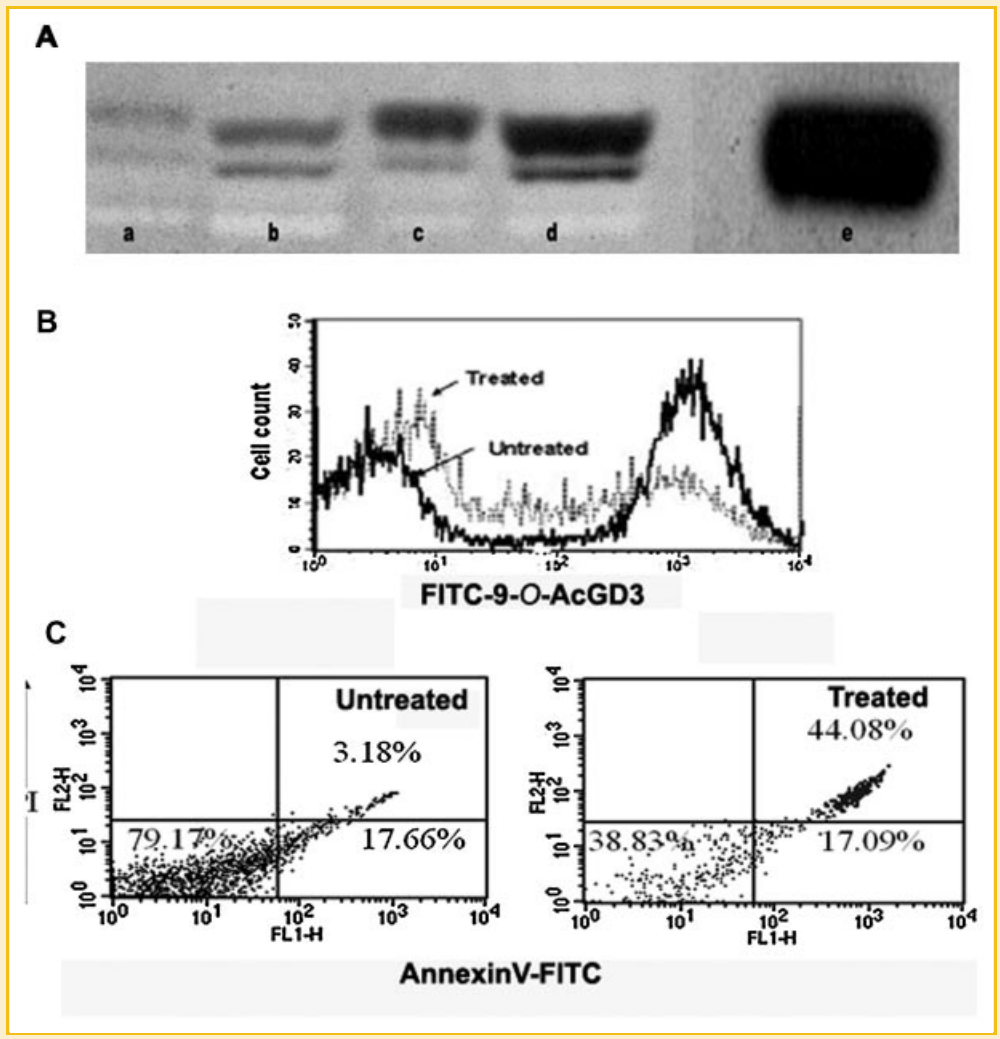

Fig. 4. In situ de-*O*-acetylation of 9-*O*-AcGD3 induces apoptosis. A: Lymphoblasts were cultured for 24 h with 5 mM salicylate for 24 h to de-*O*-acetylate the *O*-acetyl GD3. Degree of de-*O*-acetylation was determined qualitatively by immuno staining the HPTLC plates with anti-GD3 mAb as described in Materials and Methods Section before. Additionally, to confirm the specificity of the salicylate action towards de-*O*-acetylation of sialic acid, we have similarly treated the lymphoblasts with equimilar concentration of another drug "butyric acid," structurally related with salicylate, as a control. Result is representative of three independent experiments. Lanes represent accumulation of GD3 of lymphoblasts cultured with 3 μl/2 × 10^6 cells of control drug (a) 8 μl/2 × 10^6 cells of control drug (b), 3 μl/2 × 10^6 cells of sodium salicylate (c), 8 μl/2 × 10^6 cells of sodium salicylate (d), GD3 standard (3 μg; e). B: Lymphoblasts were similarly, as mentioned in (A), cultured for 24 h in absence and presence of salicylate (5 mM) for 24 h to de-*O*-acetylate the *O*-acetyl GD3. Degree of de-*O*-acetylation was determined from the decrease in 9-*O*-acetyl GD3 expression before and after treatment by FACS analysis using anti-9-*O*-AcGD3 antibody. Result is representative of three independent experiments. C: Lymphoblasts were pre-treated with 5 mM salicylate for 24 h to de-*O*-acetylate the *O*-acetyl GD3. The de-*O*-acetylated cells and untreated cells were then cultured under serum-deprived condition for 24 h and apoptosis induced was determined by annexin V and PI staining as described in Materials and Methods Section. A representative dot plot of three independent experiments is shown.

about the role of GD3 and 9-*O*-acetyl GD3 in the survival of these cancer cells.

In the present study, we have demonstrated an enhanced expression of 9-*O*-AcGD3 on lymphoblasts from childhood ALL patients, irrespective of their lineages (Fig. 1). The cellular levels of 9-*O*-AcGD3 are many fold higher in lymphoblasts from ALL patients as well as in MOLT-4 cells both by direct quantitative analysis of total lipid (Fig. 1E) as well as by ImmunoTLC (Fig. 1B,D). In contrast, minimal expression of *O*-acetyl GD3 has been observed in normal PBMC population (Fig. 1E). Thus, it may be envisaged that the *O*-acetylation machinery is upregulated in the disease condition. This is also corroborated by enhanced *O*-acetyltransferase activity in these lymphoblasts [Mandal et al., 2008]. Possibly, it is the underlying fine-tuning of *O*-acetyltransferases and *O*-acetylesterases that maintains the ratio of the *O*-acetylated and non-*O*-acetylated gangliosides and hence may be involved in apoptosis. The status of GD3 and 9-*O*-AcGD3 in ALL patients drives us to explore the functional implication of this enhanced *O*-acetylation in ALL disease biology.

An apoptotic role of GD3 was observed when freshly isolated lymphoblasts from ALL patients were exposed to exogenous GD3. This was corroborated by nuclear condensation pattern in the GD3 exposed lymphoblasts similar to the cells treated with etoposide. In contrast, no nuclear condensation was observed when these cells

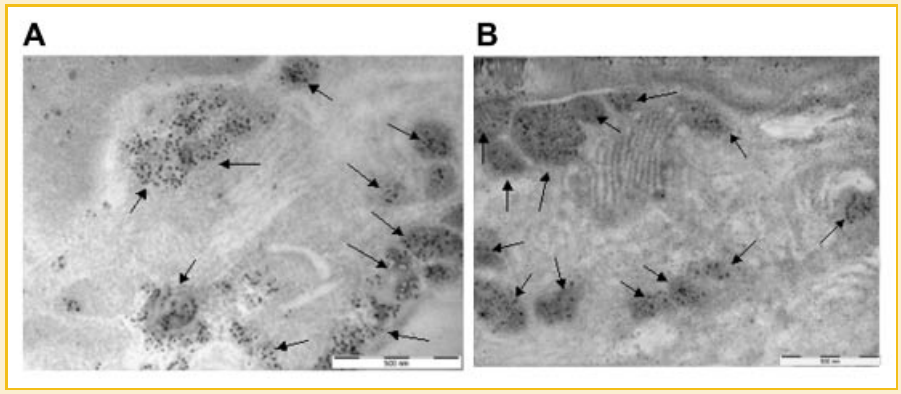

Fig. 5. Immunoelectron microscopy. Lymphoblasts were fixed and processed for the immunoelectron microscopy as described in the materials and methods. Sections were stained with anti-GD3 (A) and anti-9-*O*-AcGD3 (B) monoclonal antibodies separately followed by corresponding secondary antibodies and gold conjugated protein-A (8–10 nm). Representative photomicrographs of GD3 and 9-*O*-AcGD3 of at least five independent experiments per probe performed are shown. Bar is 500 nm. Both GD3 and 9-*O*-AcGD3 are detectable as black grains on mitochondrial membranes in the boxed region. Mitochondrial localization is evident from the pattern of grains of protein A-gold.

were exposed with an equimolar concentration of 9-*O*-AcGD3 under identical condition, suggesting opposite effect of these two gangliosides on ALL cells (Fig. 2A).

In order to select the optimum concentration of GD3, lethal to the lymphoblast, we have studied a dose-response curve at 0–200 μM concentrations. Interestingly, GD3 at 50 μM concentration could initiate the apoptotic program as denoted by the appearance of apoptotic peak at subG0/G1 region against untreated cells (data not shown) and the effects were surged at 100 μM (Fig. 2B), considered to be the upper limit of the dose. Similar range of GD3 concentration has been reported to induce early apoptotic events in isolated mitochondria [Rippo et al., 2000; Malisan et al., 2002]. To understand whether 9-*O*-AcGD3 affects the proapoptotic role of GD3, lymphoblasts were exposed to both GD3 and 9-*O*-AcGD3 simultaneously at equimolar concentration (50 μM), keeping in mind the threshold dose of GD3 that initiates the apoptosis instead of a state of saturation where the observed phenotypic changes become irreversible. Although in isolated mitochondria, the presence of *O*-AcGD3 did not influence the proapoptotic effect of GD3 when both gangliosides were added simultaneously [Malisan et al., 2002], 9-*O*-AcGD3 suppressed the apoptotic peak induced by GD3 in lymphoblasts (Fig. 2B). Therefore, it may be envisaged that *O*-acetylation can actually protect the leukaemic cells from undergoing apoptosis.

This observation was further confirmed, as GD3 exposed lymphoblasts showed collapse of the mitochondrial membrane potential, where as 9-*O*-acetyl-GD3 did not show such effect (Fig. 3A). GD3-induced apoptosis was additionally substantiated by the release of apoptotic factors like cytochrome c in the cytosol from the mitochondria. As expected, 9-*O*-AcGD3 was unable to release cytochrome *c* (Fig. 3B), did not result in the activation of caspase 9 (Fig. 3C) and caspase 3 (Fig. 3D), unlike GD3.

It has been previously shown that *O*-acetylation suppresses proapoptotic action of GD3 [Malisan et al., 2002]. In an attempt to confirm that 9-*O*-acetyl GD3 counteracts the apoptotic role of GD3 in ALL, the lymphoblasts were de-*O*-acetylated exogenously. Sodium salicylate, a nucleophilic drug, effectively de-*O*-acetylated leukaemic cell line and resulted in loss of 9-*O*-AcGD3 to pro-apoptotic GD3 (Fig. 4A,B). These de-*O*-acetylated cells were found to be more prone to apoptosis when induced by limited serum deprivation (Fig. 4C). It can be assumed that total cellular ratio of GD3 and 9-*O*-acetyl GD3 alone might not be decisive. The localization at sub cellular compartments might rather be involved in mediating the apoptotic effect. However, the protective effect of 9-*O*-AcGD3 in leukaemia is established.

It has been proposed that interaction of GD3 with the mitochondria is essential for GD3-mediated effects in intact cells. To investigate whether 9-*O*-acetyl GD3 also localizes in the mitochondria but fails to trigger apoptosis or fails to be transported to the mitochondria, we used immunoelectron microscopy. It is observed that both GD3 and 9-*O*-acetyl GD3 localize to the mitochondria in ALL blasts (Fig. 5A,B). Recent demonstration of existence of ''raft'' like micro-domains and their role as apoptotic controllers in the mitochondria is exciting and such phenomena could be expected with the *O*-AcGD3 interactions [Garofalo et al., 2007]. In other words, the study suggested that ''raft'' like micro-domains on mitochondria could represent preferential sites, where some key reactions can be catalysed contributing to cell death execution steps. It would be interesting to study the underlying events of *O*-AcGD3 in the microenvironment in eliciting the anti-apoptotic reactions. Although they are specifically located on the mitochondria, the presence of some gold labelling in the endosomes and golgi is also observed as expected. It is still an open question whether 9-*O*-AcGD3 is targeted to other cellular locations under stress where it can trigger anti-apoptotic signals.

Acetylation of GD3 prevents the mitochondrial changes either because the hypothetical mitochondrial receptor fails to recognize or has a weaker affinity to the acetylated sialic acid [Chen and Varki 2002; Morales et al., 2004]. This study shows that acetyl GD3 can bind to this hypothetical mitochondrial receptor as it actually localizes to the mitochondria.

Currently, efforts are being made to create a primary cell line from ALL patients by transfecting a viral *O*-acetyl esterase, an enzyme that specifically cleaves the *O*-acetyl moiety of sialic acid, so that the role of *O*-acetylation in ALL can be explored in further details. In conclusion, this report shows that *O*-acetylation of sialic acid can be

an effective strategy adopted by the lymphoblasts to escape GD3 accumulation and enhance survival, which emphasizes the search and stresses the importance of the search for new drugs with more specific de-*O*-acetylating property for future application in therapy.

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