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Decrease in cell surface sialic acid in etoposide-treated Jurkat cells and the role of cell surface sialidase

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The present study investigated the mechanism underlying alterations of cell surface sugar chains of Jurkat cells by inducing apoptosis with etoposide, an inhibitor of topoisomerase II. Within 3 h of etoposide treatment, flowcytometric analysis revealed a decrease in *Maackia amurensis* agglutinin recognized α2,3-linked sialic acid moieties and an increase in *Ricinus communis* agglutinin recognized galactose. The results suggested that asialo-sugar chains on glycoconjugates were rapidly induced on the etoposide-treated cell surface. To clarify the desialylation mechanism, we studied α2,3-sialyltransferase mRNA expression and the activity of sialidase on the cell surface during etoposide-induced apoptosis. The expression of hST3Gal III and hST3Gal IV mRNAs were down-regulated and sialidase activity on the cell surface increased threefold within 2 h of etoposide treatment. Moreover, the decrease in α2,3-linked sialic acid levels was significantly suppressed in the presence of 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, an inhibitor of sialidase. These results suggested that activation or exposure of sialidase on the cell surface was induced by etoposide treatment and was the main cause of the decrease in sialic acids.

Keywords: **sialidase, sialyltransferase, apoptosis, Jurkat cells, etoposide**

Introduction

Apoptosis, or programmed cell death, is a form of physiological cell death induced by various external and internal cellular signals. Apoptosis induces several cellular changes, including membrane blebbing, condensation of chromatin along the nuclear membrane and the generation of membrane-enclosed apoptotic bodies [1]. In addition, specific changes in cell surface molecules have been observed during apoptosis, including the expression of phosphatidylserine and certain proteins, and changes in sugar chains on glycoproteins and glycolipids [2–4]. These changes on the cell surface in turn contribute to a number of different biological functions. The sugar chains on many glycoproteins and glycolipids on the cell surface play important roles in such cell functions as cell-cell interaction and cell differentiation [5–10].

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Among the various sugar chains on the cell surface, sialic acids are known to be ubiquitously expressed on the non-reducing ends of sugar chains of glycoproteins and glycolipids in tissue and to be key determinants for a large variety of biological processes, including cell-cell communication and cell matrix interactions [9,10]. Additionally, sialic acids also modulate the reactivities of ligands and their receptors via their negative charges and bulky structures. The removal of sialic acids from cell surface sugar chains induces phagocytosis, which is in turn mediated by the interactions of asialoglycoconjugates and their receptors.

In the present study, we used flowcytometry to examine sialic acid and galactose levels on cell surface sugar chains of Jurkat cells, a human T cell line, undergoing apoptosis induced with etoposide, an inhibitor of topoisomerase II. In addition, we assessed the activity of cell surface sialidase and the expression levels of Galβ1,3/4GlcNAc α2,3-sialyltransferase (hST3N or hST3Gal III) and Galβ1,3GalNAc/Galβ1,-4GlcNAc α2,3-sialyltransferase (hST3O/N or hST3Gal IV) mRNA, which are predominantly expressed in Jurkat cells in the formation of α2,3-linked sialic acid [11].

Materials and methods

Materials

Diamidinophenyl indole (DAPI), 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (4-MU-NA) ammonium salt and 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) were obtained from Sigma Chemical Co. (St. Louis, MO). Etoposide was purchased from Wako Pure Chemical Co. (Osaka, Japan). The culture medium, RPMI 1640, was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan), and fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Neuraminidase (*Streptococcus 6646K*) and biotin-conjugated lectins, strongly mitogenic *Maackia amurensis* agglutinin (MAA) [12] and *Ricinus communis agglutinin* (RCA), were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Fluorescein isothiocyanate (FITC) conjugated avidin (from egg white) was obtained from Zymed Laboratories INC. (San Francisco, CA). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Cell line culture conditions

Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of cell viability and LDH release

An estimation of cell viability was performed using the trypan blue dye exclusion test. The percentage of lactate dehydrogenase (LDH) released was defined as the ratio of LDH activity in the medium to that in the sonicated whole cell suspension [13].

DNA fragmentation assays

A fragmentation assay of cellular DNA was performed as described previously [14]. After the cells had been incubated with or without 50 μM etoposide, they were harvested and lysed in 0.5% SDS solution containing 25 mM EDTA and 75 mM NaCl. The fragmented DNA and intact DNA were separated by centrifugation at 100,000 × g for 30 min at 0°C. The DNA content was measured by a fluorometric method using DAPI, as described previously [15]. Percent DNA fragmentation was defined as the ratio of the DNA content in the detergent-soluble supernatant (fragmented DNA) to that in the pellet plus supernatant (total DNA).

Flowcytometric analysis

Biotin-conjugated lectin was added to the cell suspensions (1×10^6 cells/ml) at a final concentration of 10 μg/ml in 1% BSA in PBS (1% BSA/PBS). After the cells had been left to stand on ice for 30 min, they were washed three times with 1% BSA/PBS and re-suspended in FITC-conjugated avidin solution at a final concentration of 10 μg/ml in 1% BSA/PBS. The cell suspensions were then left to stand on ice for an additional 30 min and washed with 1% BSA/PBS and 1×10^4 cells were analyzed using a CytoACE-300 flow cytometer (JASCO, Tokyo).

Northern blot analysis

Northern blot analysis for sialyltransferase mRNA was performed according to Taniguchi *et al*. [16]. Fragments generated by PCR amplification of cDNA (for hST3Gal III: K562 cDNA and for hST3Gal IV: COLO201 cDNA) using specific primers were used as the probes in the Northern blot analysis. The specific primers were 5'-ATGGGACTCTTGGTATTTGTGCGCAAT-3' and 5'-TCAGATGCCACTGCTTAGATCAGTGATGAC-3' for hST3Gal III, 5'-ATGCGTCTCTTCTACCCTGAATCT-3' and 5'-TCAGAAGGACGTGAGGTTCTTGAT-3' for hST3Gal IV. The amplification products were cloned into TA cloning vector (Invitrogen, CA) and were verified by sequence analysis. PCR was performed as described previously [16]. Five μg of total RNA, extracted by the RNA zol B (Biotex Laboratories, TX), was separated on a formaldehyde agarose gel, and capillary transferred to a nitrocellulose membrane. The sialyltransferase cDNA fragments were labeled with ^{32}P using the nick translation system (GIBCO BRL, MD). The hybridized membrane was washed twice at room temperature with 2 × SSC, 0.1% SDS for 10 min, and then twice at 42°C with 0.2 × SSC, 0.1% SDS for 30 min, and exposed to X-ray film for several days at −80°C.

Determination of cell surface sialidase activity

Sialidase activity on the cell surface of etoposide-treated Jurkat cells were determined using 4-MU-NA as a substrate, according to a previously described method [17]. Briefly, etoposide-treated Jurkat cells (5×10^6 cells) were washed with PBS and resuspended in 175 μl of PBS. The resultant cell suspension was then mixed with 25 μl of 0.5 mM 4-MU-NA, and incubated at 37°C for 2 h with occasional mixing. Reaction mixture without cells was used to determine spontaneously degradated substrate. Next, 1 ml of ethanol was added to stop the enzyme reaction, and the reaction mixture was centrifuged at 1000 × g for 15 min. Then, 100 μl of 1 M NaOH was added to 1 ml of the supernatant just prior to the fluorometric determination of released 4-MU at Ex 365 nm and Em 450 nm. The sialidase activity of all samples was estimated using a calibration curve obtained from the data using neuraminidase (*Streptococcus 6646K*).

Results

Etoposide-induced apoptosis in Jurkat cells

Jurkat cells were incubated with 50 μM etoposide, which is an inhibitor of topoisomerase II. As shown in Figure 1, etoposide treatment rapidly induced DNA fragmentation, which then led to a reduction in cell viability. DNA fragmentation occurred at

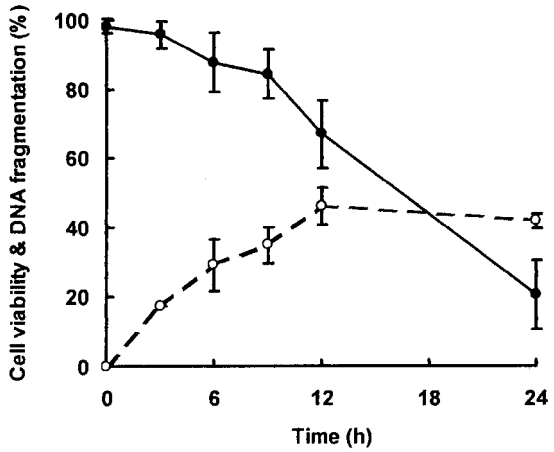

Figure 1. DNA fragmentation and cell viability of Jurkat cells cultured with etoposide. Jurkat cells were incubated with 50 μM etoposide. DNA fragmentation (open symbols) and cell viability (closed symbols) were determined at the indicated times. Data represent mean values for triplicate determinations.

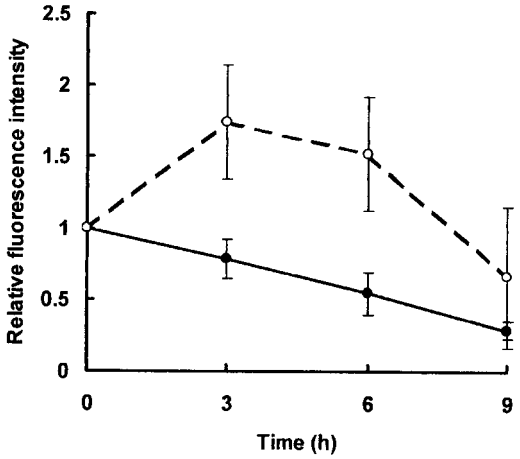

Figure 2. Alteration of cell surface sugar chains in etoposide-treated Jurkat cells. Etoposide-treated Jurkat cells were stained with MAA or RCA and analyzed using a flowcytometer. MAA (closed symbols) and RCA (open symbols) binding to Jurkat cells were determined at the indicated times. Lectin binding is estimated as the relative fluorescence intensity in comparison with those obtained in 0 h incubation as 1.0. Data represent mean values for triplicate determinations.

half the maximum level after 3 h of etoposide treatment; at which point no decrease in cell viability could be detected. Etoposide induced DNA fragmentation more rapidly than cell death.

Flowcytometric analysis of cell surface sugar chains using lectins

To determine α2,3-linked sialic acid levels on the cell surface of etoposide-treated Jurkat cells, we stained the cells using biotin-MAA and FITC-avidin, and then analyzed them using flowcytometry. Etoposide treatment induced a rapid and constant decrease in the fluorescence intensity of MAA-binding to Jurkat cells, which reached half the level of controls and accompanied with a 2-fold increase in RCA-binding after 3 h of etoposide treatment (Fig. 2). During etoposide treatment, Jurkat cells have low levels of *Sambucus nigra bark* agglutinin (SNA)-recognized α2,6-linked sialic acid. The results indicated that the decrease in α2,3-linked sialic acid levels on the cell surface was an early event in etoposide-induced apoptosis. This decrease appeared simultaneously with DNA fragmentation and just prior to cell death.

Northern blot analysis of α2,3-sialyltransferases

Cell surface sugar chains are regulated by the actions of a number of glycosyltransferases and glycosidases. Jurkat cells have been shown to predominantly express hST3Gal III and hST3Gal IV involved in the formation of the NeuAcα2-3Gal linkage, which bind to MAA [11]. In order to detect any alteration in α2,3-sialyltransferase gene expression during etoposide-induced apoptosis, mRNA from etoposide-treated Jurkat cells was probed using hST3Gal III and hST3Gal IV cDNA (Fig. 3). Decreases in expression levels of hST3Gal III

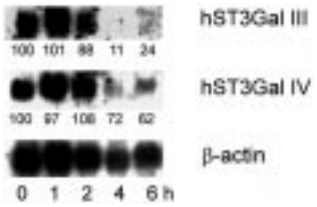

Figure 3. The mRNA expression of hST3Gal III and hST3Gal IV genes in etoposide-treated Jurkat cells. Northern blotting was performed on mRNA extracted from Jurkat cells treated with etoposide at the indicated times. The blot was probed with ^{32}P-labelled hST3Gal III (upper panel), hST3Gal IV (middle panel), or β-actin (bottom panel) cDNA. Values at the bottom of Northern blot indicate % of control (0 min) levels of each mRNA determined by densitometry.

and hST3Gal IV mRNA were detected in Jurkat cells after 2 h and 4 h of etopside treatment, respectively.

Effect of sialidase inhibitor on cell surface sialic acid levels

To estimate the contribution of sialidase to the decrease in α2,3-linked sialic acid levels on cell surface sugar chains during etoposide-induced apoptosis, we investigated the effects of the specific sialidase inhibitor, DANA. Jurkat cells were treated with 50 μM etoposide for 3 h in the presence of 10 μM DANA, and α2,3-linked sialic acid levels on the cell surface were analyzed by flowcytometry using MAA and RCA lectins (Fig. 4). DANA inhibited both the decrease in MAA-binding and the increase in RCA-binding to the cell surface of etoposide-treated Jurkat cells. These results indicated that sialidase played an important role in the decrease in α2,3-linked sialic acid levels during etoposide-induced apoptosis.

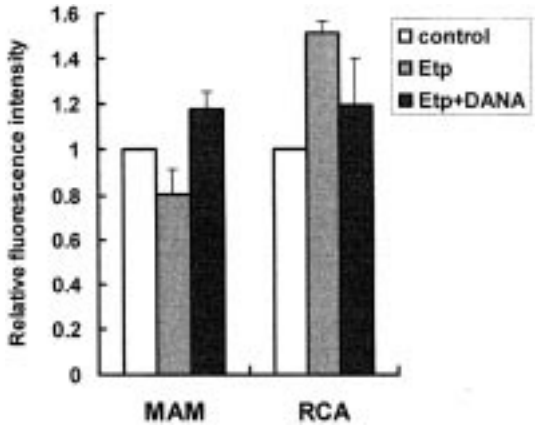

Figure 4. Effect of DANA on MAA or RCA binding to etoposide-treated Jurkat cells. Jurkat cells were cultured for 3 h with medium alone or 50 μM etoposide or 50 μM etoposide and 10 μM DANA. MAA or RCA binding to Jurkat cells were determined by flowcytometry. The values are the relative values obtained by dividing each value by those of non-treated Jurkat cells. Data represent mean values for triplicate determinations.

Determination of sialidase activity on the cell surface of etoposide-treated Jurkat cells

In previous reports, intact sialidase activities in a human myeloid leukemia cell line HL-60 [18] and human neutrophils [19], were measured using 4-MU-NA as a substrate and activated via multiple stimulations. In the present study, we determined sialidase activity on the cell surface of Jurkat cells during etoposide-induced apoptosis (Figure 5). We observed that the activity in these cells increased rapidly, attaining a three-fold increase after 2 h of etoposide treatment. This level was maintained up to 6 h post-treatment. To evaluate membrane permeability, the percentage of LDH and sialidase released were also measured in these samples. The results revealed that LDH released were less than 7% and sialidase released were not detectable in these samples. These findings strongly suggested that cell surface sialidase, modulated by etoposide-induced apoptosis, caused the decrease in α2,3-linked sialic acid levels and that a release of intracellular sialidase contributed to the sialic acid alteration in a lesser degree, if any.

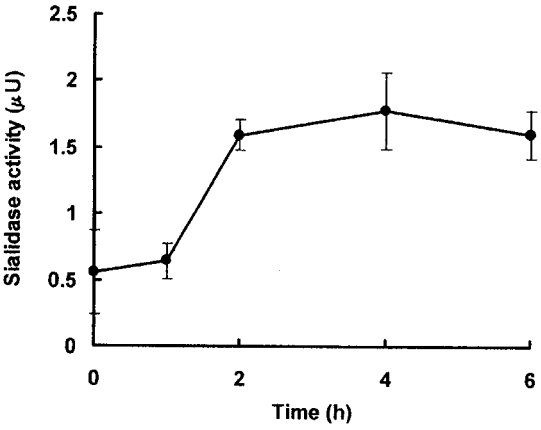

Figure 5. Sialidase activity on the intact cell surface of etoposide-treated Jurkat cells. Etoposide-treated Jurkat cells were harvested at the indicated times and cell surface sialidase activity was determined as described in the Materials and methods section. Data represent mean values for triplicate determinations.

Discussion

The present study described alterations in surface sugar chains in Jurkat cells. The changes resulted from the cells undergoing apoptosis induced by etoposide. Etoposide induced apoptosis in Jurkat cells with a concomitant rapid increase in DNA fragmentation prior to cell death, in a similar manner as we previously described in thymocyte apoptosis [15]. Jurkat cells predominantly expressed mRNA encoding α2,3-sialyltransferase, not α2,6-sialyltransferase [11], and have low levels of SNA-recognized α2,6-linked sialic acid. Recently, the induction of α2,6-linked sialic acid concerned apoptosis have been reported [20,21]. However, the SNA-binding to the cell was not increased during etoposide treatment in this study. Thus, we studied only α2,3-linked sialic acid in Jurkat cells. Flowcytometric analysis using MAA and RCA detected a decrease in α2,3-sialic acid and an increase in galactose levels on the cell surface at an early stage of etoposide-induced apoptosis. The sugar chains in glycoproteins and glycolipids are synthesized by different glycosyltranferases and degraded by different glycosidases. Thus, we investigated the mechanism underlying the decrease in α2,3-sialic acid levels on the cell surface during apoptosis, with respect to sialyltransferases and sialidase. The results demonstrated that etoposide induced a down-regulation of sialyltransferase mRNA expression, in addition to an activation of sialidase in intact cell surfaces.

Sialyltransferases, which transfer sialic acids to terminal positions of sugar chains, have been reported to include several isoenzymes with different properties and substrate specificity [22]. Jurkat cells have been shown to predominantly express two α2,3-sialyltransferases, hST3Gal III and hST3Gal IV [11], in the formation of a NeuAcα2-3Gal linkage, which bind with MAA. We demonstrated decreased expression of mRNA encoding hST3Gal III and hST3Gal IV in Jurkat cells after 2 h of etoposide treatment by northern blot analysis. For these enzymes, decreased expression of mRNA results primarily in a decrease in α2,3-sialylation of N-glycans.

Sialidases catalyzes the removal of sialic acid residues from sialo-glycoconjugates. Several sialidases are present in mammals, and these enzymes have been different in their substrate specificity and optimal pHs. Moreover, the existences of four distinct sialidases have been reported in a subcellular location in rat tissue: intralysosomal [23], cytosolic [24], lysosomal membrane and plasma membrane [25]. However, less information is available on the enzymological and physiological properties of mammalian sialidases than viral and bacterial enzymes, due to their instability and low activity. Sialidase activity in intact cell surface of HL60 cells [18] and human neutrophils [19], measured using 4-MU-NA for

fluorescence substrate, reportedly increases in response to multiple stimulation. We used this method to detect an increase in sialidase activity on the intact cell surface of etoposide-treated Jurkat cells. Recently, Miyagi *et al*. [26] cloned and characterized a plasma-membrane-associated sialidase that can release sialic acid from gangliosides, but not from 4-MU-NA. The sialidase measured using 4-MU-NA as a substrate in the present study is distinctly different from the enzyme cloned by Miyagi, in terms of substrate specificity. Although we measured the plasma membrane-associated sialidase activity in etoposide-treated Jukat cells using gangliosides as a substrate, no significant changes in sialidase activity could be detected (data not shown). Cross *et al*. [19] reported that activation of neutrophil surface sialidase is associated with a mobilization of endogeneous sialidase into the plasma membrane, suggesting that activation of intact cell surface sialidase was caused by mobilization of the enzyme on the cell surface in a relatively short period of time after etoposide treatment. In addition, early exposure of phosphatidylserine on the outer apoptotic cell membrane is caused by external flip-flop transport of internal phosphatidylserine [27]. This trans-membrane movement of phospholipid induces a change in cell membrane conformation during apoptosis. Cell membrane sialidases may be activated under such membrane conformational changes. Further studies must clarify the enzymatic properties of cell membrane-associated sialidases that are activated in early apoptosis.

The increase in RCA binding was observed in flowcytometric analysis was transient (Figure 2). This transient result may reflect the activation of other glycosidase or the inactivation of glycosyltransferase after the activation of intact sialidase at an early stage of apoptosis, given that the increase in the intact sialidase was not transient (Figure 5). At present, the mechanism underlying these changes remains unclear. Future research will need to clarify this mechanism and the function of glycosidase and glycosyltransferase.

A number of sugar chains on the cell surface have multiple functions. The desialylation of etoposide-treated Jurkat cells may unmask some portion of signal transduction through cell to cell interaction. In particular, exposed galactose on the cell surface binds to galectins, galactose-binding lectins, and to asialoglycoprotein receptors on the cell surface. Changes in cell surface sugar chains are thought to be responsible for the recognition of apoptotic cells by phagocytes [28–31]. The present results suggested that the expression of galactose in apoptotic cells is caused mainly by an activation of sialidase on intact cell surfaces and partly by a down-regulation of sialyltransferase mRNA. Thus, further research must clarify the regulatory system of sugar chains on the cell surface and the clearance mechanisms of apoptotic cells through altered sugar chains. However, our results are limited to etoposide-induced apoptosis and should not be interpreted to reflect the general process of apoptosis. Hence, the biological significance of our findings should be improved by analysis using different stimuli (e.g. Fas ligation or UV irradiation) and cell populations, in order to evaluate the relationship of this phenomenon and apoptosis.

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