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APOPTOSIS-DEPENDENT CHANGES IN EXPRESSION PATTERN OF mRNA CODING FOR SIALYL-MODIFYING ENZYMES

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mRNA expression levels of seven major cellular sialyl-modifying enzymes (Neu1, Neu2, Neu3, Neu4 sialidases and hST6Gal.I, hST3Gal.III, hST3Gal.IV sialyltransferases) during apoptotic cells death was compared. Identification of enzymes responsible for the processes of superficial desialylation at an apoptosis can become a potential biotechnology for modifying of AC phagocytosis and treatment of autoimmune disorders. Reverse-type PCR analysis to evaluate mRNA expression in human Jurkat T leukemia cells was applied. After 2 h of etoposide-induced apoptosis (when cells were still annexin V-negative), an increased mRNA expression of Neu1, Neu3, Neu4, hST6Gal.I and hST3Gal.IV, and decreased expression of hST3Gal.III mRNA were found. Expression mRNA of the above mentioned enzymes was decreased after 6 and 12 hour of apoptosis induction, when cells gained typical apoptotic characteristics. mRNA level of Neu2 was not significantly changed during the course of apoptosis. Increase of sialidase activity during early steps of apoptosis and decrease afterwards were confirmed by enzymatic fluorometric assay using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuralminic acid as substrate.

Key words: apoptosis, sialylation, neuraminidase, sialyltransferase, mRNA, glycoprotein.

In our previous works, we have shown that glycans, and particularly glycoproteins, of plasma membrane are drastically changed during apoptosis [1]. The main changes included an increase in the exposure level of α-D-mannose and α-D-galactose residues [1], which were the secondary signs to the intense desialylation process on the apoptotic cell membrane, but not in the cell milieu [1]. Decrease in sialic acid residues was also reported by other groups [2]; altered membrane glycol-pattern was proposed to be used for apoptotic cell detection [2–4] and isolation [5]. We also proved that its physiological significance involved recognition of asialylated surface glycans by macrophages and subsequent clearance of apoptotic cells, while artificial desialylation of apoptotic cells dramatically enhance their clearance [6]. Recognition of specific glycans on the surface of apoptotic cells by lectin-like molecules of the immune cells is especially important for the clearance of late apoptotic cells, while inappropriate clearance of apoptotic cells leads to the development of autoimmune disorders [7–9]. All 4 known human neuraminidases (also referred as sialidases) were shown to be associated with cancer [10], surface sialylation level was shown to be tightly connected with tumor cell invasiveness and metastatic potential [11]. These data indicated importance of cell surface sialic status during both cell death and cancer progression, thus finding of molecular target involved in apoptotic cell desialylation can be the basis of novel biotechnology aimed to facilitate apoptotic cell clearance and prevent autoimmune disorders.

Cell surface glycans are a heterogeneous group of complex compounds, most of them are sialylated at the terminal end. The amount of sialic acid derivatives can reach up to 40, while the types of their inter-connection make them a highly variable and poorly characterized group [12]. A set of different enzymes are responsible for the synthesis of this template-independent glycostructures.

Here we compared the mRNA expression levels of 7 enzymes involved in sialic acid modifications during early apoptosis process, namely of 1) sialyl-trimming enzymes, neuraminidase 1 or lysosomal sialidase (Neu1), cytosolic sialidase, N-acetyl-alpha-neuraminidase 2 (Neu2), neuraminidase 3 or N-acetyl-alpha-neuraminidase 3 (Neu3) and neuraminidase 4 or lysosomal sialidase4; and 2) of 3 sialyl-attaching enzymes — sialyltransferase 1 isoform a or CMP-N-acetylneuraminate beta-galactosamide α-2,6-sialyltransferase (hST6Gal.I),

Galα1,3/4GlcNAc α2,3-sialyltransferase (hST3Gal.III) and Galβ1,3GalNAc/Galβ1,-4GlcNAc α2,3-sialyltransferase (hST3Gal IV). Although sialyl transferases are considered to attach sialic acid residues by their transfer to galactosylic residues of glycans, there are reports that this action can be reversed, focused on trimming of terminal sialic residues [13]. The long term goal of current study is the identification of genes of sialyl-modifying enzymes with changed expression at apoptosis for development of biotechnology aimed to facilitate apoptotic cell clearance and prevent autoimmune disorders.

Materials and methods

Cell line and culture conditions. Jurkat leukemia T-cells from the Cell Culture Collection of the Institute of Cell Biology, National Academy of Sciences of Ukraine (Lviv, Ukraine) were used in the research. Cells were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, USA) which was supplemented with 10% heat-inactivated fetal calf serum (Sigma), gentamycin (50 μg/ml, Sigma) and glutamine (50 mM). Apoptosis was induced by etoposide, 1 μM (Bristol-Myers, USA) and controlled by Annexin V/PI staining.

Analysis of mRNA expression. Total RNA was isolated according to Chomchinsky [14]. The quality of RNA isolation was checked by agarose gel-electrophoresis and A260/A280 ration was found to be 1.74±0.1 as determined by NanoDrop ND1000 Spectrophotometer. mRNA was converted to cDNA using «Revert-Aid TM FirstStrand cDNA Synthesis Kit» (Fermentas, Lithuania). RT(reverse-type)-PCR analysis was performed according to [15] using following temperature modes: 4 min at 94 °C, followed by 33 cycles including 30s at 94 °C, 35s at X °C, 30s at 72 °C, afterwards 4 min at 72 °C and cooling to 4 °C; where X = 56 for Neu1, Neu2, Neu3, hST6Gal.I, hST3Gal.IV; X = 54 for Neu3′ and Neu4, X = 65 for hSTGal.III and β-actin.

The following primers were used for PCR amplification (Invitrogen, USA): NEU1, PrimerBank (pga.mgh.harvard.edu/primerbank, [16]) ID 4557791a1, F(forward) (5′ → 3′): GAGGCTGTAGGGTTTGGGTG, R(reverse): GTGTCCACTGAGCCGATCT; NEU2, PrimerBank ID4885515a1, F: CCATGCCTACAGAATCCCTGC, R: GGGTGCGTCGTAGTCTCCT; NEU3, PrimerBank ID 19923368a1, F: CAGGGCTTGAGCTGAGTTGG, R: GGTAACGGCAACGAAAAGAGG; NEU3, PrimerBank ID 19923368a3, F: TGACAGAGGGATTACCTACCG, R: AGCATCCTCATCTCTCCTCGT; NEU4, designed by us, F: CGTCTTCCTCTTCTTCATCG, R: GTAGAAGGCGAAGGAGTGAG; hST6Gal.I, PrimerBank ID 27765091a1, F: TGCTGCGTCCTGGTCTTTC, R: GCTTGAGGATACAGACTGGGAAT; hST3Gal.III [16], F: ATGGGACTCTTGGTATTTGTGCGCAAT, R: TCAGATGCCACTGCTTAGATCAGTGATGAC; hST3Gal IV [17], F: ATGCGTCTCTTCTACCCTGAATCT, R: TCAGAAGGACGTGAGGTTCTTGAT, в-actin: F: AGCATTTGCGGTGGACGATGGAG, R: GACCTGACTGACTACCTCATGA. For primer design, PerlPrimer program (sourceforge.net/projects/perlprimer/) was used, and primer mapping to genome sequence was done by BLAST.

DNA samples were loaded into the dry wells of 2% (w/v) agarose gel. Electrophoresis was carried out in 0.5x TBE buffer, pH 8.0 until the marker dye migrated 6–7 cm. Electrophoregrams were stained with ethydium bromide and screened in transilluminator under UV light and photographed.

mRNA level was estimated based on densitometry data performed with GelPro 4.5 software from Media Cybernetics, it was futher normalized to β-actin level. Significance of the difference in a typical experiment was assessed by Student's *t*-test. The level of significance was set at 0.05. Statistical interpretation of the data was done with Microcal Origin (Microcal Software, Inc., Northampton, MA, USA).

Fluorometric assay for sialidase activity. Sialidase activity was measured, as described [18] with some modification. Briefly, cells (10^7 cells/ml) were suspended in PBS with 5 mM $CaCl_2$ and $MgCl_2$, pH 7.2 in final volume of sample at 190 μl, 10 μl of 0.5 mM 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuralminic acid (4-MUNA, BioSynth, Switzerland) were added and incubated for 3 h. If needed, sialidase inhibitor DANA (Sigma, in final concentarion 15 μM) was added to protein samples. Cleavage of the substrate by sialidase yields the fluorescent product, 4-methylumbelliferone. Substrate used without lysate, served as a blank to determine the nonspecific degradation of the substrate. Cells were centrifuged, supernatant was transferred to new tubes and reaction was stopped by the addition of 1 ml of glycine-carbonate buffer, pH 10.7 and fluorescence was measured (excitation — 325 nm, and emission — 450 nm) using Turner Quantech FM109510 (USA) fluorometer. 4- Methylumbelliferyl standard (Sigma) was used for calibration.

Results and Discussion

Jurkat T-cell used for mRNA study contained the following amount of apoptotic (Annexin V+ / PI-) cells: control — 7.32±0.45%; 2h after etoposide treatment — 8.63±1.33%; 6h after etoposide treatment — 14.31±1.21% and 12 hour after etoposide treatment — 34.26±2.65%. Taking into account the limitation of the methods we considered only mRNA expression values different more than +1.5 fold or less than 1/1.5=0.67 fold of control to be valid.

Level of Neu1 mRNA was significantly (2.61 times of control) increased after 2 hours of etoposide treatment and started to decrease after 6 and 12 hour of treatment (0.48 and 1.31 times, correspondingly) (Fig.1). The level of Neu2 mRNA was not significantly changed during the course of apoptosis. Level of Neu3 mRNA was 1.53 fold increased after 2 hours of etoposide treatment and decreased afterwards. The increase was also observed for Neu4 mRNA (4.55 fold after 2h treatments) with subsequent decrease in mRNA level hST6Gal.I mRNA level was significantly increased at 2nd hour (1.81 fold) and decreased at 6th (0.35) and 12th hour (0.44) of apoptosis induction. hST3Gal.III mRNA was steadily decreased during apoptosis induction (0.80; 0.16 and 0.18 fold correspondingly). hST3Gal.IV activity was significantly increased after 2 h of apoptosis induction (12.53 fold) and decreased afterward (Table).

Determination of sialidase activity in the lysates of Jurkat cells revealed a markedly increased sialidase activity after 2h of apoptosis induction and its subsequent decline during the course of apoptosis (Fig. 2). The data of enzyme activity is in good correspondence with observed change in expression of mRNA of sialidases Neu1, 3 and 4, detected after 2h of apoptosis induction.

Current data suggest an increase in mRNA level of 3 sialidases — Neu1, Neu3 and Neu4 and sialyltransferases hST6Gal.I and hST3Gal.IV at the onset of apoptosis and subsequent decrease afterward. While the decrease of mRNA level the middle and late stages of apoptosis is a logical consequence of apoptotic machinery's action, cleaving multiple intracellular substrates, the increase in mRNA level of some specific enzymes is interesting, especially taking into account previously established fact of increased surface sialidase activity at apoptosis [19, 20]. Interestingly, that enzymatic sialidase activity of Jurkat cell during apoptosis induction correlated with

Level of expression of mRNA coding for sialyl-modyfying enzymes and its change during the course of etoposide-induced apoptosis of Jurkat T-cells

	Intact	Time after apoptosis induction		
		2 h	6 h	12 h
Neu1	1.00	2,61±0.13	0.48±0.31	1.31±0.17
Neu2	1.00	1.13±0.07	1.21±0.19	1.38±0.23
Neu3	1.00	1.53±0.11	0.50±0.15	1.28±0.08
Neu4	1.00	4,55±0.18	1.39±0.19	0.27±0.49
hST6Gal.I	1.00	1.81±0.01	0.35±0.50	0.44±0.49
hST3Gal.III	1.00	0.80±0.16	0.16±0.21	0.18±0.16
hST3Gal.IV	1.00	12,53±0.1	0.16±0.32	0.82±0.12

Notes. $M \pm m$, folds of control and also the mean value of 3 independent experiments on rt-PCT products quantification after the prior normalization to β-actin levels are presented. Significantly ($p < 0.05$) increased values are highlighted dark grey and significantly decreased — light grey.

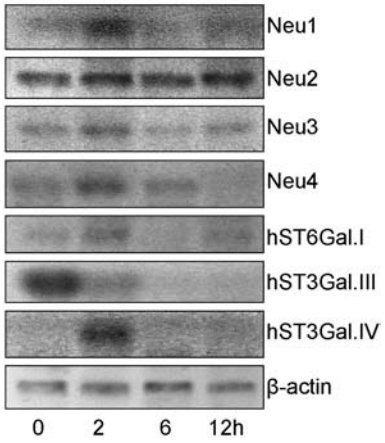

Fig. 1. **Agarose gel electrophoresis of reverse-type PCR products of genes whose products are involved in sialic acid modification during the course of etoposide-induced apoptosis in Jurkat T-cells.** One typical image of 3 replicates is presented

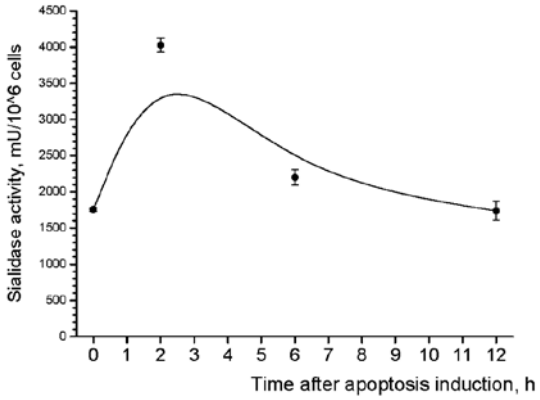

Fig. 2. **Sialidase detection in Jurkat cells suspension during the course of apoptosis induction, measurement by enzymatic digestion of fluorogenic sialidase substrate 4-MUNA.** Presented data are $M \pm m$ of 3 replicates

mRNA expression level, being 450% of control at the 2nd hour after apoptosis onset and only 120% after 12 hours, as shown on Fig.2. An increased mRNA expression level of Neu3 was also reported during apoptosis by group of Prof. Azuma by using real-time PCR and TaqMan system [21], they have also observed the increased in Neu1 mRNA level, however, the observed differences were not significant. The authors related changed sialidase mRNA expression level to ganglioside methabolism, particularly to conversion of GM3 into GD3, probably involved in apoptotic signaling, and preceding surface phosphatidylserine exposure during apoptosis. Our data demonstrated, that not only the expression of Neu3 mRNA is changed at apoptosis, but also of other sialyl-modyfing enzymes, Neu1, Neu4, hST6Gal.I and hST3Gal.IV.

Thus, we can speculate that synthesis of mRNA of some or all of these enzymes can be important at the onset of apoptosis, as sialidase activity (mediated by neuraminidase acting in forward direction or sialyltransferases acting in reverse) is important for modification of apoptotic cells surface starting after 6–12 hours after the onset of apoptosis [3, 22] and subsequent enhancement of cell clearance by macrophages [6]. At the time when cells have gained apoptotic features (phosphatidyl serine externalization, changed cell surface, etc) the mRNA level of sialyl-modifying enzymes was already declined. Other enzymes showed the decrease of their mRNA in the course of apoptosis induction, being the predicted behavior for cell death.

We demonstrated an increase of mRNA level of sialyl-modifying enzymes Neu1, Neu3, Neu4 and hST6Gal.I and hST3Gal.IV after 2 h of apoptosis induction (early apoptotic stage) and their decreased expression, as well as of hST3Gal.III, after 6 and 12 of apoptosis induction (late apoptotic stages). Potential targeting of these enzymes involved in apoptotic desialylationn can serve as novel biotechnology to facilitate apoptotic cell clearance and prevent autoimmune disorders.

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REFERENCES

1. *Bilyy R., Stoika R.* Search for novel cell surface markers of apoptotic cells // Autoimmunity. — 2007. — V. 40, N 4. — P. 249–253.
2. *Batisse C., Marquet J., Greffard A. et al.* Lectin-based three-color flow cytometric approach for studying cell surface glycosylation changes that occur during apoptosis // Cytometry A. — 2004. — V. 62, N 2. — P. 81–88.
3. *Bilyy R. O., Antonyuk V. O., Stoika R. S.* Cytochemical study of role of alpha-d-mannose- and beta-d-galactose-containing glycoproteins in apoptosis // J. Mol. Histol. — 2004. — V. 35, N 8–9. — P. 829–838.
4. *Bilyy R., Nemesh L., Antonyuk V. et al.* Apoptosis-related changes in plasma membrane glycoconjugates of peripheral blood lymphocytes in rheumatoid arthritis // Autoimmunity. — 2009. — V. 42, N 4. — P. 334–336.
5. *WO/2007/053654*. Agglutination-based method for fast detection, isolation and quantification of apoptotic cells / Stoika R., Bilyy R., Antonyuk V. PCT patent application 10/31/2006, Pub. 05/10/2007.
6. *Meesmann H. M., Fehr E.-M., Kierschke S. et al.* Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes // J. Cell. Sci. — 2010. — V. 123, N 19. — P. 3347–3356.
7. *Cline A. M., Radic M. Z.* Apoptosis, subcellular particles, and autoimmunity // Clin. Immunol. — 2004. — V. 112, N 2. — P. 175–182.
8. *Piacentini M.* Apoptosis and autoimmunity: two sides to the coin // Cell. Death. Differ. — 1999. — V. 6, N 1. — P. 1–2.
9. *Schulze C., Munoz L. E., Franz S. et al.* Clearance deficiency—a potential link between infections and autoimmunity // Autoimmun. Rev. — 2008. — V. 8, N 1. — P. 5–8.
10. *Miyagi T., Wada T., Yamaguchi K. et al.* Human sialidase as a cancer marker // Proteomics. — 2008. — V. 8, N 16. — P. 3303–3311.
11. *Miyagi T.* Aberrant expression of sialidase and cancer progression // Proc. Jap. Acad., Ser. B. — 2008. — V. 84, N 10. — P. 407–418.
12. *Gabius H.-J.* The Sugar Code: Fundamentals of Glycosciences. Weinheim: John Wiley & Sons, 2009. — 564 p.
13. *Nikonova E. Y., Tertov V. V., Sato C. et al.* Specificity of human trans-sialidase as probed with gangliosides // Bioorg. Med. Chem. Lett. — 2004. — V. 14, N 20. — P. 5161–5164.
14. *Chomczynski P., Sacchi N.* The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on // Nat. Protoc. — 2006. — V. 1, N 2. — P. 581–585.
15. *Azuma Y., Taniguchi A., Matsumoto K.* Decrease in cell surface sialic acid in etoposide-treated Jurkat cells and the role of cell surface sialidase // Glycoconj. J. — 2000. — V. 17, N 5. — P. 301–306.
16. *Spandidos A., Wang X., Wang H. et al.* PrimerBank: a resource of human and mouse

PCR primer pairs for gene expression detection and quantification // Nucl. Acids Res. — V. 38, N Database issue. — P. D792–799.

17. *Azuma H., Ikebuchi K., Yamaguchi M. et al.* Comparison of sensitivity to ultraviolet B irradiation between human lymphocytes and hematopoietic stem cells // Blood. — 2000. — V. 96, N 7. — P. 2632–2634.
18. *Warner T. G.,O'Brien J. S.* Synthesis of 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid and detection of skin fibroblast neuraminidase in normal humans and in sialidosis // Biochemistry. — 1979. — V. 18, N 13. — P. 2783–2787.
19. *Bilyy R., Tomyn A., Stoika R.* Membrane Glycoproteins During Cell Death: How And Why They Appear On The Cell Surface? // J. Physiol. Sci. — 2009. — V. 59, N S1. — P. 155–155.
20. *a201003168UA.* Method of neuraminidase activity detection in cells and cellular compartments / Bilyy R., Tomin A., Stoika R. — UA Patent application 19.03.2010; Pub. 12.07.2010, Bul. 13.
21. *Azuma Y., Sato H., Higai K. et al.* Enhanced expression of membrane-associated sialidase Neu3 decreases GD3 and increases GM3 on the surface of Jurkat cells during etoposide-induced apoptosis // Biol. Pharm. Bull. — 2007. — V. 30, N 9. — P. 1680–1684.
22. *Heyder P., Gaipl U. S., Beyer T. D. et al.* Early detection of apoptosis by staining of acid-treated apoptotic cells with FITC-labeled lectin from Narcissus pseudonarcissus // Cytometry. — 2003. — V. 55A, N 2. — P. 86–93.

АПОПТИЧНІ ЗМІНИ ЕКСПРЕСІЇ мРНК, ЩО КОДУЮТЬ СІАЛІЛМОДИФІКУЮЧІ ЕНЗИМИ

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Порівнювали рівні експресії мРНК семи основних клітинних сіалілмодифікуючих ензимів (сіалідази Neu1, Neu2, Neu3, Neu4 та сіалілтрансферази hST6Gal.I, hST3Gal.III, hST3Gal.IV) під час апоптичної загибелі клітин. Ідентифікація ензимів, відповідальних за процеси поверхевого десіалування при апоптозі, може сприяти розвиткові біотехнології для модифікації фаго апоптотичних клітир

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