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Specificity of human *trans*-sialidase as probed with gangliosides

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Abstract—It has been shown that human blood contains a soluble 67 kDa enzyme, belonging by its donor–acceptor properties to *trans*-sialidases. The enzyme is capable of both cleaving and synthesizing α2-3 and α2-6 sialosides [*Atherosclerosis* **2001**, *159*, 103]. In this work the study of donor–acceptor specificity of the new enzyme was extended. It has been demonstrated in vitro that *trans*-sialidase possesses the ability of transferring Neu5Ac residue to acceptor (asialofetuin) both from α2-3- (GM1, GM3, GD1a), and α2-8-sialylated gangliosides (GD3 and GD1b, but not GT1b and GQ1b). Transfer of radiolabeled Neu5Ac from fetuin to glycosphingolipids demonstrated that Lac-Cer > mono- and disialogangliosides > GT1b > GQ1b were acceptors for this enzyme. Two methods were used to reveal whether α2-8 bond can be formed between Neu5Ac residues during *trans*-sialylation, that is immunochemical detection using monoclonal antibodies specific to α2-8 di- and oligosialic acids, and fluorometric C_7/C_9 analysis. Both methods demonstrated the formation of Neu5Acα2-8Neu5Ac termination by *trans*-sialidase, for example, in case of the use 3′SL as sialic acid donor and Neu5Ac-PAA or LDL as acceptor. Thus, human *trans*-sialidase in vitro displays wide substrate specificity: the enzyme is capable of digesting as well as synthesizing α2-3, α2-6, and α2-8 sialosides.

1. Introduction

Low density lipoproteins (LDL) having decreased content of sialic acid were detected earlier in blood of coronary atherosclerosis patients. It was shown that desialylation of LDL took place in human blood sera. Sialic acid transfer occurred at neutral pH values and was not inhibited by Neu2en5Ac. Sialic acid removed form LDL was detected in composition of blood glycoconjugates rather than in free state. This observation leads to conclusion that the enzyme removing sialic acid from LDL transfers it to other blood components thus being a *trans*-sialidase type enzyme.[1,2] This 67 kDa *trans*-sialidase was isolated from human plasma in homogenous state and its ability to cleave and synthesize α2-3 and α2-6 sialoglycoconjugates was reported.[1]

In this work the further study of the donor–acceptor specificity of human *trans*-sialidase, that is its ability to form and cleave Neu5Acα2-8Neu5Ac glycosidic bonds was performed. Gangliosides and synthetic glycoconjugates containing mono- and disialic acids were used as substrates. Gangliosides containing one or two sialic acids proved to be preferential substrate (both donor and acceptor) for *trans*-sialidase. Formation of α2-8 sialosides was demonstrated at the example of several synthetic and natural glycoconjugates.

2. Isolation of lipoproteins

Lipoproteins and lipoprotein-deficient plasma were isolated from human plasma by method described earlier.[1] Lectin-chromatography on *Ricinus communis* agglutinin (RCA_{120}, Boehringer Mannheim GmbH, Mannheim, Germany) immobilized on agarose was used to separate LDL into sialylated (sLDL) and desialylated (dLDL) fractions.[3]

3. Preparation of radiolabeled gangliosides

Gangliosides GM3, GD3, GM1, GD1a, GD1b, GT1b, GQ1b were ^{3}H-labeled at the C-8 position of sialic acid as described by Veh et al.[4] The gangliosides isolated from bovine brain and liver were kindly provided by N. V. Prokazova (Cardiology Research Center, Moscow, Russia) and Dr. I. Mikhaliov (Institute of

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Bioorganic Chemistry, Moscow, Russia). The specific radioactivity was 0.18–10.2 mCi/mmol bound sialic acid.

4. Isolation of *trans*-sialidase by affinity chromatography

Lipoprotein-deficient serum (700 μL) was applied to a column containing 2 mL Neu5Acα2-8Neu5Ac-Sepharose FF-6 (Lectinity, Moscow, Russia). The column was washed with 20 mL of 50 mM Tris–HCl (pH 7.0). *trans*-Sialidase was eluted with 5 mL of 5 mM sialic acid in 50 mM Tris–HCl buffer and was dialyzed three times against 2000 volumes of 5 mM Tris–HCl. Enzyme was concentrated by ultrafiltration (1000 MW cut off, Amicon, USA) and stored at −70 °C. Enzyme homogeneity was proven by SDS-PAGE electrophoresis (4–20%)[5] and mass-spectrometry analysis.[6] Protein concentration was determined according to Lowry et al.[7]

5. Assay of *trans*-sialidase activity

To determine the *trans*-sialidase activity using gangliosides as sialic acid donors, the assay mixture containing 3.6 nmol of ^{3}H-labeled ganglioside, 0.5 mg/mL asialofetuin, 50 mM Tris–HCl (pH 7.2), 1 mM DTT, 2 mM $CaCl_2$ was incubated with 10 μg human plasma *trans*-sialidase preparation for 8 h at 37 °C. Incubation was stopped by addition of 1 mL 10% trichloroacetic acid. After centrifugation (10,000 g, 10 min) the pellet was delipidated consecutively with 2 mL of 2-propanol, chloroform–methanol mixture (1:1, v/v) and diethyl ester. The delipidated pellet was dissolved in 0.1 N NaOH and its radioactivity was counted.

To determine the *trans*-sialidase activity using lactosyl–ceramide or gangliosides as the acceptors, the assay mixtures contained in a final volume of 0.2 mL: 0.01 mL of ^{3}H-labeled fetuin-Sepharose (5 μg protein, 145,000 dpm), 100 nmol of lactosyl–ceramide or ganglioside, 50 mM Tris–HCl (pH 7.0), 1 mM DTT, 2 mM $CaCl_2$, and 5 μg human plasma *trans*-sialidase preparation. After incubation for 18 h at 37 °C 0.3 mL ice-cooled water was added and samples were centrifuged (5000*g*, 5 min) at 4 °C. Supernatant (0.2 mL) was used for radioactivity counting.

6. Dot blot assay for determination of di- and oligosialic acids using antibodies

The assay mixture containing 450 μg of one of the following acceptors (6′SLN-PAA, 3′SLN-PAA, SiaLe^{a}-PAA, LacNAc-PAA, Neu5Acα-PAA, sLDL, or dLDL), 1.6 mg 3′SL, 50 mM Tris–HCl (pH 7.2), 1 mM DTT, and 2 mM $CaCl_2$ was incubated with 400 μg human plasma *trans*-sialidase preparation for 18 h at 37 °C. After incubation the mixture was applied on Sepharose LH-20 column (2 × 100 mm) and eluted with acetonitrile–water 1:1 (v/v). The fraction (first 12 mL) was collected and rotary evaporated (40 °C). The residue was coated on to PVDF membrane; at three dots the concentration of the glycoconjugate differed as 1:10:100. All the used acceptors without *trans*-sialidase treatment (as a negative control) and $(Neu5Ac)_3$-PAA and $(Neu5Ac)_2$-PAA (as a positive control) were also dot-blotted on the same membrane in three concentrations (1, 5, 10 μg).

PVDF membrane was blocked with 1% BSA-PBST (10 mM sodium phosphate buffer (pH 7.2), 0.15 M NaCl, 0.05% Tween 20) at 25 °C for 1 h and incubated with primary antibodies (mAb.S2-566 at 0.51 μg/mL; mAb.OL28 at 6 μg/mL; 1E6 cultural supernatant) at 4 °C overnight. After washing with PBST, the membrane was incubated with the secondary antibody (peroxidase-conjugated anti-mouse IgG + IgM at 1:3000 dilution) at 37 °C for 1 h and washed with PBST. The membrane was visualized with ECL reagents (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's protocol.

7. Fluorometric C_7/C_9 analysis

The glycoconjugate-coated PVDF membranes were prepared as described above. The fluorometric C_7/C_9 analysis of the membrane was carried out as described previously.[8] Briefly, the membrane was cut into small pieces and 250 μL of 50 mM sodium acetate buffer (pH 5.0) and 20 μL of 0.25 M sodium periodate were added. After incubation at 0 °C for 3 h in the dark, 50 μL of 3% ethylene glycol and 320 μL of 0.2 M sodium borohydride in 0.2 M sodium borate buffer (pH 8.0) were added successively and incubated at 0 °C overnight. The membranes were washed with water and then incubated with 200 μL of 0.1 M trifluoroacetic acid at 80 °C for 1 h. The hydrolysate was dried up by SpeedVac device (SAVANT Instrument Inc., NY, USA), and subjected to derivatization with 1,2-diamino-4,5-methylenedeoxybenzene (DMB) in solution of 40 μL of 3.5 mM of DMB dihydrochloride in 7.5 mM trifluoroacetic acid, 0.5 M 2-mercaptoethanol, 9 mM sodium hydrosulfite at 50 °C for 2 h. An aliquot of the reaction mixture (20 μL) was analyzed by JASCO LC-900 HPLC system equipped with JASCO FP-920 fluorescence detector (excitation, 373 nm; emission, 448 nm), operating in isocratic mode at 1.0 mL/min at a column temperature of 26 °C. The column was TSK-gel ODS-120T (250 × 4.6 mm.) and eluted with methanol/acetonitrile/water (7:9:84, v/v/v).

8. Statistical analysis

The significance of differences between group mean values was evaluated by multiple *t*-test of one-way analysis of variance using BMDP statistical program package.[9]

The well known and studied in detail Trypanosome *trans*-sialidase has strict specificity both to donor and acceptor of sialic acid. It is capable only of cleaving and transferring sialic acid from or to terminal Lac and LacNAc of gycoconjugates cleaving or forming the α2-3 bond.[10,11] In contrast, human *trans*-sialidase acts both to α2-3 and α2-6 sialosides.[1] Using mono- and disialogangliosides and synthetic glycoconjugates

it was demonstrated in this study that in addition to the α2-3/α2-6 bonds human *trans*-sialidase was capable of forming and cleavinge α2-8 glycosidic bond. Several experimental approaches were used for this, that is (1) transfer of ^{3}H-labeled Neu5Ac, (2) detection of the formed α2-8 bonds using specific antibodies,[12] (3) confirmation of 2-8 bond formation using C_7/C_9 analysis.[8]

It is known that gangliosides contain about 10–15% of total sialic acid in plasma.[13–15] More than ten gangliosides, most of them bound to lipoproteins, were found in plasma. GM3 (about 50%) and GD3 (more than 25%) prevail in this pool. In this study both GM3 and GD3 were efficient donors of sialic acid for human *trans*-sialidase (Fig. 1). The similar transfer rate was determined for GM1, GD1a, and GD1b. Monosialylated gangliosides GM1 and GM3, and disialylated GD1a, containing only α2-3 bound sialic acid, as well as GD1b and GD3, containing only α2-8 bound terminal sialic acid, were efficient donors of sialic acid for *trans*-sialidase (Fig. 1). Thus, human *trans*-sialidase is capable of digesting all the bond types of sialic acid. *trans*-Sialylation determined for gangliosides containing three (GT1b) or four (GQ1b) sialic acids was much lower than that for mono- and disialylated gangliosides (Fig. 1). Thus, mono- and disialogangliosides are preferential substrates for human *trans*-sialidase.

In order to study glycolipids as sialic acid acceptors in *trans*-sialylation reaction we used similar test-system where native (sialylated) fetuin served as sialic acid donor. The data shown in Figure 2 demonstrate the rate of labeled sialic acid transfer from fetuin to lactosyl–ceramide and gangliosides. The highest rate of sialic acid transfer was found for lactosyl–ceramide, the rate of sialic acid transfer from fetuin onto ganliosides GM1, GD1a, and GD1b was twofold lower (Fig. 2). For GT1b the rate of sialic acid transfer was approximately twofold lower than that for gangliosides containing one

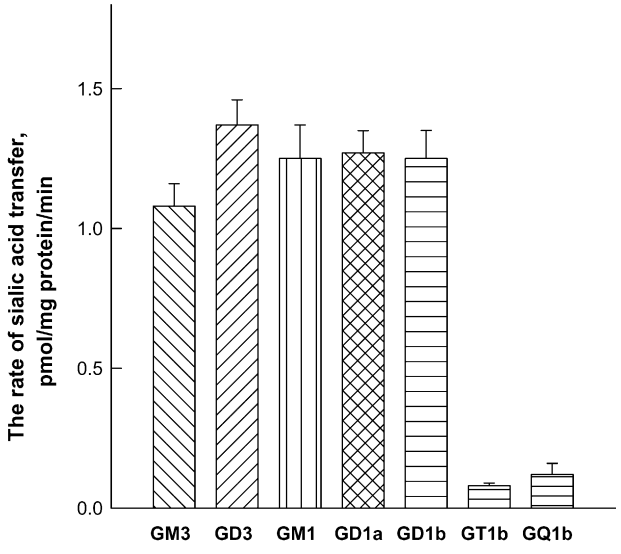

Figure 1. Sialic acid transfer from gangliosides onto asialofetuin. *trans*-Sialidase activity was measured as described in experimental procedure. Data represent an average of three determinations ± SEM.

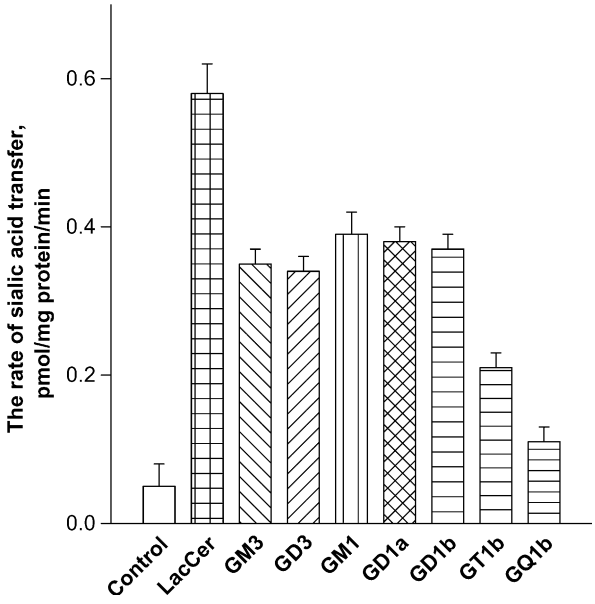

Figure 2. The rate of sialic acid transfer from ^{3}H-labeled fetuin onto lactosyl–ceramide and gangliosides. *trans*-Sialidase activity was measured as described in experimental procedure (control—assay mixture without any acceptor). Data represent an average of three determinations ± SEM.

or two sialic acids, while ganglioside GQ1b was the worst acceptor (Fig. 2). The confirmation of sialic acid transfer to lactosyl–ceramide was also performed by TLC. Radiolabeled products on chromatogram (data not shown) corresponded to standard gangliosides containing more sialic acid residues than the starting acceptor.[1]

The results obtained using radiolabeled sialic acid demonstrate the formation of oligosialic acids but do not provide any information on the type of the formed bond (α2-8, α2-9 or other). The data shown in Table 1 were obtained using monoclonal antibodies (S2-566 recognized Neu5Acα2-8Neu5Acα2-3Gal, 1E6—Neu5Acα2-8Neu5Acα, and OL28—$(Neu5Ac)_n$, $n \geqslant 4$).[12] Immunostaining gives unambiguous evidence both that *trans*-sialidase forms disialic acids and that α2-8 bonds are formed. According to Table 1 data, Neu5Acα2-8Neu5Acα fragment appeared when Neu5Acα, fetuin,

Table 1. Binding of monoclonal antibodies to glycoconjugates incubated with the human *trans*-sialidase in the presence of 3′SL

Compound	mAbs S2-566	mAbs 1E6
αNeu5Ac-PAA	++	+
LacNAc-PAA	—	—
SiaLe^{a}-PAA	±	—
3′-SLN-PAA	±	—
6′-SLN-PAA	—	±
Neu5Acα2-6GalNAcα-PAA	n.d.	±
sLDL	+	+
dLDL	—	—
Fetuin	+	+

The glycoconjugates-acceptors treated with the enzyme in presence of 3′SL were coated onto PVDF membrane and stained with mAbs. Neither glycoconjugates interacted with mAbs in absence of the enzyme. α2-8-Connected glycoconjugates $(Neu5Ac)_3$-PAA and $(Neu5Ac)_2$-PAA were used as positive control.

Table 2. Proportion of the C9 and C7 DMB derivatives revealed after treatment of the glycoconjugate with *trans*-sialidase (TS) in the presence of 3′SL followed by DMB-labeling

Compound	TS (−)			TS (+)			Results of TS treatment
	C9 (%)	C7 (%)	C9/C7	C9 (%)	C7 (%)	C9/C7	
αNeu5Ac	0	100	0	2	98	0.028	diNeuAc formed
LacNAc	n.d.	n.d.	—	2	98	0.036	diNeuAc formed
SiaLe[a]	0	100	0	1	99	0.007	diNeuAc formed
3′-SLN	0	100	0	2	98	0.018	diNeuAc formed
6′-SLN	0	100	0	1	99	0.013	diNeuAc formed
sLDL	1	99	0.005	2	98	0.033	diNeuAc formed
dLDL	n.d.	n.d.	—	0	100	0	NeuAc transferred

The data are average values of two or three independent experiments.

or sLDL were incubated with *trans*-sialidase. Antibodies OL28, specific to $(Neu5Ac)_n$ ($n \geqslant 4$) recognized $(Neu5Ac)_3$-PAA treated with *trans*-sialidase in presence of 3′SL donor (data not shown). Interaction of other glycoconjugates with *trans*-sialidase (Table 1) does not give unambiguous answer whether the fragment Neu5Acα2-8Neu5Ac is formed, that can be explained by reduced transfer rate to 8-OH (Neu5Ac) in comparison to 3-OH and 6-OH (Gal or GalNAc) according to the experiments on ^{3}H-Neu5Ac transfer and, thus, by low rate of formation of α2-8 epitopes for monoclonal antibodies. In addition, Neu5Acα-PAA (good acceptor) does not have any other potential sialylation sites except for neuraminic acid residue, whereas 3′SLN-PAA has more active potential sialylation sites (6-OH in Gal), which possibly compete with 8-OH in Neu5Ac.

In parallel, similarly prepared PVDF membranes were tested on presence of newly formed 2-8 bonds using C_7/C_9 assay, based on periodate oxidation followed by fluorescent labeling.[8] The data shown in Table 2 demonstrate that Neu5Ac2-8Neu5Ac formation takes place in case of Neu5Acα-PAA, LacNAc-PAA, SiaLe[a]-PAA, 3′SLN-PAA, 6′SLN-PAA, and sLDL as acceptors treated with *trans*-sialidase in presence of 3′SL. It was demonstrated that *trans*-sialidase treated dLDL (in contrast to sialylated part of LDL pool, sLDL) had only monomeric Neu5Ac. So, in this case sialylation by *trans*-sialidase is not rapid enough to reach the stage of di-sialylation.

Thus, in this study it was confirmed by several methods that human *trans*-sialidase is capable of both digesting and synthesizing in vitro Neu5Acα2-8Neu5Ac motif, in particular in gangliosides. Taking into consideration low rate of α2-8 transfer in comparison to α2-3 and α2-6 and higher concentration of competing potential sites for α2-3 and α2-6 sialylation, one can hardly expect for α2-8 sialylation caused in vivo by human *trans*-sialidase. However, more favorable conditions can occur for a resident (bound to LDL[2]) enzyme, namely its elevated local concentration, advantages of *cis*-sialylation (i.e., higher local concentration of acceptor), optimal pH < 7, and, possibly, presence of activator–promoter. All of this can lead to nonclassic synthesis or, oppositely, hydrolysis of Neu5Acα2-8Neu5Ac. Whether this indeed takes place on LDL surface in normal or atherosclerosis conditions should be revealed by further studies.

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