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Detection of Gangliosides as *N*-Glycolylneuraminic Acid-specific Tumor-associated Hanganutziu-Deicher Antigen in Human Retinoblastoma Cells

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Gangliosides were shown to bear the tumor-associated *N*-glycolylneuraminic acid (NeuGc)-specific Hanganutziu-Deicher (HD) antigen expressed in human retinoblastoma cells. HD antigenic gangliosides were detected by thin-layer chromatography/enzyme-immunostaining using affinity-purified chicken antibody against GM3 containing NeuGc and horseradish peroxidase-conjugated anti-chicken IgG. One to four species of the antigenic gangliosides were detected from all of 4 cell lines, Y79, WERI-Rb1, TOTL1, and YK, as well as freshly cultured retinoblastoma cells and isolated tumor tissue. All cases contained GM3(NeuGc) as an HD antigen. No HD antigenic ganglioside was detected in normal retinal tissues by the same procedure.

Key words: Gangliosides — *N*-Glycolylneuraminic acid — Tumor-associated antigen — Retinoblastoma — Hanganutziu-Deicher antigen

Hanganutziu-Deicher (HD)[*4] antigen is a novel tumor-associated antigen which is immunogenic in humans.[1] An antigenic determinant of HD antigen is *N*-glycolylneuraminic acid (NeuGc), which is absent in humans and chickens.[2] We previously demonstrated HD antigen on the cell surface of various human tumor tissues and retinoblastoma cells by means of a membrane immunofluorescence test.[3–5] Recently, we characterized HD antigenic molecules of colon cancers and melanomas as several species of gangliosides.[5–9] In this paper, we report the detection of HD antigenic gangliosides from retinoblastoma cells, including 4 cell lines.

MATERIALS AND METHODS

Retinoblastoma and Retinal Cells Human retinoblastoma cell lines Y-79, WERI-Rb1, TOTL-1, and YK, which were newly established by Sasabe *et al.*,[4] were cultured in RPMI-1640 medium supplemented with 15% human serum at 37° in a humidified atmosphere of 5% CO_2 in air for more than 9 months. Retinoblastoma cells isolated from a patient were cultured under the same conditions for 3 months. Tumor tissue and aqueous humor were isolated from an eye of another retinoblastoma patient by surgery, and serum was collected from the same patient. Normal retinal tissues were obtained from 6 eyes of 3 individuals at autopsy, performed within 24 hr of death.

Standard Gangliosides The gangliosides used as standards are listed in Table I. They were prepared as described previously.[6, 10]

Extraction and Purification of Gangliosides Glycolipid fractions were isolated from chloroform-methanol-water (4:8:3, v/v) extracts of cells by phenyl boronate agarose (PBA60, Amicon Co., Danvers, MA) column chromatography without alkaline treatment, as described previously.[6]

Analytical Procedures Gangliosides were developed on thin-layer chromatography (TLC) plates with chloroform-methanol-0.25% aqueous KCl (5:4:1, v/v; Solvent A) or chloroform-methanol-2.5*M* NH_4OH and 0.25% KCl (5:4:1, v/v; Solvent B). Enzyme-immunostaining of gangliosides on TLC plates using affinity-purified chicken anti-GM3(NeuGc) and horseradish peroxidase-conjugated rabbit anti-chicken IgG, and densitometric determination of the reactions were performed as described previously.[11]

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[*4] Abbreviations used: HD, Hanganutziu-Deicher; TLC, thin-layer chromatography. Abbreviations of gangliosides are according to the recommendations of Svennerholm[17] and the IUPAC-IUB Commission on Biochemical Nomenclature.[18]

Table I. Structures of Gangliosides Used as Standards

Abbreviation	Chemical structure
GM3(NeuGc)	NeuGc(α2–3)Gal(β1–4)Glc-Cer
NeuGc-nLc$_4$Cer	NeuGc(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc-Cer
NeuGc-nLc$_6$Cer	NeuGc(α2–3)[Gal(β1–4)GlcNAc(β1–3)]$_2$Gal(β1–4)Glc-Cer
GM2(NeuGc)	GalNAc(β1–4)Gal(β1–4)Glc-Cer \| (α2–3) NeuGc
GM3	NeuAc(α2–3)Gal(β1–4)Glc-Cer
NeuAc-nLc$_4$Cer	NeuAc(α2–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc-Cer

Glc, D-glucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Cer, ceramide (*N*-acylsphingosine).

Results

Glycolipid fractions were prepared from retinoblastoma tissue and aqueous humor of an eye and serum from a patient, as well as from retinoblastoma cell lines and normal adult retinas for comparison. In the case of two retinoblastoma cell lines, Y79 and TOTL-1, three and two batches of culture were used, respectively. All the glycolipid fractions were analyzed for total gangliosides (Fig. 1) and HD antigenic gangliosides (Fig. 2) by TLC.

As shown in Fig. 1, their ganglioside patterns were quite different from each other. The patterns of retinoblastoma cell lines differed from that of the normal adult retina, and both showed more complex patterns than those of tumor tissue and aqueous humor obtained from a patient. Judged from their mobility on TLC, the retinoblastoma cells and normal tissue expressed GM3, GM1, GD1a, GD1b and GT1 as common components, whereas the tumor tissue and aqueous humor from the patient expressed GD3 as a major component. The aqueous humor of the eye, from which the tumor had been dissected out,

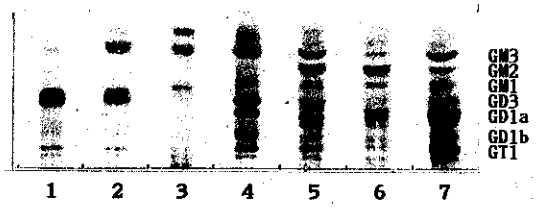

Fig. 1. Gangliosides of retinoblastomas, normal retina, aqueous humor, and serum. The glycolipid fractions were put on an HPTLC plate (J. T. Baker Chemical Co., Phillipsburg, NJ) and developed with Solvent A 4 cm from the origin, then located by the use of a resorcinol HCl spray.[12] Lanes contained glycolipid fractions of: 1, retinoblastoma tissue; 2, aqueous humor; 3, serum of a patient; 4, normal retinal tissue from 6 eyes from 3 individuals; 5, Y79; 6, WERI-Rb1; and 7, YK. No bands above GM3 showed specific resorcinol staining.

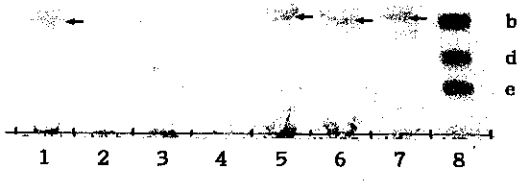

Fig. 2. HD antigenic gangliosides of retinoblastoma tissue and cell lines. The glycolipid fractions of extracts of cells or tissues were put on a plastic TLC plate (Polygram-SilG; Macherey-Nagel, Düren, FRG) and developed with Solvent A 4 cm from the origin, then located by means of enzyme-immunostaining with chicken anti-GM3(NeuGc) and peroxidase-conjugated anti-chicken IgG. Lanes 1 to 7 are the same as in Fig. 1 and lane 8 is a standard mixture containing 5 pmol of b, GM3-(NeuGc); d, NeuGc-nLc$_4$Cer; and e, NeuGc-nLc$_6$Cer. Arrows indicate specific blue purple-staining bands. Bands on lanes 3 and 4 were yellow and do not represent specific staining.

expressed GM3 in addition to GD3, which was also found in the tumor tissue.

Figures 2 and 3 show that all the retinoblastoma cell lines and the tumor tissue of retinoblastomas expressed HD antigenic gangliosides, while the aqueous humor of the tumor-bearing eye and serum from the patient did not. The HD antigenic ganglioside had the same mobility as GM3(NeuGc) which was described in a previous study.[6] The amounts of HD antigenic gangliosides determined by densitometry were in the pmol range, too low to be detected by the resorcinol-HCl reagent, and were less than 0.1% of total lipid-bound sialic acid contents (Table II). Table II also shows that freshly cultured cells from the tumor tissue contained HD antigenic gangliosides in an amount comparable to that in the tumor. All cell lines were found to express GM3(NeuGc) as an HD antigenic ganglioside, as clearly shown in Figs. 2 and 3, but one of three batches of Y79 and one of two batches of TOTL1 lines were found to contain additional antigenic gangliosides as shown in Fig. 3. The additional ganglioside of the batch of Y79 migrated with a mobility similar to that of NeuGc-nLc$_4$Cer on two-dimensional TLC (Fig. 3-A) and constituted a major HD-positive ganglioside. The TOTL1 cells contained three minor antigenic gangliosides in addition to GM3(NeuGc); one migrated slightly faster than GM3(NeuGc), the others migrated near GM2(NeuGc) and NeuGc-

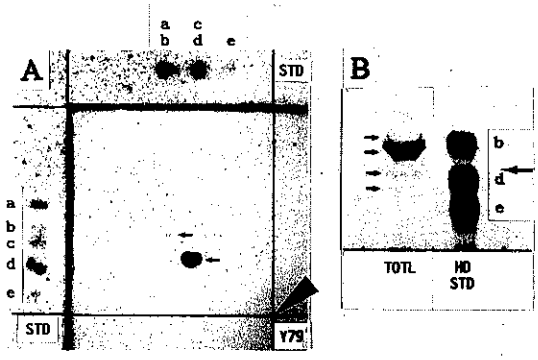

Fig. 3. HD antigenic gangliosides in retinoblastoma cell lines. A) A batch of Y79 line differing from that used in Figs. 1 and 2 was analyzed. The glycolipid fraction was developed in the first dimension (to the left) with Solvent A, and in the second dimension (upward) with Solvent B, as described previously.[10] Antigenic gangliosides were located as in the case of Fig. 2. STD, standard ganglioside mixture containing a, GM3; b, GM3-(NeuGc); c, NeuAc-nLc$_4$Cer; d, NeuGc-nLc$_4$Cer; and e, NeuGc-nLc$_6$Cer was developed in one dimension on the same TLC plate and located with resorcinol-HCl spray. An arrowhead indicates the origin. Small arrows indicate specific immunostained spots. B) The glycolipid fraction from TOTL1 cells was analyzed as described in the legend to Fig. 2. HD-STD, a mixture of b, GM3-(NeuGc); d, NeuGc-nLc$_4$Cer; and e, NeuGc-nLc$_6$Cer. Arrows indicate specifically immunostained spots. A large arrow indicates the position of GM2(NeuGc).

Table II. HD Antigenic NeuGc Content in Gangliosides of Retinoblastoma Cells by TLC Densitometry

Sample	Lot #	Glycolipid analyzed (μg)	Lipid-bound sialic acid A (nmol)	HD antigenic NeuGc detected		
				B (pmol)	B/A (%)	(pmol/mg protein[a])
Y79	1	480	7.5	2.2	0.030	0.26
	2	500	13.0	4.5	0.035	0.81
WERI-Rbl	1	520	5.7	0.1	0.0018	0.0052
	2	500	5.5	4.3	0.078	0.66
TOTL1		490	5.4	2.3	0.043	0.19
YK		500	21.1	0.30	0.0014	0.052
Freshly cultured[b]		210	1.4	0.05	0.0036	0.025
Tumor tissue		500	10.0	0.05	0.0005	0.016
Normal retina[c]		500	27.5	0 (<0.05)	0 (<0.00018)	0 (<0.015)

a) Protein contents of starting materials were determined by the method of Lowry *et al.*[13]
b) Isolated retinoblastoma cells were cultured for 3 months as described in "Materials and Methods."
c) Pool of 6 eyes from 3 individuals.

nLc_4Cer, respectively, in a neutral solvent system (Fig. 3-B). Further structural analysis of these gangliosides was not performed because of the small amounts of the compounds available. It should be noted that the other batches of the same lines did not express complex patterns of antigenic gangliosides but simple patterns with GM3(NeuGc) as a single antigenic molecule, as shown in Fig. 2.

Discussion

HD antigens were first defined by a human heterophile antibody. NeuGc, a sialic acid, which is absent in normal human and chickens, is the antigenic determinant of HD antigen.[2] As well as other sialic acids, NeuGc is present in sugar chains of various gangliosides and glycoproteins.[14,15] Our previous membrane immunofluorescence study showed that approximately 50% of various human tumor tissues contained HD antigen-positive cells.[3–5] Our recent studies showed that HD antigenic gangliosides were expressed in colon cancers and melanomas in high frequency, and some species of the gangliosides were expressed as a unique ganglioside specific to the cancers.[5–9] Further studies may clarify the relationship between individual cancers and molecular species of HD antigens.

In our previous study, we demonstrated an expression of HD antigen on the cell surface of retinoblastoma cells of Y79, WERI-Rb1, TOTL1 cell lines, and two fresh tumor isolates.[4] We have characterized in this study the antigenic molecules of all these cell lines and other retinoblastoma samples as gangliosides. GM3(NeuGc) was found to be a common antigenic ganglioside. Not all the batches of cell lines expressed identical patterns of HD antigenic and total gangliosides, even though GM3(NeuGc) was expressed in every batch. Cultured cell lines are very useful to obtain a large quantity of biological samples which are otherwise difficult to get, but they should be used with care, and the fact that they are susceptible to change in culture should be kept in mind.

In the previous complement-dependent antibody-mediated cytotoxicity test using anti-GM3(NeuGc), we observed significant lysis of cells of Y79 and TOTL1 lines but not of WERI-Rb1.[4] We were puzzled by these results, since WERI-Rb1 contained as much HD antigenic ganglioside as the two other lines (Table II).

The ganglioside composition of the normal adult retina reported in this study was clearly different from that found by Holm *et al.*[16] We detected GM3 and GM1 as major components of the retinal gangliosides in addition to GD3, GD1a, GD1b, and GT1 which they reported to occur. The discrepancy was probably caused by the difference in the isolation procedures of gangliosides. They used a phase partition method in which most of the less polar gangliosides such as GM3 and GM1 are known to be lost in the chloroform-rich lower phase.

We did not detect HD antigenic ganglioside in the aqueous humor of a tumor-bearing eye or serum of a retinoblastoma patient, although tumor tissue of the patient expressed HD antigenic ganglioside and the ganglioside pattern of the tumor tissue was similar to that of the aqueous humor. It is unknown whether or not the antigenic gangliosides of retinoblastoma shed into the surrounding fluid. It might be possible to detect the antigens in the fluid when a more sensitive method is available, and such a method could be a potential tool for clinical diagnosis.

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