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Glycosphingolipids of Human Urothelial Cell Lines with Different Grades of Transformation

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Neutral glycolipids and gangliosides from seven human urothelial cell lines, differing in grades of transformation (TGr), were characterized by fast atom bombardment mass spectrometry, exoglycosidase treatment and an immunostaining procedure. The major neutral glycolipids identified in all cell lines studied included CMH, CDH, CTH, globoside and paragloboside, the gangliosides were G_{M3}, G_{M2}, sialosylparagloboside and G_{D1a}. The following observations were made: 1. G_{M2} was the major ganglioside in the TGrII cell lines (non-tumorigenic, non-invasive), but a minor component in the TGrIII cell lines (tumorigenic, invasive). 2. All components showed C16:0 and C24:0 as major fatty acids, but in the TGrIII cell lines the fatty acid composition of CMH and some of the gangliosides were more complex showing unsaturated and hydroxy-fatty acids as well.

The malignant phenotype of transformed cells is expressed by several traits such as tumorigenicity, invasiveness and metastatic capability. It is a general assumption that these properties are, at least partially, connected with changes in cell surface components [2]. One typical example is aberrant glycosylation of cell membrane glycoproteins and glycosphingolipids [3, 4], but the functional significance of such changes for tumor progression is, however, far from an explanation.

Abbreviations: Gangliosides are named according to Svennerholm [1]; CMH, Monohexosylceramide; CDH, Lactosylceramide (Galβ1-4GlcCer); CTH, Globotriaosylceramide (Galα1-4Galβ1-4GlcCer); Globoside (GalNAcβ1-3Galα1-4Galβ1-4GlcCer); Paragloboside, (Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer); $3'L_{M1}$, Sialosylparagloboside (Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer); Asialo-G_{M2}, (GalNAcβ1-4Galβ1-4GlcCer);Asialo-G_{M1} (Galβ1-3GalNAcβ1-4Galβ1-4GlcCer); Hex, Hexosyl; HexNAc, 2-acetamido-2-deoxyhexosyl; HPTLC, high performance thin layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; TGr, transformation grade.

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Table 1. Origin and classification of human urothelial cell lines.

Line	Origin	Classification
HCV 29	Non-malignant bladder	TGrII
Hu 609	Non-malignant bladder	TGrII
Hu 1734	Transitional cell carcinoma	TGrII
HCV 29T	*In vitro* transformation of TGrII	TGrIII
Hu 609T	*In vitro* transformation of TGrII	TGrIII
Hu 456	Transitional cell carcinoma	TGrIII
Hu 549	Transitional cell carcinoma	TGrIII

Numerous comparative studies on glycoconjugate expression in normal tissue and in tumors, as well as in normal and in transformed cell lines introduced the concept of tumor-associated antigens, characteristic glycoconjugates associated with neoplastic tissues. Their detection is usually connected with increased amounts of individual glycoconjugates and/or organizational changes in the cell membrane, less frequently with neosynthesis [5, 6].

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In this study, we have examined the expression of neutral glycolipids and gangliosides in human urothelial cell lines differing in grade of transformation [7, 8]. The model used allowed comparison of glycolipids expressed by these cell lines and to relate the findings to cell growth properties *in vitro,* and *in vivo,* including invasiveness and tumorigenicity.

Numerous studies have tried to predict the invasive potential of surgical specimens from bladder cancer. These investigations were based on histological evaluation, presence of chromosomal abnormalities, expression of blood groups and other antigens and enzymatic changes [9, 10]. There is still, however, a need for new markers of potential prognostic value to improve a selection of treatment strategy.

Glycosphingolipids are known as tumor-associated antigens of many human carcinomas [3], but there is very little information available from human bladder cancer [11]. Therefore, in this study the possible role of glycosphingolipids as markers of early stages of human transitional cell carcinoma is considered.

Materials and Methods

Cell Lines and Culture Conditions

The urothelial cell lines of human origin used in the present study, established and characterized at the Fibiger Institute, Copenhagen [8], were obtained from Dr Kieler in 1982. Non-tumorigenic and non-invasive cell lines, with an indefinite life span, are defined as TGrII. Cell lines producing tumors in nude mice and invading fragments of embryonic chick hearts *in vitro* are defined as TGrIII (Table 1).

Cells were passaged using a mixture of trypsin-EDTA (Flow Laboratories, Irvine, Scotland) and maintained in plastic culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5%CO_2/ 95% air humidified atmosphere, in Fib 41B growth medium supplemented with non-essential amino-acids and 10% fetal bovine serum (Flow). Penicillin and streptomycin were added to a final concentration of 100 IU and 100 µg/ml, respectively. The cells from the passages, tested for their invasiveness *in vitro* and tumorigenicity in nude mice, were stored in 10% dimethyl sulfoxide/90% complete medium in liquid nitrogen (a tested collection of each cell line was kept frozen). For experimental use the cells were harvested from *in vitro* cultures propagated from frozen samples during a period not exceeding two months. After this period they were replaced with new samples from the frozen, tested stock. All cell lines were free of mycoplasma.

Purification of Glycosphingolipids

Glycolipids were purified essentially as previously described [12]. Material corresponding to 10^6 - 10^7 cells was extracted twice with chloroform/methanol, 2/1 and 1/2 by vol. After mild alkaline hydrolysis with 0.2 M KOH in methanol, the samples were desalted on a column of Sephadex G-25 superfine (Pharmacia, Uppsala, Sweden). Gangliosides were separated from neutral glycolipids on a DEAE-Sephadex A-25 column (Pharmacia). Gangliosides were desalted on a Sephadex G-25 column. Neutral glycolipids were purified after acetylation on a Florisil column (Fisher, Springfield, NJ, USA) according to the method of Saito and Hakomori [13].

Cell pellet corresponding to 10^8 - 10^9 cells was extracted with chloroform/methanol/water, 20/10/1, 10/20/1 and 10/10/1 by vol [14]. Combined extracts were purified as above.

Metabolic Labeling of Cells

Cell lines were grown in the presence of 1 µCi/ml D-[1-^{14}C]galactose (spec. activity = 2.15 GBq/mmol, Amersham, UK) in the Fib 41B medium [15]. Cells were harvested by mechanical scraping of the culture vessel without the use of trypsin-EDTA. The cells were then washed three times with phosphate-buffered saline (PBS; 0.01 M sodium phosphate, pH 7.2, containing 0.15 M NaCl) and pelleted.

High Performance Thin Layer Chromatography

Samples of neutral glycolipids were separated on silica 60 HPTLC plates (Merck, Darmstadt, FRG) using chloroform/methanol/water, 60/35/8 by vol, and for gangliosides chloroform/ methanol/0.2% $CaCl_2$, 60/40/9 by vol, was used. Neutral glycolipids were detected with *N*-(1-naphthyl)ethylenediamine dihydrochloride [16] (Aldrich, Milwaukee, WI, USA) and gangliosides were stained with a resorcinol reagent [17]. For autoradiography of glycolipid samples from metabolically-labelled cells the chromatogram was exposed to Kodak X-Omat film, ZAR 5 at room temperature for seven days. Gangliosides were quantified using scanning densitometry [18]. The following glycolipid standards were used. Monohexosylceramide was from Supelco, Inc., (Belefonte, PA, USA), lactosylceramide, globotriaosylceramide, globoside and GM_1-ganglioside were obtained from BioCarb (Lund, Sweden); G_{M3}, G_{M2}, G_{D1a} and sialosylparagloboside were kindly provided by Dr. T. Pacuszka, Warsaw,

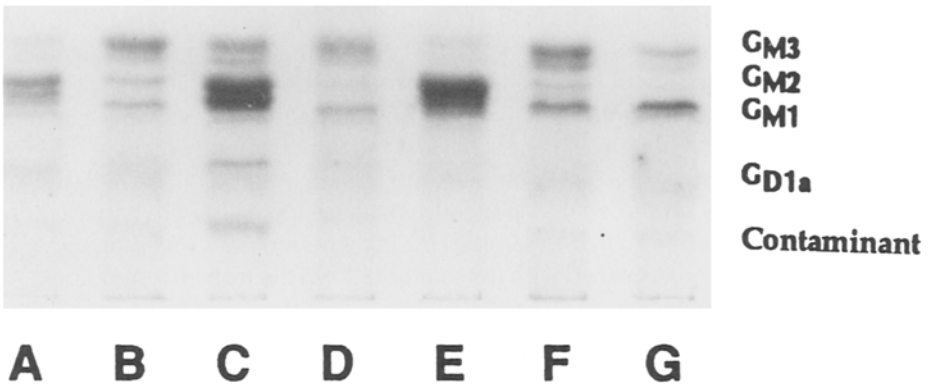

Figure 1. HPTLC pattern of total gangliosides from human urothelial cell lines of different stage of transformation. Cell lines of TGrII: HCV 29 (A), Hu 609 (C), Hu 1734 (E); cell lines of TGrIII: HCV 29T (B), Hu 609 T (D), Hu 456 (F), Hu 549 (G). Aliquots of gangliosides corresponding to 2 x 10^7 cells were separated in the solvent system chloroform/methanol/0.2% $CaCl_2$ 60/40/9 by vol. Bands were visualized by resorcinol reagent. Positions of standards are indicated to the right. The slower migrating band in lane C is a non-glycosphingolipid contaminant.

Poland. Paragloboside was obtained after desialylation of sialosylparagloboside with neuraminidase from *Vibrio cholerae*. Asialo-G_{M2} was purified from guinea pig erythrocytes as described [19].

Treatment with Exoglycosidases

Digestion of ^{14}C-labelled glycolipids with specific exoglycosidases was done essentially as previously described [20]. The following enzymes were used: neuraminidase from *Vibrio cholerae* (Serva, Heidelberg, W. Germany), α-galactosidase from *Aspergillus niger*, β-galactosidase and β-*N*-acetylhexosaminidase from jack bean (Sigma, St Louis, MO, USA).

Sialic acid linked to internal galactosyl residues and resistant to the action of neuraminidase from *Vibrio cholerae*, was hydrolyzed in 0.5 M formic acid for 1 h at 100°C [21].

Thin Layer Chromatogram Binding Assay

Monoclonal antibodies and polyclonal antisera were used in the assay performed according to the method described by Magnani *et al.* [22] and Uchigata *et al.* [23]. The monoclonal antibody 2D4 directed against asialo-G_{M2} [24] was kindly provided by Dr. W. Young, Seattle, WA, USA. the monoclonal antibody P^k002 directed against CTH [25] was kindly obtained form Dr. A. Lundblad, Lund, Sweden, and the monoclonal antibody E5C2 directed against paragloboside [23] was a gift from Dr. S. Spitalnik, New York, NY, USA.

Polyclonal antibodies against globoside were raised in rabbits, as described by Muhlradt *et al.*. [26]. The IgG fraction, obtained by precipitation with ammonium sulfate, was purified by affinity chromatography [27].

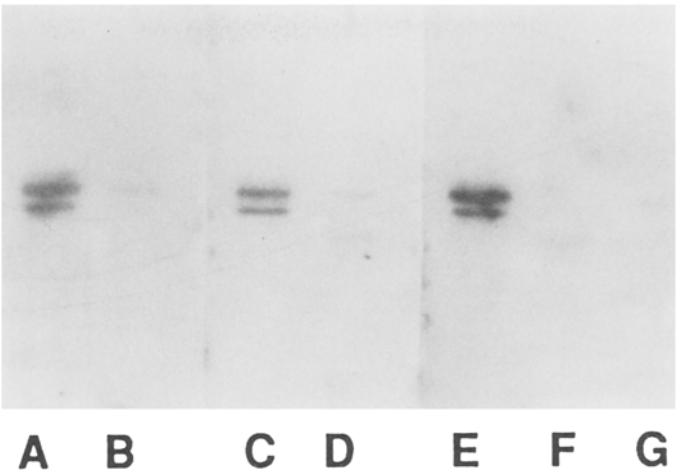

Figure 2. Thin layer chromatograms of desialylated gangliosides after mild acid hydrolysis detected by immunostaining with monoclonal antibody 2D4 directed against asialo-G_{M2}. The gangliosides were purified from cell lines of TGrII: HCV 29 (A), Hu 609 (C), Hu 1734 (E); and TGrIII: HCV 29T (B), Hu 609T (D), Hu 456 (F) and Hu 549 (G). For each cell line, aliquots of total gangliosides corresponding to 0.25 x 10^7 cells were applied to the HPTLC plate.

Mass Spectrometry

Neutral glycolipids and gangliosides from 3 x 10^8 cells were separated on HPTLC plates. The individual bands were cut out and analysed by fast atom bombardment mass spectrometry (FAB-MS) as permethylated derivatives [28] according to the method of Påhlsson and Nilsson [29].

FAB mass spectra were recorded on a VG ZAB SE instrument (VG Analytical, Manchester, UK) operated in positive ion mode. Samples were dissolved in thioglycerol (1-thio-2,3-propanediol; Ega Chemie, Steinheim, W. Germany) before being loaded on the stainless steel target. The target was bombarded with xenon atoms with a kinetic energy of 8 keV.

Results

High Performance Thin Layer Chromatography of Neutral Glycolipids and Gangliosides

In order to get an overall picture of the glycosphingolipid pattern of the human urothelial cell lines, fractions of neutral glycolipids and gangliosides were subjected to high performance thin layer chromatography together with standards.

The gangliosides found in human urothelial cell lines had mobilities corresponding to G_{M3}, G_{M2}, G_{M1} and G_{D1a}. The cell lines representing TGrII (Table 1), expressed as a major

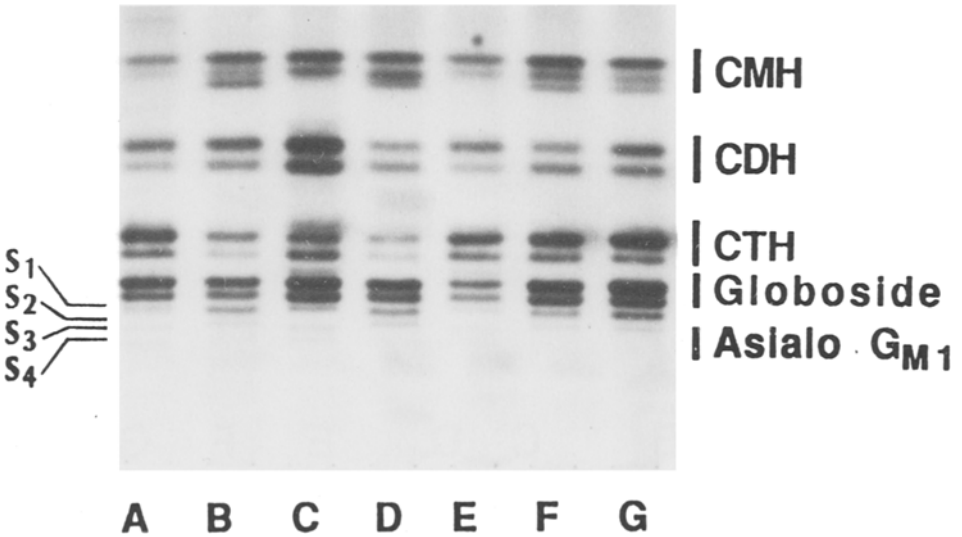

Figure 3. HPTLC pattern of total neutral glycolipids from human urothelial cell lines of different stage of transformation. Cell lines of TGrII: HCV 29 (A), Hu 609 (C), Hu 1734 (E); cell lines of TGrIII: HCV 29T (B), Hu 609T (D), Hu 456 (F), Hu 549 (G). Aliquots of neutral glycolipids corresponding to 1 x 10^7 cells were separated in solvent system chloroform/methanol/water, 60/35/8 by vol. Bands were visualized by *N*-(1-naphthyl)ethylenediamine reagent. Positions of standards are indicated to the right and positions of S_1-S_4 are indicated to the left.

ganglioside a component migrating as standard G_{M2} (Fig. 1 A, C, E). In the cell lines representing TGrIII (Table 1), this ganglioside was only seen as a minor compound (Fig. 1 B, F) or in trace amounts (Fig. 1 D, G). A semi-quantitative estimation of the content of G_{M2} ganglioside between the different cell lines was made by immunostaining of the desialylated gangliosides from all cell lines with antibody 2D4 directed against asialo-G_{M2} (Fig. 2). The autoradiogram clearly shows the difference between the cell lines of different transformation group. All cell lines synthesized G_{M3} and a ganglioside with the same mobility as G_{M1}. Weak bands corresponding to standard G_{D1a} were also found in some of the cell lines.

The neutral glycolipids found in human urothelial cell lines were represented by components with the same mobilities as standards of CMH, CDH, CTH and globoside and of two compounds (S_1 and S_2) migrating between globoside and asialo-G_{M1} (Fig 3). The component designated S_2 was only present in TGrIII cell lines. In addition, TGrII cell lines expressed two weak bands (S_3 and S_4) with mobilities as standard asialo-G_{M1} (Fig. 3 A, C, E). The glycolipids corresponding to standard CMH migrated as sets of double bands in cell lines, representing TGrII (Fig. 3 A, C, E) and as a triple bands in TGrIII cell lines (Fig. 3 B, D, F, G).

Characterization of Glycosphingolipids

Structural information about the gangliosides and the neutral glycolipids was obtained by FAB-MS, sequential treatment with exoglycosidases and an immunostaining technique. The glycosphingolipids of the cell line Hu 456 (TGrIII) and gangliosides from cell line Hu 609

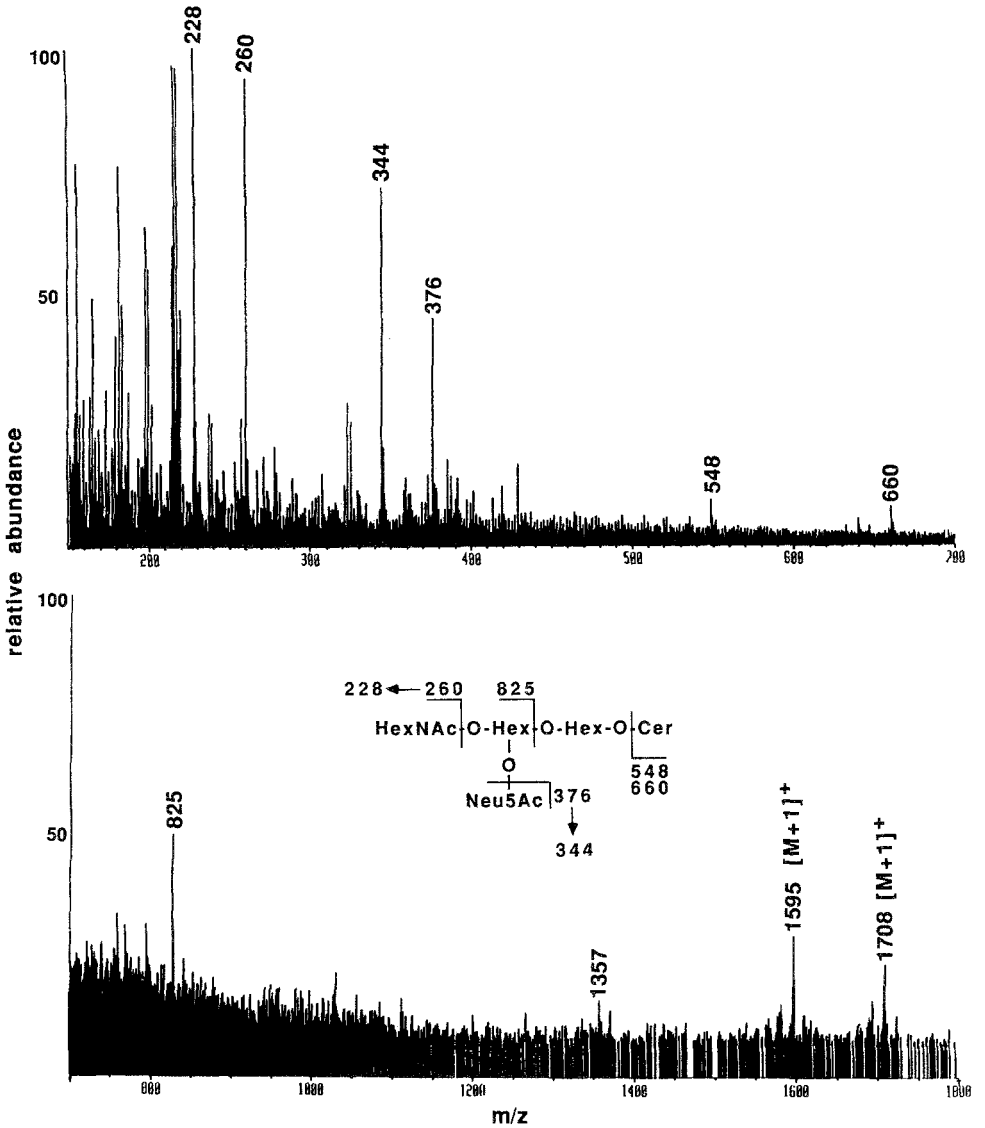

Figure 4. FAB-mass spectrum of the component, migrating as the G_{M2} standard, as a permethylated derivative. The component was derived from the cell line Hu 609. High and low mass range is shown. The primary and secondary sequence ions, as indicated, are in agreement with a G_{M2} structure. A secondary ion of m/z 1357 formed after elimination of the fatty acids from the $[M+1]^+$-ions gives a ceramide composition of C16:0 and C24:0 fatty acids and a C18:1 sphingosine base.

(TGrII) were studied in detail by the techniques above. These cell lines were chosen among the cell lines studied, due to their superior growth properties within each TGr group. However, identification of glycosphingolipid structures by immunostaining with antibodies was always performed on all seven cell lines. A previously developed method for analysis of glycosphingolipids, extracted from HPTLC plates, by FAB-MS was employed [29].

Table 2. Structural information obtained by FAB-MS of the major glycosphingolipids.

Structure	$[M+1]^+$-Ions	Ceramide composition*	Corresponding standard on HPTLC
Glycosphingolipids - TGrIII cell lines			
1. Hex-Cer	785, 815, 895, 926, 928	C16:0, C16h:0, C24:1, C24h:0, C24h:1	CMH
2. Hex-Hex-Cer	990, 1101	C16:0, C24:0	CDH
3. Hex-Hex-Hex-Cer	1193, 1305	C16:0, C24:0	CTH
4. HexNAc-Hex-Hex-Hex-Cer	1438, 1550	C16:0, C24:0	Globoside
5. Hex-HexNAc-Hex-Hex-Cer (S_1)	1438	C16:0	Paragloboside
6. Neu5Ac-Hex-Hex-Cer	1350, 1462	C16:0, C24:0	G_{M3}
7. HexNAc-[Neu5Ac]-Hex-Hex-Cer	1625	C16h:0	G_{M2}
8. Neu5Ac-Hex-HexNAc-Hex-Hex-Cer	1799, 1911, 1941	C16:0, C24:0, C24h:0	G_{M1}
9. Neu5Ac-Hex-HexNAc-[Neu5Ac]-Hex-Hex-Cer	2192, 2301	C16h:0, C24h:0	G_{D1a}
Gangliosides - TGrII cell lines			
10. HexNAc-[Neu5Ac]-Hex-Hex-Cer	1595, 1708	C16:0, C24:0	G_{M2}

* All compounds contained a C18:1 sphingosine base.

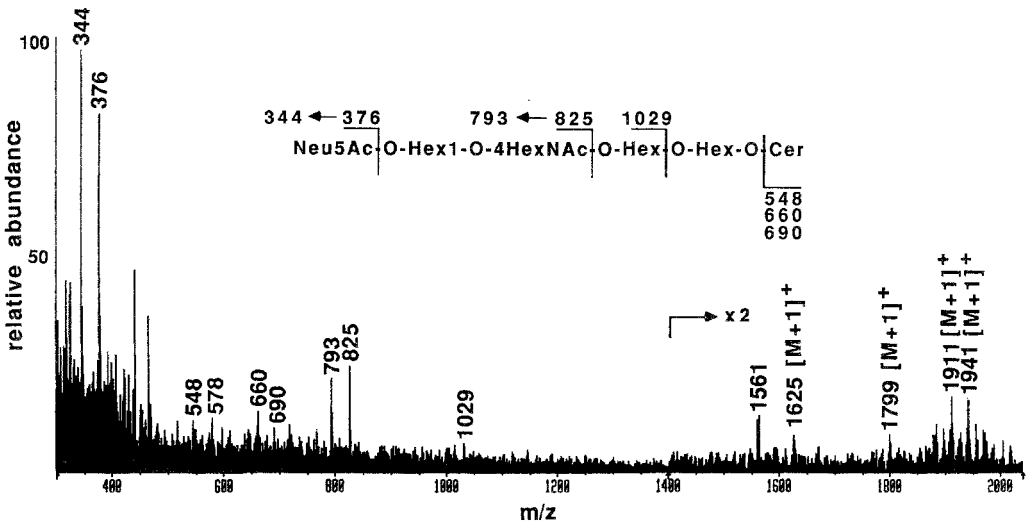

Figure 5. FAB-mass spectrum of the component, with the same mobility as standard G_{M1} as a permethylated derivative. Primary and secondary ions formed from the carbohydrate portion are indicated. From the ceramide containing ions and the $[M+1]^+$-ions together with the ion m/z 1561, formed after elimination of the fatty acids from the $[M+1]^+$-ions, a ceramide composition of a C18:1 sphingosine base with C16:0, C24:0 and C24h:0 fatty acids can be deduced. The spectrum also shows ions from a contaminating component. The ceramide ion of m/z 578 and the $[M+1]^+$ ion m/z 1625 indicate the presence of G_{M2} with a C18:1 sphingosine base and a C16h:0 fatty acid. Due to the hydroxy fatty acid this component also co-migrates with the G_{M1} standard.

FAB-MS of the permethylated glycosphingolipids gives information about the monosaccharide sequence, the ceramide composition and the molecular weight. The monosaccharide sequence is determined from the ions formed by cleavage of glycosidic linkages. Some information about binding positions could be obtained e.g. from the secondary fragments formed, by elimination, after a primary cleavage of a 2-acetamido-2-deoxyhexosyl (HexNAc) linkage. A 4-substituted HexNAc gives a secondary fragment by elimination of methanol, which is not observed for a 3-substituted HexNAc residue. The ceramide composition is deduced from the ceramide containing ions and the secondary ions formed, from the protonated molecular ion ($[M+1]^+$) by elimination of the amide linked fatty acid. A mechanism for this elimination has previously been proposed [29].

Gangliosides: Analysis by FAB-MS of the bands migrating as G_{M3} standard gave a spectrum characteristic for structure 6 (Table 2). Two $[M+1]^+$ ions indicated differences in the ceramide moiety corresponding to two different fatty acids, C16:0 and C24:0 respectively. The bands were treated in sequence with neuraminidase from *Vibrio cholerae* and β-galactosidase from jack bean giving glycolipids migrating as CDH and CMH standards, respectively.

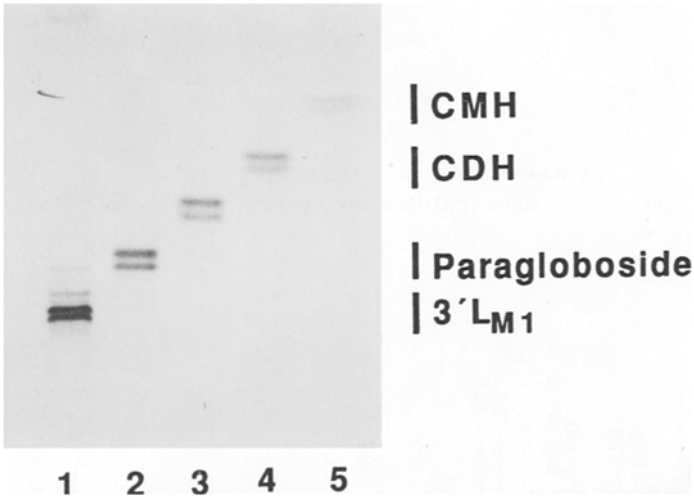

Figure 6. Degradation of the ^{14}C-labelled ganglioside with mobility corresponding to G_{M1} by exoglycosidases. Autoradiogram of the thin layer chromatogram. Lane 1 shows the native material after purification from TLC plate. This material was digested sequentially with neuraminidase from *Vibrio cholerae* (lane 2), with β-galactosidase (lane 3) and β-hexosaminidase from jack bean (lane 4) and again with β-galactosidase from jack bean (lane 5). The positions of standards: CMH, CDH, paragloboside and sialosylparagloboside (3^1L_{M1}) are indicated to the right.

The FAB-mass spectrum of the bands with the same mobility as standard G_{M2} is shown in Fig. 4. After mild acid hydrolysis the ganglioside was converted to a glycolipid migrating as standard asialo-G_{M2}. Subsequent treatment with β-*N*-acetyl-hexosaminidase from jack bean and β-galactosidase from jack bean converted this glycolipid to CDH and CMH, respectively. When the monoclonal antibody 2D4, directed against asialo-$G_{M2,}$ was bound to desialylated fractions of gangliosides corresponding to 0.25×10^7 cells, strong positive bands were seen only for TGrII cell lines (Fig. 2 A, C, E). Very faint bands were observed in the TGrIII cell lines (Fig. 2 B, D, G).

The component migrating as G_{M1} standard gave after permethylation a FAB-mass spectrum shown in Fig 5. Sialic acid was present (m/z 376 and m/z 344) and linked to an external residue determined by the ions of m/z 825 and m/z 793 characteristic for a Neu5Ac-Hex-HexNAc sequence. The m/z 793 fragment, formed by elimination of methanol from m/z 825 is characteristic for a 4-substituted HexNAc, as discussed above. The $[M+1]^+$-ions and the ceramide composition, deduced from the ceramide containing ions and the ion formed after elimination of the fatty acid from the $[M+1]^+$ ion, are listed in Table 2 (structure 8). After digestion with neuraminidase from *Vibrio cholerae* it was converted to a glycolipid migrating as paragloboside. This component was further digested with β-galactosidase from jack bean and consecutively with β-*N*-acetyl-hexosaminidase and β-galactosidase from jack bean (Fig. 6). Desialylated gangliosides from the individual cell lines gave positive double bands in overlay with the monoclonal antibody E5C2 against paragloboside (Fig. 7). The antibody also recognized an additional, slower migrating glycolipid in two of the desialylated cell lines, HCV 29 and Hu 1734 (Fig. 7 A, E). This glycolipid was not detectable by chemical staining.

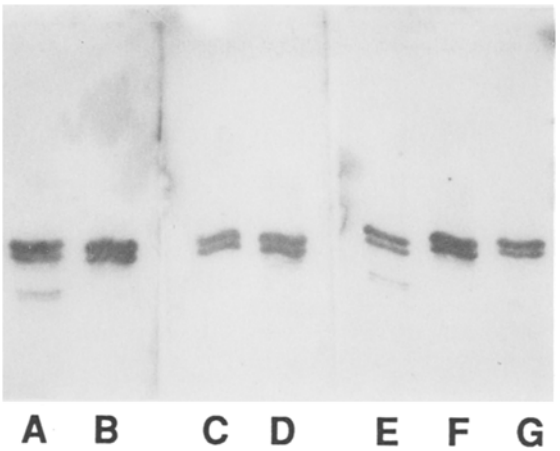

Figure 7. Immunostaining of desialylated gangliosides from urothelial cell lines separated by thin layer chromatography with anti-paragloboside antibody E5C2. Lanes A (HCV 29), C (Hu 609) and E (Hu 1734) represent cell lines of TGrII; lanes B (HCV 29T), D (Hu 609T), F (Hu 456) and G (Hu 549) represent cell lines of TGrIII. For each cell line, an aliquot of total gangliosides after mild acid hydrolysis, corresponding to 0.5 x 10^7 cells were applied to the HPTLC plate.

Analysis by FAB-MS of the component migrating as G_{D1a} standard showed a spectrum consistent with structure 9 (Table 2). After mild acid hydrolysis the component reacted with antibodies against asialo-G_{M1}.

Neutral Glycolipids: The bands migrating as standard CMH, CDH and CTH were analyzed by FAB-MS giving the structures shown in Table 2 (structure 1, 2 and 3). CMH showed considerable heterogeneity in terms of fatty acid composition and the spectrum showed unsaturated as well as hydroxy-fatty acids. Consecutive treatment of the bands, corresponding to standard CTH, with α-galactosidase form *Aspergillus niger* and β-galactosidase from jack bean yielded components with the same mobility as CDH and CMH, respectively. The neutral glycolipids from all cell lines gave positive reactions with monoclonal antibody P^k002 directed against CTH.

FAB-MS analysis of the band migrating as standard globoside showed sequence ions of m/z 260 and m/z 464 indicating a non-reducing terminal *N*-acetylhexosamine linked to a hexose. Ceramide ions, $[M+1]^+$-ions and a secondary ion formed from the $[M+1]^+$-ions by elimination of fatty acids gave the structures shown in Table 2 (structure 4). This glycolipid was digested in sequence with β-*N*-acetyl-hexosaminidase from jack bean, α-galactosidase from *Aspergillus niger* and β-galactosidase from jack bean. The bands corresponding to standard globoside were recognized by antibodies directed against globoside in the neutral glycolipid fractions from each cell line.

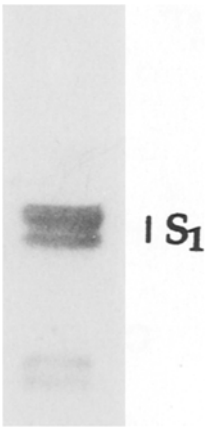

Figure 8. Immunostaining of neutral glycolipids from cell line HCV 29T separated on a thin layer chromatography plate. Staining with anti-paragloboside antibody E5C2; the major bands correspond to standard paragloboside, weaker slower migrating bands were not detectable by chemical staining. An aliquot of neutral glycolipids corresponding to 0.5 x 10^7 cells were applied to the HPTLC plate. The position of component S_1 is indicated to the right.

FAB-MS of the component designated S_1, migrating between the standards of globoside and asialo-G_{M1} gave a spectrum shown in Fig 9. The primary sequence ions of m/z 464 and m/z 668 define a sequence of Hex-HexNAc-Hex-. The secondary fragment, m/z 432, shows a 4-substituted HexNAc residue as previously discussed. The ceramide composition is shown in Table 2 (structure 5).

Immunostaining of the neutral glycolipids with antibody E5C2 which recognizes paragloboside, revealed the presence of positive double bands (Fig. 8). The lower band had the mobility of component S_1 and the upper band same mobility as standard globoside. Only one band of S_1 is seen because paragloboside is partially covered by globoside. This antibody also bound a slower migrating glycolipid, not detectable by chemical staining. The components designated S_2, S_3 and S_4 were not further characterized due to the limited amounts of material available.

Discussion

Neoplastic transformation are essentially always associated with changes of cell surface carbohydrates. Aberrant glycosylation was described for many different tumors in experimental animal systems and in humans [3, 4].

In human urinary bladder carcinomas numerous studies revealed characteristic changes in the expression of blood group antigens ABO(H) and Thomsen-Friedenreich antigen [10, 30-33]. It was shown that the disappearance of ABH antigens and appearance of Thomsen-Friedenreich determinants in the biopsy specimens of bladder carcinomas may be related to worse prognosis (recurrence, invasion). In our earlier studies a direct correlation between the grade of transformation of the urothelial cell lines analyzed in this paper, and the quantitative expression of highly branched *N*-acetyllactosamine type of glycans and differences in the expression of HLA and Thomsen-Friedenreich antigen were found [34, 35]. However, in contrast to other human tumors (adenocarcinomas, melanoma) [5] the information about the carbohydrate structures present on cell surfaces of human urinary bladder carcinomas are very limited [11] and to our knowledge, nothing was known about gangliosides expressed by these tumors.

The comparison of gangliosides expressed by human urothelial cell lines of different grades of transformation revealed characteristic, quantitative differences in the expression of G_{M2} between cells belonging to TGrII and TGrIII groups. G_{M2}, the major ganglioside of the cell lines: HCV 29, Hu 609 and Hu 1734, was only barely detectable in the remaining cell lines.

The cell lines HCV 29 and Hu 609, classified as TGrII were compared with the TGrIII cell lines HCV 29T and Hu609T which are "spontaneous" transformants of the HCV 29 and Hu 609 cell lines, respectively. The content of G_{M2} and G_{M3} in these cell lines were determined by densitometry (Table 3). The decrease of the G_{M2} ganglioside in the TGrIII cell lines was found to be accompanied by a concomitant increase in G_{M3} expression. It is possible that these changes could reflect precursor accumulation due to alterations in N-acetylglucosaminyltransferase activity.

It is interesting that these findings are not in agreement with the results obtained by Tsuchida *et al.* [36, 37] who found a positive correlation between the level of G_{M2} and tumorigenicity in human melanoma. But, as it was mentioned previously [20], specific changes in glycolipid profiles during process of carcinogenesis, can be limited to certain tissue of common germ layer origin.

Natoli *et al.* [38] used a monoclonal antibody directed against G_{M2} to define the expression of this ganglioside on the cell surface of cultured normal and malignant human cells. They investigated six lines of bladder cancer cells and they found that only one reacted with this antibody. These data are in agreement with our results that the expression of G_{M2} is suppressed in malignant urothelial cancer cells. However, it does not appear true that G_{M2} is a differentiation antigen mostly restricted to cells of neuroectodermal origin, as claimed by these authors. As it was shown by us, G_{M2} is a major ganglioside of non-malignant human urothelial cell lines. In the case of melanoma, the malignancy is connected with accumulation of G_{M2}, whereas in urothelial malignancies the expression of this ganglioside is suppressed. Therefore, it can be assumed, that G_{M2} is a differentiation antigen of urothelial cells, as well.

The biological significance of changes in G_{M2} expression for malignant phenotype remains obscure. However, it has been shown that G_{M2} induces immune response in an autologous host [39] and that gangliosides can regulate the immune system in humans [40].

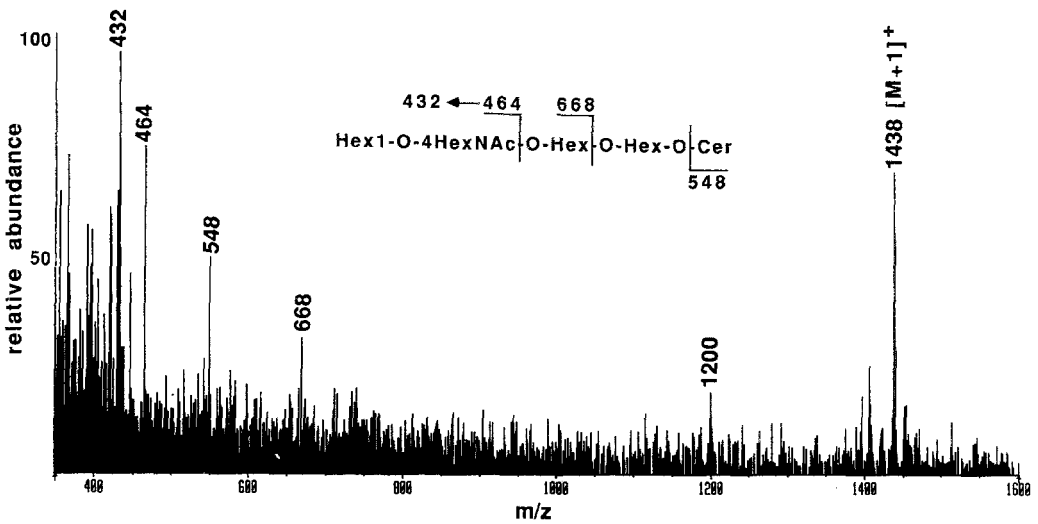

Figure 9. FAB-mass spectrum of the component migrating as paragloboside standard as permethylated derivative. The primary and secondary ions from the carbohydrate portion are indicated. A ceramide ion of m/z 548 correspond to a $[M+1]^+$-ion of m/z 1438. A secondary fragment of m/z 1200 concludes a ceramide composition of C16:0 fatty acid with a C18:1 sphingosine base.

There were no differences in the major neutral glycolipid patterns, when carbohydrate structures were compared. However, the cell lines HCV 29T, Hu 609T, Hu 456 and Hu 549 expressed an additional band in the region of CMH, suggesting the presence of a third component with different ceramide moiety. FAB-MS of CMH from TGrIII cell lines showed this component to be very heterogeneous in terms of fatty acid composition. Both unsaturated as well as hydroxy-fatty acids were found (Table 2). The hydroxy-fatty acids give rise to the slower migrating bands. Some of the gangliosides from TGrIII cell lines also showed hydroxy-fatty acids (Table 2), all other compounds tested contained only C16:0 and C24:0 fatty acids.

It was demonstrated that ceramide composition is an important factor in antigenicity of glycolipids, especially those having shorter carbohydrate chains [41-43]. Generally, the presence of fatty acids with longer chains lengths or hydroxy-fatty acids increases antigenicity of glycolipids, influencing the spatial organization of these components in the cell membrane. The additional species of CMH and some of the gangliosides seen in TGrIII cell lines can represent compounds, which may be organized in the cell membrane in a very different way. But at present a biological significance of such differences between cell lines representing different stages of transformation remains obscure.

Table 3. Content of G_{M2} and G_{M3} in human urothelial cell lines.
Gangliosides isolated from the cell lines were separated on HPTLC plates in the solvent system: chloroform/methanol/0.2% $CaCl_2$, 60/40/9 by vol, and visualized with resorcinol. G_{M2} and G_{M3} were quantified by densitometry. Values are the means ± SD from 3-4 determinations.

Cell line	TGr	G_{M2} (nmol[a])	G_{M3} (nmol[a])
HCV 29	II	3.59 ± 0.68	0.71 ± 0.13
Hu 609	II	4.43 ± 0.42	0.45 ± 0.09
HCV 29T	III	0.36 ± 0.13	2.49 ± 0.29
Hu 609T	III	0.26 ± 0.13	2.39 ± 0.46

[a] nmol of sialic acid/10^7 cells.

In conclusion, our comparative studies on glycosphingolipids expressed in human urothelial cell lines of TGrII and TGrIII revealed two major differences: (i) significant decrease or absence of G_{M2} in TGrIII cell lines, (ii) presence of additional species of CMH and some gangliosides with different ceramide moieties in TGrIII cell lines.

Studies are in progress to re-evaluate the significance of G_{M2} as a marker of potential prognostic value for bladder cancer.

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