Structural Complexity of Non-Acid Glycosphingolipids in Human Embryonic Stem Cells Grown under Feeder-free Conditions*

Angela Barone1, John Benktander2, Jonas Ångström2, Anders Aspegren1, Petter Björquist3, Susann Teneberg2,4, and Michael E. Breimer1

1From the Department of Surgery, Sahlgrenska University Hospital, S-41 345 Göteborg, Sweden
2Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, P.O. Box 440, University of Gothenburg, S-405 30 Göteborg, Sweden
3Cellectis Stem Cells, Cellartis AB, Arvid Wallgrens Backe 20, S-413 46 Göteborg, Sweden

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4To whom correspondence should be addressed: Susann Teneberg, Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, University of Gothenburg, P.O. Box 440, S-405 30 Göteborg, Sweden. Phone: +46 31 786 34 92; Fax: +46 31 413 190; E-mail: Susann.Teneberg@medkem.gu.se

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Background: Carbohydrate epitopes are often used as markers for characterization of human embryonic stem cells (hESC).

Results: Several glycosphingolipids not previously found in hESC were characterized.

Conclusion: The glycosylation of hESC is more complex than previously thought.

Significance: These findings will help to understand the immunogenicity of hESC, and might impact future applications in regenerative medicine.

Due to their pluripotency and growth capability there are great expectations of human embryonic stem cells, both as a resource for functional studies of early human development and as a renewable source of cells for use in regenerative medicine and transplantation. However, to bring human embryonic stem cells into clinical applications, their cell surface antigen expression and its chemical structural complexity have to be defined. In the present study total non-acid glycosphingolipid fractions were isolated from two human embryonic stem cell lines (SA121 and SA181) originating from left over in vitro fertilized human embryos, using large amounts of starting material (1x10⁸ cells per cell line). The total non-acid glycosphingolipid fractions were characterized by antibody and lectin binding, mass spectrometry and proton NMR. In addition to the globo series and type 1 core chain glycosphingolipids previously described in human embryonic stem cells, a number of type 2 core chain glycosphingolipids (neolactotetraosylceramide, the H type 2 pentaosylceramide, the Le⁵ pentaosylceramide, and the Le⁵ hexaosylceramide) were identified, as well as the blood group A type 1 hexaosylceramide. Finally, the mono-, di- and triglycosylceramides were characterized as galactosylceramide, glucosylceramide, lactosylceramide, galabiaosylceramide, globotriaosylceramide and lactotriaosylceramide. Thus, the glycan diversity of human embryonic stem cells, including cell surface immune determinants, is more complex than previously appreciated.

Human embryonic stem cells (hESC)¹ derived from the inner cell mass of blastocysts are pluripotent, i.e. have the capacity to transform into all derivatives of the three primary germ layers of the developing embryo, as well as the ability to replicate indefinitely (1-4). These features make hESC excellent candidates to be used in regenerative medicine, provided that the grafted cells are tolerated by the immune system of the recipient. Thus, before hESC can be brought into the clinic there is need for a deeper understanding of the molecular mechanisms underlying the
proliferation and differentiation of hESC. Carbohydrate epitopes are often used as markers for definition and characterization of hESC, and also to monitor their differentiation (5). Cell surface marker profiling of undifferentiated hESC in culture show expression of the stage-specific embryonic antigen 3 (SSEA-3) and SSEA-4, and the keratan-sulfate-associated antigens TRA-1-60 and TRA-1-81 (5-8). SSEA-3 and SSEA-4 are glycosphingolipids (globopentaosylceramide and sialyl-globopentaosylceramide, respectively), since the globo carbohydrate core is only found in glycosphingolipids.

The major part of the current knowledge about cell surface carbohydrates on embryonic stem cells originates from experiments performed on mouse embryonic cells. The majority of these studies have been done using immune labeling techniques, and chemical structural characterization of antigens are lacking. There are only two studies where the glycosphingolipids of human embryonic stem cells have been characterized (9, 10). By use of immunofluorescence, flow cytometry, MALDI-MS and MS/MS analyses of glycosphingolipids from crude lipid extracts, glycosphingolipids of the globo series (globotetraosylceramide, globopentaosylceramide/SSEA-3 and the Globo H hexaosylceramide) and with type 1 core chains (lactotetraosylceramide and fucosyl-lactotetraosylceramide/H type 1 pentaosylceramide) were identified in undifferentiated hESC, and the gangliosides found were GM3, GM1, GD1a or GD1b, sialyl-globopentaosylceramide/SSEA-4 and disialyl-globopentaosylceramide. Differentiation into neural progenitor cells led to expression of mainly gangliosides of the ganglio-series (9, 10), whereas differentiation into endodermal cells gave a predominant expression of globotetraosylceramide (10).

In order to get a comprehensive overview of the glycosphingolipid expression of cultured hESC we have in the present study isolated total non-acid glycosphingolipid fractions from two human embryonic stem cell lines (SA121 and SA181) using large amounts of starting material (1x10^9 cells per cell line). The total non-acid glycosphingolipid fractions, and isolated subfractions, were characterized with antibody and lectin binding, mass spectrometry and proton NMR. This approach allowed an increased resolution and several non-acid glycosphingolipids not previously described in human embryonic stem cells were identified, such as type 2 core chain glycosphingolipids (the H type 2 pentaosylceramide, the Le ε pentaosylceramide and Le ε hexaosylceramide), as well as a blood group A type 1 hexaosylceramide. In addition, the mono-, di- and triglycosylceramides were characterized as galactosylceramide, glucosylceramide, lactosylceramide, galabiosylceramide, globotriaosylceramide and lactotriaosylceramide.

**EXPERIMENTAL PROCEDURES**

*Expansion and harvest of human embryonic stem cells* – hESC were grown and passaged as previously described (11). In brief, two cell lines (SA121 and SA181) were generated from two separate left over human in vitro fertilized embryos. Cells were transferred from mechanically dissected cultures grown on mouse embryonic fibroblasts to the feeder free system, and expanded for four passages to achieve a frozen working cell bank. The achieved cell banks were then quality controlled according to conventional quality control criteria for human pluripotent stem cells. In order to obtain enough material for this study, each bank was thawed in passage five and expanded accordingly, with passages performed every third or fourth day. Dense flasks in passage 8, 9 and 10 were harvested using the phosphate-buffered saline-based (PBS; pH 7.3) enzyme-free cell dissociation buffer (Gibco/Invitrogen), thus minimizing the risk of destroying outer cell membrane compounds. Each harvest generated roughly 1x10^9 undifferentiated human embryonic stem cells from 30 x T175 flasks/occasion (Corning). Cells were pooled and centrifuged briefly (3 min in swing out rotor at 300xg) whereafter the supernatant was decanted and the cell pellet stored at -80 °C.

*Isolation of glycosphingolipids from human embryonic stem cell lines* – Non-acid glycosphingolipids were isolated from the two hESC lines (SA121 and SA181), essentially as described (12, 13). The cells (1 x 10^7 cells from each cell line) were extracted by addition of firstly 5 ml methanol at 70 °C. After a brief centrifugation the supernatant was removed, and the cells were extracted three times with chloroform:methanol 1:2 (by volume), and three times with chloroform:methanol 2:1 (by volume), in the same manner as above. The
supernatants were pooled and dried. Acid and non-acid glycosphingolipid fractions were then separated by DEAE-cellulose column chromatography. The non-acid fraction was subjected to mild alkaline hydrolysis and after dialysis, polar and non-polar lipids were separated on a silicic acid column. In order to separate the non-acid glycosphingolipids from alkali-stable phospholipids, the polar lipid fraction was acetylated and separated on a second silicic acid column, followed by deacetylation and dialysis. Final purifications were performed by chromatographies on DEAE-cellulose and silicic acid columns. Approximately 3 mg of total non-acid glycosphingolipids were obtained from each cell line, and these fractions were structurally characterized by thin-layer chromatography, binding of lectin and monoclonal antibodies and mass spectrometry. Thereafter, partly purified subfractions were obtained by separation of the non-acid glycosphingolipids using Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) columns (0.5 g), eluted with increasing amounts of methanol in chloroform. These subfractions were further characterized by antibody binding, mass spectrometry and proton NMR spectroscopy.

Reference glycosphingolipids – Total acid and non-acid glycosphingolipid fractions were from various human and animal tissues (13-17). The individual glycosphingolipids obtained by repeated chromatography on silicic acid columns, and by HPLC, were identified by mass spectrometry (18, 19) and proton NMR spectroscopy (20).

Thin-layer chromatography – Aluminum- or glass-backed silica gel 60 high performance thin-layer chromatography plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography, and eluted with chloroform/methanol/water (60:35:8, by volume) as solvent system. The different glycosphingolipids were applied to the plates in quantities of 1-4 µg of pure glycosphingolipid, and 20-40 µg of glycosphingolipid mixtures. Chemical detection was done with anisaldehyde (13).

Chromatogram binding assays – Erythrina cristagalli lectin was purchased from Vector Labs. Monoclonal anti-A (HE193), anti-B (HEB-29), and anti-H type 1 (17-206) antibodies were obtained from GeneTex/Abcam. Monoclonal anti-H type 2 (A583) and rabbit anti-mouse antibodies (Z0259) were from DakoCytomation Norden A/S, anti-Lea (78.FR2.3) from Dominon, anti-Leb (BG-6/T218) from Signet/Covance, anti-Globo H (MBr1) from Enzo Life Sciences, and anti-Globopenta/SSEA3 (MC-631) from eBioscience. Monoclonal anti-Lea/SSEA-1 (P12) and anti-Leb (F3) were purchased from Calbiochem.

Binding of monoclonal antibodies to glycosphingolipids on thin-layer chromatograms was performed according to Hansson et al. (21). Chromatograms with separated glycosphingolipids were dipped for 1 min in diethylether/n-hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich Chem. Comp. Inc., Milwaukee, WI). After drying, the chromatograms were soaked in PBS containing 2% bovine serum albumin and 0.1% NaN₃ (Solution A), for 2 h at room temperature. Suspensions of monoclonal antibodies diluted 1:50 – 1:500 (the dilution used for each monoclonal antibody is given in Table 1) in Solution A, were gently sprinkled over the chromatograms, followed by incubation for 2 h at room temperature. After washing with PBS followed a second 2 h incubation with 125I-labeled (labeled by the IODO-GEN method according to the manufacturer’s instructions (Pierce) rabbit anti-mouse antibodies diluted to 2 x 10⁶ cpm/ml in Solution A. Finally, the plates were washed six times with PBS. Dried chromatograms were autoradiographed for 12-24 h using XAR-5 X-ray films (Eastman Kodak).

Chromatogram binding assays with 125I-labeled lectin from E. cristagalli were done as described (16).

Binding of human serum to glycosphingolipids on thin-layer chromatograms was performed as described (22). The human blood group AB serum pool was collected from 21 healthy blood donors, and used at a dilution of 1:5. The secondary antibodies were alkaline phosphate conjugated goat anti-human polyvalent immunoglobulins (Sigma), and the reactions were visualized with BCIP/NBT chromogenic substrate (Sigma).

LC-ESI/MS and ESI/MS/MS of native glycosphingolipids – The glycosphingolipids (dissolved in methanol/acetonitrile 75:25, by volume) were separated on a 200 x 0.150 mm column, packed in-house with 5 µm polyamine II particles (YMC Europe GMBH, Dinslaken,
endoglycoceramidase digestion and LC/MS – Endoglycoceramidase II from Rhodococcus spp. (23) (Takara Bio Europe S.A., Gennevilliers, France) was used for hydrolysis of glycosphingolipids. Briefly, 50 µg of glycosphingolipids were resuspended in 100 µl 0.05 M sodium acetate buffer, pH 5.0, containing 120 µg sodium cholate, and sonicated briefly. Thereafter, 1 mU of enzyme was added, and the mixture was incubated at 37 °C for 48 h. The reaction was stopped by addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters). The eluant containing the oligosaccharides was dried under nitrogen and under vacuum.

The glycosphingolipid-derived oligosaccharides were analyzed by capillary-LC/MS and MS/MS as described (19). In brief, the oligosaccharides were separated on a column (200 x 0.180 mm) packed in-house with 5 µm porous graphite particles (HypercARB, Thermo Scientific), eluted with an acetonitrile gradient (A: 10 mM ammonium bicarbonate; B: 80% acetonitrile, 10 mM ammonium bicarbonate). The saccharides were analyzed in the negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer.

Proton NMR spectroscopy – 1H NMR spectra were acquired on a Varian 600 MHz spectrometer at 30 °C. Samples were dissolved in dimethyl sulfoxide/D2O (98:2, by volume) after deuterium exchange. Two-dimensional double quantum-filtered correlated spectroscopy (DQF-COSY) spectra were recorded using the standard pulse sequence (24).

RESULTS

Isolation of total non-acid glycosphingolipids from hESC – Total amounts of non-acid glycosphingolipids isolated from each hESC line SA121 and SA181 (1 x 10⁷ cells) were 3 mg. Thin-layer chromatography using chemical staining showed that both cell lines had a number of compounds migrating in the mono- to hexaglycosylceramide regions (Fig. 1, lanes 1-2). The total glycosphingolipid fractions were first analyzed by binding of lectin and monoclonal antibodies, as described below.

Binding of Erythrina cristagalli lectin and monoclonal antibodies to the total non-acid glycosphingolipids from hESC lines – The lectin from E. cristagalli binds to glycoconjugates with terminal Galβ4GlcNAc and Fucα2Galβ4GlcNAc sequences (16). In the non-acid fractions from the SA121 and SA181 cell lines, a binding of E. cristagalli lectin in the tetraglycosylceramide region was obtained (Fig. 1B), indicating the presence of Galβ4GlcNAc-terminated glycosphingolipid, i.e. neolactotetraglycosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer).

In addition, the monoclonal antibodies directed against the H type 1, Globo H and Globopenta/SSEA3 epitopes bound to compounds migrating in the penta- and hexaglycosylceramide regions in the non-acid fractions from the SA121 and SA181 cell lines (Fig. 1C, E and F). This suggested the presence of H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer), globopentaosylceramide (Galβ3GlcNAcβ3Galα4-Galβ4Glcβ1Cer), and Globo H hexaglycosylceramide (Fucα2Galβ3-GalNAcβ3Galβ4Glcβ1Cer).

It should here be noted that some of these monoclonal antibodies used were not entirely specific. One example is the anti-Globo H antibody which also bound to the H type 1 glycosphingolipid (Fig. 1E, lane 3). Thus, the binding of the anti-Globo H antibody in the penta-/hexaosylceramide region in the non-acid fractions from the SA121 and SA181 cell lines could be recognition of Globo H hexaglycosylceramide with a different ceramide than the reference Globo H, but could also be a cross-reactivity with the H type 1 pentaosylceramide. Also the anti-globopenta/SSEA3 antibody was highly cross-
reactive and, in addition to the globo-pentaosylceramide, it bound to globotetraosylceramide, the H type 1 pentaosylceramide and the Globo H hexaosylceramide (Fig. 1F). The cross-reactivities observed are summarized in Table 2.

Separation of the total non-acid glycosphingolipids from hESC – After these studies the non-acid glycosphingolipid fractions were separated on latrobeads columns, in order to enrich the slow-migrating glycosphingolipids. Thereby, four glycosphingolipid-containing fractions were obtained from each cell line (Fig. 2A and B). These fractions were denoted fractions 121-I, 121-II and 121:III and 181:I-IV, respectively. Thus, fractions 121:II and 181:II (0.2 mg each) contained compounds migrating in the monoglycosylceramide region, while the material in fractions 121:II and 181:II (0.2 mg each) migrated as di- and triglycosylceramides. Fractions 121:III and 181:III (0.2 mg each) contained compounds migrating as triglycosylceramides, and fractions 121:IV and 181:IV (0.7 mg each) contained triglycosylceramides and more slow-migrating compounds. After these eight fractions had been analyzed by binding of monoclonal antibodies, mass spectrometry and proton NMR, fractions 121:IV and 181:IV were further separated by latrobeads chromatography in an attempt to obtain more structural information about the slow-migrating compounds. The fractions collected were pooled according to the migration on thin-layer chromatograms giving in each case four fractions of approximately 0.1 mg. The two fractions containing the slow-migrating compounds from each separation were denoted fractions 121:IV-C, 121:IV-D, 181:IV-C and 181:IV-D, respectively.

Characterization of mono-, di and triglycosylceramides from hESC – The base peak chromatograms from ESI-MS of the native fractions 121-I, 121-II and 121:III from human embryonic stem cell line SA121 (Fig. 2C-E), indicated that the major compounds of these fractions were a monohexosylceramide, a dihexosylceramide and a trihexosylceramide, respectively, in all three cases with sphingosine long chain base and non-hydroxy 16:0 fatty acids.

Further analyses of mass chromatograms and by MS² confirmed the presence of monohexosylceramide with d18:1-16:0 in fraction 121:II (Fig. 3A). In addition, monohexosylceramide with d18:1-18:0 and d18:1-24:1 was found (Fig. 3B and C).

Fraction 121:II contained dihexosylceramides with d18:1-16:0 and d18:1-18:0 (Fig. 3D and E), and fraction 121:III had trihexosylceramides with d18:1-16:0 and d18:1-18:0 (Fig. 3F and G). Identical results were obtained by MS² of fractions 181:I-III (data not shown).

The anomeric regions of the 600 MHz proton NMR spectra of fractions 121-I, 121-II and 121:III are shown in Fig. 4 and identified structures as well as chemical shift data for the anomeric proton resonances are given in Table 3. Fraction 121-I thus contains two monohexosylceramide species identified as Glcβ1Cer (structure 1) (~ 90%) and Galβ1Cer (structure 2) (~ 10%) from the positions of the anomeric resonances at 4.075 ppm and 4.024 ppm, respectively (25). The glucose is corroborated by the observation of its H2 resonance at 2.996 ppm (data not shown). Furthermore, the ceramide structure of both compounds is consistent with a sphingosine long-chain base, as evidenced by the presence of the H4 and H5 resonances, which are shifted downfield due to the C4-C5 double bond. The fatty acids are non-hydroxy as demonstrated by the H2 and H3 resonances at 2.02 ppm and 1.44 ppm (data not shown), respectively, and the presence of a cis double bond is also evident from the triplet at 5.32 ppm.

The subsequent fraction (121-II) displays anomeric resonances from three different glycosphingolipids: globotriaosylceramide (structure 5) (~ 45%) as evidenced by resonances at 4.780 ppm (Galo4), 4.246 ppm (Galβ4) and 4.157 ppm (Glcβ1), galabiosylceramide (structure 4) (~ 10%) with anomeric resonances at 4.814 ppm (Galo4) and 4.098 ppm (Galo1), finally, and lactosylceramide (structure 3) (~ 45%) with resonances at 4.195 (Galβ4) and 4.155 ppm (Glcβ1), the latter completely overlapping the corresponding globotriaosylceramide resonance (25, 26). In fraction 121:III only one additional compound is evident, namely globotetraosylceramide (structure 6), whose minor resonances (~ 5% of the dominating globotriaosylceramide contribution) are found at 4.797 ppm (Galo4), 4.514 ppm (GalNAcβ3), 4.26 ppm (Galβ4) and 4.16 ppm (Glcβ1) (25). Both fractions 121-II and 121-III have ceramides with sphingosine long-chain
bases and non-hydroxy fatty acids, and fraction 121-II has more cis unsaturation than fraction 121-III.

The structures found in the corresponding fractions isolated from cell line SA181 were identical.

**Binding of monoclonal antibodies to the slow-migrating non-acid glycosphingolipids from hESC lines** – Probing of the non-acid fractions 121:IV and 181:IV with monoclonal antibodies directed against the blood group A epitope gave a distinct binding in the hexaglycosylceramide region in both cell lines (Fig. 5B). No binding of the anti-Le\(^a\) or the anti-Le\(^b\) antibodies was obtained (data not shown). However, slow-migrating compounds of both SA121:IV and SA181:IV were recognized by both the anti-Le\(^a\) antibody and the highly cross-reactive anti-Le\(^o\) antibody (Fig. 5C and D).

**LC-ESI/MS of the slow-migrating non-acid glycosphingolipids from human embryonic stem cell lines** – LC-ESI/MS of oligosaccharides using graphitized carbon columns gives resolution of isomeric saccharides, and the carbohydrate sequence can be deduced from series of C-type fragment ions obtained by MS\(^2\) (19). In addition, diagnostic cross-ring \(0,2A\)-type fragment ions are present in MS\(^2\) spectra of oligosaccharides with a Hex or HexNAc substituted at C-4, and thus allow differentiation of linkage positions.

The base peak chromatogram obtained from LC-ESI/MS of the oligosaccharides obtained by hydrolysis of fraction 181-IV with *Rhodococcus* endoglycosylceramidase II is shown in Fig. 6A. Molecular ions corresponding to oligosaccharides ranging from trisaccharides (detected as [M-H\(^+\)]\(^\) ions at m/z 503) to hexasaccharides (were detected as [M-H\(^+\)]\(^\) ions at m/z 1014 and 1055) were found. In addition, a number of minor oligosaccharides were detected by inspection of mass chromatograms of selected ions (see below).

**A. Major triglycosylceramide of fraction 181-IV**

MS\(^2\) of the ion at m/z 503 eluting at 13.1 min gave two C-type fragment ions (C\(_1\) at m/z 179 and C\(_2\) at m/z 341) identifying a Hex-Hex-Hex sequence (data not shown). There was a prominent \(0,2A\) fragment ion at m/z 281 demonstrating a 4-substitution of the internal Hex (19, 26, 27), and thus this trisaccharide was tentatively identified as globotriaose (Gal\(\alpha\)4Gal\(\beta\)4Glc).

**B. Tetraglycosylceramides of fraction 181-IV**

The mass chromatogram of m/z 706, corresponding to a saccharide with one HexNAc and three Hex (Fig 6B), had two sets of peaks, eluting at 13.9-14.8 min and 20.4-21.3 min, respectively. The first set of peaks eluted at the same retention time as the saccharide obtained from reference globotetraosylceramide (Fig. 6C), while the second set of peaks co-eluted with the saccharides obtained from reference lactotetraosylceramide and neolactotetraosylceramide (Fig. 6E and F).

The MS\(^2\) spectrum of the ion at m/z 706 at retention time 13.8 min (Fig. 7A) had a C-type fragment ion series (C\(_1\) at m/z 220, C\(_2\) at m/z 382, and C\(_3\) at m/z 544), demonstrating a HexNAc-Hex-Hex-Hex sequence. Cross-ring \(0,2A\)-type fragments are diagnostic for carbohydrates substituted at C-4 (19, 27, 28), and here the \(0,2A\) fragment ion at m/z 484 demonstrated a 4-substituted Hex, while the \(0,2A\) ion at m/z 646, and the \(0,2A\)-H\(_2\)O ion at m/z 628, were derived from cross-ring cleavage of the 4-substituted Glc of the lactose unit at the reducing end. The features of this MS\(^2\) spectrum were very similar to the MS\(^2\) spectrum of reference globotetra saccharide (Fig. 7B), and thus a globotetra saccharide (GalNAc\(\beta\)3Gal\(\alpha\)4Gal\(\beta\)4Glc) was tentatively identified.

MS\(^2\) of the ion at m/z 706 at the retention time 21.3 min (Fig. 7C) allowed a preliminary identification of a lactotetra saccharide (Gal\(\beta\)3GlcNAc\(\beta\)3Gal\(\beta\)4Glc). This was concluded from the C-type fragment ions (C\(_2\) at m/z 382 and C\(_3\) at m/z 544) identifying a Hex-HexNAc-Hex-Hex sequence. Here the MS\(^2\) spectrum was very similar to the MS\(^2\) spectrum of reference lactotetra saccharide (Fig. 7D). Both these MS\(^2\) spectra lacked a \(0,2A\) fragment ion at m/z 281, indicating that the HexNAc was substituted at 3-position.

The MS\(^2\) spectrum of the ion at m/z 706 at the retention time 20.7 min (Fig. 7E) was significantly different. This spectrum had a prominent \(0,2A\) fragment ion at m/z 281, and a \(0,2A\)-H\(_2\)O fragment ion at m/z 263, demonstrating a terminal Hex-HexNAc sequence with a 4-substitution of the HexNAc, i.e. a type 2 chain. In combination with the C\(_2\) ion at m/z 382 and the C\(_3\) ion at m/z 544, this suggested a neolactotetra saccharide (Gal\(\beta\)4GlcNAc\(\beta\)3Gal\(\beta\)4Glc). This suggestion...
was corroborated by the similarity between this MS2 spectrum and the MS2 spectrum of reference neolactotetra saccharide (Fig. 7F).

**C. Pentaglycosylceramides of fraction 181-IV**

The mass chromatogram of m/z 852, corresponding to a saccharide with one Fuc, one HexNAc and three Hex (Fig 8A), had a major set of peaks eluting at 19.6-20.4 min, and two minor peaks at 17.0 min and 21.8 min, respectively. The major set of peaks co-eluted with the saccharide obtained from reference Le^-pentaglycosylceramide (Fig. 8B), while the minor peaks eluted at the same retention times as the saccharides obtained from reference Le^+ pentaglycosylceramide (Fig. 8E) and H type 2 pentaglycosylceramide (Fig. 8C), respectively.

MS^2 of the ion at m/z 852 at retention time 19.5 min resulted in a series of prominent C-type fragment ions ([C_2 at m/z 325, C_3 at m/z 528, and C_4 at m/z 690] identifying a pentasaccharide with Fuc-Hex-HexNAc-Hex-Hex sequence (Fig. 9A). The features of this MS^2 spectrum were very similar to the MS^2 spectrum of reference H type 1 pentaglycosylceramide (Fig. 9B), which gave a tentative identification of a H type 1 pentasaccharide (Fucα3Galβ3GlcNAcβ3-Galβ4Glc).

The MS^2 spectrum of the ion at m/z 852 at retention time 21.8 min (Fig. 9C) suggested a H type 2 pentasaccharide (Fucα2Galβ3GlcNAcβ3-Galβ4Glc). This was concluded from the C-type fragment ion series (C_2 at m/z 325, C_3 at m/z 528, and C_4 at m/z 690) identifying a Fuc-Hex-HexNAc-Hex-Hex sequence, and the 0.2A_3 fragment ion at m/z 427, and the 0.2A_2-H_2O fragment ion at m/z 409, demonstrating a substitution of the HexNAc at C-4, i.e. a type 2 chain. This MS^2 spectrum was also very similar to the MS^2 spectrum of the reference H type 2 pentasaccharide (Fig. 9D).

A fragment ion at m/z 364 was present in the MS^2 spectrum of the ion at m/z 852 at retention time 17.0 min (Fig. 9E). This fragment ion is obtained by double glycosidic cleavage of the 3-linked branch at C_3 and Z_3, and is characteristic for an internal 4-linked GlcNAc substituted with a Fuc at 3-position (23). Taken together with the C_2 ion at m/z 528 and the C_3 ion at m/z 690, and with the similarity to the MS^2 spectrum of the reference Le^- pentasaccharide (Fig. 9F), this tentatively identified a Le^- pentasaccharide (Galβ4-(Fucα3)GlcNAcβ3Galβ4Glc).

**D. Minor compounds of fraction 181-IV**

A number of minor compounds were also found by further scrutiny of selected ions. Firstly there was a [M-H^-] ion at m/z 544, eluting at 18.0-18.1 min (data not shown). MS^2 of this ion at m/z 544 gave C-type fragment ions at m/z 220 (C_1) and at m/z 382 (C_2), identifying a HexNAc-Hex-Hex sequence. No 0.2A_2 fragment ion at m/z 322 was found, indicating that the internal Hex was 3-substituted. Taken together this tentatively identified a lacto trisaccharide (GlcNAcβ3-Galβ4Glc).

The MS^2 spectrum of the ion at m/z 868 at retention time 15.1 min is shown in Fig. 10A. A Hex-HexNAc-Hex-Hex-Hex sequence is suggested by the C-type ion series (C_2 at m/z 382, C_3 at m/z 544, and C_4 at m/z 706). The 0.2A_4 fragment ion at m/z 646, along with the 0.2A_3 fragment ion at m/z 808 and the 0.2A_3-H_2O fragment ion at m/z 790, demonstrated that the two hexoses at the reducing end were substituted at C-4 (Hex-HexNAc-Hex-4Hex-4Hex), tentatively identifying the globo pentasaccharide.

The MS^2 spectrum of the ion at m/z 998 at the retention time 18.2 min had a C_2 ion at m/z 325 indicating a terminal Fuc-Hex sequence (Fig. 10B). The prominent ion at m/z 510 is characteristic for an internal 4-linked GlcNAc substituted with a Fuc at 3-position and is obtained by double glycosidic cleavage of the 3-linked branch at C_3 and Z_3 (27). Taken together with C_4 ion at m/z 836, a Le^+ hexasaccharide (Fucα2Galβ4(Fucα3)-GlcNAcβ3Galβ4Glc) was thus suggested. This suggestion was strengthened by the similarity between this MS^2 spectrum and the MS^2 spectrum of reference Le^+ hexasaccharide (Fucα2Galβ3-GalNAcβ3Galα4Galβ4Glc).

MS^2 of the ion at m/z 1014 at the retention time 16.7 min (Fig. 10D) gave a spectrum with similar features as obtained by MS^2 of reference Globo H hexasaccharide (Fig. 10E). The series of C-type ions (C_2 at m/z 325, C_3 at m/z 528, C_4 at m/z 690, and C_5 at m/z 852) indicated a Fuc-Hex-HexNAc-Hex-Hex-Hex sequence. In addition, there was a 0.2A_3 fragment ion at m/z 792. Taken together, these spectral features indicated the presence of a Globo H hexasaccharide (Fucα2Galβ3-GalNAcβ3Galα4Galβ4Glc).
MS$^2$ of the ion at $m/z$ 1055 (Fig. 10F) gave rise to a series of C-type fragment ions (C$_2$ at $m/z$ 528, C$_3$ at $m/z$ 731, and C$_4$ at $m/z$ 893) indicating a HexNAc-(Fuc)Hex-HexNAc-Hex-Hex sequence. There was no $^6$A$_2$ fragment ion at $m/z$ 630, suggesting that the internal HexNAc was substituted at C3, i.e. a type 1 core chain. This MS$^2$ spectrum was very similar to the MS$^2$ spectrum of reference blood group A type 1 hexasaccharide (Fig. 10G). Thus, a hexasaccharide with HexNAc(Fuc)-Hex-HexNAc-Hex-Hex sequence and an internal HexNAc substituted at C-3 was indicated, most likely a blood group A type 1 hexasaccharide (GalNAcβ3(Fucα2)Galβ3-GlcNAcβ3Galβ4Glc).

In summary, LC/MS of the oligosaccharides derived from the glycosphingolipids of fraction 181-IV gave resolution of isomeric compounds and allowed a preliminary identification of a number of compounds ranging from tri- to hexa-glycosyceramides, i.e. globotriaosylceramide, lactotriaosylceramide, globotetraosylceramide, lactotetraosylceramide, neolactotetraosylceramide, blood group H type 1 pentaosylceramide, blood group H type 2 pentaosylceramide, Le$^y$ pentaosylceramide, globopentaosylceramide, Globo H type 1 pentaosylceramide (structure 5), lactotetraosylceramide (structure 6), lactotetraosylceramide (structure 7), globopentaosylceramide (structure 8), and the H type 1 pentaosylceramide (structure 9), in agreement with the mass spectrometry data described above. Neither Le$^y$, H type 2 nor blood group A structures were discernible by NMR due to the lower sensitivity of this technique compared to mass spectrometry. However, a very minor methyl doublet at 0.99 ppm, with a connectivity to a quartet centered around 4.65 ppm (H5/H6) indicating a Fuc around 4.16 ppm, signals which overlap with several other structures, the overall structure can be summed up as Fucα2Galβ3GalNAcβ3Galα4Galβ4Glcβ1Cer.

Additional glycosphingolipids identified in fraction 181:IV were globotriaosylceramide (structure 5), globotetraosylceramide (structure 6), lactotetraosylceramide (structure 7), globopentaosylceramide (structure 8), and the H type 1 pentaosylceramide (structure 9), in agreement with the mass spectrometry data described above. Neither Le$^y$, H type 2 nor blood group A structures were discernible by NMR due to the lower sensitivity of this technique compared to mass spectrometry. However, a very minor methyl doublet at 0.99 ppm, with a connectivity to a quartet centered around 4.65 ppm (H5/H6) indicating a Fucα3 residue, suggests that a small amount of Le$^x$ pentaosylceramide was present (data not shown) in accordance with the mass spectrometry data.

Further characterization of the slow-migrating glycosphingolipids of fractions 181:IV and 121:IV – The monoclonal antibodies directed against the Le$^x$ and Le$^y$ determinants gave an intense staining in the slow-migrating regions of fractions 121:IV and 181:IV (Fig. 5C and D). In an attempt to define the structures of these complex glycosphingolipids fractions 121:IV and 181:IV (approximately 0.7 mg each) were further separated by iatrobeads
chromatography. After pooling, four fractions were obtained in each case. The two fractions containing the slow-migrating compounds from each separation were denoted fractions 121:IV-C, 121:IV-D, 181:IV-C and 181:IV-D, respectively. LC/MS of the oligosaccharides obtained from these four fractions by endoglycosidase digestion, and by proton NMR, the same glycosphingolipids as in fractions 121:IV and 181:IV described above were identified. In addition, by inspection of selected ions from LC-MS a [M-H]− ion at m/z 1217, eluting at 27.1-27.4 min, was found in fractions 121:IV-C and 181:IV-C, and a [M-H]− ion at m/z 1363, eluting at 26.5-27.0.4 min, was found in fractions 121:IV-D and 18:IV-D (data not shown).

The MS² spectrum of the ion at m/z 1217 (Fig. 12A) had a fragment ion at m/z 364, which is diagnostic of an internal 4-linked GlcNAc substituted with a Fuc at 3-position (27). In addition, there was a C-type ion series (C₂ at m/z 528, C₃ at m/z 690, C₄ at 893, and C₅ at 1055), which together with the prominent 0,2A₅ fragment ion at m/z 792, and the 0,2A₃- H₂O fragment ion at m/z 774, allowed a tentative identification of a heptasaccharide with terminal Le(+© determinant and internal type 2 chain (Galβ4(Fucα3)GlcNAcβ3Galβ4-GlcNAcβ3Galβ4Glc).

MS² of the ion at m/z 1363 (Fig. 12B) gave a fragment ion at m/z 510, indicating an internal 4-linked GlcNAc substituted with a Fuc at 3-position (27). A B₃ ion was seen at m/z 656, and a series of C-type ions at m/z 836 (C₄), m/z 1039 (C₅), and m/z 1201 (C₆) was also present. Furthermore, there was a 0,2A₃ ion at m/z 878, and a 0,2A₅ ion at m/z 938. Thus, these MS² features suggested an octasaccharide with terminal Le(+© determinant and internal type 2 chain (Fucα2-Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

**Binding of human serum antibodies to the total non-acid glycosphingolipids from hESC** – Binding of natural mixed human serum antibodies to hESC non-acid glycosphingolipids is shown in Fig. 13. This mixed serum sample contains naturally occurring anti-Gal antibodies binding to the B5 glycosphingolipid (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of rabbit erythrocytes in lane 5. In addition, a distinct binding to a compound migrating in the pentaglycosylceramide region in the non-acid fractions from hESC was obtained (lane 3). The human serum also bound to reference Le© pentaglycosylceramide (lane 2).

**DISCUSSION**

Carbohydrate antigen expression in embryonic stem cells has so far been studied mainly by biological reagents such as monoclonal antibodies and lectins that do not discriminate between protein- and lipid-linked cell surface carbohydrates. A few studies have analyzed the glycosphingolipid composition of murine embryonic stem cells using immunostaining methodology, but the only glycosphingolipid identified by biochemical methods was, until recently, the ganglioside GD3, as reviewed in (30). In 2011, Liang et al. characterized the glycosphingolipid composition of two commercially available hESC lines (HES5 and H9) using both immune techniques and mass spectrometry of glycolipid extracts (9). By flow cytometry analysis, globopentaosylceramide/SSEA3, sialyl-globopentaosylceramide/SSEA4, Globo H and blood group H with type 1 core chain were identified. In addition, the crude glycolipid fraction obtained in the upper phase of Folch partition was per-methylated and subjected to mass spectrometry analysis. Thereby, the non-acid glycosphingolipids globotetraosylceramide, lactotetraosylceramide, the H type 1 pentaosylceramide, globopentaosylceramide/SSEA3, Globo H hexaosylceramide, and the gangliosides GM3, sialyl-globopentaosylceramide/SSEA4, disialyl-globopentaosylceramide, and GD1α/GD1b were identified.

In this study we have obtained a higher resolution of the glycosphingolipids present in the SA121 and SA181 hESC lines. This was due to a large amount of cell material (1x10⁹ cells) allowing several purification steps and separation of the glycosphingolipids into total non-acid and acid fractions, respectively. The glycosphingolipid material obtained also permitted us to obtain partly purified glycosphingolipid subfractions, which made it possible to identify minor compounds using a combination of mass spectrometry and proton NMR spectroscopy, giving a complete structural assignment including anomery and binding positions of the glycosidic linkages.

The short chain glycosphingolipid compounds present in the SA121 and SA181
hESC lines were identified as glucosylceramide, galactosylceramide, lactosylceramide, galabaisoacylceramide, lactotriaosylceramide and globotriaosylceramide. These compounds, along with part of the more polar ones (4-7 carbohydrate residues), are retained in the lower phase after Folch partitioning, and are therefore not included in the study of Liang et al. (9). Regarding the complex glycosphingolipids, we identified, in addition to the globo series and type 1 core glycosphingolipids, a number of glycosphingolipids not previously described in human embryonic stem cells, i.e. neolactotetraosylceramide, the H type 2 pentasaccharide, the Le^x pentasaccharide, and the Le^x hexaosylceramide. The A type 1 hexaosylceramide was also identified. In addition, a heptasaccharide with terminal Le^x determinant and internal type 2 chain (Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc), and an octaosylceramide with terminal Le^x determinant and internal type 2 chain (Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc) were tentatively identified. The identified glycosphingolipids are summarized in Table 4.

By antibody binding studies and mass spectrometry of the acid glycosphingolipid fractions from the SA121 and SA181 hESC lines, the gangliosides GM3, GD3 and sialyl-globopentaosylceramide/SSEA4 have been tentatively identified, along with some acid glycosphingolipids not previously described in human embryonic stem cells. These studies will be reported separately.

The biosynthesis of glycosphingolipids starts at the cytosolic side of the endoplasmatic reticulum with the addition of a Gal or Glc to the ceramide. After transfer to the luminal side and transport to the early Golgi apparatus a stepwise glycosylation is catalyzed by a number of glycosyltransferases (31). The Galβ4GlcNAc linkage of type 2 chains is formed by several UDP-galactose:beta-N-acetylglucosamine beta1,4-galactosyltransferases, designated β4Gal-T1, β4Gal-T2, β4Gal-T3, and β4Gal-T4, which all catalyze the transfer of galactose to neolactoseries glycosphingolipids (32, 33). The occurrence of type 2 chain glycosphingolipids in the human embryonic stem cell lines thus corresponds to the expression of one or several of these β4-galactosyltransferases, in addition to the glycosyltransferases investigated by Liang et al. (9).

The future applications for hESC in clinical medicine are yet not defined. If stem cells can be generated from the specific individual in need of treatment, the self/non-self immune barrier will not be an obstacle. However, in all therapeutic settings involving transfer of cells with a different genetic background, these cells will expose the recipient to non-self cell surface antigenic determinants that may evoke an immune response and subsequent graft rejection. The blood group ABO system is a strong immunological barrier in organ transplantation comparable to, or even stronger than, the HLA system (34). In the hESCs analyzed in this work a blood group A type 1 hexaglycosylceramide was identified. The two hESC lines were genotyped as having a blood group A1 genotype, and blood group A antigens were detected on their cell surface by immunohistochemistry (Säljö, K. and Rydberg, L., personal communications). However, selecting a blood group O phenotype hESC will eliminate the A and B antigens as an immune barrier in clinical applications of hESC.

Binding studies with mixed serum from several healthy human blood group AB blood donors demonstrated a reactivity by preformed antibodies to the pentaglycosylceramide region of the non-acid glycosphingolipid fractions of the SA121 and SA181 hESC lines. Testing of this serum against reference glycosphingolipids in the chromatogram binding assay showed binding to the Le^x pentaglycosylceramide. No other glycosphingolipids identified in the two cell lines reacted with the AB serum (data not shown), suggesting that the reactivity was due to Le^x recognition.

Similar reactivity in the pentaglycosylceramide region was also observed when using serum from a single blood group A individual, while serum from a single blood group O individual gave binding to bands in the penta- and heptaglycosylceramide region, most likely due to additional recognition of the blood group A heptaglycosylceramide (data not shown). Taken together these observations suggest a possible humoral immune response against carbohydrate antigens of hESC. Such reactivities need to be further addressed before these cells can be clinically implemented.
Culture of hESC in the presence of animal-derived compounds may lead to uptake and incorporation of the non-human sialic acid NeuGc (35), which may give an immune response due to the circulating antibodies to NeuGc that are present in most humans (36). It should be noted that the hESC lines (HES5 and H9) studied by Liang et al. (9) were cultured using mouse embryonic fibroblast conditioned media on a Matrigel surface, the latter being a complex mix of unspecified mouse material. The two hESC lines analyzed in this study were grown in a feeder cell and serum free system, and the glycosphingolipids analyzed are therefore not contaminated with compounds of animal origin. This was confirmed by the absence of binding of antibodies directed against Galα3Gal determinants (data not shown), which are found in all animal species below non-human primates in the evolutionary chain (37).

Another important aspect of comparing different hESC lines propagated in different culturing systems is the level of pluripotency. It is well known that pluripotency is a relative term, and that different culturing systems may have different efficacies in keeping the cells undifferentiated. As suggested by Liang et al., the onset of differentiation may start transition of e.g. glycosphingolipids. It would therefore be interesting to expand these studies in the future to comprise both different hESC lines and different culturing techniques.

The complex glycosphingolipids in the SA121 and SA181 cells contained a series of compounds with the Le^x determinant. This is of particular interest since it has been shown in mice that the blastocysts express Le^x and that intra-uterine injection of anti-Le^x monoclonal antibodies blocks blastocyst cell adhesion and their continuous development (38). The Le^x antigen has also been postulated to be an onco-fetal antigen involved in the carcinogenesis processes (39).

Changes in antigen expression upon differentiation of embryonic stem cells have been documented (40). Regarding carbohydrate antigens, the stage-specific embryonic antigens, SSEA-1 to 4, and the Le^x(SSEA-1)/Le^x antigens have been studied in the mouse (41, 42), while studies of human embryonic stem cells is more limited. By quantification of the mRNA for the glycosyltransferases involved in the biosynthesis of the glycosphingolipid components (9), a change in glycosphingolipid composition was found when the undifferentiated hESC were changed into embryonic body outgrowth. The compounds with globo- and lacto core chains were reduced, and ganglio-series structures were increased. This finding is similar to reports of retinoic acid induced differentiation of human embryonic carcinoma cells (43, 44), and the hESC line H7 (40), where a switch from globo-series compounds to ganglio-series compounds was found. In the 1980’s it was shown that during the early mouse embryogenesis (up to day 7) a stage-specific transition of carbohydrate epitopes from globo- to lacto- and ganglio-series occurs (reviewed in 45).

Interestingly, several of the glycosphingolipids of hESC are over-expressed in various types of human cancers (46), and it has been suggested that these carbohydrate profiles may be related to properties common to both cancer cells and embryonic stem cells, as the ability of eternal cell proliferation (47). A further suggestion is that some of these glycans may serve as markers for human embryonic stem cells and for cancers (9). However, several of the monoclonal antibodies used for detection of the glycosphingolipids were cross-reactive, which is not uncommon for monoclonal antibodies directed against carbohydrate epitopes (48). The most problematic issue is the anti-SSEA-3/Globopenta, antibody MC631, an antibody often used for characterization of human embryonic stem cells (5). In our hands this antibody also bound to globotetraosylceramide, the Globo H hexaglycosylceramide and the H type 1 pentaglycosylceramide. Thus, development of more specific monoclonal antibodies against carbohydrate epitopes is urgently needed for further studies of carbohydrate antigens as disease biomarkers or markers for human embryonic stem cells.

In summary, a number of glycosphingolipids not previously described in hESC were identified in this study, demonstrating that the glycosylation of hESC is more complex than previously appreciated. Cell-cell interactions mediated by cell surface glycoconjugates play important roles in various biological events, as embryogenesis and carcinogenesis (49-51). Thus, our findings enhance our knowledge of hESC, and provides
a platform for further studies of the functional roles of glycosphingolipids during cellular differentiation and proliferation.

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**FOOTNOTES**

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The abbreviations used are: hESC, human embryonic stem cells; ESI/MS, electrospray ionization mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; SSEA, stage-specific embryonic antigen

The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: *Eur. J. Biochem.* (1998) **257**, 293). It is assumed that Gal, Glc, GlcNAc, GalNAc, NeuAc and NeuGc are of the D-configuration, Fuc of the L-configuration, and all sugars are present in the pyranose form.

In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length and the number after the colon gives the total number of double bonds in the molecule. Fatty acids with a 2-hydroxy group are denoted by the prefix h before the abbreviation e.g. h16:0. For long chain bases, d denotes dihydroxy and t trihydroxy. Thus d18:1 designates sphingosine (1,3-dihydroxy-2-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecane).

**FIGURE LEGENDS**

**FIGURE 1.** Binding of *Erythrina cristagalli* lectin and monoclonal antibodies to non-acid glycosphingolipids of human embryonic stem cells. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of *E. cristagalli* lectin (B), the monoclonal anti-H type 1 antibody 17-206 (C), the monoclonal anti-H type 2 antibody 92FR-A2 (D), the monoclonal anti-Globo H antibody MB1 (E), and the monoclonal anti-Globo5/SSEA-3 antibody MC-813-70 (F). The lanes were: lane 1, non-acid glycosphingolipids of human embryonic stem cell line SA121, 40 µg: lane 2, non-acid glycosphingolipids of human embryonic stem cell line SA181, 40 µg: lane 3, reference H type 1 pentaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 4, reference H type 2 pentaglycosylceramide (Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 5, reference Globo H hexaglycosylceramide (Fucα2Galβ3GlcNAcβ3Galα4Galβ4Glcβ1Cer), 4 µg; lane 6, non-acid glycosphingolipids of mouse small intestine, 10 µg; lane 7, reference B type 2 hexaglycosylceramide (Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 8, non-acid glycosphingolipids of human kidney (blood group A), 20 µg. The numbers to the left of the chromatogram in (A) denotes the approximate number of carbohydrate residues in the bands. The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". Autoradiography for 12 h.

**FIGURE 2.** Thin-layer chromatograms of the isolated non-acid glycosphingolipid fractions from human embryonic cell lines SA121 and SA181, and ESI/MS of fractions 121-I-III (mono-, di- and triglycosylceramides). The lanes on (A) were: lane 1, fraction 121:I from cell line SA121, 10 µg; lane 2, fraction 121:II, 10 µg; lane 3, fraction 121:III, 10 µg; lane 4, fraction 121:IV, 20 µg, and the lanes on (B) were: lane 1, fraction 181:I from cell line SA181, 10 µg; lane 2, fraction 181:II, 10 µg; lane 3, fraction 181:III, 10 µg; lane 4, fraction 181:IV, 20 µg. The numbers to the left of the chromatogram in (A) denotes the approximate number of carbohydrate residues in the bands. The thin-layer chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and anisaldehyde was used for detection of glycosphingolipids. (C) Base peak chromatogram from ESI-MS of fraction 121-I from human embryonic stem cell line SA121. (D) Base peak chromatogram from ESI-MS of fraction 121-II from human embryonic stem cell line SA121. (E) Base peak chromatogram from ESI-MS of fraction 121-III from human embryonic stem cell line SA121. The retention times of the molecular ions are given in italics.
FIGURE 3. **MS² of mono-, di and triglycosylceramides from human embryonic stem cells (fractions 121-I-III).** The interpretation formulae show the deduced glycosphingolipid structures. (A) MS² spectrum of the ion at m/z 698 of fraction 121-I from human embryonic stem cell line SA121 (retention time 18.2 min). (B) MS² spectrum of the ion at m/z 726 of fraction 121-I from human embryonic stem cell line SA121 (retention time 18.3 min). (C) MS² spectrum of the ion at m/z 808 of fraction 121-I from human embryonic stem cell line SA121 (retention time 18.6 min). (D) MS² spectrum of the ion at m/z 860 of fraction 121-II from human embryonic stem cell line SA121 (retention time 19.7 min). (E) MS² spectrum of the ion at m/z 888 of fraction 121-II from human embryonic stem cell line SA121 (retention time 19.9 min). (F) MS² spectrum of the ion at m/z 1022 of fraction 121-III from human embryonic stem cell line SA121 (retention time 21.2 min). (G) MS² spectrum of the ion at m/z 1050 of fraction 121-III from human embryonic stem cell line SA121 (retention time 21.1 min).

FIGURE 4. **Anomeric regions of the 600 MHz proton NMR spectra (30 °C) of fractions 121-I, 121-II, 121-III derived from the human embryonic stem cell line SA121.** Samples (approximately 0.1 mg of each sample) were dissolved in dimethyl sulfoxide/D₂O (98:2, by volume). Above the spectra the six different structures (1-6) that were identified are depicted and anomeric resonances are labeled by Roman numerals. The presence of a triplet resonance due to unsaturated cis bonds in the non-hydroxy fatty acids (nFA) is indicated, as are the H4 and H5 resonances stemming from the sphingosine base.

FIGURE 5. **Binding of monoclonal antibodies to slow-migrating non-acid glycosphingolipids of human embryonic stem cells.** Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiograms obtained by binding of the monoclonal anti-blood group A antibody HE-193 (B), the monoclonal anti-Le⁰/SSEA-1 antibody P12 (C), and the monoclonal anti-Le⁴ antibody F3 (D). The lanes were: lane 1, slow-migrating non-acid glycosphingolipids of human embryonic stem cell line SA181 (fraction 181-IV), 40 µg; lane 2, slow-migrating non-acid glycosphingolipids of human embryonic stem cell line SA121 (fraction 121-IV), 40 µg: lane 3, reference A type 1 hexaglycosylceramide (GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 4, reference Le⁰ pentaglycosylceramide (Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 5, reference Le⁰ hexaglycosylceramide (Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 6, reference Le⁰ pentaglycosylceramide (Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 7, reference Le⁰ hexaglycosylceramide (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; lane 8, reference H type 2 pentaglycosylceramide (Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 µg. The numbers to the left of the chromatogram in (A) denotes the approximate number of carbohydrate residues in the bands. The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". Autoradiography for 12 h.

FIGURE 6. **LC-ESI/MS of the oligosaccharides obtained by digestion of fraction 181-IV from human embryonic stem cells, and of reference glycosphingolipids, with Rhodococcus endoglycoceramidase II.** (A) Base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181. (B) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181. (C) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from reference globotetraosylceramide (Gb4; GalNAcβ3Galβ3Galβ4Glcβ1Cer). (D) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from reference isoglobotetraosylceramide (iGb4; GalNAcβ3Galα3Galβ4Glcβ1Cer).
(E) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from reference lactotetraosylceramide (L4; Galβ3GlcNAcβ3Galβ4Glcβ1Cer).
(F) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from reference neolactotetraosylceramide (nL4; Galβ4GlcNAcβ3Galβ4Glcβ1Cer).
(G) Mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from reference gangliotetraosylceramide (GgO4; Galβ3GalNAcβ3Galβ4Glcβ1Cer).

The retention times of the molecular ions are given in italics.

FIGURE 7. MS² of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181, and from reference glycosphingolipids. RT, retention time.
(A) MS² spectrum of the ion at m/z 706 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 13.8 min).
(B) MS² spectrum of the ion at m/z 706 of reference globotetra saccharide, Gb4 (retention time 13.8 min).
(C) MS² spectrum of the ion at m/z 706 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 21.3 min).
(D) MS² spectrum of the ion at m/z 706 of reference lactotetra saccharide, L4 (retention time 21.5 min).
(E) MS² spectrum of the ion at m/z 706 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 20.7 min).
(F) MS² spectrum of the ion at m/z 706 of reference neolactotetra saccharide, nL4 (retention time 20.5 min).
(G) Interpretation formulae.

FIGURE 8. LC-ESI/MS of the oligosaccharides obtained by digestion of fraction 181-IV from human embryonic stem cell line SA181, and of reference glycosphingolipids, with Rhodococcus endoglycoceramidase II.
(A) Mass chromatogram of m/z 852 from LC-ESI/MS of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181.
(B) Mass chromatogram of m/z 852 from LC-ESI/MS of the oligosaccharides derived from reference H type 1 pentaglycosylceramide (H5-1; Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer).
(C) Mass chromatogram of m/z 852 from LC-ESI/MS of the oligosaccharides derived from reference H type 2 pentaglycosylceramide (H5-2; Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer).
(D) Mass chromatogram of m/z 852 from LC-ESI/MS of the oligosaccharides derived from reference Leα pentaglycosylceramide (Leα-5; Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer).
(E) Mass chromatogram of m/z 852 from LC-ESI/MS of the oligosaccharides derived from reference Leβ pentaglycosylceramide (Leβ-5; Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer).
The retention times of the molecular ions are given in italics.

FIGURE 9. MS² of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181, and from reference glycosphingolipids. RT, retention time.
(A) MS² spectrum of the ion at m/z 852 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 19.5 min).
(B) MS² spectrum of the ion at m/z 852 of reference H type 1 pentasaccharide, H5-1 (retention time 19.4 min).
(C) MS² spectrum of the ion at m/z 852 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 21.8 min).
(D) MS² spectrum of the ion at m/z 852 of reference H type 2 pentasaccharide, H5-2 (retention time 21.7 min).
(E) MS² spectrum of the ion at m/z 852 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 17.0 min).
(F) MS² spectrum of the ion at m/z 852 of reference Leα pentasaccharide, Leα-5 (retention time 16.8 min).
Figure 10. MS\textsuperscript{2} of the oligosaccharides derived from fraction 181-IV from human embryonic stem cell line SA181, and from reference glycosphingolipids. RT, retention time.

(A) MS\textsuperscript{2} spectrum of the ion at m/z 868 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 15.1 min).

(B) MS\textsuperscript{2} spectrum of the ion at m/z 998 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 18.2 min).

(C) MS\textsuperscript{2} spectrum of the ion at m/z 998 of reference Le\textsuperscript{y} hexasaccharide, Le\textsuperscript{y}-6 (retention time 18.2 min).

(D) MS\textsuperscript{2} spectrum of the ion at m/z 1014 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 16.7 min).

(E) MS\textsuperscript{2} spectrum of the ion at m/z 1014 of reference Globo H hexasaccharide, Globo H (retention time 16.6 min).

(F) MS\textsuperscript{2} spectrum of the ion at m/z 1055 of fraction 181-IV from human embryonic stem cell line SA181 (retention time 17.0 min).

(G) MS\textsuperscript{2} spectrum of the ion at m/z 1055 of reference A type 1 hexasaccharide, A6-1 (retention time 16.9 min).

(H) Interpretation formulae.

Figure 11. The H1/H2 region of the DQF-COSY proton NMR spectrum of fraction 181-IV from human embryonic stem cell line SA181, recorded at 600 MHz (30 °C). The spectrum at the top is the corresponding 1D spectrum of the anomeric region. The sample (approximately 0.4 mg) was dissolved in dimethyl sulphoxide-D\textsubscript{2}O (98:2, by volume) after deuterium exchange. All labeled connectivities represent H1/H2 crosspeaks, except for Gal\textalpha\textsubscript{4}H5/H6a crosspeaks which are denoted as such. The H1/H2 labels are derived from structures 5-10, and Roman numerals I-VI for individual sugars, as listed in Table 3.

Figure 12. MS\textsuperscript{2} of complex oligosaccharides in fractions 181:IV-C and 181:IV-D from human embryonic stem cell line SA181. RT, retention time.

(A) MS\textsuperscript{2} spectrum of the ion at m/z 1217 of fraction 181-IV-C from human embryonic stem cell line SA181 (retention time 27.2 min).

(B) MS\textsuperscript{2} spectrum of the ion at m/z 1363 of fraction 181-IV-D from human embryonic stem cell line SA181 (retention time 26.7 min).

(C) Interpretation formulae.

Figure 13. Binding of human serum antibodies to the total non-acid glycosphingolipids from human embryonic stem cells. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiogram obtained by binding of human blood group AB serum (B). The lanes were: lane 1, reference H type 1 pentaglycosyleramidie (Fucc2Galβ3GlcNAcβ3Galβ4Glcβ1Cer), 2 µg; lane 2, reference Le\textsuperscript{o} pentaglycosyleramidie (Galβ4(Fuccα3)GlcNAcβ3Galβ4Glcβ1Cer), 2 µg; lane 3, slow-migrating non-acid glycosphingolipids of human embryonic stem cell line SA121 (fraction 121:IV-D), 30 µg; lane 4, total non-acid glycosphingolipids of of human embryonic stem cell line SA121, 40 µg; lane 5, total non-acid glycosphingolipids of rabbit erythrocytes, 40 µg. The numbers to the left of the chromatogram in (A) denotes the approximate number of carbohydrate residues in the bands. The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures".
Table 1. Monoclonal antibodies used in chromatogram binding assays.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-blood group A</td>
<td>HE-193</td>
<td>GeneTex/Abcam</td>
<td>1:500</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-blood group B</td>
<td>HEB-29</td>
<td>GeneTex/Abcam</td>
<td>1:100</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-H type 1</td>
<td>17-206</td>
<td>GeneTex/Abcam</td>
<td>1:50</td>
<td>IgG3</td>
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<tr>
<td>Anti-H type 2</td>
<td>A583</td>
<td>DakoCytomation Norden A/S</td>
<td>1:100</td>
<td>IgM</td>
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<tr>
<td>Anti-Globo H</td>
<td>Mbr1</td>
<td>Enzo Life Science</td>
<td>1:50</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-Lewis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.FR2.3</td>
<td>Dominion</td>
<td>1:50</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-Lewis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BG-6/T218</td>
<td>Signet/Covance</td>
<td>1:100</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-Lewis&lt;sup&gt;y&lt;/sup&gt;</td>
<td>F3</td>
<td>Calbiochem</td>
<td>1:200</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-Lewis&lt;sup&gt;x&lt;/sup&gt;/SSEA-1</td>
<td>P12</td>
<td>Calbiochem</td>
<td>1:200</td>
<td>IgM</td>
</tr>
<tr>
<td>Anti-Globopenta/SSEA-3</td>
<td>MC-631</td>
<td>eBioscience</td>
<td>1:50</td>
<td>IgM</td>
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Table 2. Cross-reactivities of monoclonal antibodies

<table>
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<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Claimed specificity</th>
<th>Cross-reactivity noted</th>
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<tbody>
<tr>
<td>Anti-H type 1</td>
<td>17-206</td>
<td>H type 1: Fucα2Galβ3GlcNAc</td>
<td>Globo H: Fucα2Galβ3GalNAcβ3Galα4Galβ4Glc</td>
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<td></td>
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TABLE 3. Anomeric resonance shifts (ppm) of glycosphingolipid structures identified by NMR at 600 MHz and 30 °C for compounds isolated from human embryonic stem cell lines SA121 and SA181.

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<tr>
<th>Structure</th>
<th>Trivial name</th>
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<th>V</th>
<th>IV</th>
<th>III</th>
<th>II</th>
<th>I</th>
<th>1D NMR refs. (30°C)*</th>
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<tr>
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<td>GlcCer</td>
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<td>3</td>
<td>LacCer</td>
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<td>4</td>
<td>Galabioside</td>
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<td>Globotri</td>
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<td>7</td>
<td>Lactotetra</td>
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* NMR references do not refer to the original findings since most of these data were acquired at higher temperatures.
TABLE 4. Summary of non-acid glycosphingolipids characterized in human embryonic stem cells by binding of lectins and monoclonal antibodies in the chromatogram binding assay (CBA), mass spectrometry (MS) and proton NMR (NMR).

<table>
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<td>H type 1 pentaosylceramide</td>
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<td>A type 1 hexaosylceramide</td>
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<td>Le(^b) hexaosylceramide</td>
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<td>-</td>
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<tr>
<td>Le(^b)/SSEA-1 heptaosylceramide</td>
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<td>-</td>
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<td>Le(^c) octaosylceramide</td>
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<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

1Glycosphingolipids not previously characterized in human embryonic stem cells are in italics.
2ND, not determined.
3Glucosylceramide and galactosylceramide give identical mass spectra.
4Lactosylceramide and galabiaosylceramide give identical mass spectra.
Fig. 1

A. Chemical detection  B. *E. cristagalli* lectin

C. Anti-H type 1  D. Anti-H type 2

E. Anti-Globo H  F. Anti-Globo5/SSEA-3
Fig. 3

A. 121:I  
m/z 698

B. 121:I  
m/z 726

C. 121:I  
m/z 808

D. 121:II  
m/z 860

E. 121:II  
m/z 888

F. 121:III  
m/z 1022

G. 121:III  
m/z 1050
Fig. 4

IV  III  II  I

1  Glcβ1Cer
2  Galβ1Cer
3  Galβ4Glcβ1Cer
4  Galα4Galβ1Cer
5  Galα4Galβ4Glcβ1Cer
6  GalNAcβ3Galα4Galβ4Glcβ1Cer

5.50  5.00  4.50  4.00
PPM
Fig. 5

A. Chemical detection  B. Anti-A

C. Anti-\text{Le}^X/\text{SSEA}-1  D. Anti-\text{Le}^Y

1  2  3  4  5  6  7  8  1  2  3  4  5  6  7  8
Fig. 6

A. SA181

B. SA181

C. Gb4

D. iGb4

E. L4

F. nL4

G. GgO4
Fig. 7

A. SA181
RT 13.8 min

B. Gb4
RT 13.8 min

C. SA181
RT 21.3 min

D. L4
RT 21.5 min

E. SA181
RT 20.7 min

F. nL4
RT 20.5 min

G.

HexNAc-O-|-Hex-O-|-Hex-O-|-Hex
220 382 544
C1 C2 C3

Hex-O-HexNAc-O-|-Hex-O-|-Hex
382 544
C2 C3
Fig. 8

A. SA181

B. H5-1

C. H5-2

D. Leα-5

E. Leα-5

Relative abundance

5 10 15 20 25 30 min
Fig. 9

A. SA181
RT 19.5 min

B. H5-1
RT 19.4 min

C. SA181
RT 21.8 min

D. H5-2
RT 21.7 min

E. SA181
RT 17.0 min

F. Le\textsuperscript{x}-5
RT 16.8 min

G.
Fuc-O-Hex-O-|HexNAc-O-|Hex-O-|Hex
325 528 690
C\textsubscript{2} C\textsubscript{3} C\textsubscript{4}

Hex-O-HexNAc-O-|Hex-O-|Hex

Z\textsubscript{3}\beta 528 690
C\textsubscript{2\alpha} C\textsubscript{3\alpha}

Fuc
A. SA181
m/z 868
RT 15.1 min

B. SA181
m/z 998
RT 18.2 min

C. Le^\text{y}-6
m/z 998
RT 18.2 min

D. SA181
m/z 1014
RT 16.7 min

E. Globo H
m/z 1014
RT 16.6 min

F. SA181
m/z 1055
RT 17.0 min

G. A6-1
m/z 1055
RT 16.9 min

H.
Hex-O-HexNAc-O-|-Hex-O-|-Hex-O-|-Hex
382 544 706
C_2  C_3  C_4

Fuc-O-Hex-O-|-HexNAc-O-Hex-O-|-Hex
325 528 690 852
C_2  C_3  C_4  C_5

Fuc-O-Hex-O-|-HexNAc-O-|-Hex-O-|-Hex-O-|-Hex
325 528 690 852
C_2  C_3  C_4  C_5
Fig. 12

A. m/z 1217
RT 27.2 min

B. m/z 1363
RT 26.7 min

C.

Hex-O-HexNAc-O-|Hex-O-|HexNAc-O-|Hex-O-|Hex


Downloaded from http://www.jbc.org/ by guest on August 17, 2017
Fig. 13

A. Chemical detection  B. Human AB serum

1  2  3  4  5  1  2  3  4  5

by guest on August 17, 2017 http://www.jbc.org/ Downloaded from
Structural complexity of non-acid glycosphingolipids in human embryonic stem cells grown under feeder-free conditions
Angela Barone, John Benktander, Jonas Ångstrom, Anders Aspegren, Petter Björquist, Susann Teneberg and Michael E. Breimer

J. Biol. Chem. published online February 12, 2013

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