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Developmental changes in neutral glycosphingolipids of mouse placenta

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The mammalian placenta is a unique organ for the study of developmental changes. Placentas of laboratory animals such as the mouse allow for the determination of the exact stage of pregnancy, which cannot be achieved with human placenta. In this study, neutral glycosphingolipids were isolated from mouse (inbred strain C57BL/6) placentas, from day 10 to day 18 of gestation, and were separated by high performance thin layer chromatography. Densitometric measurements after orcinol staining showed, at day 10 of gestation, the presence of mono-, tetra-, tri- and dihexosylceramide in decreasing quantities, as well as four unidentified spots. On day 12, the glycosphingolipid composition changed with the disappearance of the unidentified spots and the appearance of an orcinol positive spot migrating similarly to the Forssman antigen; no further changes occurred between days 12 and 18 of gestation. The identity of the Forssman-like glycosphingolipid with the Forssman antigen was established by binding of ^{125}I labelled *Helix pomatia* agglutinin (α-GalNAc specific) to glycosphingolipids separated on high performance thin layer chromatography plates, and by the reaction of the isolated glycosphingolipid with a monoclonal anti-Forssman antibody. The appearance of the Forssman antigen at day 12 of gestation coincided with the day of final maturation of the mouse placenta and subsequent cessation of growth, suggesting a possible role of the glycosphingolipid during embryonic development.

Keywords: Forssman antigen, neutral glycosphingolipids, mouse placenta, developmental changes

Abbreviations: asialo-GM$_1$, Gal β3GalNAcβ4Galβ4Glcβ1Cer; BCIP, 5-bromo-4-chloro-3-indolylphosphate; DHC, lactosylceramide, Galβ4Glcβ1Cer; Forssman antigen, GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer; globoside, GalNAcβ3Galα4Galβ4Glcβ1Cer; GSL, glycosphingolipids; HPA, *Helix pomatia* agglutinin; HPTLC, high performance thin layer chromatography; MHC, galactosylceramide, Galβ1Cer; MHC*, glucosylceramide, Glcβ1Cer; PBS, phosphate-buffered saline; PNA, peanut agglutinin; PVP, poly(vinylpyrrolidone), mol. wt 40 000; SBA, soybean agglutinin; THC, trihexosylceramide, Galα4Galβ4Glcβ1Cer.

Introduction

Glycosphingolipids (GSL) are present in most tissues of vertebrates, and appear to be confined mainly to the outer surface of plasma membranes [1]. During the last decade it became evident that these compounds play a special role in cell growth and differentiation, in cell recognition and in regulation of the immune response [2, 3]. The involvement of GSL in these (and other) physiological processes is highlighted by the remarkable changes in their expression, which is associated with stages of cellular differentiation, development and oncogenesis. Thus, the stage specific embryonal antigen Le^x (SSEA-1) appears only during specific phases in early embryonic development, and mediates embryonic compaction, possibly via carbohydrate–carbohydrate interactions [4].

The placenta is a rapidly growing organ, unique from the viewpoint of cell differentiation and immune response. Neutral GSL [5, 6], as well as gangliosides [5–8], from human placenta have been widely investigated. The importance of placental GSL as targets for maternal antibodies mediating spontaneous abortions (due to maternal–fetal blood group incompatibility within the P blood group) has been demonstrated [9]. However, most, if not all, of these studies involved human placenta of full-term pregnancies. It was therefore of interest to follow changes in placental GSL as a function of placental maturation. Since a systematic study of developmental changes in the course of pregnancy in

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humans is not practicable, we used mice as a model system.

Materials and methods

Materials

Mouse inbred strain C57BL/6 was used, and placentas were excised at days 10, 12, 14, 16 and 18 of pregnancy. *Helix pomatia* agglutinin (HPA), peanut agglutinin (PNA), poly(vinylpyrrolidone) (PVP, mol. wt 40 000) and orcinol were purchased from Sigma (St. Louis, MO, USA). Soybean agglutinin (SBA) was purified as described [10]. Sephadex G-50, Sephadex LH-20 and DEAE-Sephadex A-25 were from Pharmacia (Uppsala, Sweden). Iatrobeads were from Iatro Laboratories (Tokyo, Japan) and poly(isobutylmethacrylate) was from Polysciences (Warrington, PA, USA). Individual standards of neutral GSL were from BioCarb Chemicals (Lund, Sweden). Silica gel 60 precoated high performance thin layer chromatography (HPTLC) plates (20 cm × 20 cm, aluminium backed, No. 5547 and 10 cm × 10 cm, glass backed, No. 5633, both without fluorescence indicator), as well as Florisil (0.15–0.25 mm particle size), were from Merck (Darmstadt, Germany); monoclonal rat IgG2c anti-Forssman antibody was a generous gift from Dr U Bethke (Biotest Pharma, Dreieich, Germany), alkaline phosphatase conjugated goat anti-rat IgG was from Dianova GmbH (Hamburg, Germany), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was from Biomol (Hamburg, Germany). All other chemicals were of analytical grade.

Isolation of neutral glycosphingolipids

The freshly excised placentas were briefly homogenized, and lyophilized. Pulverized samples of 400 mg were extracted with chloroform:methanol mixtures, 2:1, 1:1, 1:2, and the solvents were evaporated. Neutral GSL were separated from gangliosides by DEAE-Sephadex A-25 chromatography [11]. Further purification of neutral GSL was performed by acetylation, Florisil chromatography, deacetylation [12] and finally by chromatography on Iatrobeads [11]. The column was eluted with two successive 5 ml portions of chloroform:methanol, 1:1. The purification of the individual compounds was performed by preparative HPTLC [13] with freshly distilled solvents, using silica Gel-60 plates in solvent system (chloroform:methanol:water, 60:35:8 by vol). GSL were visualized by a brief exposure to iodine, eluted with chloroform:methanol:water (50:50:10 by vol) and rechromatographed to demonstrate homogeneity. The final step was by chromatography on a micro-column (5 mm × 60 mm) of Iatrobeads [11]. GSL were eluted with 10 ml chloroform:methanol, 1:1. Neutral reference GSL were isolated from sheep red blood cell membranes by standard procedures [14]. Final purification of peracetylated derivatives was by Florisil chromatography [12] followed by deacetylation.

Glycosphingolipid composition analysis

Preliminary identification of the isolated neutral GSL was based on HPTLC separation in a chloroform:methanol:water, 60:30:5 by vol, solvent system, staining with orcinol [15] and comparison with known standards. Quantitative measurements of the separated GSL were by scanning densitometry, after staining with orcinol, in a Video Densitometer (Model 620, Bio-Rad, Richmond, CA, USA), and the area corresponding to each spot was recorded.

Iodination and overlay assay with radio-iodinated lectins

Lectins were iodinated by the chloramine T method [16] under mild conditions [17]. Briefly, the reaction mixture contained 50 μg lectin in 150 μl phosphate buffered saline (PBS) with 0.2M galactose, 500 μCi $Na^{125}I$ (carrier-free, from Amersham, UK), and 20 μg chloramine T (2 mg ml^{-1}). After 1 min at 4 °C, the reaction was stopped by addition of 20 μl sodium metabisulfite (4 mg ml^{-1}), and 30 s later, 45 μl 0.01M KI were added. The [^{125}I]lectins were separated from free ^{125}I and galactose on a Sephadex G-50 column in PBS–0.1% bovine serum albumin. The specific activity of [^{125}I]lectins used was 3–5 × 10^6 counts min^{-1} μg^{-1}. A modified version [17] of the lectin overlay assay, introduced by Magnani *et al.* [18], was performed as follows. After drying, the developed HPTLC plates were dipped in a 0.1% solution of poly(isobutylmethacrylate) in ether, air-dried and made hydrophylic by soaking for 2 h at 4 °C in PBS containing 2% bovine serum albumin, 2% PVP and 0.01% NaN_3. The overlay was performed with radio-iodinated lectin, 70–80 μl cm^{-2} of chromatogram area, 1 × 10^6 counts min^{-1} ml^{-1} in the same buffer. Incubation was for 5 h at 4 °C in a humidified atmosphere. The plates were then washed in cold PBS containing 0.05% PVP, dried in air and exposed to Agfa-Gevaert Curix X-ray films for 6–18 h at −70 °C.

Immunostaining with anti-Forssman antibody on HPTLC plates

Neutral GSL were separated on glass backed silica gel 60 HPTLC plates in the solvent system described above, the silica gel was fixed with poly(isobutylmethacrylate), and immunostained with an anti-Forssman antibody essentially as described [19] but with some modifications [20]. The supernatant of a hybridoma which produces a monoclonal rat IgG2c antibody against Forssman GSL, and alkaline phosphatase-conjugated goat anti-rat IgG antibody were diluted 1:20 and 1:2000, respectively, in PBS containing 1% bovine serum albumin and 0.02% NaN_3. Specifically bound antibodies were visualized by overlaying the plate with a 0.05% w/v solution of BCIP in 0.1M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4.

Results and discussion

Thin layer chromatograms of neutral GSL from mouse placenta at five developmental stages and their densitometric quantitation are shown in Figs 1 and 2, respectively. The major orcinol-positive band corresponded in migration to a mixture of glucosyl- and galactosylceramides, in contrast to human placenta, where globoside was found to be the major neutral GSL [5]. Other orcinol-positive bands corresponded to globoside, tri- and dihexosylceramide in decreasing quantities, with the last mentioned present only in trace amounts. On day 10 some unidentified spots were seen, which disappeared from day 12. On the other hand, a neutral GSL, with the chromatographic behaviour of Forssman GSL, appeared from day 12, whereas the composition and content of the other GSL changed only slightly from day 10 to day 18.

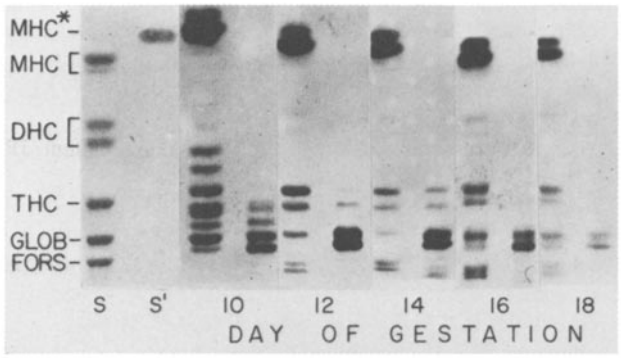

Figure 1. HPTLC separation of neutral GSL isolated and purified from mouse placenta (C57BL/6 strain) at days 10, 12, 14, 16 and 18 of gestation. Lane S: mixture of standard neutral GSL (5 μg each) containing galactosylceramide (MHC), dihexosylceramide (DHC), trihexosylceramide (THC), globoside (GLOB) and Forssman antigen (FORS). Lane S': glucosylceramide (MHC*; 5 μg). GSL (14–18 μg each), from two successive eluates from the Iatrobeads column, were applied to the HPTLC plates.

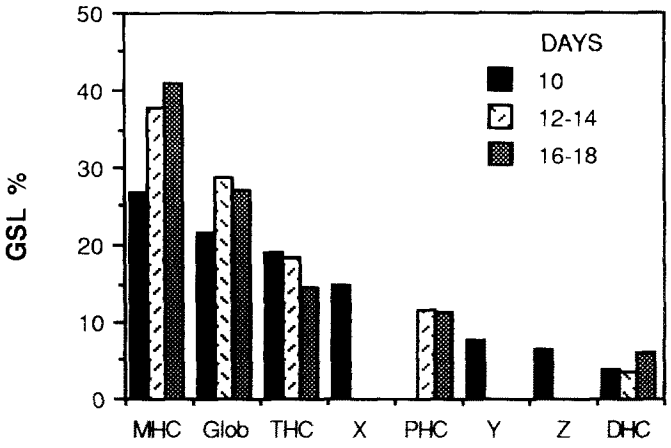

Figure 2. Mean values of individual GSL during embryonal development, calculated from densitometric measurements. MHC, monohexosylceramide, the sum of galacto- and glucosylceramide; Glob, globoside; THC, trihexosylceramide; X, unknown GSL (from day 10 of gestation) between globoside and trihexosylceramide; PHC, pentahexosylceramide, Forssman-like GSL appearing from day 12; Y and Z, unknown GSL (found only at day 10) between trihexosylceramide and dihexosylceramide; DHC, dihexosylceramide.

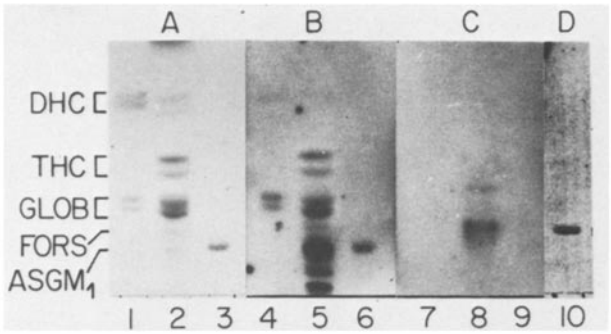

Figure 3. Overlay assay with [^{125}I]SBA and [^{125}I]HPA; panel A, staining with orcinol; panel B, overlay with [^{125}I]SBA; panels C and D, overlays with [^{125}I]HPA; lanes 1, 4 and 7, globoside (GLOB) and dihexosylceramide (DHC) (5 μg each); lanes 2, 5 and 8, GSL from placenta, day 18 (17 μg); lanes 3, 6 and 9, asialo-GM$_1$ (ASGM$_1$) (5 μg); lane 10, Forssman antigen (FORS) (4 μg).

Further identification of the GSL separated on HPTLC plates was achieved by binding of ^{125}I labelled lectins employing the solid phase autoradiographic (overlay) technique [17, 18]. These overlays (Fig. 3) were done only on GSL from day 18 since the GSL pattern of placenta changed very little between days 12 and 18. Staining of mouse placenta GSL with [^{125}I]SBA (Fig. 3, panel B) revealed GSL having terminal galactose or *N*-acetylgalactosamine, which co-chromatographed with THC, globoside, Forssman antigen and asialo-GM$_1$, as shown by orcinol staining (Fig. 3, panel A). However, no staining was found with [^{125}I]PNA (data not shown), which is highly specific for the latter GSL [21]. It was therefore concluded that the GSL detected with [^{125}I]SBA is not asialo-GM$_1$. Upon staining with [^{125}I]HPA, specific for terminal nonreducing αGalNAc (Fig. 3, panel C), only one large spot, migrating parallel to Forssman GSL, was observed. Since HPA has a high affinity for the Forssman antigen [22], the latter finding strongly suggests that the GSL, appearing in mouse placenta from day 12 of gestation, is indeed the Forssman GSL. To obtain further evidence, the Forssman-like GSL was purified by preparative HPTLC [13] and analysed by the immunostaining technique [19]. This method is highly reliable and more sensitive than fast atom bombardment mass spectrometry, which made it possible to work with the minute amounts of GSL present. The immunostaining of the mouse placenta Forssman-like GSL with anti-Forssman antibody [19, 20] was compared with immunostaining of GSL isolated from sheep erythrocytes having the Forssman antigen as the major GSL [22]. The positive staining of the parallel migrating GSL (Fig. 4) is in excellent agreement with the results of the [^{125}I]HPA

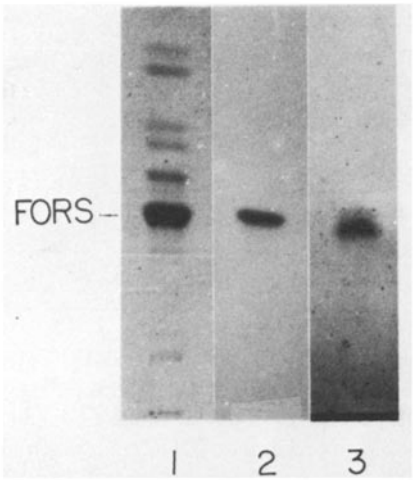

Figure 4. Immunostaining of Forssman GSL from sheep erythrocytes and Forssman-like GSL from mouse placenta: lane 1, 10 μg sheep neutral GSL stained with orcinol; lane 2, 1 μg sheep neutral GSL overlaid with monoclonal anti-Forssman antibody; lane 3, about 20 ng of purified Forssman-like GSL from mouse placenta overlaid with monoclonal anti-Forssman antibody.

overlay. The data demonstrate convincingly the presence of Forssman GSL in mouse placenta, not previously reported. Moreover, the abrupt appearance of this GSL on day 12 of gestation coincides with the end of differentiation and growth of the placenta [23]. GSL have been shown to act as regulators of cell proliferation [3]. It is therefore possible that the Forssman antigen is involved in the cessation of growth of mouse placenta.

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