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Oxygenation alters ganglioside expression in rat liver following partial hepatectomy ☆

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Abstract

Gangliosides from livers of weanling rats were analyzed after 15% partial hepatectomy (PH) and different pre- and post-operative hyberbaric oxygenation (pre- and postHBO). Neu5Ac was the predominant ganglioside-derived sialic acid (>85%) compared to Neu5Gc. Almost identical low total sialic acid content (Neu5Ac + Neu5Gc) of the control and operated nonHBO animals opposed a 6.4- to 7.6-fold increase in pre- and postHBO animals (69.26 and 81.64 pmol/mg wet weight, respectively). NanoESI-QTOF mass spectrometry combined with HPTLC immunostaining revealed GM3(Neu5Ac) and GM3(Neu5Gc) as major gangliosides, correlating with the respective sialic acid concentrations. Minor neolacto-series gangliosides were enhanced in preHBO and postHBO, but GM1-core gangliosides only in preHBO rats. GM2 and GalNAc-GM1b were clearly detectable in oxygenated rats compared to traces in the control and nonHBO animals. These results point at a functional role of gangliosides in liver growth regulation and reconstitution after PH combined with pre- and post-operative HBO treatment.

Keywords: HPLC; HPTLC; Immunostaining; NanoESI-QTOF mass spectrometry; Sialic acid; Partial hepatectomy; Oxygenation; Gangliosides

☆ *Abbreviations:* ESI-QTOF MS, electrospray ionization quadrupole time-of-flight mass spectrometry; GSL(s), glycosphingolipid(s); HBO, hyperbaric oxygenation; HPTLC, high-performance thin-layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; PH, partial hepatectomy. The designation of the GSLs follows the IUPAC-IUB recommendations [47] and the nomenclature of Svennerholm [48]. Lactosylceramide or LacCer, Galβ1-4Glcβ1-1Cer; gangliotriaosylceramide or Gg3Cer, GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or Gg4Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; gangliopentaosylceramide or Gg5Cer, GalNAcβ1-4Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; neolactotetraosylceramide or nLc4Cer or nLc4, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; neolactohexaosylceramide or nLc6Cer or nLc6, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; GM3, II^3Neu5Ac-LacCer; GM2, II^3Neu5Ac-Gg3Cer; GM1, II^3Neu5Ac-Gg4Cer; GM1b, IV^3Neu5Ac-Gg4Cer; GalNAc-GM1b, IV^3Neu5Ac-Gg5Cer; GD3, $II^3(Neu5Ac)_2$-LacCer; GD1a, IV^3Neu5Ac,II^3Neu5Ac-Gg4Cer; GD1b, $II^3(Neu5Ac)_2$-Gg4Cer; and GT1b, IV^3Neu5Ac, $II^3(Neu5Ac)_2$-Gg4Cer. Only Neu5Ac-substituted gangliosides are presented in this list of abbreviations.

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Gangliosides, sialic acid-containing glycosphingolipids (GSLs), are a structurally varied class of complex carbohydrates and ubiquitous, highly conserved membrane components [1]. The parent sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), which play crucial roles in various biological functions [2]. Gangliosides are involved in cell surface recognition phenomena [3,4] and exhibit modulatory potential toward a variety of membrane-associated proteins [5]. They are assembled as ''rafts'' [6] or ''glycosynapses'' [7] in the outer leaflet of the plasma membrane, but are also associated with intracellular organelles [8]. The membrane clustered gangliosides are suggested to execute many biological activities [7]. In particular, the simple and in mammalian tissue most widely distributed ganglioside GM3 has been shown to be involved in cell growth regulation [9,10], T-cell activation [11], signal transduction [12], and insulin signaling [13].

Hepatocytes, which are normally quiescent in the adult animal, are readily induced to proliferate by partial hepatectomy (PH). PH causes mitochondrial oxidative stress [14] and the remnant liver demands an increased amount of oxygen for mitochondrial oxidative phosphorylation to restore hepatic energy [15]. During rat liver regeneration after 68% PH, the ganglioside content and distribution have been reported in previous studies to undergo significant changes [16,17]. Resections of less than 40% of tissue in the adult animals elicit little DNA synthesis, suggesting that the functional deficit produced by removal of a small amount of tissue is not sufficient to trigger regeneration [18]. In contrast to young adult rats, this threshold is not apparent in weanling animals in which the proportionality between the amount of tissue removed and the level of DNA synthesis is maintained even for small resections [18]. Hyperbaric oxygenation (HBO) protects hepatocytes against carbon tetrachloride-induced injury [19] and improves regeneration of the predicted remnant liver after portal vein embolization [20]. Recently, we described beneficial effects of HBO treatment before 15% PH upon the starting process of liver mass restitution in weanling rats [21].

In the present study, we performed for the first time a detailed structural investigation of the altered ganglioside expression in partially hepatectomized rats, which were kept under different pre- and post-operative oxygen pressure. HBO before or after PH revealed a tremendous increase in ganglioside-derived sialic acids correlating with an enhanced expression of GM3 and some less abundant gangliosides. The detailed structural characterization of major GM3(Neu5Ac) and GM3(Neu5Gc), and less abundant neolacto-series gangliosides of rat liver was performed by nanoESI-QTOF mass spectrometry in combination with HPTLC immunostaining. Minor GM2, GM1-, and GM1b-type gangliosides were identified by immunostaining using a panel of GSL-specific antibodies. Our data suggest that the ganglioside metabolism may play an important role in the process of liver regeneration.

Materials and methods

Animals and partial hepatectomy. Experiments were performed with male Wistar rats raised under controlled conditions (temperature, 22 ± 1 °C; light schedule: 14 h of light and 10 h of dark) at the Split University Animal Facility. Laboratory food and tap water were supplied ad libitum. Animals were bred and maintained according to the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources, Commission on Life Sciences, National Research Council, 1996) and the protocol has been approved by the Split University Medical School Ethics Committee. One-month-old male Wistar rats weighing 73–102 g were submitted to 15% partial hepatectomy (PH) by removing the left middle lobe. The operation lasted 15 min with diethyl ether as an anesthetic. Ten animals, non-operated and without hyperbaric oxygenation (HBO) treatment, served as references (control or group 1). Thirty rats underwent 15% PH and they were divided into three groups (each containing 10 animals) according to the housing conditions before and after operation: recovered under normal ambient conditions of oxygen pressure after operation (defined as nonHBO or group 2), pretreated with hyperbaric oxygen before operation and recovered under normal ambient conditions (preHBO or group 3), and recovered under hyperbaric oxygen after operation (postHBO or group 4). The preHBO group was treated at day 2 (48 h), day 1 (26 h), and day 0 (4 h) before PH with HBO (100% O_2, 45 min/day at 2 atm). The postHBO group was treated at day 0 (4 h), day 1 (26 h), and day 2 (48 h) after PH with HBO (100% O_2, 45 min/day at 2 atm). The pressure of 2 atm (=202,650 Pa) was selected as the HBO regimen because this pressure is within the safety range of HBO protocols in humans. The HBO-exposure took place in a Comex hyperbaric chamber (Comex, Marseilles, France). The oxygen and carbon dioxide concentrations in the chamber during HBO-exposure were controlled by a Servomex Oxygen Analyzer 570A (Servomex, Houston, TX, USA) and by a Carbon dioxide Gas Analyzer Infrared (Industries Inc., Santa Barbara, CA, USA). All animals were sacrificed 54 h after operation with prolonged anesthesia (diethyl ether).

Isolation of gangliosides from rat livers. The livers were dissected from 10 animals of each group, pooled, minced with a scalpel, and suspended in distilled water in a 1/2 ratio(w/v). The organs were homogenized for 10 min with a dispersing tool (Polytron PT1200C, Kinematica AG, Littau/Luzern, Switzerland) and GSLs were isolated according to standard procedures [22]. Briefly, tissue homogenates were extracted with chloroform/methanol (2:1, v/v), chloroform/methanol (1:1, v/v), and chloroform/methanol (1:2, v/v) (10-fold volumes of the tissue wet weight) for 30 min with sonication. The combined extracts were evaporated, resuspended in chloroform/methanol/water (30:60:8, by vol.), and gangliosides were isolated by anion exchange chromatography on DEAE–Sepharose CL-6B (Pharmacia, Freiburg, Germany) as reported by Müthing et al. [23]. The ganglioside fraction was incubated for 1 h at 37 °C in aqueous 1 N NaOH to saponify phospholipids, followed by neutralization with acetic acid and dialysis. Gangliosides were further purified by adsorption chromatography on Iatrobeads 6RS-8060 (Macherey-Nagel, Düren, Germany) [24]. After column chromatography purifications, final ganglioside fractions were adjusted to defined volumes of chloroform/methanol (2:1) corresponding to 2 mg liver wet weight per microliter.

High-performance thin-layer chromatography and reference gangliosides. Gangliosides were separated on silica gel 60 precoated high-performance thin-layer chromatography plates (HPTLC-plates, size 10 cm × 10 cm, thickness 0.2 mm, Merck; Art. No. 5633) using chloroform/methanol/water (120:85:20, each by vol.) with 2 mM $CaCl_2$,

and visualized with resorcinol [25]. The following reference gangliosides (standards S1 to S6) were used. S1: rat brain ganglioside preparation from one-month-old male Wistar rats (control group 1, see above) composed of GM1, GD1a, GD1b, and GT1b as major constituents; S2: GM3(Neu5Ac) from CHO cells (Alexis, Läufelfingen, Switzerland; product ID ALX-302-018-MC05); S3: GM3(Neu5Gc) from mouse hybridoma cells (Alexis; product ID ALX-302-019-MC01) [26]; S4: human granulocyte ganglioside mixture composed of GM3(Neu5Ac), IV^3Neu5Ac-nLc4Cer (IV^3nLc4), IV^6Neu5Ac-nLc4Cer (IV^6nLc4), and VI^3Neu5Ac-nLc6Cer (VI^3nLc6) [27,28]; S5: GM2 (Pallmann GmbH, Munich, Germany); and S6: GM1b- and GalNAc-GM1b-type gangliosides from murine T lymphoma YAC-1 cells [29].

Determination of sialic acids. Neu5Ac and Neu5Gc of gangliosides were identified as their fluorescent derivatives by HPLC according to [30]. Sialic acids were released from gangliosides with 50 mM H_2SO_4 (80 °C, 120 min) and converted with 1,2-diamino-4,5-methylenedioxybenzene (DMB; Sigma, Deisenhofen, Germany; 18 mM DMB, 45 mM $Na_2O_4S_2$, and 1 M β-mercaptoethanol; 50 °C, 3 h) into fluorescent derivatives. The sialic acid derivatives were separated by isocratic elution with water/acetonitrile/methanol (84:9:7, by vol.) on an RP_{18} column (Kromasil ODS, 4.0 mm × 150 mm, 5 μm spherical silica, VDSoptilab, Berlin, Germany) with a flow rate of 0.5 ml/min. Reference Neu5Ac (Cat. No. A-0812) and Neu5Gc (Cat. No. G-2755) were from Sigma. Experiments were run in duplicate.

Antibodies. The polyclonal chicken anti-GM3(Neu5Ac), anti-GM3(Neu5Gc), and anti-nLc4Cer antibodies (the latter used for the detection of neolacto-series gangliosides after neuraminidase treatment, see below) have been characterized in previous papers [26,31]. The anti-nLc4Cer antibody recognizes the Galβ1-4GlcNAc-residue and thus reacts with nLc6Cer and nLc8Cer as well. The antibody shows some cross-reactivity with Galβ1-4Glcβ1-1Cer (lactosylceramide). The polyclonal chicken anti-GM2(Neu5Ac) and rabbit anti-GM1(Neu5Ac) antibodies were produced according to the method of Kasai et al. [32]. The anti-GM1 antibody was further used for the detection of gangliosides with GM1-core (namely GD1a, GD1b, and GT1b) after neuraminidase treatment (see below).

The polyclonal rabbit anti-Gg4Cer antibody, used for the detection of GM1b after neuraminidase treatment (see below), has been described in previous publications [33,34]. The specificity of the chicken polyclonal anti-GalNAc-GM1b antibody has been reported earlier [35].

The mouse IgG3 monoclonal antibody (mab) R24 was used for the detection of GD3. Cell culture supernatant was used 1:20 diluted in solution A [36].

HPTLC immunostaining procedure. The overlay technique was carried out as previously described with some modifications [37,38]. All GSL-specific antibodies were used at 1:1000 dilutions in phosphate-buffered saline (PBS) supplemented with 1%(w/v) bovine serum albumin and 0.02% NaN_3(w/v) (=solution A) [31]. Secondary rabbit anti-chicken IgG, goat anti-rabbit IgG, and goat anti-mouse IgG and IgM antibody, all affinity chromatography-purified and labeled with alkaline phosphatase (0.6 mg/ml), were purchased from Dianova and used in 1:2000 dilution in solution A [37]. Briefly, the silica gel was fixed with polyisobutylmethacrylate and unspecific protein binding was blocked by a 15 min incubation of the plate with solution A [33]. The plates were then overlaid for 1 h with anti-GSL antibodies, and unbound antibodies were removed by washing each plate five times with solution B (0.05% Tween 21, 0.02% NaN_3 in PBS). After 1 h incubation with secondary antibodies, the plates were washed again, followed by 2-fold rinsing with glycine buffer (0.1 M glycine, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, pH 10.4), to remove phosphate. Bound antibodies were visualized with 0.05%(w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) in glycine buffer. Each antibody analysis was performed twice, with identical results.

NanoESI-QTOF mass spectrometry. Dried aliquots from liver ganglioside preparations were dissolved in 20 μl pure distilled methanol (Merck, Darmstadt, Germany) to an estimated concentration of approximately 0.05 μg/μl. NanoElectrospray ionization (ESI) mass spectrometry was carried out on a quadrupole time-of-flight (QTOF) instrument equipped with a nanospray manipulator (Micromass, Manchester, UK) essentially as described before [39,40]. Negative ion mode was applied exclusively for the mass spectrometric investigation of liver gangliosides; nitrogen was used as desolvation and nebulizer gas. Capillaries were made in-house from borosilicate glass (Hilgenberg, Malsfeld, Germany) using a vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, CA USA). High voltage was applied via a steel wire to the sample solution. The capillary voltage was set to 1.1 kV and the voltage on the counter electrode was set to 140 V.

Results

This study presents a comparative investigation of gangliosides from livers of rats after partial hepatectomy and recovery after operation under different pre- and post-operative oxygen pressure.

Non-operated and non-oxygenated animals served as references (control or group 1). The second group of animals was hepatectomized and allowed to recover under normal ambient conditions (nonHBO or group 2). The third group was treated with oxygen before operation and recovered under normal ambient conditions (preHBO or group 3). The fourth group received oxygen after operation and recovered under hyperbaric oxygen conditions (postHBO or group 4). Gangliosides were isolated from the livers of 10 male rats of each group (Table 1).

To analyze the qualitative and quantitative differences between Neu5Ac and Neu5Gc substitution, ganglioside-derived sialic acids were quantified as their DMB derivatives. The structural characterization of liver gangliosides was performed by nanoESI-QTOF mass spectrometry in combination with HPTLC immunostaining using a panel of GSL-specific antibodies.

Resorcinol staining of rat liver gangliosides

The resorcinol-stained HPTL-chromatogram of liver gangliosides from the four groups of rats studied and the reference gangliosides used as positive controls for the following HPTLC-immunostains are shown in Figs. 1A and B, respectively. For direct comparison, ganglioside amounts corresponding to 30 mg liver wet weights from each group were applied. A remarkable quantitative difference between the control group (group 1) and the nonHBO group (group 2) on the one hand and the oxygen-treated animals of the preHBO- and postHBO-group (groups 3 and 4) on the other hand was apparent from this initial stain. The most obvious difference between the 4 groups was a dominant double band at the position of GM3 in the ganglioside fractions of the oxygen-treated animals (Fig. 1A, lanes 3 and 4). Minor compounds separating below GM3 were slightly increased in livers of these animals, too. No further

Table 1
Total wet weights and liver regeneration-related factors of rat livers from non-operated and partially hepatectomized rats after different oxygen treatments

Variable/No.[a]	1. control[d]	2. nonHBO[e]	3. preHBO[f]	4. postHBO[g]
Total wet weight[b] (g)	44.43	38.54	41.05	39.67
Liver wet weight/body weight[c] (g/g)	0.0697 ± 0.0013	0.0517 ± 0.0016	0.0618 ± 0.0084	0.056 ± 0.0014
Lipid peroxides (nM/g MDA)[h]	110.1 ± 4.3	318.1 ± 24.6	119.7 ± 4.9	288.3 ± 12.4
Albumin plasma level (g/L)[h]	40.4 ± 0.3	33.5 ± 0.7	20.0 ± 2.4	25.3 ± 0.4
ALT activity (U/L)[h]	23.3 ± 4.2	53.3 ± 4.4	30.3 ± 5.1	67.8 ± 9.6
AST activity (U/L)[h]	43.3 ± 5.4	137.5 ± 6.3	101.1 ± 12.1	218.4 ± 40.6

[a] Numbering of ganglioside fractions from differently treated rats.
[b] Total wet weights of livers obtained from 10 male rats.
[c] Ratio of the wet weight of the remnant liver lobes to the body weight calculated from excised livers after partial hepatectomy and 54 h recovery under different oxygen conditions; $p < 0.05$ vs control assessed by Kruskal–Wallis test followed by Dunn's test.
[d] Non-operated and non-oxygenated rats.
[e] Partially hepatectomized and recovered under normal ambient conditions.
[f] Treated with oxygen before operation and recovered under normal ambient conditions.
[g] Treated with oxygen after operation and recovered under hyperbaric oxygen conditions.
[h] According to Kurir et al. [21]; MDA, malonyldialdehyde; ALT, alanine transaminase; and AST, aspartate transaminase.

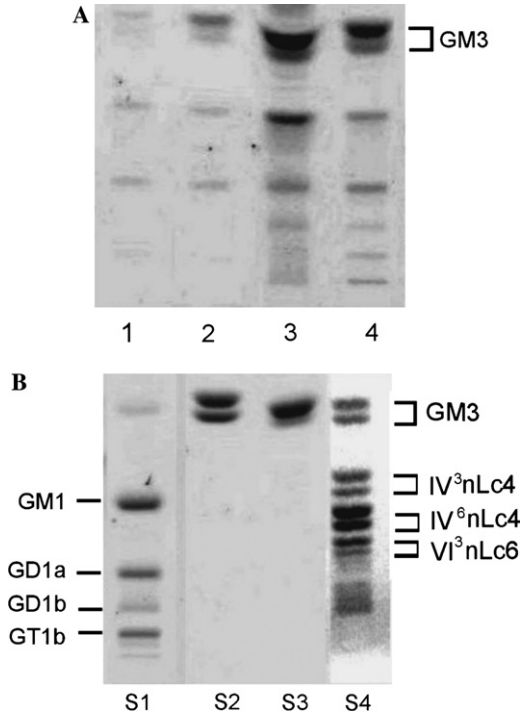

Fig. 1. Resorcinol stain of HPTLC-separated liver ganglioside fractions from partially hepatectomized rats (A) and reference gangliosides (B). (A) Lane 1, non-operated and non-oxygenated (control or group 1); lane 2, operated and recovered under normal ambient conditions (nonHBO or group 2); lane 3, treated with oxygen before operation and recovered under normal ambient conditions (preHBO or group 3); and lane 4, treated with oxygen after operation and recovered under hyperbaric oxygen conditions. Ganglioside amounts corresponding to 30 mg liver wet weight were chromatographed and stained with resorcinol. (B) S1: 5 μg of gangliosides from rat brain, S2: 5 μg GM3(Neu5Ac), S3: 5μg GM3(Neu5Gc), and S4: 15 μg of human granulocyte gangliosides. IV^3nLc4, IV^3Neu5Ac-nLc4Cer; IV^6nLc4, IV^6Neu5Ac-nLc4Cer; and VI^3nLc6, VI^3Neu5Ac-nLc6Cer.

conclusions concerning the structural differences between the four groups could be drawn from the resorcinol stains.

Ganglioside-derived sialic acids from rat liver

To analyze the qualitative and quantitative differences between Neu5Ac and Neu5Gc substitution, lipid-bound sialic acids were quantified as their DMB-derivatives. The ganglioside-derived sialic acid concentrations of the four animal groups investigated are listed in Table 2. Single sialic acid profiles of the ganglioside fractions from nonHBO animals (group 2) and postHBO animals (group 4) are shown as examples in Figs. 2B and C, respectively. Neu5Ac was found to be the predominant sialic acid vs Neu5Gc in all ganglioside fractions, ranging from 87.4% (control group) to 94.6% (postHBO) vs rather low concentrations of Neu5Gc ranging from 12.6% (control group) to 5.4% (postHBO group). Compared to very similar quantities of Neu5Gc in the control, nonHBO, and preHBO group (average 11%), a notable reduction in Neu5Gc expression was detectable for the postHBO group (5.4%). However, the total sialic acid content (Neu5Ac plus Neu5Gc) of the two non-oxygenated and the two oxygenated animal groups differed considerably. Almost identical quantities of group 1 and 2 animals (10.28 and 10.80 pmol/mg wet weight, respectively) oppose an approximate 6.4- to 7.6-fold increase compared with the preHBO (69.26 pmol/mg) and the postHBO animals (81.64 pmol/mg). Thus, pre- and post-operative oxygenation led to a remarkable increase of total ganglioside content of the rat liver.

In the next set of experiments, we used a panel of GSL-specific antibodies and well-characterized reference gangliosides to study the expression of prevalent GM3 and minor neolacto- and ganglio-series gangliosides. The resorcinol stains of GM3(Neu5Ac) and GM3(Neu5Gc) are shown in Fig. 1B, lanes S2 and S3, respectively, and the neolacto- and ganglio-series gangliosides in Fig. 1B, lanes S4 and S1, respectively.

Table 2
Ganglioside-derived sialic acid concentrations in livers of partially hepatectomized rats after different oxygen treatments

No.[a]	Treatment	Neu5Ac[b] (pmol/mg)	Percentage	Neu5Gc[b] (pmol/mg)	Percentage	Total[c] (pmol/mg)
1	Control[d]	8.98	87.4	1.30	12.6	10.28
2	nonHBO[e]	9.82	90.9	0.98	9.1	10.80
3	preHBO[f]	61.28	88.5	7.98	11.5	69.26
4	postHBO[g]	77.26	94.6	4.38	5.4	81.64

[a] Numbering of ganglioside fractions from differently treated rats.
[b] pmol sialic acid per milligram liver wet weight, repeat determination.
[c] Total ganglioside-derived sialic acids (Neu5Ac + Neu5Gc), given as pmol per milligram liver wet weight.
[d] Non-operated and non-oxygenated rats.
[e] Operated and recovered under normal ambient conditions.
[f] Treated with oxygen before operation and recovered under normal ambient conditions.
[g] Treated with oxygen after operation and recovered under hyperbaric oxygen conditions.

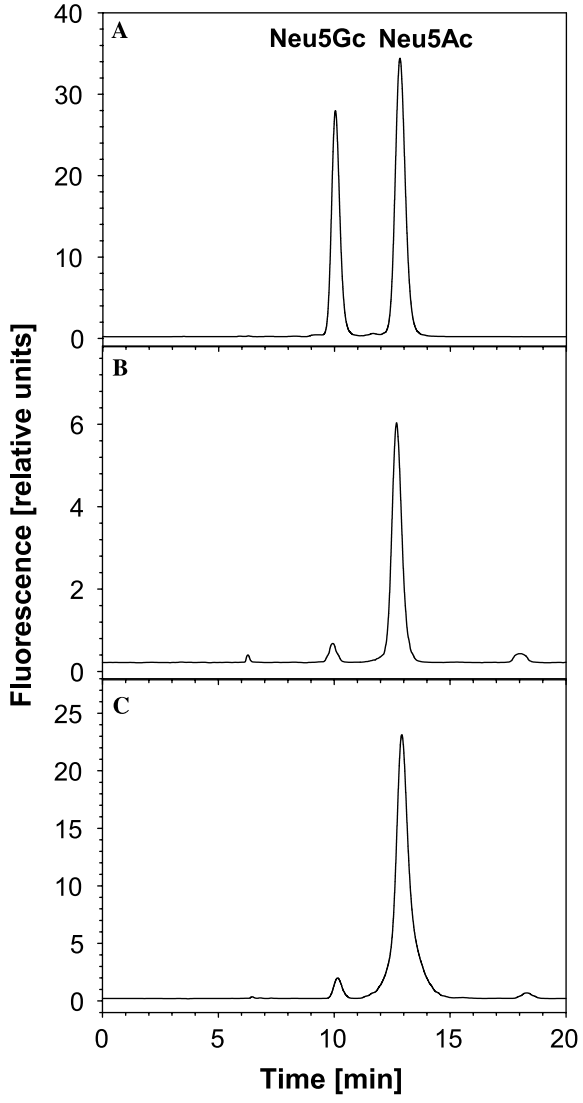

Fig. 2. HPLC elution profiles of fluorescent DMB derivatives of sialic acids released from rat liver gangliosides. (A) Neu5Gc and Neu5Ac standards (250 pmol each). (B) Sialic acids from rat liver gangliosides of group 2. (C) Sialic acids from rat liver gangliosides of group 4. Ganglioside-released sialic acids correspond to 4 mg liver wet weight. For specific oxygen treatment of animals from groups 2 and 4 see Table 1. The ganglioside-derived sialic acid concentrations of all 4 animal groups are listed in Table 2.

Structural characterization of GM3(Neu5Ac) and GM3(Neu5Gc) by combined HPTLC-immunostaining and nanoESI-QTOF mass spectrometry

The HPTLC immuno overlay assays of the ganglioside fractions of the four groups of rats with anti-GM3(Neu5Ac) and anti-GM3(Neu5Gc) antibodies are shown in Figs. 3A and B, respectively. Group 3 and 4 animals showed significant higher expression of both types of GM3, whereas only weak positive reactions were observed in the group 1 and 2 animals. These results correlate well with the differing ganglioside-released sialic acid concentrations as shown above (see Table 2). The characteristic GM3 double band binding patterns of both antibodies suggest abundant substitution of both GM3 species (Neu5Ac and Neu5Gc) with long chain (C22- and/or C24, upper band) and short chain fatty acids (C16- and/or C18, lower band) as known for many mammalian cell types.

NanoESI mass spectrometry revealed basically very similar spectra of the ganglioside fractions from all four animal groups without nameable qualitative differences, indicative for a homogeneous and stable ganglioside

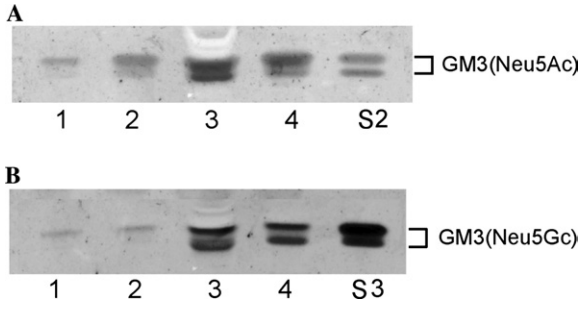

Fig. 3. HPTLC-immunodetection of GM3(Neu5Ac) (A) and GM3(Neu5Gc) (B) in the liver of rats. Lanes 1–4 correspond to group numbering as depicted in Tables 1 and 2. Ganglioside amounts corresponding to 10 mg liver wet weight were chromatographed together with 0.5 μg GM3(Neu5Ac) (S2) and 0.5 μg GM3(Neu5Gc) (S3).

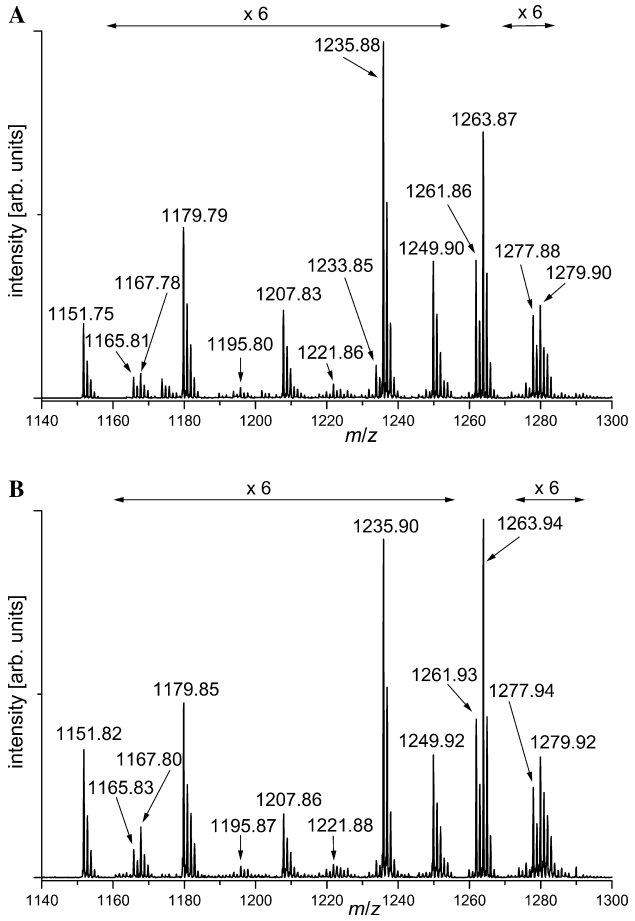

Fig. 4. Section of nanoESI-QTOF mass spectra in the negative ion mode of rat liver GM3-gangliosides from group 2 (A) and group 4 (B). The *m/z*-values and the corresponding proposed structures are listed in Table 3. For specific oxygen treatment of animals from groups 2 and 4 see Table 1.

expression in the rat livers. Fig. 4 shows as examples the parts of the spectra with the GM3 species detected within the liver from animal group 2 (Fig. 4A) and animal group 4 (Fig. 4B). The experimentally derived *m/z*-values together with the corresponding proposed structures are listed in Table 3. In both spectra, a complete series of various GM3 species carrying different fatty acids (from C16:0 to C24:0, see Table 3) are detected. In correlation to the quantification of ganglioside-released sialic acids (Table 2) the majority of peaks can be attributed to GM3 species with Neu5Ac in the sugar moiety. The most abundant molecular ions at *m/z* 1261.86 and 1263.87 in Fig. 4A, and 1261.93 and 1263.94 in Fig. 4B represent GM3(Neu5Ac), substituted with C24:1 and C24:0 fatty acid, respectively, which is in good agreement with the HPTLC immunostaining (see Fig. 3A). The less abundant molecular ions at *m/z* 1277.88 and 1279.90 (Fig. 4A), and 1277.94 and 1279.92 (Fig. 4B) with 16 mass units more than the GM3(Neu5Ac) species with C24:1 and C24:0 fatty acid are assigned to GM3(Neu5Gc, C24:1) and GM3(Neu5Gc, C24:0),

Table 3
Experimental *m/z*-values of the molecular ions of GM3-species and their proposed structures from liver gangliosides investigated by nanoESI-QTOF mass spectrometry in the negative ion mode

$[M-H]^-$ [*m/z*]		Proposed structure[b]
nonHBO[a] (group 2)	postHBO[a] (group 4)	
1151.75	1151.82	GM3(Neu5Ac)(d18:1, C16:0)
1165.81	1165.83	GM3(Neu5Ac)(d18:1, C17:0)
1167.78	1167.80	GM3(Neu5Gc)(d18:1, C16:0)
1179.79	1179.85	GM3(Neu5Ac)(d18:1, C18:0)
1195.80	1195.87	GM3(Neu5Gc)(d18:1, C18:0)
1207.83	1207.86	GM3(Neu5Ac)(d18:1, C20:0)
1221.86	1221.88	GM3(Neu5Ac)(d18:1, C21:0)
1233.85	1233.88	GM3(Neu5Ac)(d18:1, C22:1)
1235.88	1235.90	GM3(Neu5Ac)(d18:1, C22:0)
1249.90	1249.92	GM3(Neu5Ac)(d18:1, C23:0)
1261.86	1261.93	GM3(Neu5Ac)(d18:1, C24:1)
1263.87	1263.94	GM3(Neu5Ac)(d18:1, C24:0)
1277.88	1277.94	GM3(Neu5Gc)(d18:1, C24:1)
1279.90	1279.92	GM3(Neu5Gc)(d18:1, C24:0)

[a] For specific oxygen treatment of animals from groups 2 and 4 see Table 1.
[b] The corresponding HPTLC resorcinol and immunostains are shown in Figs. 1 and 3, respectively.

respectively. Additionally, small amounts of GM3(Neu5Gc) carrying C16:0 and C18:0 fatty acids in the ceramide moiety are detected at *m/z* 1167.78 and 1195.80 (Fig. 4A), and 1167.80 and 1195.87 (Fig. 4B).

Structural characterization of neolacto-series gangliosides combined with HPTLC-immunostaining and nanoESI-QTOF mass spectrometry

The detection of terminally α2-3- and α2-6-sialylated neolacto-series gangliosides such as IV^{3}Neu5Ac-nLc4Cer and VI^{3}Neu5Ac-nLc6Cer or IV^{6}Neu5Ac-nLc4Cer and VI^{6}Neu5Ac-nLc6Cer is feasible with an anti-nLc4Cer antibody after *Vibrio cholerae* neuraminidase treatment [41]. The antibody, which has been raised by immunizing a chicken with HPLC-purified nLc4Cer, binds to the Galβ1-4GlcNAc-terminus of neutral GSLs with neolacto-core structures, e.g., nLc4Cer and nLc6Cer. The HPTLC-immunodetection of terminally sialylated neolacto-series gangliosides within the ganglioside fractions of the four groups of rats is shown in Fig. 5 (lanes 1–4). A reference ganglioside mixture from human granulocytes, comprising as major gangliosides IV^3-Neu5Ac-nLc4Cer, IV^{6}Neu5Ac-nLc4Cer, and VI^3-Neu5Ac-nLc6Cer (for resorcinol stain see Fig. 1B, lane S4), served as positive control in the overlay assays. Each of these gangliosides shows a characteristic double band pattern (Fig. 5, lane S4) due to substitution of the sphingosine with long chain (C24 and C22, upper band) and short chain fatty acids (C16, lower band). The positive binding of the antibody at the position of GM3 indicates a cross-reactivity with Galβ1-4Glcβ1-

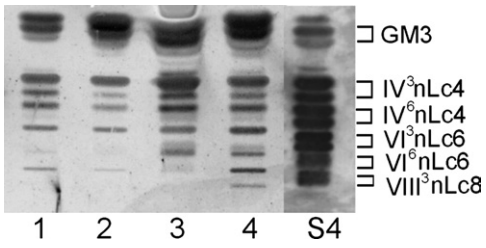

Fig. 5. Detection of neolacto-series gangliosides in the liver of rats. Lanes 1–4 correspond to group numbering as depicted in Tables 1 and 2. Ganglioside amounts corresponding to 30 mg liver wet weight were chromatographed together with 2.5 μg of human granulocyte gangliosides (S4). The HPTLC immunostaining was performed with anti-nLc4Cer antibody after *V. cholerae* neuraminidase treatment. IV^3nLc4, IV^3Neu5Ac-nLc4Cer; IV^6nLc4, IV^6Neu5Ac-nLc4Cer; and VI^3nLc6, VI^3Neu5Ac-nLc6Cer.

1Cer after enzymatic desialylation of GM3. Neolacto-series gangliosides were detectable in the livers of the 4 animal groups (Fig. 5, lanes 1–4) and the most likely structures could be attributed to the positive bands at this stage of investigation. These are double-banded IV^3Neu5Ac-nLc4Cer with long chain (upper band) and short chain fatty acids (lower band) and single IV^6Neu5Ac-nLc4Cer. In addition to these gangliosides with nLc4Cer core, one positive band was detected at the position of VI^3Neu5Ac-nLc6Cer in all animals and one at the position of VI^6Neu5Ac-nLc6Cer in group 3 and 4 animals (see Fig. 5). The tentative ganglioside $VIII^3$Neu5Ac-nLc8Cer was exclusively found in postHBO rats (Fig. 5, lane 4). The most prominent quantitative differences in the expression of those gangliosides detected in all rats were the somewhat enhanced expression of double banded IV^3Neu5Ac-nLc4Cer and single IV^6Neu5Ac-nLc4Cer and VI^3Neu5Ac-nLc6Cer in preHBO rats (Fig. 5, lane 3) and the elevated expression of the upper band of IV^3Neu5Ac-nLc4Cer and single IV^6Neu5Ac-nLc4Cer and VI^3Neu5Ac-nLc6Cer in postHBO rats (Fig. 5, lane 4) compared to the non-oxygenated animals, respectively (Fig. 5, lanes 1 and 2).

The part of the nanoESI mass spectra containing the molecular ions of neolacto-series gangliosides with nLc4Cer core, detected within the livers of all four animal groups, are exemplified for nonHBO (group 2) and postHBO rats (group 4) in Figs. 6A and B, respectively. The experimentally derived *m*/*z*-values together with the corresponding proposed structures are listed in Table 4. The spectra confirm the results of the HPTLC immunostain (Fig. 5, lanes 2 and 4) where neolacto-series gangliosides with long chain fatty acids (upper bands) give rise to the most intensive bands. The most abundant molecular ions at *m*/*z* 1601.01, 1615.02, 1627.05, and 1629.04 in Fig. 6A, and 1601.08, 1615.09, 1627.07, and 1629.12 in Fig. 6B, are assigned to Neu5Ac-substituted nLc4Cer species carrying C22:0, C23:0, C24:1, and C24:0 long chain

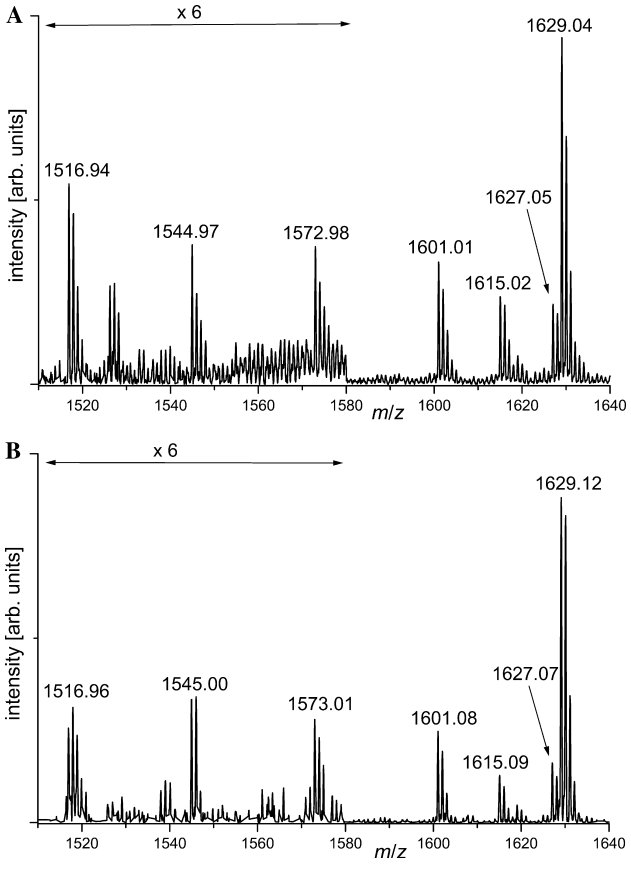

Fig. 6. Section of nanoESI-QTOF mass spectra in the negative ion mode of rat liver neolacto-series gangliosides with nLc4Cer core from group 2 (A) and group 4 (B). The *m*/*z*-values and the corresponding proposed structures are listed in Table 4. For specific oxygen treatment of animals from groups 2 and 4 see Table 1.

Table 4
Experimental *m*/*z*-values of the molecular ions of neolacto-series gangliosides and their proposed structures from liver gangliosides investigated by nanoESI-QTOF mass spectrometry in the negative ion mode

$[M-H]^-$ [*m*/*z*]		Proposed structure[b]
nonHBO[a] (group 2)	postHBO[a] (group 4)	
1516.94	1516.96	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C16:0)
1544.97	1545.00	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C18:0)
1572.98	1573.01	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C20:0)
1601.01	1601.08	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C22:0)
1615.02	1615.09	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C23:0)
1627.05	1627.07	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C24:1)
1629.04	1629.12	$IV^{3/6}$Neu5Ac-nLc4Cer(d18:1, C24:0)

[a] For specific oxygen treatment of animals from groups 2 and 4 see Table 1.
[b] The corresponding HPTLC resorcinol and immunostains are shown in Figs. 1 and 5, respectively.

fatty acids in the ceramide moieties, respectively. The presence of neolacto-series gangliosides with short chain fatty acids (C16:0, C18:0, and C20:0) is indicated

by the molecular ions assigned in the enlarged section of the spectra (see Table 4).

HPTLC-immunodetection of ganglio-series gangliosides

A polyclonal anti-GM1 antibody was used for the detection of the ganglio-series gangliosides GM1, GD1a, GD1b, and GT1b. The latter 3 gangliosides are detectable with the anti-GM1 antibody on the HPTLC plate after *V. cholerae* neuraminidase treatment. The enzyme removes sialic acids of di- and trisialogangliosides (and higher sialylated gangliosides with GM1-core) with the exception of the inner galactose-linked sialic acid, leaving the intact GM1-core. The HPTLC-immunodetection of GM1-core gangliosides within the ganglioside fractions of the 4 groups of rats is shown in Fig. 7 (lanes 1–4). A reference ganglioside mixture from rat brain, comprising as major gangliosides GM1, GD1a, GD1b, and GT1b (for resorcinol stain see Fig. 1B, lane S1), served as positive control in the overlay assays. Livers of group 3 rats showed a slightly enhanced expression of GM1, GD1a, and GD1b, but they lacked GT1b, which was present in the three other groups (Fig. 7, lanes 1, 2, and 4). An unknown impurity in the ganglioside preparation of preHBO rats is most likely the reason for the slightly altered HPTLC of GD1a and GD1b (Fig. 7, lane 3).

HPTLC-immunodetection of GM2, GalNAc-GM1b, GM1b, and GD3

A sharp band of GM2 was clearly visible in livers of oxygenated animal group 3 and 4 (Fig. 8A, lanes 3 and 4, respectively) compared to trace quantities in the two non-oxygenated animal group 1 and 2 (Fig. 8B, lanes 1 and 2, respectively).

GalNAc-GM1b, the pendant of the GM1b-pathway with identical terminal trisaccharide and outer β1-4-linked GalNAc like GM2, was also found to be preferentially expressed in group 3 and 4 rats (Fig. 8B, lanes 3 and 4, respectively). The GalNAc-GM1b single band chromatographs at position of GalNAc-GM1b(Neu5Ac,

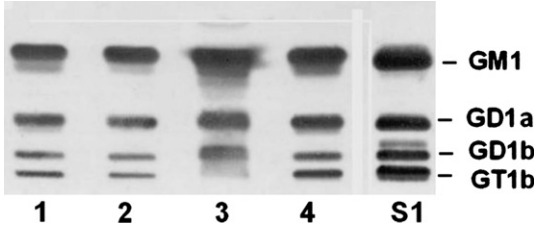

Fig. 7. Detection of gangliotetraose-type gangliosides in the liver of rats. Lanes 1– 4 correspond to group numbering as depicted in Tables 1 and 2. Ganglioside amounts corresponding to 30 mg liver wet weight were chromatographed together with 2 μg gangliosides from rat brain (S1). The HPTLC immunostaining was performed with anti-GM1 antibody after *V. cholerae* neuraminidase treatment.

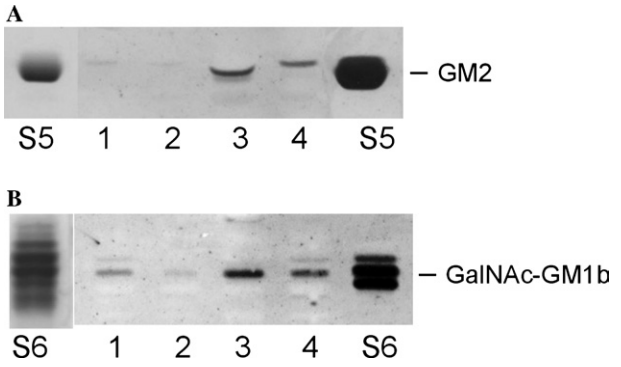

Fig. 8. HPTLC-immunodetection of GM2 (A) and GalNAc-GM1b (B) in the liver of rats. Lanes 1–4 correspond to group numbering as depicted in Tables 1 and 2. Ganglioside amounts corresponding to 30 mg liver wet weight were chromatographed. (A) Left lane S5: resorcinol stain of 6 μg reference GM2, right lane S5: immunostain of 2 μg reference GM2. (B) Left lane S6: resorcinol stain of 10 μg reference YAC-1 gangliosides, right lane S6: immunostain of 1 μg reference YAC-1 gangliosides.

C16-fatty acid) deduced from the reference monosialoganglioside fraction of YAC-1 cells (Fig. 8B, lane S6) [29]. Highest expression was detected in the liver of preHBO rats (Fig. 8B, lane 3).

GM1b, the precursor structure of GalNAc-GM1b, was completely absent from all of the livers examined (data not shown). Only trace quantities of GD3 were detectable in group 4 rats and none in the group 1, 2, and 3 animals (data not shown).

Discussion

Several authors have described in the past beneficial effects of HBO treatment upon liver regeneration triggered by embolization, reperfusion injury after ischemia or injury caused by toxic agents [19,20]. Recently, we described a significant increase in the starting process of the liver mass restitution (determined as liver weight/body weight ratio) in the preHBO-treated rats [21]. Animals of this group were without signs of liver dysfunction, evaluated by alanine transaminase and aspartate transaminase activities (Table 1). Liver lipid peroxide concentration was the lowest in the preHBO group and the light microscopy findings revealed that the lobule composition of preHBO group liver was the closest to normal histological features [21]. These results suggested that HBO pretreatment was undoubtedly beneficial for the recovery of rats after PH. In postHBO group liver mass restitution was lower compared to preHBO-treated animals. Lipid peroxide content was 2.4-fold higher and activities of liver enzymes were approximately 2-fold increased in postHBO group compared to preHBO animals, suggesting less efficient liver regeneration in postHBO rats [21].

The present paper reports on the altered expression of predominant GM3 and less abundant neolacto-series, GM1- and GM1b-type gangliosides in the liver of rats, which were kept under different housing conditions, related to oxygen pressure before or after PH. The enhanced expression of the major gangliosides GM3(Neu5Ac) and GM3(Neu5Gc) in preHBO and postHBO correlated with the respective total ganglioside-released sialic acid content (Neu5Ac plus Neu5Gc) in terms of low concentrations in the non-oxygenated rats (control and nonHBO) and high concentrations in oxygenated rats (preHBO and postHBO). The relative contribution of Neu5Gc to total sialic acids in the control rats amounting to 12.6% was found to be in good agreement with previously reported 11% in rat liver glycoconjugates [42]. Hyperbaric oxygenation provoked a 6.4- to 7.6-fold total sialic acid increase in the oxygenated animals revealing more or less stable molar Neu5Ac/Neu5Gc ratios of 6.9 in control, 10.0 in nonHBO, and 7.7 in preHBO rats, but a remarkably elevated quotient of 17.6 in postHBO animals due to reduced quantity of Neu5Gc. The biosynthesis of Neu5Gc occurs via hydroxylation of Neu5Ac at the level of the CMP-Neu5Ac glycoside [43] and the expression of Neu5Gc has been found to be related to the level of CMP-Neu5Ac hydroxylase mRNA [44]. It is suggested that hydroxylation of CMP-Neu5Ac controls the expression of Neu5Gc in GM3 ganglioside, as reported by Bouhours and Bouhours [45] for small intestine of inbred rats. The expression of Neu5Ac in GM3 was found to depend on a single autosomal gene and was correlated with the activity of CMP-Neu5Ac-hydroxylase. Thus, the activity of CMP-Neu5Ac-hydroxylation is assumed to play a key role in the regulation of the biosynthesis of Neu5Gc-containing gangliosides. The hydroxylation is a monooxygenase type of reaction and is carried out by several factors including the soluble form of cytochrome b_5 [46]. Remarkably reduced expression of Neu5Gc in postHBO rats is reasonable concerning our previous finding of 2.4 higher concentration of lipid peroxides in postHBO group compared to preHBO-treated animals [21].

Significant changes in the ganglioside content and distribution during rat liver regeneration after PH have been reported [16,17]. Riboni et al. used Fisher strain male inbred rats (200–220 g body mass) and removed approximately 70% of the liver mass. Total liver gangliosides increased until the 4th day after surgery and progressively decreased to reach control values at the 12th day. At day 2 after operation, only marginal differences could be determined for GM3 and GM1-core gangliosides being in good agreement with our findings. Fishman et al. [16] used 68% hepatectomized Sprague–Dawley rats (150–200 g body mass) and detected a significant increase in GM1-core gangliosides at day 2 after operation, whereas changes in GM3 were negligible. In our study, one-month-old male Wistar rats (73–102 g body mass) were submitted to 15% PH, because even small liver resections are sufficient to trigger regeneration in weanling rats [16]. Animals were sacrificed at day 2.25 (54 h) after operation. Changes in GM3 expression and total ganglioside-derived sialic acids were marginal in partially hepatectomized nonHBO animals compared to the control group. A vast increase of GM3 and corresponding ganglioside-derived sialic acids amounting to factor 6.4 and 7.6 was determined for the pre- and post-operatively oxygenated rats compared to the operated but non-oxygenated animals, respectively. However, despite these quantitative changes, the GM3 mass spectra evidenced a stable expression pattern in all four animal groups in terms of the various GM3(Neu5Ac) or GM3(Neu5Gc) species within the ganglioside fractions of oxygenated rats (preHBO and postHBO).

GM3, the most prominent and widely distributed ganglioside in mammalian cells, has been shown to exert a variety of biological activities being involved in cell adhesion and signal transduction events (for review see [3] and references therein). In particular, recent publications point to an important role of GM3 in cell growth regulation [9,10], T-cell activation [11], signal transduction [12], and insulin signaling [13]. Extracellular signals that mediate liver regenerative process after PH act initially at the plasma membrane, where binding of receptors to endocrine and paracrine agents, and to elements of the extracellular matrix, transport of nutrients, and interactions with neighboring cells occur. Since liver regeneration appears to involve a large number of hormone–receptor systems and cellular changes (i.e., oncogene expression, TGF-α expression), modulation of the ganglioside content of hepatocyte plasma membrane could provide a means for upregulating cellular responsiveness to a variety of factors. Thus, these functional assumptions throughout liver regeneration of GM3 as an integral part of microdomains or ''glycosynapses'' in the plasma membrane, organized with cell adhesion molecules and coupled with signal transducers, remain to be determined.

Along with the progress in analytical techniques we were able to unequivocally characterize the less abundant ganglioside species besides predominant GM3 in rat livers by means of HPTLC immunostaining combined with nanoESI-QTOF mass spectrometry. This study contains the first report in respect of the existence and detailed structural characterization of neolacto-series gangliosides in rat liver, namely IV^{3}Neu5Ac-nLc4Cer and IV^{6}Neu5Ac-nLc4Cer, and the immunochemical identification of terminally sialylated nLc6Cer and nLc8Cer species. Unlike Neu5Gc substitution in the GM3 variants, only Neu5Ac- and no Neu5Gc-substituted neolacto-series species were detected. The nanoESI-QTOF mass spectra revealed stable expression

pattern in all four animal groups and no specific alterations of certain MS-detectable nLc4Cer-core gangliosides in oxygenated rats (preHBO and postHBO). HPTLC immunostainings of less abundant gangliosides revealed a faint increase of GM1-type gangliosides in preHBO rats and a slight enhancement of GM2 and GalNAc-GM1b in the oxygenated preHBO and post-HBO animals. Since certain sialyl-, galactosyl- or *N*-acetylgalactosaminyltransferase activities are responsible for the final steps of the biosynthesis of GM3, neolacto-series, GM1-core, and GM1b-type gangliosides, our results point at a functional role of glycosyltransferases in the regulation of liver growth and reconstitution after PH combined with pre- and post-operative HBO treatment.

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