Physico-Chemical Proofs Concerning the Existence of Ganglioside GM3 in Normal Human LDL

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Low density lipoproteins (LDL) were separated from normal human serum by a standard procedure. Total lipids of LDL were recovered by chloroform-methanol extraction and submitted to Folch partition. Lipids from upper layer were dialysed, submitted to ion-exchange chromatography on DEAE-Sephadex A-25 and the fractions containig lipid bound sialic acid passed on a column of silica gel. Thin layer chromatography of crude mixture, in comparison with adequated standards suggested the presence of ganglioside GM3. A compound isolated from LDL lipids, by techniques currently used in gangliosides separation, produced a compound co-migrating with ganglioside GM3. Desialysation of this compound produced a glycolipid which co-chromatographed with standard lactosyl-ceramide. ^{1}H NMR Spectra of the isolated compound indicated that it contained all the structural ingredients of ganglioside GM3: sialic acid, D-galactose, D-glucose, sphingosine, fatty acid. Concentration of lipid bound sialic acid in normal human LDL was 8.5 nmols sialic acid/mg of protein.

Key words: ganglioside GM3, lactosyl-ceramide, low density lipoproteins, human serum, atherosclerosis

The knowledge of the chemical composition of human blood and vessels constitutes an essential condition in the combat with atherosclerosis and its consequences. In comparison with cholesterol esters, triglycerides and phospholipids, gangliosides are relatively less studied in these tissues [1]. Low density lipoproteins became a standard constituent of human health since was discovered that human people devoid of cellular receptor for LDL present a genetically transmissible disease, familial hypercholesterolemia – an altered metabolism of cholesterol [2]. Gangliosides are glycosphingolipids containing glycosidic sialic acid in their molecules [3]. The term ganglioside was coined by E. KLENK for a group of compounds isolated from ganglia [4]. Glycosphingolipids present an outstanding molecular variety, more than 300 representatives being isolated and characterized at this moment [5, 6]. Gangliosides have been involved in numerous fundamental physiological and cellular processes: growing, differentiation, ageing, transformation [7]. The association between gangliosides and different diseases have been made since their discovery [8]. In the course of their investigations, a question was raised concerning their physico-chemical compatibility, as lipids, with hydrosoluble enzymes metabolizing them [9]. The answer consisted in the discovery of a new group of compounds – saposins or activator proteins [10] having an emulsifying action on glycosphingolipids. Their defficiency, similarly with the deficiency of the respective enzymes, are the direct cause of some diseases, i. e., Tay-Sachs disease can be equally determined, although not at the same frequency, by an altered β-hexosaminidase and/or by an altered saposin [8]. As cellular constituents amphiphylic molecules, gangliosides are inserted in the membrane with their hydrophobic (ceramide) part, while the sugar moieties protrude outside the cell forming, together with carbohydrates of glycoproteins, the so-called glycocalyx, a characteristic matrix of young and specialized cells [11]. The knowledge of ganglioside structure and metabolism as well as of their interactions with other molecules generated clear explanations for a series of pathological phenomena. Guillain-Barre syndrome, manifested by an unusual high titer of antibodies in the tissues of the patient, is caused by the elaboration, by the respective microorganism, of oligosaccharides imitating the structure of gangliosides [12, 13]. Cells of the host are confused by this similarity and they produce antibodies by an uncontrolled manner [14]. Cholera infection is mediated by an exceptional affinity of a toxin from microbe wall (cholera toxin) and constituent gangliosides of brush border of small intestine. This binding is promtly followed by a perturbation of normal physiological functions of digestive tract [15]. Gangliosides have unique implications in feeding processes. Having a relatively high proportion of ceramides they contribute to the protection of colon against tumors caused by physical, chemical or genetic factors [16]. On the other hand, infant babies nourished by breast milk acquired a higher proportion of sialic acid in their brain, in comparison with babies that received formula milk [17].

In this paper, the presence of ganglioside GM3 in normal human LDL has been proved by using exclussively physico-chemical means: colorimetric methods, chromatography, spectroscopic methods.

Experimental part

The compounds and materials used in this work were purchased from Merck (Germany): hydrochloric acid, anthrone, resorcinol, feric chloride, silica gel for column chromatography, silica gel coated glass plates. The following standard compounds have been prepared in our laboratory: galactocerebroside from bovine brain [18] and ganglioside GM4 (sialosyl-α-3-β-D-galactopyranosyl-1'ceramide), ganglioside GM3 (sialosyl-α-3-β-D-galactopyranosyl-4-βD-glucopyranosyl-1'ceramide) and lactosyl-ceramide from rooster testes [19]. LDL was separated from normal human serum by ultracentrifugation and processed immediately [20]. More precisely, it was extracted with chloroform-methanol mixture and submitted to Folch partition [18, 19]. Lipids of upper layer were dialyzed and the content of dialysis bag

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was passed successively on two columns of DEAE Sephadex A-25 and silica gel, respectively. Sialic acid was determined colorimetrically by resorcinol reaction [19] and protein concentration by Lowry method [21]. The following solvent systems (SS) were used for thin layer chromatography (TLC): SS 1, chloroform-methanol-water, 60/25/4 (v/v); SS 2, chloroform-methanol-water-conc. ammonia, 70/30/4/1 (v/v); SS 3, isopropanol-water-conc. ammonia, 6/3/2 (v/v); SS 4, chloroform-methanol-acetone-acetic acid-water, 10:2:4:2:1 (v/v). Three reagents were used for plates visualisation: resorcinol for gangliosides, orcinol for neutral glycosphingolipids and phosphomolybdenic acid as a universal reagent [18, 19]. Mild alkaline hydrolysis of gangliosides mixture was made as indicated [18]. Desialysation of gangliosides was made by heating them in 0.05 M hydrochloric acid by 80^0C for 1 h [19].

NMR Spectra Registration

Ganglioside isolated from normal human LDL, in native form was used for ^{1}H NMR spectra registration as a solution in dimethyl sulfoxide-$^2H_6$/^2H_2O (98:2, v/v). Concomitantly, ^{1}H and ^{13}C NMR spectra of the following reference compounds were registered: galactocerebroside, lactosyl ceramide, ganglioside GM3. NMR experiments were performed on a Bruker ACE-300 NMR at 300 and 75 MHz, respectively.

Results and discussion

Concentration of lipid bound sialic acid in normal human LDL was 8.5 nmols sialic acid/mg of protein. Complexity of chemical composition of LDL, both in upper and lower layer of Folch partition as well as in total mixture, is illustrated in figure 1. It is quite evident from the chromatogram of figure 1 that Folch partition brings a clearing of separation in the sense that less polar lipids and glycolipids are found in lower layer while lipids having higher polarity, i. e., gangliosides, are found in the upper layer. In this stage of separation, it was possible to determine the concentration of lipid bound sialic acid. After dialysis, lipids of upper layer were submitted to mild alkaline hydrolysis in order to destroy some minor derivatives of gangliosides, especially lactones [22] and acetate esters [23]. TLC of whole lipids from upper Folch layer, in comparison with gangliosides GM4 and GM3 (fig. 2), suggested the presence of ganglioside GM3. This hypothesis was confirmed for the purified ganglioside from LDL (fig. 3). However, there is an equivocal situation in

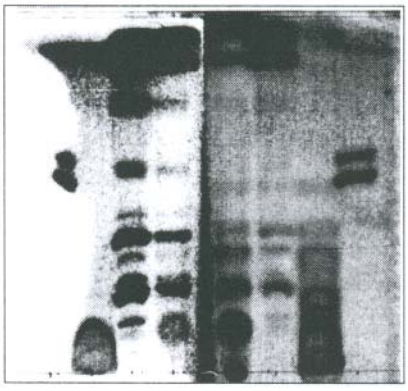

Fig. 1. TLC Analysis of total lipids from normal human LDL by comparative staining with phosphomolybdic acid (left half of the plate) and orcinol/$FeCl_3$ (right half). Lane 1 and 8, galactocerebrosides from bovine brain; lane 2 and 7, upper layer lipids; lane 3 and 6, lipids of lower layer; lane 4 and 5, total lipids, before Folch partition. Migration, SS 1.

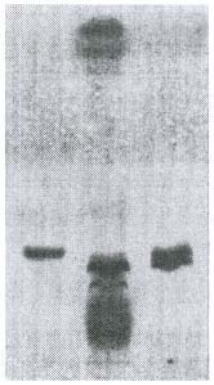

Fig. 2. Chromatographic comparison between LDL lipids from upper layer (lane 2) and ganglioside GM4 (lane 1) and a mixture of ganglioside GM4 (faster spot) and GM3 (lane 3). Migration, SS 2; visualisation, resorcinol

this point, due to the relatively low chromatographic resolution of TLC plates concerning glycosphingolipids having alternatively normal chain fatty acid or 2-D-hydroxy fatty acid. In this situation, the so-called twin spots are obtained by TLC, with a difference in R_F value less than 0.05. Consequently, LDL could contain either GM3 or GM4 hydroxylated in a variable degree in ceramide moiety. This equivocal situation is completely eliminated by desialysation of gangliosides. The selectivity of this reaction is based on the fact that glycosidic bond of sialic acid is more labile in acidic environment than glycosidic bond of hexosides. By desialysation, ganglioside GM4 is converted to galactocerebroside and GM3 to lactosyl-ceramide (β-D-galactopyranosyl-4-β-D-glucopyranosyl-1'ceramide) and these two neutral glycosphingolipids present a significant difference of R_F values by TLC (fig. 4). (The twin spot is regained after desialysation if only one ganglioside, either GM4 or GM3 is present). It is evident from the latter figure that the ganglioside isolated from normal human LDL produces a compound comigrating with lactosyl-ceramide and not with galactosyl-ceramide (galacto-cerebroside). In this way, a number of arguments have been gathered that the ganglioside from human LDL is GM3: co-migration with standard GM3 in four different solvent systems; producing, by desialysation, of a compound co-migrating with lactosyl-ceramide. This hypothesis was confirmed by ^{1}H NMR spectra.

^{1}H NMR Spectra of LDL ganglioside

Due to the scarcity of biological material, only ^{1}H NMR spectra of the ganglioside isolated from normal human

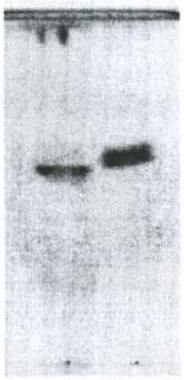

Fig. 3. TLC Migration of the ganglioside isolated from normal human LDL (lane 1) and a mixture of gangliosides (lane 2): GM4 (faster spot) and GM3. Migration, SS 3; visualisation, resorcinol

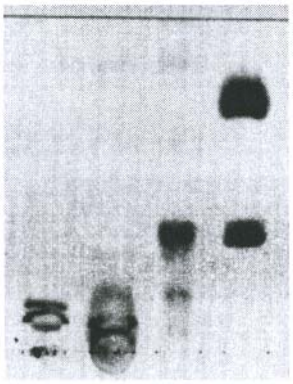

Fig. 4. Ganglioside isolated from normal human LDL produces a compound co-migrating with lactosyl-ceramide (asialo-GM3). Lane 1, mixture of standard gangliosides (GM4 and GM3); lane 2, ganglioside separated from LDL; lane 3, product obtained by desialysation of LDL ganglioside; lane 4, standard glycosphingolipids: galactocerebroside (upper spot) and lactosyl-ceramide. Migration, SS 4; visualisation, orcinol

LDL could be registered. However, the interpretation of the results was much facilitated by the registration of ^{1}H and ^{13}C NMR spectra of other glycosphingolipids: galacto-cerebroside from bovine brain and lactosyl ceramide and ganglioside GM3 from rooster testes.

(Dimethyl sulfoxide-d_6/D_2O (98:2, v/v)); δ ppm; *J* Hz): 0.86 (t, H-18 and H-18') (fig. 5), 1.25 (s, methylene groups of sphingosine and fatty acid), 1.89 (s, H-11''''), 5.35 (m, H-4), 5.53 (m, H-5), 4.14 (d, 7.2, H-1''), 4.17 (d, 7.3, H-1'''), 2.71 (H-3''''(e)), 1.35 (H-3''''(a)).

Sofisticated biological chemical methods are currently used for localization and determination of gangliosides in biological materials. Incorporation of ganglioside GD3 in CHO chinese hamster ovary cells transfected with GD3 synthase was visualised with mouse IgG3 monoclonal antibodies R24 while 9-O-acetylated GD3 produced by the same cells was visualised with monoclonal antibodies 27A [24]. In fact, pure gangliosides or their derivatives (lactones, esters, etc.,) are used for elaboration of monoclonal or polyclonal antibodies produced by animal laboratories, usually mice or rabbits. To another antibody, usually goat IgG or IgM, produced by using the above-mentioned antibodies as antigens, an enzyme is coupled (ELISA) or fluorescein (fluorometry) [25]. Radioisotopes have been also extensively used for tracing of gangliosides in cell culture or laboratory animals. In this sense, a diversity of methods have been used for labeling: de-acetylation of sialic acid and reacetylation with [^{3}H] acetyl chloride [26], catalytic hydrogenation of double bond of sphingosine with ^{3}H [27], preparation of the corresponding lyso-derivative by removing the fatty acid of the N-amide and its replacement with a fatty acid labeled with ^{3}H or ^{14}C [28], enzymatic oxidation of primary hydroxy group of D-galactose in the oligosaccharidic part to aldehyde and its subsequent reduction with $NaB[^{3}H]_4$ [28], selective oxidation of C-3 hydroxy group of sphingosine and its reduction with ^{3}H [28]. Spin label of gangliosides with nitroxide was also accomplished and used for investigations concerning its incorporation into fibroblast cell membranes [29]. On the other hand, physico-chemical methods are constantly refined and improved and such refinements and improvements endow them with sensitiveness and swiftness. In secondary ion mass spectrometry, an amount of 1μg of sample containing ganglioside being sufficient for complete registration of spectra [30]. We proved in this paper that some physico-chemical methods are adequate for a conclusive analysis in a relatively less accessible material based on human blood, i. e., LDL. In a previous paper we demonstrated that metabolic relationships concerning gangliosides biosynthesis in turkey testis can be inferred by ^{1}H NMR spectroscopy [31].

Synthesis of gangliosides was considered an important and challenging task of chemical synthesis. It has been accomplished in four steps: (A) construction of oligosaccharide backbone; (B) sialosylation of the oligosaccharidic backbone; (C) construction of sphingolipid moiety; (D) coupling of the sialo-oligosaccharide fragment and sphingolipid moiety [32, 33, 34]. In the course of gangliosides synthesis, an essential step is glycosylation reaction. One of the most used reaction of this type is Schmidt glycosylation [35]. At the same time, by using sugars as precursors for sphingosine, a new argument for the knowledge of chiral centers of the

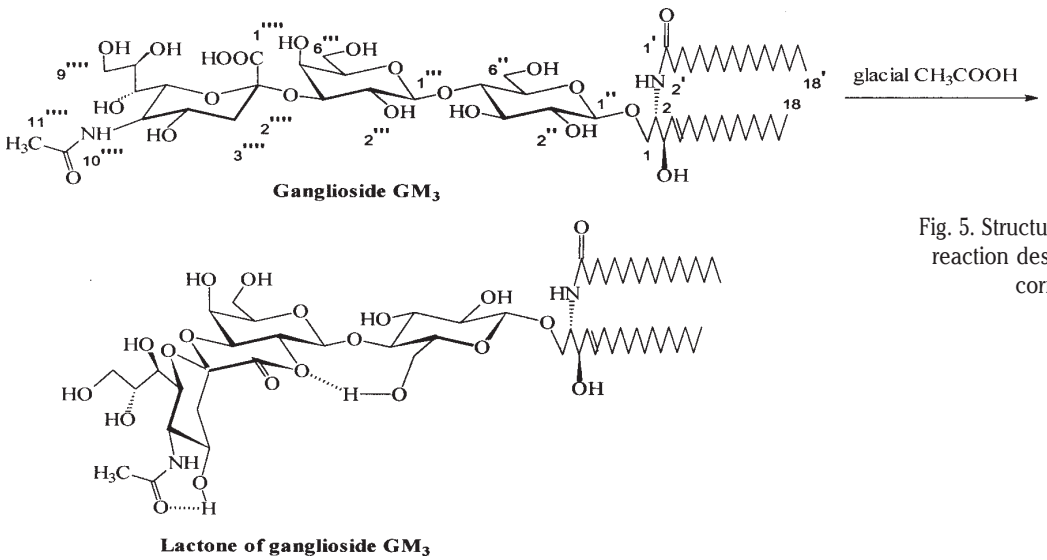

Fig. 5. Structure of ganglioside GM3 and the reaction describing its conversion to the corresponding lactone.

long chain base was brought [36]. An extremely beneficial biological activity of some gangliosides is their neuritogenic function, especially if we take into consideration the fact that nervous cells are the most difficult to be maintained in culture, in comparison with other types of animal or vegetable cells. A neuritogenic ganglioside has been isolated from the sea cucumber *Stichopus chloronotus* its structure being NeuGcα-6-D-Galβ-1'Cer with a ceramide formed of C16-isosphingosine and 2-D-hydroxynervonic acid [37]. A problem inevitably appears in this point: biological material of marine origin is relatively less accessible even for countries having a developed fishing industry. By using galactocerebroside as starting material, a precursor for the neuritogenic ganglioside has been prepared, i. e., β-*D*-galactopyranosyl (2,3,4-tri-O-acetyl)-1'(3'-O-acetyl)ceramide [38].

Conclusions

The major ganglioside in normal human LDL is GM3, its concentration being 8.5 nmols sialic acid/mg of protein.

Physical chemical methods, i. e., colorimetry, chromatography, spectroscopy, proved adequate for conclusive results in approaching a relatively scarce material, LDL.

The use of reference compounds proved of essential value in isolation and characterization of ganglioside GM3 from normal human LDL.

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Manuscript received: 5.01.2009