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Activation of Ganglioside GM3 Biosynthesis in Human Monocyte/Macrophages during Culturing *in vitro*

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Abstract—We found that GM3 levels in human peripheral blood monocytes and cultured monocyte-derived macrophages were 0.37 and 2.7 μg per million cells, respectively. GM3 synthase of monocytes and to a greater extent of monocyte-derived macrophages was shown to be able to sialylate endogenous substrate, lactosylceramide (LacCer), to form GM3. With exogenously added LacCer, GM3 synthase activity was 57.1 and 563 pmol/h per mg protein in monocytes and monocyte-derived macrophages, respectively. The revealed changes in ganglioside GM3 biosynthesis are specific as the activity of some other sialyltransferases under these conditions was not altered. Human anti-GM3 synthase antibody detected in monocytes a main protein with molecular weight of 60 kD and minor proteins with molecular masses of 52 and 64 kD. In monocyte-derived macrophages the amounts of 60 kD protein and especially 64 kD protein sharply rose. Thus, the increase in ganglioside GM3 levels, GM3 synthase activity, and the enzyme amounts during culturing of monocyte/macrophages may be one of the mechanisms of *in vivo* increased ganglioside GM3 levels in arterial atherosclerotic lesions.

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Gangliosides are ubiquitous plasma membrane constituents of all eukaryotic cells. The main function of gangliosides is participation in reception of various molecules, regulation of cell cycle, cell differentiation, and cancer transformation [1]. In human peripheral blood lymphocytes and monocytes, monosialoganglioside GM3 represents the main ganglioside constituent of cell plasma membrane [2, 3]. In lymphocytes, it is involved in formation of plasma membrane microdomains where among surface antigens, protein kinases, phospholipases, and other regulatory molecules are located [4]. GM3-enriched membrane domains of T lymphocytes are associated with the integral membrane glycoprotein CD4 and its linked Src family tyrosine kinases. These domains are implicated in cell–cell interaction, antigen recognition, cell activation, and signal transduction [5].

Our previous immunohistochemical studies on sections of human atherosclerotic aorta revealed high levels of ganglioside GM3 and GM3 synthase expression by cells that originated from monocyte/macrophages and abundantly infiltrated aortic intima in atherosclerosis. In non-diseased aortic intima wherein these cells are absent, the expression of ganglioside GM3 and GM3 synthase was very low. Further *in vitro* experiments showed that monocyte-derived macrophages and dendritic cells as well as the cells of aortic atherosclerotic lesions expressed high levels of GM3 synthase [6]. Based on these facts, we suggested that a 5-fold increase in ganglioside GM3 levels in human atherosclerotic aorta is due to excessive influx of blood monocytes into aortic wall with their subsequent transformation into macrophages. To test this assumption, we determined ganglioside GM3 amounts and GM3 synthase activity in isolated human blood monocytes and in cultured monocyte-derived macrophages. It was found that ganglioside GM3 amount

Abbreviations: BSA) bovine serum albumin; CMP[^{14}C]NeuAc) cytidine 5′-monophospho[^{14}C]N-acetylneuraminic acid; HPTLC) high performance thin layer chromatography; LacCer) lactosylceramide; PBS) phosphate buffered saline; PMA) phorbol 12-myristate 13-acetate; PTA) phosphotungstic acid; ST) sialyltransferase; TCA) trichloroacetic acid; TLC) thin layer chromatography.

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was 4.1 times greater and GM3 synthase activity was 10 times higher in monocyte-derived macrophages than in monocytes.

MATERIALS AND METHODS

Materials. Chemicals of analytical grade from Sigma (USA), Serva (Germany), and of domestic production were used in the present work; cytidine 5′-monophospho[^{14}C]N-acetylneuraminic acid (CMP[^{14}C]NeuAc; 287 mCi/mmol) was from Amersham (Great Britain). Human liver ganglioside GM3 was isolated according to the method described earlier [7].

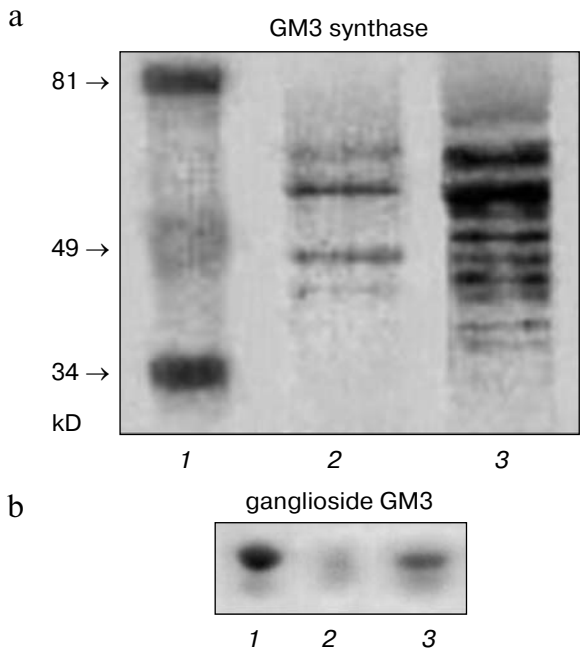
Human monocyte isolation and macrophage generation. Peripheral blood mononuclear cells were isolated from leukocyte rich plasma prepared by leukophoresis of blood from HIV and hepatitis B and C seronegative donors by centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Sweden) gradients using standard procedures. Monocytes were purified from peripheral blood mononuclear cells by an additional centrifugation over Percoll (Amersham Biosciences) according to the procedure described in [8]. The purity of monocytes averaged 75% as determined by flow cytometry after staining cells with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated monoclonal antibodies to CD45/CD14 and isotypic mice IgGs (all from BD Pharmingen, USA). The cell pellet was resuspended in minimal volume of distilled water, sonicated for 10 sec, and the cell lysate was stored at –70°C.

To obtain monocyte-derived macrophages, the suspension of mononuclear cells in DMEM medium (Gibco-BRL Life Technologies, USA) containing 10% fetal calf serum (ICN, USA), 1% antibiotic mixture (penicillin, streptomycin) (Invitrogen, Sweden), 2 mM glutamine, and 3.5 μg/ml insulin (Novo Nordisk A/S, Denmark) was seeded at 10^6 cells per plate in 100-mm plastic tissue plates (Corning Costar, USA) and cultured at 37°C in a 5% humidified CO_2-incubator with the culture medium being replaced every two days. After 14 days, the plates with macrophages were washed with PBS and the cells were harvested by scraping with a polyethylene cell scraper. The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers ($CD14^+/CD206^+$) (anti-CD14-FITC, anti-CD206-PE; Immunotech, France) by flow cytometry that was performed as described above. The cell pellet was resuspended in minimal volume of distilled water, sonicated for 10 sec, and the cell lysate was stored at –70°C.

Sialyltransferase activity assay. Sialyltransferase (ST) activity was assayed according to the procedure described earlier [9]. All experiments were carried out in duplicate. Assay mixtures (final volume 50 μl) contained 0.2 mM LacCer (GM3 synthase substrate) or 0.2 mM GM3 (GD3 synthase substrate), 0.4 mM CMP[^{14}C]NeuAc (total activity 3.2 mCi), 1 mM $MnCl_2$, 0.75% Triton X-100, 150 mM cacodylate buffer, pH 6.2, and monocyte or macrophage lysates (80-200 μg cell protein). The same reaction mixtures but without LacCer or GM3 (depending on the enzyme assayed) were used as blanks in every determination. After incubation of the mixtures at 37°C for 1.5 h, the reaction was stopped by addition of 0.5 ml 0.05 M acetate buffer, pH 4.4. Then the mixtures were applied onto 5 × 10 mm Sep-Pak C_{18} cartridges (Waters, USA). Free CMP-NeuAc and other water soluble components were removed by washing the column subsequently with 1 ml 0.05 M acetate buffer, pH 4.4, 10 ml water, and 4 ml mixture of methanol–water (1 : 4). Gangliosides were eluted with 1 ml methanol, 6 ml mixture of chloroform–methanol (1 : 2), and 6 ml mixture of chloroform–methanol (2 : 1). The eluates were pooled and dried, and the reaction products were separated by TLC on HPTLC plates (Merck, Germany) using solvent system chloroform–methanol–0.25% $CaCl_2$ (55 : 45 : 10). The plates were visualized with resorcinol reagent. Zones corresponding to chromatographic mobility of authentic GM3 (when GM3 synthase activity assayed) or GD3 (when GD3 synthase activity assayed) were scraped into scintillation vials. Radioactivity was measured on a 1215 Rackbeta II liquid scintillation counter (LKB, Sweden). In parallel with this, one replicate chromatogram of each experiment was exposed at –70°C for 7-14 days to an X-ray Retina film (Fotochemische Werke GmbH, Germany). ST activity was calculated as pmol NeuAc transferred to LacCer (or GM3) in 1 h by 1 mg protein of cell lysate.

ST activity was also assayed using the glycoprotein asialofetuin as the substrate [10]. A 200-μl sample of monocyte or macrophage lysate (50-120 μg cell protein) was added to 30 μl solution of CMP[^{14}C]NeuAc (total activity 50 nCi, 0.03 mM) and 0.4 mg asialofetuin in 0.1 M cacodylate buffer, pH 6.8. Under the same conditions two reaction mixtures, without asialofetuin or without cell lysate, were used as blanks. After incubation of the mixtures at 37°C for 1 h, the reaction was stopped by addition of 1% phosphotungstic acid (PTA) in 5% trichloroacetic acid (TCA) on ice and left overnight at 3°C. The precipitates were filtered through GF/C filters on 3 MM Whatman paper presoaked in PTA–TCA mixture and washed with 10 ml of this mixture. Ethanol–ether mixture (3 ml; 2 : 1) was used for the last washing. The pellets bound to GF/C filters were air-dried. Radioactivity was measured in the 1215 Rackbeta II liquid scintillation counter. ST activity was calculated as pmol NeuAc transferred to asialofetuin in 1 h by 1 mg protein of cell lysate.

Quantitative analysis of GM3. Lysates of monocytes ($(2\text{-}3)\cdot10^6$ cells) or macrophages ($(1\text{-}2)\cdot10^6$ cells) were twice extracted by chloroform–methanol mixture (2 : 1). The extracts were pooled and dried. The lipids were dis-



Changes in GM3 synthase and ganglioside GM3 amounts during transformation of human peripheral blood monocytes into macrophages. a) Cell lysates (20 μg protein) of monocytes (*2*) or monocyte-derived macrophages (*3*) were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with anti-GM3 synthase antibody. *1*) Protein markers stained by Ponceau S (on the left, their molecular masses). b) Gangliosides were isolated from cell lysates and separated by HPTLC using chloroform–methanol–0.25% $CaCl_2$ (50 : 40 : 10) solvent system. *1*) GM3 standard; *2*) monocytes (28 μg protein of initial lysate); *3*) macrophages (25 μg protein of initial lysate)

solved in chloroform–methanol mixture (2 : 1), so that the quantity of lipids in 20-25 μl solution that was applied onto HPTLC plates (Merck, Germany) corresponded to 25-90 μg protein of the extracted initial cell lysate, and then chromatographed in solvent system chloroform–methanol–0.25% $CaCl_2$ (55 : 45 : 10) using 0.5-1 μg authentic GM3 as a standard. The fractionated components were visualized with resorcinol reagent. GM3 quantitative analysis on chromatographs was performed using a Sorbfil densitometer (Russia) by computer program Sorbfil 1.0 (figure and Table 1). Ganglioside amount was expressed as μg GM3 per mg cell protein or per 10^6 cells.

Protein concentration was assayed by a modified Lowry procedure [11] using BSA as a standard.

Immunoblotting of GM3 synthase. Proteins (20 μg) from monocyte or macrophage lysates were resolved by electrophoresis on a 10% SDS-polyacrylamide gel according to the method of Laemmli [12] and then transferred to a nitrocellulose membrane. Nonspecific protein sorption was blocked in 5% low-fat milk on TBS-T buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.2% Tween-20) for 1 h. Membrane strips were washed in TBS-T buffer and incubated with anti-GM3 synthase antibody (R27C1 [9]) (dilution 1 : 100) in 1% low-fat milk on TBS-T buffer for 1 h. Then the strips were washed in TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit secondary antibody (dilution 1 : 10,000). Antibody binding was detected by nonradioactive ECL reagent (Amersham Pharmacia Biotech, England) according to the manufacturer's recommendations.

RESULTS

To determine whether transformation of monocytes into macrophages is associated with changes in the cellular level of ganglioside GM3 and GM3 synthase activity, monocytes were purified from peripheral blood of healthy donors and maintained in culture conditions that promoted differentiation into macrophages. GM3 amounts in monocytes ($CD14^+$, $CD206^-$) and macrophages ($CD14^+$, $CD206^+$) are shown in Table 1. It follows from the data obtained that the amount of ganglioside GM3 in macrophages was greater than in monocytes (approxi-

Table 1. Amount of GM3 in monocytes and monocyte-derived macrophages

Donor	Monocytes		Macrophages	
	μg GM3 per million cells	μg GM3 per mg protein	μg GM3 per million cells	μg GM3 per mg protein
1	0.43	6.0	2.7	26.7
2	0.3	6.3	2.6	25.6
3	n.d.	7.0	2.76	27.6
Mean ± S.D.	0.365 ± 0.09	6.4 ± 0.5	2.7 ± 0.08*	26.6 ± 1.0*

Note: n.d., not determined; S.D., standard deviation.
* $p < 0.001$ vs. monocytes.

Table 2. GM3 synthase activity in monocytes and monocyte-derived macrophages, pmol/h per mg protein

Donor	Monocytes			Macrophages		
	incorporation into endogenous substrate	total activity	incorporation into exogenous substrate*	incorporation into endogenous substrate	total activity	incorporation into exogenous substrate*
1	62	105	43	323	953	630
2	109	159	50	397	920	523
3	50	128	78	864	1404	540
Mean ± S.D.	73.7 ± 31.2	130.7 ± 27.0	57 ± 18.5**	528 ± 293**	1092 ± 271	564 ± 77.8**

Note: S.D., standard deviation.

* The values are derived by subtracting the incorporation into endogenous substrate from the total activity.

** $p < 0.001$ vs. monocytes.

mately 7-fold if calculated per cell or 4.1-fold if calculated per mg cell protein).

GM3 synthase activity determinations showed that in the absence of exogenously added LacCer, a significant incorporation of CMP[^{14}C]NeuAc into endogenous substrate (endogenous activity) to form [^{14}C]GM3 occurred both in monocytes and macrophages (Table 2). The level of endogenous activity varied markedly from donor to donor, but in macrophages it increased, on average, 7-fold in comparison to monocytes. In the presence of exogenously added LacCer, total incorporation of CMP[^{14}C]NeuAc into substrate of monocytes was 131 pmol, and this incorporation increased to 1091 pmol in macrophages. The difference between total and endogenous activity (GM3 synthase activity) was 57.1 and 563 pmol/h per mg protein in monocytes and macrophages, respectively. It should be noted that the exogenous activity did not vary from donor to donor. These data provide evidence that GM3 synthase activity in macrophages is enhanced markedly (almost 10-fold).

To assess if the revealed increase in GM3 synthase activity is specific, activities of some other sialyltransferases of Golgi multienzyme complex were determined [13]. Under conditions wherein a substantial increase in GM3 synthase activity was observed, no activity of GD3 synthase (the enzyme transferring CMP[^{14}C]NeuAc on ganglioside GM3) was detected either in monocytes or in macrophages. To estimate activity of sialyltransferases transferring CMP[^{14}C]NeuAc to a glycoprotein, asialofetuin, was chosen as an acceptor as it contains N- and O-glycosidic bonds in oligosaccharide chains and can be sialylated on positions 3 and 6 of terminal galactose [10]. Both monocytes and macrophages had similar asialofetuin-ST activity that was 750 pmol/h per mg protein.

To determine whether the increase in GM3 synthase activity in macrophages is associated with a corresponding increase in amount of the enzyme protein, cell proteins were resolved by SDS-PAGE and then analyzed by immunoblotting using polyclonal antibody specific to human GM3 synthase [9]. In monocytes, anti-GM3 synthase antibody revealed proteins with molecular masses of 52, 60, and 64 kD (figure). In macrophages, the amount of proteins with molecular masses of 60 and, especially, 64 kD markedly increased with appearance of additional highly stained bands. These data demonstrate that the absolute amount of proteins that were revealed by anti-GM3 synthase antibody increased as monocytes were cultured.

Thus, there is an increase in amounts of ganglioside GM3 and GM3 synthase as well as in activity of this enzyme in macrophages in comparison with monocytes.

DISCUSSION

We first have shown that ganglioside GM3 amount, GM3 synthase activity, and the amount of this enzyme were much greater in macrophages than in monocytes. The revealed changes in biosynthesis of ganglioside GM3 are specific as the activities of some other sialyltransferases were not altered under these conditions.

With the increase in GM3 synthase activity, the intensity of staining of protein bands that were revealed by anti-GM3 synthase antibody was much higher in macrophages than in monocytes. It is necessary to note that the antibody in both cell types revealed more than one protein band; especially many bands were observed for macrophages. We also observed multiple bands in HL60 cells differentiated into monocyte/macrophage phenotype in contrast to other human tissues (liver, aorta, brain) investigated by us [9]. Five GM3 synthase mRNA isoforms are known to be present in human tissues and cells [14-17]. Recently it has been shown that two GM3 synthase mRNA isoforms were differentially expressed during differentiation of HL60 cells into monocyte/macrophage type [14]. It is possible that multiple protein bands revealed by anti-GM3 synthase antibody are related to expression pattern of GM3 synthase mRNA iso-

forms in the certain tissue and to the stage of pathology development (cancer, atherosclerosis, inflammation) the tissue is in.

The increased ganglioside levels are found during cell differentiation in embryogenesis and cancer transformation [18-21]. In particular, an elevation of ganglioside GM3 levels has been detected during monocyte/macrophage differentiation in human myelocytic and monocytic leukemia cell lines (HL60, K562, KG-1, ML1, HO, and U937) after the treatment with phorbol esters [20]. In HL60 cells, monocyte/macrophage differentiation is accompanied by a 10-fold increase in activity of GM3 synthase [21]. Investigation of mechanisms for GM3 synthase activation in HL60 cells induced by phorbol ester PMA (phorbol 12-myristate 13-acetate) has shown that PMA produced cell differentiation through a PKC/ERK-dependent signal transduction pathway. Activation of PKC/ERK led to the phosphorylation of CREB, a transcription factor. In PMA-stimulated HL60 cells, this transcription factor regulated expression of GM3 synthase, inducing synthesis of ganglioside GM3. The increased ganglioside GM3 through activation of a PKC/ERK/CREB-dependent pathway by PMA and by treatment of HL60 cells with an exogenous ganglioside GM3 as well, induced expression of CD11b known as a monocyte/macrophage differentiation marker. Inhibitor of ganglioside synthesis (PDMP) decreased not only PMA-induced elevation of GM3 but also CD11b expression and cellular adherence. Based on these data, the authors concluded that ganglioside GM3, whose synthesis is enhanced through activation of the PKC/ERK/CREB-dependent pathway, is involved in differentiation of HL60 cells into monocyte/macrophage phenotype inducing expression of CD11b [22]. In this connection, the revealed increase in ganglioside GM3 biosynthesis in macrophages in contrast to monocytes suggests that ganglioside GM3 may be involved in differentiation of human peripheral blood monocytes into macrophages.

The recruitment of peripheral blood monocytes into the intima of main arteries is observed in many animals and in man and considerably increased in atherosclerosis as well as in experimental models of hypercholesterolemia. As this takes place, monocytes become tissue macrophages that beyond a doubt are precursors of foam cells formed in vascular wall in atherosclerosis [23]. Hence, cell differentiation into macrophage phenotype takes place in the process of atherosclerotic plaque formation.

Earlier we showed that human aortic intima isolated from atherosclerotic lesions contains gangliosides in amounts 5 times greater than intima from non-diseased vascular areas [24]. The cells that were isolated from atherosclerotic plaques contained 2 times more ganglioside GM3 than cells of non-diseased intima [25, 26].

In this study, we aimed to test our hypothesis that just monocyte/macrophage differentiation is responsible for the increased levels of ganglioside GM3 in human atherosclerotic lesions [27]. As monocytes and monocyte-derived macrophages are the most adequate models of cellular elements that form human atherosclerotic lesions *in vivo*, the presented data can be extrapolated with certain probability to the situation that is in the wall of main human arteries during development of atherosclerotic lesions. Thus, we have shown that the increase in ganglioside GM3 and activation of GM3 synthase in macrophages derived *in vitro* from human peripheral blood monocytes may be a mechanism of enhanced levels of this ganglioside in atherosclerotic lesions.

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