Incorporation and localisation of ganglioside GM3 in human intimal atherosclerotic lesions

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Abstract

Immunohistochemical examination showed that sections of intimal atherosclerotic plaques contained cells and cell clusters as well as areas of extracellular matrix specifically stained with antibodies against ganglioside GM3. No immunohistochemical staining was observed in areas bordering the plaques where there was no histological evidence of atherosclerosis. To determine whether the ganglioside GM3 deposits in the intimal plaques derived directly from plasma or were synthesised by intimal cells, intimal plaque and plasma LDL were assayed for ganglioside GM3 fatty acid composition. This assay showed that more than 50% of the fatty acids of GM3 isolated from both atherosclerotic and normal intima are either minor fatty acids or those absent from LDL GM3. We conclude that the GM3 deposits present in intimal plaque arise in intimal cells and do not derive from plasma LDL. © 1997 Elsevier Science B.V.

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1. Introduction

A major feature of atherosclerosis is an increase in the lipid content of the arterial wall. Most pronounced is an increased cholesterol concentration, especially cholesteryl esters, but there are also changes in the concentration and composition of all lipid classes. Our previous findings [1,2] as well as data from other investigators [3–7] demonstrated a significant increase in the content of gangliosides and changes in their composition when atherosclerotic lesions of human aortic intima were compared with normal intima.

How the main lipid classes accumulate and localise in atherosclerotic lesions has been extensively investigated [8,9]. In contrast, the accumulation of gangliosides in atherosclerotic lesions has been little studied and their origins are unclear. In particular, it is unknown whether gangliosides in plaques are derived from plasma or whether they form through metabolic processes in the intima.

In the present study we used polyclonal antibodies raised against ganglioside GM3 to investigate ganglioside accumulation in tissue sections prepared from human arterial atherosclerotic lesions and normal intima. To determine whether ganglioside GM3 in atherosclerotic plaques derived directly from the plasma or were synthesised in situ, both athero-

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sclerotic plaques and unaffected intimal areas were assayed for their ganglioside fatty acid compositions. These assays were compared to analogous compositions of LDL taken from healthy donors and patients with hypercholesterolaemia.

2. Materials and methods

The present investigation conformed with the principles outlined in the Declaration of Helsinki [10].

2.1. Tissues

For ganglioside fatty acid analysis, thoracic aortas from 40–60 year old men and women who had died of myocardial infarction were obtained at autopsy within 3 h of sudden death. The atherosclerotic lesions (mainly plaques) occupied about half the total inner surface of the aortas. The intima was mechanically separated from the media along the secondary internal limiting membrane as described earlier [11]. Grossly normal areas (three specimens from each aorta, 1–1.5 g wet tissue) and uncomplicated plaques (four specimens from each aorta, 1–2 g wet tissue) were excised.

For immunohistochemistry, arterial segments from 9 aortas and 14 carotid arteries were obtained from patients whose ages ranged from 38 to 69 years. The aortic specimens were obtained during aortic reconstruction and the carotid artery specimens by endarterectomy. These arterial segments included atherosclerotic lesions with adjacent macroscopically normal areas. Arterial samples were embedded in an OCT compound, rapidly frozen in liquid nitrogen and stored at $-70°C$ until cryostat sectioning.

2.2. Low density lipoproteins

Human LDL ($d = 1.019–1.063$ g/ml) were obtained by sequential ultracentrifugation as described earlier [12] from the fresh plasma of normal volunteers (average serum cholesterol, 2.2 mg/ml) and the plasma of patients with hypercholesterolaemia (average serum cholesterol, 4.2 mg/ml) which were taken during plasmapheresis.

2.3. Extraction and analysis of lipids

The aortic tissues were homogenised and extracted with chloroform/methanol [13]. Lipid extraction of LDL and the quantitative determination of lipid phosphorous and total cholesterol were carried out as described earlier [14,15].

GM3 was isolated by alkaline treatment of lipid extracts, dialysis and chromatography on DEAE-Sephadex A-25 (Pharmacia, Sweden) with TLC monitoring as described earlier [1]. Quantitative determination of the total gangliosides was carried out according to the Svennerholm method [16].

Fatty acid methyl esters were prepared by acid methanolysis of GM3 followed by extraction with hexane and separation by gas chromatography using a methyl silicone capillary column (12 m $\times$ 0.2 mm $\times$ 0.33 μm) [1]. The initial oven temperature was maintained at 150°C for 1 min and then increased at a rate of 10°C/min until 220°C was reached. Gas chromatography/mass spectrometry analysis was performed on a Hewlett-Packard 5890 gas chromatograph with HP5970B mass-selective detector. Methyl esters of margarinic acid were used as an internal standard.

The results were expressed as means $\pm$ SE. Statistical evaluation of the data was performed by the Student's t-test with a value of $p < 0.05$ being considered significant.

2.4. Characterisation of anti-GM3 antibodies

Anti-GM3 serum was obtained in our laboratory by 2–5 immunisations of rabbits with human liver GM3-methylated BSA conjugate. Fatty acid composition of human liver GM3 was: 16:0–10%, 18:0–6%, 18:1–1%, 20:0–5%, 22:0–32%, 22:1–3%, 23:0–9%, 24:0–19% and 24:1–15% [15].

An immunoglobulin fraction with GM3-binding activity was isolated by affinity chromatography on an octyl-Sepharose CL 4B (Pharmacia, Sweden) coated with human liver GM3. The column was first washed with 0.01 M PBS, pH 7.4 and 1 M NaJ in PBS. The anti-GM3 antibodies were eluted with 3 M NaSCN in PBS [17].

The activity and specificity of anti-GM3 immunoglobulins were tested by ELISA as described earlier [18]. The antigen specificity of anti-GM3 was studied using GM1, GD1a, GD1b and GT1b gangliosides from bovine or human brain prepared in our laboratory. The amount of each ganglioside in the microtiter well was 1 nmol. Concentrations of anti-GM3

antibodies and control rabbit γ-globulins (Sigma) were 2.5 and 5.0 μg/ml. The binding of anti-GM3 antibodies to GM3 from human liver and aorta was about threefold higher than with other tested gangliosides. Rabbit γ-globulins (Sigma) used as a negative control showed low binding levels to GM3 and other gangliosides studied.

2.5. Immunohistochemistry

Sections of arterial wall were cut at 5–7 μm thickness, air dried for 45 min and then fixed in acetone for 10 min. After elimination of endogenous peroxidase activity by 1% hydrogen peroxide for 5 min, the sections were tested by the ABC method [19] as previously described [20]. In brief, the sections were incubated for 60 min with anti-GM3-antibodies (100 μg/ml). After washing in Tris-phosphate buffered saline (TPBS, pH 7.4, 10 min) the sections were incubated for 20 min with a biotin-labeled secondary antibody (goat anti-rabbit-VEC-TOR BA-1000). The sections were then washed in PBS for 5 min and the avidin–biotin complex (ELITE-ABC, VECTOR PK61000) was added for 30 min. After washing for 10 min in TPBS, brown staining was produced by a 5 min treatment with 3,3′-diaminobenzidine (DAB). All the incubations were completed at room temperature. For negative controls, the first antibody was omitted or the sections were treated with an immunoglobulin fraction of non-immune goat serum (VECTOR S-1000) as a substitute for the primary antibody. None of the negative control sections showed positive immune staining. Counterstaining was performed with Mayer's haematoxylin.

3. Results

3.1. Ganglioside localisation in intimal atherosclerotic plaques

Immunohistochemical examination clearly demonstrated that sections of intimal atherosclerotic plaques contained cells and cell clusters as well as areas of extracellular matrix specifically stained with antibodies against ganglioside GM3. Staining with anti-GM3 antibodies was found in atherosclerotic plaques from both aortas (Fig. 1a–c) and carotid arteries (Fig. 1d). Intimal areas without any histological signs of atherosclerosis which were adjacent to the lesions did not stain with the antibodies (Fig. 1d). Binding of anti-GM3 antibodies within the atherosclerotic plaques was irregular with areas manifesting intense staining bordering on areas without staining. The intensity of anti-GM3 antibody binding with the cells varied and was usually stronger in cells which formed clusters than in single cells (Fig. 1a). Antibody-positive cell clusters were observed more often than antibody-positive single cells, the majority of which looked like foam cells (Fig. 1b). It was apparent that not only cells, but the extracellular matrix also was specifically stained with anti-GM3 antibodies (Fig. 1c and d). The extracellular matrix was usually diffusely stained. Diffuse extracellular binding of GM3-antibody was detected in all atherosclerotic plaques but large extracellular GM3 positive deposits with a diameter no less than 50 μm were also found in atherosclerotic plaques in one aorta (Fig. 1c) and in five carotid arteries (Fig. 1d). The matrix areas binding anti-GM3 antibodies often surrounded cholesterol crystals (Fig. 1b).

3.2. Lipids of aortic intima and LDL

To characterise the intima and plasma specimens we assayed their lipid composition. The content of cholesterol, phospholipid and ganglioside concentrations in intimal atherosclerotic lesions amounted to 28.9 ± 7.01, 8.55 ± 1.2 and 0.161 ± 0.03 μmol/g wet tissue, respectively. The content of these components in unaffected intima were 12.7 ± 3.0, 5.7 ± 0.9 and 0.046 ± 0.01 μmol/g wet tissue, respectively. This data is similar to that of other investigators [3,4,8,9].

The content of gangliosides in plasma LDL of hypercholesterolaemic patients and healthy donors did not differ significantly (4.1 and 4.2 nmol/mg of protein, respectively) which is similar to the findings of others [14].

3.3. GM3 fatty acid composition

To determine whether ganglioside GM3 deposits in intimal plaques originate from plasma or are synthesised by plaque cells in situ, we assayed athero-
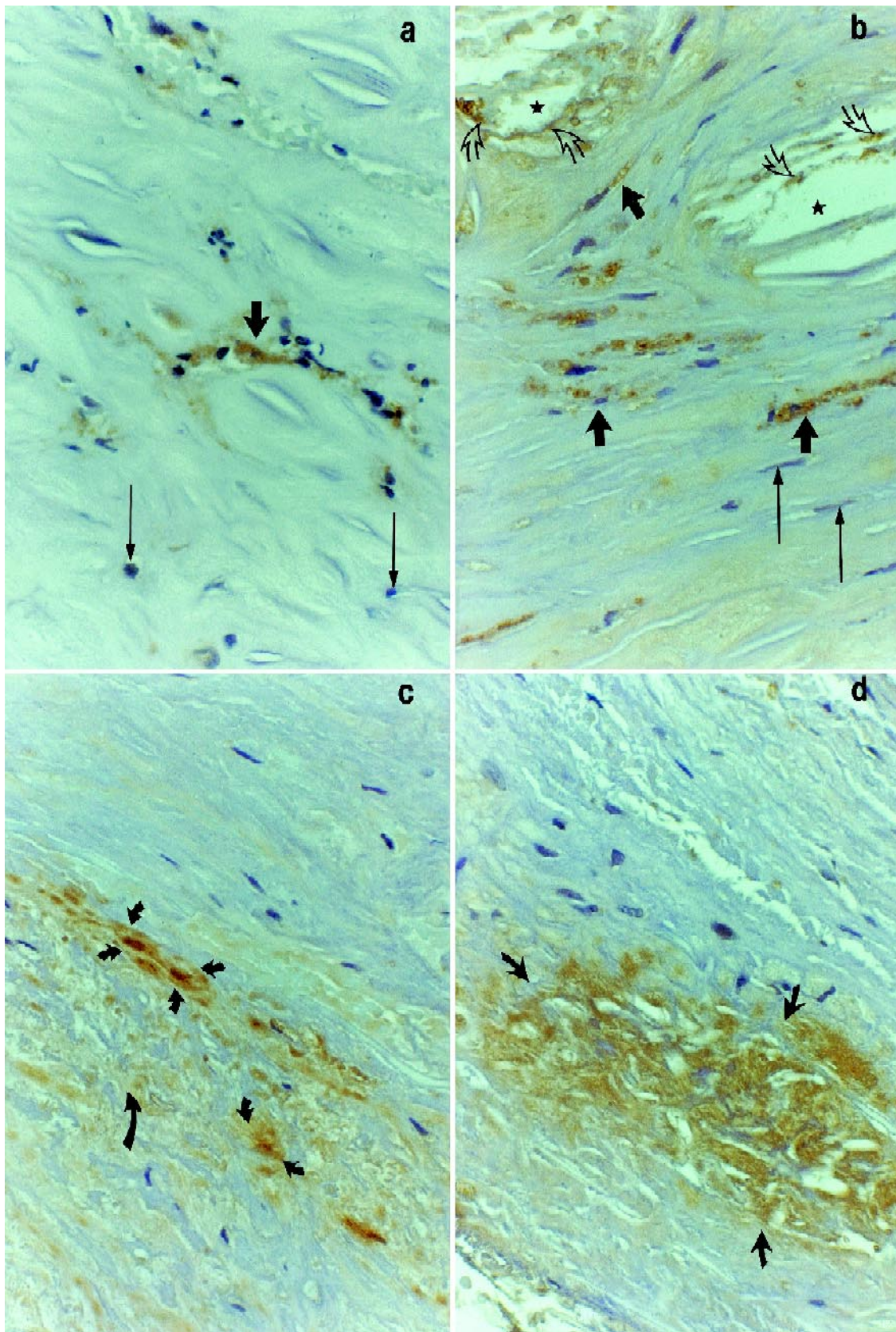
Table 1

Fatty acid composition of GM3 ganglioside from normal and atherosclerotic aorta intima and LDL of healthy donors and patients with hypercholesterolaemia (mean $\pm$ SE)

Fatty acids	LDL		Aortic intima	
	patient ($n = 3$)	donor ($n = 2$)	lesions ($n = 4$)	unaffected areas ($n = 3$)
14:0	4 ± 2	1 ± 0.4	tr.	1 ± 0.2
16:0	39 ± 9	40 ± 2	14 ± 1	9 ± 1
18:2	10 ± 3	6 ± 1	5 ± 1	7 ± 1
18:1	20 ± 3	28 ± 4	5 ± 1	5 ± 1
18:0	19 ± 5	18 ± 4	8 ± 2	7 ± 2
20:0	1 ± 0.3	1 ± 0.2	1 ± 0.3	3 ± 0.5
22:0	1 ± 0.5	0.5 ± 0.1	11 ± 2	15 ± 2
23:0	2 ± 0.4	0.5 ± 0.05	8 ± 1	7 ± 1
24:0	—	—	20 ± 5	17 ± 4
24:1	—	—	23 ± 8	16 ± 4
Other	4 ± 1	5 ± 2	5 ± 1	13 ± 3

sclerotic intimal samples and plasma LDL for their GM3 fatty acid composition. There were no differences in the fatty acid compositions of GM3 in plasma LDL when the normal and hypercholesterolaemic groups were compared ($p < 0.001$). In GM3 from the intimal plaques, the contents of palmitic acid (16:0) and 24:1 acids were approximately 1.5-fold higher than in GM3 from the uninvolved areas while the content of 22:0 acid was lower (Table 1).

There were, however, considerable differences in the fatty acid composition of GM3 from the intima and from LDL. Palmitic acid was the most common fatty acid found in LDL GM3 while the actual content of this acid was relatively low in intimal gangliosides. Stearic (18:0), oleic (18:1) and linoleic (18:2) acids accounted for 50% of GM3 fatty acids in LDL. In the intima, these made up about 20% of GM3 fatty acids while C22–24 acids (saturated and monoenic) were the highest, constituting 62 and 55% of fatty acids in GM3 from plaques and uninvolved areas, respectively. In LDL GM3, these long chain fatty acids were only minor components or were practically absent (Table 1).

4. Discussion

Considerable attention has been directed to how human vascular wall gangliosides accumulate in atherosclerotic lesions. The content and composition of these functionally important components of cells and the extracellular matrix has been studied by chemical and immunological methods [1–7] but the origin of ganglioside deposits and where they localise in atherosclerotic lesions remain unclear. The present study shows that the ganglioside GM3 accumulation in intimal atherosclerotic plaques is both intracellular and extracellular.

Atherosclerotic plaques represent areas of lipid storage usually associated with elevated plasma LDL and increased influx of lipids into the arterial wall. GM3 is the major ganglioside in all extraneural tissues including the intima. GM3 comprises 66% of gangliosides in normal intima, 93% of gangliosides in atherosclerotic intima [1,2] and 45% of gangliosides in human plasma LDL [21]. Whether GM3 accumulates in plaques because of a significantly higher influx of LDL in atherosclerotic intima [22–24] or because of changes in local ganglioside metabolism and trafficking in atherosclerotic intimal cells, is not known.

The data of Kundu et al. [25] on human plasma gangliosides and our findings concerning the structure of gangliosides in the human aortic intima [1], highlight considerable differences between the fatty acid composition of plasma and intimal GM3. We hypothesised that a comparison of the fatty acid composition of GM3 isolated from normal intimal areas and atherosclerotic plaques with that of GM3 from plasma LDL of both healthy subjects and hypercholesterolaemic patients would help elucidate the origin of GM3 deposits in atherosclerotic intima. Our study showed no differences in the fatty acid composition of LDL GM3 from donors and patients, which agrees with the findings of Kundu et al. [25]. The higher palmitic acid content of GM3 isolated from

Fig. 1. GM3-positive staining of atherosclerotic intima in the aorta (a–c) and carotid artery (d). (a) Large arrows show GM3-positive cells. Small arrows show GM3-negative cells in atherosclerotic intima. (b) Large arrows show GM3-positive cells which appear to be foam cells. Outlined arrows indicate the association of GM3 with cholesterol clefts which are marked by stars. (c) Focal and diffuse locations of specific deposits of GM3-positive in the extracellular matrix are shown by small and large arrows, respectively. (d) Focal locations of specific deposits of GM3-positive in the extracellular matrix are shown by arrows. ABC immunoperoxidase technique, counterstaining with Mayer's haematoxylin. Original magnification: $\times 400$.

atherosclerotic plaques (in comparison with normal intima) may be due to influx from the plasma. However, most fatty acids ($>50\%$) of GM3 in atherosclerotic and normal areas of intima represented C22–24 acids which are virtually absent from LDL GM3. These differences imply that GM3 deposits in atherosclerotic intima are of local cell origin. This conclusion is supported by the fact that in contrast to other lipids (phospholipids and cholesteryl esters), there is no exchange of fatty acid in the ceramide moiety of gangliosides. No enzymes capable of cleaving fatty acid from whole ganglioside molecules have been identified. Some cell types are said to contain very small amounts of lysogangliosides (0.1% of the total ganglioside content) [26].

Nevertheless, influx of LDL may be involved in the accumulation of GM3 in the intima. Chatterjee et al. [27] showed that in proximal tubular cells from LDL receptor-negative subjects, LDL stimulated the synthesis of lactosylceramide, a precursor of GM3 and the effects of LDL were not related to their glycosphingolipid content. Metabolic studies with exogenous gangliosides labeled by sphingosine also showed that ceramide can be utilised by cells for the synthesis of their own gangliosides [28].

Our immunohistochemical findings show that intimal atherosclerotic plaques contain cells intensively reacting with anti-GM3 antibodies as well as antibody-negative cells. In the areas bordering atherosclerotic plaques without histological signs of atherosclerosis, the intimal cells did not react with anti-GM3 antibodies. Three possibilities might explain this phenomenon. First, the GM3 content might be higher in immunoreactive cells than in immunonegative cells. Second, GM3 at the surface of the immunoreactive cells might be more highly exposed and its organisation in the membrane might be unique for immunoreactive cells compared with GM3 in immunonegative cells. Third, the immunoreactive cells might belong to a cell type absent from uninvolved areas.

The first explanation is supported by our previous finding that the GM3 content is higher in cells from atherosclerotic lesions cells than in cells from normal areas [2]. The second possibility is consistent with the data of other authors [29–31]: GM3 is found in virtually all types of cells and tissues but only those cells with a relatively high content of GM3 show positive immunoreactivity with anti-GM3 antibodies.

Furthermore, since there is a threshold concentration of GM3 exposed at the cell surface, a high concentration of GM3 in the plasma membrane may induce a novel conformational change that is recognised by anti-gangliosides. The third possibility cannot be excluded but we did not determine the type of GM3-positive cells in this study. Judging from their lipid inclusions, some of the GM3-positive cells appeared to be foam cells but their origin was not established.

Comparing the fatty acid compositions of GM3 from atherosclerotic intima with that from LDL suggests that the extracellular matrix-bound gangliosides are also of local cell origin. Significant amounts of gangliosides are contained in the substrate-attached material of cultured cells [32,33] as well as in the culture medium [34]. The appearance of gangliosides in the extracellular space might relate to plasma membrane shedding, a process which can be characterised as exfoliation of whole portions of the plasma membrane into the extracellular environment. This fragmentation is likely to affect the most rigid domains of the plasma membrane. Apical membranous fragments have the highest cholesterol content and thus are highest in lipid microviscosity which makes these fragments more prone to shedding than other areas of the membrane [35]. GM3 and higher gangliosides are localised in the outer surface of the plasma membrane and often occupy the apical areas. Cells from atherosclerotic intima contain an increased cholesterol:phospholipid ratio in comparison to normal cells which makes their membranes even more rigid [8]. Shedding might be a way of removing rigid lipids from the cell surface and this process might occur through the formation of microvesicles from the plasma membrane. Exfoliation of the apical membranous fragments containing gangliosides is thought to occur in many different normal and neoplastic cells [35–37]. Li et al. [38] related shedding to cellular ganglioside content and showed that highly expressed gangliosides of neuroblastoma tumor cells are shed at a rapid rate.

Shedding of microvesicles might be important in some physiological and pathophysiological processes [37,38]. In atherosclerosis, for example, shedding of vesicles is thought to be involved in the mineralisation processes [39]. Tanimura et al. [39] showed that a major mechanism in the development of atherosclerotic calcification is the deposition of calcium

phosphate within matrix vesicles which appear to derive from intimal smooth muscle cells by budding from their cytoplasmic membranes.

In mammalian cells, newly synthesised gangliosides translocate from the Golgi complex to the plasma membrane while membrane gangliosides flow in the opposite direction from the plasma membrane to intracellular organelles [40]. Shedding membranous fragments thus excludes some portions of cell gangliosides from the turnover pathway. Influx of cholesterol into the vascular wall might intensify this process and facilitate the formation of ganglioside deposits in atherosclerotic lesions. The specific staining of intimal material around cholesterol crystals found in the present study can thus be associated with the destruction of some cells in the intima which in turn is followed by the release of cellular components into the extracellular space [41].

The processes leading to the formation of atherosclerotic lesions in the intima, such as influx of lipid components from plasma, cholesterol incorporation, changing of cell phenotype and cell destruction, can all promote the accumulation of GM3 in atherosclerotic lesions [42]. Shedding GM3 with the membranous fragments, itself depending on an increased cholesterol content of intimal cells, may influence the change in cell phenotype [43], induce cell proliferation [44] and production of antiganglioside antibodies [18]. Impregnation of the extracellular matrix with gangliosides might initiate adhesion and chemotaxis of lymphoid cells [45], binding of Ca^{2+} [46,47], growth factors [44] and a number of other processes promoting the formation of advanced atherosclerotic plaques.

Acknowledgements

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