

Analysis of Serum Sphingomyelin Species by Uflc-Ms/Ms in Patients Affected with Monoclonal Gammopathy

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Abstract

Cancer cells are hungry of cholesterol incorporated from serum with avidity and used to favour the expressions of proteins involved in cell proliferation such as RNA polymerase II, STAT3, PKC α and cyclin D1. Numerous studies have shown that exists a strong interaction between unesterified cholesterol and saturated fatty acid sphingomyelin which arises from the Van der Waals interaction. Since sphingomyelin and cholesterol association is responsible for the formation of membrane lipid raft involved in cell signalling, we studied the possible hyposphingomyelinemia associated to hypocholesterolemia in the patients with cancer. The blood of 23 patients with monoclonal gammopathy were analyzed for cholesterol, 12:0 sphingomyelin, 16:0 sphingomyelin and 18:1 sphingomyelin content. The results demonstrated that only the patients with very low level of cholesterol (65-99 mg/dl) had low amount of sphingomyelin and, in particular, of saturated sphingomyelin specie (16:0 sphingomyelin). The possibility that the hypocholesterolemia in cancer was secondary to hyposphingomyelinemia was discussed.

Keywords: Cholesterol; Sphingomyelin; MS/MS-UFLC; Blood lipids; monoclonal gammopathy

Introduction

Lipids are structural and functional molecules localized in cell membranes and in the nucleus where interact with active chromatin and play specific roles in cell proliferation, differentiation, apoptosis and cancer [1]. Hypocholesterolemia is common in patients with various malignant diseases since cancer cells are hungry of cholesterol (CHO) [2]. In fact, CHO is incorporated in cancer cells from serum with avidity and used to favour the expressions of proteins involved in cell proliferation such as RNA polymerase II, STAT3, PKC α and cyclin D1 [2]. In cells, CHO is not randomly distributed over the membranes but forms lipid microdomains thank to its link with saturated fatty acid of sphingomyelin (SM) by Van der Waals interaction [3]. The lipid microdomains, also known as lipid rafts, are platforms for specific proteins involved in cell signaling if they are localized in cell membrane and are platforms for DNA duplication and transcription if they are associated to the inner nuclear membrane [4,5]. It has been demonstrated that lipid rafts play important mediating roles in cell migration, metastasis, cell survival and tumor progression [6]. In particular CHO has an important role in regulating the synthesis of *invadopodia*: protrusion in the cell membrane of some cells, displaying properties of SM and CHO-enriched membranes or lipid rafts frequently seen in invasive and metastatic cancer cells that invade surrounding tissues [7]. It remains unclear how the rafts are formed in cancer cells, i.e., if using lipids that incorporate from the blood, reducing their physiological values. There is evidence that low levels of serum CHO-phospholipids are associated with the antiphospholipid antibodies in monoclonal gammopathy [8]. However, while the hypocholesterolemia is widely studied in cancer nothing is known about the possible change in the content of SM in the blood of cancer patients. Thus, we aimed to study the possible association of hypocholesterolemia with hyposphingomyelinemia in the patients with monoclonal gammopathy (MG). We analysed the main species of SM by SM/SM-UFLC in order to highlight the changes of saturated fatty acid SMs that interact specifically with CHO.

Materials and Methods

Patients

Blood samples from patients affected by monoclonal gammopathy (MG), with diagnosis from the "Laboratorio Centralizzato di analisi chimico-cliniche Ospedale Silvestrini, Perugia", were collected over a 24 month period. Young and without poor nutrition state or extremely dietary control patients were chosen as experimental model. From all patients (62), only those with a low level of total CHO (23 patients) were analyzed. The population was composed of 13 males and 10 females, average age was 36 yrs. The control group was composed of 20 healthy blood donors: 12 males and 8 females, average age 39 yrs, with normal serum CHO levels.

Lipid extraction

Lipid extraction was performed according to Matyash et al. [9]. 1 mL of serum was diluted with 1 mL methanol. 3 mL ultra pure water and 3 mL methyl tert -butyl ether (MTBE) were added. Each Sample was vortexed for 1 min and centrifuged at 3000 g for 5 min. The supernatant was recovered. The extraction with MTBE was repeated on the pellet and the supernatant was added to the first. The organic phase was dried under nitrogen flow and resuspended in 500 μ L of methanol.

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| TOTAL CHOLESTEROL | | |
|-------------------|---------|-------|
| 160-220 | 100-159 | 65-99 |
| C | | P 1 |
| | P 2 | |
| | P 3 | |
| | P 4 | |
| | P 5 | |
| | P 6 | |
| | | P 7 |
| | P 8 | |
| | | P 9 |
| | P10 | |
| | | P11 |
| | P12 | |
| | P13 | |
| | P14 | |
| | P15 | |
| | P16 | |
| | P17 | |
| | P18 | |
| | | P19 |
| | P20 | |
| | P21 | |
| | P22 | |
| | P23 | |

Table 1: Content of cholesterol in control and patients affected with monoclonal gammopathy.

Ultra-Fast Liquid chromatography tandem mass spectrometry (UFLC- MS/MS)

Lipid standards SM 18:1 12:0, SM 18:1 16:0 and SM 18:1 SM 18:1 were prepared according to Matyash et al. [9]. Standards were dissolved in chloroform/methanol (9:1 v/v) at 10 µg/mL final concentration. The stock solutions were stored at -20°C. Working calibrators were prepared by diluting stock solutions with methanol to 500:1, 250:1, 100:1, 50:1 ng/ml final concentrations. 20 µL of standards or lipids extracted from serum were injected after purification with specific nylon filters (0.2 µm)

Analyses were carried out according to [10] by using Ultra Performance Liquid Chromatography system tandem Mass Spectrometer Applai biosistem (Shimadzu Italy s.r.l., Italy). The lipid species were separated, identified and analysed by following the methods of Rabagny et al. [10]

Statistical analysis

Data are expressed as mean ± S.D. of three analysis and *t* test was used for statistical analysis. **p*< 0.001.

Results and Discussion

MG patients with low level of CHO in serum were chosen as experimental model. The comparison of the data was performed with the serum of healthy blood donors with normal level of CHO. Of 23 GM patients, 18 (78%) were found to have low level of CHO (100-159 mg/dl) and 5 (22%) very low level of CHO (65-99 mg/dl), exactly the patients number 1,7,9,11,19 (Table 1).

The analysis of SM was performed by UFLC-MS/MS. Chromatograms displaying mass transitions for SM showed more than one peak. The correct peak of the main species under study were identified by using available standards (SM 12:0, SM 16:0 and SM:18:1). Since CHO is linked by saturated SM species [3], the species under

study were chosen to the aim to highlight the behavior of saturated and unsaturated fatty acids. Among saturated fatty acid SM species, were chosen one present in the cells in low concentration (SM:12:0) and another present in high concentration (SM:16:0). The analysis of total concentration of SM species showed that of the 18 patients with low level of CHO, none had low level of SM and three patients (number 4, 5, and 21) had values higher than control samples (Figure 1). Differently of the 5 patients (number 1,7,9,11 and 19) with very low level of CHO, 100% had values of SM lower than control patients (Figure 1). The value of 12:0 SM, 16:0 SM and 18:1 SM in serum of donor controls were 14.63 ± 6.54, 2975.00 ± 238.60 and 402.04 ± 124.25, respectively. Since the content of 12:0 SM was 203 times lower than that of 16:0 SM, its value has no bearing on the behavior of the total SM. The analysis

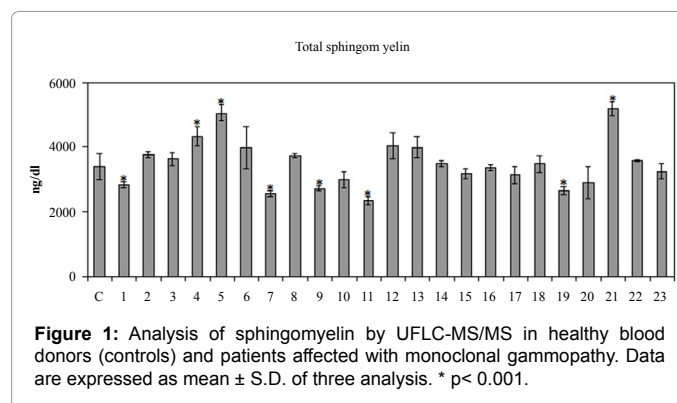


Figure 1: Analysis of sphingomyelin by UFLC-MS/MS in healthy blood donors (controls) and patients affected with monoclonal gammopathy. Data are expressed as mean ± S.D. of three analysis. * *p*< 0.001.

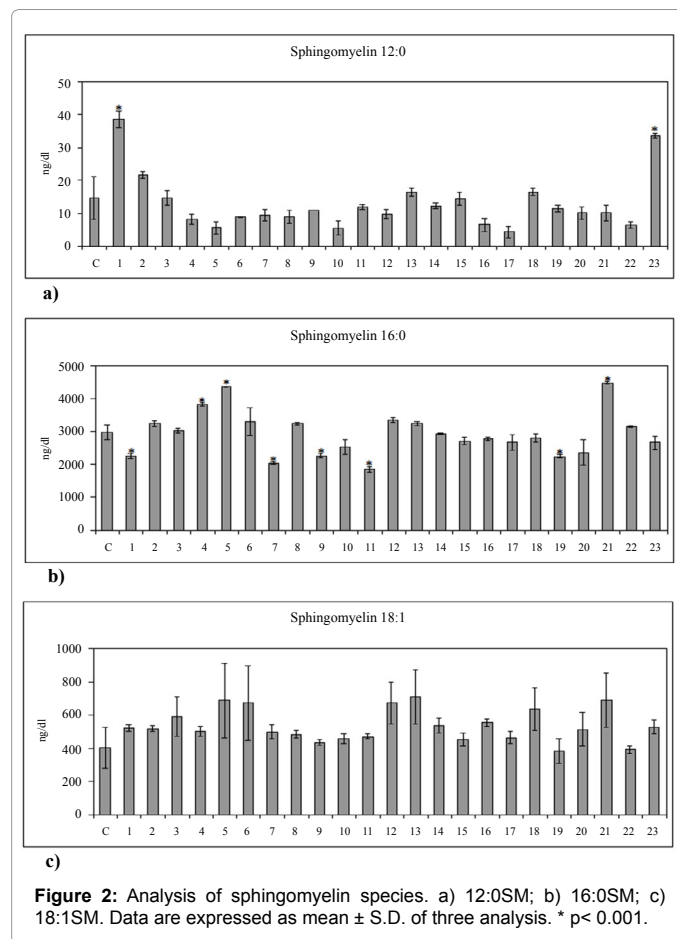


Figure 2: Analysis of sphingomyelin species. a) 12:0SM; b) 16:0SM; c) 18:1SM. Data are expressed as mean ± S.D. of three analysis. * *p*< 0.001.

of 12:0 SM in GM patients showed that none sample had highly significant difference in comparison with controls with the exception of number 1 and 23 whose values increased 2.63 and 2.30 times, respectively (Figure 2a). The results of the 16:0 SM analysis showed a behavior similar to that obtained with the analysis of total SM, lower values in 1,7,9,11, and 19 and higher values in 4, 5, and 21 patients in comparison with donor controls (Figure 2b). No change was reported for 18:1 SM (Figure 2c). The increase in patients 1 and 23 of 12:0 SM was difficult to interpret at the time. It's really important to note that there was no change in the unsaturated SM (18:1), while the saturated and strongly represented SM (16:0) decreased significantly only in the samples with very low CHO content. Since CHO is bound by saturated SM [3], it is possible to hypothesize that in patients with cancer CHO follows the fate of saturated SM. It is well known that in patients with cancer hypocholesterolemia is due to a strong incorporation of CHO from the tumor cell [2]; our data suggest the possibility that the hypocholesterolemia is secondary to hyposphingomyelinemia (5/23 total patients have hypocholesterolemia and hyposphingomyelinemia). The idea of screening and following patients with malignancy by CHO and SM blood based tests is appealing from several points of view as its ease, economic advantage, non invasiveness, and possibility of repeated sampling.

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