TÜBİTAK

ORIGINAL ARTICLE

Turk J Med Sci
2008; 38 (4): 293-299
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E-mail: medsci@tubitak.gov.tr

The Relationship between Carnitine Levels and Lipid Peroxidation in Glial Brain Tumors

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Received: November 30, 2007
Accepted: May 05, 2008

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Aim: It is assumed that reactive oxygen species and lipid peroxidation cause cell membrane damage and play a role in oncogenesis. Carnitine and acyl esters, which play a role in intracellular short-, medium-, and long-chain fatty acid metabolism, are one of the defense mechanisms against free radical toxicity. It is thought that the protective effects of carnitine are related with its role in lipid metabolism. In our study, we analyzed the relation between malondialdehyde (MDA) and free carnitine, and C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 carnitine levels in glial tumors [glioblastoma multiforme (GBM) (n = 29), high-grade astrocytoma (n = 8) and low-grade astrocytoma (n = 8)] to determine whether there is a relation between carnitine-acyl carnitines and lipid peroxidation in carcinogenesis.

Materials and Methods: The present study examined the free carnitine and C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 levels of glial tumors in tandem mass spectrometry. We measured MDA levels using HPLC system.

Results: There was a significant correlation between MDA and C20:4 carnitine levels in GBM. C20:4 carnitine levels increased with increasing MDA levels ($p = 0.000$, $r = 0.916$), but no significant correlation was found in the other groups.

Conclusions: In conclusion, measurement of only carnitine-MDA relation did not reflect any significant correlation. Detailed studies, including measurement of parameters showing antioxidant status and tumor cellular metabolism, are necessary.

Key Words: Carnitine, lipid peroxidation, tandem mass spectrometry, HPLC

Glial Beyin Tümörlerinde Karnitin Düzeyleri ve Lipid Peroksidasyonu Arasındaki İlişki

Amaç: Serbest oksijen radikallerinin ve lipid peroksidasyonunun hücre zarı hasarına neden olduğu ve onkogenezde rol oynadığı düşünülmektedir. Hücre içi kısa, orta ve uzun zincirli yağ asidi metabolizmasında rol oynayan karnitin ve açil esterleri, serbest radikal toksisitesine karşı savunma mekanizmalarından birisidir. Karnitinin koruyucu etkilerinin lipid metabolizması üzerindeki rolüyle ilişkili olduğu düşünülmektedir. Çalışmamızda, karsinogenezisde karnitin-açilkarnitinlerin lipid peroksidasyonuyla arasında bir ilişki olup olmadığını açıklamak için glial tümörlerdeki (glioblastoma multiforme (GBM) n = 29, yüksek grade astrositoma n = 8 and düşük grade astrositoma n = 8) MDA ve serbest karnitin, C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 karnitin düzeyleri arasındaki ilişkiyi inceledik.

Yöntem ve Gereç: Glial tümörlerdeki serbest karnitin ve C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 karnitin düzeyleri ardışık kütle spektrometrisinde çalışıldı. MDA düzeyleri ise HPLC sistemi kullanılarak ölçüldü.

Bulgular: GBM' de MDA ve C20:4 karnitin düzeyleri arasında anlamlı bir korelasyon bulundu. MDA düzeyleri artarken C20:4 karnitin düzeylerinin de arttığı görüldü ($p = 0.000$, $r = 0.916$). Fakat diğer gruplar arasında anlamlı bir korelasyon görülmedi.

Sonuç: Sonuç olarak sadece MDA-karnitin ilişkisinin ölçümü anlamlı bir korelasyon yansıtmamaktadır. Antioksidan yapılar ve tümör hücre metabolizmasını gösteren parametreleri de içeren daha kapsamlı çalışmalarla konunun incelenmesi gerekmektedir.

Anahtar Sözcükler: Karnitin, lipid peroksidasyonu, ardışık kütle spektrometrisi, HPLC

Introduction

Reactive oxygen species (ROS) are known to be implicated in signaling pathways regulating cell growth (1) and cell status redox control. However, when produced in excess, ROS exert detrimental effects that are able to damage cell macromolecules, such as DNA, lipids or proteins (2). Among these targets, lipid peroxidation is particularly more damaging because it facilitates propagation of free radical reactions. It is assumed that ROS and lipid peroxidation cause cell membrane damage and play a role in oncogenesis. ROS may act in several steps in carcinogenesis. It has been shown that the oxidative mechanism may have a potential role in the initiation, progression and malignant transformation of carcinogenesis (3,4). ROS are involved in the pathogenesis of three common tumors: lung cancer, hepatocellular carcinoma, and carcinoma of prostate (5).

Lipid peroxidation is a harmful chain reaction. It directly affects cell membrane structure. In addition, it produces reactive aldehydes, and thusly damages other cell components. Peroxidation of three or more pair bonds causes destruction of lipid hydroperoxides and produces malondialdehyde (MDA). MDA causes transversal binding and polymerization of membrane components. MDA is a good parameter of the degree of lipid peroxidation (6).

The brain is dependent on aerobic energy production, which requires constant flow of oxygen. The high level of polyunsaturated fatty acids in cell membranes (7) makes the brain particularly vulnerable to free oxygen radical damage. Iron-rich areas in the brain also play an essential role in generating ROS (8). The sensitivity of brain tissue to oxidative stress and the effect of free radicals in oncogenesis led us to consider the relation between brain tumors and oxidative stress.

Carnitine has two main functions: transporting long-chain fatty acids into the mitochondrial matrix for ß oxidation to provide cellular energy and modulating the rise in intramitochondrial acyl-CoA/CoA ratio, which relieves the inhibition of many intramitochondrial enzymes involving glucose and amino acid catabolism (9).

The carnitine shuttle in brain mitochondria revealed a different substrate specificity than in other tissues; the system was found more specific towards short- than long-chain acyl derivatives of carnitine (10). It is postulated that its major role in the adult brain would be translocation of acetyl moieties from mitochondria into the cytoplasm for acetylcholine synthesis. Acetylcholine synthesis is stimulated by carnitine and choline in a synergistic way in cells utilizing glucose as the main energetic substrate (11). Thus, it seems reasonable to claim for acetyl-L-carnitine (C2 carnitine) a pivotal role in the regulation of carbohydrate and lipid metabolism in the central nervous system (9). In some experiments, it is suggested that C2 carnitine has neurotrophic and neuroprotective activities (12).

Carnitine and carnitine esters protect cells, which play a role in attenuating oxidative damage (13). Carnitine contributes to the depression of oxygen radical formation by their regulatory effects on cell energy metabolism and stabilization effects on cell membrane phospholipids. Thus, it is concluded that both endogenous scavenging systems and regulation of energy metabolism play an important role in neuroprotective effects against oxygen free radicals (14). Carnitine is also associated with buffering of excess acyl-Co A, which is potentially toxic to the cells, and it was reported that L-carnitine has a protective effect on lipid peroxidation by reducing the formation of hydrogen peroxide.(15) L-carnitine also improved antioxidant status in rats and showed free radical scavenging activity as well (16).

There is sufficient detailed information about the effect of carnitine in cancer patients. The small number of studies about carnitine and its acyl esters in brain tumor cells expose the need for a new clinical approach on this subject. In our study, we analyzed the levels of MDA and free carnitine, and C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 carnitine in glial tumors to demonstrate if there is any relation between carnitine-acyl carnitines and lipid peroxidation in carcinogenesis.

Materials and Methods

With the approval of the local ethics committee, specimens used in this study were obtained from patients with glial tumors who were seen at Gazi University, Department of Neurosurgery (Table 1). Forty-five

Table 1. Working groups included in the study according to pathological diagnosis (brain tumor tissues of patients at Gazi University, Department of Neurosurgery).

Tissue Groups	n	Female	Male	Age Interval
Glioblastoma multiforme	29	11	18	32-87
High-grade astrocytoma	8	3	5	18-75
Low-grade astrocytoma	8	3	5	34-51
Total	45	17	28	–

patients were enrolled in the study following provision of informed consent. All tumor groups underwent surgical resection after a full clinical and radiological evaluation. The tumor was resected microsurgically as a gross total. The diagnosis was confirmed by histopathologic evaluation. They were obtained during surgery and stored at –80°C.

Tissue MDA levels were detected as MDA-TBA (thiobarbituric acid) complexes in high performance liquid chromatography (HPLC) using Agarwal et al.'s (17) method. 50 mg tumor tissues were homogenized with 0.95 ml 1.95% KCl. Samples were centrifuged at 14000 rpm for 10 min at 4°C and then supernatants were separated. Standards were prepared from 1,1,3,3-tetraethoxypropone (TEP) at 20 µM, 10 µM, 5 µM, and 2.5 µM concentrations.

50 µl supernatant or 50 µl standard, 50 µl 0.05% BHT, 400 µl 0.44 M H_3PO_4, and 100 µl TBA were added into tubes, vortexed and closed tightly. They were incubated at 100°C for 1 h. They were treated with 250 µl n-butanol after cooling. Tubes were vortexed for 5 min and centrifuged at 14000 rpm for 3 min. A 20 µl sample from the n-butanol layer was injected into HPLC (Waters). Fluorescence detector was used for HPLC. A 20 µl sample was injected into the device at 1 min intervals. Methanol/phosphate buffer (40/60, v/v) was used for mobile phase with 1 ml/min flow rate. The fluorescence detector was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. The column was 5 µl ODS 100x4.6 mm placed in a column warmer set to 37°C.

Free carnitine and acylcarnitine derivatives were measured according to Chace et al.'s (18) tandem mass spectrophotometric (TMS) method. Free carnitine and acylcarnitine derivative levels in tissue (19) were detected at Micromass Quatro II TMS using ESI ionization source. Samples were given to the system with a Jasca PU-980 pump and a Jasca AS-950 auto sampler. Nitrogen and argon gases were used for analyses. Free and acylcarnitine reference standard (Cambridge Isotope Laboratories, USA) was used as the standard.

50 mg tissue was homogenized with 2 ml methanol. Materials were placed on shaker for 30 min and left at -80°C for another 30 min for deproteinization. They were centrifuged at 14000 rpm for 5 min. 100 µl supernatant was transferred into microtiter plate, where 100 µl of 1/245 diluted internal standard was added. Samples were evaporated under nitrogen for derivatization. 60 µl butanyl HCl (acetyl chloride/n-butanol, 1/10) was added and the plate was covered and incubated at 65°C for 20 min. After evaporation under nitrogen, 100 µl acetonitril/H_2O (80/20) was added to samples.10 µl of solution was injected into TMS for analysis.

Statistical Analysis

SPSS software for Windows (version 11.0) was used for evaluating data about acylcarnitine levels of brain tumors, statistical calculations of the groups and standard deviations (±SD), and for determining the significance of the differences between the groups' mean values. Mann–Whitney U test was applied to compare differences between the groups. Spearmen correlation test was used to show if there was a significant relation between MDA and carnitine levels. A p value of less than 0.05 ($P < 0.05$) was considered statistically significant.

Results

MDA levels of glioblastoma multiforme (GBM) and high- and low-grade astrocytoma tumor groups were calculated as nmol/g damp tissue (Table 2).

There was no statistically significant difference between MDA levels in the tumor groups ($P > 0.05$).

Table 2. MDA levels of tumor groups (mean ± SD).

GROUPS	n	MDA Levels (nmol / g damp tissue)
Glioblastoma multiforme	29	29.9 ± 8.1
High-grade astrocytoma	8	27.6 ± 4.5
Low-grade astrocytoma	8	26.5 ± 4.3

Free carnitine, acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), dodecanoylcarnitine (C12), tetradecanoylcarnitine (C14:1), myristoylcarnitine (C14), palmitoleoylcarnitine (C16:1), palmitoylcarnitine (C16), oleoylcarnitine (C18:1), stearoylcarnitine (C18), and arachidonoylcarnitine (C20:4) levels of GBM, and high- and low-grade astrocytoma tumor groups are given and compared in Table 3.

C2, C18 and C18:1 carnitine levels were significantly decreased in low-grade astrocytomas when compared with high-grade astrocytomas. C16:1 carnitine was increased; however, there were no apparent differences in other carnitine levels. Most of the carnitine levels showed decrease in astrocytomas compared with the GBM group (except C16:1 level in high-grade astrocytomas).

MDA and C20:4 carnitine levels were increased in GBM according to Spearman correlation analysis (Figure). No significant correlation was found between the other parameters.

Table 3. Carnitine levels of glioblastoma multiforme, high-grade astrocytoma, and low- grade astrocytoma tumor groups (mean ± SD).

Acylcarnitine derivatives	Glioblastoma Multiforme (nmol/g damp tissue)	High-Grade Astrocytoma (nmol/g damp tissue)	Low-Grade Astrocytoma (nmol/g damp tissue)
Free Carnitine	152.0 ± 82.0	67.1± 31.0 [b]	52.0 ± 19.0 [b]
C2	65.7 ± 26.8	56.7 ± 16.9	37.5 ± 9.4 [b,c]
C3	6.5 ± 2.9	1.1 ± 0.5 [b]	1.1 ± 0.4 [b]
C4	8.1 ± 3.7	4.1 ± 1.8 [b]	2.8 ± 0.9 [b]
C5	3.3 ± 1.2	1.3 ± 0.8 [b]	1.0 ± 0.3 [b]
C6	1.5 ± 0.6	0.8 ± 0.2 [b]	0.8 ± 0.4 [b]
C8	0.13 ± 0.05	0.08 ± 0.03 [b]	0.08 ± 0.04 [a]
C10	0.19 ± 0.08	0.11 ± 0.05 [a]	0.10 ± 0.03 [b]
C12	0.95 ± 0.50	0.39 ± 0.18 [b]	0.36 ± 0.09 [b]
C14:1	0.51 ± 0.20	0.43 ± 0.24 [d]	0.35 ± 0.10 [a]
C14	1.5 ± 0.6	1.4 ± 0.8	0.9 ± 0.3 [b]
C16:1	1.15 ± 0.58	1.60 ± 0.86	0.73 ± 0.28 [c]
C16	11.6 ± 5.5	8.5 ± 4.0	5.9 ± 1.4 [b]
C18:1	5.4 ± 1.7	4.8 ± 1.3	2.9 ± 0.5 [b,d]
C18	9.7 ± 3.1	4.3 ± 1.3 [b]	1.6 ± 0.4 [b,d]
C20:4	0.34 ± 0.09	0.10 ± 0.05 [b]	0.10 ± 0.04 [b]

* Glioblastoma multiforme tumor group compared with high-grade astrocytoma, low-grade astrocytoma tumor [a] $P < 0.05$ - [b] $P < 0.01$.

* High-grade astrocytoma group compared with low-grade astrocytoma tumor group [c] $P < 0.05$ - [d] $P < 0.01$.

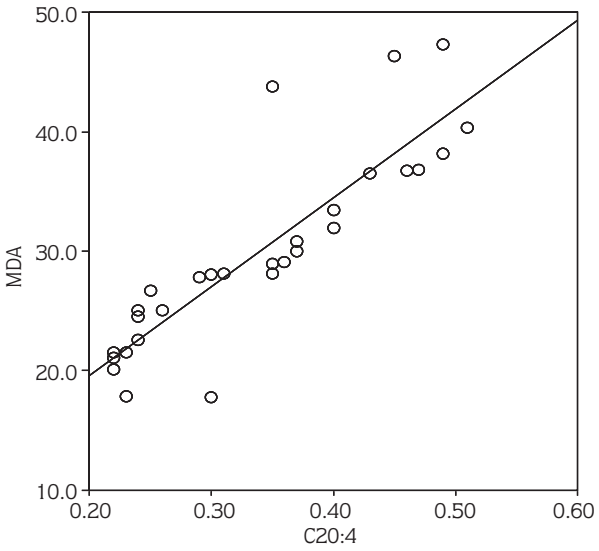

Figure. The correlation between MDA levels and C20:4 carnitine in GBM.

Discussion

In recent years, carnitine and its acyl esters have been considered as part of the defense biochemical network devoted to protection against free radical toxicity in various treatment areas of medicine. Findings in several recent studies have suggested that carnitine and some of its acyl esters may have a beneficial effect in those pathologic conditions where the free radicals are blamed as potential mediators of cellular damage (20). L-carnitine can increase the metabolic efficiency of mitochondria and cause a redistribution of the metabolic workload, resulting in increased cellular efficiency and possibly a decrease in the rate at which mitochondrially derived oxidants are produced (21). In addition, *in vitro* evidence would support the concept that acetyl-L-carnitine might posses a direct antioxidant activity (22). It is demonstrated that acetyl-L-carnitine plays an important role in carbohydrate and lipid metabolism in the central neural system (12). In many studies, it was shown that acetyl-L-carnitine has neurotrophic and neuroprotective activities (23). It is known that carnitine and its species improve myocardial function and metabolic parameters developed in myocardial ischemia and ischemia-reperfusion damage (14). Reznick et al. (23) showed L-propionyl carnitine protects the heart via two mechanisms: 1) it combines the iron that is necessary for the production of hydroxyl radicals, and 2) it serves as a source of energy. All these are supported by carnitine, which decreases heart lipid peroxidation stimulated by doxorubicin (13), and propionyl carnitine, which decreases lipoperoxide production that occurs during ischemia reperfusion damage in the rat heart (24). Furthermore, the protective effects of carnitine in the brain were shown, but the characteristics of these protective effects can not be clarified. Koudelova et al. (25) found that carnitine protects the rat brain from lipid peroxidation and also partially improves neuron excitability after low pressure hypoxia. Carnitine contributes to the depression of oxygen radical formation by its regulatory effects on cell energy metabolism and stabilization effects on cell membrane phospholipids. Thus, it is concluded that both endogenous scavenging systems and regulation of energy metabolism play an important role in the neuroprotective effects against oxygen free radicals (14).

Especially in advanced lung and breast cancer patients, serum acylcarnitine concentrations were decreased and renal clearance of acylcarnitine was elevated (26). Since it is generally recognized that serum and urine carnitine profiles change with the metabolic state, the higher acylcarnitine clearance and acid insoluble acylcarnitine (AIAC) excretion in cancer patients may reflect their metabolic state (27). It is found that the concentration of acid soluble acylcarnitine (ASAC) was low in cancer patients, whereas nonesterified carnitine (NEC) and AIAC levels were similar to those of non-cancer patients (26). The decreased concentrations may have been due to increased utilization of lipids, decreased production and increased excretion of ASAC or a combination of these (26,27). The changes in serum carnitine levels may not always be indicative of the tissue carnitine concentrations. It is found that total carnitine concentrations in rats bearing pituitary tumor Mt T-F4 were significantly higher than those in non-tumor animals, whereas the serum NEC concentration was significantly decreased (28).

Palmitoylcarnitine has been reported to inhibit proliferation of melanoma cells by its interaction with protein kinase C. Low concentrations of palmitoylcarnitine induce rapid differentiation of neuroblastoma NB-2a cells in culture. Further, adding palmitoylcarnitine inhibits proliferation of neuroblastoma and melanoma (29); however, the potential effect of palmitoylcarnitine on brain tumor cells necessitates clinical trials on this issue.

In the present study, C2, C18 and C18:1 carnitine levels were significantly decreased in low-grade astrocytomas when compared with levels in high-grade astrocytomas. C16:1 carnitine was increased; however, there were no apparent differences in the other carnitine levels. Most of the carnitine levels showed decrease in astrocytomas compared with the GBM group (except C16:1 level in high-grade astrocytomas). Sandikci et al. (9) showed that acid soluble carnitine levels of grade II and grade III astrocytomas were found statistically lower than of glioblastoma. They also stated that acid insoluble carnitine levels of astrocytomas were lower than of glioblastoma. In this respect, these findings support our study.

In our study, we analyzed the relation between MDA and free carnitine, and C2, C3, C4, C5, C6, C8, C10, C12, C14:1, C14, C16:1, C16, C18:1, C18, C20:4 carnitine levels in glial tumors. We found that there was a significant correlation only between MDA and C20:4 carnitine levels in GBM. As MDA levels increased, C20:4 levels also increased ($p = 0.000$, $r = 0.916$). This supports the claim that the unsaturated fatty acids in the membrane structure are the most affected in lipid peroxidation. No significant correlation was found between the other groups.

As a result, measurement of only carnitine-MDA relation did not reveal any significant correlation. Detailed studies including antioxidant structures and other parameters that are related with tumor cell metabolism are necessary.

Acknowledgement

This study was supported by grants from Gazi University Scientific Research Projects Department.

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