ORIGINAL ARTICLE

Asymmetric dimethylarginine in experimental breast cancer; action of Vitamin C and E

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Abstract

Objective: To investigate the arginase-nitric oxide synthase paradox through asymmetric dimethylarginine, symmetric dimethylarginine and nitric oxide levels, and to see the effect of antioxidant vitamins on this mechanism of cancer action.

Methods: The animal-based study was conducted at Trakya University, Turkey, in 2008, and comprised mice that were divided into five equal groups. Group 1 had healthy controls, while in the other four groups breast cancer was induced. Group 2 received saline solution, group 3 received 200 mg/kg/day vitamin C (tumour +vit C), group 4 received 300 mg/kg/day vitamin E (tumour +vitE) and group 5 received both 200 mg/kg/day vitamin C and 300 mg/kg/day vitamin E (tumour +vit C+vit E) for 15 days intra-peritoneally.Arginine, asymmetric dimethylarginine, symmetric dimethylarginine and nitric oxide levels were determined in each group.

Results: The 50 mice in the study were divided into five groups of 10(20%) each. Plasma arginine levels were significantly decreased, asymmetric dimethylarginine and symmetric dimethylarginine levels were increased, while plasma nitric oxide level was significantly decreased in group 2. There was no statistically significant difference in treatment groups for all parameters ($p>0.05$ each).

Conclusion: Understanding of the mechanism may help to develop new anti-cancer agents.

Keywords: Breast cancer, Arginine, ADMA, SDMA, NO, Vitamin C, Vitamin E. (JPMA 65: 829; 2015)

Introduction

Breast cancer is the commonest type of cancer in women. It accounts for about 20% of all cancer-related mortality in the European Union.[1] Therefore, great medical and scientific efforts are constantly invested into understanding the disease's pathology and in finding new methods for its early diagnosis, prevention and treatment.

In 1992, a study first described asymmetric dimethylarginine (ADMA) as an endogenous inhibitor of nitric oxide synthase (NOS). Increased ADMA level leads to reduced endothelium-derived nitric oxide (NO) synthesis.[2]

Endogenous arginine analogues are the result of the degradation of nuclear proteins containing methylated arginine residues.[3] Methylation of the terminal nitrogen atom(s) of arginine residues in proteins is catalysed by a family of enzymes termed protein-arginine methyltransferases (PRMTs). One or two methyl groups are added to the guanidino nitrogen (NG)of arginine with the synthesis of NG-monomethyl L-arginine, also calledL-NG-Monomethylarginine (L-NMMA), ADMA and symmetric dimethylarginine (SDMA), respectively.[3] ADMA formation is characterised by the addition of two methyl groups on a single guanidino nitrogen of the arginine residue. SDMA, the stereoisomer of ADMA, is obtained by the addition of one methyl group on either guanidino nitrogen of the arginine residue.[3]

Increased ADMA plasma levels, including renal dysfunction, were also reported in several conditions, including hypercholesterolemia, hyperhomocysteinemia, peripheral arterial disease, diabetes mellitus type 2, hypertension, coronary heart disease, heart failure, stroke, hyperthyroidism and pre-eclampsia.[4] ADMA is accepted as a marker of organ dysfunction and mortality in intensive care patients.[4] ADMA and SDMA are also found to be increased in women with polycystic ovary syndrome (POS).[5] There is limited information about ADMA in cancer. It has been found to be elevated in adults with haematological malignancies, including acute lymphoblastic leukaemia.[4] In a study, ADMA level was shown to be higher and was reduced with taxane-based adjuvant chemotherapy in patients with breast cancer.[6]

NO production depends on NOSs which are the enzymes that synthesise the oxidation of arginine to NO and citrulline. Three different NOS types are available: neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3). eNOS and nNOS are believed to be constitutive (cNOS).[7] NO has several aspects in cells

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and its action may vary depending on its level.[8] Studies have proposed that in cancer growth, NO may have an important action as a preventative and therapeuticagent. The use of NO donors as cancer therapeutics had been shown as a new venue that was not appreciated in the past, because NO was primarily used for treatment of blood vessel-related disorders and in other non-cancer conditions. On cancer cells, the indication of NO-mediated cytotoxicity through its anti-proliferative and chemo-sensitising behaviour, supported the idea of their usage in cancer therapy.[8]

In cells, L-arginine is embraced in protein synthesis. Arginine is also used by arginase, arginine decarboxylase, NOSs and glycine transaminase. Arginase is a crucial enzyme chargeable for nitrogen metabolism. Arginine is its main substrate and from which it creates urea and L-ornithine.[9] There are two types of arginase. Arginase I is cytosolic and is mostly found in the liver. It is considered to be primarily liable for ammonia's detoxification. The other isoenzyme, arginase II, is engaged in ornithine creation. Ornithine is the precursor of some products which take a place in cell growth. Those are glutamate, proline and polyamines (spermine, spermidine and putresine).[10] As polyamines are crucial for cell proliferation, it is probable that the elevated level of ornithine, due to increased arginase activity, could lead to the development of cancer.[11]

Vitamin C performs a potent water-soluble antioxidant activity in biological fluids by scavenging physiologically relevant reactive oxygen and reactive nitrogen species (ROS). These contain free radicals, aqueous peroxylradicals, superoxide anion and nitrogen dioxide as well as non-radical species such as hypochlorous acid, ozone, singlet oxygen and nitrosating species.[12]

Moreover, the antioxidant capacity of vitamin E has prompted many to study its ability to prevent chronic diseases, especially those believed to have an oxidative stress (OS) component such as cardiovascular diseases, atherosclerosis and cancer. Vitamin E takes on specific roles beyond that of its antioxidant function.[13]

To better understand cancer metabolism and cancer development, the current study was planned to investigate the arginase-NOS paradox through ADMA, SDMA and NO levels. We also planned to assess the effect of antioxidant vitamins on this mechanism of cancer action.

Material and Methods

The animal-based study was performed in the Experimental Animal Breeding and Research Unit of the University of Trakya, Turkey, in 2008, after approval by the institutional animal ethics committee. Adult male inbred BALB/c mice 8 weeks of age were used in the study.

The sample size was based on power analysis. The minimum detectable difference in ADMA levels was 0.2±0.12, an alpha level of 5% and power 80%.

In the eighth week, five equal groups were formed. Group 1 comprised healthy controls. Ehrlich ascites tumour cells derived from a spontaneous murine mammary adenocarcinoma were used to induce breast carcinoma in the other four groups. Tumour development was assessed after 9 days by measuring their footpads' thickness. On the 10th day, treatments were started. Group 2 received saline solution (tumour control), group 3 received 200 mg/kg/day vitamin C (tumour +vit C), group 4 received 300 mg/kg/day vitamin E (tumour +vitE) and group 5 received both 200 mg/kg/day vitamin C and 300 mg/kg/day vitamin E (tumour +vitC +vit E) for 15 days intraperitoneally (ip). At the end of treatment period, the animals were sacrifised under anaesthesia. Tissues were extracted and stored at -80 C.

Measurement of arginine, ADMA and SDMA levels were performed using a high-performance liquid chromatography (HPLC) system.[14] The system is one of the best and reliable methods for the measurement of arginine, ADMA and SDMA levels in biological samples.

The HPLC method is suitable for the simultaneous analysis of ADMA, SDMA and arginine in plasma and other biological samples. In this method, Waters Alliance 2690 separation module, Model 474 fluorescence detector and Waters Millennium 32 software were used. Standards and samples were derivatised according to the original procedure. O-phthalaldehyde (OPA) was used as a derivatisation reagent.[14]

Chromatography was performed on a Symmetry C18 column (3.9 x 150 mm, 5µm particle size) with a guard column (3.9 x 20 mm) packed with the same stationary phase (Waters, USA). Mobile phase A consisted of 50mM potassium phosphate buffer (pH: 6.5), containing 8.7% acetonitrile, and mobile phase B was acetonitrile/water (50/50, v/v). Fluorescence was measured at excitation and emission wavelengths of 340 and 455nm, respectively.

Nitrite and nitrate concentrations were measured spectrophotometrically.[15] Results were expressed as µmol/L for plasma or µmol/mg protein for tissue samples.

Mean ± standard error of mean were calculated. Normality of continuous variables were measured by Shapio-Wilk's test and these variables were found to be

skewed. Plasma and tissue arginine, ADMA, SDMA and NO levels in the tumour and treatment groups were compared using Kruskal Wallis test and then Mann Whitney U test was applied. P<0.05 was considered statistically significant.

Results

The 50 mice in the study were divided into five groups of 10(20%) each. Plasma arginine levels were significantly decreased, ADMA and SDMA levels were increased, while plasma NO level was significantly decreased in group 2 (Table).

Table: Plasma and tissue levels of arginine, ADMA, SDMA and NO (mean ± SEM).

	Control	Tumour	Vit. C	Vit. E	Vit. C+E	P*
Plasma						
Arginine (µmol/L)	100±13.35	52.2±8.07p=0.009	48.4±8.39p=0.721	53.0±6.89p=0.959	36.0±6.72p=0.105	0.004
ADMA (µmol/L)	0.97±0.07	1.38±0.43p=0.965	1.23±0.13p=0.442	1.11±0.07p=0.505	1.12±0.07p=0.442	0.579
SDMA (µmol/L)	0.21±0.05	0.45±0.17p=0.016	0.36±0.03p=0.328	0.30±0.02p=0.878	0.31±0.02p=0.574	0.004
NO (µmol/L)	8.36±0.01	6.27±0.48p=0.001	4.66±0.28p=0.017	7.96±1.57p=0.710	5.23±0.87p=0.181	0.015
	Tumour	**Vit. C**	**Vit. E**	**Vit. C+E**		**P***
Tissue						
Arginine (µmol/mg pr.)	6.20±0.92	6.41±0.47p=0.721	7.91±2.37p=0.959	6.74±1.0p=0.645		0.912
ADMA (µmol/mg pr.)	1.54±0.27	2.15±0.23p=0.195	2.12±0.27p=0.328	1.73±0.18p=0.645		0.393
SDMA (µmol/mg pr.)	0.25±0.04	0.32±0.03p=0.161	0.29±0.03p=0.574	0.27±0.03p=0.721		0.618
NO (µmol/mg pr.)	5.45±0.78	5.17±0.37p=0.867	5.52±0.47p=0.867	7.34±1.13p=0.121		0.371

*Kuruskal Wallis p value.
Mann-Whitney comparisons were between Control and Tumour, Tumour and Vit. C, Tumour and Vit. E, Tumour and Vit. C+E.
ADMA: Asymmetric dimethylarginine
SDMA: Symmetric dimethylarginine
NO: Nitric oxide. Vit: Vitaman.

Vitamin C treatment significantly decreased plasma NO levels (p=0.017). In tumour tissues, there was no significant difference between cancer and treatment groups (p>0.05 each).

Discussion

NOSs and arginase can compete for their common substrate, L-arginine; this interaction between these two enzymes represents a potential important factor in the regulation of NO production. Elevated arginase activity could limit NO synthesis by reducing L-arginine availability for NOS.[16] It has been documented that arginase activity is 5-fold greater than NOS activity and arginase was found to be present in the major pathway of L-arginine metabolism in nephritic glomeruli.[17] Likewise, it was suggested that while arginase activity increases, NOS activity decreases in the erythrocytes of the patients with chronic renal failure.[18] It was also reported that for the mammalian arginase, the Michaelis constant(Km) for L-arginine was 2-20mM and 1-20 M for the various NOS isoenzymes.[19] However, at physiological pH the Vmax of each one of the NOS enzyme was less than 1000 times of arginase enzyme[20] and, therefore, it was concluded that both enzymes, NOS and arginase, could use arginine.[21] Previously, we also showed that there was a negative relation between arginase enzyme activities and NO levels in breast cancer.[22]

NO is a pervasive molecule that employs many biological outcomes.[8] The decisive effect of NO on tumour growth are complicated and remain largely unclear. While low levels of NO have been suggested to stimulate tumour cell proliferation and growth,[23] and to increase the metastatic ability of tumour,[24] high NO concentration may inhibit tumour growth,[25,26] and induce apoptosis in tumour cells.[27] Studies report that the duration, expression level and timing of NO delivery; the microenvironment; the cell type and the genetic background might regulate NO sensitivity and the total effect of NO.[28] NO can be used as a chemo-sensitising and an immuno-sensitising agent,[8] and thus, we believe its clinical application may help treatment of cancer.

Arginase has been found to be present in many non-hepatic tissues such as the mammary gland, intestine, kidney, brain and lungs.[29] Extra-hepatic arginase could be connected in the regulation of tissue repair and cell growth.[9] Elevated arginase activity was found in a variety

of established human breast cancer cells.[30] Previous studies have demonstrated high levels of serum arginase activity in several different carcinomas, including gastric, colorectal, large bowel, prostate, lungs and breast cancers, suggesting that this enzyme might serve as a useful biomarker in cancer and cancer progression.[30] L-ornithine could be converted to proline and glutamate by ornithine aminotransferase.[31] In mammalian cells, ornithine is substrate for the formation of glutamate, proline and polyamines.[32] Polyamines (putrescine, spermine and spermidine) have also been indicated to play an important part in cell proliferation and growth.[31,32] They have also been associated with carcinogenesis.[33,34] The exact mechanism(s) for the arginase activity in cancer tissues are unknown. On the other hand, one possible mechanism would be to depend upon elevated concentration of ornithine, a precursor of polyamines, and might result from an increase in extra-hepatic arginase. Increased tissue arginase enzyme activity and ornithine levels together appear to enlarge in polyamine formation, which would lead to cancer development.

ADMA is the major endogenous inhibitor of NOS, the enzyme which forms NO, a molecule endowed with important anti-cancerous properties. The closely related compound, SDMA, does not inhibit NOS activity.[35] However, as arginine, ADMA and SDMA share a common pathway for entry into cell. SDMA may directly reduce NO production by competing arginine for cellular uptake in high plasma concentrations.[35] ADMA and SDMA are molecules that inhibit NOS enzyme, the increase in the concentrations of these molecules in breast cancer may reduce NO production. Much more arginine may be used in the ornithine production and this may lead to formation of polyamines which have been previously shown to be closely related to cancer progression.

One of the limitations of this study is not determining the NOS enzyme activities. The other one is not measuring the polyamine production. With those parameters, we would have had greater opportunity to understand this complex mechanism and might have got some of the pieces of this huge puzzle. This is also a starting point of our future studies.

Inhibition of NO synthesis involves ADMA in the cancer metabolism and, therefore, elevated plasma levels of ADMA will mostly inhibit the NO production and this may have complex impact on cancer biology, including inhibition of angiogenesis or inhibition of cytotoxic effects of NO on breast cancer cells.[4]

The major goal of the present study was bringing a new insight to understand NOS-arginase paradox, adding the ADMA molecule in the story. We have shown, like other studies, that ADMA and SDMA levels increase in cancer, leading to inhibition of NOS enzyme. As a result of this inhibition, plasma NO level is going to be decreased. This may also explain an increase in the ornithine and polyamine production through the arginase activity. This may lead to cancer progression.

Conclusion

Inhibition of arginase enzyme would be one of the strategies of cancer treatment. However, one should also consider the presence of ADMA as inhibition of this molecule would be more useful for cancer progression. While there was an increase in ADMA levels, plasma NO levels were decreased in the study. One of the possible ways to stimulate NO production may depend on ADMA inhibition and this point deserves further research. It is very difficult to hypothesise that either vitamin C or E have any beneficial effects on this metabolism.

Acknowledgements

We are grateful to University of Trakya Research Project for financial assistance.

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