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Original Article

Biochemistry Section

The Relation of Serum Arginine Levels with Serum Arginase and Nitric Oxide Synthase Activity in Patients with Breast Cancer

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ABSTRACT

Introduction: There is a significant disparity between metabolism in normal individuals and cancer patients which have resulted in metabolism based diagnosis. Several types of tumours have abnormalities in arginine and arginine metabolic enzymes. It is now becoming apparent that the two key enzymes of arginine metabolism: arginase and Nitric Oxide Synthase (NOS) in mammals play key roles in regulation of most aspects of arginine metabolism in cancer.

Aim: To evaluate arginine levels, arginase and nitric oxide synthase activity from serum of breast cancer patients of different stages and healthy controls and to find out the diagnostic use of these parameters for early breast cancer detection.

Materials and Methods: Fifty histopathologically proved cases of breast cancer of any stage (Stage I to Stage IV) in the age group of 35-70 years and 25 age and sex matched healthy controls were recruited for this prospective case control study. Intravenous blood sample was obtained to evaluate study parameters. Serum arginine levels were estimated by Sakaguchi method, serum arginase activity was estimated by Roman and Ray method while serum NOS activity was measured by Cortas and Wakid method.

Results: The results of this study showed significant decrease in serum arginine levels and significant increase in the activities of serum arginase and NOS in patients of all stages when compared with controls ($p<0.01$). Serum arginine levels further decreased ($p<0.05$) and activity of serum arginase ($p<0.01$) and of serum nitric oxide synthase ($p<0.05$) was found to be significantly increased in final stage (Stage III+IV) patients when compared with patients of initial stage (Stage I+II).

Conclusion: Complex interrelationship exists between the two important arginine metabolic pathways: arginase and NOS which may profoundly influence tumour growth and biology. The estimation of these parameters can give additional insight regarding disease progression.

Keywords: Apoptosis, Tumour, Tumour node metastasis

INTRODUCTION

Breast cancer is the most frequent cause of cancer death in women in developed and developing countries. It is estimated that during the year 2012, about 144,937 new cases of breast cancer in women occurred in India, which accounts for 27.0% of all malignant cases (an incidence rate of 25.8 per 100,000 population). About 70,218 women died of this cancer in 2012; mortality rate being 12.7 per 100,000 population, ranking number one killer in women [1]. Cancer cells may differ from their normal counterparts in the concentration of various metabolites or activities of certain enzymes. That difference may act as a useful biological marker of malignancy and/or aggressiveness in tumours. There is widespread interest in the involvement of L-arginine in multiple metabolic processes that play important roles in tumour progression. L-arginine and its two important catabolic enzymes: arginase and NOS are known to influence either cancer progression or apoptosis. High expression and/or activity of arginase as well as reduced levels of L-arginine are often observed in the blood of cancer patients [2,3]. Nitric Oxide ($NO^{\bullet}$) derived from L-arginine via NOS has been found to play a significant role in many of the specific events that lead to cancer. Role of $NO^{\bullet}$ in tumour biology is extremely complex. $NO^{\bullet}$ has been shown to have conflicting roles with seemingly opposite effects in tumour initiation, promotion, and progression [4]. Most importantly, various studies have shown that all three isoforms of NOS, {endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)}, have been detected in tumour cells from a wide range of isolates [5-8]. NOS activity has been observed to be increased in human tumour cell lines and cells from tumour biopsies.

L-arginine is a semi-essential amino acid in the human diet. Arginine is required for defence against viruses, bacteria, fungi, and malignant cells in mammals. Adequate provision of arginine is required for lymphocyte proliferation and development, and dietary arginine supplementation enhances immune responses in various models of immunological challenges. Arginine enhances T-lymphocyte proliferation, the cytotoxicity of specific cells, IL-2 production, IL-2 receptor expression on T- lymphocytes, and the delayed type hypersensitivity response [9]. Arginase is a manganese containing enzyme that catalyses the hydrolysis of L-arginine to urea. There are two isoforms of arginase: I and II. Arginase I is cytosolic type and is abundantly present in the liver. It is primarily responsible for ammonia detoxification in the form of urea. A second isoenzyme, arginase II is mitochondrial type and expressed at lower levels in kidney, brain, small intestine, mammary gland, and macrophages. It is involved in the production of ornithine as a precursor to proline, glutamate or polyamines which are essential for cellular growth [2,9]. NOS is a family of isoenzymes responsible for converting arginine to $NO^{\bullet}$. There are three isoenzymes of NOS: nNOS, eNOS and iNOS. NOS expressed in neuronal and vascular cells are calcium dependent. $NO^{\bullet}$ produced by them serves as signal transduction mechanism while iNOS is induced by cytokines and is calcium independent. Cytokines induce sustained production of $NO^{\bullet}$ which mediates cytostatic and cytotoxic effects of the immune system [5].

Recent research [3-4,8-10] has demonstrated the existence of a complex relationship between immunity, tumourigenesis and L-arginine metabolism, where the enzymes arginase and NOS metabolise L-arginine to polyamines and $NO^{\bullet}$ which are essential

products for tumour growth and tumour cytolysis respectively. Thus, whether arginine suppresses or enhances tumour growth depends on the relative activities of arginase and NOS pathways and expression of these enzymes varies with the stage of carcinogenesis. Till date stage-wise study of changes in arginine levels and alteration in the activities of arginase and NOS in serum in breast cancer patients has not been carried out. So, we attempted to find out how the arginine bioavailability changes in serum. We also determined arginase and NOS activity which decides the tumour growth and lysis in the initial and final stages of breast cancer patients.

MATERIALS AND METHODS

The study was carried out at the Department of Biochemistry, BJ Medical College after the approval from Institutional Ethical Committee during June 2011 to December 2013. However, the samples were collected from patients admitted in Sassoon Hospital which is attached to B.J. Government Medical College, Pune, Maharashtra, India.

Study group: Included total of 50 subjects with breast cancer in the age group of 35-70 years. These patients were divided as Stage I, Stage II, Stage III, Stage IV as per Tumour, Node and Metastasis (TNM) system of classification. Stages were grouped as initial Stage-I+II and final Stage-III+IV. All the cases were clinically diagnosed and histopathologically proven for breast cancer.

The clinicopathological characteristics of the patients are given in [Table/Fig-1].

Exclusion criteria: Subjects with other systemic diseases, taking chemotherapy, radiotherapy, any medications/antioxidant supplementation were not included in the study.

A detailed case history of the patient was taken. A case history format was filled, with an informed consent which was duly signed by each patient.

Control group: Twenty five age and sex matched healthy adults without breast cancer was included.

Collection of serum: About 5 mL of venous blood samples of the subjects was collected in fasting state, centrifuged to separate the serum and stored at -80ºC till the analysis was done.

Estimation of serum arginine levels by Sakaguchi method [11]: Arginine is a basic amino acid containing guanidine group. Sakaguchi's reaction is based on the red colour which arises from treatment of an alkaline solution of guanidine compound with 8-hydroxyquinoline and sodium hypobromite. Urea lengthens the time of colour fading sufficiently. The standard curve was prepared for concentrations of 20-100 µmol/L.

Estimation of serum arginase activity by Roman and Ray method [12]: Ninhydrin reacts with ornithine formed by arginase action in the presence of $MnCl_2$ giving a pink coloured ornithine-ninhydrin complex which is read spectrophotometrically at 530 nm. Concentration was determined using standard graph. The standard curve was prepared for concentrations of 0-25 IU/L.

Estimation of serum nitric oxide synthase activity by Enzychrom Nitric Oxide Synthase Assay Kit [13]: Enzychrom Nitric Oxide Synthase Assay Kit involves two steps: a NOS reaction step during which $NO^{\bullet}$ is produced followed by an $NO^{\bullet}$ detection step. Since the $NO^{\bullet}$ generated by NOS is rapidly oxidized to nitrite and nitrate, the $NO^{\bullet}$ production is measured following reduction of nitrate to nitrite using an improved Griess method. The standard curve was prepared for nitrite concentrations of 500-0 µmol/L.

Unit definition: One unit of NOS catalyzes the production of 1 µmole of $NO^{\bullet}$ per minute under the assay conditions (pH 7.5 and 37°C).

For all assays, the amount of analyte in each sample was determined by extrapolating OD values against the corresponding standard concentrations using the standard curve.

Clinicopathological characteristics	
Number of patients	50
Age range	35-70 years
Mean age	52.13±9.90
Status	
Premenopausal	14
Postmenopausal	36
Diagnosis	
Invasive ductal carcinoma	37
Invasive lobular carcinoma	13
TNM Stage	
Initial Stage (I+II)	25
Final Stage (III+IV)	25

[Table/Fig-1]: Clinicopathological characteristics of 50 breast cancer patients.

Stage	Arginine (µmol/L)	Arginase (IU/L)	NOS (IU/L)
Control	70.94±12.47	2.65±1.39	12.39±1.44
Initial	30.69±8.56*	22.31±2.65*	20.72±3.14*
Final	24.88±8.29*#	29.59±6.35*##	22.50±3.09*#

[Table/Fig-2]: Serum arginine levels and serum arginase, NOS activities in healthy controls and in initial stage and final stage breast cancer patients.
The data was expressed as mean±SD
Comparison with control: * p<0.001
Comparison with initial stage: #p<0.05, ##p<0.001

Variable	Initial stage	Final stage
Arginase/Arginine	r=-0.79	r=-0.88
NOS/Arginine	r=-0.84	r=-0.82

[Table/Fig-3]: Correlation analysis of each variable value with other variables in initial stage and final stage breast cancer patients.

STATISTICAL ANALYSIS

Results are presented as mean±Standard Deviation (SD) value and statistically analysed by ANOVA and Student's unpaired t-test. A p-value <0.05 was considered significant.

RESULTS

[Table/Fig-2] shows the mean serum arginine levels, arginase activity and NOS activity in healthy controls and in initial stage and final stage breast cancer patients (initial stage I + II = 25, final stage III + IV = 25). Statistically highly significant decrease was observed (p<0.001) in serum arginine levels of initial and final stage breast cancer patients as compared with control group. These levels further decreased significantly (p<0.05) in final stage breast cancer patients as compared to initial stage. Statistically highly significant increase was observed (p<0.001) in serum arginase activity of initial and final stage breast cancer patients as compared with control group. This activity further rose significantly in final stage patients compared with initial stage (p<0.001). Highly significant rise was found (p<0.001) in serum NOS activity of initial and final stage breast cancer patients as compared with controls. Serum NOS activity further rose (p<0.05) in final stage of breast cancer patients as compared with initial stage [Table/Fig-2].

[Table/Fig-3] shows a correlation analysis of the parameters measured in initial and final stage patients. Using bivariate correlation analysis of the measured parameters, we observed a significant negative correlation between serum arginase and serum NOS activity with serum arginine levels indicating that increase of activities of these enzymes decreases the levels of serum arginine. Serum arginase and serum NOS activity showed positive correlation with each other [Table/Fig-3].

DISCUSSION

Cancer is a disease in which abnormal arginine levels or arginine metabolism act as contributory factors. It has been shown that

cancer interferes with arginine metabolism to combat the immune responses. Lamb J et al., showed that arginine deficiency disrupts the DNA synthesis phase of G1. Arginine levels were found to be altered in various malignancies [14]. Bedoya AM et al., observed high levels of arginase activity with a consequent decrease of arginine in patients with cervix cancer [15]. According to them, immunosuppressive microenvironment observed during human cervical carcinogenesis may at least be partially due to the arginase activity and a concomitant degradation of arginine. Kaplan I et al., found that in patients with oesophageal cancer, plasma arginine concentration was significantly lower than the control group [16].

We demonstrated that serum arginine concentrations were substantially low in breast cancer patients than in controls ($p<0.01$) and the levels further decreased in advance stages ($p<0.05$) [Table/Fig-1]. This means that in physiological conditions exogenous and endogenous arginine meet the needs of the adult human body. However, during cancer, the body's requirement for arginine is increased and de novo synthesis of arginine is inadequate. During cancer, the hepatic citrulline uptake limits the amount of gut derived citrulline reaching the kidney, so less citrulline is taken up by the kidneys and renal arginine production is decreased and at the same time its utilisation is more. This results in reduced extracellular arginine. Reduction of extracellular arginine level arrests the proliferation of activated T-cells in the G0-G1 phase of the cell cycle and blocks the re-expression of the CD3γ chain. Inflow of innate immature myeloid cells is one of the strategies of immune suppression; thus, cancer and subsequent chronic inflammation itself create a mechanism for antitumour immune response of the host. Due to imbalance in proinflammatory mediators, cytokines and chemokines, these immature cells develop into a tumour promoting phenotype [17]. Myeloid Derived Suppressor Cells (MDSC) accumulate in the tumour environment and produce arginase and NOS; important catabolic enzymes of arginine. Thus, arginine levels remain low [Table/Fig-2].

Arginase is a crucial enzyme that is required for nitrogen metabolism as well as for polyamine synthesis in terms of ornithine. Different studies conducted on arginase levels in the serum and tissues of cancer patients have associated arginase enzyme activity with cancer. Some researchers even emphasised arginase enzyme activity as an important determinant even in patients with breast cancer [18]. High levels of serum arginase activity in several different carcinomas including gastric, colorectal, large bowel, prostate, lungs and breast cancers, suggests that this enzyme might serve as a useful biomarker in cancer and cancer progression [10]. We also found elevated levels of serum arginase activity in the serum of breast cancer patients than controls and this activity was further raised with the advancement of stage ($p<0.01$) [Table/Fig-1]. Our study is supported by Boniface J et al., Geyikli I et al., and Perez G et al., [3,19,20]. Boniface J et al., in their study on early stage cancer patients reported that despite a low tumour burden, arginase I expression was significantly increased in blood-derived myeloid cells of breast cancer patients and it got decreased after tumour resection and was associated with tumour grade [3]. Geyikli I et al., found that activity of arginase was high in the early stages (Stage I+II, $p<0.01$), and higher in the advanced states (Stage III+IV, $p<0.001$) of the malignant group in comparison with those of the normal subjects [19]. Perez G et al., observed that the mean activity of arginase in plasma was significantly higher in breast cancer patients than in healthy volunteers [20].

The exact mechanism for effect of increased arginase activity in cancer is unknown. One possible mechanism would be the elevated concentration of ornithine, a precursor of polyamines, which result from an increase in extrahepatic arginase. Polyamines play an important part in cellular proliferation and growth. They have also been associated with carcinogenesis. It has been suggested that oestrogens modulate the growth of certain breast cancer cell lines by increasing the expression of Ornithine Decarboxylase (ODC), thereby increasing the synthesis of polyamines. Thus, increased arginase enzyme activity may suggest cancer progression [3,10,18].

Increased NOS activity is also associated with metaplastic changes in the breast. The present study showed that NOS activity was high in serum samples of breast cancer patients compared with normal ($p<0.01$). Furthermore, the activity of the enzyme was significantly greater ($p<0.05$) in case of final stage patients (Stage III+IV) compared with the initial stage (Stage I+II) [Table/Fig-2]. During tumour growth, NOS activity may be increased by proinflammatory cytokines and hypoxia present in the tumour microenvironment. NOS isoforms may be involved in tumour cell proliferation, survival, migration, and invasiveness. Ranganathan S et al., Ridnour LA et al., had detected increased NOS activity in breast cancer cell lines and correlated its expression with tumour grade and proliferation rate [21,22]. In numerous studies elevated iNOS expression has been identified in precancerous lesions and numerous studies which have suggested a potential use of iNOS as a predictive cancer biomarker [22–24]. iNOS produces micromolar levels of $NO^{\bullet}$, which damage DNA and modify protein structure/function [8]. However, eNOS, which produces nanomolar levels of $NO^{\bullet}$, also plays a role in all the stages of cancer. Protumourigenic agents, such as oestrogen, have been shown to induce eNOS expression in tumour cells. eNOS plays an essential role in endothelial cell proliferation and is a stimulator of Vascular Endothelial Growth Factor (VEGF) and Prostaglandin E2 (PGE2). VEGF increases angiogenesis while PGE2 increases endothelial cell sprouting (the first step in neoangiogenesis) through the $NO^{\bullet}$/cGMP pathway. Several groups have observed that low concentrations of $NO^{\bullet}$ released by eNOS, stimulate cancer cell cycle progression and proliferation [5,6,21,22]. In studies performed by Xu W et al., they found strong co-expression of iNOS and eNOS in in situ ductal carcinomas [25].

These findings suggest that regulation of these pathways represents the mechanism to limit arginine-derived $NO^{\bullet}$ production and polyamines. There exists a complex interplay between arginase and NOS as they use a common substrate. A negative correlation between serum arginase and serum NOS activity with serum arginine levels [Table/Fig-3] indicate that increase of activities of these enzymes decreases the levels of serum arginine being a common substrate for these enzymes. Both the enzymes try to check activities of each other and increase its own activity. These complex interrelationships between the various arginine metabolic pathways may profoundly influence tumour growth and biology.

LIMITATION

The sample size was small, further larger series studies are needed to confirm our findings.

CONCLUSION

Exogenous arginine and the arginine de novo synthesis fail to meet the high demand for arginine. Bioavailability of arginine is dependent on arginase and NOS activity. Both these enzymes play a role in the tumourigenesis in one way or the other. So, their activity might be of use as markers for the diagnosis of breast cancer.

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