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Evaluation of serum arginase I as an oxidative stress biomarker in a healthy Japanese population using a newly established ELISA

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ABSTRACT

Objective: We reported previously that serum arginase I increased in asthmatic patients and was associated with oxidative stress in a small healthy population. However, the exact association of arginase I with oxidative stress is not known. The present study aimed to analyze the association of arginase I with oxidative stress in a larger healthy population by a newly established ELISA.

Design and methods: The new ELISA for the measurement of human arginase I was established by generating recombinant arginase I protein in human arginase I gene-transfected *Escherichia coli* via an ARG1 cDNA fragment-inserted vector and -specific antibody in rabbits. Serum arginase I was evaluated in a cross-sectional study on a healthy population (n = 721) by comparing a commercial ELISA kit with the new ELISA.

Results: The mean levels of serum arginase I were 20.3 ± 0.7 ng/mL and 4.7 ± 0.2 ng/mL using the commercial ELISA kit and the new ELISA, respectively. Arginase I was correlated with WBC, RBC, hs-CRP, 8-OHdG, HDL-c, ALT, and BMI. Logistic regression analysis showed independent positive associations of arginase I with WBC, RBC, and urinary 8-OHdG and inverse independent associations with serum insulin and age. The association of arginase I with hs-CRP was not independent.

Conclusion: The independent associations of arginase I with urinary 8-OHdG and serum insulin may reflect its involvement in oxidative stress and diabetes mellitus.

Introduction

Arginase has two distinct isoforms, arginase I and II, which differ in their subcellular localization. Arginase I is predominantly localized in the cytosol of hepatic cells as a key enzyme for the urea cycle, while arginase II is expressed in the mitochondria of extra-hepatic cells and is encoded by a different gene [1]. Both isozymes, constitutively expressed in cells and tissues, indirectly regulate nitric oxide (NO) generation from nitric oxide synthase (NOS) by competition for a common enzyme substrate, L-arginine, which is metabolized to L-citrulline by NOS and to L-ornithine by arginase [2–4]. Therefore, the induction of arginase has been focused in the context of inflammatory cells of asthmatic lung as pathophysiological evidence that the consumption of L-arginine by arginase may lead to the depletion of NO and thus the enlargement of bronchial smooth muscle associated with airway hyperresponsiveness [5–8].

Serum arginase levels were evaluated in various diseases by activity assay and the ELISA method. In asthmatic patients, although no agreement was established in terms of serum activity for arginase [9–12], serum levels of arginase I were elsewhere shown to be elevated by ELISA [13]. In sickle cell disease, asthma and pulmonary hypertension were also shown to be induced by high hemolysis-induced levels of serum arginase [14,15]. In colorectal cancer, serum ELISA levels of arginase I increased [16]. In renal cell carcinoma and pancreatic cancer, an increase in myeloid-derived suppressor cells (MDSC) was found to be associated with T-cell dysfunction by the depletion of L-arginine due to a high level of arginase I released from MDSC [17]. However, little is known about arginase I in healthy populations [18], although its association with diabetes mellitus and oxidative stress has been demonstrated [19].

Many factors, including Th2 cytokines such as IL-4 and IL-13, growth factors, endotoxin, cAMP-elevating agents, oxygen tension, and reactive oxygen species (ROS), were shown to be involved in the induction of arginase I in various rodent cells [20,21]. However, in human cells,

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although there is little evidence of inducers of arginase I, nicotine in cigarette smokers [22] and pregnancy-specific glycoprotein 1α have been identified [23]. Although inductions of arginase I by IL-4 or IL-13 were shown to be mediated by a nuclear transfer factor, STAT 6, in the lung of an asthmatic mouse model [24], induction of arginase I was not demonstrated by IL-13 or IL-4 in human cells [9].

8-Hydroxydeoxyguanosine (8-OHdG), a product of the oxidatively modified DNA base guanine, is the most representative product that may reflect oxidative DNA damage induced by ROS [25]. 8-Isoprostanes, a group of bioactive prostaglandin F2-like compounds generated by oxidatively catalyzed reactions of arachidonic acid, are recognized as reliable markers of lipid peroxidation in vivo [26]. In a previous study on healthy populations, arginase I was found to be associated with urinary 8-OHdG and 8-isoprostanes [13].

Therefore, in this study, we evaluated the interaction of arginase I with several clinical parameters including oxidative stress biomarkers in a healthy population by a newly established ELISA.

Materials & methods

Establishment of ELISA system

Human arginase 1 complementary DNA (cDNA) was cloned from *Homo sapiens* arginase from the liver (ARG1) as transfection-ready DNA (OriGene Technologies, Inc., Rockville, MD, USA) by polymerase chain reaction. DNA fragments were ligated into pCR Blunt II vector (Invitrogen, Carlsbad, CA, USA). Recombinant human ARG1 protein recovered from ARG1-transformed *Escherichia coli* BL21 (DE3)pLysS Competent Cells (Merck KGaA, Darmstadt, Germany) was purified by Ni-Resin chromatography (Invitrogen, Tokyo, Japan), eluted from Ni-Resin using 50 mM Tris–HCl (pH 8.0) with 100 mM NaCl, and passed through a HiTrap SP column (GE Healthcare Bioscience) with elution from this column with a 150 mM–1 M gradient of NaCl in FPLC. After the judgment of its purity by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), highly purified human arginase I was used for immunization (polyclonal antibody) [27].

The ELISA system was prepared as follows: anti-human arginase I IgG (2.5 μg/mL) dissolved in 0.1 M $NaHCO_3$ containing 0.025% Proclin 300 was coated on a Nunc 96-well microplate (Thermo Fisher Scientific, Hudson, New Hampshire) for 2 h, discarded, and supplemented with 0.1% BSA/PBS/0.025% Proclin 300 for blocking. After discarding the blocking buffer, 100 μL of buffer 1 (0.1% BSA/PBS/0.05% Tween-20/0.025% Proclin 300) was added and then 50 μL of various concentrations of arginase I (R&D Systems Inc., Minneapolis, MN, USA) for standards or serum samples was incubated for 2 h. The plates were then washed 3 times with 3 M NaCl/1% Tween-20 and supplemented with 0.1 μg/mL biotin-conjugated anti-human arginase I IgG in buffer 1 for 1 h. Washing was performed 3 times with 3 M NaCl/1% Tween-20 and then 0.1 μg/mL HRP-conjugated streptavidin was added for 1 h. After 5 washes with 3 M NaCl/1% Tween-20, TMB solution (ScyTek Laboratories, Inc., Logan, Utah, USA) was added for coloration at 450 nm.

Study design

From 847 individuals who undertook a worksite lifestyle study in Okayama city, 721 healthy individuals without a previous history of cancer, stroke, diabetes, ischemic heart disease, liver disease, diabetes mellitus, asthma, and with serum not appearing to be hemolytic were finally selected. The ethics committee of Okayama University approved the study, and all subjects gave informed consent.

Measurement of parameters for whole blood and serum

Blood samples were collected after overnight fasting. Whole blood and separated serum were measured for hematological parameters (RBC and WBC) and serum alanine aminotransferase (ALT) because arginase I is constitutively expressed in granulocytes, red blood cells, and liver cells. Low-density lipoprotein (LDL-c), uric acid, and high-sensitivity C-reactive protein (hs-CRP) were measured to evaluate the correlation of arginase I with atherosclerosis. Hemoglobin A1c (HbA1c) was measured for the evaluation of diabetes mellitus. Serum arginase I levels were determined using ELISA kits of Hycult Biotechnology b.v. UDEN, and a newly established ELISA system. Other clinical examinations involving hematological parameters were performed using automated XE-2100 (Sysmex, Japan) and H7700 (Hitachi High-Technologies, Japan). Serum hs-CRP was measured by a highly sensitive CRP assay (Behring Latex-Enhanced using the Behring Nephelometer BN-100; Behring Diagnostics, Westwood, MA, USA). NOx (NO_2^- + NO_3^-) levels in the serum were determined with an NO analyzer (model-280i NOA with the Purge Vessel; Sievers, Boulder, CO) to evaluate the correlation of arginase I with nitric oxide (NO) generation [28]. Serum was treated with nitrate reductase (Sigma-Aldrich, St. Louis, MO) to convert nitrate to nitrite for 30 min at room temperature. Nitrite was further reduced to NO in the Purge Vessel containing the reducing agent potassium iodide in acetic acid, and NO was subsequently detected by the ozone-chemiluminescence method. Information on lifestyle factors including cigarette smoking, past history, and present steroid medication was obtained using self-reported questionnaires or clinical records.

Analysis of urinary oxidative stress biomarkers

Urinary 8-isoprostane and 8-OHdG were determined using a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI) and an ELISA kit from the Japan Institute for the Control of Aging (Fukuroi, Shizuoka, Japan), respectively. Spot urine samples stored at −80 °C before analysis were used because spot urine for 8-isoprostane did not exhibit significant variation from the levels measured in 24-h urine samples in the same healthy individuals by radioimmunoassay [29], and the correlation coefficient of 8-OHdG by ELISA between spot and 24-h urine samples was 0.87 [30]. The intra-assay and inter-assay CV were 5.4% and 11.0% for 8-isoprostane and 5.2% and 8.1% for 8-OHdG, respectively. Values for 8-isoprostane and 8-OHdG were normalized per milligram of creatinine (Cre) measured in urine (Creatinine Test Kit, R&D Systems, Minneapolis, MN).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0c for Mac (GraphPad Software, Inc., San Diego, CA) and PASW Statistics 18 for Mac. Results are expressed as means ± SEM. Spearman's correlation was used to examine the relationship between serum arginase I and clinical parameters. Logistic regression analysis was performed to test the relationship between arginase I levels and the variables that had a significant correlation as tested using Spearman's correlation, as well as variables that are presumably confounding for arginase I, such as age and sex. A p value < 0.05 was considered statistically significant.

Results

New ELISA system

The standard curve of arginase I is shown in Fig. 1. The detection limit for arginase I by the new ELISA was 0.085 ng/mL. The upper limit of detection was 500 ng/mL near the anchor point by a standard curve. The intra-assay and inter-assay CV for the new ELISA were 2.6% and 6.7%, and those for the commercial ELISA were 5.4% and 11.0%, respectively. The arginase I concentrations of serum samples kept at room temperature and at 4 °C for 24 h did not differ significantly. Four sessions of freezing and thawing of serum samples did not affect the concentration. For dilution testing, arginase I values of three times