Serum Levels of S-Glutathionylated Proteins as a Risk-Marker for Arteriosclerosis Obliterans

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Background Oxidative stress plays a role in the development of chronic peripheral arterial disease (PAD) because under these conditions redox regulation is impaired, inducing the S-glutathionylation of proteins. A method of estimating the levels of S-glutathionylated proteins has been developed using biotinylated glutathione S-transferase, which allows the study of their crucial role in the oxidative stress-related progression of PAD.

Methods and Results The serum levels of S-glutathionylated proteins were examined in 41 patients with arteriosclerosis obliterans (ASO) and 38 age-matched non-ASO patients using biotinylated glutathione S-transferase. The levels were higher in the patients with ASO, even early on, and positively correlated with the ankle/brachial index. In vitro, the levels of S-glutathionylated proteins were reduced in the presence of glutathione and glutaredoxin.

Conclusions Serum levels of S-glutathionylated proteins are a sensitive risk-marker for ASO at an early stage. (Circ J 2007; 71: 100–105)

Key Words: Arteriosclerosis obliterans; Oxidative stress; S-glutathionylation

The number of patients suffering from arteriosclerosis obliterans (ASO) is anticipated to increase, accompanying the increase in incidence of risk factors such as obesity, hypercholesterolemia, diabetes, and hypertension.¹ Pathologically, ASO derives from atherosclerosis, and complete occlusions by fresh or old thrombi are often observed. Treatment includes anticoagulants, antiplatelet drugs, and vasodilators, and in the advanced stages percutaneous transluminal angioplasty, bypass surgery, and prosthetic arterial grafts have been used. New approaches include intravenous administration of prostaglandin E₁² or gene therapy with hepatocyte growth factor,³ both aimed at increasing peripheral blood flow. Most patients with ASO have no apparent clinical symptoms early on, but diagnosis at the early stage is essential for preventing progression. Unfortunately, there are currently no specific and sensitive markers for ASO, so the aim of this study was to find a new risk-marker for the diagnosis of ASO in the earlier stages.

The development of atherosclerosis is induced by severe damage to endothelial cells from various pro-inflammatory cytokines, adhesion molecules, or sheer stress, for example.^{4–6} Furthermore, oxidative stress is believed to play a

crucial role in the progression of peripheral arterial disease (PAD),⁷ because it induces modifications of cellular components such as proteins, lipids, and DNA, leading to cell dysfunction or apoptosis. Most of the risk factors for PAD, such as smoking, obesity, hypertension, diabetes, and hypercholesterolemia, create oxidants that damage endothelial cells.⁷ The cysteine thiols of proteins are easily modified by oxidative stress when the antioxidative systems are suppressed and under oxidative stress caused by reactive oxygen species or nitrogen oxide species, it is the sulfhydryl residues of proteins that are most susceptible. In response, the sulfhydryl groups are oxidized to form disulfides in a reaction with the reduced form of glutathione disulfide (GSSG) or converted irreversibly to sulfenic, sulfinic, and sulfonic acid derivatives.¹¹ S-Glutathionylated proteins reported to date include glyceraldehyde-3-phosphate dehydrogenase,¹² annexin A2,¹³ protein kinase C,¹⁴ and carbonic anhydrase III.¹⁵ The S-glutathionylation of proteins is initiated in the presence of GSSG.⁸ The S-glutathionylation of the sulfhydryl groups changes a protein's function, and the process is regulated by thioredoxin (TRX) or glutathione (GSH)/glutaredoxin (GRX). Such modifications of protein-thiols by oxidative stress are speculated to occur in patients with PAD; however, no data on changes in the levels of serum S-glutathionylated proteins have been reported for patients with peripheral or cardiovascular diseases such as stroke, coronary artery disease, and end-stage renal disease. We are interested in the S-glutathionylation of proteins in ASO patients and so the aim of the present study was to evaluate the serum levels in these patients, as a risk-marker for ASO in the early stages, because elevation supposedly reflects redox imbalance.

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Table 1 Characteristics of Patients With ASO

	Non-ASO patients	ASO patients (Fontaine)		
		Stage 1	Stage 2	Stage 3
Patients, n	38	9	22	10
ABI (mean±SD)	0.94±0.02	0.72±0.07	0.41±0.13	0.21±0.06
Age (mean±SD), years	66±11	73±8	72±7	71±5
Male sex, n (%)	15 (39)	6 (67)	16 (73)	8 (80)
Risk factors, n (%)				
Hypertension	17 (45)	7 (78)	19 (86) [†]	9 (90) [†]
Diabetes	23 (61)	3 (33)	6 (27) [‡]	3 (30)
Smoking	9 (24)	6 (67) [§]	15 (68) [§]	5 (50)
Hypercholesterolemia	16 (42)	4 (44)	9 (41)	5 (50)
Chronic renal failure on hemodialysis	0	0	0	0
Angina	4 (11)	2 (22)	4 (18)	3 (30)
Lipid profile (mean±SD)				
Total cholesterol, mg/dl	253±45	198±23*	205±28*	202±17*
LDL-C, mg/dl	153±46	111±24 [†]	119±23 [†]	116±14 [†]
hs-CRP (mean±SD), ng/ml	2.19±0.42	3.17±1.01	3.57±0.85 [#]	4.30±0.99 [#]

Values for hs-CRP were transformed in logarithm of 10. One-way ANOVA was followed up with Tukey-Kramer pairwise comparisons among means.

[†]*p*<0.05 for comparison with non-ASO patients; [‡]*p*<0.05 for comparison with non-ASO patients; [§]*p*<0.05 for comparison with non-ASO patients; **p*<0.005 for comparison with non-ASO patients; [†]*p*<0.05 for comparison with non-ASO patients; [#]*p*<0.05 for comparison with non-ASO patients.

ASO, arteriosclerosis obliterans; ABI, ankle/brachial index; LDL-C, low-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein.

Methods

Patient Sample

We enrolled 41 patients diagnosed with ASO. All of them had the characteristic complaints of chronic limb ischemia, including intermittent claudication, rest pain, or non-healing ischemic ulcers (Fontaine I, *n*=9; Fontaine II, *n*=22; Fontaine III, *n*=10) as confirmed by angiography. Of the patients visiting hospital without apparent PAD, we recruited 38 age-matched controls. All participants gave written informed consent and prior to the commencement of the present study, the protocol was approved by the ethics committees of all the participating universities and hospitals.

Measurement of Ankle/Brachial Index (ABI)

Blood pressure, heart rate, and ABI were measured using the Form pulse wave velocity (PWV)/ABI non-invasive vascular screening device (Nihon Colin Inc, Tokyo, Japan) after the subject had rested supine for at least 20 min. ABI was calculated 2 or 3 times for both legs and averaged; an ABI <0.9 was considered to indicate the presence of disease.

Immunoblot Analysis

Unless otherwise indicated, 20 µg samples of serum was used. Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Samples were electrophoresed on 5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) in the absence of dithiothreitol (DTT) and the proteins in the gels were transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (10 mmol/L Tris-HCl [pH 7.5] and 0.15 mol/L NaCl; TBS) containing 0.05% Tween 20, *v/v* (TBST), and 5% (w/v) nonfat dry milk, then reacted with primary antibodies in TBST containing 3% (w/v) bovine serum albumin overnight with constant agitation at 4°C. After several washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-IgG antibodies. Proteins

in the membranes were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions. Levels of high-sensitivity C-reactive protein (hs-CRP) were determined in the same serum samples used for sLOX-1, with a commercially available electrochemiluminescent immunoassay kit (F. Hoffman-La Roche Ltd).

Detection of S-Glutathionylated Proteins by Biotin-Glutathione S-Transferase (GST) on Blotted Membranes

Serum levels of S-glutathionylated proteins were estimated according to the methods described by Cheng et al⁸ using biotinylated GST. Serum samples were collected serially and stored at -80°C until assays were performed. Of each sample, 20 µg/lane were subjected to 5% SDS-PAGE under non-reducing conditions. The proteins in the gels were transferred onto nitrocellulose membranes, which were blocked in phosphate buffered saline (PBS) containing 0.1% Tween 20, *v/v*, and 5% nonfat dry milk, then treated with BSA containing 5% (w/v) bovine serum albumin for 2 h at room temperature and further incubated with 30 mg/ml biotin-GST overnight. After several washes with PBS, the membranes were incubated with HRP-conjugated streptavidin (1:1,000 dilution) for 1 h at room temperature. Peroxidase activity was detected after treatment with 2 mmol/L hydrogen peroxide and 0.6 mg/ml 4-chloro-1-naphthol in PBS.

Statistical Analysis

Statistical analysis was performed using Stat-View (version 4.5, Abacus Concepts Inc, Calabasus, CA, USA) and R. The 1-way ANOVA was used to compare continuous variables, with the Tukey-Kramer test for multiple comparisons, and 2-way cross-tabulation with the chi-square test was used for binary variables, when appropriate, to compare differences between groups. Statistically significant differences among groups were analyzed by the Kruskal-Wallis test with Dunn's test. When S-glutathionylated proteins

Fig 1. Serum levels of S-glutathionylated proteins and high-sensitivity C-reactive protein (hs-CRP). S-glutathionylated protein levels are expressed as relative intensity compared with non-ASO patients (A), and hs-CRP ($\mu\text{g/ml}$) levels (B) were determined in 41 ASO patients (9 at Fontaine stage 1, 22 at stage 2, and 10 at stage 3) and 38 non-ASO patients. Data are indicated in box plots. Center horizontal lines indicate median values; upper and lower edges of outer boxes, 25th and 75th percentiles; and lower and upper bars, 10th and 90th percentiles. Statistically significant differences among the 4 groups by Kruskal-Wallis test with Dunn's test. * $p < 0.0001$ vs non-ASO patients; ** $p < 0.05$ vs non-ASO patients; *** $p < 0.05$. ASO, arteriosclerosis obliterans.

were undetectable by immunoblot analysis, a score of 0 was assigned. Any association between S-glutathionylated proteins and hs-CRP, total cholesterol or low-density lipoprotein (LDL)-cholesterol (C) was evaluated with Spearman's rank correlation coefficient. Logarithmic values of hs-CRP were used as variables for statistical analyses. The effect of S-glutathionylated proteins on ABI was analyzed by using a multiple linear regression model with hyperlipidemia, hypertension, smoking, diabetes mellitus, and hs-CRP as covariates. The squared multiple correlation coefficient (R^2) was calculated as a goodness-of-fit measure. Values of $p < 0.05$ were considered statistically significant.

Results

Clinical Characteristics of the Study Group

Table 1 summarizes age, gender, conventional vascular risk factors, ABI, lipid profile, and levels of hs-CRP. Pa-

tient characteristics, including age and the incidence of hypercholesterolemia and angina, were comparable between the ASO and non-ASO groups. The ratio of males to females was higher in the ASO groups. Of the risk factors, the rate of hypertension was higher at Fontaine stages 2 and 3 in the ASO groups than in the non-ASO group ($p < 0.05$), and the rate of smoking was higher at Fontaine stages 1 and 2 in the ASO groups ($p < 0.05$). There was no difference in the rate of angina between the ASO and non-ASO groups. In this study, patients with chronic renal failure on hemodialysis were excluded. The serum concentrations of total cholesterol and LDL-C were lower in the ASO groups than in the non-ASO group ($p < 0.05$).

Serum Levels of S-Glutathionylated Protein

Fig 1A shows the estimated S-glutathionylated protein levels in serum samples from the ASO groups. Statistically significant differences were found among the 4 groups (Kruskal-Wallis test). The median level of S-glutathionylated proteins was 1.06 in non-ASO patients, 2.46 at stage 1, 2.62 at stage 2, and 3.97 at stage 3. The number of males in the non-ASO group was less than in the ASO groups; however, in a preliminary study, there was no difference in the levels of S-glutathionylated proteins between the sexes (data not shown). The levels were increased at every stage of ASO compared with the non-ASO patients ($p < 0.0001$). A significant difference in the levels of S-glutathionylated proteins was observed between stages 2 and 3 ($p < 0.05$). Table 1 and Fig 1B show the serum levels of hs-CRP in the ASO groups; they were higher than in the non-ASO patients ($p < 0.0001$) and increased as the disease developed ($p < 0.05$).

Fig 2 shows a typical result of the analysis of S-glutathionylated proteins using biotin-GST. SDS-PAGE profiles did not differ between sera from non-ASO patients and sera from ASO patients under reduced (Fig 2A, lanes 2, 3) or non-reduced (lanes 4, 5) conditions. S-glutathionylated protein bands were detected more in ASO patients than in non-ASO patients under non-reduced conditions (Fig 2B). In vitro, levels of S-glutathionylated proteins were reduced weakly in the presence of the GSH/GSSG system (Fig 2C, lane 2), and strongly in the presence of the GSH/GSSG system and GRX (lane 3). This suggests that the increase in the serum levels of S-glutathionylated proteins reflects a reduced redox regulation in ASO patients. Immunoprecipitation of proteins by anti-apolipoprotein B100 (apoB100) and treatment with biotin-GST revealed that apoB100 is S-glutathionylated in ASO (Fig 2D), which suggests that the S-glutathionylation of proteins in serum involves apoB100.

The serum levels of total cholesterol and LDL-C were higher in the non-ASO patients than in the ASO patients; however, there was no correlation between the levels of S-glutathionylated proteins and those of total cholesterol or LDL-C. Similarly, the levels of S-glutathionylated proteins did not relate to the levels of triglyceride in serum (data not shown).

Relationship Between S-Glutathionylated Proteins and ABI

The relationship between S-glutathionylated proteins and ABI was analyzed using a multiple linear regression model with covariates (Table 2). The coefficient, standard error, and p-value of S-glutathionylated proteins were -0.0455 , 0.0173 , and 0.0105 , respectively. Similarly, the p-value of both hypertension and smoking was less than 0.05. The data suggest that formation of S-glutathionylated

Fig 2. Characteristics of S-glutathionylated proteins. Serum samples from a patient with ASO and another with non-ASO was subjected to 5% SDS-PAGE and analyzed using biotin-GST. (A) CBB stain. Lane 1, molecular weight standard; lane 2, non-ASO with DTT; lane 3, patient with DTT; lane 4, non-ASO without DTT; lane 5, patient without DTT. (B) Staining using biotin-GST under reducing conditions (1 and 2) and non-reducing conditions (3 and 4). (C) Effect of the GSH/GRX system on the S-glutathionylated proteins. From the serum sample of a patient with ASO 1 μ l was treated in buffer containing components of the GSH/GRX system for 30 min at 30°C. GSH/GSSG, 1 mmol/L GSH and 0.05 mmol/L GSSG; NADPH/GSSG reductase, 1 mmol/L NADPH and 1.2 units of GSSG reductase; 1 μ g of GRX (16 samples were subjected to 5% SDS-PAGE under non-reducing conditions and analyzed using biotin-GST. (D) Serum was immunoprecipitated by anti-apoB100 antibody, subjected to 5% SDS-PAGE under non-reducing conditions, and analyzed using biotin-GST. Lane 1, non-ASO patient; lane 2, ASO patient. The arrow indicates a band corresponding to apoB100 protein. ApoB100, apolipoprotein B100; ASO, arteriosclerosis obliterans; CBB, coomassie brilliant-blue; DTT, dithiothreitol; GRX, glutaredoxin; GSH, glutathione; GST, glutathione S-transferase; GSSG, the reduced form of glutathione; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels.

Table 2 Multiple Linear Regression Model for ABI

Variables	Coefficient	Standard error	p value
S-glutathionylated proteins (relative intensity)	-0.0455	0.0173	0.0105
Hypertension*	-0.189	0.0622	0.00338
Diabetes*	0.0275	0.0503	0.587
Hypercholesterolemia*	-0.0229	0.0514	0.657
Smoking*	-0.170	0.0512	0.00142
hs-CRP (ng/ml)**	-0.0513	0.0286	0.0774

*Positives are defined as 1. Negatives are defined as 0. **Values for hs-CRP were transformed in logarithm of 10. Abbreviations see in Table 1.

proteins in serum is involved in the progress of ASO.

Discussion

As to the diagnosis of ASO, several tests, such as angiography, estimations of ABI and PWV, and measurements of circulating levels of hs-CRP, have been used to

detect PAD. However, these estimations are not sufficient to predict the development of ASO in its earlier stages.

Oxidative stress is a principle cause of aging and the development of diseases such as inflammation, infection, cancer, and cardiovascular disorders.^{9,10} Exogenous or endogenous sources of oxidative stress and weakened anti-oxidative defenses can damage macromolecules such as

DNA, lipids, and proteins. The levels of molecules modified by oxidative stress can be estimated; however, there are currently no sensitive and specific methods to evaluate the oxidative stress-induced development of cardiovascular diseases.

The redox system regulates certain protein functions and protects cells from H₂O₂-induced apoptosis!¹⁶ TRX is a protein that is ubiquitously expressed in all living cells and which fulfils a variety of biological functions related to cell proliferation and apoptosis!¹⁷ Increases in serum TRX levels have been found in patients with various coronary risk factors, such as smoking, hypertension, and hypercholesterolemia!¹⁸ Increases in S-glutathionylated proteins have been found in ischemic preconditioned hearts!¹⁹ Those reports suggest that chronic oxidative stress may be involved in the progression of the coronary diseases associated with risk factors. As to the role of GSH/GRX, we previously found that the anti-apoptotic activity of Akt is regulated by the GSH/GRX system inside the cell!¹⁶ which led us to speculate that an imbalance of the redox state in serum reflects an impairment of circulatory compartments by oxidative stress, and we became interested in estimating the levels of S-glutathionylated proteins in serum as a marker for the risk of developing peripheral vascular damage. In the present study, serum levels of S-glutathionylated proteins were elevated in the earlier stages of ASO (Fig 1A). Levels of S-glutathionylated proteins in sera from ASO patients were reduced in the presence of the GSH/GRX system (Fig 2C). These results strongly suggest that during the development of ASO, chronic oxidative stress induces an imbalance of the redox state and protein thiols are oxidized in the serum of patients with ASO, although the mechanism of redox regulation to maintain the reduced form of cysteine thiols is not well understood. The application of anti-oxidant therapies, such as α -tocopherol!¹⁸ statins!²⁰ or exercise!²¹ may improve the redox imbalance and reduce the levels of S-glutathionylated proteins in ASO. If so, estimation of S-glutathionylated proteins is useful as a marker for the success of therapies and trials may be warranted.

Redox-active cysteine residues in the albumin of human serum have been reported;²² however, under the experimental conditions used in the present study, we could not identify S-glutathionylated albumin (data not shown). We found that apoB100 protein is S-glutathionylated (Fig 2D). At present, it is unclear if the thiol-modification of apoB100 affects its function.

The method we used for the estimation of S-glutathionylated proteins used biotinylated GST. However, methods using electrophoresis are neither simple nor sensitive. Attempts have been made to detect S-glutathionylated proteins by a proteomic approach using ³⁵S-labeled GSH in vitro, but this is not a convenient method!²³ Further development of a widely applicable method, such as enzyme-linked immunosorbent assay, is required for use with clinical samples.

ASO is an atherosclerotic peripheral occlusive disease. Oxidized LDL (ox-LDL) appears to play a key role in atherogenesis!²⁴ A circular Ox-LDL, a product of oxidative stress, has been reported in patients with hyperlipidemia!^{25,26} In the present study, there was no relation between lipid metabolism and levels of S-glutathionylated proteins (data not shown), and a pathological comparison of S-glutathionylated proteins with ox-LDL was not conducted.

The relationship between various risk-markers and the development of ASO was analyzed with a multiple regression model (Table 2). The data suggested that levels of

S-glutathionylated proteins in serum are a risk-marker for ASO. Similarly, cigarette smoking was found to correlate with a decrease in ABI, which is consistent with a report that smoking induces low-grade inflammation and thrombogenicity;²⁷ as well as chronic obstructive pulmonary disease!²⁸ It should be taken into account that there are many other factors influencing the progress of ASO, such as drugs, duration of accompanying diseases, genetic background, etc and the analysis here is not sufficient to rule out other risk factors; however, the increase in the serum levels of S-glutathionylated proteins may, at least in part, reflect progression of ASO.

In summary, the S-glutathionylation of proteins in serum may reflect the redox imbalance induced by oxidative stress and play a role in the development of ASO.

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