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Original Contribution

Association of body iron stores with low molecular weight iron and oxidant damage of human atherosclerotic plaques

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Abstract

The association between iron, an oxidant catalyst, and atherosclerosis is controversial. In particular, it is unknown whether: (1) stored iron, namely serum ferritin, is correlated with catalytic iron and oxidant damage of human atherosclerotic plaques; (2) catalytic iron is related to oxidative injury within such plaques; (3) plaque oxidant burden is associated with the severity of atherosclerosis. Thus, we assessed low molecular weight iron (LMWI), which represents the metal catalytically active form, together with fluorescent damage products of lipid peroxidation (FDPL) and lipid hydroperoxides (LOOH), in 38 atherosclerotic plaques surgically removed from 38 patients who had undergone selective carotid endarterectomy. In each patient, the levels of serum ferritin were measured and correlated with those of plaque LMWI and lipoperoxides by the Spearman rank correlation test with Spearman rank correlation coefficient (r_S) calculation. Moreover, in patients selected from the same study population, we compared plaque analyte levels between two groups with different severity of atherosclerotic carotid stenosis, i.e., <90% (group A, $n = 25$) or ≥90% (group B, $n = 13$), and between another two groups without (group C, $n = 27$) and with (group D, $n = 11$) associated contralateral carotid stenosis ≥50%, indicative of "extensive" and more severe atherosclerotic disease. In group A patients, serum ferritin was directly and significantly correlated with plaque LMWI ($r_S = 0.46$, $P < 0.025$) and FDPL ($r_S = 0.58$, $P < 0.005$), while its correlation with plaque LOOH, albeit direct, did not attain statistical significance. Moreover, a direct and significant relationship was evident between the plaque content of LMWI and that of both FDPL ($r_S = 0.61$, $P < 0.0025$) and LOOH ($r_S = 0.51$, $P < 0.025$), suggesting a prooxidant role of catalytic iron within human atherosclerotic plaques. Considering the 13 patients of group B, a positive and significant correlation was observed between the levels of serum ferritin and those of plaque LMWI ($r_S = 0.83$, $P < 0.0001$); on the other hand, serum ferritin, as well as plaque LMWI, showed no significant correlation with either plaque FDPL or LOOH, conceivably reflecting the small number of patients belonging to group B. Finally, plaque LMWI, FDPL, and LOOH content was significantly higher in group B than in group A, and in group D than in group C. These data suggest a role for catalytic iron in atherosclerotic plaque oxidation and in the severity of atherosclerosis, which appears indeed associated with plaque oxidant burden.

Keywords: Atherosclerosis; Ferritin; Iron; Oxidant damage

Introduction

In recent years substantial evidence has accumulated indicating that oxidant-mediated vascular injury and lipoprotein oxidation are involved in the pathogenesis of atherosclerosis [1–3]. Iron is an essential catalyst of oxidant generation leading to biomolecular oxidative damage such as lipid peroxidation [3]. To foster oxidant injury, however, iron must be in a protein-unbound free form, i.e., complexed by low molecular weight

Abbreviations: FDPL, fluorescent damage products of lipid peroxidation; FOX, ferrous oxidation-xylenol orange assay; IQR, interquartile range; LMWI, low molecular weight iron; LOOH, lipid hydroperoxides; URF, units of relative fluorescence.

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ligands like organic acids and nucleotides [3]; this catalytic iron form is so named low molecular weight iron (LMWI). On the other hand, iron is physiologically bound in a safe, redox-inactive form to proteins, such as ferritin, which is present both intracellularly and in the plasma environment [3–7]. Ferritin has received much attention as a potential prooxidant, since its iron can be pathologically mobilized and induce oxidative damage [3,8], including lipoprotein oxidation [9]. Remarkably, serum ferritin concentration is directly related to the amount of storage iron, thus basically representing an adequate measure of body iron stores [4–7]. Increased serum ferritin levels and body iron stores have been shown to represent significant risk factors of myocardial infarction and atherosclerosis owing to iron-mediated oxidative damage [7,10–15], albeit conflicting data have also been reported [7,12,16–19]. Moreover, even though most experimental studies have indicated that iron has prooxidant/proatherogenic properties [7,12,20], such properties have not always been confirmed [12]. Thus, the association between iron and atherosclerosis is still controversial; in particular, it is unknown whether: (1) stored iron, namely serum ferritin, is correlated with LMWI and oxidant damage of human atherosclerotic plaques; (2) LMWI is related to tissue oxidative injury within such advanced arterial lesions; (3) plaque oxidant burden is associated with the severity of atherosclerosis.

We have therefore studied the relationship among the levels of serum ferritin and those of LMWI and lipoperoxides of human atherosclerotic plaques surgically removed from stenosed carotid arteries in patients subjected to selective endarterctomy. A possible association between plaque oxidant burden and the severity of atherosclerosis was also investigated.

Materials and methods

Study subjects

Carotid atherosclerotic plaques were surgically removed from 38 patients (29 men and 9 women, median age 66.5 [IQR 61–71] years) scheduled for elective carotid artery endarterectomy and evaluated preoperatively also by intraarterial digital subtraction angiography. Institutional review board approval was obtained for plaque procurement, and informed consent was given by all study participants. As expected for endarterectomy specimens, plaques represented advanced fibrofatty lesions, without, however, significant calcification and/or thrombosis. Patients had no clinical or laboratory evidence of inflammation, showing indeed no leukocytosis and normal values of C reactive protein and erythrocyte sedimentation rate. There was also no clinical or laboratory evidence of hepatic diseases, as judged by normal values of liver analytes such as serum bilirubin, γ-glutamyltranspeptidase, and transaminases, and absence of hepatic structural changes at abdominal ultrasonography. Moreover, no patient had hematologic, neoplastic, infectious, and/or renal diseases, nor was there alcohol abuse, and nobody took iron or antioxidant supplements. All subjects were from the same geographical area (Chieti, Pescara; Abruzzo; Italy), and had a similar dietary pattern.

Biochemical analyses

Reagents and diagnostic kits were from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise indicated. All glassware was steeped overnight in 5 mol/L HCl to remove metal contaminants and then repeatedly rinsed in glass-bidistilled water. Solutions were prepared using Chelex 100 resin and deionized, glass-bidistilled water.

Plaque oxidant damage was evaluated assessing fluorescent damage products of lipid peroxidation (FDPL) and lipid hydroperoxides (LOOH); the former, which result from the interaction of lipoperoxidation aldehydes like 4-hydroxynonenal with biomolecular primary amino groups, are sensitive indicators of lipid peroxidation in vivo with the tendency to remain at oxidant burden sites [21–23], while the latter are primary products of oxidant-driven lipid peroxidation [3,24,25]. To extract lipids and their oxidized forms, a plaque portion was homogenized in butylated hydroxytoluene-containing chloroform/methanol (2/1, v/v), followed by addition of 0.05 mol/L NaCl solution and centrifugation at 1300 *g* to separate aqueous and chloroform phases [21–23]. An aliquot of the lower chloroform layer was dried under a stream of argon, and the resulting lipid residue resuspended in chloroform/methanol (10/1, v/v) for spectrofluorometric assessment of FDPL at 360/430 nm excitation/emission [21–23]. Another aliquot of the chloroform layer was similarly dried under a stream of argon. The resulting lipid residue was resuspended in methanol to measure total cholesterol by a commercial enzymatic kit, and LOOH by the spectrophotometric methanolic ferrous oxidation-xylenol orange (FOX) assay [24]; signal of authentic LOOH was confirmed using 2.5 mmol/L triphenylphosphine as the specific LOOH reductant [25]. Lipoperoxide values were normalized for tissue cholesterol content, expressing FDPL and LOOH as, respectively, units of relative fluorescence (URF) and nanomole LOOH per milligram cholesterol.

To assess LMWI, another plaque portion was homogenized in ice-cold 20 mmol/L [tris(hydroxymethyl)aminomethane]/HCl buffer, pH 7.9. After centrifugation at 105,000 *g*, the supernatant was subjected to ultrafiltration through Amicon 10,000 M_r cutoff filters and then treated with trichloroacetic acid before specific spectrophotometric assay with the highly sensitive iron colorimetric detector ferene S as previously reported [26]. LMWI was normalized for plaque protein concentrations, which were measured by Bradford's method [26].

Serum ferritin was determined by enzyme-linked immunosorbent assay (Boehringer Mannheim, Mannheim, Germany).

Statistics

Data are expressed as median and interquartile ranges (IQR). Correlations were studied using nonparametric statistics, namely the Spearman rank correlation test with Spearman rank correlation coefficient (r_S) calculation [27]. The Mann-Whitney *U* test was also used as appropriate [27]. A *P* value <0.05 was considered as statistically significant [27].