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Acute phase proteins and oxidised low-density lipoprotein in association with ischemic stroke subtype, severity and outcome

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Abstract

Objectives: The goal of our study was to investigate the associations of oxidized LDL (apoB100 aldehyde-modified form) and acute phase proteins (fibrinogen, CRP) with acute ischemic stroke severity and outcome.

Materials and Methods: The study included 61 ischemic stroke patients and 64 controls. Strokes were subtyped according to TOAST criteria, the severity and outcome of stroke (at 1 year) were measured.

Results: The mean triglyceride, fibrinogen, CRP and glucose values were significantly higher among cases. The median oxLDL value for patients with large artery atherosclerosis (LAA) type of stroke was significantly higher than for other subtypes. The oxLDL values did not correlate with age, stroke severity and outcome.

Conclusions: Inflammatory markers (fibrinogen and CRP) predicted the stroke severity and outcome whereas elevation of oxLDL levels did not. Our data refer to possibility that there may exist some links between the LAA subtype of stroke and elevated oxLDL (apoB100 aldehyde-modified form).

Keywords: *Lipoprotein, oxidative stress, oxLDL, stroke*

Introduction

The basic mechanism of ischemic stroke is known to be atherosclerosis. Atherosclerosis is a multifactorial, multistep disease that has recently been found to closely associate with chronic inflammation and oxidative stress [1–3]. The need for early identification and treatment of atherosclerosis is becoming more and more important [4]. Thrombotic strokes are characterized by the elevated generation of free radicals and oxidative injury, leading to the promotion of lipid peroxidation [5], also DNA and protein oxidation [6]. In addition, development of atherosclerosis and oxidative stress causes mitochondrial dysfunction which could be associated with many pathological processes, including neurodegeneration [7–8]. Recent studies have used different markers (lipid hydroperoxides, thiobarbituric acid reactive substances, oxidised low density lipoprotein (oxLDL) etc.) to measure the magnitude of oxidative stress in cerebral ischemia [5,9–13]. The modifications of LDL is now considered to be one of the key events in the biological process that initiates and accelerates the development of the atherosclerotic lesion [14]. Furthermore, Uno et al. have shown that a free radical scavenger edaravone lowers oxLDL levels and thus inhibits brain damage in stroke [15]. However, the role

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of elevated oxLDL and its role (especially the role of its specific forms) in ischemic stroke pathogenesis and outcome remains to be elucidated.

It is known that inflammation is both a risk factor and consequence of stroke and a source of oxidative stress in this disease process [1]. Similarly, elevated levels of C-reactive protein (CRP) and fibrinogen are known to be associated with inflammation and atherosclerosis [1–2,16–18].

The goal of our study was to investigate the associations of oxLDL (a marker of systemic oxidative stress and atherosclerosis in cardiovascular disease), via assessment of the apoB100 aldehyde-modified form of oxLDL, and acute phase proteins (via determination of fibrinogen and CRP) with the ischemic stroke risk factors, stroke severity, subtype and outcome in the acute phase of stroke and compare to this information with healthy controls.

Subjects and methods

Study population

Cases. Patients, less than 70 years of age, consecutively admitted to Tartu University Clinics' Department of Neurology and Neurosurgery between March 2002 and September 2003, suffering from first-ever ischemic stroke were included in the study. Stroke was defined according to WHO criteria. All patients underwent brain computerised tomography scan on admission.

Baseline demographic data, history of risk factors, concomitant diseases and smoking status were obtained.

For the classification of ischemic stroke subtype, the TOAST criteria [19] were used: (1) *large-artery atherosclerosis* (LAA); (2) *lacunar stroke* or *small-artery occlusion*; (3) *stroke of other determined cause*; (4) *stroke of undetermined cause*. Patients with cardioembolic strokes were excluded.

The Scandinavian Stroke Scale (SSS) [20] was used to assess neurological deficit at certain time points after stroke. It was performed by a study neurologist on admission (SSS0) and on the 7th day (SSS7) in hospital.

Outcome of patients was assessed using the Barthel Index (BI; score 0 to 20) at the mean of 15 months after stroke with a questionnaire sent by mail [21].

Blood samples were drawn approximately after a week from stroke onset.

Controls. The control population consisted of 64 healthy middle-aged subjects in the age range 40–65 years who were recruited as volunteers from general population. Men and women were agitated to participate in the study where their health was thoroughly evaluated. All subjects passed a medical evaluation including a complete history and physical examination, electrocardiography and blood tests. Subjects who suffered from overt coronary artery disease, valve pathologies, arterial hypertension, cerebral and/or peripheral atherosclerotic disease, diabetes, malignancies, chronic degenerative diseases, endocrine pathologies, were excluded.

Blood samples for oxLDL analyses (collected after an overnight fast) were placed in vacuum tubes containing EDTA. Plasma was separated by centrifugation at 3000*g* for 15 min, within 30 min of venipuncture and stored at −25°C until analysis. OxLDL levels were measured using an enzyme-linked immunosorbent assay kit (Mercodia, AB, Uppsala, Sweden). OxLDL levels were measured using an enzyme-linked immunosorbent assay kit (Mercodia, AB, Uppsala, Sweden). This kit uses monoclonal antibody 4E6 [22,23] directed against a conformational epitope in the Apo B100 moiety of LDL that is generated as a consequence of substitution of at least 60 lysine residues of Apo B100 with aldehydes. This number of substituted lysines corresponds to the minimal number required for scavenger-mediated uptake of oxidized LDL. Substituting aldehydes can be produced by peroxidation of lipids of LDL, resulting in the generation of oxidized LDL. However, lipid peroxidation is not required. Indeed, aldehydes that are released by endothelial cells under oxidative stress or by activated platelets may also induce the oxidative modification of Apo B100 in the absence of lipid peroxidation of LDL. Kit's arbitrary upper reference limit is 117 U/L.

Other blood analyses included total cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), fibrinogen and CRP and were conducted immediately. Lipid levels were measured by the Hitachi 912 analyser. Plasma LDL cholesterol and HDL cholesterol (Roche Diagnostics, Germany), total cholesterol (Human, Germany), and triglycerides (Biocon, Germany) were measured. Fibrinogen was measured by clotting method after Clauss using the Stago Compact analyser (Diagnostica Stago, France). CRP was determined by a validated high-sensitivity assay by using a latex particle-enhanced immunoturbidimetric assay (Roche Diagnostics GmPh, Germany) with the automated analyser Hitachi 912.

Reference values of other markers were as follows: total cholesterol < 6.5 mmol/L, triglycerides < 2.3 mmol/L, HDL > 1 mmol/L, LDL < 3.3 mmol/L, fibrinogen < 4 g/L and CRP < 5 mg/L.

The diagnostic criteria for hypertension were blood pressure $> 140/90$ mmHg in three consecutive measurements or the use of antihypertensive medication. Diabetes was diagnosed as fasting blood sugar values > 5.5 mmol/L in repeated measurements. The diagnoses of cardiac diseases were based on case history and on the results of electrocardiogram and/or echocardiography. Cigarette smoking was considered as risk factor in current smokers.

None of the patients used vitamin and/or iron supplementation prior to stroke.

All patients and control subjects gave informed consent before entering the study. The project was

Table I. Main characteristics of ischemic stroke patients ($n = 61$) included in the study.

Mean age, years (range)	59.0 (30–70)
Men, *n* (%)	41 (67%)
History of	
Hypertension, *n* (%)	32 (52%)
Diabetes, *n* (%)	9 (15%)
Ischemic heart disease, *n* (%)	17 (28%)
Current smoker, *n* (%)	21 (34%)
Median SSS 0	44 (MAD ± 11)
Median SSS 7	54 (MAD ± 5)
Diagnosis	
Large-artery atherosclerosis, *n* (%)	22 (36%)
Lacunar stroke, *n* (%)	21 (34%)
Stroke of undetermined cause, *n* (%)	16 (26%)
Stroke of other determined cause, *n* (%)	2 (4%)

approved by the Ethics Review Committee on Human Research of the University of Tartu.

Statistical methods

For the variables of interest, the mean (in case of normal distribution) or median and standard deviation (SD) or median absolute deviation (MAD) were calculated separately for the control and case group. When appropriate, the 95% confidence interval (CI) was added. The T-test and Wilcoxon ranksum test were used to compare the values of controls and cases. For oxLDL linear models were considered with different sets of explanatory variables. Spearman and Kendall rank correlations and their *p*-values were found to study association between variables. All calculations were done using R [24] and SAS [25].

Results

Cases

A total of 61 patients with first-ever ischemic stroke were included. Patient characteristics are shown in Table I. Two patients (3%) died within 28 days of stroke onset.

Blood analyses. The mean time of blood sample collection was 5.7 (SD ± 2.4) days from the onset of stroke.

OxLDL. The results for biochemical markers are shown in Table II. A total of 48% patients (18 men and 11 women) had oxLDL levels above the reference value. Twenty-two men (55%) and 10 women (48%) had plasma oxLDL levels in a normal reference range (< 117 U/L). The median plasma oxLDL value for hypertensive patients (121 U/L) was slightly higher compared to other patients (113 U/L), but this finding was not statistically significant ($p = 0.3$). The median oxLDL value (134 U/L) for patients with LAA type of stroke was significantly higher compared to patients with other types of ischemic stroke (113 U/L; $p = 0.01$). The levels of oxLDL were not statistically significantly different when the samples taken during the first 5 days and samples taken later than 5 days were compared ($p = 0.91$). The oxLDL values did not correlate with age, stroke severity or outcome.

Other analyses. All the mean (or median) triglyceride, LDL and CRP levels were above the reference range (Table II). Six patients (10%) had a plasma total cholesterol value above 6.5 mmol/L, whereas 31 patients (51%) had elevated LDL and 22 patients (36%) had HDL levels < 1 mmol/L. The mean LDL/HDL ratio was 3.3 (SD ± 1.2). High triglyceride values were found in 19 patients (31%). Triglyceride levels correlated positively with stroke severity ($r = 0.3$; $p = 0.05$). Twenty nine (48%) of the patients had elevated CRP levels and high fibrinogen concentration was detected in 24 patients (39%). Fibrinogen levels were negatively correlated with the SSS0 score ($r = -0.2$; $p = 0.01$).

Stroke outcome data is available for 52 (85%) patients. The mean time of assessment was 15 ± 6 (12–28) months. The mean BI score was 13 ± 8 points (mode = 20). The BI scores correlated positively with SSS7 ($r = 0.7$; $p < 0.001$). There was

Table II. Fasting plasma mean (± SD) or median (± MAD)⋆ values of analyzed biochemical markers in ischemic stroke patients and control subjects.

	Men		Women		Total	
	Cases $n = 40$	Controls $n = 42$	Cases $n = 21$	Controls $n = 22$	Cases $n = 61$	Controls $n = 64$
OxLDL, U/L⋆	116 ± 36	110 ± 28	124 ± 20	109 ± 30	117 ± 31	110 ± 28
Fibrinogen, g/L⋆	3.9 ± 0.8	3.0 ± 0.4†	3.7 ± 0.5	3.1 ± 0.3†	3.8 ± 0.7	3.0 ± 0.3†
CRP, mg/L	10.4 ± 24	1.7 ± 1.6†	9.0 ± 11.0	1.9 ± 2.7†	10.0 ± 20.5	1.7 ± 2.0†
Cholesterol, mmol/L	5.1 ± 1.2	5.3 ± 0.9	5.4 ± 0.8	5.5 ± 1.0	5.2 ± 1.1	5.4 ± 1.0
LDL, mmol/L	3.2 ± 1.0	3.5 ± 0.8	3.5 ± 0.8	3.4 ± 0.8	3.3 ± 0.9	3.4 ± 0.8
HDL, mmol/L	1.0 ± 0.3	1.4 ± 0.3	1.3 ± 0.4	1.7 ± 0.4	1.1 ± 0.4	1.5 ± 0.4
Triglycerides, mmol/L⋆	1.8 ± 0.6	1.0 ± 0.3†	1.7 ± 0.5	0.9 ± 0.3†	1.8 ± 0.6	1.0 ± 0.4†

† Significant difference between cases and controls ($p < 0.05$).

no correlation between oxLDL values with BI or SSS7. The patients with lower CRP values had better outcome compared to those with higher values ($r = -0.5$; $p = 0.001$). Similar correlation was found between fibrinogen and BI ($r = -0.3$; $p < 0.001$). Other studied parameters (triglycerides, total cholesterol, stroke subtype and concomitant diseases) had no significant affect on stroke outcome.

Controls. Sixty-four control subjects with mean age of 53.2 (SD ± 7.4) were assessed. Forty-two of them (66%) were men and 22 (34%) were women. All controls demonstrated normal findings at physical and biochemical examinations, and had normal BP values. Seven subjects (11%) were current smokers.

OxLDL. No differences in oxLDL levels between sexes were found ($p = 0.7$; see Table II). Twenty-four men (57%) and 14 women (64%) had plasma oxLDL levels in a normal range. A total of 41% patients (18 men and 8 women) had oxLDL levels above the reference value. The oxLDL values did not correlate with age.

Other analyses. All the mean (or median) blood values of biochemical markers are presented in Table II. The majority of other blood markers were within the normal range. Seven subjects (11%) had plasma total cholesterol value above 6.5 mmol/L, while 33 patients (52%) had abnormal LDL and 3 patients (5%) had HDL levels < 1 mmol/L. The mean LDL/HDL ratio was 2.5 (SD ± 0.9). High triglyceride values were found only in two subjects (3%). Three subjects (4%) had elevated CRP levels and high fibrinogen concentration was detected in one patient (2%).

Cases vs. controls. The LDL/HDL ratio was significantly higher in stroke patients ($p < 0.0001$). The oxLDL values did not differ between cases and controls. OxLDL levels both for patients and for controls correlated positively both with total cholesterol and with LDL values ($r = 0.53$; $r = 0.55$; $p < 0.001$).

The linear regression models showed that increased glucose, cholesterol and fibrinogen levels were related to higher oxLDL values. The multiple linear regression model showed that higher triglyceride (OR = 20.1; 95%CI 2.1–148.5), CRP (OR = 1.5; 95%CI 1.0–2.3) and fibrinogen (OR = 4.6; 95%CI 1.2–18.0) levels are associated with the increased risk of stroke. The mean triglyceride, fibrinogen, CRP and glucose values were significantly higher among patients ($p < 0.001$). The concomitant diseases did not affect the levels of studied parameters in linear regression analysis.

Discussion

We have conducted a case-control study that shows significant differences in triglyceride, CRP and fibrinogen concentrations, but not in median oxLDL (the aldehyde-modified form) values between control subjects and acute stroke patients less than 70 years of age.

A recent meta-analysis has clearly shown that oxLDL is a suitable marker for identifying patients at risk for cardiovascular disease [4]. Uno et al. have shown that acute ischemic stroke patients have two times higher oxLDL levels compared to control subjects and that the peak rise in plasma oxLDL was on the 3rd day following stroke and not correlated with the severity of stroke [5]. In their recent study, an association of elevated oxLDL levels and increased infarct size were shown and also an association with stroke severity was detected [12]. Our goal was to investigate the role of oxLDL at a more conditionally subacute period of stroke (5–6 days). The significantly higher oxLDL values of stroke patients compared to controls was not confirmed in our sample. But similarly to the study by Faviou et al. [14], the oxLDL levels correlated positively both with LDL and total cholesterol values. The mean time of oxLDL determination was 5.7 days and therefore we could have missed the peak concentration reported by Uno et al. On the other hand, Polidori et al. showed that the peak concentration of lipid peroxidation index occurred on day 5 following stroke and it was positively correlated with the severity of stroke [10]. Forty-one percent of our healthy controls had elevated oxLDL values which could indicate a relevant problem considering the pathogenesis of atherosclerosis in the whole population. This could be associated with high incidence and case-fatality of stroke in our country [26]. In addition, we showed that patients with LAA type of stroke had significantly higher oxLDL values compared to other subtypes. This has also been shown by other studies [5,9]. The values did not depend on concomitant risk factors of stroke patients. The systemic review conducted by Lobbes et al. [4] showed, that the relative risk of death and coronary events increases with increasing levels of oxLDL (OR 1.9–3.2) according to some case-control and cohort studies. Our follow-up period was shorter compared to previous studies and the levels of oxLDL (the apoB100 aldehyde-modified form) in our study did not predict stroke outcome.

The elevation of CRP and fibrinogen in stroke patients is anticipated and related to the systemic changes and the development of ischemic brain damage. The role of CRP in stroke has been evaluated both in cohort [18] and cross-sectional [17,27]

studies. Elevated CRP levels in healthy subjects increase the risk of stroke by two to three times and higher levels in stroke patients predict poor outcome [1,17,18,27,28]. Meta-analysis of several studies indicates two patterns of CRP in stroke patients: gradually decreasing and gradually increasing levels. This finding suggests that CRP is not only associated with the acute phase of stroke but also indicates persistent inflammation [1].

Fibrinogen participates in clot formation by stimulating the adhesion of leucocytes to the vascular endothelium via the mechanisms of expression of intracellular adhesion molecules [1] and is the main determinant of blood viscosity [29]. Thus, this glycoprotein has a major role in the pathogenesis of cardiovascular diseases representing the inflammatory component of atherosclerosis [1,2]. The persistent enhanced levels of fibrinogen in stroke survivors support the idea of the link between inflammation and atherosclerosis [1,2]. In our study sample only 1 control subject had an elevated fibrinogen level compared to 24 patients. Both high fibrinogen and CRP levels were associated with unfavourable outcome at 1 year in our study sample. Similar findings were also reported by Di Napoli et al. [28]. Moreover, both markers increased the risk of stroke and fibrinogen levels, also correlated with stroke severity, in our study sample. As we studied patients with acute stroke, it is not known to what extent the acute phase accounts.

Two studies have demonstrated that lower triglyceride values in stroke patients predict poor outcome and more severe stroke [30,31]. In our sample, we found a weak positive correlation between stroke severity and triglyceride levels and no association of triglyceride levels with the outcome of stroke.

One possible limitation of our study could be the age-difference of cases and controls. Nevertheless, in none of the multiple regression models age was found to be in an association with the studied biochemical markers. Moreover, the reference values of biochemical markers are the same regardless of patient's age.

It can be concluded that conventional inflammatory markers (CRP and fibrinogen) are important factors predicting the severity and outcome of stroke. At the same time a certain elevation of oxLDL (the apoB100 aldehyde-modified form) levels did not act as a direct sign for first-ever ischemic stroke severity and outcome. Hence, we found that there may exist some links between the LAA subtype of ischemic stroke and elevated oxLDL levels. We chose to use the validated ELISA method [22,23] for the detection of plasma oxLDL in stroke patients. It is possible that evidently some other form(s), not the apoB100 aldehyde-modified form, of LDL reflects directly severity and outcome of acute ischemic stroke. Thus, the role of oxLDL, especially its different forms, as a possible acute stroke-linked marker of systemic oxidative stress must be thoroughly identified in further studies assessing stroke severity, subtype and outcome.

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