

Abnormal levels of serum anti-elastin antibodies in patients with symptomatic carotid stenosis



Plamen Tzvetanov^a, Vish Hegde^a, Jasem Y. Al-Hashel^c, Milena Atanasova^b, Aman P.S. Sohal^a, Rossen T. Rousseff^{c,*}

^a Department of Neurosciences, University Hospitals, Coventry and Warwickshire NHS Trust, Clifford Bridge Road, Coventry CV2 2DX, UK

^b Department of Biology, Medical University of Pleven, Kliment Ohridski st 1, Pleven 5800, Bulgaria

^c Department of Neurology, Ibn-Sina Hospital, pob 25427, Safat 13115, Kuwait

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ABSTRACT

Background and objective A correlation between the levels of antibodies to alpha-elastin (alpha-AEAb) and tropoelastin (tropo-AEAb) and the corresponding peptide concentration is found in human serum in health and disease. Serum elastin peptide and anti-elastin antibodies (AEAb) levels are age-related and vary with the stages of atherosclerotic vascular damage. This study aims to determine if elastin metabolism (assessed by the ratio of tropo-AEAb to alpha-AEAb) differs in patients with symptomatic carotid stenosis versus subjects with asymptomatic stenosis.

Patients and methods: Alpha-AEAb and tropo-AEAb were measured by ELISA in blood sera of 65 patients with ultrasound verified high-grade symptomatic carotid stenosis (resulting in stroke 1–7 days before measurement) compared to 51 patients with asymptomatic stenosis.

Results: Serum anti-alpha-elastin IgG levels are extremely increased in symptomatic versus asymptomatic carotid stenosis. The ratio of tropo-AEAb (reflecting elastin synthesis) to alpha-AEAb (a function of elastin degradation) was 3.7 in symptomatic stenosis versus 14.2 in asymptomatic stenosis ($p < 0.001$).

Conclusions: There is a significant difference in elastin metabolism in patients with symptomatic carotid stenosis versus asymptomatic stenosis. The ratio of tropo-AEAb to alpha-AEAb as an index of elastin synthesis/degradation proves useful in investigation of atherosclerotic lesions and may represent a new immunologic marker for carotid plaque destabilization.

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1. Introduction

Elastin is a major component of the extracellular matrix. Changes of its metabolism are involved in pathophysiology of destructive lesions of elastin-rich organs, such as blood vessels, kidney, skin and lungs. Elevation of serum elastin-derived peptides (EDPs) levels is observed in emphysema, abdominal aortic aneurysm and atherosclerosis [1–3].

Mature atherosclerotic lesions develop a fibrous cap composed of dense extracellular matrix containing collagen and elastin. Degradation of elastin in arterial walls is a characteristic feature of atherogenesis [4,5]. The products of degradation are EDPs that have been detected and quantified in circulating blood [3,6]. Their serum levels are increased only in ulcerative, but not in occlusive atherosclerotic lesions [3].

Serum EDPs are immunogenic and provoke the synthesis of anti-elastin antibodies (AEAbs) [6]. These secondary immune and

inflammatory responses to elastin might lead to further elastinolysis and production of more EDPs triggering a vicious circle which causes further degradation of the fibrous cap [4,5].

We undertook this study in an attempt to compare elastin turnover in stroke patients with symptomatic carotid stenosis (resulting in stroke 1–7 days before measurement) versus subjects with asymptomatic stenosis. We measured the absolute levels of serum tropo-AEAb (that correlates with elastin synthesis) and alpha-AEAb (that parallels with elastin degradation) and used the ratio of tropo-AEAb to alpha-AEAb as an index of elastin metabolism.

2. Patients and methods

2.1. Patients

The study enrolled 116 patients (mean age: 62.5; SD: 11.3; range: 28–85 years; 53 women) with ultrasound verified internal carotid stenosis as a part of research project. The study procedures are approved by a Local Ethic Committee. All the patients

* Corresponding author. Tel.: +96566379970; fax: +96524948226/96524849226.
E-mail address: rossentrouseff@yahoo.co.uk (R.T. Rousseff).

or their representatives gave informed consent for participation in the study in accordance with the provision of Helsinki Declaration.

Exclusion criteria were infection, systemic diseases, neoplasm, emphysema and pneumonia.

All patients underwent CT investigations on a Siemens Somatom ARC. According to the protocol of our stroke unit, persons with normal CT on entry were repeatedly scanned between 3rd and 7th hospital day.

At the initial evaluation, colour duplex scan was performed by 2 operators using Phillips SONOS 5500 equipment. The patients and control subjects underwent colour duplex carotid scan on the right and left common and internal carotid arteries in a supine position. High resolution B-mode, colour Doppler and pulsed-wave Doppler were done with an ultrasound linear array 5–10 MHz transducer.

In accordance with the consensus for Doppler ultrasound criteria for the diagnosis of internal carotid stenosis [7], carotid stenosis severity was classified into: significant (>70%) identified by a peak systolic velocity >230 cm/sec and non-significant (<50%) with a peak systolic velocity <125 cm/sec. Plaque morphology was assessed using the modified Gray-Weale's criteria [8]. According to their structural appearance plaques were classified as heterogeneous and homogeneous [9]. Plaque surface morphology was also assessed and classified as regular (smooth) or irregular [10].

Clinical characteristics and biochemical parameters of patients are shown in Table 1.

According to clinical and imaging criteria [11–13] patients were divided into two groups.

The symptomatic stenosis group included 65 patients (mean age: 55.7; SD: 9.7; range: 28–74 years; 28 women) with clinically diagnosed and CT proven stroke involving the homolateral brain hemisphere. The asymptomatic stenosis group included 51 subjects without brain infarction (mean age: 54.8; SD: 14.6, range: 22–80 years, 25 women). In 15 patients the stenosis was significant. Ten of patients had multiple non-significant stenosis of extracranial arteries.

2.2. Measurement of anti-elastin antibodies

In symptomatic stenosis group, serum samples were obtained between days 1 and 7 (mean 3 days) after the strokes.

Using the method described by Mechem and Lange [14], alpha-elastin was prepared from human cadaver aortas. Tropoelastin was prepared from porcine aorta and purified from copper-deficient swine by modified method proposed by Sandberg et al. [15].

Detection of alpha-AEAb and tropo-AEAb in serum samples was carried out by enzyme-linked immunosorbent assay (ELISA). Ninety six microliter well plates have been coated with 100 μ l of alpha- or tropoelastin antigens which were dissolved in a carbonate buffer (pH 9.6) at a concentration of 15 μ g/ml. Then 100 μ l of 0.1% bovine serum albumin in phosphate buffered saline (PBS, pH 7.2) was added for blocking the unoccupied binding sites and placed in adjoining wells as a control for each antigen, and 100 μ l carbonate buffer was added as an assay control. The plates were sealed and incubated overnight at 4 °C. The plates were washed three times with PBS-Tween (0.05%) before adding 100 μ l of patient serum diluted at 1:100 with PBS that contained 10% heat inactivated fetal calf serum. Phosphate buffered saline buffer (100 μ l) was added to all control wells. Plates were sealed and incubated overnight at 4 °C. The wells were washed three times with PBS-Tween. Goat anti-human IgG alkaline phosphatase (Sigma; St. Louis, MO) secondary Ab was diluted with PBS-Tween at 1:1000 and added to all wells and incubated at 37 °C for 1 h. The wells were again washed three times with PBS-Tween and then 100 μ l of p-nitrophenyl phosphate (substrate) made up in diethanolamine buffer (pH 9.6) at a concentration of 1 mg/ml, was added to all wells and incubated at a room temperature for 15 min to 2 h depending on maximum colour

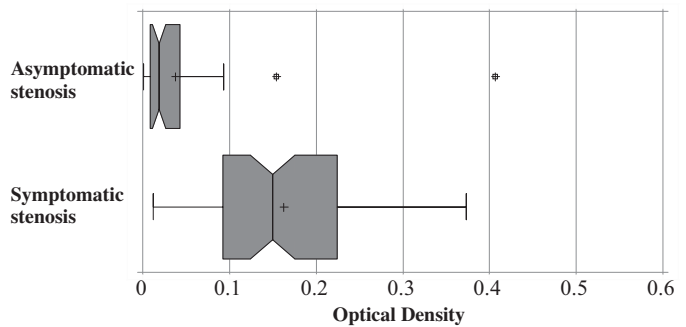


Fig. 1. Serum levels of anti-alpha-elastin antibodies in asymptomatic and symptomatic carotid stenosis.

development. The results were recorded as absorbance A405 nm for the conversion of the substrate to p-nitrophenol expressed as the difference between the test sample and the serum response to carbonate buffer alone. All sera were assayed simultaneously on a Titertek Multiskan MC ELISA reader. The specificity of the immunoconjugates was verified using a competitive version of this ELISA [16]. Optical density readings were selected from the linear portion of the time curve for colour development of alkaline phosphatase.

2.3. Statistical methods

The Statgraphic Plus Version 2.1 statistical system was used for data analysis. For categorical data the χ^2 and Kruskal–Wallis tests and one-way ANOVA for dispersion analysis were applied. For numerical data along with descriptive methods we used regression analysis (Pearson's r correlation coefficient). Difference between diseased and controls were considered significant for P values less than 0.05.

3. Results

Comparison of clinical, demographical and biochemical parameters between the patient groups is presented in Table 1.

Thirty five (54%) symptomatic patients had significant carotid stenosis, while the remaining 30 patients (46%) had multiple non-significant stenotic changes of extracranial arteries. Six patients (9.6%) had large infarctions (with involvement of at least 80% of the superficial territory or superficial/deep lesions) and the rest 59 patients (90.4%) had non-large infarction [11,12].

According to the Oxford Community Stroke Project classification [13] 5 of the symptomatic patients (8%) suffered total anterior circulation stroke, 46 (71%) had partial anterior circulation strokes, and 14 (21%) had lacunar strokes.

In 15 (30%) of asymptomatic patients the stenosis was significant. Ten (19%) patients had multiple non-significant stenoses of extracranial arteries.

ELISA-measured levels of antibodies to alpha-elastin were significantly higher ($p < 0.0001$) in symptomatic patients than in asymptomatic patients (Fig. 1). There was no difference in serum levels of anti-tropoelastin antibodies between the two groups (Fig. 2).

No significant correlation was found between either the number or the size of ischemic lesions and the levels of anti-elastin antibodies ($p > 0.05$).

The ratio of synthesis to degradation was 14.2 in patients with asymptomatic stenosis and 3.7 in symptomatic stenosis ($p < 0.001$) (Table 2). The abnormally lower ratio reflects the increased degradation of elastin fibers in patients with symptomatic carotid stenosis.

Table 1
Demographical, clinical and biochemical patient parameters.

	Asymptomatic stenosis (n = 51)	Symptomatic stenosis (n = 68)	P
Age (years)	54.8 ± 14.6	55.7 ± 9.6	NS
Male n (%)	23 (45.1)	44 (67.7)	0.001
Systolic arterial pressure (mmHg)	152 (24.1)	158 (32.8)	0.08
Duration of hypertension (months)	130 (108–240)	120 (60–240)	NS ^a
Diabetes mellitus type I n (%)	9 (17.7)	18 (29)	NS
Diabetes mellitus type II n (%)	42 (82.4)	44 (71)	NS
Duration of diabetes (years)	8 (6–11)	13 (8–30)	NS ^a
Canadian scale	–	9.9 ± 2.6	–
Rankin	–	2 (1–4)	–
MMS	–	20 ± 7.0	–
Previous stroke n (%)	2 (3.9)	25 (40)	0.0001
Smoking (cigarettes/day)	3 (0–20)	0 (0–4)	0.005 ^a
Alcohol consumption (g/week)	0 (0–350)	50 (0–350)	0.04 ^a
BMI	28.7 ± 5.6	27.3 ± 6.6	0.08
Total cholesterol (mg/dl)	5.0 ± 1.7	5.2 ± 1.8	NS
LDL cholesterol (mg/dl)	3.1 ± 1.3	3.5 ± 1.4	NS
Triglycerides (mg/dl)	1.7 (1.1–2.4)	1.5 (1.1–2.1)	NS ^a
CRP (mg/dl)	0.8 (0.5–4.4)	2.5 (0.7–10)	0.0001

^a Kruskal-Wallis Test with non-normal distribution; n, number; NS, non-significant; BMI, body mass index; MMS, mini mental state examination scale.

Table 2
Ratio of synthesis to degradation in asymptomatic and symptomatic carotid stenosis.

Subjects	Number	Anti-tropo-elastin Abs	Anti-alpha-elastin Abs	Ratio ^a
Asymptomatic stenosis	47	M.O.D. ± SD 0.524 ± 0.19	M.O.D. ± SD 0.037 ± 0.06	14.2
Symptomatic stenosis	63	0.596 ± 0.28	0.163 ± 0.09	3.7
P-Value		P > 0.05	P < 0.0001	P < 0.001

^a Ratio of synthesis (anti-tropo-elastin) to degradation (anti-alpha-elastin) Abs; M.O.D. = mean optical density; SD = standard deviation.

4. Discussion

Our study detected a significant elevation of serum alpha-AEAb in patients with carotid stenosis leading to stroke, compared with asymptomatic stenosis patients. Moreover, the plaque destabilisation is not accompanied by significant change in elastin synthesis as the tropo-AEAb serum levels did not differ between the two groups. The significant decrease in the ratio of anti-tropoelastin to anti-alpha-elastin antibodies reflects the prevalence of elastin degradation over elastin synthesis in patients with symptomatic stenosis.

Although the degree of lumen obstruction is still a marker of stroke risk [17–19], there is increasing evidence that plaque vulnerability plays more important role in the pathogenesis of atherothrombotic stroke [5]. The carotid artery plaque which is responsible for acute ischemic events has a thin fibrous cap, a large lipid pool and macrophage-dense inflammation on or beneath its surface [20,21]. The fibrous cap of more mature atherosclerotic lesions is composed by extracellular matrix proteins containing collagen and elastin [23]. These extracellular matrix molecules are

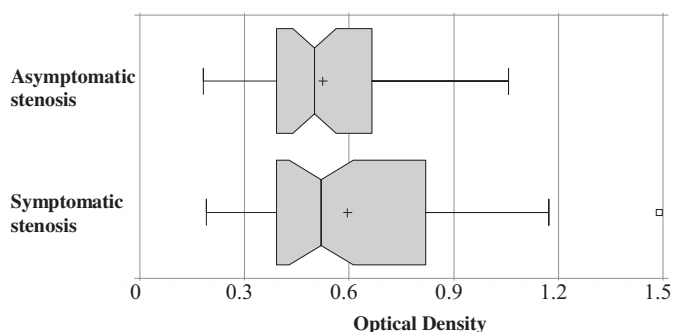


Fig. 2. Serum levels of anti-tropo-elastin antibodies in asymptomatic and symptomatic carotid stenosis.

produced by smooth muscle cells that migrate from the tunica media through the internal elastic lamina into the intima. However, the myocytes together with activated white cells also release mediators and proteinases that degrade extracellular matrix in the plaque's fibrous cap [4,23].

Elastases break down elastin and products of this degradation – elastin-derived peptides (EDPs) appear in the blood serum [24] of patients with a dilatatory or/and an ulcerative but not with an occlusive manifestation of atherosclerosis [3]. Serum concentrations of EDPs correlate with the intensity of the elastinolysis in atherosclerotic lesions of the vasculature [24,25] and were shown to possess several important biological properties as chemotactic activity to monocytes and fibroblasts [26], activation of ion fluxes [27], and induction of AEAb synthesis [6].

On the other hand abnormal neovascularisation associated with an increase of tropoelastin levels [28] is considered to precipitate plaque rupture [4,5]. This is confirmed by a post-mortem study [22] which found inflammatory infiltrate and microvascular network in the arterial wall of the symptomatic carotid artery plaque.

Elastin antigenicity and circulating anti-elastin antibodies were discovered by Stein et al. [29] in 1965 and confirmed by several immunological studies [6,30,31]. Tropoelastin is a precursor molecule of the insoluble mature elastin which converts into soluble alpha-elastin by oxalic acid hydrolysis. On the surface of the elastin molecule two major sites have been recognised as antigen determinants; one species-specific and a second site which has broad cross-species reactivity [30].

Elastin synthesis and degradation in tissue and body fluids can be measured with antisera against both tropoelastin and alpha-elastin [30]. Both alpha-AEAb [6] and tropo-AEAb [31] levels in human serum are age-related. The highest antibody levels were found in the neonatal and prepubertal periods [6,31].

The ratio of serum tropo-AEAb to alpha-AEAb reflects the balance between elastin synthesis and degradation. It may be used as an indicator of elastin turnover in systemic lupus erythematosus, systemic sclerosis, fibromyalgia and polymyalgia rheumatica

[32,33]. To the best of our knowledge this study is the first application of ELISA for measuring the elastin turnover in patients with symptomatic carotid stenosis.

The present study has some limitations in view of its retrospective design and the definition of carotid stenosis as symptomatic based on the presence of homolateral CT-documented brain ischemia. Could ischemic stroke itself lead to changes in elastin turnover? Our previous results established only a mild activation of elastin degradation in patients with recurrent strokes [34]. Besides, the serum samples were obtained early after stroke, while the elevated alpha-AEAb belong to the IgG class, consistent with prolonged antigen stimulation preceding the ischemic lesion. On these grounds we believe that the increase of serum alpha-AEAb is an index of atherosclerotic vascular lesion rather than an epiphenomenon of stroke.

The patient groups differ significantly in gender, but to the best of our knowledge there are no literature data on the impact of gender on serum elastin metabolites.

A further study in a prospective population-based setting seems warranted to avoid the weaknesses of our current design.

In conclusion, this study is the first application of ELISA for assessment of elastin turnover in patients with symptomatic versus asymptomatic carotid stenosis. A significant increase in serum alpha-AEAb is observed in symptomatic cases. The measurement of ratio of elastin synthesis (tropo-AEAb) to elastin degradation (alpha-AEAb) may provide a useful clinical tool for investigation of atherosclerotic lesions and a new immunologic marker for carotid plaque destabilization.

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