International Journal of Cardiology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Serum levels of osteopontin predict major adverse cardiovascular events in patients with severe carotid artery stenosis

Federico Carbone [a,*,1,2,3], Fabio Rigamonti [b,1,2,3], Fabienne Burger [c,3,4], Aline Roth [c,3,4], Maria Bertolotto [a,3,4], Giovanni Spinella [d,3,4], Bianca Pane [d,3,4], Domenico Palombo [d,3,4], Aldo Pende [a,3,4], Aldo Bonaventura [a,3,4], Luca Liberale [a,e,3,4], Alessandra Vecchié [a,3,4], Franco Dallegri [a,f,3,4], François Mach [b,c,3,4], Fabrizio Montecucco [a,f,g,2,3]

[a] First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa School of Medicine, 6 viale Benedetto XV, 13132 Genoa, Italy
[b] Division of Cardiology, Department of Medical Specialties, Geneva University Hospitals, 4 rue Gabrielle-Perret-Gentil, 1211 Geneva, Switzerland
[c] Division of Cardiology, Foundation for Medical Researches, Department of Medical Specialties, University of Geneva, 64 Avenue de la Roseraie, 1211 Geneva, Switzerland
[d] Vascular and Endovascular Surgery Unit, Department of Surgery, Ospedale Policlinico San Martino, 10 Largo Benzi, 16132 Genoa, Italy
[e] Center for Molecular Cardiology, University of Zürich, 12 Wagistrasse, 8952 Schlieren, Switzerland
[f] Ospedale Policlinico San Martino, 10 Largo Benzi, 16132 Genoa, Italy
[g] Centre of Excellence for Biomedical Research (CEBR), University of Genoa, 9 viale Benedetto XV, 16132 Genoa, Italy

ARTICLE INFO

Article history:
Received 29 June 2017
Received in revised form 1 December 2017
Accepted 2 January 2018
Available online xxxx

Keywords:
Atherosclerosis
Osteopontin
Macrophage
Inflammation

ABSTRACT

Background: Inflammatory mediators in the blood stream and within plaques are key determinants in atherogenesis. Here, we investigated serum osteopontin (OPN) as a potential predictor of poor outcome in patients with severe carotid atherosclerosis.
Methods: Carotid plaques and serum were collected from patients asymptomatic ($n = 185$) or symptomatic ($n = 40$) for ischemic stroke. Plaques were stained for lipids, smooth muscle cells, neutrophils, M1 and M2 macrophage subsets and matrix metallopropteinase-9 (MMP-9). Serum levels of OPN and interleukin-6 (IL-6) were determined by colorimetric enzyme-linked immunosorbent assays.
Results: Symptomatic patients showed a two-fold increase in serum OPN levels. In both symptomatic and asymptomatic patients, OPN levels positively correlated with intraplaque count of neutrophils, total macrophages, and MMP-9 content. In asymptomatic patients, OPN levels also positively correlated with lipids and M1 macrophage subsets. Receiver operating characteristic curve analysis identified serum OPN concentration of 70 ng/ml as the best cut-off value to predict major adverse cardiovascular events (MACEs). Patients with high OPN levels had more vulnerable plaque phenotype and reduced levels of HDL-cholesterol and IL-6 as compared to low OPN levels. Kaplan–Meier curve confirmed that patients with OPN levels >70 ng/ml had more MACEs at a 24-month follow-up. In the multivariate survival analysis, OPN levels >70 ng/ml predicted MACEs, independently of age, gender, and symptomatic status.
Conclusion: High circulating OPN levels were strongly correlated with vulnerability parameters within plaques and predict MACEs in patients with severe carotid artery stenosis. Although confirmation is needed from larger trials, OPN could be a promising clinical tool to assess atherosclerotic outcomes.

Abbreviations: OPN, osteopontin; ACS, acute coronary syndromes; MACE, major adverse cardiovascular events; IL-6, interleukin-6; CEA, carotid endarterectomy; ECST, European Carotid Surgery Trial; ACST, Asymptomatic Carotid Surgery trial; IS, ischemic stroke; York NYHA, Heart Association; low-density lipoprotein cholesterol, LDL; high-density lipoprotein cholesterol, HDL; hsCRP, high sensitivity C-reactive protein; MMP, matrix metalloproteinase.

* Corresponding author at: First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa School of Medicine, 6 viale Benedetto XV, 13132 Genova, Italy.
E-mail address: federico.carbone@edu.unige.it (F. Carbone).

[1] These authors equally contributed as first authors to this work.
[2] Federico Carbone, Fabio Rigamonti and Fabrizio Montecucco contributed to the conception and design of the study, as well as in the analysis and interpretation of data.
[3] All authors contributed to the final approval of the manuscript.
[4] Fabienne Burger, Aline Roth, Maria Bertolotto, Giovanni Spinella, Bianca Pane, Domenico Palombo, Aldo Pende, Aldo Bonaventura, Luca Liberale, Alessandra Vecchié, Franco Dallegria, François Mach contributed to acquisition of data.

https://doi.org/10.1016/j.ijcard.2018.01.008
0167-5273/

1. Introduction

The magnitude of inflammatory processes is nowadays recognized as a critical determinant of atherosclerosis progression to the vulnerable plaque, which is associated with higher risk of rupture and thrombosis [1,2]. Although the role of many inflammatory mediators (*e.g.* cytokines, oxidative stress markers and inflammatory cells) has been widely established, the research field on inflammatory processes in atherogenesis is still investigating both intraplaque and plasma biomarkers [3,4]. Among them, osteopontin (OPN) was demonstrated to be involved in several atherosclerotic steps (from initiation to calcification).

OPN is an aspartic acid-rich, N-linked glycosylated phosphoprotein that interacts with several surface receptors, including integrins and CD44. The OPN gene was primarily described as particularly up-regulated in bone cells, such as osteoblasts and osteoclasts [5,6]. However, OPN expression was later demonstrated in several other cell types and tissues, including immune cells. Especially macrophages have been described as a major source of OPN within inflamed atherosclerotic plaques [7,8]. However, the role of OPN in atherogenesis is still controversial. OPN deficiency has been shown to decrease atherosclerotic burden in mice, but inhibitory effects of OPN on vascular calcification were also demonstrated [8]. Nevertheless, results from clinical studies indicate a pro-atherosclerotic role of OPN, as suggested by the linear association with atherosclerotic burden and plaque instability. Indeed, increased serum levels of OPN were detected in patients with clinical and ultrasound features of plaque vulnerability, as well as in those with acute coronary syndrome (ACS) [9,10]. Conversely, few studies have so far investigated OPN as a long-term predictor of major adverse cardiovascular events (MACE) [11–13]. This study has been then designed to assess the predictive ability of serum OPN toward the occurrence of MACE over a 24-month follow-up. Potential correlations between circulating OPN and plaque composition will also be investigated.

2. Methods

2.1. Enrolment and clinical assessments of patients

From March 2008 to June 2014, 225 patients with extra cranial high-grade internal carotid stenosis (>70% luminal narrowing) [14] were consecutively enrolled in an observational single center (Ospedale Policlinico San Martino, Genoa, Italy). Among the total cohort, no serum sample was missing, leaving all serum samples ($n = 225$) available for analyses of serum OPN and interleukin (IL)-6 that were included in the present sub-study. As previously described [15], all patients underwent elective carotid endarterectomy (CEA) according to the recommendations published by the North American Symptomatic Carotid Endarterectomy Trial (NASCET) [16], the European Carotid Surgery Trial (ECST) [17], and the Asymptomatic Carotid Surgery trial (ACST) [18]. As previously published [15,19], patients ($n = 40$) were defined as "symptomatic" if they had a first episode of ipsilateral ischemic stroke, which had occurred in the period between 30 and 10 days before endarterectomy. Ischemic stroke was defined as ipsilateral focal neurological deficit of acute onset lasting >24 h. Control subjects ($n = 185$) were defined as "asymptomatic" when they had no personal history of recent ischemic symptoms, but they presented a severe internal carotid stenosis incidentally diagnosed at ultrasound Doppler during the same time period. The Ethics Committee of Ospedale Policlinico San Martino in Genoa (Italy) approved this protocol, performed in accordance to the guidelines of the Declaration of Helsinki. Patients gave informed consent before entering in the study. The day prior to CEA, serum samples were obtained to measure circulating markers of cardiovascular (CV) vulnerability. Carotid plaques were shortly processed (within 10 min on ice temperature) after the endarterectomy. Medications reported in Supplementary Table 1 were not modified in the two months prior to enrolment. Exclusion criteria were: spontaneous cerebral embolism up to 30 min preoperatively or during the dissection phase of the operation, malignant hypertension, acute coronary artery disease, any cardiac arrhythmias, congestive heart failure (II, III, and IV New York Heart Association [NYHA] classes), liver or renal disorder or function abnormalities, acute and chronic infectious diseases, autoimmune and rheumatic diseases, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, and hormone, cytokine or growth factor therapies.

2.2. Study endpoints and power estimation

The present clinical study was designed to investigate serum levels of OPN. The primary end-point of the study was to determine whether circulating OPN might predict the occurrence of MACE (defined as a composite fatal/non-fatal myocardial infarction or IS) over a follow-up of 24 months [20]. Study power calculation was performed considering a 4-fold increase of MACE incidence, as already observed in patients with high levels of OPN after a 24 month-follow-up [21]. According to our power calculation for the LogRank test, the minimal sample size requested to detect a 4-fold increase in MACE risk (2% *vs.* 8%) with a power of 80% and with a two-sided alpha error of 5% was of 185 patients. As secondary endpoint, potential correlations between circulating OPN and plaque histological features were investigated. Two independent investigators who were blinded to the biochemical and histological analyses adjudicated the study endpoints. Information was obtained during a check-up visit at 24 months and was further confirmed by checking patients' medical file, targeting medical history relevant to the study endpoint, as noted in their medical file.

2.3. Detection of biochemical and inflammatory biomarkers

Routine auto-analyzers were used to assay hematological parameters and blood chemistry including total cholesterol, low- and high-density lipoprotein cholesterol (LDL and HDL, respectively), triglycerides, glycaemia, and fibrinogen. Serum levels of OPN, high sensitivity C-reactive protein (hsCRP) and IL-6 were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D systems, Minneapolis, MN). The limits of detection were 62.50 pg/ml for OPN, 31.25 pg/ml for hsCRP, and 0.20 pg/ml for IL-6. Mean intra- and inter-assay coefficients of variation were <8% for all markers measured by ELISA.

2.4. Oil Red O staining for lipid content

Sixteen sections per plaque separated by 105 μm from each other were stained and counterstained with Mayer hemalune's solution, as previously described [15,19]. Quantifications were performed with MetaMorph™ 6 software. Data were calculated as ratios of stained area on total lesion area.

2.5. Immunostaining of endarterectomy specimens

Sixteen sections per plaque separated by 105 μm from each other were fixed in acetone at room temperature and immunostained with specific antibodies anti-human smooth muscle actin (smooth muscle cells, diluted: 1:100; Dako Corporation, Glostrup, Denmark), anti-human CD66b (neutrophils, diluted: 1:50; Beckman Coulter, Nyon Switzerland) and anti-human matrix metalloproteinase (MMP)-9 (diluted 1:250; Southern Biotech, Birmingham AL). For detecting total macrophage content anti-human CD68 was used (diluted: 1:100; Dako Corporation). For identifying the pro-inflammatory M1 macrophages, the sections were immunostained for anti-human CD86 (diluted: 1:100; GeneTex Inc., Irvine, CA) or anti-human HLA-DR (diluted 1:100; Dako Corporation). The anti-inflammatory phenotype M2 was detected by the immunostaining for anti-human CD163 (diluted: 1:50; AbD Serotec, Oxford, UK) [22,23]. Quantifications were performed using MetaMorph™ 6 software. Data were presented as cells/mm^2 (neutrophils) or percentages of stained area on total lesion area (other parameters).

2.6. Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 21.0 (IBM CO., Armonk, NY). Categorical data are presented as relative and absolute frequencies. Continuous variables were expressed as median and interquartile range (IQR) since the normality assumption was not demonstrated. Intergroup comparisons were drawn by Fisher's exact test and Mann–Whitney *U* test, as appropriate. Ranked Spearman correlation coefficients were performed to establish correlations between OPN and intraplaque biomarkers. The prognostic ability of OPN toward 24-month MACE occurrence was assessed in a post-hoc manner based upon receiver operating characteristic (ROC) curve (MedCalc 12.5, MedCalc Software, Ostend, Belgium). The area under the curve (AUC) was given with 95% confidence interval (CI) and the cut-off point of OPN was calculated maximizing the sensitivity in accordance to Youden's index. Kaplan–Meier survival analysis with the LogRank test was performed to estimate cumulative event rate during 24 months after CEA and to calculate the corresponding risk difference according to categorized OPN. Finally, Cox proportional hazards models were used to estimate the effect of circulating OPN on risk of composite outcome (MACE) and the single events fatal/non-fatal ACS or IS. Results were expressed as hazard ratios (HR) and 95% CI. In the multivariate model, we adjusted for age, gender and symptomatic status. In the basic research study, data were presented as mean ± standard deviation (SD). Statistical analyses were performed using the Mann–Whitney *U* test. For all statistical analyses a 2-sided p-value <0.05 was considered as statistically significant.

3. Results

3.1. Patients' characteristics

Clinical characteristics and medications, as well as laboratory parameters in asymptomatic and symptomatic patients are described in Supplementary Table 1. Classical risk factors for IS did not differ in the two study groups, except for the carotid artery lumen stenosis, which

Please cite this article as: F. Carbone, et al., Serum levels of osteopontin predict major adverse cardiovascular events in patients with severe carotid artery stenosis, Int J Cardiol (2018), https://doi.org/10.1016/j.ijcard.2018.01.008