Assessment of extracellular matrix-related biomarkers in patients with lower extremity artery disease

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ABSTRACT

Background: The prevalence of lower extremity artery disease (LEAD) is high (20%-25%) in the population older than 65 years, but patients are seldom identified until the disease is advanced. Circulating markers of disease activity might provide patients with a key opportunity for timely treatment. We tested the hypothesis that measuring blood-specific fragments generated during degradation of the extracellular matrix (ECM) could provide further insight into the pathophysiologic mechanism of arterial remodeling.

Methods: The protein profile of diseased arteries from patients undergoing infrainguinal limb revascularization was assessed by a liquid chromatography and tandem mass spectrometry, nontargeted proteomic approach. The information retrieved was the basis for measurement of neoepitope fragments of ECM proteins in the blood of 195 consecutive patients with LEAD by specific enzyme-linked immunosorbent assays.

Results: Histologic and proteomic analyses confirmed the structural disorganization of affected arteries. Fourteen of 81 proteins were identified as differentially expressed in diseased arteries with respect to healthy tissues. Most of them were related to ECM components, and the difference in expression was used in multivariate analyses to establish that severe arterial lesions in LEAD patients have a specific proteome. Analysis of neoepitope fragments in blood revealed that fragments of versican and collagen type IV, alone or in combination, segregated patients with mild to moderate symptoms (intermittent claudication, Fontaine I-II) from those with severe LEAD (critical limb ischemia, Fontaine III-IV).

Conclusions: We propose noninvasive candidate biomarkers with the ability to be clinically useful across the LEAD spectrum. (J Vasc Surg 2018;■:1-8.)

Keywords: Atherosclerosis; Biomarker; Collagen; Extracellular matrix; Neoepitopes; Peripheral artery disease; Versican

Atherosclerosis is a progressive, age-related disease that may simultaneously affect multiple arteries. The interest in noncoronary atherosclerosis is increasing because patients with manifestations in several vascular beds have poorer prognosis than those with just one territory affected.[1] This association is particularly evident in patients with lower extremity artery disease (LEAD; also known as peripheral artery disease, PAD). There are >200 million patients with LEAD in industrialized countries, and the convergent epidemics of diabetes and obesity suggest bleak prospects.[1,2] Despite its major prognostic impact, limited information is available on asymptomatic PAD. The life expectancy of a patient with either intermittent claudication (IC) or critical limb ischemia (CLI) is low, and once diagnosed, patients have significantly less chance of receiving risk factor modification than patients with coronary disease.[3,4] The challenge is to establish whether lesions in lower extremity arterial occlusive disease have a specific proteome and to propose noninvasive surrogates to anticipate prevention strategies.

Locoregional hemodynamic and rheologic factors favor the progression of atherosclerotic lesions in lower extremity arteries, and reduction in lumen caliber governs the course of symptoms in patients with LEAD.[5] Vessel wall remodeling and angiogenesis in peripheral arteries appear to be crucial processes to understanding of the overall response to atherosclerotic injuries.[6] In this scenario, the

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extracellular matrix (ECM) provides a mechanical scaffold and support to cell migration, which is regulated by the correct functioning of cytokines, enzymes such as matrix metalloproteinases (MMPs), and growth factors.[7-9] Atherosclerosis-associated remodeling and disrupted cytoskeletal architecture are the consequence of inflammatory cell activity, lipid deposition, and changes in ECM balance.[10-14] We address the proteome composition and the relative expression of ECM components in severely affected peripheral arteries to evaluate different neoepitope biomarkers of ECM degradation measured in serum from PAD patients to assess whether these markers might be associated with disease activity.

ARTICLE HIGHLIGHTS

- **Type of Research:** Histologic and proteomic analyses of human arteries removed during surgical revascularization
- **Take Home Message:** Compared with healthy controls, multiple extracellular matrix proteins were identified and validated by enzyme-linked immunosorbent assay to confirm identity and expression levels. In particular, fragments of versican and collagen type IV allowed discrimination of peripheral artery disease severity.
- **Recommendation:** Data suggest that multiple extracellular matrix serum profile screening in peripheral artery disease patients may have utility in discriminating mild to severe disease.

METHODS

Participants and study design. The local Ethics Committee and Institutional Review Board approved the procedures involved in this study (Epinols/12-03-09/3proj6; Inflamet/15-04-30/4proj6). First, histologic and proteomic analyses were performed in portions of superficial femoral arteries that included the entire artery wall from patients requiring infrainguinal limb revascularization ($n = 18$) and controls ($n = 3$) obtained from road accident victims of similar age. Written informed consent was obtained from participants or next of kin. Demographic and cardiovascular risk profiles of control individuals and PAD patients used for these analyses can be found in the Supplementary Methods and Supplementary Table I (online only). To limit likely sex differences and because the disease is more prevalent in men, we then recruited men with an established diagnosis of PAD attending our Department of Vascular Surgery. There were 195 participants included. Serum was collected at the time of inclusion, identified according to Fontaine classification,[15] and stored at −80°C until analyses. Exclusion criteria were clinically assessed; patients with infected lesions, evidence of recent neoplastic disease, chronic kidney disease, liver disease, or inflammatory disease (or receiving anti-inflammatory drugs) were excluded. Ankle-brachial index (ABI) was measured per standard technique in both lower limbs, and imaging techniques were performed according to the standard of care.

Histologic examination. To examine tissue morphologic features, serial sections of tissue were obtained from samples fixed in 10% neutral buffered formalin and embedded in paraffin. Hematoxylin and eosin staining (Sigma-Aldrich, Steinheim, Germany) was used to identify different cellular structures. Masson trichrome staining (Bio Optica, Milan, Italy) was used to assess collagen fibers, smooth muscle cells, nucleus, and cytoplasm, and sirius red staining (direct red 80; Sigma-Aldrich) was used to identify collagen fibers. Images were obtained at 200× magnification, and the intima-media ratio was obtained by dividing the thickness of the intima by the thickness of the media measured using an optical microscope (Eclipse E600; Nikon, Madrid, Spain) equipped with image analysis.

Untargeted proteomics. To explore the proteome composition of the arteries, we used a nondirected proteomic approach. Proteomics experiments were exploratory, with extensive mapping of digested peptides to identify and to quantify as many proteins as possible, and performed using chemical labeling to differentiate groups. Methods were similar to those previously used to analyze the protein secretion profile of carotid atherosclerotic plaques.[16] Specific details may be found in the Supplementary Methods (online only). Briefly, sample arteries were cut into pieces and homogenized in the presence of type 1 collagenase (Sigma-Aldrich). Following different rounds of centrifugation and chemical treatment, precipitated proteins were vacuum dried and dissolved. Samples were then sequentially denatured, reduced, and alkylated. For digestion, samples were incubated with sequencing-grade trypsin overnight at 37°C. We used a liquid chromatography-mass spectrometry (MS) approach for quantification by performing isobaric tag for relative and absolute quantitation (iTRAQ) labeling with iTRAQ 8-plex reagent kits (SCIEX, Madrid, Spain), as previously described.[17] Labeled peptides were then purified using an SCX column (Strata SCX 55 μm, 70Å; Phenomenex, Torrance, Calif), desalted and concentrated through a C18 Sep-Pak column (Waters, Bedford, Mass), and analyzed by using a C18 reversed phase nanocolumn coupled to a trap nanocolumn for real-time ionization and peptide fragmentation on an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, Calif). To identify proteins, information was obtained from tandem mass spectra with the aid of Proteome Discoverer (version 1.4.0.288; Thermo Fisher Scientific). All MS and tandem MS (MS/MS) samples were analyzed using Mascot (version 2.4.1.0; Thermo Fisher Scientific). Protein quantification was performed