Identification by a Differential Proteomic Approach of Heat Shock Protein 27 as a Potential Marker of Atherosclerosis

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Background—We hypothesized that normal and pathological vessel walls display a differential pattern of secreted proteins. We have recently set up the conditions for comparing secretomes from carotid atherosclerotic plaques and control arteries using a proteomic approach to assess whether differentially secreted proteins could represent markers for atherosclerosis.

Methods and Results—Normal endartery segments and different regions of endarterectomy pieces (noncomplicated/complicated plaques) were incubated in protein-free medium, and the released proteins were analyzed by 2D electrophoresis (2-DE). Among the differently secreted proteins, we have identified heat shock protein-27 (HSP27).

Surprisingly, compared with control arteries, HSP27 release was drastically decreased in atherosclerotic plaques and barely detectable in complicated plaque supernatants. HSP27 was expressed primarily by intact vascular cells of normal arteries and carotid plaques (immunohistochemistry). Plasma detection of soluble HSP27 showed that circulating HSP27 levels are significantly decreased in the blood of patients with carotid stenosis relative to healthy subjects (0.19 [0.1 to 1.95] versus 83 [71.8 to 87.8]) ng/mL, P<0.0001).

Conclusions—HSP27 secretion is decreased in complicated atherosclerotic plaques, and sHSP27 plasma levels are decreased in atherosclerotic patients compared with healthy subjects. Plasma sHSP27 levels could be a potential index of atherosclerosis, although further validation is needed in large patient cohorts. (Circulation. 2004;110:***-***.)

Key Words: plasma ■ cells ■ muscle, smooth ■ electrophoresis

A therothrombosis is the leading cause of death in developed countries. Beyond the classic risk factors (dyslipidemias, diabetes, and hypertension), humoral markers of plaque vulnerability related primarily to inflammation (eg, high-sensitivity C-reactive protein, interleukin-6, -10, and -18, CD40L) or reflecting pathological vascular remodeling (eg, immune activation, apoptosis, extracellular matrix degradation) have recently been highlighted. Emerging noninvasive imaging techniques for assessment of subclinical atherosclerosis permit measurement of intima-media thickness or peripheral flow-mediated dilatation, which are inversely correlated with coronary artery diseases. Despite these achievements, intermediate phenotypes between risk factors and clinical complications are needed to target vulnerable patients. We hypothesized that the patterns of protein secretion are different between atherosclerotic plaques and normal endarteries. Whereas the existing markers were found by monitoring the variations of a candidate protein related to the pathology, our strategy is to compare the secretome from normal and pathological arteries using a differential proteomic approach to identify new biological markers potentially released by the arterial wall within the plasma. The incubation of complicated and noncomplicated endarterectomy samples or control endarteries in a serum-free culture medium allowed us to harvest separately the proteins released from lesioned and healthy areas. Two-dimensional electrophoresis (2-DE) enabled us to analyze these secretomes globally and to identify, among the differently secreted proteins, heat shock protein 27 (HSP27) as a potential marker of atherosclerosis. Confirming these results, plasma HSP27 was markedly decreased in atherosclerotic patients relative to healthy subjects.

Methods

Tissue Sampling
Twenty-eight patients (carotid stenosis >70%, 21 men/7 women; age, 68±9 years; 86% hypertensive, 39% diabetic, 54% hyperlipidemic) undergoing carotid endarterectomy at our institutions were included. Informed consent was obtained before enrollment. Blood samples were collected from these patients the day of endarterectomy.
my and from 12 healthy volunteers without significant differences for sex (6 men/6 women) and age (62±8 years). Conditioned media from 36 control endarteries (24 mammary, 12 radial) and 35 atherosclerotic endarterectomies (10 femoral, 25 carotid) were collected. The study was approved by the local Ethical Committees in accordance with institutional guidelines.

**Tissue Culture**

Carotid endarterectomy samples were dissected as described previously, separating the stenosing complicated zone (origin of the internal carotid artery) from the adjacent plaque (common and external carotid endartery). Histological analysis showed that complicated plaques had ruptured and contained an intraplaque hemorrhage with a variable but important proportion of inflammatory cells. The adjacent area, considered noncomplicated plaque, was composed of fibrous thickening with a variable content of vascular smooth muscle cells (VSMCs). Femoral endarterectomies exhibited a high degree of calcification but no intraplaque hemorrhage. For mammary and radial arteries, the adventitia was removed before incubation of the intima-media. Samples were cut and incubated separately for 24 hours in serum-free RPMI medium at 37°C. Conditioned media were collected and centrifuged, and protein concentration was determined by Bradford method. Tissue secretion was measured as medians and interquartile ranges and were analyzed by the Mann-Whitney test. Differences between noncomplicated and complicated plaques were analyzed by Wilcoxon paired test.

**Proteomic Analysis**

Conditioned media (600 μg of protein) were precipitated by use of 10% trichloroacetic acid in acetone with 0.07% 2-mercaptoethanol (1 hour, −20°C) and washed with cold acetone (−20°C, 20 minutes) before 2-DE was performed. The spots of interest were treated as described previously. The peptides were analyzed in a Voyager DE STR MALDI-TOF mass spectrometer (Perspective Biosystem). Proteins were identified by use of the MASCOT program and comparing with SWISS-PROT and NCBI protein sequence databases. Phosphopeptides were characterized by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS) and by immobilized metal affinity chromatography (IMAC) combined with MALDI Q-TOF MS/MS.

**Western Blot**

Equal amounts of conditioned-medium proteins (15 μg) were loaded into a 12% polyacrylamide gel and electrophoresed as described previously. Blots were incubated with anti-HSP27 (sc-9012, Santa Cruz), anti-HSP60 (sc-1052), and anti-HSP70 (SPA-812 Stressgen) antibodies.

**ELISA**

Plasma and tissue conditioned media levels of soluble HSP27 (sHSP27) were measured with a commercially available kit (Oncogene).

**Immunohistochemistry**

Specimens were fixed with paraformaldehyde and embedded in paraffin, and immunohistochemistry was performed on 4-μm sections as described previously using polyclonal goat anti-human HSP27 antibody (sc-1049) and anti–SMC-α-actin (clone 1A4, Dako).

**Statistical Analysis**

Statistical analysis was performed with SPSS 8.0. ELISA data are expressed as medians and interquartile ranges and were analyzed by the Mann-Whitney test. Differences between noncomplicated and complicated plaques were analyzed by Wilcoxon paired test.

**Results**

**HSP27 Secretion Is Drastically Decreased in Atherosclerosis**

The 2-DE analysis of the secretomes of carotid endarterectomy samples versus control mammary endarteries led us to the identification of HSP27 by MS, as differentially secreted from both arteries (Figure 1, A and B). HSP27 isoforms were characterized by LC-MS/MS analysis. Signals corresponding to the peptide QLpS82SGVSEIR (m/z, 578.24 [2+] for HSP27 protein in spot 1 and QLS82SGVSEIR (m/z, 538.25 [2+]) in spot 2 demonstrated that HSP27 in spot 1 was phosphorylated. These results were confirmed by IMAC and MALDI MS/MS sequencing.

Western blot analysis showed that HSP27 secretion is lower in atherosclerotic plaques (femoral, F; carotid noncomplicated plaques, NCP; carotid complicated plaques, CP) compared with control arteries (mammary, M; radial, R) (Figure 1, C and D). These data were confirmed by quantitative ELISA: M, 1243 (734–1909); R, 910 (505–2508); F, 303 (138–526); NCP, 315 (119–515); CP, 33 (17–111) (Figure 1D). We tested the secretion of other HSPs and found a diminished sHSP70 level in atherosclerotic samples, whereas sHSP60 showed the opposite pattern (Figure 1C). Whereas HSP60/70 exhibited a diffuse trend, diminished HSP27 release was clearly correlated to the complexity of the plaque.

**Plasma HSP27 Is Decreased in Atherosclerotic Patients Relative to Healthy Subjects**

To confirm our hypothesis that plasma protein content can reflect arterial wall secretion, we measured sHSP27 level in the plasma of patients with carotid stenosis and healthy controls. Circulating HSP27 levels were decreased 20-fold in patients with carotid atherosclerosis relative to healthy subjects (0.19 [0.1 to 1.95] versus 83 [71.8 to 87.8] ng/mL, P<0.0001) (Figure 1D).

**Tissue HSP27 Immunostaining**

By immunohistochemistry, we found that both human atherosclerotic plaques and mammary arteries expressed HSP27 protein. Immunostaining for HSP27 (Figure 2, A and C) and SMC-α-actin (B and D) in serial tissue sections showed that HSP27 was expressed primarily by VSMCs. No staining was obtained in negative controls (not shown).

**Discussion**

In a preliminary study, we have validated an original approach analyzing the secreted proteomes from atherosclerotic plaques and nonatherosclerotic arterial wall by 2-DE: incubation of the tissue in a serum-free medium allows the accumulation of proteins and their subsequent analysis without interference of plasma proteins. In the present study, using the same procedure, we have identified HSP27, for which production by the arterial wall correlates negatively with atherosclerotic plaque complexity.

HSPs are ubiquitous proteins serving as molecular chaperones, and their cytoprotective functions rely on intracellular mechanisms. HSPs can also be secreted and released into the bloodstream, where their role in this soluble form remains unknown. In cardiovascular diseases, HSP expression is modulated both at the lesion site and in plasma. HSP70 has been suggested to protect VSMCs from oxidative aggression. Furthermore, increased levels of sHSP70 have been correlated with decreased intima/media thickness and with low risk of coronary artery disease. HSP60 was detected in aorta and carotid arteries, correlating with atherosclerosis severity. Moreover, sHSP60 could be a marker of atherosclerosis. To the best of our knowledge,
Figure 1. HSP27 secretion: from arterial wall to plasma. 2D gels of secretomes from control endartery (A) and complicated plaque (B). Circles show 2 spots corresponding to HSP27, corresponding to different phosphorylation states. C, Western blot for HSP27, HSP60, and HSP70 in conditioned media samples (#, subject/patient number). D, sHSP27 quantification by ELISA. Left, Conditioned media samples (HSP27 levels normalized by protein concentration; *P<0.005 M, R vs F, NCP; †P<0.0001 M, R, F, NCP vs CP). Inset, corresponding Western blot for HSP27. Right, plasma levels of atherosclerotic patients (n=28) and controls (n=12) (*P<0.0001). Boxes represent 25th and 75th percentiles; line within boxes, median. Error bars mark 10th and 90th percentile.
nothing has yet been reported on HSP27 in atherosclerosis. HSP27 is expressed by both endothelial cells and VSMCs and is able, in its phosphorylated form, to bind and stabilize actin microfilaments, favoring the formation of actin stress fibers.16

We have shown that HSP27 colocalizes with VSMCs in human atherosclerotic plaques and mammary arteries, probably as a physiological response to hemodynamic or biomechanical stress. Indeed, hemodynamic stress increases the expression of HSP27 in VSMCs.8 Pharmacological induction of HSP27 attenuates intimal hyperplasia in vivo.47 HSP27 could also interfere with the atherosclerotic inflammatory response by inhibiting nuclear factor-κB activation.18 Finally, HSP27 can downregulate the apoptotic signaling pathway19 and could thus contribute to stabilize atherosclerotic lesions. We show for the first time that HSP27 is secreted by the undiseased vascular wall and that its release markedly diminishes according to the complexity of the plaque. Although this differential secretion does not seem to be specific for a vascular territory, because both mammary and radial endarteries secrete a higher amount of HSP27 than carotid or femoral atherosclerotic endarterectomies, this possibility cannot be excluded. Moreover, whereas sHSP27 is detected in the blood of 100% of healthy individuals, sHSP27 levels were almost undetectable in a large number of atherosclerotic patients. Despite a limited number of patients analyzed in this study, the marked statistical significance confers considerable value to our findings.

The cause and the biological significance of this important diminution of plasma HSP27 in atherosclerotic patients remain to be elucidated. Whether soluble HSP27 has an atheroprotective role or whether it is the reflection of a pathological vascular remodeling process is not known and requires further investigation. Our results strongly suggest that low levels of plasma HSP27 could serve as a potential marker for atherosclerosis and should be validated in larger cohorts.

Disclosure
The authors are named as coinventors on pending patents filed by the Fundacion Jimenez Diaz that relate to the use of biomarkers on cardiovascular disease.

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