

Circulating Human Monocytes in the Acute Coronary Syndrome Express a Characteristic Proteomic Profile

María G. Barderas,^{†,△} José Tuñón,^{‡,§,△} Verónica M. Dardé,[†] Fernando De la Cuesta,[†]
María C. Durán,[†] José J. Jiménez-Nácher,[⊥] Nieves Tarín,[○] Lorenzo López-Bescós,[⊥]
Jesús Egido,^{§,||} and Fernando Vivanco^{*,†,#}

Department of Immunology, Fundación Jiménez Díaz, Madrid, Spain, Department of Cardiology, Fundación Jiménez Díaz, Madrid, Spain, Department of Medicine, Universidad Autónoma de Madrid, Spain, Renal and Vascular Laboratory, Fundación Jiménez Díaz, Madrid, Spain, Cardiology Unit, Fundación Hospital de Alcorcón, Madrid, Spain, Department of Cardiology, Hospital de Móstoles, Madrid, Spain, and Department of Biochemistry and Molecular Biology I, Universidad Complutense, Proteomic Unit, Madrid, Spain

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Abstract: We examined the proteome of circulating monocytes of patients with acute coronary syndrome at different times in comparison to that of patients with stable coronary artery disease. On admission, the expression of 18 spot proteins was altered, 10 of which were totally absent. This pattern changed progressively, and at 6 months, there were no differences with the monocyte proteome of stable patients.

Keywords: acute coronary syndromes • monocytes • coronary artery disease • two-dimensional electrophoresis • mass spectrometry • atherosclerosis

Introduction

Atherosclerosis is a chronic disease that begins with endothelial dysfunction, which allows lipids and other molecules from blood to enter the vessel wall.¹ In addition, an inflammatory response takes place, and circulating monocytes migrate into the intima, where they differentiate to macrophages, which uptake lipids and become foam cells.¹ The presence of different growth factors will induce the formation of a fibrous cap, composed of collagen and other matrix proteins produced by vascular smooth muscle cells. This cap covers a central lipid core, containing mainly foam cells and extracellular lipids. Once these lesions are formed, and when they narrow significantly the vessel lumen, ischemia of the subsidiary organ may occur.

Atherosclerotic plaques may be suddenly complicated by a thrombus that may occlude the vessel lumen, causing an acute coronary syndrome (ACS), such as unstable angina or myocardial infarction. Plaque thrombosis is triggered, in most cases,

by the rupture of the fibrous cap, that allows blood to contact the lipid core, which is highly thrombogenic.² Although this rupture is ultimately due to the hemodynamical forces, previous weakening of the fibrous cap by metalloproteinases and other enzymes released by the macrophages seems to play a key role.¹

Despite the great advances produced in the study of the pathophysiology of ACS, its mechanisms have not been completely elucidated. As a consequence, we still have a poor capacity to predict which patients, or even healthy subjects, are at risk of suffering this disorder. Although in the last years plasmatic levels of several inflammatory markers have been found to predict the possibility of developing an ACS,^{3,4} none of them have been adopted unanimously by the clinicians due to either a limited predictive power or a lack of enough studies confirming their value.⁵ This is very important because many patients suffering an ACS may die before they reach the hospital and receive treatment.

Given the central role of macrophages in plaque rupture and thrombosis, their behavior is of great interest in the study of ACS. Although these cells are not easily accessible in human coronary atherosclerotic plaques, it is possible to focus on their precursors, blood-circulating monocytes. These cells are in contact with the diseased endovascular lumen and, as such, may serve as reporters. In addition, it has been recently shown that a certain population of macrophages foam cells migrates from atherosclerotic plaques, which permits their re-entry into the blood.⁶ Monocytes are activated during ACS⁷ and can then yield information about the processes occurring in this setting. In addition, identifying the proteins in this syndrome with altered expression in circulating monocytes may disclose new potential biomarkers that can help the clinician to assess the future risk of having a recurrent ACS. Searching for such biomarkers directly in plasma could be a complicated task, because the plasmatic proteome is, probably, the most complex in the human body.⁸

In this work, we have studied the variation of the protein expression in the circulating monocytes of patients suffering a non-ST elevation ACS. The protein expression pattern was compared with that of patients with stable coronary artery

* Author for correspondence: Professor Fernando Vivanco, Department of Immunology, Fundación Jiménez Díaz, Avda. Reyes Católicos 2, 28040 Madrid, Spain. E-mail: fvivanco@fjd.es. Phone: 34-915498446. Fax: 34-915448246.

[†] Department of Immunology, Fundación Jiménez Díaz.

[‡] Department of Cardiology, Fundación Jiménez Díaz.

[§] Department of Medicine, Universidad Autónoma de Madrid.

[△] Both authors contributed equally to this work.

^{||} Renal and Vascular Laboratory, Fundación Jiménez Díaz.

[⊥] Cardiology Unit, Fundación Hospital de Alcorcón.

[○] Department of Cardiology, Hospital de Móstoles.

[#] Department of Biochemistry and Molecular Biology I, Universidad Complutense.

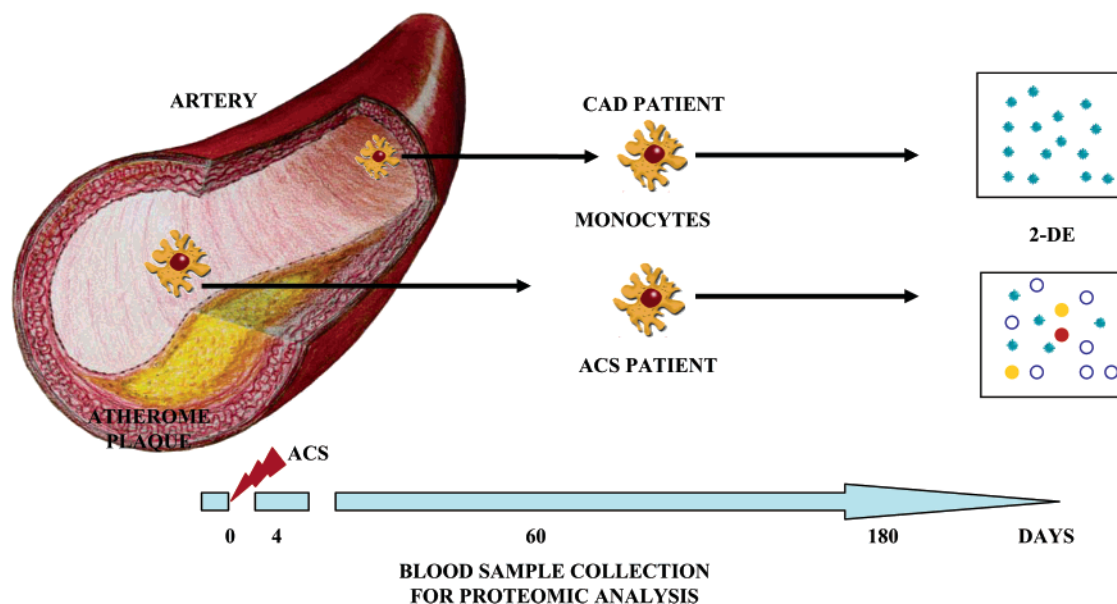


Figure 1. Schematic representation of blood sample collection for monocyte purification at different times and the subsequent proteomic analysis by 2-DE. Samples obtained from ACS patients at admission (day 0) are shown by the red ray.

disease (CAD) to search for proteins potentially related to plaque thrombosis.

Materials and Methods

Patient Selection. Eighteen patients admitted into the Fundación Hospital de Alcorcón suffering a non-ST elevation ACS, defined as anginal pain with troponin elevation and/or ST-T changes on the electrocardiogram, entered the study. The exclusion criteria were inflammatory or neoplastic disease, coagulation disorders, other significant heart disease except left ventricular hypertrophy secondary to hypertension, chronic treatment (except drugs for pre-existing clinical atherosclerosis or its risk factors), and having suffered surgical procedures, major traumatism, thromboembolic events, or revascularization procedures in the previous 6 months. Patients with ejection fraction less than 0.45 were also excluded, given that left ventricular dysfunction may enhance inflammation, adding a potential confounder to the results.⁹

At the moment the diagnosis was made, the patients were asked to participate in the study (Figure 1). In case of acceptance, they signed the informed consent, and 28 mL of blood was withdrawn for analysis and introduced in EDTA-prepared collection tubes (Venoject, Terumo Europe). In cases diagnosed after the evening, blood was extracted at 9 a.m. the next morning. Monocyte isolation must be performed within 4 h, and the samples could not be stored during the night to await this process. Management included coronary angiography in all cases. A second blood sample was taken on day 4 to investigate if there was a fast reversion of the changes observed in monocyte proteome on admission. The last blood sample was performed at 6 months, as we hypothesized that changes could be normalized, given that evidence of inflammation has been shown to cease at this time.¹⁰ An intermediate blood sample was withdrawn at 2 months. Thus, at each time point, 18 patients were studied. The study had been previously approved by the Hospital Ethics Committee. Blood was also collected from 15 healthy subjects and from 10 stable CAD patients without significant differences in age and sex. Stable CAD patients must have been diagnosed by the existence of a

Table 1. Baseline Characteristics of ACS Patients^a

Mean age	60.0 years (50.3–68.5)
Sex (male/female)	67%/33%
Current smoking	39%
Hypertension	44%
Diabetes	22%
Hyperlipidemia	77%
Obesity/overweight	28%/33%
Family history of early CAD	28%
Total cholesterol	215 (194–225)
LDL	142 (127–158)
HDL	39 (34–44)
Triglycerides	159 (129–202)
Body mass index	28.4 (25.3–32.86)
Previous ACS	11%
Statin	6%
Antithrombotic therapy	6%

^a Quantitative data are expressed as median (interquartile range). Statin and antithrombotic therapy refer to the treatments received by the patients previously to the present event. Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease.

previous myocardial infarction or by coronary angiography and could not have a history of acute cardiovascular event in the previous year to the inclusion in the study.

Monocyte Isolation. Isolation of circulating monocytes has been previously described.¹¹ Blood was centrifuged at 600g (GS-GR Beckman Coulter) for 10 min at room temperature. The cell pellet was mixed with the same volume of FACS FLOW buffer (Becton Dickinson), layered over 10 mL of Ficoll (ICN Biomedicals, Irvine, CA), and centrifuged at 700g for 30 min. A band corresponding to peripheral blood mononuclear cells (PBMCs) was recovered at the interface over Ficoll, and then they were washed three times in FACS FLOW buffer and with PBS, pH 7.2. Monocytes were isolated by passing the PBMCs through a magnetic cell separation system (AutoMACS; Miltenyi Biotec). The purity of the monocytic cells obtained was evaluated by flow cytometry. The purified monocytes (10^7 cells) were solubilized immediately using 200 μ L of lysis buffer.^{11,12} Finally, the sample was sonicated and treated with benzonase nuclease (Novagen) to degrade all DNA and RNA forms (single

strand, dual strand, linear and circular). The total proteins obtained were quantified using the Bradford method.¹³

Two-Dimensional Electrophoresis (2-DE). An individual gel was run for each blood sample, from each patient (at four different times: on admission, 4 days, 2 months, and 6 months). The low number of cells (10^7 monocytes) that can be obtained with >95% of purity from 28 mL of blood yields only about 200–220 μ g of protein that was loaded on each individual gel. Protein samples (200 μ g) were diluted in rehydration solution (350 μ L final volume) containing 8 M urea, 0.5% CHAPS, 10 mM dithiothreitol (DTT), and 0.2% Pharmalyte pH 3–10. For the first dimension of 2-DE (isoelectric focusing, IEF), immobilized pH gradient gel strips (IPGs, pH 4–7 range; Bio-Rad) were used. The IEF was developed horizontally in a protean IEF cell system (Bio-Rad). After completed IEF, the strips were equilibrated in 1.5 M Tris buffer, pH 8.8.¹¹ Second dimension of 2-DE was performed by applying the equilibrated strips (IPGs) onto polyacrylamide gels (17 $\times$ 20 cm) in the presence of SDS, as previously described.^{11,12,14} Afterward, the gels were fixed in 5% (v/v) acetic acid and 30% (v/v) ethanol and silver-stained using the Silver Staining protein Kit (Pharmacia Biotech), following the instructions of the manufacturer.

Image Analysis of 2D Gels. The stained gels were scanned and digitized using a DUOScan HiD (AGFA Geavaert, Marstel, Belgium). The images obtained with two-dimensional gels were analyzed using a PDQuest 2DE Gel Analysis Software (Bio-Rad version 6.2). The position of the points was detected with the same software, followed by characterization with respect to their isoelectric point (pI) and apparent molecular weight (MW). The coordinates were expressed in relation to a reference point in each 2-DE gel. Spot intensities of patients within the same group (ACS) were calculated using the PDQuest 6.2 which includes a statistical package. To compensate for any variation in protein loading and development level of silver stain, spots quantities were normalized based on the total staining density of the image. Spots were tested with Student's *t*-test comparing the ACS group, at different stages, with the healthy group, yielding the values corresponding to protein spots whose expression was found to be changed. These values were analyzed using statistical software packages SPSS 13.0 and GraphPAD Instant (GraphPAD software). The mean values and coefficient of variation (CV) of the differentiated points (as expression or presence/absence) were calculated using the same software.

In-Gel Digestion of Proteins and Sample Preparation for Mass Spectrometric Analysis. Protein spots were excised manually and then digested using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol used was that of Schevchenko et al.¹⁵ with minor variations: gel plugs were washed with 25 mM ammonium bicarbonate and acetonitrile (ACN) prior to reduction with 10 mM DTT in 25 mM ammonium bicarbonate and alkylation with 100 mM iodoacetamide in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and ACN and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI) at a final concentration of 16 ng/ μ L in 50 mM ammonium bicarbonate was added to the dry gel pieces, and the digestion proceeded at 37 °C for 6 h. The reaction was stopped by adding 0.5% trifluoroacetic acid (TFA) for peptide extraction. A 0.4 μ L aliquot of matrix solution (5 g/L 2,5-dihydroxybenzoic acid in 33% aqueous ACN and 0.1% TFA) followed by 0.4 μ L of the above digestion solution

were automatically deposited into a 400 μ m AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature.

MALDI Peptide Mass Fingerprinting and Database Searching. Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) in positive ion reflector mode using 140 ns delayed extraction and a nitrogen laser (337 nm), as previously described.¹⁴ The laser repetition rate was 50 Hz, and the ion acceleration voltage was 25 kV. Mass measurements were performed automatically using fuzzy logic-based software. Each spectrum was internally calibrated with the mass signals of two trypsin autolysis ions: [VATVSLPR + H]⁺ (m/z = 842.510) and [LGEHNIDVLEGNEQFINAAK + H]⁺ (m/z = 2211.105) to reach a typical mass measurement accuracy of $\pm$ 30 ppm. Known trypsin and keratin mass signals, as well as potential sodium adducts (+21.982 Da) or signals arising from methionine oxidation (+15.995 Da), were removed from the peak list. The measured tryptic peptide masses were transferred using the MS BioTools program (Bruker-Daltonics) as inputs to search the NCBI database using Mascot software (Matrix Science, London, U.K.). The identifications were accepted if they represented the highest-ranking hit, had MOWSE scores over 64, and if the sequence coverage was at least 15–30% depending on protein size.^{14,16} No restrictions were placed on the species of origin of the protein, and no variable modifications were allowed. Up to one missed tryptic cleavage was considered, and a mass accuracy of 40 ppm was generally used for tryptic mass searches. Detailed analysis of peptide mass mapping data was performed using flexAnalysis software (Bruker-Daltonics).

ESI-IT and TOF/TOF MALDI (MS/MS). All the proteins were identified by PMF, and three of them were confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (protein disulfide isomerase, vimentin, and pyruvate kinase) using an ESI-IT (Deca-XP, Thermo-Finnigan) or a 4700 Protein Analyzer TOF-TOF (Applied Biosystems).¹⁴ In the first case (LC-Ion Trap), peptides mixtures were dried in a Speed-Vac and resuspended in 0.5% acetic acid. The chromatographic separation was accomplished by loading the peptide samples into an HPLC fitted with a reverse-phase C₁₈ column (150 μ m, i.d.) at a flow rate of 1.5 μ L/min. Sequential elution of peptides was accomplished using a linear gradient from 0.5% acetic acid to 0.5% acetic acid and 80% ACN.

Samples for MS/MS sequencing in the 4700 Protein Analyzer (1 μ L) were mixed with 0.4 μ L of α -cyano matrix (3 mg/mL in 50% ACN and 0.1% TFA) and spotted on stainless steel plates. Spectra were obtained in reflector mode using an acceleration voltage of 1 kV. Desorption and ionization was performed with an Nd:YAG laser operating at 355 nm. Fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in Data Explorer and further analyzed with GPS Explorer software (Applied Biosystems).

Antibodies. Mouse anti-human calgranulin A monoclonal antibody and mouse anti-vimentin monoclonal antibody were from Chemicon International, Inc. Mouse anti-paraoxonase was a gift from Dr. Richard W. James, Hôpitaux Universitaires de Genève. Goat polyclonal anti-cathepsin D and goat polyclonal to PARK 7 (Oncogen DJ1) antibodies were from Abcam. Goat polyclonal heat-shock protein 70 (HSP70) antibody was from Santa Cruz Biotechnology, Inc.

Western Blotting. Protein samples obtained from monocytes were analyzed using a 12% SDS-PAGE in a Bio-Rad Miniprotein

Table 2. Changes in Monocyte Protein Expression (Fold Regulation and *p*-Value)^a

spot	protein	stable	ACS day 0	ACS day 4	ACS 2 m	ACS 6 m
1702	Nuclease	—	D 0.36 <i>p</i> < 0,05 vs Stable/ H	—	—	—
2402	Chain A triosephosphate isomerase	—	A <i>p</i> < 0,001vsStable/H	—	—	—
2403	Chain A triosephosphate isomerase	D 0.049 <i>p</i> < 0.001 vs H	D 0.2 <i>p</i> < 0.001 vs H	D 0.19 <i>p</i> < 0.001 vs H	D 0.13 <i>P</i> < 0.001 vs H	D 0.049 <i>P</i> < 0.001 vs H
7208	RNA binding regulatory subunit oncogene DJ1	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H
1201	Chain A nuclease DNase, RNase sugar nonspecific	—	A <i>p</i> < 0.01 vs Stable/H <i>p</i> < 0.05 vs ACS 6 m	A <i>p</i> < 0.01 vs Stable/H <i>p</i> < 0.05 vs ACS 6 m	—	—
7405	Chain A endonuclease	—	D 0.05 <i>P</i> < 0,01 vs Stable/H	—	—	—
5401	Paraoxonase I (PON I)	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H
1504	60kDa Heat Shock protein	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs Stable/H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H
8503	70 kDa Heatshock protein (44sub)	A <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs Stable/H	A <i>p</i> < 0.01 vs H	D 0.28 <i>p</i> < 0.01 vs H	A <i>p</i> < 0.01 vs H
1714	Mannose 6 phosphate receptor binding protein	—	A <i>p</i> = 0.007vs Stable/ H	—	—	—
2202	MY032 protein	—	—	D 0.1 <i>p</i> < 0.01vs Stable/ H	—	—
3109	Vimentin	—	U 4.7 <i>p</i> = 0.001vs Stabl/ H	—	—	—
2814	Thymidine phosphorylase precursor	—	A <i>p</i> = 0.004vs Stable/ H	—	—	—
3916	Albumin precursor	—	A <i>p</i> = 0.028vs Stable/ H	—	—	—
4802	Protein disulfide isomerase ER60	—	A <i>p</i> < 0.05 vs Stable/ H	A <i>p</i> < 0.05 vs Stable/ H	A <i>p</i> < 0.05vs Stab/H	—
8106	Protein disulfide isomerase ER60	—	D 0.35 <i>p</i> = 0.001vs Stable/ H	—	—	—
5206	Glutathione transferase	—	A <i>p</i> = 0.003vs Stable/ H	—	—	—
5302	Alpha enolase	—	U 8.2 <i>p</i> < 0.001 vs Stable/ H <i>p</i> = 0.001 vs 2 m	—	—	—
2114	Alpha enolase	—	U 3.5 <i>p</i> < 0.001vs Stable/ H	—	—	—
5102	Alpha enolase	—	U 10.5 <i>p</i> < 0.05 vs Stable/H	—	U 2.3 <i>p</i> < 0.05vs Stab/ H	—
8006	S100 Calcium binding protein A8 Calgranulin	—	A <i>p</i> = 0.018 vs Stable/H	—	—	—
5712	Uracil DNA glycosylase	—	D 0.202 <i>P</i> < 0.001vs Stable/ H	—	—	—
4308	Cathepsin D proform	—	A <i>p</i> < 0.05 vs Stable/ H	A <i>p</i> < 0.05 vs Stable/ H	A <i>p</i> < 0.0 vs Stab/H	—
2115	MatureCathepsinD	—	—	U 2.13 <i>P</i> < 0,01 vs Stable/ H	—	—
5708	Pyruvate Kinase M2 isozyme	—	A <i>P</i> < 0.001v Stable/ H	—	—	—

^a Empty cells correspond to spots whose expression is not significantly altered as compared to healthy or stable subjects. Stable patients show changes in the expression of only five proteins vs healthy patients. Conversely, ACS patients show additional variations in the expression of all the remaining proteins at one or more time-points. A, absent; ACS, acute coronary syndrome; D, downregulated; H, healthy subjects; U, upregulated; —, no variation.

III electrophoresis unit, run at a constant current of 25 mA, applied during 1 h. After SDS-PAGE, the proteins were electrotransferred to a nitrocellulose membrane under a constant voltage of 15 V for 20 min. The membranes were then blocked with PBS containing 7.5% non-fat dry milk powder and 0.1% Tween 20 during 1 h.¹⁷ The membranes were then incubated overnight with the first antibody in PBS-T containing 5% non-fat dry milk, and finally incubated with the specific horseradish peroxidase (HRP)-conjugated secondary antibody or with mouse true-blot (eBioscience) secondary antibody in PBS-T containing 5% of non-fat dry milk. Detection was performed by enhanced chemiluminescence (ECL kit, Amersham Biosciences) following the instructions of the manufacturer.

Statistical Analysis. Proteomic analysis was done using PD-Quest 6.2 software which includes a statistical package. This program was used to compare the spots of stable CAD and ACS

patients, at different stages, with those of healthy subjects and yielded the values corresponding to protein spots whose expression was found to be changed. These values were analyzed using statistical software packages SPSS 13.0 and GraphPAD Instat (GraphPAD software). A Kolmogorov–Smirnov test did not demonstrate significant differences for any of the proteins as compared to a normal distribution. Then, a Student's *t*-test could be performed to compare spot values for which the PD-Quest gave data for only two groups of patients. For spots in which this software yielded three or more values as potentially different, a Levene test for homogeneity of variance was performed. When variances were not significantly different, the data could be studied by an ANOVA followed by a Scheffé test. In case equality of variance was not present, we used a nonparametric Kruskal–Wallis followed by

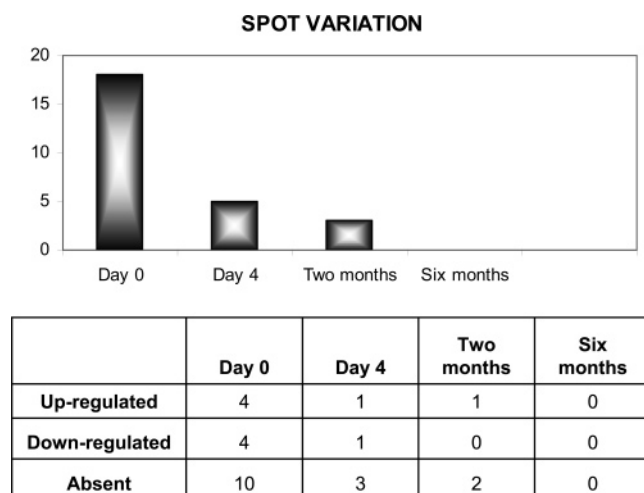


Figure 2. Number of proteins abnormally expressed in ACS patients' monocytes at different times in comparison with stable patients. The analysis was carried out using PDQUEST 2-D version 6.2.1 software (Bio-Rad, Hercules, CA).

a Dunn's test. Statistical significance was accepted when $p < 0.05$ (two-tailed).

Results

Patients. The basal characteristics of the patients are described in Table 1. The final diagnosis was unstable angina in 11%, and non-Q wave myocardial infarction in 89%. All patients underwent coronary angiography: the percentages of patients with 1-, 2-, and 3-vessel disease were 44%, 17%, and 17%, respectively; 22% did not have severe coronary lesions at the time of angiography. The ejection fraction was normal in all cases. Fifty percent of the patients were treated with percutaneous coronary intervention, 11% with coronary artery bypass graft, and 39% received only medical treatment. At discharge, 94% received aspirin and/or clopidogrel, 83% were treated with a statin, and 22% with antidiabetic therapy.

Protein Expression Profile in PBMCs of Patients with ACS. Blood was extracted a median of 8 h (8, 12) after admission. Monocytes were isolated with high purity for the 2-DE analysis by a method which removes albumin and other contaminants.¹¹ Moreover, the expansion of a region of the pH gradient 4–7 to the full length of the IEF strip caused a marked increase in resolution in the two-dimensional gels. For this reason, we selected the pH range 4–7 for the study, detecting more than 1000 spots. Reproducibility was tested by comparison of the variation within the different gels in the same group. An analysis of 1015 spots revealed $CV < 50\%$ in 89.3% of the spots in same-group gels. Among these, a $CV < 30\%$ was obtained in 61.5% of the spots. These data confirmed the high reproducibility of the gels obtained with the method used. Although silver staining methods provide a reduced linear response with over a 10- to 40-fold range in protein concentration,¹⁸ the range of variations found in our 2-D gels were within these limits, since the protein with the higher upregulation (α -enolase, one protein spot) increases 10.5-fold (see Table 2).

Figures 2–4 and Table 2 show the result of the 2-DE analysis of the proteomes of circulating monocytes of ACS patients at different times. On admission, the expression of 18 protein spots was altered in comparison to that of stable patients (4 proteins were upregulated, 4 downregulated, and 10 absent). At day 4, this number was reduced to 5 proteins (1 upregulated,

1 downregulated, and 3 absent). At 2 months, 3 proteins displayed an altered expression (1 upregulated and 2 absent). Finally, at 6 months, there was not differential expression of any protein as compared to chronic CAD patients. These patients showed an altered expression of 5 proteins as compared to healthy subjects (Table 2). In the whole study, we found differences in the expression of 20 protein spots in monocytes of ACS patients as compared to stable CAD patients. Of interest, five of the protein spots showing changes in their expression in ACS corresponded to two proteins: protein disulfide isomerase (PDI) ER60 showing two different spots each one, and α -enolase, with three different spots (Table 2). Then, the total number of proteins whose expression was changed during ACS was 17.

Functional Groups Identified. Table 3 shows the theoretical M_r and pI , the access key, the sequence coverage according to PMF, and the main function of the identified proteins. Attending to their function, the proteins differentially expressed in ACS compared with stable CAD may be classified in different groups:

(a) Energetic and metabolism proteins: chain A triosephosphate isomerase (one protein spot), mannose 6 phosphate receptor, PDI (two protein spots), piruvate kinase M2 isozyme, alpha 1 enolase (three protein spots), chain A nuclease DNase/RNase sugar nonspecific, chain A endonuclease, nuclease, MY032, and glutathione transferase (HGSTP1).

(b) Hydrolytic enzymes: mature cathepsin D, cathepsin D proform, and uracil DNA glycosylase.

(c) Contractile and structural proteins: vimentin.

(d) Proteins involved in other functions (miscellaneous proteins): S100 calcium binding protein A8 (MRP8), thymidine phosphorylase precursor, and albumin precursor.

Among the five proteins differentially expressed in stable CAD patients as compared to healthy subjects, four [paraoxonase I (PON I), heat-shock protein-70 (HSP70), HSP60, and oncogene DJ1] were stress proteins, and one (an isoform of chain A triosephosphate isomerase) belonged to the subgroup of energetic and metabolism proteins.

Identification and Confirmation of Several Proteins of Interest with Specific Antibodies. To further confirm the proteomic results, a group of six proteins were analyzed by unidimensional Western blot. In the S100 calcium binding protein A8 Western blot, a band of 10 kDa corresponding to this protein was detected in healthy subjects but was not detected in ACS patients at day 0 (Figure 5). The same results were obtained by 2-DE Western blot (data not shown). The antibody recognized a protein of 10 kDa with a 6.5 theoretical pI in healthy subjects, in ACS patients at day 4, 2 months, and 6 months, and in stable CAD patients, but again, this protein was not present in ACS patients at day 0. Both results were similar to those obtained by proteomic analysis with the PD QUEST software. Assessment of oncogene DJ1, HSP70, and PON I expression yielded identical results. Finally, unidimensional Western blot showed an increase in vimentin expression in ACS patients versus healthy subjects, and Western blot also evidenced an enhanced expression of mature cathepsin D but not of its precursor form, confirming the results obtained by proteomic analysis.

Discussion

Monocyte-macrophages play a capital role in the formation and complication of the atherosclerotic plaques, and have been the focus of extensive research. However, we still do not have

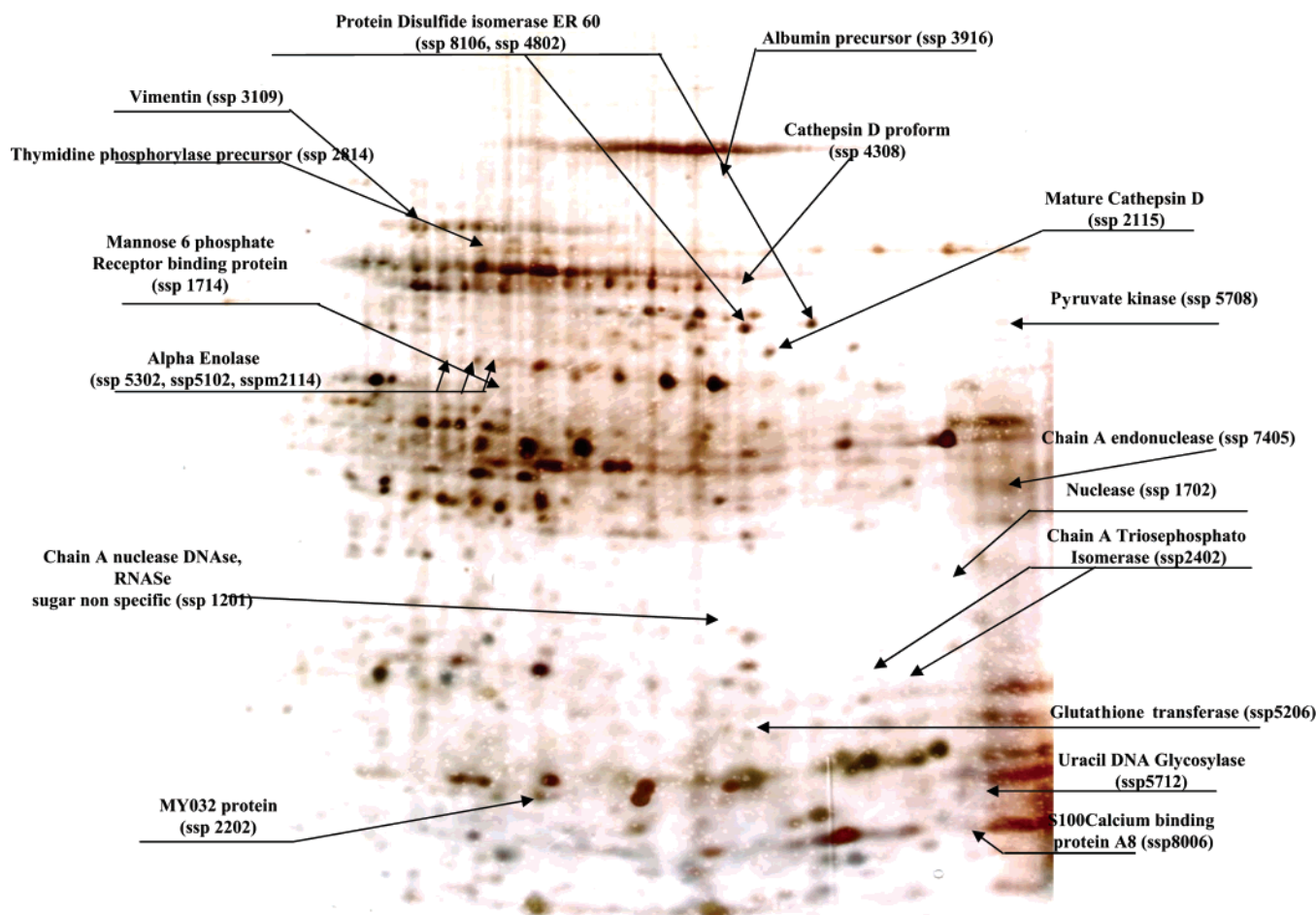


Figure 3. Representative image of 2-DE silver-stained gel of circulating monocytes (IEF: 4–7 pH range, second dimension, 12.5% acrylamide). Labeled arrows indicate protein spots that change in abundance in ACS patients versus stable subjects.

a complete understanding of their functions in atherothrombosis. Thus, we have addressed the proteomic study of circulating monocytes in patients with ACS and compared it with the proteome of patients with stable CAD.

The aim of this work was to identify novel proteins potentially involved in the development of ACS that could be the focus of future mechanistic studies. In addition, those proteins expressed differentially during this disorder may be new candidates to be tested in clinical trials as prognostic biomarkers.¹⁹ Of interest, we chose to study patients with non-ST-elevation ACS because, in this setting, myocardial necrosis is minimal or even absent. In the other subtype of ACS known as ST-elevation myocardial infarction, there is a large amount of myocardial necrosis that can induce systemic inflammatory changes. In this case, the alterations observed in the proteome of monocytes could be a consequence of myocardial necrosis, rather than being potentially related to the pathophysiology of ACS.

2-DE maps showed 20 spots with different expression levels in ACS as compared with stable CAD. Five of them corresponded to two proteins having several potential isoforms, probably due to post-translational modifications, then reducing to 17 the number of proteins whose expression was modified during ACS. The number of spots with altered expression was maximal on admission and decreased progressively. Six months after the ACS, the proteome of circulating monocytes stabilized and became similar to that of chronic patients. When compared

to healthy subjects, this proteome differed in five proteins. The protein spots showing an altered expression are grouped according to their functions for discussion purposes.

Energetic and Metabolism Proteins. Chain A triosephosphate isomerase, mannose 6 phosphate receptor, PDI, and pyruvate kinase are mitochondrial proteins. Mitochondria provide the energy that fuels the maintenance, repair, and turnover of cellular components. Impairment of mitochondrial function is thought to play a major role in many diseases resulting from the damage caused by reactive oxygen species.²⁰ Mitochondrial oxidative stress has been involved in heart disease including ischemia, myocardial preconditioning, and other pathologies. With the exception of one isoform of chain A triosephosphate isomerase, which was underexpressed similarly in ACS and chronic patients, all the proteins of this group were underexpressed specifically in ACS, but not in stable patients. Therefore, a reduction in the expression of these proteins could be related to the triggering of ACS. If these changes are secondary to this syndrome or if they are implicated in its pathophysiology must be the subject of future work.

PDI is a multifunctional protein which catalyzes the oxidation, reduction, and isomerization of disulfide bridges within a molecule during the post-translational process.²¹ It has a critical role in immune and inflammatory responses, and its overexpression can suppress NF- κ B-dependent transcriptional activity.²² NF- κ B upregulates the expression of many proinflammatory and prothrombotic proteins,²³ and its expression

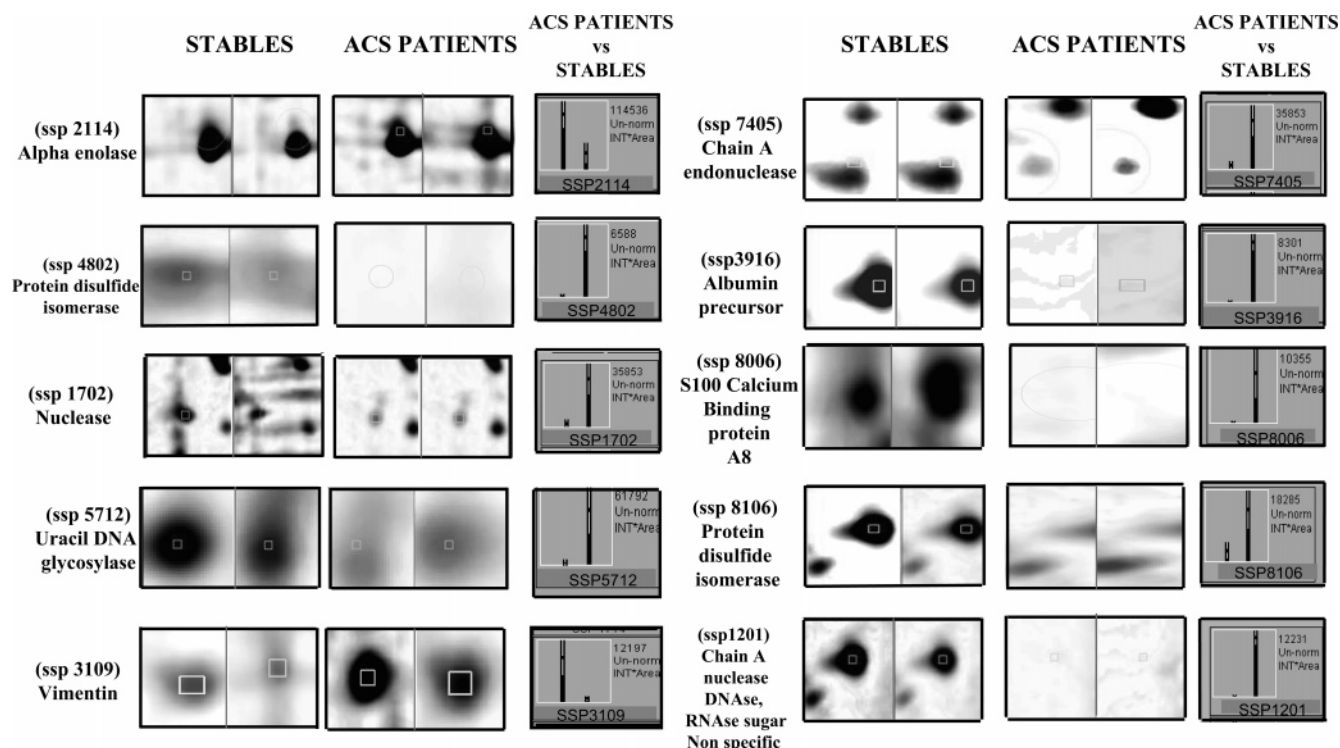


Figure 4. Differential protein expression in monocytes of ACS patients at day 0 compared with stable subjects. The selected area of the spots shows intensity difference between both groups.

is enhanced in atherosclerotic plaques responsible for an ACS²⁴ and in circulating PBMCs during this disorder.²⁵ In our study, monocytes from ACS patients at day 0, but not from stable subjects, showed a significant decrease of an isoform of PDI and complete absence of another isoform, the latter keeping absent at least during 2 months. This raises the hypothesis that, in ACS, the enhanced activity of NF- κ B could be due to a lack of negative regulation by PDI.

The glycolytic pathway was upregulated in ACS patients, as, on admission, there was an increase in the expression of alpha 1 enolase that was not present in stable subjects and which normalized at day 4. These results suggest a balance favoring this pathway over fatty acid oxidation. It has been shown previously that the expression of this enzyme increases in mouse peritoneal macrophages when they are transformed into foam cells through cholesteryl ester accumulation.²⁶ Thus, during ACS, circulating monocytes display biochemical features predisposing them to become foam cells once they enter the vascular wall. We found also an important downregulation in the expression profile of enzymes that catalyze the hydrolysis of nucleic acids and play an important role in cell defense.²⁷

Glutathione transferases have a protective function, modulating oxidative stress. Transfection with cDNA of glutathione S-transferase A4-4 protects endothelial cells from H₂O₂-induced apoptosis,²⁸ a biological process related to atherosclerotic plaque instability.^{29,30} In addition, the induction of this and other antioxidant enzymes in rat smooth muscle cells increases their resistance to oxidative vascular injury.³¹ Glutathione transferase is also involved in the detoxification of harmful substances from tobacco smoke, such as epoxides. The genetic lack of glutathione S-transferase predicts an increased incidence of stroke and myocardial infarction in diabetic smokers.³² The absence of expression of this enzyme in the monocytes of ACS patients raises the possibility of a reduction in the

antioxidant capacity of the organism as a contributor to the triggering of this syndrome, since its expression was normal in stable CAD patients.

Hydrolytic Enzymes. Cathepsin D is a protease responsible for the degradation of intracellular and endocytosed proteins.³³ It is biosynthesized as precursor cathepsin D and activated to a single chain mature form by proteolytic changes in different cellular compartments. After it is released from macrophages, the mature form may modify LDL, leading to foam cell formation³⁴ and inducing apoptosis,³⁵ then favoring plaque instability. ACS, but not stable, patients displayed absence of the expression of cathepsin D proform. However, the expression of the active enzyme was enhanced as demonstrated by proteomic and Western blot approaches. This suggests that during ACS there is an enhanced activation of cathepsin D. Given that this enzyme is a marker of differentiation of monocytes into macrophages,³⁶ our results may indicate that circulating monocytes in ACS patients have begun this process in part before entering the arterial wall.

Contractile Proteins. The expression of the contractile protein vimentin was upregulated in ACS patients on admission, returning later to normal values. This protein is the most ubiquitous component of the intermediate filaments that build up the cytoskeleton of almost all eukaryotic cells and is the first to be expressed during cell differentiation. Vimentin binds to oxidized LDL in macrophages, where it could play a role in the intracellular processing of this lipoprotein.³⁷ It is also involved in vascular smooth muscle cell differentiation.³⁸ Vimentin expression has been described recently by proteomic analysis to be upregulated in THP-1 human monocytes stimulated by oxidized LDL.³⁹ The enhanced expression of this protein in ACS patients on admission opens the possibility of a role for this protein in the triggering of ACS, although mechanistic studies must be performed to explore this matter.

Table 3. Characteristics of the Proteins Differentially Expressed in ACS versus Stable Patients^a

Protein	Mr Th/ (experimental)	pI Th/ (experimental)	access key	sequence coverage (%)	function/cellular localization
Nuclease (1702)	35.4 (35)	8.42 (6.7)	P61221	45	Hydrolyzes DNA/nucleus
Chain A triosephosphate isomerase (2402)	26.5 (26)	7.7 (6.3)	Q6FHP9	37.9	Glycolysis/Mitochondria
Chain A triosephosphate isomerase (2403)	26.5 (26)	7.7 (6.3)	Q6FHP9	38.3	Glycolysis/Mitochondria
RNA binding regulatory subunit ONCOGEN DJ1 (7208)	19.9 (20)	6.4 (6.2)	Q99497	42.9	Transforms cells when coexpressed with H-Ras/ Cytoplasm and or nucleus
Chain a nuclease, DNase, RNase, sugar nonspecific (1201)	26.5 (26)	6.2 (5.8)	Q99575	78	Hydrolyzes DNA/Nucleus
Chain endonuclease (Serratia) (7405)	35.4 (35)	8.4 (7)	P13717	48.2	Hydrolyzes DNA/Nucleus
Paraoxonase I (PON I) (5401)	27.5 (32)	5.7 (5.5)	P27169	33.7	Pentose phosphate pathway. Antioxidant/Plasma Membranes, Extracellular, Mitochondria/Microsomes
Heat shock protein (60 kDa) (1504)	60 (60)	5.48 (5.4)	P10809	12	Transport of membrane components through the cell/Mitochondria and or Cytoplasm
70 kDa Heat shock protein (44 kDa subunit) (8503)	44 (44)	5.48 (5.4)	P08107	16	Chaperone/Cytoskeleton, Nucleus
Mannose 6 phosphate receptor binding protein I (1714)	47 (45)	5.3 (5.2)	O60664	23.5	Proteins transport from endosomes to the trans-Golgi/Cytoplasm
MYO32, ATP synthase D chain, mitochondrial (2202)	18.4 (20)	5.22 (5)	O75947	44.5	Mitochondrial ATPase complex/Mitochondria
Vimentin (3109)	53.6 (59)	5.06 (5)	P08670	49.8	Class III filaments/Cytosol, Mitochondria,
Thymidine phosphorylase precursor (Endothelial cell growth factor 1 platelet derived) (2814)	50 (58)	5.2 (5)	P19971	13.2	Maintenance of blood vessels. Angiogenic factor/Cytosol
Albumin precursor (3916)	69.4 (72)	5.8 (5.6)	P02768	99	Regulation of blood colloidal osmotic pressure/ Extracellular, Mitochondria
Protein disulfide isomerase ER60 (4802)	56.8 (57)	5.9 (5.5)	P07237	42.2	Rearrangement of disulfide bonds/Endoplasmic reticulum, Golgi apparatus
Protein disulfide isomerase ER60 (8106)	56.8 (57)	5.9 (5.5)	P07237	29.9	Rearrangement of disulfide bonds/ Endoplasmic reticulum, Golgi apparatus
Glutathione transferase (HGSTP1) (5206)	25 (25)	5.3 (5.4)	P08263	41.3	Glutathione conjugation. Detoxification
α Enolase (5302)	47 (50)	7.7 (5)	P06733	30.4	Glycolysis, oxidative stress/Cytosol
α Enolase (5102)	47 (50)	7.7 (5.2)	P06733	34.6	Glycolysis, oxidative stress/Cytosol
α Enolase (2114)	47 (50)	7.7 (5.2)	P06733	34.6	Glycolysis, oxidative stress/Cytosol
Uracil DNA glycosylase (5712)	29.2 (18)	9.1 (7)	P16769	28.1	Protease. Prevents mutagenesis/Mitochondria, Nucleus
S100 Calcium binding protein A8 (MRP8) (8006)	10.8 (10)	6.5 (6.8)	P05109	53.8	Macrophage activation, regulated myeloid cell mutation, fatty acid metabolism./Mitochondria, Cytoplasm, Nucleus
Cathepsin D mature (2115)	32 (38)	5.2 (5.5)	Gi/494296	32.8	Proteolytic enzyme. Intracellular protein breakdown/Cytosol, Nuclear, Extracellular, Lysosomal
Cathepsin D preproform (4308)	55 (52)	6.7 (5.6)	P07339	52.3	Proteolytic enzyme. Intracellular protein breakdown/Cytosol, Nuclear, Extracellular, Lysosomal
Pyruvate kinase M2 isozyme (5708)	57.8 (55)	7.95 (7)	P14618	21.7	Glycolysis. Presented as antigen by dendritic cells/Mitochondria

^a Mr Th, theoretical molecular weight/experimental molecular weight; pI Th, theoretical isoelectric point/experimental isoelectric point.

Miscellaneous Proteins. Thymidine phosphorylase has been shown recently to inhibit vascular smooth muscle cell proliferation,⁴⁰ a biological phenomenon involved in the pathophysiology of atherosclerosis. In addition, it enhances neoangiogenesis,⁴¹ a process which alleviates the consequences of CAD, since the neovessels formed may supply blood and oxygen to myocardial regions jeopardized by atherosclerotically narrowed

coronary arteries. Then, the absence of expression of its precursor in the monocytes from ACS patients may represent a lack of these protective effects.

S100 are a family of calcium-binding proteins that have been reported to be useful markers for stroke and other brain injuries.^{42,43} They are also ligands for the receptors for advanced glycation end products, which are important mediators of

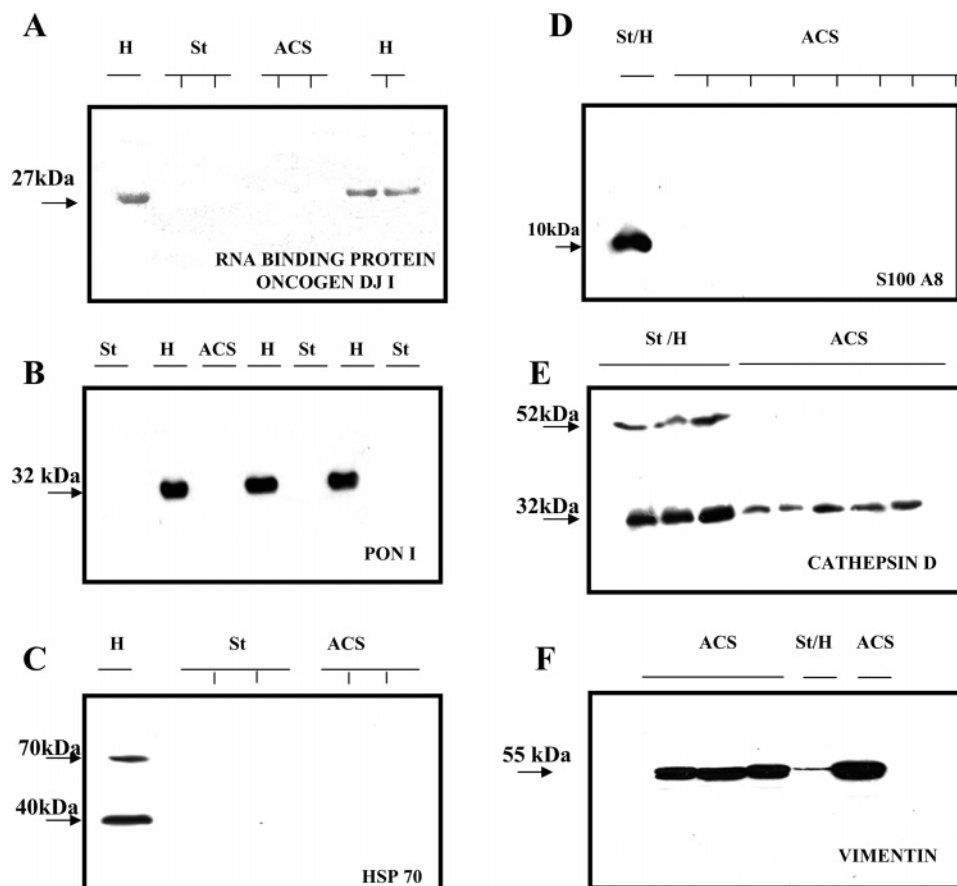


Figure 5. Confirmative Western blots. (A) RNA binding protein oncogene DJ1, a band of 27 kDa, was detected in healthy patients (H) but did not appear in ACS patients (ACS) and in stable patients (St). (B) PON I shows similar results that the RNA binding protein oncogene DJ1, a band of 32 kDa, was detected in healthy patients (H) but did not appear in ACS patients (ACS) and in stable patients (St). (C) HSP-70; the antibody detected two bands (44 and 70 kDa) in healthy subjects (H), but these bands did not appear in ACS patients (ACS) and in stable patients (St). (D) S100 A8 protein: a band of 10 kDa was detected in a healthy subject and in stable patients (H/St) but did not appear in ACS patients (ACS). (E) Cathepsin D is detected in two bands in healthy subjects and in stable patients (H/St) corresponding to the precursor (52 kDa) and mature forms (32 kDa) of the enzyme. Only the mature form is detected in ACS patients (ACS), confirming the results obtained by proteomic analysis. (F) Vimentin Western blot showed an increase in expression in ACS patients (ACS) versus healthy and stable subjects (H/St).

vascular injury, and had been shown to accelerate atherogenesis in murine models.^{44,45} S100A8 is expressed constitutively in high concentrations by granulocytes and during early differentiation stages of monocytes.^{44,45} It regulates phosphorylation, NADPH-oxidase activity, and fatty acid transport in monocytes and neutrophils.⁴⁶ This protein has also chemoattractant properties and has been related to inflammatory conditions, including atherosclerosis,⁴³ and is present in the macrophages, foam cells, and neovessels from human atheroma.⁴⁶ However, we have found that S100 A8 is not expressed by monocytes from ACS patients on admission, while it was expressed normally in chronic patients. Moreover, this result was confirmed by 2-DE and Western blot analysis. Then, the disappearance of S100 A8 expression could be related to ACS triggering. This is an unexpected result given the proatherogenic properties of this protein. Further studies are needed to elucidate if these results are due to a reduction in the expression of S100 A8 or to its secretion from monocytes to plasma during stress conditions.

Stress Proteins. With the exception of one isoform of chain A triosephosphate isomerase, all the proteins discussed above were differentially expressed in ACS as compared to chronic patients. However, four proteins involved in the cell response

to stress were differentially expressed both in chronic and acute patients as compared with healthy subjects. Thus, they seem not to be implicated in plaque complication, but could have a role in the pathogenesis of atherosclerosis, as a working hypothesis for future studies.

PON I is partially responsible for the antioxidant effects of HDL and has been reported to be protective against atherothrombosis.⁴⁷ Polymorphisms of PON I with lower capacity to protect from LDL oxidation are associated with an increased incidence of CAD.⁴⁸ PON I serum activity is reduced in patients with myocardial infarction and in subjects with different cardiovascular risk factors.⁴⁹ Its absence in the monocytes of stable CAD patients could indicate a lack of antioxidant activity in chronic coronary atherosclerosis, a disorder in which oxidative stress plays a major role.

HSP-70 protects endothelial function and is involved in antiapoptotic mechanisms preserving the myocardium from hypoxia.^{50,51} Then, patients with CAD lack the protective properties of HSP-70. In this regard, it has been reported that increased serum levels of this protein are associated with low risk of CAD.⁵¹

HSP-60 is involved in atherogenesis; activates endothelium, smooth muscle cells, and macrophages; and is present in

human atheroma.^{52,53} Moreover, serum levels of HSP-60 are augmented in patients with previous myocardial infarction.⁵⁴ The absence of detectable HSP-60 in the monocytes of all patients with CAD in our study could represent the release of this protein into the plasma, as its proinflammatory activity remains unexpressed while it is sequestered inside the cells.⁵⁴ In this regard, the assessment of HSP-60 plasma levels in ACS patients deserves further research.

Finally, oncogen DJ1, was also absent in stable and ACS patients. This protein is a cochaperone of HSP-70, and they prevent LPS and IFN-gamma-induced apoptosis in macrophages.⁵⁵ Thus, the absence of the chaperone pair HSP-70/DJ1 could enhance apoptotic death of macrophages, favoring the atherogenic process.⁵⁶

Conclusions

In this work, we have shown that the expression of 20 proteins is altered in the circulating monocytes of patients with ACS. The number of proteins whose expression was modified decreased during follow-up, and at 6 months, the pattern of monocyte protein expression was identical to that of patients with stable CAD. These findings open the possibility to explore in future studies if the reported variations of protein expression in monocytes of ACS patients play a role in the pathophysiology of this disorder. Also, they bring the opportunity to investigate if some of the proteins whose expression was altered could be potential candidates for risk assessment in ACS. Nevertheless, prospective studies correlating their plasmatic levels with the evolution of patients are needed to confirm this issue.

Abbreviations: ACS, acute coronary syndrome; CAD, coronary artery disease; CV, coefficient of variation; MS, mass spectrometry; PBMC, peripheral blood mononuclear cells; PDI, protein disulfide isomerase; PON I, paraoxonase I; 2-DE, two-dimensional electrophoresis.

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