[Frontiers in Bioscience E2, 796-804, June 1, 2010]

Proteomics reveals high levels of vitamin D binding protein in myocardial infarction

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1. ABSTRACT

The pathogenic mechanisms underlying the disease processes in cardiovascular disease are likely to involve significant alterations in myocardial gene and protein expression. Proteomics analysis can define new protein and peptide changes associated with myocardial infarction (MI). The aim of the present study was to analyze serum proteome of patients with ST-Elevation MI (STEMI). Serum samples were collected from STEMI patients (age 65.0+/-10.3) at 5.3+/-2.7 hours after the onset of typical chest pain and before initiating standard therapy. Ten age- and sex-matched donors were used as controls. The samples were albumin- and IgG-depleted. Isotope-coded affinity tag method was employed to label cysteine residues and liquid chromatography-Tandem Mass Spectrometry analysis was performed to measure the labelled proteins. Our proteomic approach identified increased levels of vitamin D-binding protein precursor (VDB) in the serum from STEMI patients compared to control donors. Western blot analysis confirmed the increase in VDB protein in STEMI patients. Moreover, fresh thrombotic plaques, obtained during primary angioplasty, showed high expression of VDB protein. Finally, VDB protein reduced the aggregation rate and prolonged coagulation time.

2.INTRODUCTION

Arterial thrombosis precipitates coronary occlusion and myocardial infarction characterized by ST-segment elevation (STEMI) (1). When plaque disruption occurs, a sufficient quantity of thrombogenic substances is exposed, and the coronary artery lumen may become obstructed by a combination of platelet aggregates, fibrin and red blood cells. At a pathophysiological level, STEMI is distincted by abrupt plaque destabilization, platelet aggregation and coronary thrombus formation, as demonstrated by profound benefit induced by prompt aggressive antiaggregatory regimens for such patients. Several studies addressed the role of inflammation, atherogenesis and, ultimately, atherothrombosis as pivotal pathogenetic determinants of plaque rupture (2).

As the mechanisms and pathways involved in the processes of plaque rupture, thrombosis, and response to injury are defined, a logical evolution would be to use this opportunity to better define risk of future events and complications (3). Although genomic approaches provide information on all the possible ways in which an organism may express its genes, it does not provide insights into the ways in which an organism may modify its pattern of gene expression in response to particular conditions (5).

Table1. Clinical characteristics of the groups studied

	STEMI patients (n=10)	Control donors (n=10)	STEMI patients (n=10)
Male	70	70	90
Age, y	65.0+/-10.3	63.8+/-12.6	52.5+/-12.9
Hypertension	60	0	60
Hyperlipidemia	60	0	50
Diabetes Mellitus	40	0	10
Smoking	60	0	40
Familial CAD[1]	40	0	30
Hystory of chest pain	30	0	20
Previous PTCA[2]	20	0	20
ST-segment elevation	anterolateral	no	anterolateral
EF[3]	43.0+/-9.9	54.6+/-8.2	43.7+/-8.7

Patients demographics for proteomics study (left and middle columns) and immunoblotting analysis on thrombi (right column). Data are expressed as mean +/- SD where appropriate. Values are percentages unless otherwise indicated. Descriptions: [1]coronary atherosclerotic disease; [2]percutaneous transluminal coronary angioplasty; [3]ejection fraction

Therefore, use of proteomic markers of coronary thrombosis in acute coronary syndromes (ACS) to predict the likelihood of recurrence or even appropriate response to therapy may facilitate targeted therapeutic strategies based on a comprehensive molecular risk profile rather than on demographic and clinical characteristics (6).

Currently available markers, such as troponins and myoglobin, reflect myocyte injury but do not pinpoint the specific mechanism of injury and are not active contributors to the pathophysiology of ACS (7).

It has been demonstrated that plasma levels of fibrinogen and other inflammation-sensitive plasma proteins such as haptoglobin, orosomucoid, alfa-1-antitrypsin, and ceruloplasmin, are associated with incidence of myocardial infarction and death by determining acute and chronic inflammatory response of the plaque (8). However, in order to gain insight into the identification of patients at earlier stage of disease atherosclerotic lesions destabilization, new markers that can detect endothelial damage, thrombus formation, and platelet aggregation need to be investigated.

Proteomic approaches, such as two-dimensional gel electrophoresis, and mass spectrometry, have been extensively employed to investigate cancer and other diseases, but still few reports in the cardiovascular scenarios are available (9, 10). In the absence of specific targets or processes, mass spectrometry-based proteomics offers an unbiased high-content approach to identify disease-associated compositional changes (11). By using proteomics, recent studies demonstrated different protein isoforms changings in plasma during acute coronary events, thus adding experimental data on the proinflammatory cascade seen in the ACS setting (12, 13).

Accordingly, the aim of the present study was to use proteomic analysis to identify new protein changes associated with STEMI.

3. MATERIALS AND METHODS

3.1. Sample preparation

Serum samples were obtained from 10 normal individuals (age 63.8+/-12.6) and 10 patients (age 65.0+/-10.3) with ST-elevation myocardial infarction at 5.3+/-2.7 hours after the onset of typical chest pain and before standard medical therapy was initiated (Table 1). According to the Declaration of Helsinki the locally appointed ethics committee has approved the research protocol and all informed consents have been obtained from the subjects.

The serum was aliquoted and stored at –80 °C until further analysis. Using a ProteoExtract™ Albumin/IgG Removal Kit (Calbiochem) as previously described (9, 12), a 35 microL aliquot of serum was diluted 10-fold with Binding Buffer (kit reagent), before loading it onto the gravity-flow column. The depletion procedure was performed according to manufacturer protocol instructions. Flow-through contained the albumin and IgG-depleted serum fraction. The column was further washed with 600 microL of ProteoExtract™ Albumin/IgG Binding Buffer and the elute fractions collected were combined with the flow-through depleted serum fraction. Total protein content of crude and depleted serum samples were detected using the dye-binding protein assay (Bio-Rad) with bovine serum albumin as standard curve. SDS-PAGE analysis of human serum before and after depletion was performed to evaluate albumin and IgG removal. The gel was stained with colloidal blue stain (EZBlue™ Gel Staining Reagent, Sigma).

3.2. ICAT assay

The depleted serum samples were mixed with cold-acetone in 1:7 v/v ratios and the proteins were precipitated at –20°C over night. The precipitated proteins were then centrifuged at 13.000 *g* for 15 min at 4°C and resuspended in 50 mM Tris and 0,1% SDS buffer pH 8,5. The complex protein samples (100 microg) were reduced, alkylated and digested according to the protocols supplied in the Cleavable ICAT Reagent Kit for protein Labeling (Applied Biosystems).

Sera from the 10 patients were individually labelled with the ICAT heavy reagent, while the 10 control samples were pooled and labelled with the ICAT light reagent.

Samples were cleaned-up by the ICAT Cartridge-Cation Exchange to remove detergents, reagents and trypsin. Tryptic digest peptides were acidified (pH 3,0) by the addition of 100mM formic acid. Cysteine-containing

peptides were isolated by the ICAT Reagent Cartridge-Avidin followed by TFA treatment to remove the linker containing the biotin moiety as described in the Cleavable ICAT Reagent Kit for protein labelling (Applied Biosystems).

3.3. Protein sequencing and identification

Chromatography was performed on an Ultimate nano LC system from Dionex (Sunnyvale, CA). All chromatographic columns used were also from Dionex. The ICAT-labelled peptide mixture was dissolved in 200 microL of loading pump solvent, consisting of H_2O/acetonitrile/trifluoroacetic acid (TFA) 98/2/0.1 (v/v/v). 20 microL of the peptide solution were then injected for nanoscale LC-MS analysis. Peptides were loaded onto a 0.3 X 5 mm Pepmap C_{18} trapping column at a flow rate of 30 microL/min (using the loading pump solvent). Trapping column wash time was 5 minutes. For gradient elution of the peptides through the trap/analytical column, two mobile phases were used. Mobile phase A was H_2O/acetonitrile/formic acid (FA)/TFA 97.9:2:0.08:0.02 (v/v/v/v); mobile phase B was H_2O/acetonitrile/FA/TFA 4.9:95:0.08:0.02 (v/v/v/v). Gradient was from 5 to 45% B in 80 minutes at 300 nL/min flow rate. Then, after 5 minutes at 95% B, the column was re-equilibrated at 5% B for 20 minutes.

MS detection was performed on a QSTAR XL hybrid LC-MS/MS from Applied Biosystems (Foster City, CA) operating in positive ion mode, with nanoelectrospray potential at 1800 V, curtain gas at 15 units, CAD gas at 3 units. Information-dependent acquisition (IDA) was performed by selecting the two most abundant peaks for MS/MS analysis after a full TOF-MS scan from 400 to 1600 m/z lasting 1.5 seconds. Both MS/MS analyses were performed in enhanced mode (1.5 seconds/scan). Threshold value for peak selection for MS/MS was 20 counts.

Protein quantification and identification were performed using the ProICAT™ software from Applied Biosystem. For protein identification, data were searched on the Swiss-Prot database accessed on November 2005. Protein hits having at least one peptide identified at 95% confidence were retained. The following parameters were used: MS tolerance 0.15 Da; MS/MS tolerance 0.5 Da; possible methionine oxidation; enzyme trypsin; max. missed cleavages 1.

3.4. Western blotting analysis on whole serum

Data obtained by proteomics analysis were confirmed using Western blot: 80 microg of entire serum proteins from different pathologic samples and a mix from 10 control samples were loaded and separated by SDS-PAGE, transferred on a nitrocellulose filter through trans-blot (Biorad) and hybridized with VDB protein antibody (SantaCruz Biotechnology). Gel was normalized using Coomassie Brilliant Blue (Bio-Rad).

3.5. Immunoblotting analysis in thrombus extracts

In order to correlate the increased levels in VDB protein with plaque rupture, fresh coronary thrombi were retrieved in additional 10 consecutive STEMI patients who were admitted in the catheterization laboratory and treated with a thrombectomy catheter (Export™ Aspiration Catheter, Medtronic Inc.) within three hours of onset of chest pain. Thrombotic material was digested in lysis buffer and stored at -80º C until further analysis. Proteins extracted from coronary thrombi, together with a positive control, were loaded and separated by SDS-PAGE, transferred on a nitrocellulose filter through trans-blot (Biorad) and hybridized with VDB protein antibody (SantaCruz Biotechnology). Gel was normalized using Coomassie Brilliant Blue (Bio-Rad).

3.6. Platelet Aggregation Study

Platelet aggregation was determined within 2 h of blood being drawn. Briefly, VDB protein at the dose of 200 microg/ml was infused into the withdrawn blood. Then platelet-rich plasma (PRP) was prepared by centrifugation of blood at 150 *g* for 8 min, whereas platelet-poor plasma (PPP) was prepared by centrifuging PRP at 1500 *g* for a further 20 min. Platelets in PRP were counted using a Neubauer haemocytometer (Merck Eurolab, Lutterworth, UK) and, if necessary, the cell count was adjusted to 2.5+/-0.5 x 10^5 cells/mL by dilution with PPP. Aggregation was performed in a PAP-4 Platelet Aggregation Profiler (Bio/Data, Horsham, PA), which was first calibrated using PRP (0% aggregation) and PPP (100% aggregation). Aggregation was carried out at 37°C in 0.2 mL of PRP in microvolume tubes and was initiated by adding ADP (stock 200 micromol/L) to final concentrations of 8 micromol/L. The aggregation curves were recorded and analyzed using the associated Bio/Data software for total percentage aggregation.

3.7. Coagulation assays

All blood specimens to measure PT, aPTT, fibrinogen, I.N.R. and D-dimer were collected into 5-mL vacutainer tubes (Becton Dickinson, Oxford, UK) containing 0.129 mol/l (3.8%) sodium citrate giving a specimen mixture of one part of citrate and nine parts of blood. Blood was centrifuged for 10 min at 3500 rpm and 4°C as soon as possible after collection (within 30 min). For absolute determinations, VDB protein at the dose of 200 microg/ml was added to platelet-rich plasma, while for evaluation of a dose-dependent effect of VBD protein on coagulation cascade, increasing amount of VDB protein (75, 150, 300, and 600 microg/ml) where used. All parameters were measured directly after sampling by using STA-R (Diagnostica Stago, Asnières Cedex, France) coagulation analyzer.

3.8. Data analyses and statistics

All data are shown as means +/- SE. Differences between groups were calculated by independent *t* test, 1-way ANOVA corrected with Bonferroni method. A *p* value less than 0.05 was considered significant.

4. RESULTS

4.1. Proteomic analysis revealed novel serum fingerprints

Both gel-based and non-gel-based proteomics methods for protein resolution and identification were used,

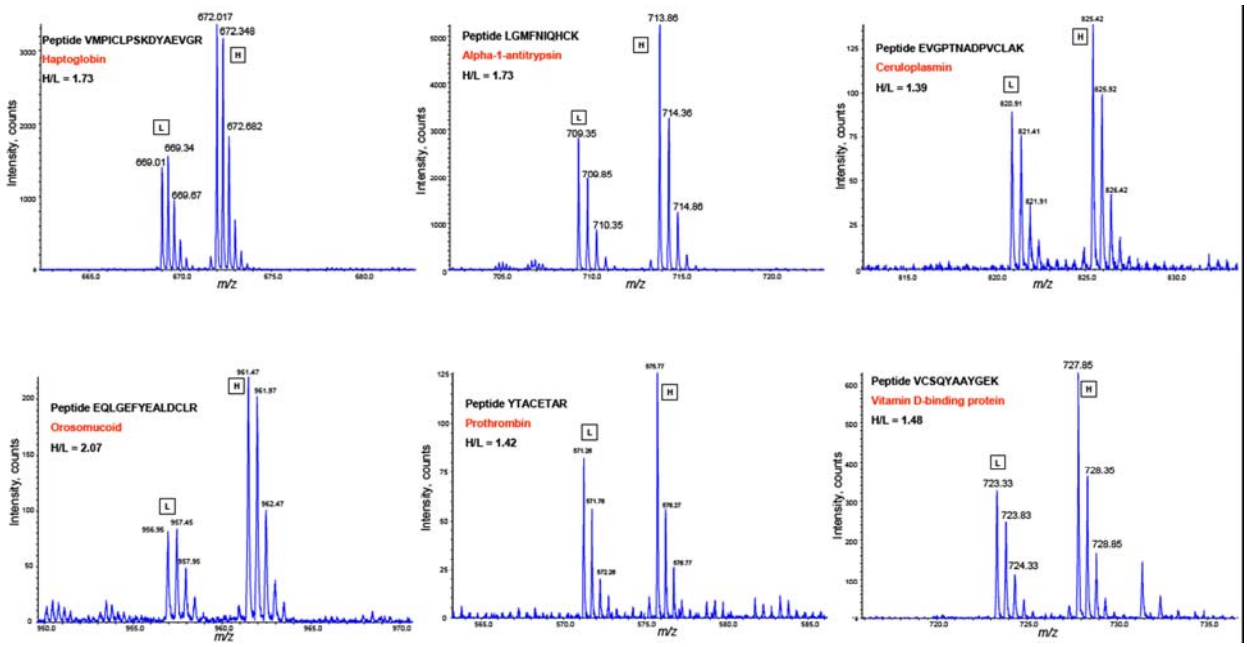

Figure 1. Mass spectrometry in proteomic analysis of blood samples from ST segment elevation myocardial infarction (STEMI) patients. Actual mass spectrometry analysis of six different peptides, whose sequences are shown in each plot, derived from protein fragmentation. The relative abundance of each fragment is shown in the spectrum as a function of its mass-to-charge ratio (m/z), and has been found increased in all the STEMI samples.

aimed at enhancing the coverage and reliability of the analysis. A total of 300 proteins were highly defined by the described proteomic method in the 10 pathological samples together with an additional pool of control samples. Ten of these proteins, haptoglobin precursor, prothrombin precursor (Coagulation factor II), alfa-1-antitrypsin, vitamin D-binding (VDB) protein precursor, hemopexin precursor, Lg-alpha-1 chain C regions, complement factor B precursor, ceruloplasmin precursor, complement C4 precursor and serum albumin precursor were identified in 100% of the analyzed samples. Mass spectrometric analysis demonstrated a significant increase in concentration of haptoglobin, alfa-1-antitrypsin, ceruloplasmin, and orosomucoid-1 (Figure 1) in the samples from patients with acute STEMI compared to controls. These data well reproduce similar findings reported in recent studies. However two novel proteins were identified in patients with STEMI. Remarkably, we observed a significant increase of VDB protein and prothrombin in the serum from acute STEMI patients compared to control donors (Figures 1 and 2). Monodimensional gel confirmed the higher amount of VDB protein and prothrombin in serum from patients after STEMI (Figures 3 and 6).

4.2. VDB protein is increased in thrombi extracts

In order to correlate the increased levels of VDB in the serum after STEMI with coronary thrombosis, thrombus extracts from coronary arteries of an additional subgroups of STEMI patients undergoing coronary angiography were collected and processed for protein extraction. Surprisingly, immunoblotting of lysates from thrombi demonstrated increased levels of VDB protein compared to a positive control (Figure 4), thus suggesting a possible role of VDB protein in the pathogenesis of plaque rupture.

4.3. VDB protein administration decreased platelet aggregation

The extent of platelet aggregation in response to ADP was significantly reduced from 79+/-13% to 48+/-13% after VDB protein release at the dose of 200 microg/ml directly to control blood samples (Figure 5, $p<0.001$). This was more dramatic at an intermediate concentration of ADP (8 micromol/L) reaching a level of 70-80%. At ADP concentrations more than or equal to 8 micromol/L, total percentage aggregation was submaximal and it was not significantly affected by VDB protein administration (data not shown).

4.4. VDB protein administration affects coagulation cascade at different levels

In order to evaluate both intrinsic and extrinsic coagulation pathways, chronometric and immunological tests were performed. VDB protein (200 microg/ml concentration) was released before testing coagulation properties of each sample, therefore aPTT and PT were evaluated. Both coagulation time indexes, as well as I.N.R., were significantly increased (15+/-0.9 sec. vs. 13+/-0.5 sec., p less than 0.01; 35.4+/-2.3 sec. vs. 29.6+/-0.7 sec., p less than 0.001; 1.25+/-0.13 vs. 1.02+/-0.07, p less than 0.01, respectively, Figure 7 from A to C).

Moreover, fibrinogen and D-dimer plasma concentrations were significantly reduced after VDB administration in all the evaluated

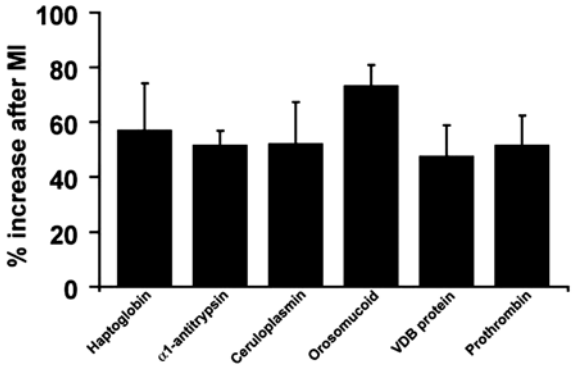

Figure 2. Percent change of plasma proteins in serum from STEMI patients. Isotope-coded affinity tag (ICAT) assay and liquid chromatography and mass spectrometry (LC-MS/MS) analysis revealed an increase in haptoglobin, alfa-1-antitrypsin, ceruloplasmin, and orosomucoid-1 levels in albumin/immunoglobulins depleted serum from STEMI patients compared to controls. Interestingly, we observed also a significant increase in Vitamin D Binding Protein (VDB) and Prothrombin levels.

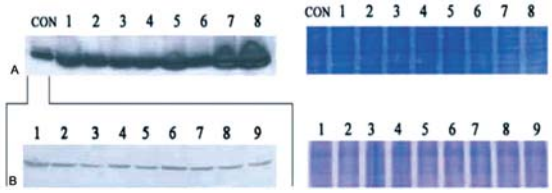

Figure 3. Monodimensional gel evaluation of VDB levels in STEMI patients. VDB increased significantly in patients suffering from an acute cardiac event. 80 microg of proteins extracted from whole serum of STEMI patients and controls were loaded, separated in SDS-PAGE, transferred on nitrocellulose filter and blotted with VDB antibody (*upper panel*). Normalization was obtained using Coomassie Brilliant Blue (*lower panel*).

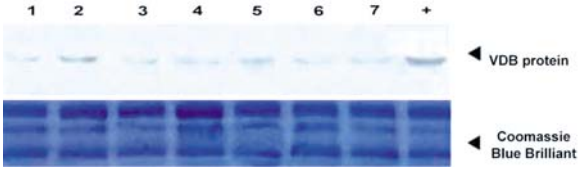

Figure 4. Immunoblotting for VDB protein in coronary arteries thrombi extracts. Thrombotic material was digested in Tris/EDTA buffer (pH 7.4). Proteins extracted from coronary thrombi, together with a positive control, were loaded and separated by SDS-PAGE, transferred on a nitrocellulose filter through trans-blot (Biorad) and hybridized with VDB protein antibody. Remarkably, detectable VDB was found in all samples (*upper panel*). Gel was normalized using Coomassie Brilliant Blue (*lower panel*).

samples compared to controls (240.8+/-31.1 mg/dl vs. 335.3+/-61.8 mg/dl, *p* less than 0.05; 0.23+/-0.025 microg/ml vs. 0.38+/-0.070 microg/ml, *p* less than 0.001, respectively, Figure 7 D and E). In summary, coagulation times of both intrinsic and extrinsic pathways were significantly prolonged after VDB protein release, indicating a possible implication of VDB protein in the common pathway of the coagulation cascade.

5. DISCUSSION

The present study examines changes in the serum proteome in patients suffering of an acute myocardial infarction at the time of their first admission to the emergency unit. The major findings of this study were: a) the serum proteomic analysis employed in this study was able to identify VDB protein variations as novel indicators of coronary thrombosis in acute STEMI; b) VDB protein levels have been found augmented in freshly isolated coronary thrombi extracts: c) both aggregation and coagulation properties were significantly affected by VDB protein administration in *ex vivo* extracts.

Despite improvements in the diagnosis and management of coronary atherosclerotic disease, heart failure resulting from myocardial infarction (MI) remains one of the leading causes of morbidity and mortality in the United States (14). Although scientific community is mainly involved in finding a specific marker with the ability to promptly detect patients at higher risk of developing an ACS, the question about which patients are at major risk requiring intervention or aggressive pharmacological therapy is still controversial (3). Therefore risk stratification in patients with ACS remains a major objective for the selection of optimal medical and interventional treatment regimens (8). Considerable evidence supports the concept that endothelial damage elicited by different stimuli such as certain bacterial products or diverse risk factors such as dyslipidemia, vasoconstricting substances, glycoxidated reactants, or proinflammatory cytokines, can trigger coronary thrombosis and, consequently, acute vessel's occlusion (4).

It has been recently described that endothelial cells under stress release paracrine mediators that facilitate accumulation of vascular smooth muscle cells (VSMCs) at sites of vascular injury (15). One of these growth factors, Vitamin D Binding Protein (VDB), has been involved in actin scavenging and modulation of inflammatory and immune mechanisms together with the ability to bind the principal vitamin D metabolites: 25-hydroxyvitamin D (25(OH)D; calcidiol), the major circulating metabolite, and 1,25-dihydroxyvitamin D (1,25(OH)2D; calcitriol), the most active metabolite of the vitamin (16).

Vitamin D-binding protein is a monomeric, multifunctional glycoprotein first identified as the group-specific component of serum or Gc-globulin (17). It is essential to the transport of vitamin D sterols in the blood and to the removal of plasma actin monomers released to the blood subsequent to tissue damage (18, 19). VDB protein also contributes to complement C5a-mediated chemotaxis, macrophage activation, and fatty acid transport (20, 21).

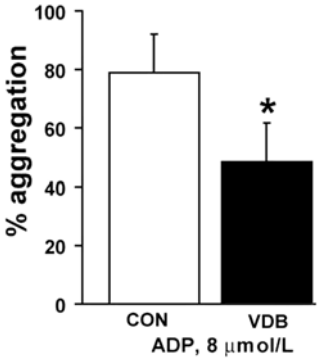

Figure 5. VDB protein reduced platelet percent of aggregation. ADP-induced platelet aggregation in human blood samples was evaluated in 9 consecutive control donors before and after VDB protein administration (200 microg/ml) and it was found reduced from 79 +/- 13% to 48 +/- 13%. Platelet aggregation was initiated by the addition of 8 micromol/L ADP to platelet-rich plasma (PRP). Values are means +/- SEM (p less than 0.001).

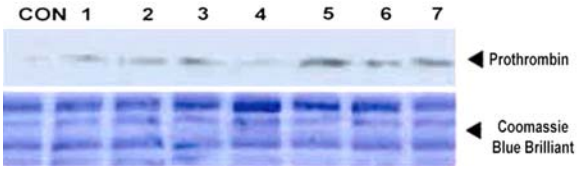

Figure 6. Monodimensional gel evaluation of prothrombin levels in STEMI patients.Prothrombin increased significantly in patients suffering from an acute cardiac event compared to a pool of control samples. 80 microg of proteins extracted from whole serum of STEMI patients and controls were loaded, separated in SDS-PAGE, transferred on nitrocellulose filter and blotted with prothrombin antibody which binds activation peptide fragment 1, activation peptide fragment 2, thrombin light chain, thrombin heavy chain (*upper panel*). Normalization was obtained using Coomassie Brilliant Blue (*lower panel*).

We previously demonstrated that under stressing conditions VSMCs can proliferate from the media layer to the lumen of the vessels and create a new structure called "neointima" (22, 23). This new formation is mainly related to the activation of specific intracellular pathways (24, 25, 26, 27). Although VSMC proliferation is poorly involved in the phenomenon of coronary thrombosis in native vessels, the extensive use of percutaneous revascularization with drug eluting stents, not followed by appropriately aggressive dual antiplatelet therapy with both aspirin and clopidogrel for an extended time, could paradoxically increase the incidence of vessel thrombosis in the near future, due to either a direct effect of the coating materials (28) or to different antiproliferative drugs employed (29).

Our study demonstrates for the first time an augmentation of VDB protein in freshly isolated coronary thrombi aspirated from percutaneously treated patients suffering of an acute myocardial infarction. According to a previous report, we found correlation among inflammation, platelet aggregation and coronary disease (33). Although higher thrombi levels of VDB protein were found in patients affected by acute myocardial infarction compared to control subjects, no relationship was found between VDB protein levels in sera compared to thrombi from same patients. Moreover, *in vitro* experiments using blood samples withdrawn from healthy donors without cardiovascular risk factors demonstrated that VDB administration to platelet-rich plasma fractions was able to reduce percent of aggregation and to increase coagulation time after ADP stimulation, hence corroborating the role of VDB protein in lowering the extent of platelet aggregation and thrombosis.

It is of relevant importance that patients included in our study were all homogeneous for type and extension of myocardial infarction together with infarction-related reduction in cardiac performance (Table 1). To this regard, no correlation has been found between seric levels of VDB protein and clinical parameters of acute ischemic conditions such as TIMI frame count before PTCA, and/or ejection fraction at admission.

Proteomics is an emerging approach capable to investigate the serum *in toto,* allowing to identify novel proteins in patients with STEMI. By using proteomic analysis, we identified changings of serum proteome in patients with acute myocardial infarction, showing a significant increase of Vitamin D-Binding Protein levels compared with the serum of control donors. Recent reports have extensively elucidated the need for new markers of cardiac risk and the prognostic significance of the identification of high molecular weight protein involved in coagulation cascade and/or thrombogenesis (30, 31, 32). However, our study demonstrated that other proteins and factors are able to modulate both coagulation and thrombogenesis without a direct correlation with these biological phenomena and that a proteomic approach is able to reveal novel roles and functions of molecules not strictly related to cloth or thrombus, such as VDB protein. Interestingly, this protein has been identified in several works as being present in the general circulation in at least three different isoforms, this phenomen perhaps due to alternative splicing processing (6, 33). However, there was no change in the plasma expression of any of the VDB isoforms in our study, as opposed to alfa-1-antitrypsin modifications in other studies, and no correlation with one specific VDB isoform has been detected so far in the STEMI setting. Therefore, our data could be relevant to establish a specific serum fingerprint, based on the proteomics, for the diagnosis and prognosis of the acute coronary syndromes and to investigate about the physiopathologic role of novel biomarkers in the myocardial damage in patients with STEMI.

A limitation of the present study is the small sample size due to logistic difficulties in performing extensive proteomic analysis on many samples obtained at the same time. Furthermore, the discriminating detection of 2-D gel electrophoresis might make evident only the most obvious changes in highly expressed proteins while obscuring changes in other proteins with lower expression

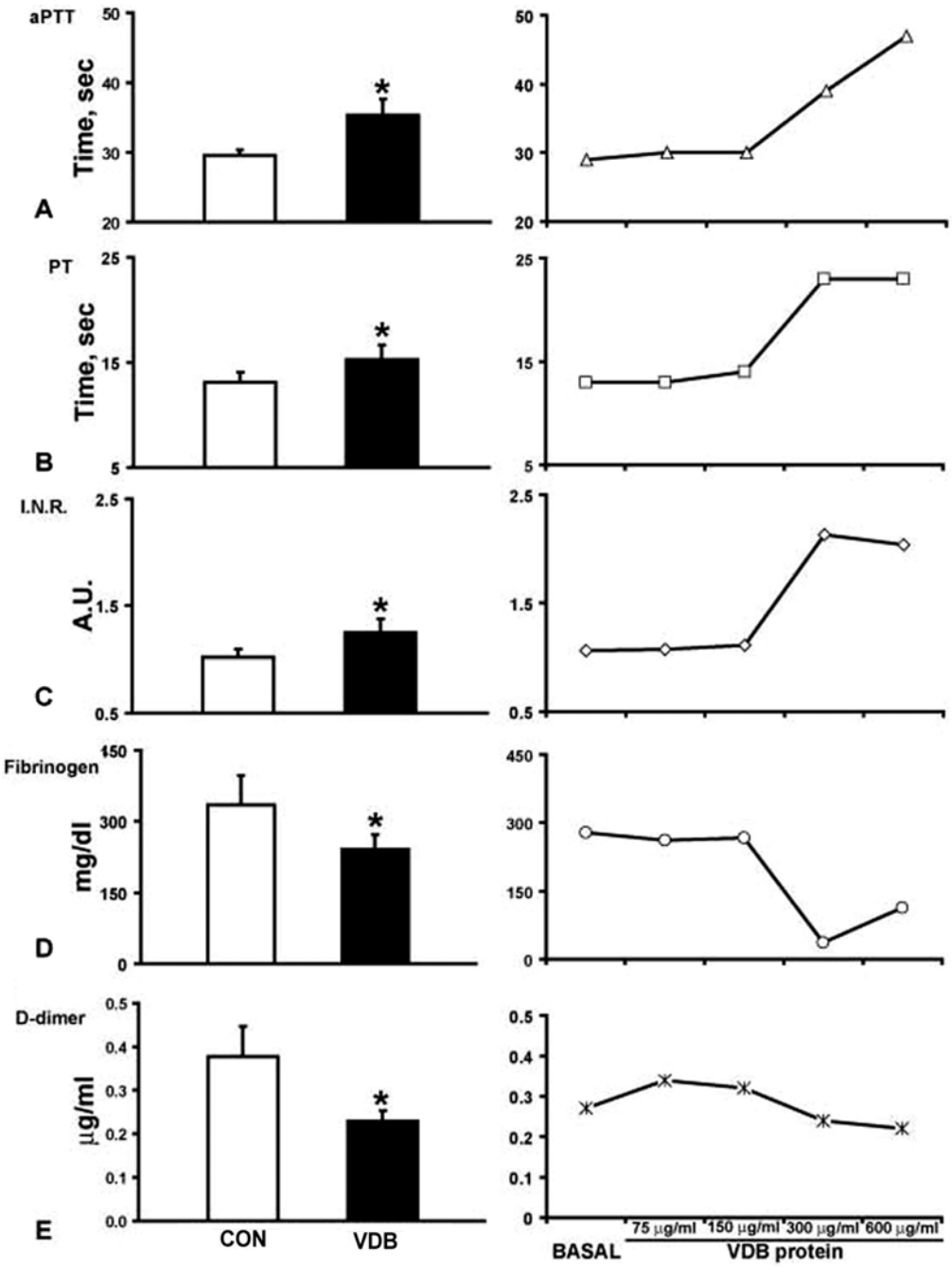

Figure 7. Effects of exogenous VDB protein administration on common pathway of the coagulation cascade. After *in vitro* administration of VDB protein (200 microg/ml) significantly prolonged coagulation time parameters such as prothrombin time, activated partial thromboplastin time and I.N.R. (15+/-0.9 sec. vs 13+/-0.5 sec., p less than 0.01; 35.4+/-2.3 sec. vs 29.6+/-0.7 sec., p less than 0.001; 1.25+/-0.13 vs 1.02+/-0.07, p less than 0.01, respectively) and reduced fibrinogen and D-dimer levels (240.8+/-31.1 mg/dl vs 335.3+/-61.8 mg/dl, p less than 0.05; 0.23+/-0.025 microg/ml vs 0.38+/-0.070 microg/ml, p less than 0.001, respectively) compared to controls.

levels. Other laboratory methodologies, such as ELISA and RIA assays, are currently employed to detect minimal changes in circulating molecular determinants of different diseases. These techniques easily overcome the fact that proteomic analysis is currently limited by sensitivity, specificity, and throughput. Moreover, quantification using two-dimensional electrophoresis is not an exact method, because gel spots can contain multiple proteins and mass spectrometry is not quantitative. However, this field and its methodologies are rapidly developing. Future studies with

an increased sample size of different ACS patients and normal coronary arteries will further validate our conclusion and perhaps might add newer interpretation of the role of VDB protein in coronary thrombosis in patient subset of increased risk of thrombosis such as DES, ACS/NSTEMI, and unstable angina.

In conclusion, an increase in VDB protein levels was found in patients with STEMI. Moreover, VDB protein was highly expressed in fresh thrombotic plaques of patients with STEMI. Finally, a reduced aggregation rate together with a prolonged coagulation time were found *in vitro* after VDB protein administration.

6. ACKNOWLEDGMENTS

Cosimo Gasparri and Antonio Curcio contributed equally to this work. This study was supported by an unrestricted grant from the Italian Health Ministry to the Tuscany Region for the Finalized Medical Research Program 2007 and by a Grant from Regione Calabria 2003.

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Abbreviations: STEMI: ST-segment elevation myocardial infarction; NSTEMI: non-ST-segment elevation myocardial infarction; ACS: acute coronary syndromes; VSMCs: vascular smooth muscle cells; VDB: vitamin D binding protein; PRP: platelet rich plasma; PPP: platelet poor plasma; ADP: adenosine diphosphate; ICAT: Isotope-coded affinity tag; LC-MS: liquid chromatography and mass spectrometry; TOF: time of flight; PT: prothrombin time; aPTT: activated partial thromboplastin time; ANOVA: analysis of variance; DES: drug eluting stents.

Key Words: proteomics, STEMI, Coronary Thrombosis, Vdb Protein, Atherosclerotic Plaque, Mass Spectrometry, Coagulation, Platelet Aggregation

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