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MECHANISMS OF INTRACORONARY THROMBUS FORMATION DURING ACUTE MYOCARDIAL INFARCTION: A COMPREHENSIVE MORPHOLOGICAL EVALUATION OF CULPRIT LESIONS, PROTEOMIC ANALYSIS AND SYSTEMS BIOLOGY

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1. Summary

Hypothesis

Multiple mechanisms, both local and systemic, are involved in the formation of the intracoronary thrombus related with acute myocardial infarction. Our hypothesis is that proteomic analysis of the intracoronary thrombus obtained from patients in the acute phase of myocardial infarction and the analysis with intracoronary image of the culprit lesions could reveal systemic and local pathophysiological triggers of thrombus formation. The integration of clinical, proteomic and systems biology data could facilitate the identification of potential biomarkers and new pharmacological targets.

Objectives

Primary objective: To know the molecular process associated with the formation of intracoronary thrombus during acute myocardial infarction (AMI) and its relationship with the degree of instability of the culprit lesion, in order to identify possible therapeutic objectives.

Secondary objectives:

1. Identify the differential expression of the proteins in intracoronary thrombus in AMI according to the mechanism of disruption of the culprit lesion (rupture versus non-rupture).
2. Integrate the clinical data of the patients with the proteomic data from the thrombus and plasma exosomes to determine the causal variability of the observed groups.
3. Identify the differential protein expression in the AMI according to the mechanism of disruption of the culprit lesion (rupture vs. no rupture).
4. Correlate the coronary substrate with inflammatory markers and system biology results.

Methodology

Thirty-nine patients with AMI with ST segment elevation between 50 and 80 years old, less than 6 hours of evolution from the onset of symptoms and undergoing percutaneous primary angioplasty and manual thromboaspiration have been prospectively included. The aspirated intracoronary thrombi were divided into two parts, one for histopathological analysis to confirm the nature of the thrombus and

another for its proteomic analysis. Likewise, intracoronary and peripheral blood samples were taken for analysis. Twenty patients were able to undergo optical coherence tomography (OCT) to determine the possible mechanism of disruption of the culprit plaque (rupture vs. no rupture - erosion or calcium nodule). Optical coherence tomography images have been analyzed in a centralized and accredited laboratory. The quantitative analysis included reference luminal area, luminal area of the lesion, thrombus length, thrombus area, total thrombus volume of each lesion, minimum luminal area and length of the responsible segment. The qualitative analysis evaluated type of plaque, presence or absence of breakage or erosion, and type of thrombus.

Proteomic and exosomal analysis.- Proteins were extracted in 7M urea, 2M thiourea and 2% SDS, and were reduced and digested using the FASP approach with trypsin / LysC and the peptides were cleaned with C18 upper tip microcolumns. Finally, the cleaned peptide solutions were dried and stored at -20° C and analyzed by LC-MSMS. All samples were analyzed in a single batch with the Maxquant software (1.6.3.4) and the Andromeda built-in search engine. Samples were classified according to the number of quantified proteins. Plasma exosomes were prepared and centrifuged at 1500 g for 10 min (4° C) and then at 10,000 g for 20 min (4° C) and purified using the Izon columns (qEVoriginal / 70 nm). The proteins were extracted with 150 ml of 6 M urea / 200 mM ammonium bicarbonate and precipitated. The resulting clean precipitate was resuspended in 6 M urea / 0.1 M Tris-HCl, vortexed and sonicated in an ultrasonic bath for 10 minutes. Proteins were quantified, digested with trypsin / lys C and the peptides desalted with C18 columns. The resulting peptides were analyzed by LC-MSMS, as for thrombi.

All the clinical and periprocedure characteristics of the patients were collected and a one-year clinical follow-up was performed.

2. Results obtained

Analysis of clinical characteristics and proteomic results of the intracoronary thrombus.

1. There were no statistically significant differences between the two clusters (A and D) in any of the clinical, angiographic and OCT characteristics, except for a slight association in diabetes mellitus.

2. A moderate correlation was detected between platelet factor 4 (PF4, CXCL4) with ischemia time (R2 = 0.584, p = 0.0085) and with PCR (R2 = 0.677, p = 0.002) (Figure 1a and Figure 1b). Platelet factor 4 is a cytokine released in alpha platelet granules that appears to have a procoagulant effect by moderating the effects of heparin. These results could suggest that PF4 would have an early role in thrombus formation, being consumed during its evolution, once the mechanisms of systemic inflammation reflected by high CRP values have been activated and amplified.

Figure 1a

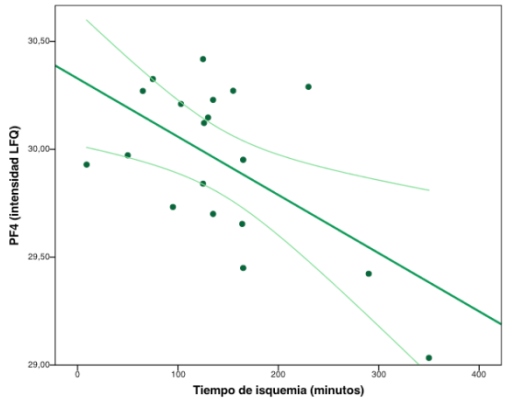
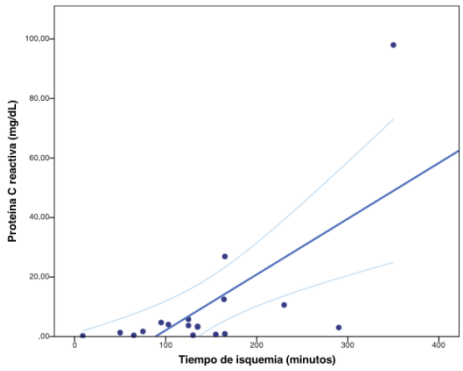


Figure 1b



These results will be the subject of future research and a plasma validation will be carried out to determine if PF4 is secreted into the bloodstream and could be considered a possible diagnostic biomarker of hyperacute infarction. At the same time,

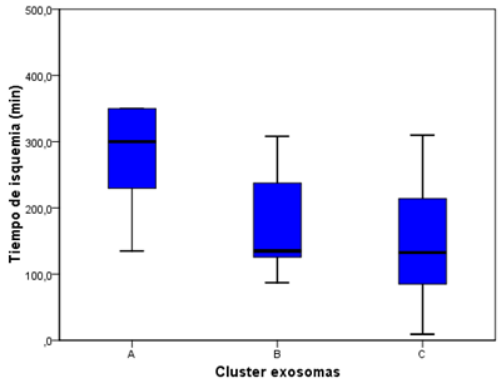
it could be the subject of an investigation of its potential role as a therapeutic target in hyperacute infarcts.

Analysis of the clinical characteristics and results of proteomics of exosomes

After the analysis for principal components of the proteins detected in plasma exosomes, 3 differentiated clusters were identified.

In the analysis of clinical and procedural characteristics of these patients it was documented that cluster A only differed from B and C in that it presented a significantly longer ischemia time (300 [164-350] min vs. 132 [103-232] min; p = 0.006) Figure 2. The ischemia time is a variable that determines the characteristics of the thrombus due to its extremely dynamic nature. Therefore, a comparison was performed of A vs. combined B + C, and in a second analysis comparison of B vs C.

Figure 2. Differences in ischemia time between the 3 clusters of exosomes.



Clusters B and C presented several differential clinical characteristics. B presented a higher proportion of classical cardiovascular risk factors (arterial hypertension: 92% vs. 25%; p = 0.001, diabetes mellitus: 50% vs. 13%; p = 0.044) (Figure 3a and Figure 3b). The ischemia time was similar and a higher concentration of C-reactive protein (8.2 [3.9-14.8] vs 1.4 [0.9-3.0]; p = 0.003) (Figure 3c) was documented reflecting a clearly different clinical profile.

Figure 3a. Differences in the incidence of AHT among the 3 exosome clusters (B vs C; p = 0.001).

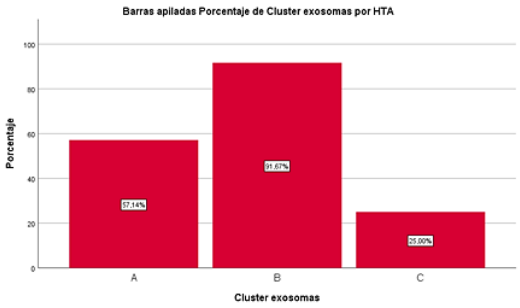


Figure 3b. Differences in the incidence of diabetes among the 3 exosome clusters (B vs C; p = 0.04).

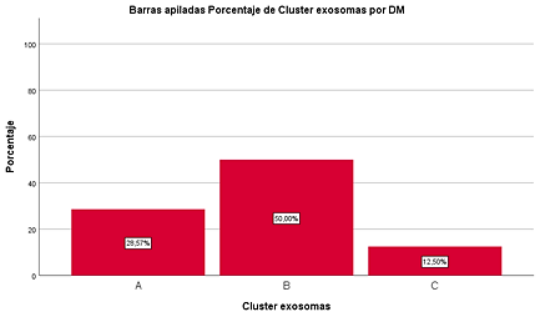
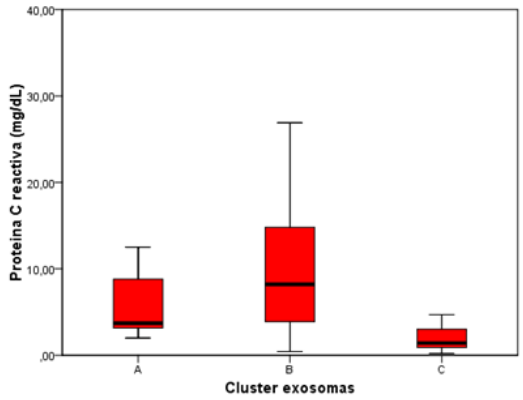


Figure 3 c. Differences in the concentration of C-reactive protein between the 3 clusters of exosomes (p = 0.003).



In the analysis of differentially expressed proteins between clusters B and C, an analysis of the different metabolic differential pathways was performed. The complement pathway was found to be overrepresented in cluster B which, together with a higher concentration of C-reactive protein, could hypothesize that the

thrombotic trigger in these patients depends more on a systemic proinflammatory state than local plaque instability. On the other hand, in cluster C (patients with fewer risk factors and a lower concentration of C-reactive protein) the pathways linked to lipoprotein metabolism are overexpressed, which could be linked to greater atherogenicity and greater atheroma plaque burden, despite LDL levels similar to cluster B. In fact, apolipoprotein B100 and A1 concentrations are clearly related to the incidence of acute myocardial infarction, regardless of LDL values.

Data analysis allows us to know the pathways and molecules represented in the two groups of samples defined in the analysis of main components. With the objective of giving an additional and complementary value to the study, we also analyzed the exosome samples from the plasma of the same patients. These vesicular structures are key in the transport of functionally relevant molecules and we hope that their content can give us relevant information to explain the observed grouping of the samples.

Exosome analysis and plasma validation technique

Circulating exosomes have recently emerged as biomarkers for prognosis, diagnosis, risk stratification and therapy in many diseases, from cancer to cardiovascular disease. During myocardial infarction, circulating exosomes change not only in their number but also in the charges composed of miRNA, lipids and proteins. For this reason, we are validating in plasma using Luminex xMAP technology. This technology allows the detection of up to 50 different biomarkers at the same time in biological and fluid samples. This technology can be configured to perform protein assays quickly, cost-effectively and accurately.

3. Relevance and possible future implications

Cardiovascular disease is the leading cause of mortality in western countries, and ischemic heart disease is its most frequent entity that gives patients a limited prognosis. Acute myocardial infarction is the first clinical manifestation in a significant number of patients with ischemic heart disease. AMI is associated with a very high initial mortality and determines in many of the survivors a limited long-term prognosis. The mechanisms involved in the genesis and progression of the intracoronary thrombus

in patients with AMI are not entirely known. At the same time, it is not known what is the exact nature that causes the instability of the culprit lesion and the subsequent formation and progression of the intracoronary thrombus (rupture, erosion or undetectable alterations).

This project analyzes the potential mechanisms of formation and progression of intracoronary thrombosis in the context of AMI. The very initial evaluation of the degree of instability of the responsible lesion (plaque rupture, erosion, absence of injury) by very sensitive intracoronary imaging techniques (OCT image) can allow a more precise identification of the degree of participation of the different triggers. The relationship of these factors and the proteomic composition of the intracoronary thrombus can identify or clarify aspects in relation to the mechanism of the genesis and progression of the thrombi. These aspects can facilitate the incorporation of new pharmacological approaches or preventive or very early mechanists looking for more targeted or personalized treatments. Given the prevalence and prognostic and social impact of AMI, its potential clinical application could be very important.

Proteomic analysis. In the proteomic analysis of the thrombi obtained from the patients, only a slight association with diabetes mellitus ($p = 0.04$) was documented since the diabetic patients are grouped together, although this group included samples of non-diabetic patients who are also similar, in terms of the proteins identified and quantified.

Exosomic analysis. Our study documented a moderate correlation between platelet factor 4 (PF4, CXCL4) and ischemia time ($R2 = 0.584$, $p = 0.0085$) and with CRP concentrations ($R2 = 0.677$, $p = 0.002$). These results may suggest that PF4 would have an early role in thrombus formation, being consumed during its evolution once the mechanisms of systemic inflammation, reflected by high CRP values, have already been activated and amplified. These results, in our series, will be the subject of future research since a validation will be carried out in patients' plasma to determine if PF4 is segregated to the bloodstream and can be considered a possible diagnostic biomarker of hyperacute infarction. On the other hand, and this point is also important, the absence of correlation between the components of the thrombus and the plaque instability in the OCT evaluation (severe plaque rupture, fissure or erosion) suggests limited relevance of the initial trigger in the final composition of the thrombi

In the analysis of the clinical and procedural characteristics of the patients grouped by clusters of exosomes, it was documented that one of the clusters differed from the others because it presented a significantly and markedly longer ischemia time (300 min vs. 132 min; p = 0.006). The ischemia time is a determining variable in the characteristics of the formation, progression and final composition of the thrombus, due to its extremely dynamic nature. Therefore, comparative evaluations were performed between the different groups of exosomes identified. The complement pathway was overrepresented in one of the clusters (B). This aspect, together with a higher concentration of C-reactive protein, could hypothesize that the thrombotic trigger in these patients would depend more on a systemic proinflammatory state than on a local plaque instability. On the contrary, cluster C (patients with fewer risk factors and a lower concentration of C-reactive protein) has overexpressed pathways linked to lipoprotein metabolism. This aspect could be linked to a greater atherogenicity and a greater load of atheroma plaque although LDL levels are similar to those of cluster B.

Additional research scenarios created in conjunction with other projects. The significant amount of thrombus obtained in our population of patients with acute myocardial infarction and the results of their proteomic analysis will allow comparative analysis with thrombus obtained by aspiration in different vascular territories of patients presenting with acute thrombotic events. We are making a comparative assessment in the proteomic composition of coronary thrombus versus thrombus of the cerebral vascular territory in patients who have presented acute thrombotic episodes.

4. Scientific bibliography generated

An important aspect to mention is the reasons why the completion of the study has required a longer period than was initially established. The beginning of the project was delayed approximately 6 months by an exclusively bureaucratic problem due to the difficulty in obtaining the approval of the 2 institutions (Bellvitge Hospital-ICS and IDIBELL) because it was a coordinated project.

Additionally, a 1-year extension was requested and a series of amendments were made and justified due to the difficulty in collecting the necessary samples (thrombus) for the

project. One of the initial main objectives of the study was to characterize the molecules involved in the generation of the thrombus formed in the coronary arteries of diabetic and non-diabetic patients, at the time of acute myocardial infarction. A very relevant point during the development of the project has been the important difficulty in obtaining intracoronary thrombus samples by aspiration in diabetic patients, at the time of infarction, of sufficient weight and measure to be studied and analyzed. This finding has been absolutely unexpected. There are no series that have described this difficulty. The difficulty in obtaining coronary thrombus that we have observed in diabetic patients, although with maximum reserve, may suggest a different mechanism in the pathophysiology of acute myocardial infarction in these patients. Patients with diabetes have more fibrous and more rigid coronary plaques and may experience more progressive and less abrupt plaque ruptures where the thrombotic load is less or the thrombus when being formed progressively is much more organized. This aspect may explain the greater resistance and difficulty in its extraction through intracoronary aspiration. In contrast, the important thrombotic burden that is detected in infarcts of non-diabetic patients where coronary lesions are less fibrous and with a greater lipid component and, possibly, with more recent thrombus can explain the ease in obtaining thrombotic material by aspiration. This has been one of the aspects that, on the other hand, has delayed the inclusion of patients in the study and that has also forced us to modify certain objectives of the same.

List of publications that are currently in preparation:

1. Mechanisms of formation and progression of thrombosis in acute myocardial infarction. Proteomic analysis of thrombi and intracoronary plasma.

2. Trigger mechanism of acute myocardial infarction with ST elevation: greater systemic proinflammatory status and lower local plaque instability.

3. Value of platelet factor 4 (PF4) as a plasma biomarker of the most initial phase of acute myocardial infarction.

4. Blocking of the chemokine ligand 4 (CXCL4) as a therapeutic target in acute myocardial infarction with ST segment elevation

5. Role of the complement system in the acute pathophysiology of acute myocardial infarction with ST elevation in patients with high classical cardiovascular risk

6. Coronary secretion vs. systemic exosomal apolipoproteins in acute myocardial infarction with ST segment elevation.