

Inflammation, fibrinogen and thrombin generation in patients with previous myocardial infarction

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Background and Objectives. It has been reported that the influence of plasma fibrinogen on the incidence of myocardial infarction is related to inflammatory processes. The aim of this study was to investigate the relationship between inflammatory activity, fibrinogen and thrombin generation in patients 5 years after the acute phase of myocardial infarction.

Design and Methods. Sixty-seven patients 5 years after a myocardial infarction and 67 control subjects were studied. Plasma fibrinogen protein (Fg-protein) and function (Fg-function), prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), total sialic acid (TSA) and C-reactive protein (CRP) were measured.

Results. The levels of Fg-protein, Fg-function, F1+2, TAT, TSA and CRP were significantly higher in patients than in the control subjects. Plasma TSA correlated with CRP ($r=0.31$, $p<0.05$). There was a significant correlation between TSA and Fg-protein or Fg-function ($r=0.48$, $p<0.01$). CRP correlated with Fg-function ($r=0.32$, $p<0.05$) while there was no correlation between CRP and Fg-protein. CRP also correlated with F1+2 and TAT ($r=0.4$, $p<0.01$).

Interpretation and Conclusions. Five years after myocardial infarction there was clear evidence of low-grade inflammation that was accompanied by an increase in thrombin formation. The increase of the plasma fibrinogen level is mainly related to TSA and the increase of CRP, which is associated with thrombin generation.

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Key words: fibrinogen, sialic acid, C-reactive protein, thrombin generation, myocardial infarction, inflammation.

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After a myocardial infarction, a thrombogenic and low grade-inflammatory response may contribute to the occurrence, extent and persistence of coronary thrombosis and its clinical sequelae.¹⁻² The plasma concentration of fibrinogen has been identified in several epidemiological studies as an independent predictor of cardiovascular events.³⁻⁴ Previous reports have shown that increased levels of plasma fibrinogen in patients with coronary artery disease are associated with an increase in the incidence of recurrent ischemic events.⁵⁻⁹ Hyperfibrinogenemia, independently of other risk factors, is associated with a specific histologic composition of atherosclerotic plaques that predisposes them to rupture and thrombosis.¹⁰ In addition to these pathologic roles fibrinogen appears to be associated with an acute and chronic inflammatory process¹¹ and inflammation seems to be involved in atherosclerosis and in the progression of coronary heart disease.¹² It has recently been reported that the influence of plasma fibrinogen on the incidence of myocardial infarction is related to inflammatory processes.¹³ Consistent with this concept, plasma C-reactive protein and sialic acid, as markers of inflammatory activity, have attracted recent interest as indicators of cardiovascular risk since they have been associated with the progression of atherosclerosis.¹⁴⁻¹⁶ It has also been reported that an elevated level of C-reactive protein is associated with an increased risk of recurrent coronary events.¹⁷⁻¹⁸ Sialic acid levels rise in acute¹⁹⁻²⁰ and chronic⁹ ischemic coronary syndromes. Elevated serum levels of sialic acid predicted both death from coronary heart disease and stroke in men and women, independently of age.¹⁴ However, markers of inflammation in relation to procoagulant activity five years after myocardial infarction have been poorly evaluated. Procoagulant activity has been observed two years after myocardial infarction.²¹⁻²² Therefore, the presence of blood factors that reflect enhanced throm-

bogenic activity might not only be associated with the thrombotic process but also with atherogenesis and an inflammatory process.

The present study was thus designed to examine whether raised plasma levels of inflammatory activity markers (total sialic acid and C-reactive protein) correlate with fibrinogen and thrombin generation markers (prothrombin fragment 1+2 and thrombin-antithrombin complex) in patients 5 years after the acute phase of a myocardial infarction.

Design and Methods

Patients and study design

Sixty-seven patients with a previous myocardial infarct were referred for study between March-July 1999; these participants were aged between 48 to 79 years old. The selection of patients was based on the following entry criteria: 1) a confirmed (clinical signs, electrocardiogram, enzymes) acute myocardial infarction 5 years before entering the study and 2) the absence of acute or chronic inflammatory disease, malignancy, history of major surgery within the previous month, or known hemostatic defects. Eleven percent of patients had suffered a confirmed secondary coronary ischemic event. The new coronary ischemic events were identified by applying clinical and electrocardiographic criteria. All patients were treated with 200 mg of aspirin daily. The control group was formed of 67 subjects without a history of myocardial infarction, and were age- and sex-adjusted. The controls were studied at the same time as the patients, between March-July 1999 (case-control study).

The study was approved by the Hospital Clinic Research Ethics Committee. All the patients and healthy subjects gave their informed consent to the protocol.

Blood sampling and processing

Blood samples were collected into Vacutainer tubes containing 0.13 mol/L sodium citrate. The ratio of anticoagulant to blood was 1/9 (vol/vol). Each sample was immediately centrifuged at 3,000 g for 15 min at 4°C. Plasma samples were tested immediately or frozen in aliquots at -80°C until use. Samples from patients and controls were processed simultaneously.

The plasma fibrinogen protein (Fg-protein) level was measured by the heat precipitation method.^{9,23} In brief, fresh plasma was incubated at 56 °C for 15 min and the turbidity was measured at 350 nm. The fibrinogen protein concentration was calculated

using a standard curve of fibrinogen concentration against the turbidity values. The results were expressed in mg of fibrinogen/dL of plasma. This assay had an intra-assay coefficient of variability of less than 8%.

The level of plasma fibrinogen function (Fg-function) was obtained by measuring the plasma fibrin formation rate by a turbidity assay.^{9,24} In brief, 10 µL of a solution of human thrombin (25 IU/mL) was added to 490 µL of diluted fresh plasma (1/50, v/v), and a kinetics study was performed at 350 nm. The results were expressed in mg of fibrinogen/dL of plasma. This transformation was carried out using a standard curve of fibrinogen protein concentration against the fibrin formation rate value obtained at different dilutions of a pool of normal plasmas. This assay had an intra-assay coefficient of variability of less than 5%.

The plasma prothrombin fragment 1+2 (F1+2) and thrombin antithrombin complex (TAT) levels were determined by enzyme immunoassay kits (Enzygnost F1+2 kit and Enzygnost TAT kit, Dade-Behring, Germany). The intra-assay coefficients of variation of the F1+2 and TAT techniques were less than 8 and 6%, respectively.

High sensitivity assays for plasma C-reactive protein (CRP) were assayed using the N Latex CRP mono kit with immunonephelometry (Dade-Behring, Germany). This assay had an intra-assay coefficient of variability of less than 4.3%.

The total plasma sialic acid (TSA) level was measured by a colorimetric assay for an enzymatic method (Sialic acid Farbstest, Boehringer Mannheim, Germany). This method uses an enzyme assay reaction, incorporating neuraminidase and pyruvate oxidase linked to a peroxidase dye system. This assay had an intra-assay coefficient of variability of less than 3.8%.

Cardiovascular risk factors: hypertension (systolic blood pressure >150 mmHg, diastolic blood pressure >90 mm Hg), hyperlipidemia (total cholesterol >250 mg/dL and/or triglycerides >150 mg/dL), hyperglycemia (glucose > 120 mg/dL), obesity (body mass index >30 kg/m²) and smoking (daily consumption of >1 cig / day) were evaluated.

Cholesterol, triglyceride and glucose were measured by enzymatic techniques (RA-1000 Bayer Diagnostic).

Statistical analysis

Statistical comparisons between the patients and control subjects were performed by means of a one-way analysis of variance. Differences in percentages between cases and controls for categoric

variables were performed by the χ^2 test. Bivariate correlation and multiple linear regression analysis were used to measure the linear association among variables. The upper reference limit levels of the parameters studied were calculated by ascertaining the 97.5 percentile of the distribution in the control group and were set at: Fg-protein, 350 mg/dL (1.5% of the control group had values higher than the upper limit); Fg-function, 373 mg/dL (1.5% of the control group had values higher than the upper limit); F1+2, 2.0 nmol/L (2.9% of the control group had values higher than the upper limit); TAT, 4.2 μ g/mL (5.5% of the control group had values higher than the upper limit); TSA, 78 mg/dL (1.5% of the control group had values higher than the upper limit); and CRP, 3.3 mg/L (2.9% of the control group had values higher than the upper limit). All statistical calculations were performed with statistical package SPSS computer software. All the data are expressed as mean \pm standard deviation. Values of $p < 0.05$ were considered as statistically significant.

Results

Clinical characteristics of the study participants

Table 1 shows the clinical characteristics of the patients and the control subjects matched for age and sex. No significant differences in hypertension, hyperlipidemia, obesity and current smoking were found between the patients and the control group. A high total cholesterol serum level (>250 mg/dL) was observed in 22% of patients and in 10% of controls. A high serum level of triglycerides (>150 mg/dL) was observed in 25% of patients and controls. The glucose serum level was significantly higher in patients than in controls. An elevated glucose level (>120 mg/dL) was present in 25% of patients and in 5% of controls ($p < 0.001$).

Fibrinogen, thrombin generation and inflammatory activity markers

The mean plasma concentrations of both fibrinogen protein and function were significantly higher in patients than in control subjects (Table 2). Nine percent of patients had higher plasma levels of fibrinogen-protein than the 97.5 percentile of the distribution in the control group (≥ 350 mg/dL) and 16% of patients had elevated plasma levels of fibrinogen-function (≥ 373 mg/dL).

The mean plasma concentrations of F1+2 and TAT were significantly higher in patients than in control subjects (Table 2). Twelve percent of patients had plasma levels of F1+2 higher than the 97.5 percentile of the distribution in the control

Table 1. Clinical characteristics of 67 patients with a myocardial infarction 5 years before entering the study compared with 67 age- and sex-matched control subjects.

	Patients (n=67)	Control subjects (n=67)	p value
Age (y)	62.1 \pm 6.7	61.0 \pm 6.7	
Male sex (%)	91	91	
Current smoking (%)	5.9	16.4	0.09
Hypertension (%)	29.8	20.9	0.32
Total cholesterol (mg/dL)	222 \pm 38	217 \pm 35	0.43
HDL cholesterol (mg/dL)	48 \pm 12	49 \pm 10	0.60
LDL cholesterol (mg/dL)	146 \pm 33	147 \pm 34	0.86
Triglycerides (mg/dL)	126 \pm 63	108 \pm 52	0.07
Glucose (mg/dL)	117 \pm 47	94 \pm 16	<0.001
Body mass index (Kg/m ²)	27.4 \pm 5	28 \pm 4	0.44

Data are present as mean \pm SD or as percentages.

Table 2. Fibrinogen, and markers of thrombin generation and inflammation in patients with previous myocardial infarction and controls adjusted for age and gender.

	Patients (n=67)	Controls (n=67)	p value
Fg-protein (mg/dL)	282 \pm 45	263 \pm 44	<0.05
Fg-function (mg/dL)	317 \pm 63	266 \pm 52	<0.001
F1+2 (nmol/L)	1.2 \pm 0.6	1.02 \pm 0.4	<0.05
TAT (μ g/mL)	2.9 \pm 2.2	2.3 \pm 0.8	<0.05
TSA (mg/dL)	62 \pm 15	50 \pm 15	<0.001
CRP (mg/L)	3.8 \pm 3.6	1.2 \pm 0.9	<0.001

Values are mean \pm SD. Fg-protein, fibrinogen protein; Fg-function, fibrinogen function; F1+2, prothrombin fragment 1+2; TAT, thrombin-antithrombin complex; TSA, total sialic acid; CRP, C-reactive protein. The p value is for the statistical comparison between patients and controls adjusted for age and gender.

group (≥ 2.0 nmol/L) and 15% of patients had elevated plasma levels of TAT (≥ 4.2 μ g/mL).

The mean plasma levels of TSA and CRP were significantly higher in patients than in control subjects (Table 2). Sixteen percent of patients had higher plasma levels of TSA than the 97.5 percentile of the distribution in the control group (≥ 78 mg/dL) and 39% of patients had elevated plasma levels of CRP (≥ 3.3 mg/L).

When the subgroup of 7 patients who had suffered recurrent coronary ischemic events was compared with the group of 60 patients without these episodes, Fg-function level (354 \pm 58 vs 304 \pm 62 mg/dL, $p < 0.05$) was significantly increased in patients with recurrent ischemic events. Plasma levels of Fg-protein (300 \pm 39 vs. 278 \pm 45 mg/dL, $p = 0.19$), F1+2 (1.6 \pm 0.9 vs. 1.1 \pm 0.5 nmol/L, $p = 0.79$),

TAT (4.0 ± 3.2 vs. 2.6 ± 1.9 $\mu\text{g/mL}$, $p=0.09$), TSA (71.0 ± 18 vs. 60.6 ± 13.9 mg/dL , $p=0.07$) and CRP (5.1 ± 1.8 vs. 3.4 ± 3.2 mg/L , $p=0.17$) were also higher in patients with recurrent coronary ischemic events than in patients without these episodes but the differences were not significant.

Analyses of correlation between increasing levels of inflammatory markers of inflammation (TSA or CRP), thrombin generation (F1+2 or TAT) and fibrinogen (protein or function)

Markers of inflammatory activity assessed in previous myocardial infarction patients demonstrated a significant correlation except for plasma fibrinogen protein and C-reactive protein (Table 3). The highest correlation was seen between plasma sialic acid and fibrinogen-function ($r=0.48$, $p<0.01$) and fibrinogen-protein ($r=0.48$, $p<0.01$). Plasma sialic acid in patients was correlated with C-reactive protein ($r=0.31$, $p<0.05$).

There was a significant correlation between plasma C-reactive protein and F1+2 ($r=0.4$, $p<0.01$) and TAT ($r=0.4$, $p<0.01$) levels. However, plasma sialic acid did not correlate with F1+2 and TAT levels (Table 2). F1+2 showed a significant correlation with TAT ($r=0.4$, $p<0.01$) but there was no correlation between fibrinogen and F1+2 and TAT.

Correlation analysis of markers of inflammation and thrombin generation in patients with new coronary ischemic events showed a strong correlation between C-reactive protein and F1+2 ($r=0.76$, $p<0.05$) and TAT ($r=0.71$, $p<0.05$).

A multiple regression analysis allowed determination of the specific influence of inflammatory activity parameters on plasma fibrinogen concentration and function. Three models were tested that systematically included plasma sialic acid and C-

Table 3. Correlations of fibrinogen protein, fibrinogen function, F1+2, TAT levels with total sialic acid and C-reactive protein in 67 patients with previous myocardial infarction.

Variable	TSA	CRP
Fibrinogen protein	0.48**	0.28
Fibrinogen function	0.48**	0.32*
Total sialic acid	-	0.31*
C-reactive protein	0.31*	-
F1+2	0.16	0.40**
TAT	0.16	0.40**

* $p<0.05$; ** $p<0.01$; F1+2, prothrombin fragment 1+2, TAT, thrombin antithrombin complex.

Table 4. A multiple regression analysis of fibrinogen, inflammatory activity parameters, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and glucose measured in 67 patients with previous myocardial infarction.

	Model 1		Model 2		Model 3	
	P1	P2	P1	P2	P1	P2
<i>Variables in model for fibrinogen protein and fibrinogen function</i>						
Total sialic acid	0.000	0.001	0.004	0.002	0.006	0.007
C-reactive protein	0.31	0.21	0.59	0.43	0.62	0.22
Age	-	-	0.050	0.14	0.37	0.56
Total cholesterol	-	-	-	-	0.71	0.39
HDL cholesterol	-	-	-	-	0.69	0.53
LDL cholesterol	-	-	-	-	0.39	0.67
Triglycerides	-	-	-	-	0.62	0.64
Glucose	-	-	-	-	0.59	0.23

P1, p values in model for fibrinogen protein; and P2, p values in model for fibrinogen function. Variables in model for fibrinogen protein: Model 1: $R^2=0.26$, $p<0.001$; Model 2: $R^2=0.32$, $p<0.001$; Model 3: $R^2=0.35$, $p<0.05$. Variables in model for fibrinogen function: Model 1: $R^2=0.31$, $p<0.001$; Model 2: $R^2=0.34$, $p<0.001$; Model 3: $R^2=0.38$, $p<0.05$.

reactive protein (first model), age (second model) and triglyceride and cholesterol (third model as covariates). In these three models the relative importance of each independent variable on plasma fibrinogen concentration and function were only significant for plasma sialic acid (Table 3). Thus, plasma sialic acid levels emerge as a significant independent factor influencing plasma fibrinogen concentration. About 26% of the variance of fibrinogen protein and 31% of that of fibrinogen function levels could be explained by the influence of this independent variable (Table 4).

Discussion

The results of this study demonstrate that patients with previous myocardial infarction have elevated fibrinogen-protein and function, parameters that can be related to chronic low inflammatory activity. The role of fibrinogen as a risk factor for atherothrombotic disorders is influenced directly by both its protein concentration and functional activity.^{8,9} In the present study it was observed that the patients with new ischemic events have higher levels of markers of inflammatory and thrombin generation than patients without these events, but this increase was not statistically significant. The elevated plasma fibrinogen levels found in patients were usually accompanied by an increase of C-reactive protein and sialic acid. The multiple regression analysis of the data showed

that sialic acid plasma level emerged as a significant independent factor influencing fibrinogen protein and functional plasma concentration, while a poor correlation was found between C-reactive protein and fibrinogen.

The increased levels of plasma fibrinogen and markers of inflammation may reflect activity of the atherosclerotic process. However, the different correlations between fibrinogen, sialic acid and C-reactive protein suggest that several biological mechanisms involved in the pathogenesis of atherosclerosis regulate the acute phase response. It has been suggested that fibrinogen is a marker of cytokine secretion which, in turn, stimulates fibrinogen biosynthesis and angiogenesis inducing plaque rupture and atherothrombosis.^{10,25} In addition, a possible source of the elevation in plasma sialic acid content could be some type of acute phase reaction associated with a risk of atherosclerosis.²⁰ In this regard, an elevated sialic acid is correlated with arterial intima-wall thickness and may reflect the existence or the activity of an atherosclerotic process.²⁶ One could assume that the strong correlation between the elevated fibrinogen and sialic acid would be due to a common type of acute phase reaction related to the atherosclerotic lesion.

On the other hand, it has now been suggested that generation of C-reactive protein is up-regulated in atherosclerotic plaques²⁷ and elevated C-reactive protein is associated with elevated serum levels of cytokines which may be the mechanism of C-reactive protein generation. It has also been reported that C-reactive protein concentration is a marker of atherosclerotic activity rather than extent of atherosclerosis.²⁸ If so, the elevation in plasma C-reactive protein levels, which correlated with the elevation in thrombin generation (F1+2 and TAT), would be due to some type of acute phase reaction which is associated with the risk factor of the thrombotic process. In fact, when the markers of inflammation were correlated with those of the generation of thrombin, a stronger correlation was observed between C-reactive protein and F1+2 and TAT in those patients with new coronary ischemic events than in patients without these events. Tissue factor is a glycoprotein that is considered to be the primary cofactor of cellular origin involved in coagulation activation and is strongly induced in an inflammatory process.²⁹ Tissue factor is a candidate molecule linking plaque inflammation with arterial thromboembolism.³⁰ The finding that thrombin generation was slightly increased in patients implies that the formation of thrombin was associated with this inflammatory process.

In conclusion, 5 years after the acute phase of a myocardial infarct, patients have clear evidence of low-grade inflammation. The increase in the plasma fibrinogen level is mainly related to TSA and the increase in CRP is associated with thrombin generation. This chronic inflammatory activity reflects the progression of the atherosclerotic lesion five years after myocardial infarction. Future studies are warranted to investigate whether these inflammatory markers may be useful in routine cardiovascular risk assessment and in monitoring new pharmacologic therapies after myocardial infarction.

Contributions and Acknowledgments

ER, VV, VM contributed to the conception and design of the study, carried out part of the analytical assays and contributed with the analysis and interpretation of the results. AV was involved in the clinical management of the patients and contributed to the conception and design of the study. JA contributed to the conception and design of the study and critically corrected the different versions of the manuscript. The order in which the names appear is based on the time spent by each contributor on this research. The authors wish to thank Ursula Salinas, Guadalupe Manzano, Aurelia Royo and Rosa Ferrer for their expert technical assistance and also to Dr. Jesus Aznar (MD) for the analysis of the C-reactive protein assays.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlap with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Diego Ardissino, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Ardissino and the Editors. Manuscript received January 14, 2002; accepted May 6, 2002.

What is already known on this topic

There is extensive literature about the correlation between inflammation and thrombosis. This paper is a further demonstration of the existing link.

What this study adds

The novel aspect of the present investigation relates to the duration of the activation of coagulation system after an episode of acute myocardial infarction. Previous studies have evaluated this activation after shorter periods; here the assessment was done after 5 years and was the coagulation system found to be still activated.

Potential implications for clinical practice

The persistent activation of the coagulation system requires an adequate antithrombotic therapy. It is therefore plausible that the combination of oral coagulation and aspirin is the best antithrombotic therapy after acute myocardial infarction as proved in the WARIS II trial.

Diego Ardissino, Associate Editor