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Factors Involved in Sudden Coagulation Observed in Patients with Acute Myocardial Infarction

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Abstract. *Coronary artery diseases (CAD) evolving into acute myocardial infarction (AMI) is associated with coagulation and thrombotic occlusion of coronary vessels in the presence of unstable atheroma. The atheromatous plaque becomes unstable when it is infiltrated by monocytes, macrophages and neutrophils capable of secreting proteases that induce plaque erosion, rupture and initialize the coagulation process. The aim of this study was (a) to analyse the plasma of patients with AMI for the presence of proteases that may activate rapid coagulation, (b) to evaluate coagulation markers as prothrombin fragment (F1+2) and antithrombin III and (c) to find an interrelation between proteases and coagulation markers. The examined plasma showed high values of prothrombin fragment (F1+2) and low levels of antithrombin III. These markers showed a highly significant negative-correlation. The plasma also exhibited increased levels of matrix metalloproteinase-9 (MMP-9) which were positively-correlated with the prothrombin fragment (F1+2). MMP-9 seems to cause the coagulation activity by increasing the level of prothrombin fragment (F1+2) and the consumption of antithrombin III. The examined plasma also exhibited high levels of neutrophil gelatinase-associated lipocalin (NGAL), which is known to modulate MMP-9 activity. The high plasma levels of MMP-9 and NGAL can be attributed to plaque instability and appear to activate sudden coagulation. MMP-9 and NGAL, in the presence of altered values of prothrombin fragment (F1+2) and antithrombin III in AMI patients, seem to be suitable markers to be studied in unstable plaque patients, for the prediction and prevention of acute coronary syndrome.*

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Coronary artery disease (CAD) evolving into acute myocardial infarction (AMI) is associated with thrombotic occlusion of coronary vessels in the presence of atheromatous plaque (1). Recent studies have shown that inflammation plays a fundamental role in mediating the stages of progressive plaque instability and thrombotic complications (2). Under inflammatory conditions, monocytes/macrophages, T-cells and neutrophils are recruited to the arterial wall (3).

Atherectomy specimens of atheromatous plaques are rich in macrophages, monocytes and neutrophils, all of which are capable of degrading the extracellular matrix by secreting proteolytic enzymes such as metalloproteinases, collagenases and gelatinases (4). These proteases may weaken the fibrous cap and predispose it to rupture (5). Plaque erosion or rupture lead to rough surfaces that favour platelet adhesion and stimulate the development of thrombosis, thus increasing the risk of an acute ischemic syndrome in patients who may die immediately or shortly after episodes of unstable angina or myocardial infarction (6-8).

As the proteases involved in destabilising the plaque are released in the plasma, it is possible to check the evolution of the plaque by monitoring their levels in CAD patients, with the aim of avoiding sudden episodes of acute ischemic coronary syndrome by stabilising the atheromatous plaque. It is known that monocytes/macrophages play a role in producing metalloproteinases such as matrix metalloproteinase-9 (MMP-9) (9), whereas neutrophils play a role in producing gelatinases such as NGAL at the site of vascular inflammation (10), infiltrated by neutrophils chemo-attracted to the plaque (11). It can be hypothesised that these proteases could act as predictors of CAD complications and could be used to estimate the risk of myocardial infarction.

We studied the plasma of a number of AMI patients in order to investigate the presence of MMP-9, which is over-expressed in unstable plaques (12) and of NGAL, which is expressed in atheromatous plaques and in myocardial infarctions (13). Some coagulation markers have been found to be altered in AMI patients and/or unstable angina cases. In particular, prothrombin fragment (F1+2) has been described to be increased (14, 15) and antithrombin III, which is highly consumed during coagulation was reported to be decreased in AMI patients or in unstable angina subjects (16, 17).

The aim of this study was to investigate whether the presence of MMP-9 and NGAL may be associated with altered values of prothrombin fragment (F1+2) or antithrombin III and whether they may be involved in causing or maintaining coagulation.

Patients and Methods

Eight randomly selected male patients with AMI aged 50-65 years and eight healthy males of the same age were selected in order to verify whether these markers are interrelated in priming the coagulation and thrombosis that leads to AMI. This preliminary study was approved by the Ethical Committee of Niguarda Ca' Granda Hospital Milan, Italy.

The presence of AMI was demonstrated by measuring the specific marker c-TnT (18) in the plasma using a commercial kit provided by Roche (Milan, Italy) (18). MMP-9 was assayed using a BenderMed System flow cytomix simplex kit from Prodotti Gianni (Milan, Italy). These were selected as representative examples of technologies currently used for high-thoughput immunoanalysis (19). NGAL was analysed using an enzyme-linked immunosorbent assay (ELISA) (20), supplied by Bioporto Diagnostic (Verona, Italy), in accordance with the manufacturer's instructions. Plasma prothrombin fragment (F1+2) was measured using an immuno-enzymatic procedure named Enzygnost F1+2 monoclonal, provided by Siemens (Milan, Italy) (21). Plasma antithrombin III was measured using a colorimetric method, based on a chromogenic substrate (S-2765:N-Z-D-ARG-GLY-ARG-pNA) using a kit provided by Instrumentation Laboratory (Milan, Italy) (22).

Statistical analyses. The plasma values of the various biochemical markers were compared using analysis of variance (ANOVA), with the Tukey's test being used to determine the statistical significance of any differences between the groups. Statistical significance was set at $p<0.05$ (23).

The correlations between prothrombin fragment (F1+2) and MMP-9 or prothrombin fragment (F1+2) and antithrombin III in the plasma of healthy individuals and patients with AMI were determined using the Pearson's correlation coefficient (r); statistical significance was set at $p=0.05$ (23).

Results

The AMI patients in comparison with healthy subjects showed altered values of c-TnT, prothrombin fragment (F1+2), antithrombin III, MMP-9 and NGAL.

Figure 1 exhibits the levels of c-TnT in AMI patients and healthy subjects. The c-TnT values are very high in comparison to the reference controls (3.20±0.79 *vs*. <0.01±0.001 ng/ml; $p<0.001$).

Figure 2 (left panel) shows the levels of prothrombin fragment (F1+2) observed in healthy subjects and in AMI patients. The values of prothrombin fragment (F1+2) in AMI patients, when compared with healthy subjects, appear to be significantly different (1220.75±239.05 *vs*. 196.38±19.96 pmol/L; $p<0.001$).

Figure 2 (right panel) shows the levels of antithrombin III in healthy subjects and AMI patients. In AMI subjects the antithrombin III values appear to be significantly lower in comparison with healthy subjects (94.50±2.61 *vs*. 108.50±2.58 mg/dl; $p<0.004$).

A negative highly-significant correlation is observed between prothrombin fragment (F1+2) and antithrombin III: r=–0.628; $p=0.0036$.

Figure 3 (right panel) exhibits the levels of MM9-9 observed in healthy subjects and in AMI patients. The values of MMP-9 observed in AMI patients appear to be more elevated in comparison with those of healthy subjects (32.37±0.88 *vs*. 29.29±0.77 ng/ml; $p<0.034$).

Figure 3 (left panel) shows the levels of NGAL found in healthy subjects and AMI patients. The values of NGAL detected in AMI in comparison with healthy subjects appear to be significantly highly increased (201.13±51.43 *vs*. 67.58±9.06 ng/ml; $p<0.004$).

It should be noted that a positive correlation exists between MMP-9 and prothrombin fragment (F1+2): r=0.603; $p=0.013$.

Discussion

A myocardial infarction occurs due to the thrombotic occlusion of coronary vessels in the presence of atheromatous plaques (1). The occurrence of AMI in our patients was demonstrated by the high levels of the specific marker c-TnT (18) (Figure 1). The plasma of our AMI patients contained increased levels of prothrombin fragment (F1+2) and decreased levels of antithrombin III, as markers of coagulation (Figure 2). The increased values of prothrombin fragment (F1+2) in AMI was also observed in other studies (14, 15). The decrease of antithrombin III found, was also described by others (16,17). In this article prothrombin fragment (F1+2) and antithrombin III exhibit a highly significant negative correlation (r=–0.682; $p=0.0036$).

The lower levels of antithrombin III observed in AMI patients (Figure 2) can be attributed to the increased antithrombin III consumption (16), caused by prothrombin activation (14). It is important to note that AMI patients also have higher levels of MMP-9 (Figure 3), as this has been also observed in other studies (24,25). Thus, there is a

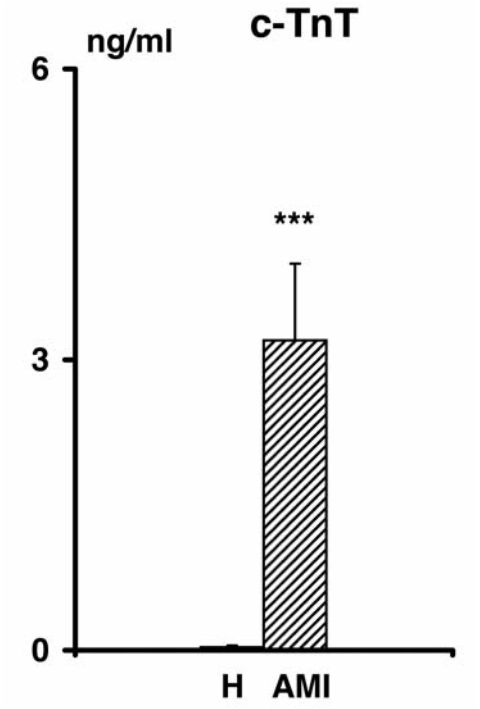

Figure 1. *Plasma levels of c-troponin T (c-TnT) observed in healthy subjects (H) and myocardial infarction patients (AMI). Each column represents the mean value±SE of eight individuals. Significance levels ***p<0.001.*

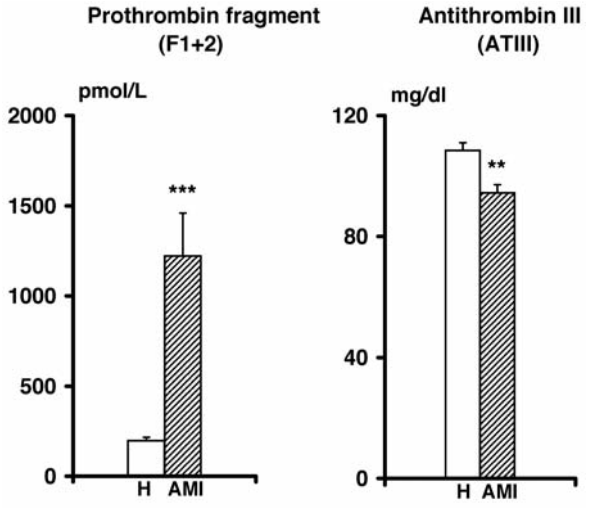

Figure 2. *Plasma levels of prothrombin fragment (F1+2) (left panel) and antithrombin III (ATIII) (right panel) observed in healthy subjects (H) and AMI patients (AMI). Each column represents the mean values±SE of eight individuals. Significance levels **p<0.01; ***p<0.001.*

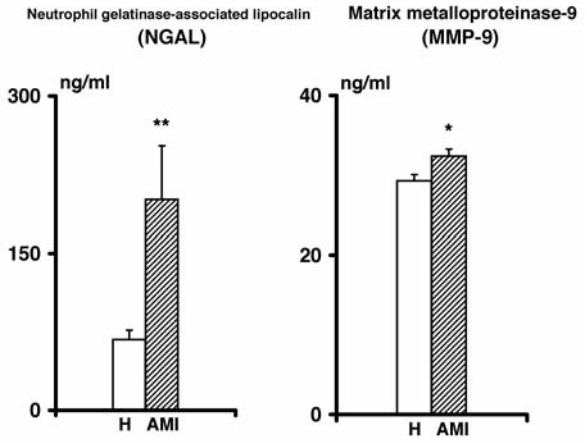

Figure 3. *Plasma levels of neutrophil gelatinase-associated lipocalin (NGAL) (left panel) and matrix metalloproteinase-9 (MMP-9) (right panel) observed in healthy subjects (H) and myocardial infarction patients (AMI). Each column represents the mean value±SE of eight individuals. Significance levels *p<0.05; **p<0.01.*

significantly positive-correlation between prothrombin fragment (F1+2) and MMP-9 (r=0.603; p=0.013). This observation indicates an interaction between MMP-9 and the prothrombin fragment (F1+2). There is also a trend of an inverse relationship between MMP-9 and antithrombin III, which may suggest that MMP-9 affects the levels of antithrombin III. The effects of MMP-9 on coagulation factors agree with the observation that the over-expression of MMP-9 promotes intravascular coagulation and thrombus formation (26).

The process of coagulation is activated by MMP-9, which cleaves tissue factor inhibitor (27, 28) and allows the tissue factor to interact with factor VIIa: *i.e.* the clot starts with the MMP-9 priming activity (29). The histological examination of the unstable atheromatous plaque has revealed the over-expression of MMP-9 to be associated with the infiltration of inflammatory cells such as macrophages, which are known to secrete proteases, particularly MMP-9 (12). This induces not only collagen breakdown in the fibrous caps of atherosclerotic plaques, as well as plaque rupture (5), but also the priming coagulation and thrombosis, as the over-expression of MMP-9 promotes intravascular thrombus formation (in porcine) coronary arteries "*in vivo*" (26). The plasma MMP-9 concentration can therefore be considered as a novel predictor of high cardiovascular risk in CAD patients, in agreement with other authors (30).

The same AMI patients also had higher NGAL levels (Figure 3) in comparison with stable angina subjects (31). Other authors have observed increased systemic levels of NGAL in AMI patients and in atheromatous plaque, and the two phenomena seem to be related (32). NGAL has been

also found to be associated with the severity of coronary artery disease (20). In particular NGAL seems to play an important role in MMP-9 activity, which has been reported to be markedly high in injured vessels in the presence of NGAL (11). NGAL/MMP-9 complexes are also specifically released by neutrophils infiltrating the plaque (33). Since NGAL has been found to co-localise with MMP-9 in areas of high proteolytic activity associated with macrophages, it has been suggested that it plays a role in maintaining plaque instability and erosion (13). NGAL/MMP-9 complexes are capable of protecting MMP-9 from degradation in a dose-dependent manner, thus preserving its enzymatic activity and modulating and maintaining its coagulation activity (34).

Conclusion

Exposition of the lipid rich core after atherosclerosis plaque erosion/rupture into the arterial lumen triggers the formation of unstable platelet aggregates with fibrin deposition, due to activated coagulation, which may lead to coronary thrombus formation (6). Plaque erosion and rupture are caused by the local release of metalloproteinases (5), which causes rapid coagulation (27). A clot starts with MMP-9 priming activity (29), which is modulated by NGAL (34). Although it is also possible that the inflammatory cells, found in the morphological changes that occur 12-24 h after an AMI (1), may contribute to the release of factors affecting coagulation, the origin of the markers studied here (NGAL and MMP-9) may be also attributed to inflammatory cells infiltrating the atherosclerotic plaque (1, 4, 5, 26).

We suggest that the markers MMP-9 and NGAL, found in the presence of altered coagulation markers (prothrombin fragment (F1+2) and antithrombin III) in AMI patients, may be used to reveal a risk of acute coronary syndrome and to offer therapeutic solutions.

Conflicts of interest

None of the Authors have any conflicts of interest in relation to this manuscript.

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