Delayed neutrophil apoptosis in patients with unstable angina: relation to C-reactive protein and recurrence of instability

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Aims
To investigate spontaneous polymorphonuclear neutrophils (PMNs) apoptosis in unstable angina (UA) and its association with recurrence of instability.

Methods and results
We compared PMNs apoptotic rate at 4 and 24 h in patients with UA, stable angina (SA), and controls (H) with two different protocols by flow cytometry. We measured apoptotic rate of isolated PMNs (Protocol 1) in 30 UA patients, 13 SA patients, and 34 H; and apoptosis of PMNs in whole blood culture (Protocol 2) in further 10 UA patients, 7 SA patients, and 6 H. Serum high-sensitivity C-reactive protein was also measured. Polymorphonuclear neutrophils of UA patients showed a decreased apoptotic rate compared with SA patients and H at 4 h in Protocol 1 (both \( P < 0.01 \)), and at 24 h in Protocol 2 (\( P < 0.05 \) and \( < 0.01 \), respectively). In overall population, a negative correlation was found between apoptotic rate at 4 h and high-sensitivity C-reactive protein levels (\( P < 0.01 \)). Six among 40 patients with UA had early recurrence of symptoms and their apoptotic rate was significantly reduced compared with UA patients without recurrence of symptoms (\( P = 0.024 \)).

Conclusions
Our study demonstrates delayed PMN apoptosis in UA. This alteration might be involved in the persistence of inflammatory activation and affects recurrence of instability.

Keywords
Apoptosis • Polymorphonuclear neutrophils • Unstable angina • Inflammation

Introduction
A growing body of evidence supports a pivotal role of inflammation in acute coronary syndromes (ACS), from endothelial dysfunction, and plaque progression to plaque instability: inflammatory cells such as neutrophils are principal players in this setting. However, the mechanisms involved are still largely unknown.1

A key mechanism in regulation of tissue damage in many inflammatory conditions is neutrophil apoptosis. Several previous studies have demonstrated that in patients with ACS, circulating polymorphonuclear neutrophils (PMNs) are functionally activated and infiltrate culprit coronary lesions.2–5 Activated PMNs release a variety of proteolytic enzymes, all of which have the potential for tissue destruction, which might be exacerbated by a prolonged survival.6

Spontaneous PMNs apoptosis represents a constitutive mechanism of cell death regulating the toxic potential of PMNs and the resolution of inflammatory process.6–7 Delayed PMNs apoptosis has been described in several severe inflammatory diseases, including systemic inflammatory response syndrome (SIRS),8 bacterial pneumonia,9 Kawasaki disease,10 and inflammatory bowel disease.11 In SIRS, a delayed PMNs apoptosis is considered one of the mechanisms leading to persistence of disease and potentially to death.8 However, data on PMNs apoptosis in patients with ACS are scant. A previous study by Garlichts et al.12 showed that circulating PMNs death may be delayed in patients with ACS. More recently, Narducci et al.13 have found that PMNs telomerase, an enzyme involved in delaying cell senescence and apoptosis, normally absent in PMNs, is reactivated in PMNs from plaques of patients.
with unstable angina (UA), but not in peripheral PMNs from UA patients nor in PMNs from plaques of stable angina (SA) patients. These data suggest a direct involvement of PMNs telomerase reactivation as a mechanism of apoptotic resistance, in plaque rupture and coronary thrombosis. They also suggest that in patients with ACS different mechanisms might be involved in PMNs survival in the coronary plaque milieu and in peripheral blood.

To shed further light on this issue, we assessed the death rate of peripheral PMNs due to apoptosis in patients with UA, in patients with SA, and in healthy controls (H) using two different protocols. We also evaluated the relationship between apoptotic rate and the recurrence of instability.

Methods

Population

We studied 40 consecutive patients with Braunwald’s class IIIB UA admitted in our Coronary Care Unit (CCU) within 12 h from the last ischaemic event, 20 patients with chronic stable effort angina (SA) as a first manifestation of ischaemic heart disease lasting more than 2 years and 40 age- and sex-matched healthy controls (H). We excluded all patients with evidence of inflammatory or infectious diseases, malignancies or immunologic or haematologic disorders, as described elsewhere. A blood sample was obtained from all patients at admission to the hospital before any drug administration. In patients with UA, early in-hospital recurrence of angina (within 48 h) was also obtained. Healthy controls had no clinical signs of atherosclerosis and had not received any medication for at least 2 weeks. The study was approved by the Ethics Committee of the Catholic University of Rome and all patients gave their written, informed consent to participate.

Assessment of polymorphonuclear neutrophils apoptosis

Polymorphonuclear neutrophil apoptotic rate was evaluated with two different protocols. In protocol 1, conducted on 30 patients with UA, 13 patients with SA, and 34 H, we studied apoptosis of freshly isolated peripheral PMNs. This design allowed us to measure PMNs apoptosis in the absence of other blood elements and soluble factors like cytokines. In the attempt to reproduce an environment that more closely mimics the in vivo situation, we also conducted protocol 2. In this protocol, we measured PMNs apoptosis in whole blood of 10 patients with UA, 7 patients with SA, and 6 H.

Protocol 1

Aliquots of heparinized peripheral blood were used to isolate PMNs by a single-step density gradient procedure using Polymorphprep™ separation medium (Nycomed Pharma AS, Oslo, Norway). Following centrifugation at 500g for 30 min at 20°C, the mononuclear cell band was discarded and PMNs were retrieved and washed twice in PBS. Contaminating erythrocytes were removed by hypotonic lysis. Polymorphonuclear neutrophilis (2.5 × 10^6/mL) were resuspended in RPMI 1640, supplemented with 10% fetal calf serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 0.2 mmol/L L-glutamine. Cells were incubated for up to 24 h in polypropylene tubes to prevent adherence, under sterile condition at 37°C and in atmosphere containing 5% CO2.

At 4 and 24 h, PMNs (2.5 × 10^6 cells) were labelled with fluorescein isothiocyanate conjugate (FITC) Annexin-V and propidium iodide (Immunotech, Marseille, France) in binding buffer for 10 min at 4°C protected from light and cell fluorescence was analysed by flow cytometry (Coulter Epics cytometer). Spontaneous PMN apoptosis was expressed as percentage of Annexin-V-positive cells on the total sample of 2.5 × 10^6 cells. The percentage of apoptotic cells was assessed by counting a minimum of 5000 events in each sample.

Protocol 2

Aliquots of 200 mL of heparinized whole blood were taken in polypropylene tubes and incubated for up to 24 h, under sterile condition at 37°C and in atmosphere containing 5% CO2. At 4 and 24 h, a total of 100 μL of whole blood were stained with CD16-PE (Beckman Coulter, USA), a mouse monoclonal antibody against human CD16 antigen expressed on neutrophils, purified by affinity chromatography and labelled with R-Phycocerythrin, for 15 min and then with fluorescein isothiocyanate conjugate (FITC) Annexin-V (Immunotech, Marseille, France) in binding buffer for 10 min at 4°C protected from light. After lysis of red blood cells and washing, cell fluorescence was analysed by Coulter Epics flow cytometer and PMNs apoptosis was expressed as percentage of double CD16-Annexin-V-positive cells. The percentage of apoptotic cells was assessed by counting a minimum of 5000 events in each sample.

Laboratory assay

High sensitivity C-reactive protein (hs-C-reactive protein) was measured in all patients using a latex-enhanced immunonephelometric assay by BN II analyser (Siemens Healthcare Diagnostics, Deerfield, USA) as described elsewhere.14

Statistical analysis

Data distribution was assessed by the Shapiro–Wilk’s test. Clinical variables were compared by ANOVA or χ² test, as appropriate. Proportions were compared by χ² test. Apoptotic rates were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics of study patients (Protocol 1)</th>
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<tbody>
<tr>
<td></td>
<td>UA (n = 30)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66 ± 12.5♭</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>21 (70)</td>
</tr>
<tr>
<td>Risk factors, n (%)</td>
<td></td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>10 (33)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (20)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>11 (37)</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>13 (43)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>11 (37)</td>
</tr>
<tr>
<td>Medication, n (%)</td>
<td></td>
</tr>
<tr>
<td>β-Blockers</td>
<td>8 (27)</td>
</tr>
<tr>
<td>Ca-antagonist</td>
<td>6 (20)</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>25 (83)</td>
</tr>
<tr>
<td>Other antiplatelet agents</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Statins</td>
<td>21 (70)</td>
</tr>
<tr>
<td>Multi-vessel disease, n (%)</td>
<td>7 (23)</td>
</tr>
</tbody>
</table>

UA, unstable angina; SA, stable angina. ¶Values are mean ± SD.
non-normally distributed and were expressed as median and interquartile range (IQR) and compared by Kruskal–Wallis test. As hs-C-reactive protein values were strongly negatively skewed, they were log transformed. Correlations between PMNs apoptotic rate and Log hs-C-reactive protein or recurrence of instability were assessed by Spearman’s rank test. Linear multivariate regression analysis was performed to identify variables independently predicting PMNs apoptosis. Statistical analysis was performed by SPSS software, version 15.0 (SPSS Inc., Chicago, IL).

**Results**

Patient characteristics are summarized in Table 1 for protocol 1 and in Table 2 for protocol 2. Leucocytes and PMNs counts were similar in overall groups. In protocol 1, the apoptotic rate at 4 h of incubation, was significantly lower in UA than in SA and H [10.1% (7.18–19) vs. 28.5% (17.42–47.05) and 41.8% (27.75–58.52), respectively; \( P < 0.01 \) for UA vs. SA and UA vs. H] (Figure 1A). At 24 h apoptotic rate was lower in UA and in SA than in H [70% (48.95–78.8) and 72.9% (43.52–85.92) vs. 82.55% (79.1–87.32), respectively; \( P < 0.01 \) for UA vs. H and \( P < 0.05 \) for SA vs. H] (Figure 1B).

In protocol 2, at 4 h apoptotic rate was lower in UA and in SA than in H [6.04% (3.79–12.36) and 5.4% (2.75–27.03) vs. 42.08% (26.54–66.55), respectively; \( P < 0.001 \) for UA vs. H and \( P < 0.01 \) for SA vs. H] (Figure 2A). At 24 h, the apoptotic rate was significantly lower in UA than in SA and in H [14.79% (11.47–18.57) and 36.7% (26.76–51.33) vs. 47.5% (38.75–81.56), respectively; \( P < 0.05 \) for UA vs. SA and \( P < 0.01 \) for UA vs. H] (Figure 2B).

At linear multivariate regression analysis, diagnosis of UA was the only independent predictor of PMNs apoptosis (Table 3).

The median levels of hs-C-reactive protein were significantly higher in UA than in SA and in H [7 mg/L (0.88–44) vs. 2.6 mg/L (0.7–32) and 1.9 (0.8–3.8)], respectively; \( P < 0.05 \) for UA vs. SA and UA vs. H]. In the overall population, a weak but significant negative correlation was found between PMNs apoptotic rate at 4 h and log hs-C-reactive protein levels \((R = −0.26, P < 0.01; \text{Figure 3})\).

Six out of 40 patients with UA had recurrence of symptoms within 48 h; PMNs apoptotic rate at 4 h of these patients was significantly lower than that of UA patients without early recurrence of symptoms [5.8 (2–12) vs. 12.6% (12–41), \( P = 0.024 \)].

**Discussion**

Our study demonstrates a delayed apoptosis of peripheral PMNs in UA patients than in SA patients and H. In UA patients,
delayed PMNs apoptosis correlates with hs-C-reactive protein levels and with recurrence of symptoms at 48 h, suggesting a new contributory mechanism to coronary instability.

Previous clinical studies have demonstrated PMNs activation in ACS. Increased PMNs release of elastase and myeloperoxidase and expression of activation molecules have been detected in the peripheral and in the coronary circulation of patients with ACS. Polymorphonuclear neutrophils infiltration of culprit lesions has also been described in ACS. Furthermore, recent studies suggest that PMNs-platelet aggregates may play a role in vascular response to injury that occurs after erosion or rupture of an atherosclerotic plaque or during coronary angioplasty or stent replacement. Garlichs et al. have previously shown a delay of PMNs death, evaluated as percentage of propidium-staining positive cells, in patients with ACS. However, this technique allows only detection of dying cells, but not of the type of death. For this reason, in our study, we used a method, staining of PMNs with Annexin-V, which specifically assesses PMNs death due to apoptosis. This is a first difference between Garlichs’ study and ours. Furthermore, in our study we have also explored the clinical relevance of PMNs delayed apoptosis, evaluating the relationship between PMNs apoptotic rate and the early recurrence of coronary instability. We also assessed the apoptotic rate of PMNs in the absence and in the presence of modulatory factors present in whole blood.

Finally, in our study we found a negative correlation between spontaneous PMNs apoptosis and hs-C-reactive protein, suggesting that cytokines responsible for the induction of acute phase proteins, in particular IL-6 which is the major inducer of C-reactive protein, might also play a role in determining PMNs survival. IL-6 indeed has well-known anti-apoptotic properties. On the other hand, although C-reactive protein is involved in pro-apoptotic pathways and acts as an opsonin on apoptotic cells, it has also been shown, when in monomeric form, to have anti-apoptotic properties.
More recently, Narducci et al. have demonstrated high telomerase activity in PMNs from coronary plaques of patients with UA, but not in peripheral blood PMNs of UA patients nor in patients with SA. Telomerase is normally absent in differentiated cells such as PMNs, but it can be reactivated under mitogenic stimulation and it might represent a way to overcome replicative senescence, resulting in a prolonged survival and toxic potential of these inflammatory cells. Notably, in patients with UA, the only predictor of telomerase reactivation in coronary atherosclerotic plaques was a short time interval from symptom onset to PMNs collection, supporting a possible role of plaque PMNs delayed apoptosis in the early phases of coronary instability.

In this study, we found a significantly delayed PMNs apoptosis in patients with UA as compared with patients with SA and H in both protocols, but with some intriguing differences in the timing of PMNs apoptosis. In protocol 1, the apoptotic rate was significantly lower in UA patients vs. SA patients and H at 4 h of incubation, while it was similar to that of SA patients at 24 h. In contrast in protocol 2, apoptotic rate in UA patients was lower than in SA and H at 24 h, while it was similar to that of SA patients at 4 h. These results could be accounted for by the presence of cytokines and blood elements in whole blood and support the notion that blood components, either soluble factors, such as cytokines or growth factors, or other blood elements, are able to delay PMNs apoptosis by activation of survival signals. Indeed in several studies, IL-6, GCSF, INF-gamma, and TNF-alfa, the latter with different effects according to its concentration, have been shown to reduce apoptotic rate. Recently, reduced PMNs apoptosis has been showed associated with soluble TNF receptor 2 (sTNFR2) plasma levels in congestive heart failure.17

Prolonged PMNs survival can translate in higher inflammatory activity of PMNs in coronary instability, as we have previously shown, reporting an elevated PMNs activation in UA patients analysed by intracellular myeloperoxidase index. The association between instability and delayed apoptosis is supported by the observation that six patients with recurrent angina within 48 h of admission had a lower PMNs apoptotic rate at 4 h than those without recurrence of angina.

Finally, this study is in agreement with the significant correlation between time from symptoms onset and PMNs’ telomerase activity found by Narducci and supports the hypothesis of a role of PMNs apoptotic delay in the early phases of instability.15 At variance from the current study, however, Narducci et al. did not find telomerase activity, which is a marker of PMNs apoptotic delay, in peripheral bloods of UA as well as SA patients. Thus, different PMNs survival pathways may be active at peripheral and local level. It may be speculated that in peripheral blood, circulating PMNs are exposed to relatively low levels of cytokines, as GM-CSF and IL-6, with anti-apoptotic properties on PMNs, while at plaque level the micro-environment is likely to be very different, with high concentrations of cytokines, including GM-CSF and G-CSF, known to induce telomerase expression, and stronger cell–cell interaction.

This study has some limitation. First, the sample size is small, although differences are highly significant, therefore our data should be confirmed by further controlled studies enrolling larger patient cohorts. Second, we did not evaluate apoptotic rate of PMNs isolated from coronary sinus, it could help to better understand the mechanisms at the basis of local instability.

In conclusion, our study demonstrates that patients with UA exhibit a remarkable delay of apoptosis. A prolonged survival of peripheral PMNs suggests an enhanced and prolonged inflammatory activity of these cells, which may represent a novel contributory pathway in the pathogenesis and maintenance of instability. Other studies are required to establish whether regulation of PMNs apoptosis in ACS might yield new therapeutic strategies.

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Conflict of interest: L.M.B. received consultancy fees from Sanofi-Aventis, Pfizer, Siemens Diagnostics. All other authors have no conflict of interests to declare.

References

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Real-time three-dimensional transoesophageal echocardiography showing sequential events of the percutaneous mitral clip procedure

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A surgical technique, called edge-to-edge technique or Alfieri’s stitch, approximating the middle scallops of the mitral leaflets to create a double orifice with improved leaflet coaptation was introduced in the early 1990s. Recently, a percutaneous method to create the same type of repair was developed. Real-time 3D transoesophageal echocardiography (RT 3D TEE) is a new technique which allows a consistently fine image quality of mitral valve from a left atrium perspective. We present RT 3D TEE sequential images showing the most relevant steps of this transcatheter mitral valve repair procedure. Panel A shows a guide-wire passing into the left atrium and in Panel B, the transseptal apparatus is exchanged for the guide catheter. In Panel C, the clip delivery system is advanced into the left atrial chamber and centred over the mitral orifice. Panel D shows as the arms of the clip are opened and oriented perpendicularly to the long axis of the leaflet edges. Panel E shows the achievement of a double-orifice mitral valve because of leaflet insertion into the closed clip arms. Panel F shows the final result once two clips are sequentially released from the clip delivery system and the delivery system and guide catheter are withdrawn. Real-time 3D TEE colour Doppler images before (Panel G) and after (Panel H) the procedure show a significant improvement in mitral regurgitation severity. Finally, panel I shows a laminar diastolic flow through the two orifices thus indirectly confirming the absence of obstruction.

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