Serum From Patients With Acute Coronary Syndromes Displays a Proapoptotic Effect on Human Endothelial Cells
A Possible Link to Pan-Coronary Syndromes

Marco Valgimigli, MD; Laura Agnoletti, MSc; Salvatore Curello, MD; Laura Comini, PhD; Gloria Francolini, BSc; Francesca Mastrorelli, MD; Elisa Merli, MD; Roberto Pirani, MD; Gabriele Guardigli, MD; Pier Giovanni Grigolato, MD; Roberto Ferrari, MD, PhD

Background—Endothelial apoptosis of atherosclerotic lesions is a possible determinant for the stable-to-vulnerable plaque transition. Recent data support the notion that plaque activation may be a pan-coronary process, advocating the existence of circulating triggers.

Methods and Results—Serum from 40 healthy subjects (group 1) and 73 patients with stable angina (n=32; group 2) or acute coronary syndromes (n=41; group 3) was incubated with human umbilical vein endothelial cells. The percentage of apoptosis by flow cytometry and Fas, Bax, and Bcl-2 protein expression by immunoblotting were evaluated at entry in patients and control subjects and repeated after 12 months in group 3. At baseline, apoptotic nuclei were higher in group 3 (14±6%) than in group 2 (3.3±1.8%) and group 1 (1.35±0.8%) (P<0.001). Fas and Bcl-2 were increased in group 3 with respect to groups 1 and 2 (P<0.01). Coincubation of group 3 serum with anti–tumor necrosis factor-α and anti–interleukin-6 monoclonal antibodies did not affect the human umbilical vein endothelial cell apoptotic process, whereas addition of Trolox decreased apoptosis to <50%. The percentage of apoptosis in group 3 significantly correlated to the numbers of coronary complex lesions at angiography (r=0.58, P<0.0005). In group 3, apoptosis and the Bax/Bcl-2 ratio decreased at 1 year (P<0.0001, P<0.05 respectively).

Conclusions—Serum from patients with acute coronary syndromes displays a proapoptotic effect on human endothelial cells, supporting the theory of the existence of circulating triggers potentially able to activate atherosclerotic lesions.

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Key Words: apoptosis ■ inflammation ■ plaque ■ interleukins ■ endothelium

The transition of intracoronary atheromatous plaques from stable to unstable is thought to be the basis for acute coronary syndromes (ACS). The mechanisms for such a transition are not fully understood.

The demonstration of additional unstable lesions other than the culprit lesion in ≈20% of patients with acute myocardial infarction (AMI) supports the notion that plaque instability may not simply be a localized vascular accident but rather a generalized pan-coronary process. The finding that patients immediately after AMI are at a localized vascular accident but rather a generalized pan-coronary (AMI) supports the notion that plaque instability may not simply be fully understood.

Circulating factors might induce plaque activation during ACS through apoptotic death of endothelial cells.

We evaluated whether incubation with serum from patients with ACS is proapoptotic on human umbilical vein endothelial cells (HUVECs) compared with that from patients with stable angina (SA) and from healthy subjects. To gain further insights into this mechanism, the roles of cytokine activation and of oxidative stress have been investigated by in vitro addition of anti-human cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-6) monoclonal antibodies and of the antioxidant Trolox on HUVECs incubated with serum from patients with ACS.

Methods

Study Population
Forty healthy subjects and 73 patients were enrolled. Their clinical and biochemical profiles are presented in Table 1. Local ethics
TABLE 1. Baseline Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 (n=40) Healthy control Subjects</th>
<th>Group 2 (n=32) Stable Angina</th>
<th>Group 3 (n=41) Unstable Angina (n=14)</th>
<th>Group 3 (n=41) Infarction (n=27)</th>
<th>( P_1 )</th>
<th>( P_2 )</th>
<th>( P_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>65±6</td>
<td>68±11</td>
<td>69±9</td>
<td>64±9</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>24/16</td>
<td>21/11</td>
<td>9/5</td>
<td>20/7</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Smokers, y/n</td>
<td>22/18</td>
<td>8/24</td>
<td>8/6</td>
<td>17/10</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension, y/n</td>
<td>0/40</td>
<td>19/13</td>
<td>6/8</td>
<td>19/8</td>
<td>0.0003</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Family history of CVD, y/n</td>
<td>19/21</td>
<td>20/12</td>
<td>11/3</td>
<td>20/7</td>
<td>0.03</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hypercholesterolemia, y/n</td>
<td>0/40</td>
<td>17/15</td>
<td>5/9</td>
<td>10/17</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes, y/n</td>
<td>0/40</td>
<td>10/22</td>
<td>7/7</td>
<td>15/12</td>
<td>0.0002</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>LDL, mg/dL</td>
<td>...</td>
<td>133±41</td>
<td>141±37</td>
<td>126±34</td>
<td>...</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>...</td>
<td>38±12</td>
<td>41±10</td>
<td>47±9</td>
<td>...</td>
<td>0.05</td>
<td>NS</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>...</td>
<td>121±23</td>
<td>131±53</td>
<td>111±50</td>
<td>...</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Prior MI, y/n</td>
<td>0/40</td>
<td>0/32</td>
<td>2/12</td>
<td>5/22</td>
<td>0.002</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Prior PCI or CABG, y/n</td>
<td>0/40</td>
<td>0/32</td>
<td>1/13</td>
<td>9/18</td>
<td>0.0006</td>
<td>0.001</td>
<td>0.01</td>
</tr>
<tr>
<td>KILLIP class &gt;1(%)</td>
<td>...</td>
<td>0</td>
<td>16</td>
<td>37</td>
<td>...</td>
<td>0.0001</td>
<td>0.02</td>
</tr>
<tr>
<td>CK peak, U/L</td>
<td>...</td>
<td>...</td>
<td>68±98</td>
<td>3026±2276</td>
<td>...</td>
<td>...</td>
<td>0.000037</td>
</tr>
<tr>
<td>CK-MB peak, ng/mL</td>
<td>...</td>
<td>...</td>
<td>8±14</td>
<td>369±209</td>
<td>...</td>
<td>...</td>
<td>0.0005</td>
</tr>
<tr>
<td>Troponin I peak, ng/mL</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>149±201</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>C-reactive protein, mg/dL</td>
<td>...</td>
<td>0.7±0.2</td>
<td>3±3</td>
<td>3.4±4</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>WBC, 10³/mm³</td>
<td>...</td>
<td>9.1±3.3</td>
<td>11±3</td>
<td>9.3±4</td>
<td>...</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>...</td>
<td>14±1.1</td>
<td>13±2</td>
<td>13±1.7</td>
<td>...</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glycemia, mg/dL</td>
<td>...</td>
<td>111±19</td>
<td>141±44</td>
<td>129±40</td>
<td>0.02</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>...</td>
<td>49±8</td>
<td>45±10</td>
<td>40±15</td>
<td>...</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic volume, mL/m²</td>
<td>...</td>
<td>78±19</td>
<td>85±12</td>
<td>94±9</td>
<td>...</td>
<td>0.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

TG indicates triglycerides; MI, myocardial infarction; CK, creatine kinase; WBC, white blood cells; LV, left ventricular; and CVD, cardiovascular disorders. CABG: Mean duration 7±3 years.

Patients and control subjects were well-matched for sex and age but differed for prevalence of cardiovascular risk factors, previous cardiovascular events, and factors related to the extension of infarct area such as CK and troponin release.

*Group 1 vs group 3, †group 2 vs group 3, ‡group 3A vs group 3B.

Each group 3 patient was treated with a standard regimen of aspirin, \( \beta \)-blocker, statin, and ACE-I. After hospital discharge, patients of group 3 underwent 2 follow-up visits (after 6 months and 1 year).

Angiographic Analysis and Plaque Evaluation

All group 3 patients underwent coronary angiography during hospital phase. Two independent angiographers analyzed coronary angiograms. The results were compared and, in the case of disagreement, the final decision was made by consensus. All substantial lesions were measured by quantitative analysis. Complex coronary plaques were identified according to standardized criteria.1,14–16

Cytokines and Cytokine Receptor Levels

Antigenic TNF-\( \alpha \) and its soluble receptors were assayed as previously described.17 IL-6 and IL-1Ra levels were assessed by an ELISA kits (R&D Systems).

Oxidized LDL

Oxidized LDL (ox-LDL), which stimulates endothelial apoptosis18 and could therefore be responsible for increased apoptotic activity of serum either alone or in combination with TNF-\( \alpha \),19 was measured in plasma by an ELISA kit (Mercodia AB).

Cell Culture

HUVECs were isolated from umbilical cords according to Jaffe et al.20 and resuspended in M199-RPMI 1640 1:1 containing 20%
pooled human serum and seeded on culture flasks without additional growth factors. HUVECs were von Willebrand factor positive and showed typical cobblestone morphology.

As positive control, at third split some of the cells were treated without serum for 48 hours. Others were incubated for 72 hours with 20% serum from either healthy subjects or patients.

To test the specific role of TNF-α, IL-6, and oxidative radicals on apoptosis, in separate groups of experiments, HUVECs were incubated with serum from group 3 patients in the presence of 1 μg/mL of monoclonal anti-human TNF-α, IL-6 antibodies (R&D Systems), or 1 mmol of a water-soluble α-tocopherol (vitamin E) model (S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Quantitative Assessment of Apoptosis by Flow Cytometry

Apoptotic cells were detected by flow cytometry following the method of Nicoletti et al., slightly modified. Briefly, endothelial cells were fixed in cold methanol and then incubated with 50 μg/mL propidium iodide (PI) and RNAse, 0.1% Triton X-100 in sodium citrate 0.1%, pH 7.4. Analysis was performed by means of a Coulter Epics XL-MCL flow cytometer equipped with an argon laser at 488 nm wavelength. A minimum of 10,000 cells was analyzed from each sample. Apoptotic cells were detected on a PI histogram of cells as PI-positive apoptotic cells showing typical nuclear fragmentation, a fluorescence microscope was used for qualitative assessment of apoptosis. For viewing nuclei. Incubation with serum from healthy subjects (group 1) showed 3% positive control showed an average of 22% of apoptotic nuclei. Incubation with serum from patients with SA (group 3) slightly but not significantly increased the rate of apoptosis in respect to healthy subjects (group 1) (from 1.3±0.8% to 3.3±1.8%, NS). Incubation with serum from patients with ACS resulted in a significant increase of the rate of apoptosis (from 1.3±0.8% to 14.3±6%, P<0.001). Within patients of group 3, there was no significant difference between the apoptotic effect of serum from patients with UA or AMI (14.8±7%, 13.5±6%, respectively).

TABLE 2. Cytokines and Oxidized LDL Cholesterol in the Study Population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 (n=40) Healthy Subjects</th>
<th>Group 2 (n=32) Stable Angina</th>
<th>Group 3 (n=41) Acute Coronary Syndromes</th>
<th>P0†</th>
<th>P1†</th>
<th>P2‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α, pg/mL</td>
<td>18.2±4</td>
<td>22.5±8</td>
<td>34±17</td>
<td>0.002</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sTNFR-I, pg/mL</td>
<td>978±775</td>
<td>1297±985</td>
<td>2090±1082</td>
<td>0.003</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>STNFR-II, pg/mL</td>
<td>1947±1403</td>
<td>2489±1403</td>
<td>3278±1508</td>
<td>0.005</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>4.3±8</td>
<td>3.4±13</td>
<td>42±7</td>
<td>0.001</td>
<td>0.005</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1-ra, pg/mL</td>
<td>224±112</td>
<td>306±227</td>
<td>1224±1089</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>ox-LDL, μL</td>
<td>35±10</td>
<td>39±20</td>
<td>55±25</td>
<td>0.04</td>
<td>0.09</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Group 1 vs group 3; †group 2 vs group 3; ‡group 1 vs group 2.

All tested parameters were increased in group 3 in respect to group 1 and group 2, except for ox-LDL, which did not differ significantly between groups 3 and 2.

Qualitative Assessment of Apoptosis by Fluorescence Microscopy

After treatments, cells were washed and spun on slides. For viewing PI-positive apoptotic cells showing typical nuclear fragmentation and chromatin condensation, a fluorescence microscope was used (Nikon Optiphot-2 equipped with mercury lamp excitation [HBO 100 W] and a 510- to 560-nm long-pass filter for the PI fluorescence detection).

Western Blot Analysis

Western blotting was performed by using anti-human Fas and Bax (R & D Systems, 1:500 and 1:1000, respectively) and Bcl-2 (Serotec, 1:1000) as primary antibodies. To compare baseline with follow-up protein expression, results are expressed as a ratio to the internal control obtained by serum subtraction. Results are also reported as Bax/Bcl-2 ratio.

Statistical Analysis

Values are expressed as mean±SD. Comparisons between two groups were performed with the Student’s t test or Mann-Whitney test in the case of nonparametric variables. Fisher’s exact test was used for categoric variables. Comparisons among more than two groups were performed by 2-tailed ANOVA and post hoc comparisons by Turkey honest significance difference test. Correlations between variables were tested by Pearson analysis. Probability was significant at a level of <0.05.

Results

The clinical and biochemical characteristics of the studied population are shown in Table 1. In group 3, coronary angiographic evaluation was performed at 3±2 days from hospital admission. No patient had significant obstruction of the left main artery: 15 patients (36%) had 3-vessel disease, 11 (27%) had 2-vessel disease, and 12 (29%) had 1-vessel disease. Three patients (7%) had no significant obstruction of the coronary lumen. Complex coronary plaques were present in 32 patients of group 3, 18 having a single complex coronary plaque.

Cytokines and ox-LDL

Cytokines and ox-LDL levels are shown in Table 2. In group 3 patients, all the tested parameters were significantly higher than those from healthy subjects and patients of group 2. There were no significant differences between subgroups 3A and 3B.

Occurrence of Apoptosis

Representative cytofluorometric recordings of the HUVECs treated either with serum subtraction (positive control) or with serum from healthy subjects (group 1) and patients with SA (group 2) or ACS (group 3) are shown in Figure 1A. Mean data are reported in the lower panel of Figure 1B. The positive control showed an average of 22% of apoptotic nuclei. Incubation with serum from patients with SA (group 2) slightly but not significantly increased the rate of apoptosis in respect to healthy subjects (group 1) (from 1.3±0.8% to 3.3±1.8%, NS). Incubation with serum from patients with ACS resulted in a significant increase of the rate of apoptosis (from 1.3±0.8% to 14.3±6%, P<0.001). Within patients of group 3, there was no significant difference between the apoptotic effect of serum from patients with UA or AMI (14.8±7%, 13.5±6%, respectively). Occurrence of apoptosis, characterized by typical nuclear fragmentation, has been confirmed by fluorescence microscopy (Figure 2).
Fas, Bcl-2, and Bax protein expression in HUVECs after incubation with serum from each group are shown in Figure 3. Fas and Bcl-2 were increased when cells were incubated with serum from patients of group 3 (P<0.05), whereas Bax showed a borderline elevation (P=0.06).

Role of Cytokine Activation and Oxidative Stress in Inducing Apoptosis

The effect of the coincubation of serum from patients of group 3 with anti-human TNF-α and IL-6 antibodies, either separately or together, are reported in Figure 4. Clearly, the apoptotic effect of the serum so treated is unchanged. On the contrary, coincubation with the antioxidant Trolox showed a reduction of apoptotic nuclei by >50% (from 14±6% to 5.5±3.8%, P<0.001).

Correlations

There were several linear correlations between cytokine activation, left ventricular ejection fraction, and C-reactive protein (Table 3).

The percentage of apoptosis was significantly correlated only with the number of complex lesions (r=0.58, P<0.005).

Figure 1. Serum-induced HUVEC apoptosis in control subjects and patients. A, Representative cytfluorometric recordings of HUVECs treated without serum (positive control) or with serum from either healthy control subjects (group 1) and patients with SA (group 2) or ACS (group 3). Arrows in A mark the hypodiploid peak. Mean data are reported in B. *P<0.001 vs group 1.

Figure 2. Representative image showing PI-positive endothelial cells at fluorescence microscopy, with typical nuclear fragmentation (arrows) (magnification ×40).
This finding was further confirmed by direct comparison of rate of apoptosis and the number of complex lesions. Patients with single complex lesion showed a 10%/4.7% rate of apoptosis, significantly less than the 17.5%/4.6% of patients with multiple complex lesions (P<0.0001).

Apoptosis and Cytokine Activation After 1 Year
Data of patients of group 3, when reassessed after 1 year in stable conditions, are reported in Table 4.

The rate of HUVEC apoptosis was markedly reduced and no longer different from that of healthy subjects. Fas protein expression was unchanged; Bax and Bcl-2 showed a borderline reduction, the ratio of Bax/Bcl-2 being significantly reduced (P<0.05). TNF-α, sTNFRI, and sTNFRII levels did not differ significantly from the corresponding levels at entry, whereas IL-6 and IL-1Ra levels were significantly decreased.

Discussion

Original Findings
Serum from patients with ACS displays a proapoptotic effect on HUVECs. The proapoptotic effect is not related to the degree of necrosis (ie, UA versus AMI) or to the level of cytokine activation, and it is not significantly affected by coincubation with anti-TNF-α or anti-IL6 human antibodies. However, the apoptotic effect is related to the activation of the atherosclerotic plaque: The effect disappears once clinical conditions are stabilized, and it is not present in patients with SA. Interestingly, the level of endothelial cell apoptosis is correlated with the number of complex coronary lesions.

When taken together, these data indicate that the acute setting of coronary events is not necessarily a localized vascular insult because the systemic proapoptotic activity of the serum could exert deleterious effects at distance. This could contribute to the explanation of the pan-coronary syndrome.1,2

It has been suggested that increased endothelial apoptosis leads to atheroma denudation and subsequent coronary thrombosis.5–9,24 Endothelial apoptosis could also increase susceptibility toward coronary thrombosis through the release of apoptotic cell debris into the blood stream, which is known to directly activate coagulation cascade.11–13 Lutgens et al25 have reported that the number of apoptotic cells in the plaque is related to the stage of plaque development, being higher in advanced lesions, suggesting a possible cause-effect relation. On the other hand, it is also known that the plaque itself could release, once activated, biochemical substances such as, for instance, pro-oxidants,26–28 which, in turn, can stimulate the apoptotic process.29–31 The already accepted association between oxidative stress and ACS32 together with the present finding that coincubation with Trolox markedly reduced the proapoptotic effect of serum would support this hypothesis.

Putative Mechanisms
Factors released in serum during ACS, responsible for its increased apoptotic activity, are still speculative at the moment. In patients with UA, circulating CD 4 T lymphocytes undergo a change in functional profile, become perform-
expressing and have increased oxygen radical production, and acquire cytotoxic capability toward HUVECs.\textsuperscript{3,3} Perforin and oxygen radicals could be released in serum and exert injury at a distance, explaining the finding of coexistence of multiple activated coronary plaques. In addition, C-reactive protein was found to sensitize endothelial cells to this cytotoxic process, further providing a link between immune activation and plaque rupture.

Results on Bax and Bcl-2 reinforce data on apoptosis, whereas the persistence of Fas elevation at follow-up possibly reflects unchanged levels of TNF-\(\alpha\).

Our previous data indicate that blood withdrawn from patients with advanced heart failure (HF) exerts a similar pro-apoptotic effect on HUVECs.\textsuperscript{22} Interestingly, in HF, TNF-\(\alpha\) activation induces a downregulation of endothelial constitutive nitric oxide synthase (eNOS) and has a causal effect on apoptotic process as incubation with an anti-TNF-\(\alpha\) antibody with serum significantly reduces its apoptotic activity.\textsuperscript{22} On the contrary, serum from patients with ACS without HF has a pro-apoptotic effect, which is independent from proinflammatory cytokine levels and does not downregulate eNOS (0.43±0.14 in control subjects versus 0.44±0.21 in patients of group 3 at Western blot analysis; data not shown). We have no clear explanation for this discrepancy that could be related to the different pattern of cytokine elevation in these two different clinical situations: TNF-\(\alpha\) elevation in HF higher and persistent in comparison to the lower and more transient in ACS.

**Study Limitations**

The sample size of this study is too small to properly assess whether the serum-induced apoptotic rate is an independent predictor for clinical outcome in ACS patients. This will require prespecified investigations.

We cannot exclude that actions not related to the antioxidant effect of Trolox could partially explain our results on apoptosis.

**Conclusions**

Our study gives evidence for the first time that serum from patients with ACS displays a proapoptotic effect on endothelial cells, disappearing once clinical stabilization is restored. These and other findings,\textsuperscript{34} together with the positive correlation between the degree of serum-induced HUVECs apoptosis and the number of coronary complex lesions, suggest that coronary events are not necessarily a localized vascular insult and contribute to the pathophysiological explanation of the pan-coronary syndrome.

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**References**


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