TUMOR NECROSIS FACTOR-ALPHA AS TRIGGER OF PLATELET ACTIVATION IN PATIENTS WITH HEART FAILURE.

Author’s list:
Pasquale Pignatelli\textsuperscript{\textregistered}, Luciano De Biase\textsuperscript{*}, Luisa Lenti\textsuperscript{\textregistered\textregistered}, Giuliano Tocci\textsuperscript{*}, Alessandra Brunelli\textsuperscript{#}, Roberto Cangemi\textsuperscript{\textregistered}, Silvia Riondino\textsuperscript{\textregistered\textregistered}, Susanna Grego\textsuperscript{\textregistered}, Massimo Volpe\textsuperscript{*} and Francesco Violi\textsuperscript{\textregistered}.

\textsuperscript{\textregistered}Divisione IV Clinica Medica, \textsuperscript{\textregistered\textregistered}Dipartimento di Medicina Sperimentale e Patologia, Policlinico Umberto I, Università “La Sapienza” di Roma; \textsuperscript{#}Dipartimento di Scienze dell’Invecchiamento, Policlinico Umberto I, Università “La Sapienza” di Roma; \textsuperscript{*}Ospedale Sant’Andrea, Cardiologia, II Facoltà di Medicina e Chirurgia, Università “La Sapienza” di Roma.

Short title: platelet activation by TNF-\textalpha\ in HF

Corresponding Author:

Dr. Francesco Violi
University of Rome “La Sapienza”
Divisione IV Clinica Medica
Policlinico Umberto I
Viale del Policlinico
00185, Rome, Italy
Tel.: +39-06-4461933
Fax: +39-06-4940594
e-mail: francesco.violi@uniroma1.it

Words: 1326
ABSTRACT

Clinical history of patients with Heart Failure (HF) is complicated by arterial thromboembolism. Platelet activation was reported in this setting, but the underlying mechanism has not been clarified. 42 patients with HF scored according to NYHA classification had higher collagen-induced platelet aggregation, platelet tumor necrosis factor-alpha (TNF-α) receptor expression, serum thromboxane B₂ and circulating levels of TNF-α than 20 healthy subjects. Coincubation of platelets from HF patients with an inhibitor of TNF-α receptors significantly reduced collagen induced platelet aggregation. In vitro study demonstrated that TNF-α amplified the platelet response to collagen; this effect was inhibited by (a) TNF-α receptor antagonist and (b) inhibitors of arachidonic acid metabolism. This study shows that TNF-α behaves as a trigger of platelet activation via stimulation of the arachidonic acid pathway.

Key Words: platelet, TNF-α, Heart Failure, Arachidonic Acid Pathway
INTRODUCTION

Chronic congestive Heart Failure (HF) represents an important cause of morbidity and mortality in the western population.\(^1\) HF predisposes to thromboembolism, in particular to ischemic stroke, in a relatively high percentage of patients.\(^1\) The association between HF and thromboembolic stroke is higher in patients with severe HF compared to patients with mild-to-moderate HF;\(^2\) but the mechanisms favoring thromboembolism have not been fully clarified.\(^3\)

The proinflammatory cytokine tumor necrosis factor-alpha (TNF-\(\alpha\)) is upregulated in HF patients and seems to be involved in the pathophysiology of this disease.\(^4,5\) As previous in vitro studies have demonstrated that TNF-\(\alpha\) may promote platelet aggregation\(^6,7\) we investigated if circulating levels of TNF-\(\alpha\) could have a role in eliciting platelet aggregation in HF patients.
MATERIALS AND METHOD

Patients

We analyzed 42 patients with a diagnosis of HF, enrolled from the outpatient clinic of our facilities, and 20 healthy subjects (HS). The study population’s characteristics are reported in Table 1. Inclusion and exclusion criteria as well as diagnostic methods have been previously reported. HF was scored according to NYHA classification.

All patients gave informed consent to participate in the study.

Platelet activation

Agonist-induced platelet aggregation (PA) (Born's method) was performed in washed platelets according to and expressed as light transmission (LT%) difference between Platelet Rich Plasma and platelet poor plasma 3 min. after the addition of the agonist. Collagen-induced platelet thromboxane B₂ (TxB₂) was measured as previously described. Serum TxB₂ was measured as previously described.

Plasma TNF-α levels were determined using TNF-alpha ELISA-assay kits.

Flow cytometry analysis of TNF-α receptors

TNF-α receptors (TNFR)1 and TNFR2 expression on platelets membrane was analysed using the specifics FITC-labelled monoclonal antibodies anti-TNFR1 and TNFR2 (Mab) (R and D System). All assays included samples to which an irrelevant isotype-matched antibody was added.

Twenty microlitres of Mab were added to 200 µl of platelet suspension (2x10⁸/mL) previously fixed with (2%) paraphormaldeide (0.1% BSA) and incubated for 60 min at 4°C. The unbound Mab was removed by the addition of 0.1% BSA phosphate buffer saline (PBS) and centrifugation at 5000 x g for 3 min (twice). Fluorescence intensity was analysed on an Epics XL-MCL Cytometer (Coulter Electronics, FL, USA) equipped with an argon laser at 488 nM. For every histogram, 50,000 platelets were counted to evaluate the percentage of positive platelet. Antibody reactivity is reported as mean fluorescence.
IN VITRO STUDY

PA and TxB\textsubscript{2} formation were measured as above reported\textsuperscript{9} in platelets taken from aspirin-free patients and incubated at 37°C for 15’ with TNF-\textalpha (20-40 pg/ml) and subthreshold concentration of collagen\textsuperscript{11} (0.5 µg/ml) or ADP (0.4 µM) or thrombin (0.01 U/ml) and added or not with 14 µM AACOCF\textsubscript{3}, an inhibitor of PLA\textsubscript{2} enzyme,\textsuperscript{9} or 100 µM aspirin, an inhibitor of cyclooxygenase enzyme\textsuperscript{9} or 1 µM WP9QY, an inhibitor of TNF-\textalpha receptors.\textsuperscript{13} For these experiments AACOCF\textsubscript{3}, aspirin and WP9QY were incubated 10 min at 37°C before the addition of agonists.

Statistical Analysis

Data are reported as mean ± SD. The comparison between variables was analysed by Student’s t-test for unpaired data.

When comparing >2 groups, two way ANOVA test followed by Kruskall-Wallis as a post-hoc test was used. If a significant difference was found, the Mann-Whitney U test (2-tailed) was used to determine the differences between each pair of groups. Correlation analysis was performed by Pearson’s correlation coefficient. Significance was accepted at a P<0.05 level.
RESULTS AND DISCUSSION

Clinical characteristics and drug therapy of patients with HF divided according to NYHA classification are reported in Table 1.

Compared to HS, HF patients had higher PA (LT 72% vs 40%; p<0.001) and serum TxB2 (377 ng/ml vs 284 ng/ml; p<0.001); after excluding four aspirin-treated patients such increases were still evident and associated with HF severity (fig. 1A). These findings were not observed using other agonists such as ADP or thrombin and were not influenced by HF etiology (data not shown).

TNF-α was significantly higher in patients than in HS (39.6 ± 12 pg/ml vs 8.5 ± 3 pg/ml, p<0.001), progressively increased from mild to severe HF (fig. 1B) and significantly correlated with both PA (r=0.72; p<0.001) and serum TxB2 production (r=0.53; p<0.001), suggesting a possible cause-effect relationship between the increase of this cytokine and platelet function.

Recent studies demonstrated, that, upon activation, platelet express TNFR and that engagement of TNF-α with its receptors elicits platelet activation. Therefore flow cytometry analysis was done in a subset of 20 HF patients and in 10 HS matched for sex and age (fig. 1C). Compared to controls, patients with HF had higher expression of TNFR1 depending on HF severity; TNFR2 were also increased in patients compared to controls but the difference was less marked. A significant correlation was observed between platelet TNFR1 and PA (fig. 1D), suggesting that upregulation of TNFR1 is implicated in the activation of platelets.

In order to explore this issue, PA was measured in platelets added or not with WP9QY. While the TNFR inhibitor did not effect aggregation of platelets from HS, it significantly inhibited PA in HF patients, with a higher percentage inhibition in those with severe HF (fig. 1E). Taken together these findings suggested that in HF patients platelet hyperactivation could be related to the increased levels of TNF-α, that, upon interaction with its receptors, enhanced platelet responsiveness to the agonist.

To explore if TNF-α behaved in vitro as platelet agonist, it was added at concentration up to 40
pg/ml to platelets taken from HF patients but no change of PA could be observed (not shown). This finding is apparently at variance with previous studies showing that TNF-α per se is a platelet agonist,6,7 however the order of magnitude of TNF-α used in those studies is much higher (1-50 ng/ml) than that we used (20-40 pg/ml), indicating, that at physiologic concentration, TNF-α is not an aggregating agent. Conversely TNF-α elicited PA and TXb2 formation in collagen-primed platelets (fig.1F).

On the basis of the previous study suggesting that TNF-α elicits activation of PLA2 upon interaction with its receptor TNRF115 we hypothesized that stimulation of arachidonic acid pathway could have a key role in TNF-α-mediated platelet activation. This hypothesis was supported by showing that an inhibitor of PLA2 and aspirin significantly inhibited TNF-α-induced PA (fig.1F) Amplification of platelet response to collagen was dependent upon TNF-α interaction with its receptors as the addition of TNFR antagonist significantly inhibited PA (fig. 1F). The specificity of this effect was corroborated by showing no influence of TNFR antagonist in platelets stimulated with collagen alone (not shown).

TNF-α (40 pg/ml) amplified the response to collagen also in platelets taken from HS (untreated platelets; PA 3%±1 LT; subtreshold collagen-treated platelets PA 43%±4 LT); addition of platelets with TNFR or arachidonic acid pathway inhibitors significantly inhibited PA (not shown). Amplification of platelet response to TNF-α was not observed with thrombin or ADP likely because of minor role of arachidonic acid pathway in platelet activation induced by these agonists16 (not shown).

In conclusion the study provides evidence of a progressive increase in platelet aggregation and TxB2 formation form mild to severe HF. Enhanced circulating levels of TNF-α may account for platelet hyperfunction as TNF-α is able to activate platelets via stimulation of arachidonic acid pathway. These findings give new insight into the mechanism that could favor thromboembolism in patients with HF.
ACKNOWLEDGEMENTS

This work was partially supported by public grant (MIUR 2000) to Francesco Violi.
REFERENCES


United Kingdom. 1101 pp.


Table 1. Clinical characteristics of study population

<table>
<thead>
<tr>
<th>NYHA Class</th>
<th>HS (n=20)</th>
<th>MILD HF (n=19) (NYHA I-II)</th>
<th>SEVERE HF (n=23) (NYHA III-IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MALES/FEMALES</td>
<td>18/2</td>
<td>18/1</td>
<td>20/3</td>
</tr>
<tr>
<td>AGE (years)</td>
<td>58±5</td>
<td>59±12</td>
<td>58±8</td>
</tr>
<tr>
<td>Cigarette smoking (M/F)</td>
<td>2/1</td>
<td>1/0</td>
<td>3/1</td>
</tr>
<tr>
<td>Causes of Heart Failure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated Cardiomyopathy</td>
<td>/</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Coronary Artery Disease</td>
<td>/</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Valve Disease</td>
<td>/</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>56.2±5.5</td>
<td>29.0±4.8</td>
<td>26.9±7.2</td>
</tr>
<tr>
<td>Total cholesterol (mg/ml)</td>
<td>184.1±38.6</td>
<td>207.3±41.4</td>
<td>201.7±49.9</td>
</tr>
<tr>
<td>Diabetes (M/F)</td>
<td>0/0</td>
<td>0/0</td>
<td>1/0</td>
</tr>
<tr>
<td>Therapeutic agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretics</td>
<td>/</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>/</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Calcium channel antagonists</td>
<td>/</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>/</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>ATI receptor antagonists</td>
<td>/</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Anti-coagulant drugs</td>
<td>/</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Lipid lowering agents</td>
<td>/</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Digitalis</td>
<td>/</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Aspirin</td>
<td>/</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>/</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
FIGURE LEGEND

Fig. 1

Panel A. Collagen-induced platelet aggregation (PA) and serum TxB₂ production in patients with mild and severe HF and in the healthy subjects (HS) (*=p<0.001 vs mild HF, §=p<0.001 vs HS).

Panel B. TNF-α plasma levels in patients with mild and severe HF and in HS (*=p<0.001 vs HS, §=p<0.001 vs mild HF).

Panel C. TNFR1 and TNFR2 expression on platelet surface in patients with mild (n=10, 9 males, 1 female, age 58±10) and severe HF (n=10, 9 males, 1 female, age 58±6) and in HS (n=10, 9 males, 1 female, age 58±7) (*=p<0.001 vs HS; §=p<0.001 vs mild HF).

Panel D Correlation between platelet TNFR1 and PA (r=0.79, p<0.001) in 20 patients with HF and 10 HS.

Panel E Collagen-induced PA in aspirin-free patients with mild (n=10) or severe (n=10) HF and in HS (n=10) in presence or not of the TNFR inhibitor WP9QY (*=p<0.01, **=p<0.001)

Panel F: Effect of WP9QY or AACOCF3 or ASA on TNF-α-induced activation of collagen(0.5 µg/ml) -primed platelets taken from 3 patients with mild HF and 2 with severe HF. (§=p<0.001 vs TNF-α free platelets, *=<0.001 vs platelets added with collagen and TNF-α). Data (n=5) are expressed as mean ± SD.
Fig. 1

Panel A
Serum TxB2 (nM/ml)

Panel B
TNF-α (pg/ml)

Panel C
mean fluorescence

Panel D
TNFR1 (mean fluorescence)

Panel E

Panel F

subthreshold collagen

+ TNF-α 40 pg/ml
+ WP9QY 1 µM
+ AACOCF3 14 µM
+ ASA 100 µM
Tumor necrosis factor-alpha as trigger of platelet activation in patients with heart failure

Pasquale Pignatelli, Luciano De Biase, Luisa Lenti, Giuliano Tocci, Alessandra Brunelli, Roberto Cangemi, Silvia Riondino, Susanna Grego, Massimo Volpe and Francesco Violi