Serum levels of RIPK3 and troponin I as potential biomarkers for predicting impaired left ventricular function in patients with myocardial infarction with ST segment elevation and normal troponin I levels prior percutaneous coronary intervention

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Summary

The current study examined the serum levels of receptor-interacting protein kinase 3 (RIPK3) in 51 patients with New York Heart Association (NYHA) class III-IV heart failure, 53 patients with myocardial infarction with ST elevation (STEMI), and 19 healthy subjects serving as a control group. An enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of RIPK3 expression in serum. The area under the receiver operating characteristic curve (AUC) was then used to evaluate the predictive performance of RIPK3 and troponin I in patients with STEMI. In patients with normal levels of troponin I prior to percutaneous coronary intervention (PCI), serum levels of RIPK3 and troponin I after PCI were sufficient to differentiate patients with a preserved left ventricular ejection fraction (LVEF) from those with impaired left ventricular function after PCI (AUC = 0.780 (95% CI: 0.565-0.995, p = 0.043) with a sensitivity of 76.9% and a specificity of 71.4% vs. AUC = 0.735 (95% CI: 0.530-0.941, p = 0.038) with a sensitivity of 88.2% and a specificity of 63.6% at the optimal cutoff values, respectively). Moreover, elevated levels of troponin I after PCI were associated with an increased risk of an LVEF < 50% prior to discharge (odds ratio, 1.014; 95% CI, 1.001 to 1.027; p = 0.03), while elevated levels of RIPK3 were not associated with such a risk. The current findings suggest that in patients with normal levels of troponin I prior to PCI, serum levels of RIPK3 and troponin I can serve as a potential marker to identify patients with a decreased LVEF, thus possibly allowing an early shift to more intensive therapy.

Keywords: Receptor-interacting protein kinase 3 (RIPK3), marker, percutaneous coronary intervention (PCI), left ventricular ejection fraction (LVEF)

1. Introduction

The prevalence of acute and chronic ischemic heart disease and heart failure is as high as 8% in Western countries; these conditions account for more than one-third of all human mortality and remain the leading causes of death worldwide (1). The myocardium consists
of differentiated cardiomyocytes that are responsible for its contractile function. The heart is an organ with limited capacity for regeneration and repair. In cardiovascular diseases such as myocardial infarction and congestive heart failure, there is a significant loss of cardiomyocytes (2). Hence, new biomarkers that indicate early cardiac cell death need to be found. Much attention has been focused on understanding the mechanism of cell death in acute and chronic heart diseases in order to improve patient outcomes. All types of cell death - autophagy, apoptosis and necrosis - participate in the progression of heart diseases - with a great uncertainty as to which of them prevails (3,4).

Recently, a novel type of cell death called "programmed necrosis" or necroptosis has been reported to be involved in the pathogenesis of heart disease (5). Similar to apoptosis, this process is tightly regulated by distinct molecules and has morphological features of both necrosis and inflammation (3,6-9). Nevertheless, the exact pathways activating this programmed necrosis are not fully understood (10). A study has shown that tumor necrosis factor alpha (TNF-α) is a factor that triggers the formation of a complex, called necrosome, in the cytoplasm between receptor-interacting protein kinase 1 (RIPK1) and receptor-interacting protein kinase 3 (RIPK3) (7). In the process of the formation of this complex, RIPK3 phosphorylates RIPK1, which is an essential step in inducing necroptosis (11).

The current study examined the serum levels of RIPK3 in 51 patients with New York Heart Association (NYHA) class III-IV heart failure, 53 patients with myocardial infarction with ST elevation (STEMI), and 19 healthy subjects. This study sought to determine the dynamics of the release of RIPK3 after myocyte injury within 48 hours after the onset of symptoms in all patients with STEMI. This study examined the association between serum levels of RIPK3 and patient outcomes like a decreased left ventricular ejection fraction (LVEF), mortality, and further hospitalization.

2. Materials and Methods

2.1. Ethics Statement

All procedures were approved by the Scientific Research Ethics Committee of the Prof. Dr. Paraskev Stoyanov Medical University of Varna. Blood samples were collected from 53 patients with STEMI (myocardial infarction with ST elevation), 51 patients with NYHA class III-IV heart failure, and 19 healthy individuals at St. Marina University Hospital, Varna after an informed consent form (ICF) was obtained from all study participants.

2.2. Patient selection

From October 2014 to April 2016, 123 subjects were enrolled - 51 with NYHA class III-IV heart failure, 53 with STEMI, and 19 healthy individuals serving as controls. Every possible attempt was made to ensure that selected groups, including healthy controls, were matched by age and sex. Patients with STEMI were treated with percutaneous coronary intervention (PCI). All patients with STEMI had a post-procedure Thrombolysis in Myocardial Infarction (TIMI) flow grade of 3. Serum levels of RIPK3 were measured upon admission on Day 0 (< 12 hours of the onset of symptoms) and on Day 1 (24-48 hours after the onset of symptoms). Serum levels of RIPK3 were not evaluated on Day 2 or later after PCI. Inclusion criteria for patients with myocardial infarction were: chest pain with significant (minimum 2-mm) ST segment elevation according to at least 2 contiguous electrocardiogram (ECG) leads, a significant increase in cardiac markers (troponin I > 0.2 ng/mL), and < 12 hours of the onset of symptoms to primary PCI. All of the patients with myocardial infarction underwent cardiac catheterization and were treated with primary angioplasty and stent replacement.

Serum from 51 hospitalized patients with NYHA class III-IV heart failure was collected to measure RIPK3. The current study defined heart failure in accordance with the definition of the European Society of Cardiology (12), which defines heart failure as a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling, and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles, and peripheral oedema) caused by a structural abnormality resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress. Seven (13.7%) of the 51 patients had NYHA class IV heart failure. For most patients (34/51, 66.7%), symptoms of heart failure were due to ischemic heart disease or previous myocardial infarction. In 9/51 patients (17.6%), heart failure was due to idiopathic dilated cardiomyopathy, and in 8/51 patients (15.6%) heart failure was due to some other reason – e.g. heart valve disease or arrhythmia. All patients had a decreased LVEF (mean 34.4% ± 8.07). Exclusion criteria for all groups were: a current infection, an inflammatory disease, or an oncological disease.

2.3. Echocardiography

LVEF was measured using the biplane method of disks (Simpson’s rule) in accordance with the European Association of Cardiovascular Imaging (13). LVEF was evaluated prior to PCI and upon discharge. The LVEF upon discharge (49.5% ± 9.5) improved significantly compared to the LVEF prior to PCI (47.3% ± 11.6).

2.4. Measurement of RIPK3 and troponin I in serum

For patients with STEMI, peripheral venous blood was drawn at the beginning of the procedure and then again
of demographic variables. Median follow-up was 10 months for patients with STEMI and patients with heart failure. After the follow-up, 5 patients with STEMI and 5 patients with heart failure died due to cardiac causes. Four patients with STEMI were hospitalized again during follow-up due to symptoms of heart failure.

3.2. RIPK3 levels in patients with heart failure or STEMI and its predictive value

The current study sought to determine if RIPK3 is released extracellularly and if it could serve as a marker of necroptosis and cellular injury. RIPK3 was measured in 51 patients with NYHA class III-IV heart failure and 19 healthy subjects. Serum levels of RIPK3 did not differ significantly in healthy persons (mean 0.253 ng/mL ± 0.2) and patients with NYHA class III-IV heart failure (mean 0.236 ng/mL ± 0.4) (Figure 1). Patients with NYHA class III-IV heart failure who died during the follow-up (n = 5) had RIPK3 levels that were not significantly higher than those in patients who did not die. Linear and logistic regression were used to evaluate the potential association between baseline variables such as the number of previous hospitalizations due to heart failure, the decrease in the LVEF, and the serum level of RIPK3. No significant associations were noted.

Figure 1. Bar graph, representing serum levels of RIPK3 in healthy controls, patients with class III-IV NYHA heart failure (HF), and patients with STEMI. The Wilcoxon paired test was used to detect significant differences in serum levels of RIPK3 in patients with STEMI on Day 0 and Day 1. Two-tailed p-values (< 0.05) were considered significant. (RIPK3: receptor-interacting protein kinase 3; STEMI: myocardial infarction with ST elevation.)

2.5. Statistical analysis

Statistical analysis was performed with SPSS Statistics v.23 using descriptive statistics. Categorical variables were summarized with frequencies and percentages. Variables are expressed as the mean ± standard deviation (SD). The Mann-Whitney U test, Wilcoxon paired test, Pearson correlation, and \( \chi^2 \) test were used to compare and estimate correlations between serum levels of RIPK3 and troponin I and demographic and clinical characteristics such as gender and age. The specificity and sensitivity with which serum levels of RIPK3 and troponin I were able to differentiate patients with a preserved LVEF (≥ 50%) from patients with impaired left ventricular function after STEMI upon discharge were evaluated with receiver operating curve (ROC) analysis. The diagnostic accuracy of biomarkers was also determined by obtaining the largest possible area under the curve (AUC) in ROC analysis. Simple logistic regression was used to estimate the odds ratios with which to predict a decreased LVEF after PCI and prior to discharge. Two-tailed p-values (< 0.05) were considered significant.

3. Results

3.1. Patient characteristics and outcomes

Demographic characteristics of patients with STEMI (n = 53) and heart failure (n = 51) are shown in Table 1. There were no significant differences between healthy control subjects and patients with STEMI in terms of age. There were no significant differences between patients with heart failure and STEMI in terms of gender, hypertension, diabetes, and hypercholesterolemia. However, patients with heart failure had a significantly higher percentage of patients on statins compared to patients with STEMI. Patients with STEMI had a significantly lower LVEF and a higher percentage of patients with a history of heart failure. There were no significant differences in the percentage of patients with a prior myocardial infarction or a prior PCI. The percentage of patients with a prior PCI was lower in patients with heart failure compared to patients with STEMI.

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>Patients with heart failure (n = 51)</th>
<th>Patients with STEMI (n = 53)</th>
<th>Significance</th>
<th>Healthy control subjects (n = 19)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs.</td>
<td>67.2 ± 9.3</td>
<td>63.9 ± 12.9</td>
<td>( p = 0.32 )</td>
<td>59.5 ± 10.5</td>
<td>( p = 0.11 )</td>
</tr>
<tr>
<td>Male, %</td>
<td>72.9</td>
<td>57.4</td>
<td>( p = 0.43 )</td>
<td>73.7</td>
<td>( p = 0.12 )</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>18.6</td>
<td>15.7</td>
<td>( p = 0.68 )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypercholesterolemia, %</td>
<td>64.8</td>
<td>70.2</td>
<td>( p = 0.13 )</td>
<td>-</td>
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</tr>
</tbody>
</table>

STEMI: myocardial infarction with ST elevation.

**Table 1. Baseline characteristics of patients with STEMI, patients with heart failure, and healthy control subjects**

Between 24-48 hours after the onset of symptoms, samples were collected from patients with heart failure, peripheral blood was drawn once during hospitalization. From healthy subjects, samples were collected once from 8-12 AM. Blood was collected in 5-mL containers and no more than 15 min later it was centrifuged at 2,500 g for 20 min. Serum was stored at -80°C. Samples were analyzed for RIPK3 using an ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions. RIPK3 levels were not used to render clinical decisions about patients. Levels of troponin I in serum were measured with Immulite 2000 (Siemens, Erlangen, Germany).
in 53 patients with STEMI on Day 0 following admission to the intensive care unit. All patients underwent PCI and a blood sample was collected on Day 1 to measure levels of RIPK3 and troponin I (Table 2). Serum levels of RIPK3 on Day 0 did not differ significantly from those in patients with NYHA class III-IV heart failure. However, serum levels of RIPK3 on Day 0 (mean 0.264 ng/mL ± 0.5) and Day 1 (mean 0.660 ng/mL ± 1.3) did differ significantly (Figure 1). Troponin I was more sensitive than RIPK3 at detecting early biochemical evidence of cardiac myocyte death. The ROC curve was plotted for highest levels of troponin I and RIPK3 and the LVEF prior to discharge. Neither serum levels of RIPK3 nor troponin I were sufficient to differentiate patients with a preserved LVEF from those with impaired left ventricular function after STEMI. Patients with STEMI who died (n = 5) or were hospitalized again (n = 4) during follow-up had RIPK3 or troponin I levels that were not significantly higher than those in surviving patients with STEMI.

The current study divided patients with STEMI (n = 53) into two groups - one with increased troponin I levels (n = 25) and another with normal troponin I levels (n = 28) prior to PCI. In patients with normal levels of troponin I prior to PCI, serum levels of RIPK3 and troponin I after PCI were sufficient to differentiate patients with a preserved LVEF and those with impaired left ventricular function after PCI (AUC = 0.780 (95% CI: 0.565-0.995, p = 0.043) with a sensitivity of 76.9% and a specificity of 71.4% vs. AUC = 0.735 (95% CI: 0.530-0.941, p = 0.038) a sensitivity of 88.2% and a specificity of 63.6% at the optimal cutoff values, respectively) (Figure 2). Upon discharge, serum levels of RIPK3 (mean 0.860 ng/mL ± 1.6) and troponin I (mean 139.1 ng/mL ± 55.8) in the patients with an LVEF < 50% were significantly higher than those in patients with a preserved LVEF (Figure 3). Simple logistic regression analysis showed that elevated levels of troponin I after PCI were associated with an increased risk of an LVEF < 50% prior to discharge (odds ratio, 1.014; 95% CI, 1.001 to 1.027; p = 0.03), while elevated levels of RIPK3 were not associated with such a risk. There was a weak correlation between serum levels of troponin I and RIPK3, but that correlation was not significant.

4. Discussion

Cell death plays a critical role in the pathogenesis of the major syndromes that affect the heart: heart failure and myocardial infarction. The magnitude and time of cell death in these syndromes differ significantly. Patients with heart failure exhibit ongoing myocyte death over months, but patients with myocardial infarction have a spike in cell death for several hours only (14).

RIPK3 is a novel regulator of programmed cell death. The goal of the current study was to examine the serum levels of RIPK3 expression in patients with NYHA class III-IV heart failure and patients with STEMI in comparison to levels in healthy control subjects. There were no significant differences in the serum levels of RIPK3 in patients with NYHA class III-IV heart failure and patients with STEMI up to 12 hours after the onset of symptoms in comparison to levels in healthy controls. However, RIPK3 and troponin I were
found to have a potential role in differentiating patients with preserved left ventricular function from patients with impaired left ventricular function after STEMI among patients with normal serum levels of troponin I prior to PCI.

Programmed necrosis mediated by RIPK3 has recently been defined as a novel mechanism of cell death with major functional importance in several organs, including the heart (15). Cell culture experiments in human cell lines have shown that an association between RIPK1 and RIPK3 in response to TNF-α stimulation represents the crucial initial step in programmed necrosis (5). Although the exact mechanisms of necroptosis remain unknown, necroptosis is believed to lead to rapid plasma membrane permeabilization, release of cell contents, and exposure of damage-associated molecular pattern molecules (16). This provokes a strong inflammatory response, resulting in impaired left ventricular function (17). RIPK3 is also responsible for generation of reactive oxygen species, which may be another mechanism for organ damage due to myocardial ischemia (5).

In a study using an in vivo model of myocardial infarction 24 hours after permanent ligation of the left anterior descending coronary artery, overexpression of RIPK3 was detected via an immunoblot test in mouse hearts (10). In that study, RIPK3-deficient mice had a significantly better ejection fraction and less hypertrophy in magnetic resonance imaging (MRI) studies 30 days after experimental infarction in comparison to wild-type mice. Moreover, the hearts of RIPK3-deficient mice were found to have lower levels of B-type natriuretic peptide (BNP) and those mice were found to have lower serum levels of troponin T (10). Another in vivo study found that pharmacological inhibition of necroptosis reduces infarct size 24 hours after induction of ischemia (18).

The widespread use of PCI has hampered the diagnosis of myocardial necrosis and infarction. Given these circumstances, whether a biomarker alone is sufficient to define myocardial infarction without angiographic evidence of ischemia is still uncertain (19). After PCI, levels of troponin are often elevated without clinical symptoms. Several mechanisms, such as distal embolization of plaques disrupted by a stent or balloon, platelet-rich microthrombi, and vasospasms, have been proposed as an explanation (20). Restoring blood flow can paradoxically induce cardiac injury, but PCI is nonetheless the most effective strategy for improving clinical outcomes. Recently, important differences in the underlying mechanism of myocardial ischemia and reperfusion injury after PCI have been noted (21). The current results suggest that RIPK3 is at least partially involved in this post-procedure injury. This is consistent with previous studies that reported that a deficiency in RIPK3 provides profound protection against cardiac injury induced by reperfusion (22). Several studies in patients with normal levels of troponin prior to PCI have found that an increase in troponin levels after PCI is associated with myocardial necrosis according to MRI and a poor outcome (23-25). The current results suggest that in patients with normal levels of troponin prior to PCI, serum levels of RIPK3 and troponin I after PCI can differentiate patients with preserved left ventricular function and impaired left ventricular function (AUC = 0.780 (95% CI: 0.565-0.995, p = 0.043) with a sensitivity of 76.9% and a specificity of 71.4% vs. AUC = 0.735 (95% CI: 0.530-0.941, p = 0.038) with a sensitivity of 88.2% and a specificity of 63.6% at the optimal cutoff values, respectively) (Figure 2). RIPK3 is not specific to the heart, so other reasons besides myocardial infarction for elevated levels of RIPK3 on Day 1 cannot be ruled out. Only a few studies of human pathology have examined levels of RIPK3 expression and their association with clinical outcomes (26,27). The current results suggest that the serum level of RIPK3 increased significantly 24 hours after the onset of symptoms. Unfortunately, the current results provide no direct evidence that necroptosis is the dominant form of cell death in myocardial damage after PCI. Troponin I is a more specific and sensitive marker than RIPK3 in detecting the level of cardiomyocyte death, but whether existing biomarkers alone and to what extent they are sufficient enough to define PCI-related myocardial injury without the presence of angiographic ischemia is uncertain (24). Significantly elevated serum levels of RIPK3 on Day 1 (after PCI) may indicate myocardial injury after PCI. Therefore, RIPK3 may have the potential to be specific marker for myocardial injury after PCI. However, larger studies are necessary to determine whether RIPK3 can serve as a specific marker of necroptosis and to define its role as a biomarker in patients with myocardial infarction.

A study has indicated that apoptosis is a key factor in the pathogenesis of heart failure (28). The prevalence of necrosis in heart failure has not been studied intensively. Several mice models suggested that necrosis plays a role in the progression of heart failure (29,30). A limitation of the current study, besides its relative small sample, is that most patients had serum levels of RIPK3 close to the minimum detection limits of the assay used. The expected difference in the serum levels of RIPK3 in patients with heart failure and healthy controls was in a very low range that the assay was able to detect. This means that the sensitivity issue is unresolved (31).

In summary, the current results suggest that RIPK3 is significantly elevated in patients with STEMI after PCI, implying that RIPK3 may be involved in post-procedure injury. In patients with normal troponin I levels prior to PCI, elevated levels of RIPK3 and troponin I can serve as a potential marker with which to identify patients with a decreased LVEF prior to discharge. This may allow an early shift to more intensive therapy to improve clinical outcomes.