

Characterization of the psycho-neuro-immunological state in patients with coronary artery disease

Ph.D. Thesis (Summary)

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LIST OF PAPERS RELATED TO THE SUBJECT OF THE THESIS

- I. Margit Keresztes, Tamás Horváth, Imre Ocsovszki, Imre Földesi, Gyöngyi Serfőző, Krisztina Boda, Imre Ungi
ACTH- and cortisol-associated neutrophil modulation in coronary artery disease patients undergoing stent implantation
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- II. Gyöngyi Serfőző, Tamás Horváth, Imre Földesi, Beatrix Rafael, Tamás Forster, Imre Ungi, Roland von Känel, Margit Keresztes
The monocyte-to-lymphocyte ratio correlates with psycho-neuro-inflammatory factors in patients with stable coronary artery disease
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INTRODUCTION

1. Coronary artery disease

It is widely known, that coronary artery disease (CAD) is among the three leading causes of death in most countries worldwide. Although its mortality rate reduced since the latter part of the 20th century, it is expected to remain the number 2 killer in 2030 globally, closely after cancer. Basically, CAD occurs as a consequence of atherosclerosis of the coronary arteries, which leads to limited blood flow and results in an imbalance between myocardial oxygen demand and supply and results in angina pectoris symptoms. Beside its well-known traditional risk factors, the recognition of novel, psychological risk factors, like psychosocial stress and depression, is also emerging in CAD.

2. The process of atherosclerosis – main points

Atherosclerosis is a chronic inflammatory disease of the arteries. Initial steps in its pathogenesis start at young age and it develops silently over decades before it leads to serious cardio- and cerebrovascular consequences.

On the very onset of this disease, endothelial dysfunction develops due to certain atherogenic factors; then lipoprotein particles and smaller plasma molecules get through the endothelium into the subendothelial space, accumulate, undergo an oxidative modification and become proinflammatory. Specifically, oxidized LDL is the most significant proatherogenic contributor to the initiation of atherosclerosis.

The endothelium becomes activated, upregulates the expression of adhesion receptors and recruits monocytes, T-cells and other blood-borne cells to the atherosclerotic lesion. Within intima, monocytes differentiate into macrophages and phagocytose the proatherogenic lipoproteins via scavenger receptors and become fully lipid-loaded foam cells. Overloading and mass apoptosis of macrophages contribute to the formation of a soft lipid-rich core in the lesion called the fatty streak, which is still asymptomatic, although activation and death of macrophages result in the release of a great variety of inflammatory, chemotactic and growth factors.

Intimal smooth cells mediate a fibroproliferative response that leads to plaque formation and contributes to narrowing of the lumen, reduced blood flow and ischemia. In response to

this, remodelling of the artery takes place to maintain a nearly normal lumen and additional hypoxia-induced formation of collaterals will occur.

Death of the myofibroblasts results in a thin, fragile fibrous cap and leads to the development of a vulnerable plaque and eventually to plaque rupture that ends in the formation of atherothrombus.

3. Roles of neutrophils and monocytes in coronary artery disease

Activated neutrophils are involved both in the initiation and progression of atherosclerosis through their major contribution to oxidative stress and inflammation. Several studies showed that high neutrophil count or neutrophil-to-lymphocyte ratio is associated with coronary stenosis and may predict CAD and major adverse cardiac events such as myocardial infarction. Neutrophil activation is widely observed in unstable angina, however, the state of neutrophils in stable angina pectoris patients appears to be controversial and requires more investigation.

Activated monocytes and macrophages have a central role in the process of atherosclerosis through foam cell formation and inflammatory factor production. Monocyte count may predict the severity of atherosclerotic stenosis and coronary plaque progression in acute coronary syndromes; furthermore, of all white blood cell types, it showed the strongest positive and independent relationship with CAD risk in asymptomatic adults. A recent paper found that low lymphocyte-to-monocyte ratio – i.e. an elevated monocyte-to-lymphocyte ratio (MLR) – was associated with CAD and prior myocardial infarction. However, the clinical importance of the monocyte-to-lymphocyte ratio in atherosclerosis, especially in CAD, is still largely unexplored.

4. Psychosocial stress and coronary artery disease (psycho-neuro-immunology)

Psychosocial stress (e.g. social isolation, workplace stress or childhood abuse) and depression are becoming increasingly recognized as major risk factors for incident coronary artery disease and as independent predictors of poor cardiac prognosis. Psycho-neuro-immunology (PNI) is a relatively new field of research investigating the interactions between stress (psychological state), the neuroendocrine and the immune system and health outcomes.

When facing with stressors, organisms respond with the ‘general adaptation syndrome’, which is governed primarily by the sympathetic-adrenomedullary system (resulting

catecholamine release) and the hypothalamic-pituitary-adrenocortical /HPA/ axis (resulting corticotropin-releasing hormone /CRH/, adrenocorticotropic hormone /ACTH/ and cortisol release). As chromogranin A (CgA) is co-stored and co-released with catecholamines in adrenal medulla, therefore, its elevated level is also a reliable indicator of general neuroendocrine overactivity including elevated sympathoadrenal activity.

Majority of the immune and inflammatory cells/leukocytes (e.g. neutrophils and monocytes) have stress hormone receptors, therefore, psychological stress could affect their activation. Several stress hormones exert immunomodulatory effects that can result in cytokine production that may act back on the hypothalamus and augment the activity of the HPA axis even more. Among these cytokines, especially interleukin-6 (IL-6) was found to be a highly potent stimulator of HPA axis. Exploring the details and associations of this network is a major topic of psycho-neuro-immunology.

5. Blood plasma inflammatory factors in CAD

Cytokines are small proteins that have a central importance in the PNI system. Proinflammatory cytokines (especially IL-1, IL-6 and TNF- α) take part not only in the process of atherosclerosis and CAD, but also in inducing stress and depression. IL-6 is a main regulator of the systemic inflammatory response and it is a potent inducer of the acute phase reaction including C-reactive protein (CRP) synthesis. Both IL-6 and CRP are major biomarkers of vascular inflammation in CAD and both were found to be elevated in psychosocial stress and depression. Neutrophils produce several kinds of cytokines (e.g. in response to stenting), and on the other hand, they are affected by several cytokines as various cytokine/chemokine receptors are present in their plasma membrane.

Lactoferrin, a specific activation marker of the neutrophil cell surface, can gain access to the blood and can be regarded as a soluble inflammatory marker as well; its appearance in plasma reflects neutrophil activation/degranulation, which results in the release of proinflammatory cytokines in addition to oxygen radicals (among others).

LL-37, the only member of the human antimicrobial cathelicidin family, participates in inflammation and atherosclerosis. It can be expressed by several cells and was found that it could be produced also by macrophages in atherosclerotic lesions. LL-37 provides a chemotactic effect: stimulates inflammatory cell recruitment to the atherosclerotic plaque and induces angiogenesis via endothel cell activation.

AIMS

From the aspect of psycho-neuroendocrine background, our knowledge is still incomplete on the activation state of neutrophils in CAD. Since neutrophils have a fundamental role in coronary atherosclerosis, it is considerably important to reveal the possible stress-related regulation of their activation in CAD patients before and after PCI (percutaneous coronary intervention) i.e. stenting. While participation of monocytes/macrophages in atherosclerosis is well-known, associations of the monocyte-to-lymphocyte ratio with the psycho-neuro-immune network are largely unexplored in CAD.

Therefore, in our studies, we were particularly interested in the PNI characteristics of our CAD patients and in the potential associations among stress markers, depressive symptoms and inflammatory markers with a special focus on neutrophil activation state and the monocyte-to-lymphocyte ratio.

More precisely, we set out to the following investigations:

Study I.: in stable angina pectoris (SAP) and acute coronary syndrome (ACS) patients in connection with stenting (PCI) (before, directly after and the next day of PCI)

- assessment of the psycho-neuroendocrino-immune state (assayed stress hormones: ACTH and cortisol and a general inflammatory marker: IL-6; all tested in plasma)
- analysis of the activation state of granulocytes (analysed cell surface markers: L-selectin, CD15 and neutrophil-specific lactoferrin; plasma lactoferrin)
- examination of the possible associations between granulocyte (neutrophil) activation markers and stress markers (plasma ACTH and cortisol)

Study II.: in SAP patients: cross-sectional investigations

- analysis of the possible associations of the MLR with neuroendocrine markers (cortisol and CgA), inflammatory parameters (IL-6, CRP, LL-37) and psychosocial factors (depressive symptoms and stress-coping scores)
- investigations on further associations of the PNI network: among psychological factors, inflammatory parameters and neuroendocrine markers

MATERIALS AND METHODS

- Subjects/Patients: 21 stable angina pectoris (SAP) and 20 acute coronary syndrome (ACS) patients participated in study I. and 23 patients with stable angina pectoris took part in study II.
- Fasting blood samples were collected from patients before, immediately after and on the following day of PCI (percutaneous coronary intervention, i.e. stenting) in study I., one day before PCI in study II.
- Routine blood parameters: blood cell counts, total cholesterol, triglycerides, HDL-cholesterol and CRP (in study II. only) were determined in automatic analyzers, LDL-cholesterol and the MLR (in study II.) were calculated.
- For special plasma assays, samples were collected into cooled EDTA-Vacutainer tubes and stored in aliquots in -80°C. Tests for special plasma parameters included measurements of neuroendocrine markers: ACTH (only in study I.), cortisol and chromogranin A (only in study II.); inflammatory markers: lactoferrin (only in study I.), LL-37 (only in study II.) and IL-6. ACTH was measured by a chemiluminescent assay, cortisol and CgA were analysed with radioimmunoassay kits, lactoferrin was assayed using an 'in-house' ELISA kit, LL-37 and IL-6 were measured using commercial ELISA kits.
- Surface granulocyte activation markers (study I.): L-selectin, CD15 and lactoferrin were determined by flow cytometry with an indirect immunofluorescent method using a FACStar Plus Becton Dickinson equipment. Granulocytes were separated according to their typically high side and forward scatter characteristics. Granulocytes with lactoferrin expressed on their surface were identified as activated neutrophils. Ratios of labelled granulocytes (% of granulocytes bearing labelled markers on cell surface) and mean fluorescence intensities (MFI, associated to mean quantity of labelled molecules/cells) were given from the analysis data.
- Psychosocial measures: the Hungarian version of the 21-item Beck Depression Inventory (BDI) was used to measure the severity of the depressive symptoms and Rahe's Brief Stress and Coping Inventory (BSCI) was employed to evaluate everyday stress level and coping capacity of the patients in study II. From BSCI coping subscales, the social support subscale measures the degree of a subject's social network; the meaning of life subscale shows to what extent participants feel their life 'worth living'.

- Statistical analysis: two-sample Student's t-test and Fisher's exact test were used to compare the general characteristics of the groups in study I. Two-way repeated measurements ANOVA was applied for over time comparisons (pre-PCI, post-PCI, and 1d-PCI samples), and Spearman's coefficient of correlation and its significance were calculated to assess the relationship between the stress hormones and selected inflammatory markers in study I. In study II., bivariate and multivariate Pearson correlation analyses with adjustments for age, sex and BMI were performed to examine the associations of the MLR with psychosocial factors, neuroendocrine and inflammatory markers; and similarly, to estimate the associations among psychosocial factors and neuroendocrine or inflammatory markers. CgA models were additionally adjusted for intake of proton pump inhibitors and NYHA grade (New York Heart Association functional classification: classifies the extent of heart failure), and models with BDI scores were additionally corrected for sedative use. Statistical analyses were performed using IBM-SPSS (version 15.0 and 20.0, SPSS Inc., Chicago, IL, USA) and SAS (v9.1, SAS institute, Cary, NC) softwares.

RESULTS AND DISCUSSION

1. Stress and inflammatory parameters in CAD patients undergoing stenting

The highly elevated plasma cortisol level of ACS patients indicated that they were in an extremely stressed state before PCI; the substantially reduced plasma cortisol values on the day after PCI reflects a more relaxed state. Plasma cortisol value of SAP patients remained mostly unreactive and did not change noticeably that may be due to a chronically overloaded, exhausted HPA axis. The final low ACTH value of the ACS patients suggests that the initially overactivated HPA axis returned to a much more decreased, normal functioning state after stenting. The moderately decreased 1d-PCI plasma ACTH concentration of SAP patients could indicate a relief after stenting.

Leukocytes constitutively express L-selectin in their plasma membrane. In case of leukocyte activation, it is being cleaved from the cell surface. However, its increased cell surface presence may also accompany cell activation, e.g. after a mechanical trauma. The ratio of L-selectin-bearing granulocytes in SAP patients increased slightly during PCI, then it decreased moderately; although these differences were not substantial, they were found to be statistically significant. These changes may reflect homeostatic alterations in these activated granulocytes (shedding balanced by reappearance).

CD15 (Lewis-X) is a tetrasaccharide ligand to selectins on the surface of all myeloid cells; neutrophils can mobilize it from their azurophilic/primary granules upon stimulation. The ratio of CD15-carrying cells increased slightly directly after PCI (post-PCI samples of both groups). This statistically significant, though not considerable elevation may reflect modest late granulocyte activation (affecting primary granules) during stenting, independently of group. The slight decrease on the following day suggests a less activated state.

Lactoferrin is a specific activation marker of neutrophils: following cell activation and release from secondary granules, it can bound to cell surface and gain access to the circulation. Similarly to the changes in the ratio of CD15-bearing cells, the proportion of lactoferrin-carrying neutrophils increased mildly directly after PCI in both groups. In parallel, plasma lactoferrin elevated slightly in both types of post-PCI samples, which indicates slight neutrophil activation. Our pre-PCI values of surface lactoferrin-carrying neutrophils were also considerably high (>25%), showing an elevated ratio of activated neutrophils in CAD. One day after the intervention, marked reductions in cell surface (percentage of carrier cells, MFI) and

plasma lactoferrin values were observed in both patient groups that indicates a less activated state of neutrophils.

Since both plasma lactoferrin and the percentage of surface lactoferrin-bearing cells changed markedly and in a similar way in our patient groups, both might be used as ‘neutrophil activity sensors’ in CAD patients. However, from a practical point of view, determining lactoferrin from plasma samples seems to be more suitable. (Blood plasma tests are less expensive, less complicated and they could be probably automated.)

In both patient groups, plasma IL-6 increased during the intervention and enhanced especially on the following day of PCI, when activation level of neutrophils was already reduced; this could be a consequence of the onset of acute phase reaction. Circulating level of IL-6 may also have a strong link with psychological stress.

2. ACTH- and cortisol-associated neutrophil modulation in CAD patients undergoing stenting

We found that plasma ACTH and/or cortisol could be associated with the activation state of neutrophils in CAD.

We showed inverse correlations between ACTH and surface lactoferrin-bearing granulocytes and plasma lactoferrin the following day of PCI in both groups. The supposition that ACTH could be capable to suppress neutrophil activation/degranulation is consistent with the concept of tonal inhibition of immune/inflammatory responsiveness by ACTH.

Cortisol and its analogues are well-known anti-inflammatory agents and immune suppressors, therefore, correlation of cortisol with plasma lactoferrin before stenting in ACS appears to be controversial. It could probably reflect an association of cortisol with neutrophil activation in extremely stressed CAD patients as glucocorticoids may be capable of activating human neutrophils owing to the high ratio of α to β type glucocorticoid receptors on them. Cortisol may be proposed as a proatherogenic stress factor, as morning plasma cortisol levels were found to be associated with coronary atherosclerosis severity (e.g. in US Air Force aircrew members).

3. The monocyte-to-lymphocyte ratio: a PNI correlate in stable CAD

An elevated monocyte fraction and MLR reflects an inflammatory state in our stable CAD patients; this is supported by the correlations found between the MLR and plasma LL-37, IL-6 and CRP. Interestingly, the reciprocal of the MLR value ($1/0.32 = 3.125$) of our patients

is almost equal to the critical lymphocyte-to-monocyte ratio of 3.1, characteristic for patients with a high risk of severe (atherosclerotic) limb ischemia, which is often accompanied by CAD.

The elevated circulating levels of chromogranin A (CgA) may suggest a general neuroendocrine overactivity with increased sympathoadrenal activity. It should be noted, that we controlled our CgA models for the effect of proton pump inhibitors and myocardial release of CgA, as these factors could also lead to the elevation of CgA levels in plasma. Recently, a study showed that elevated sympathetic activity may be directly related to monocytosis through the enhanced release of hematopoietic cells from the bone marrow that was followed by increased monocytopoiesis in the spleen. Monocyte release into blood stream could lead to the progression of atherosclerosis via promoting inflammation especially following myocardial infarction. Accordingly, increased plasma CgA could reflect sympathetic activation that can lead to monocytosis in blood. This may be in the background of the highly significant correlation between CgA and the MLR. Furthermore, activated monocytes and macrophages are also capable to produce chromogranins.

About one-third of the participants had clinically relevant levels of depressive symptoms and the average global stress-coping score was in the 'sufficient' range only, suggesting limited coping skills under stress amongst our patients. Patients with more severe depressive symptoms had higher plasma CgA levels, this could be explained by the increased activity of the neuroendocrino-sympathoadrenal system that accompanies depressive symptomatology. Previous studies found that both depressed and chronically stressed subjects show sympathoadrenal hyperactivity that may lead to monocyte/macrophage activation via β -adrenergic receptors in CAD. Elevated CgA associated with low social support as well, suggesting that limited emotional/social support may lead to increased psychosocial stress and to elevated sympathoadrenal activity.

Proinflammatory cytokines (particularly IL-1) released by activated monocytes/macrophages may be involved in the background of the association between the MLR and depressive symptoms. These cytokines could contribute to the development of depressive mood via interfering with neurotransmitter functioning. Correlations of the MLR with IL-6 and CRP probably reflect the activated state of monocytes and their secretion of IL-6, the most potent inducer of CRP synthesis and activator of acute phase response in general. IL-6 and CRP are both major proinflammatory markers that are proposed as links between stress/depression and CAD. Depressed CAD patients could exhibit considerably higher plasma

CRP and lower cortisol levels compared to CAD patients without depression, according to the literature. Similarly, considerably high CRP and relatively low plasma cortisol levels were found in our patients that could be a result of long-term negative feedback in chronic stress.

The association observed between the MLR and LL-37 is not surprising, as monocytes and derived macrophages are known to release LL-37 in atherosclerotic lesions. Interestingly, LL-37 correlated significantly with depressive symptoms in our CAD patients; this supports the concept that a possible connection could exist between inflammation and depression.

CONCLUSION

To conclude, a simplified picture of a vicious PNI network may appear: psychological tension or low psychosocial support could lead to raised HPA activity and predominant sympathetic activity, which then may facilitate neutrophil activation and could also lead to the elevation of the monocyte count and activity. Proinflammatory cytokines and other factors secreted by these activated neutrophils and monocytes could facilitate the development of CAD and/or depression; with the progression of CAD, the activity of the mentioned PNI network may be further elevated. Our findings appear to underline the importance of stress management programs and psychosocial interventions, which are capable to reduce chronic stress, to boost psychosocial support and stress-coping skills and to alleviate depressive symptoms. These programs/interventions could favourably influence the vicious PNI cycle of interacting psychosocial factors, neuroendocrine and inflammatory factors, and thereby, they could effectively improve both the mental and physical health of the CAD patients.

We suggest that while plasma lactoferrin could be employed as a ‘neutrophil activity sensor’ in CAD, the MLR – the other easily available and inexpensive laboratory parameter tested by us – could be used as a PNI correlate marker in the routine clinical monitoring of stable CAD patients.

SUMMARY OF NEW FINDINGS

1. We found that in stable and acute CAD patients, both pre- and post-PCI states could be associated with increased ratio of activated/degranulated neutrophils shown by the elevated lactoferrin parameters (proportion of lactoferrin-carrying neutrophils and plasma lactoferrin), which declined well below the initial values the next day of PCI indicating a substantial decrease in neutrophil activation.
2. We revealed correlations between plasma ACTH, cortisol and neutrophil activation/degranulation state in CAD patients undergoing stenting. We found that the 1d-PCI lactoferrin values (percentage of lactoferrin-carrying neutrophils and plasma lactoferrin) correlated inversely with plasma ACTH in both stable and acute CAD groups, that suggests a negative association between neutrophil activation and plasma ACTH the day after the intervention. Additionally, we found a correlation of cortisol with plasma lactoferrin in patients with acute coronary syndrome before stenting that appears to reflect a positive association of plasma cortisol with neutrophil activation state in extremely stressed acute CAD patients before the intervention.
3. We showed that in stable CAD patients, increased MLR could be associated with the severity of the depressive symptoms, with increased neuroendocrino-sympathetic activity (marked by elevated plasma chromogranin A) and with elevated plasma levels of the tested inflammatory factors: LL-37, IL-6 and CRP that are thought to be involved in the initiation and progression of atherosclerosis.
4. Additionally, we found correlations between increased neuroendocrino-sympathetic activity and low social support and high-score depressive symptomology that could reflect the association of elevated sympathoadrenal activity with limited psychosocial support and with depressive symptom severity in stable CAD.

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