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Soluble CD40 ligand, interleukin (IL)-6, and hemostatic parameters in metabolic syndrome patients with and without overt ischemic heart disease

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Abstract *Background and aim:* Soluble CD40 ligand (sCD40L, also known as CD154) is a marker for platelet activation which could increase coagulation and inflammation. In this case-control study, we aimed to assess the levels of plasma sCD40L, IL-6, and some hemostatic parameters in patients with metabolic syndrome (MetS) whether or not associated with overt ischemic heart disease (IHD).

Subjects and methods: We measured plasma sCD40L (an index of platelet activation), interleukin (IL)-6 (a proinflammatory cytokine), and some hemostatic parameters (tissue factor [TF], thrombin–antithrombin [TAT] and D-dimer) in 47 patients with metabolic syndrome (21 with and 26 without overt IHD) versus 25 comparable healthy control subjects.

Results: Significantly higher levels of sCD40L, IL-6, and thrombotic markers (TF, D-dimer and TAT) were found in patients with metabolic syndrome compared to healthy controls. The levels of IL-6 and sCD40 were highest in patients with overt IHD. Strong positive correlations existed

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between sCD40L and IL-6 ($r = 0.67$, $p = 0.003$), TF ($r = 0.59$, $p = 0.008$), and platelets count ($r = 0.64$, $p = 0.005$).

Conclusion: Higher levels of sCD40L, IL-6, and thrombotic markers exist in MetS patients, particularly those with IHD. The strong positive correlations between sCD40L and IL-6, TF, and platelets count support a link between the CD40–CD40L system and the underlying inflammatory and hypercoagulable state in MetS patients.

1. Introduction

CD40L (L stands for ligand), a trimetric transmembrane protein of the tumor necrosis family also known as CD154, was originally identified on the cells of the immune system.[1] It binds to CD40 on cells such as endothelial cells, monocytes, and dendritic cells.[2] CD40L is also stored in platelets and translocated to the platelet surface upon stimulation within seconds after activation in vitro and in vivo.[3] Surface-expressed CD40L is then cleaved and/or released from the platelet over a period of minutes to hours, subsequently generating a soluble fragment, soluble CD40L (sCD40L).[4] sCD40L can promote inflammatory or thrombotic response by causing further platelet activation.[5] This can occur through binding to platelet CD40 or to the integrin.[6,7] More than 95% of the circulating CD40L exists in platelets, and the role of platelets was already explained as inflammatory cells.[8,9]

First, platelets release a wide range of inflammatory mediators from the intracellular stores such as several chemokines. Second, platelets not only express and release inflammatory mediators but upon activation may also induce the expression of such substances in other cells (e.g., monocytes/macrophages and granulocytes). Finally, platelets may themselves respond to inflammatory mediators. In fact, platelets recently have been found to express several chemokine receptors that upon stimulation enhance platelet activation. Hence, platelets may not only promote thrombus formation during activation but also seem to release and express inflammatory mediators. Brandt et al.[10] concluded from their study that the generation of inflammatory signals by platelets may occur following acute mechanical damage of the endothelium or infection of the vascular system. Platelet-associated CD40L – on exposure to CD40-expressing vascular cells (including endothelial cells) – induces the expression of adhesion molecules, the release of inflammatory cytokines, e.g., interleukin (IL)-6, and the procoagulant tissue factor (TF).[11] Surface-expressed CD40L is subsequently cleaved to generate the soluble fragment sCD40L. This soluble fragment retains much of its biological activities by virtue of binding to glycoprotein IIb/IIIa and the induction of signaling reactions when bound to the receptors.[12] Like soluble P-selectin (sP-sel), circulating sCD40L is believed to derive predominantly from the activated platelets and hence may reflect platelet activation.[13] Therefore, these data suggest a link between platelet activation, thrombogenesis, and the inflammatory state in metabolic syndrome (MetS).[14]

MetS has been identified as a significant and multifaceted risk factor for cardiovascular disease (CVD) by the United States National Cholesterol Education Program Adult Treatment Panel (ATP)-III report.[15] MetS includes the clustering of abdominal obesity, insulin resistance, microalbuminuria, dyslipidemia, and elevated blood pressure and is associated with other abnormalities including the prothrombotic and proinflammatory states, non-alcoholic fatty liver disease, and reproductive disorders.[16] MetS doubles coronary heart disease mortality, after adjustment for age, sex, cholesterol level, physical activity, and smoking.[17] In Ischemic Heart Disease Risk Factor Study, men with MetS had a threefold increase in coronary death, after adjusting for age, LDL cholesterol, smoking, and family history.[18] MetS is associated with an approximate doubling of the cardiovascular disease risk and a fivefold increased risk for incident type 2 diabetes mellitus. Although it is unclear whether there is a unifying pathophysiological mechanism resulting in the MetS, abdominal adiposity and insulin resistance appear to be central to this syndrome. Life style modification and weight loss should therefore be the cornerstone for treating or preventing MetS and its components.[19] In addition, there is a general consensus that the other cardiac risk factors should be aggressively managed in individuals with MetS. Finally, MetS is an evolving concept that continues to be data-driven and evidence-based with revisions forthcoming.[20] The aim of the present study was therefore designed to assess the behavior of plasma sCD40L levels in patients with MetS. Furthermore we sought to determine if a relationship could exist between sCD40L and hemostatic abnormalities in MetS patients.

2. Subjects and methods

Blood samples were obtained from 25 healthy volunteers (controls) not taking any antiplatelet medications within 7 days before sampling; and 47 MetS patients who were admitted to our hospital (26 without and 21 with overt ischemic heart disease [IHD]). Informed consent was obtained from all subjects.

None of the subjects had any clinical evidence of the peripheral vascular disease or inflammatory conditions. Potential patients with infection, tumors, diabetes, hypertension, liver or kidney disease were also excluded. All patients with overt IHD were those with unstable angina who had experienced ischemic chest pain at rest within the preceding 48 h but had no evidence of myocardial necrosis by enzymatic criteria.Both patients and controls were subjected to:

1. Thorough history and clinical examination
2. Routine laboratory investigations: Complete blood count, fasting lipogram (total cholesterol, HDL-C, LDL-C, TG), fasting serum glucose level, Troponin T, and HbA1c (measured by liquid chromatography [BioRad]).[21]
3. Specific investigations included

Table 1 Comparison of means of cytokine levels and hemostatic variables in the studied groups.

	Controls (n = 25)	MS with IHD (n = 26)	MS without IHD (n = 21)	p-value
sCD40L (pg/ml)	76.7 ± 38.1	501.8 ± 181.7	807.2 ± 264.5	0.001*
IL-6 (pg/ml)	246.2 ± 131.7	683.3 ± 134.6	690.8 ± 138.7	0.001*
Tissue factor (pg/ml)	392.7 ± 111.6	645.3 ± 242.7	1405.6 ± 264.5	0.001*
TAT (ng/ml)	11.9 ± 4.8	22.8 ± 7.3	26.4 ± 9.6	0.055
D-Dimer (ng/ml)	188.2 ± 62.7	316.7 ± 89.6	694.0 ± 271.9	0.001*

* Significant at $p < 0.01$.

(a) Enzyme immunoassay (EIA) for sCD40L: Concentrations of sCD40L were determined using a commercially available enzyme immunoassay (Bender Med Systems GmBH, Austria) with a detection limit of 95 pg/ml (0.095 ng/mL). The following antibodies were used: mouse-anti-human-CD40, mouse-anti-human- CD40L, and mouse IgG-PE-conjugated.[22]

(b) ELISA (Enzyme-Linked Immuno Sorbent Assay) for IL-6: ELISA kits (AviBion Human IL-6, ELISA, V-antaa, Finland) containing the micro titration plate with adsorbed conjugate of the mouse monoclonal antibody against determined antigens and nonspecific horseradish peroxidase were used. Every plate contained a doublet of 7 standards for the construction of the calibration curve and blank. The pH of the washing phosphate-buffered saline solution with 1% tween 20 was adjusted to 7.4. A tetramethyl benzidine (TMB) solution served as the substrate for the redox reaction. Activity of the enzyme was stopped by 1 M phosphoric acid and absorbance was measured at 4-50 nm filter. The detection limit was < 500 pg/ml.[23]

(c) Hemostatic parameters: Automated analysis of thrombin–antithrombin (TAT) complexes levels took place in the coagulation analyzer (Dad Behring, Marburg GmbH agnostica Stago, Inc.) which implements an antibody that reacts with the modified antithrombin (AT)-III found in the AT-III/protease complexes. The major component of the AT-III protease complexes is the combination of AT-III with thrombin. The reference range for this assay is < 20 ng/ml.

(d) Dimer and tissue factor (TF) MeasurementPlasma D-dimer levels were measured by the Mini-VIDAS ELISA method (bioMerieux-France).

2.1 *Statistical analyses*

Data analyses were performed with SPSS for Windows version 13 (SPSS Inc., Chicago, IL). Results are expressed as the mean ± standard deviation of the mean for continuous data. Comparison of the means of different continuous variables in the three studied groups was performed with one-way ANOVA test. A two-sided significance level of 0.05 was used for all analyses.

3. Results

As shown in Table 1, the mean values of sCD40L, IL-6, TF, TAT, and D-dimer showed a significant trend to increases as we move from "*control*" to "*MS with IHD*" to "*MS without IHD*". Besides, the five variables showed a significant difference regarding their levels in controls when compared to the MS patients. In other words, the mean value in MS patients (whether or not having IHD) was significantly higher compared to controls. Besides, the mean value of these variables within the MS patients was significantly higher in the IHD patients when compared to those without IHD (apart from IL-6 and TAT, where the difference was not statistically significant).

Regarding the correlations, we found a strong positive correlation between sCD40L and IL-6 ($r = 0.67$, $p = 0.003$), TF ($r = 0.59$, $p = 0.008$), and platelets count ($r = 0.64$, $p = 0.005$).

4. Discussion

sCD40L is an important link between inflammation, atherosclerosis and thrombosis. Consistent with the previous reports, these results showed higher levels of sCD40L in patients with MetS compared with controls. This is explained by Garlichs et al.[24] who reported that platelets express the transmembrane signaling protein CD40L within seconds of activation. After activation with thrombin, adenosine diphosphate (ADP), or collagen; a soluble form of CD40L is also released. Entmann et al.[25] demonstrated that CD40L binds to platelet CD40 leading to further cleavage of the membrane-bound CD40L and the release of soluble CD40L. The present study shows that MetS patients with unstable angina had significantly higher levels of sCD40L and IL-6 compared with both controls and patients without IHD. Grewal et al.[26] believed that the increased serum levels of sCD40L in patients with unstable angina are not only a marker of immune activation, but may also be involved in the pathogenic processes in these patients. sCD40L in the circulation may pass through damaged atherosclerotic endothelium and come into direct contact with the cells inside the lesion. However, sCD40L may activate circulating leukocytes to enhance the release of proinflammatory cytokines,[27] increase the expression of adhesion molecules, and enhance the release of CC-chemokines (i.e., monocyte chemoattractant protein [MCP]-1) in mononuclear cells. Such an activation of the circulating leukocytes may be facilitated at the endothelium outside an atherosclerotic plaque with up-regulation of the adhesion molecules. These inflammatory responses mediated by sCD40L may further promote the infiltration of the activated leukocytes into the atherosclerotic lesion. This in turn may directly activate macrophages and T-cells inside the vascular wall.[28] Higher CD40L levels in patients with unstable angina may reflect important pathogenic aspects in these patients. The rupture of an atherosclerotic plaque, which again triggers thrombosis, is an important pathogenic event in the development of acute coronary

syndromes.[29] Second, we observed increased level of IL-6 in patients with MetS with and without IHD, being highest in patients with overt IHD. This is explained by Volpato et al.[30] who reported that CD40L stimulation increases the expression of adhesion molecules, chemokines, and inflammatory cytokines especially IL-6, IL-1 and IL-8 from the vascular endothelial cells leading to the recruitment and activation of leukocytes within the atherosclerotic plaque. Kazes et al.[31] reported that CD40 ligation on monocytes and dendritic cells results in: (1) an enhanced survival of these cells; (2) the secretion of cytokines (such as IL-6, IL-1, IL-8, IL-10, IL-12, TNF-α) and enzymes such as matrix metalloproteinase (MMP); (3) enhanced monocyte tumoricidal activity. Increased level of IL-6 in patients with IHD was explained by Loppnow et al.[32] who reported that MCP-1 regulates monocyte and macrophage migration and infiltration to the sites of active inflammation. MCP-1 further induces proinflammatory cytokines, chemokines, and MMPs. Then, the proinflammatory IL-6 promotes vascular smooth muscle cell proliferation at the inflamed plaques.

This study also demonstrated an increase in the hemostatic parameters in patients with MetS especially if associated with overt IHD, in line with a previous report.[33] Ridker et al.[34] hypothesized that CD40L over-expression may be a stimulus for clotting activation. This hypothesis is based on previous studies[35–38] demonstrating that in monocytes, endothelial cells, and smooth muscle cells CD40L over-expression facilitates atherosclerotic plaque rupture; exposing collagen and von Willebrand factor on the damaged endothelium to create an extensive surface for platelet adhesion. Adherent platelets subsequently aggregate, following the release of a variety of platelet intracellular signaling molecules including thromboxane A_2, ADP, serotonin, and epinephrine in addition to the systemically circulating thrombin. Additional platelets are recruited to the site of injury and adhesion where conformational changes in platelet cytoskeletal proteins result in up-regulation and expression of Gp IIb/IIIa on the platelet receptors surfaces.[36] The binding of Gp IIb/IIIa receptors to fibrinogen causes extensive cross-linking, which facilitates thrombus formation.[37]

In patients without overt IHD, sCD40L showed non-significant correlations with any of the hemostatic factors or IL-6; while in patients with IHD, we found strong positive correlations between sCD40L and IL-6 ($r = 0.67$, $p = 0.003$), TF ($r = 0.59$, $p = 0.008$), and platelets count ($r = 0.64$, $p = 0.005$); hence supporting a link between the CD40–CD40L system and the underlying inflammatory and hypercoagulable state in these patients. Also, the positive correlation between sCD40L and platelet count raises a question about the origin of this mediator. Major studies on the cellular distribution of CD40L indicate that >95% of the circulating sCD40L exists in platelets. In fact, activated platelets have been found to stimulate the adhesion molecule and MCP-1 production in endothelial cells through the enhanced sCD40L secretion and direct platelet–endothelium contact mediated by CD40L expression on the platelet surface.[38] In addition, the increase in sCD40L in patients with overt IHD expressing high levels of TF and IL-6 has been associated with more adverse cardiovascular outcomes and the development of coronary events compared to subjects without clinically established IHD.

5. Conclusion

High levels of sCD40L, IL-6, and thrombotic markers exist in patients with MetS syndrome compared to healthy individuals; particularly in MetS patients with IHD .Thus, the elevated plasma sCD40L levels in patients with MetS (especially those with overt IHD) can be reduced by the intensive application of a package of multifactorial cardiovascular risk intervention strategy.

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