ORIGINAL ARTICLE

Serum soluble CD40L concentration depending on the stage of multiple myeloma and its correlation with selected angiogenic cytokines

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KEY WORDS

ABSTRACT

INTRODUCTION   Little is known about the CD40L–CD40 pathway in hematologic malignancies, especially in multiple myeloma (MM).

OBJECTIVES   The aim of the current study was to evaluate serum soluble CD40 ligand (sCD40L) concentrations in patients with newly diagnosed MM prior to treatment at different stages of disease, compared with healthy controls. To assess the clinical significance of sCD40L, we assessed correlations between the levels of sCD40L and those of angiogenic cytokines: interleukin 6 (IL-6), soluble receptor of IL-6 (sIL-6R), tumor necrosis factor α (TNF-α), soluble vascular cell adhesion molecule 1 (sVCAM-1), and platelet-derived growth factor AB (PDGF-AB), as well as with well-established biomarkers of MM activity (lactate dehydrogenase activity and percentage of bone marrow plasma cells) and with a marker of platelet activation (β-thromboglobulin).

PATIENTS AND METHODS   The study group consisted of 41 patients with newly diagnosed MM; the control group consisted of 30 healthy subjects. The level of sCD40L was determined using an enzyme-linked immunosorbent assay.

RESULTS   The level of sCD40L was significantly higher in patients with MM than in controls and increased with the stage of the disease. Moreover, it significantly correlated with the levels of IL-6, sIL-6R, sVCAM-1, PDGF-AB, as well as the levels of MM activity markers and β-thromboglobulin.

CONCLUSIONS   Our findings indicate that increased serum sCD40L levels may be related to angiogenesis in patients with MM. This protein has potential clinical usefulness in MM and may be considered as an additional prognostic marker. The correlation of sCD40L with β-thromboglobulin may indicate that in patients with MM sCD40L derives from activated platelets.

INTRODUCTION   Multiple myeloma (MM) is a neoplastic disease characterized by the proliferation of a single clone of plasma cells derived from B cells in the bone marrow.[1] The diagnosis depends on the identification of abnormal monoclonal plasma cells in the bone marrow, M-protein in serum or urine, osteolytic lesions, and a clinical picture consistent with MM.[1,2] MM accounts for 1% to 2% of all malignancies and about 10% of hematologic malignancies.[3]

The CD40 ligand (CD40L), which is also known as CD154, gp39, or tumor necrosis factor (TNF)-related activation protein (TRAP), is a member of the TNF superfamily. CD40L is a type II transmembrane protein predominantly expressed on activated CD4+ T cells and platelets (over 95% of circulating CD40L is released from α granules after platelet activation).[4,5]

CD40L is a natural ligand for the CD40 receptor, which is type I protein presenting on the surface of B cells and on the membrane of the antigen-presenting cells, epithelial cells, smooth muscle cells, endothelial cells, blood platelets, and tumor cells including MM cells.[5,6] It should be

noted that CD40 is not expressed on normal matured plasma cells, but only on human MM cells.[7,8]

CD40L–CD40 interactions play a crucial role for the immune response regulation in inflammation, autoimmune diseases, and hemostasis.[9] CD40 ligation leads to the production of cytokines, chemokines, adhesion molecules, and metalloproteinases by the endothelial cells and fibroblasts. Both the proinflammatory and prothrombotic roles of CD40L–CD40 pathway were revealed in various diseases.[10]

It has been suggested that in human MM cells CD40 ligation induces the secretion of vascular endothelial growth factor (VEGF) by the p53 pathway, which stimulates interleukin 6 (IL-6) secretion in bone marrow stromal cells and thus increases MM cell growth.[9] Studies have indicated that inhibition of CD40L-depended production of VEGF and IL-6 via specific VEGF antibodies leads to the toxic effect on proliferation and survival of MM cells.[11] Interestingly, the CD40L–CD40 system activation induces the apoptosis of CD40-positive cancer cells but not the healthy cells.[10] Moreover, a varied expression of CD40 is associated with cancer stage and usually decreases with the progression of disease.[12,13] Therefore, monitoring alterations in sCD40L concentrations could provide useful information. Unfortunately, little is known about sCD40L concentrations and their biologic role in patients with hematologic malignancies, especially those with MM.

To the best of our knowledge, circulating levels of sCD40L in patients with MM have been evaluated so far only by Tsirakis et al[5] and Hock et al.[14] For that reason, the present study was designed to evaluate serum sCD40L concentrations in patients with newly diagnosed MM prior to treatment at different stages of disease, compared with healthy controls. In the next step, sCD40L was correlated with selected angiogenic cytokines (interleukin 6 [IL-6], soluble receptor of IL-6 [sIL-6R], tumor necrosis factor α [TNF-α], soluble vascular cell adhesion molecule 1 [sVCAM-1], and platelet-derived growth factor AB [PDGF-AB]) to establish whether this protein may be recognized as a biomarker of angiogenesis in patients with MM. To assess its clinical significance, sCD40L was also correlated with well-established biomarkers of MM activity: β_2-microglobulin, albumin concentrations, lactate dehydrogenase (LDH) activity, the percentage of plasma cells in the bone marrow , and also platelet count and β-thromboglobulin concentrations as markers of platelet activation. Analyzing serum circulating levels of sCD40L and their clinical significance in patients with MM undoubtedly provides more information regarding the role of sCD40L in this condition.

PATIENTS AND METHODS **Patients** The study group included 41 patients (18 women and 23 men; mean age, 68 years; range, 47–86 years) with newly diagnosed MM, prior to treatment.

Patients were diagnosed at the Department of Hematology of the Clinical Hospital of the Medical University of Bialystok, Białystok, Poland, according to the World Health Organization criteria, including an increased number of abnormal, atypical, or immature plasma cells in the bone marrow or histological evidence of plasmocytoma, the presence of the M protein in serum or urine, and bone lesions.[15] The whole study group was classified depending on the stage of the disease according to the Durie and Salmon staging system[16]: 34% patients (n = 14) were in stage I; 42% patients (n = 17), in stage II; and 24% patients (n = 10), in stage III. The median percentage of bone marrow plasma cells in the whole study group was 19.4% (median, 3.1% in patients with stage I; 15.7% in those with stage II; and 48.5% in those with stage III of MM). Serum protein electrophoresis revealed that the median monoclonal protein concentration in the whole study group was 3.79 g/dl (median, 3.01 g/dl in patients with stage I; 3.75 g/dl in those with stage II; and 7.95 g/dl in those with stage III of MM). The detection and typing of monoclonal antibodies or immunoglobulins by means of serum immunofixation demonstrated immunoglobulin G in 66% of all patients with MM, immunoglobulin A in 27%, and light chains (Bence-Jones protein) in 7%. The clinical characteristics of patients with MM are presented in TABLE 1.

The control group consisted of 30 healthy volunteers (15 women and 15 men; mean age, 66 years; range, 45–77 years). The study was approved by the Bioethics Committee on human research of the Medical University of Bialystok (permission number: R-I-002/112/2009).

Blood samples from all study participants were drawn between 6 AM and 7 AM, following a fasting period of 10 to 12 hours. Tubes with the blood collected without an anticoagulant were allowed to clot for 30 minutes before centrifugation (15 minutes at 1000 × *g*); obtained serum samples were stored at a temperature of –75°C until further analysis. Blood collected into tubes containing citrate, theophylline, adenosine, and dipyridamole anticoagulant was left in the ice bath for at last 15 minutes, following which the tubes were centrifuged (20 minutes at 2500 × *g*, at a temperature of 2°C to 8°C) within 1 hour from blood collection. After the first centrifugation, one-third of the obtained plasma was removed from the middle region of the supernatant and centrifuged for the second time (20 minutes at 2500 × *g*, at a temperature of 2°C to 8°C). Once again, one-third of the obtained plasma was removed from the middle region of the supernatant, aliquoted into several portions of 200 μl, and frozen at –20°C until the assay.

Methods Serum concentrations of sCD40L were measured using an enzyme-linked immunosorbent assay (ELISA), Quantikine® Human sCD40L Immunoassay kit (Catalog number: DCDL40; R&D Systems Europe Ltd., Abingdon, United

TABLE 1 Characteristics of patients with multiple myeloma

Parameter	Median (lower and upper quartile)
WBC count, $\times 10^3/\mu l$	5.81 (4.54–8.15)
RBC count, $\times 10^6/\mu l$	3.59 (3.09–3.87)
red blood cell aggregates, % of patients	29
hemoglobin, g/dl	10.7 (9.0–12.4)
platelet count, $\times 10^3/\mu l$	212 (157–267)
LDH activity, U/l	206 (174–253)
β_2-microglobulin, mg/l	3.57 (2.22–8.40)
albumin, g/dl	3.74 (3.35–4.07)
serum calcium, mmol/l	2.36 (2.22–2.61)
serum creatinine, mg/dl	1.47 (0.82–1.30)

Conversion factors to SI units are as follows: for WBC, 1.0; for RBC, 1.0; for hemoglobin, 10.0; for platelets, 1.0; for LDH, 0.01667; for albumin, 10; for calcium, 0.2495; and for creatinine, 88.4.

Abbreviations: LDH, lactate dehydrogenase; RBC, red blood cell; WBC, white blood cell

Kingdom). According to the ELISA protocol, serum sCD40L was 5-fold diluted with the assay diluent. The manufacturer of assay kits referred to the intra-assay coefficient of variation (CV%) as 5.1% at a mean sCD40L concentration of 430 pg/ml (SD = 21.8 pg/ml).

Serum β-thromboglobulin activity was measured using a commercially available ELISA (Catalog number: 00950; ASSERACHROM ® β-TG, Diagnostica Stago S.A.S., France), according to the manufacturer's instruction. Samples were not diluted before analysis. The manufacturer of assay kits referred to the intra-assay coefficient of variation (CV%) as 6.6% at a mean β-thromboglobulin activity of 28.7 IU/ml (SD = 1.9 IU/ml).

IL-6, sIL-6R, TNF-α, sVCAM-1, and PDGF-AB levels, platelet count, β_2-microglobulin and albumin concentrations, serum LDH activity, as well as the percentage of bone marrow plasma cells were measured using methods described elsewhere.[17]

Statistical analysis The obtained results were statistically analyzed with the use of the STATISTICA 10.0 PL software (StatSoft Inc., Tulsa, Oklahoma, United States). The concentrations of sCD40L did not follow a normal distribution in the preliminary statistical analysis (χ^2-test), thus a nonparametric statistical analysis was used. The Mann–Whitney test was used to compare 2 independent samples, and analysis of variance rank Kruskal–Wallis test was used for the comparison of 3 samples. The post hoc Dwass–Steele–Critchlow–Fligner test was conducted to assess which groups were different if significant differences were found. The values for each measured variable were presented as medians and interquartile ranges (IQRs). Differences were considered statistically significant at a P value of less than 0.05. Correlation coefficients were obtained by applying the Spearman's rank method. Receiver operator characteristic (ROC) curves were generated to calculate the areas under the ROC curves (AUCs). The Youden index, a function of

sensitivity and specificity, was estimated to indicate an optimal threshold value (cut-off point) for sCD40L. The Youden index is a commonly used measure for evaluating the effectiveness of new biomarkers, which gives equal weight to sensitivity and specificity for the concentrations of the biomarker tested.[18]

RESULTS Median serum concentrations of sCD40L in the whole study group were significantly higher (1373 pg/ml; IQR, 805–1781 pg/ml) as compared with control subjects (530 pg/ml; IQR, 335–901 pg/ml) (**FIGURE 1**). Moreover, the concentrations of sCD40L were significantly increasing with the stage of the disease (P <0.0001). Median sCD40L concentrations in stage I of MM were 595 pg/ml (IQR, 283–805 pg/ml); in stage II of MM, 1592 pg/ml (IQR, 1032–1843 pg/ml); and in stage III of MM, 1790 pg/ml (IQR, 1607–2188 pg/ml). The post hoc test showed significant differences in sCD40L concentrations between stages I and II of MM and stages I and III of MM as well as between stage II of MM and controls and between stage III of MM and controls. The differences were not significant between stages II and III of MM and between stage I of MM and controls (**FIGURE 2**).

As shown in our previous study, the serum concentrations of selected angiogenic cytokines (IL-6, sIL-6R, TNF-α, sVCAM-1, and PDGF-AB) in the whole study group of patients with MM were significantly higher compared with those in healthy subjects.[17] Additionally, as described previously, median serum concentrations of the above cytokines were significantly increasing with the stage of the disease.[17]

Median β-thromboglobulin concentrations were significantly higher in patients with MM (median, 114 IU/ml; IQR, 113–117 IU/ml) compared with controls (median, 29 IU/ml; IQR, 23–33 IU/ml) (**FIGURE 3**). β-thromboglobulin concentrations did not change depending on the stage of the disease (data not shown).

FIGURE 1 Serum sCD40L concentrations in patients with multiple myeloma (MM) in comparison with the control group
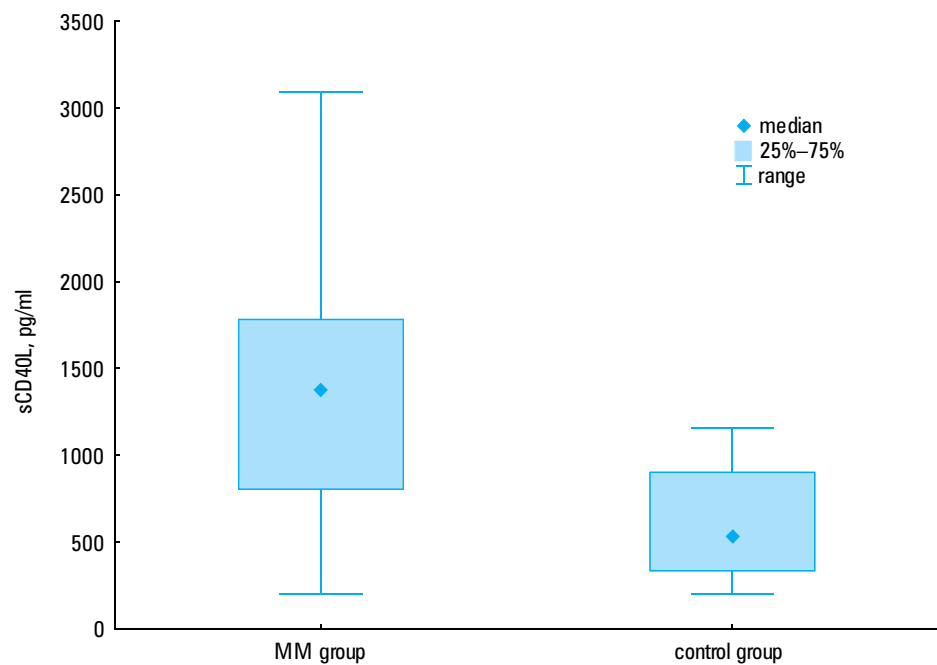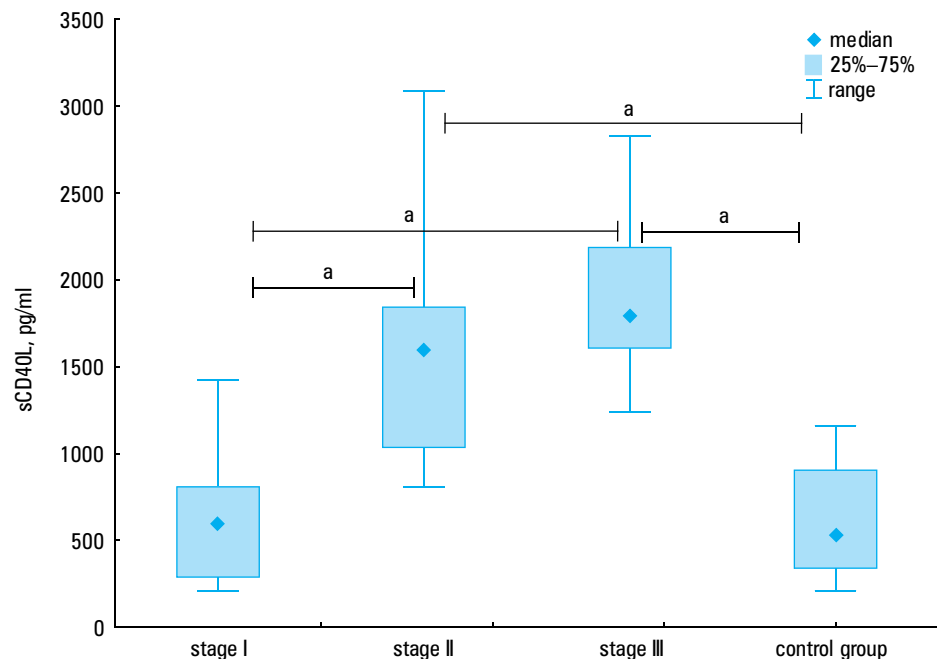$P <0.0001$

FIGURE 2 Serum sCD40L concentrations in patients with multiple myeloma depending on the stage of the disease, as compared with controls
a $P <0.0001$

Significant correlations were found between the levels of sCD40L and those of IL-6, sIL-6R, sVCAM-1, and PDGF-AB. On the contrary, no significant correlation was found between sCD40L and TNF-α levels (**TABLE 2**). Moreover, the Spearman's rank method revealed a significant positive correlation between sCD40L levels and the percentage of plasma cells in the bone marrow and β-thromboglobulin levels, whereas a negative correlation was shown with LDH activity and platelet count. No significant correlations were found between the levels of sCD40L and those of albumin or $β_2$-microglobulin (**TABLE 3**).

The AUC for sCD40L revealed the clinical significance of the protein (**FIGURE 4**). The diagnostic usefulness of serum sCD40L concentrations in patients with MM is presented in **TABLE 4**.

DISCUSSION There is no consensus regarding the role of CD40L in cancers, including MM; studies have indicated its ambivalent role in malignant diseases.[12-14] The signals delivered after membrane CD40 ligation are undoubtedly involved in tumor immunity, which has been well established in animal tumor models and in vitro studies.[14] Therefore, monitoring alterations in CD40 ligating agents, such as sCD40L, is within the scope of clinical interests and may provide useful prognostic information. According to our knowledge, circulating levels of sCD40L in MM patients

FIGURE 3 Plasma β-thromboglobulin levels in patients with multiple myeloma (MM) in comparison with the control group *P* <0.0001
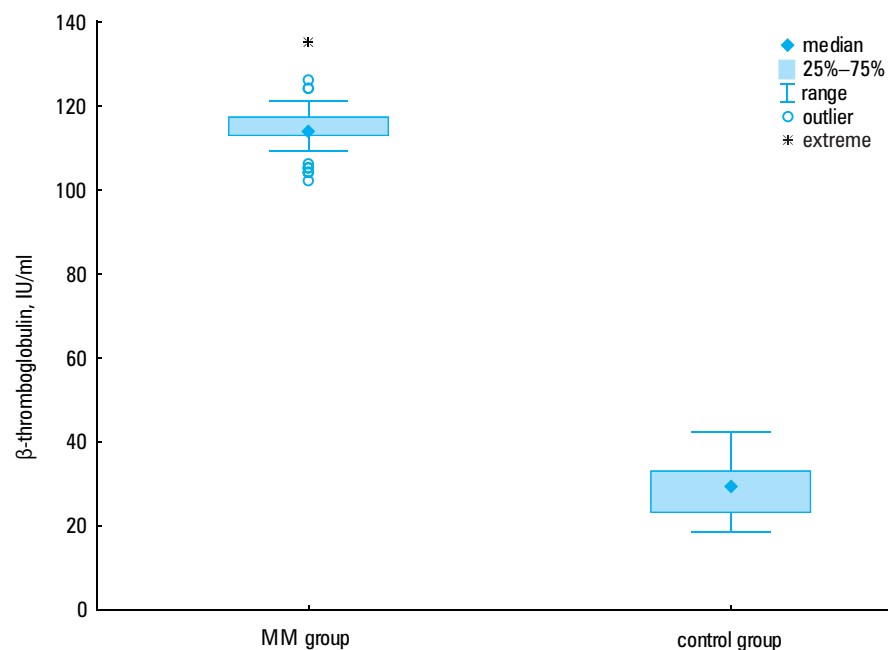
TABLE 2 Correlations of serum sCD40L levels with selected angiogenic cytokines in patients with multiple myeloma

Parameter		IL-6, pg/ml	sIL-6R, pg/ml	TNF-α, pg/ml	sVCAM-1, pg/ml	PDGF-AB, pg/ml
sCD40L	*r*	0.682	0.610	NS	0.682	0.699
	P	<0.0001	<0.0001		<0.0001	<0.0001

Abbreviations: IL-6, interleukin 6; NS, nonsignificant; PDGF-AB, platelet-derived growth factor AB; TNF-α, tumor necrosis factor α; sIL-6R, soluble receptor of interleukin 6; sVCAM-1, soluble vascular cell adhesion molecule 1

TABLE 3 Correlations of serum sCD40L levels with markers of multiple myeloma activity, platelet count, and β-thromboglobulin

Parameter		Bone marrow plasma cells, %	Albumin, g/dl	$β_2$-microglobulin, mg/l	LDH activity, U/l	Platelet count, x 10 ^{3}/μl	β-thromboglobulin, IU/ml
sCD40L	*r*	0.682	NS	NS	−0.496	−0.477	0.434
	P	<0.0001			0.0013	0.0016	0.005

Abbreviations: see **TABLE 1**

TABLE 4 Diagnostic usefulness of sCD40L in the whole study group of patients with multiple myeloma

Parameter	Cut-off[a]	Youden index	AUC	SE	Sensitivity, %	Specificity, %	PPV, %	NPV, %	ACC, %
sCD40L	1236.55	0.585	0.802	0.052	59	100	100	64	76

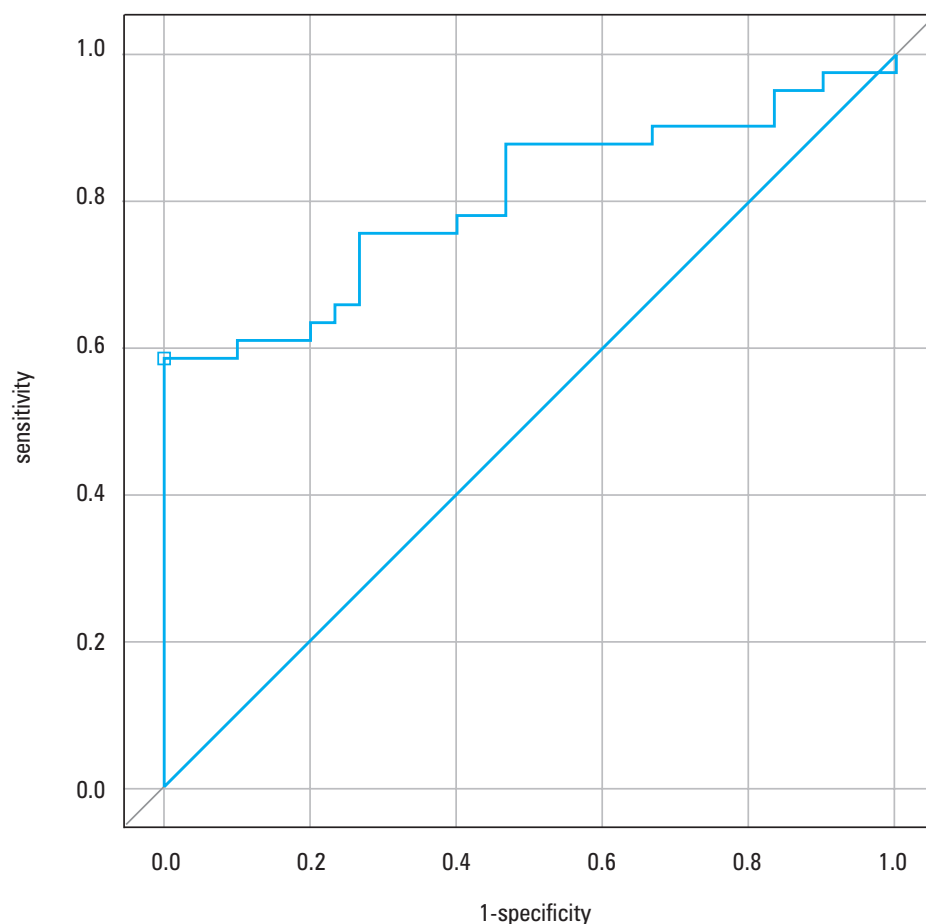**a** cut-off (based on the highest Youden index)

Abbreviations: ACC, diagnostic accuracy; AUC, area under the receiver-operating characteristic curve; NPV, negative predictive value; PPV, positive predictive value; SE, standard error

have been evaluated so far only by Tsirakis et al[5] and Hock et al.[14]

In the current study, we indicated that patients with newly diagnosed MM, prior to treatment, had a 2.6-fold higher median serum sCD40L concentration in comparison with controls. Of all patients with MM, 54% had sCD40L concentrations above the upper limit observed in healthy individuals; a further analysis revealed that 83% of patients with MM had sCD40L concentrations above the median sCD40L concentration obtained in healthy subjects (data not presented in the Results section). Our findings are in line with the results obtained by Tsirakis et al[5] and Hock et al,[14] which also revealed increased sCD40L concentrations in patients with MM as compared with

control subjects. In the next step of their analysis, Hock et al[14] divided MM patients (based on the established optimal cut-off point) into 2 groups: with high sCD40L concentrations and with low sCD40L concentrations. Data obtained by Hock et al[14] indicated that patients with MM with high sCD40L concentrations had a significantly shorter survival than those with low sCD40L concentrations.[14] Moreover, a multivariate analysis indicated that sCD40L concentrations provide additional prognostic information to that obtained by the evaluation of β_2-microglobulin concentrations.[14] Well-established markers of MM activity are β_2-microglobulin concentrations, albumin concentrations, LDH activity, and the percentage of plasma cells in the bone marrow.[17] However, in contrast to data obtained by Hock et al,[14] our results did not show a correlation between sCD40L and β_2-microglobulin concentrations; however, a significant correlation was found between sCD40L and LDH activity and percentage of bone marrow plasma cells. Additionally, our findings revealed that sCD40L concentrations were significantly increasing with the stage of the disease, which can be additional information supporting the hypothesis that sCD40L may be involved in the development and progression of MM and may be recognized as a marker of poor prognosis.

Once again, we would like to highlight that the involvement of the CD40L–CD40 pathway in neoplastic diseases is controversial.[10] Moreover, the anti- and proneoplastic activity of the CD40L–CD40 interaction in MM has not been clarified so far.[9] The CD40 is present on cancer cells of the breast,[19] ovary intestines, liver, nasopharynx, melanoma, or lymphoma.[10] CD40 is also expressed on human MM cells.[7,20] Tong et al[19] revealed that the use of soluble recombinant CD40L therapy in patients with MM resulted in the blockage of the disease progression.[19] On the other hand, Young et al[21] indicated that decreased expression of CD40L by MM cells may contribute to the ligand-dependent loss of epithelial growth regulation and thus uncontrolled inflammation and infection, which directly lead to the increased susceptibility of patients to the development of neoplastic diseases.

Because our findings showed increasing sCD40L concentrations with the stage of MM, we suggest its role as a proneoplastic protein. To support this hypothesis, we would like to point that significant correlations were found between sCD40L and IL-6, sIL-6R, sVCAM-1, and PDGF-AB, which are recognized as proangiogenic cytokines.[17,22] It should be emphasized that this is the first study estimating correlations between sCD40L and adhesion molecule (sVCAM-1) and growth factor (PDGF-AB) in patients with MM. It is well known that tumor growth depends on angiogenesis and lymphangiogenesis.[23] It has been proposed that pathologic angiogenesis results from an increased production of proangiogenic factors or lack of function of antiangiogenic molecules. In vitro and in vivo studies have pointed to

a major role of the CD40L–CD40 pathway in angiogenesis.[24] Tai et al[25] demonstrated that CD40 induces migration of MM cells and VEGF secretion, which suggests the role of CD40 ligation in MM homing and angiogenesis. Moreover, in our previous study, we reported a significant increase in the levels of IL-6, sIL-6R, TNF-α, sVCAM-1, and PDGF-AB in patients with MM as compared with controls; in addition, the levels significantly increased with the stage of the disease.[17] Increased concentrations of the above proteins and their correlations with sCD40L concentrations provide evidence that increased serum sCD40L concentrations may be related to the process of angiogenesis in patients with MM. A significant correlation between seleced angiogenic cytokines and sCD40L in patients with MM was also observed by Tsiraksis et al.[5] Moreover, the authors indicated a strong correlation between sCD40L concentrations and the stage of MM. Additionally, the posttreatment sCD40L concentrations were higher in patients with increased activity of the disease.[5]

CD40 as well as soluble CD40L may be involved in platelet activation.[26,27] Above 95% of circulating CD40L is derived from α granules of platelets after their activation and degranulation.[4] The clotting process also may lead to sCD40L release, which was proved by increased serum levels of sCD40L, compared with plasma levels.[28] In our previous study, we observed that platelet count was lower in patients with MM than in controls and significantly decreased with the stage of the disease (however, thrombocytopenia was observed only in patients with stage III of MM).[29] Well-established biomarkers of platelet activation in vivo are platelet products such as thromboxane B2, P-selectin, and β-thromboglobulin. To this purpose, we analyzed β-thromboglobulin in patients with MM at different stages, compared with control subjects. Our study revealed that median serum β-thromboglobulin levels in the whole group of MM patients was significantly higher than in controls; however, the analysis of sCD40L levels depending on the stage of the disease did not reveal significant differences. Moreover, the Spearman's rank method revealed a significant positive correlation of sCD40L levels with β-thromboglobulin, and a negative one with PLT. Increased sCD40L concentrations and biomarkers of platelet activation were also noted in other malignancies.[30,31]

In conclusion, our findings demonstrated that serum sCD40L levels were significantly higher in patients with MM than in controls and were related to the stage of the disease. The correlation of sCD40L with β-thromboglobulin may indicate that in MM sCD40L derives from platelets after their activation. Moreover, sCD40L significantly correlated with some angiogenic cytokines (IL-6, sIL-6R, sVCAM-1, and PDGF-AB), which provides evidence that increased serum sCD40L levels may be related to angiogenesis in patients with MM. Additionally, the AUC for sCD40L was

significantly higher than 0.05, which may indicate that this protein may have potential clinical usefulness in MM. Increasing sCD40L concentrations with the stage of MM, together with a significant correlation between sCD40L and markers of MM activity (LDH activity and percentage of bone marrow plasma cells), indicate that the evaluation of sCD40L levels may provide additional prognostic information. Certainly, further studies are needed to explain whether sCD40L may be recognized as a biomarker of angiogenesis and poor prognosis in MM.

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ARTYKUŁ ORYGINALNY

Stężenie sCD40L w surowicy w zależności od stadium zaawansowania szpiczaka mnogiego oraz w porównaniu z wybranymi cytokinami angiogennymi

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SŁOWA KLUCZOWE

cytokiny angiogenne, czynnik wzrostu śródbłonka naczyniowego, interleukina 6, sCD40L, szpiczak mnogi

STRESZCZENIE

WPROWADZENIE   Niewiele wiadomo na temat szlaku CD40L/CD40 w nowotworach układu krwiotwórczego, a zwłaszcza w szpiczaku mnogim (*multiple myeloma* – MM).

CELE   Celem niniejszej pracy było określenie stężenia sCD40L w surowicy u pacjentów z nowo zdiagnozowanym MM przed rozpoczęciem leczenia w różnych stadiach choroby, w porównaniu z osobami zdrowymi. W celu oceny znaczenia klinicznego sCD40L sprawdzono, czy stężenie sCD40L korelowało ze stężeniem angiogennych cytokin: interleukiną 6 (IL-6), rozpuszczalnym receptorem dla interleukiny 6 (sIL-6R), czynnikiem martwicy nowotworu α (*tumor necrosis factor α* – TNF-α), rozpuszczalną cząsteczką adhezji komórkowej naczyń 1 (*soluble vascular cell adhesion protein 1* – sVCAM-1) oraz płytkowym czynnikiem wzrostu AB (*platelet-derived growth factor AB* – PDGF-AB), a także z uznanymi biomarkerami aktywności MM (aktywność dehydrogenazy mleczanowej i odsetek procentowy komórek plazmatycznych w szpiku kostnym) oraz z markerem aktywacji płytek krwi (β-tromboglobulina).

PACJENCI I METODY   Grupa badana składała się z 41 nowo zdiagnozowanych pacjentów z MM; grupę kontrolną stanowiło 30 zdrowych osób. Stężenie sCD40L oznaczono za pomocą metody ELISA.

WYNIKI   Stężenie sCD40L był istotnie wyższe u chorych na MM niż w grupie kontrolnej i wzrastało wraz z zaawansowaniem choroby. Ponadto stężenie istotnie korelowało ze stężeniem IL-6, sIL-6R, TNF-a, VCAM-1, PDGF-AB oraz ze stężeniem markerów aktywności MM i β-tromboglobuliną.

WNIOSKI   Nasze badania wskazują, że zwiększone stężenie sCD40L w surowicy może być związane z procesem angiogenezy u chorych na MM. Białko to ma potencjalną przydatność kliniczną w MM i może być brane pod uwagę jako dodatkowy czynnik prognostyczny. Korelacja sCD40L z β-tromboglobuliną może wskazywać, że źródłem sCD40L u pacjentów z MM są zaktywowane płytki krwi.