The Levels of Soluble CD40 Ligand and C-Reactive Protein in Normal Weight, Overweight and Obese People

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Objective: Obesity has been suggested as an independent risk factor for cardiovascular disease. Increasing evidence shows that engagement of soluble CD40 ligand (sCD40L) with its receptor plays a crucial role in the pathogenesis of atherosclerosis. The aim of the present study was to test whether obesity is associated with low-grade systemic inflammation as measured by serum high-sensitive C-reactive protein (hsCRP) and sCD40L concentration.

Methods: Serum hsCRP and sCD40L concentrations were measured in 148 nondiabetic people. The participants were divided into three groups depending upon their body mass index (BMI) levels: Group 1 (normal weight), BMI < 25 kg/m²; Group 2 (overweight), BMI 25 kg/m² to 29.9 kg/m²; and Group 3 (obese), BMI ≥ 30 kg/m².

Results: Obese people had more elevated hsCRP levels than both their normal weight and overweight counterparts ($P=0.000$ and $P=0.000$, respectively). Similarly, serum concentrations of sCD40L were significantly higher, statistically, in obese subjects compared with normal weight subjects ($P=0.003$). In addition, obese subjects had higher values of sCD40L than overweight subjects, but the difference did not reach statistical significance ($P=0.063$). The levels of high-density lipoprotein cholesterol were significantly lower in obese subjects compared to normal weight subjects ($P=0.048$). The analysis of platelet count disclosed a statistically significant difference between obese subjects and normal weight subjects ($P=0.028$). The levels of BMI were positively correlated with the serum levels of hsCRP and sCD40L in all subjects ($r=0.514$, $P=0.000$ and $r=0.283$, $P=0.000$, respectively). Levels of hsCRP were positively correlated with waist circumference, fasting glucose, total cholesterol, triglyceride, low-density lipoprotein cholesterol, leukocytes, platelets, systolic and diastolic blood pressure. Similarly, soluble CD40L levels were positively correlated with waist circumference, fasting glucose and leukocytes.

Conclusion: Obese patients showed a significant increase of hsCRP and sCD40L levels compared with normal weight subjects, which might contribute to the known proinflammatory milieu found in these patients.

Keywords: Body mass index; CD40 ligand; C-reactive protein; Inflammation; Obesity

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Obesity has been suggested as an independent risk factor for cardiovascular disease.^{1,2} The levels of several acute phase response proteins and proinflammatory cytokines are significantly increased in both atherosclerosis and obesity.³ Adipose tissue is now recognized as an endocrine organ involved in regulating physiologic and pathologic processes including inflammation. It synthesizes and secretes a variety of products known as adipokines, including cytokines and chemokines. The release of adipokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and monocyte chemoattractant protein-1 (MCP-1) leads to a chronic subinflammatory state that could play a central role in the increased risk of cardiovascular disease associated with obesity.⁴⁻⁶

Serum high-sensitive C-reactive protein (hsCRP) is the principal mediator of the acute phase response. Previous studies have shown increased levels of CRP in patients with obesity.^{3,7-9} In addition, decrease in the levels of CRP after weight loss was reported in these patients.¹⁰⁻¹⁴ It has been reported that adipose tissue is an important determinant of a low level, chronic inflammatory state as reflected by levels of CRP.⁸

Besides the classical inflammatory markers, CD40 ligand (CD40L), being a trans-membrane protein and member of the tumor necrosis family, was introduced as a new inflammatory marker. It has been identified on T-helper cells, platelets and vascular smooth muscle cells.¹⁵ Studies on the cellular distribution of CD40L indicate that >95% of the circulating CD40L exist in platelets. Platelets express CD40L on their surface upon stimulation; CD40L is then cleaved and circulates as soluble CD40L (sCD40L). When expressed on the surface of platelets and exposed to CD40-bearing vascular cells, platelet-associated CD40L triggers the expression of various proinflammatory mediators, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, IL-1, IL-6, IL-8, IL-12, TNF- α , interferon- γ , and MCP-1.¹⁶⁻¹⁸ During recent years the CD40-CD40L system has been implicated in the pathophysiology of chronic inflammatory diseases, including atherosclerosis.¹⁶⁻¹⁹ There is evidence that the sCD40L level is a strong predictor of cardiovascular risk.²⁰ In literature, enhanced levels of CD40L were reported in patients with hypercholesterolemia,²¹⁻²³ diabetes mellitus,^{24,25} and acute coronary syndromes.²⁶⁻²⁹ Few studies have reported the relationship between obesity and sCD40L.³⁰⁻³² Therefore, our aim was to test whether obesity is associated with low-grade systemic inflammation as measured by serum hsCRP and sCD40L concentration.

Methods

Subjects

Patients were recruited from the Department of Internal Medicine at the Dokuz Eylul University Faculty of Medicine hospital. We enrolled 148 patients between the ages of 27

years and 80 years in the study. All subjects gave written informed consent, and the study protocol was approved by the Ethics Committee of Dokuz Eylul University. A standardized health questionnaire was completed by a physician covering the subjects' past medical history, current medications and information about other diseases. Height, weight, and waist and hip circumferences were recorded with subjects wearing light clothing without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the height in meters squared. Waist circumference was measured at the natural indentation between the 10th rib and the iliac crest (minimum waist). Hip circumference was measured over the widest part of the gluteal region, and the waist-to-hip ratio (WHR) was calculated. Arterial blood pressure was measured using a mercury sphygmomanometer on the right arm with the subjects in a sitting position after a 5-minute rest.

The participants were divided into three groups depending upon their BMI: Group 1, BMI < 25 kg/m² (n=30); Group 2, BMI 25 kg/m² to 29.9 kg/m² (n=62); and Group 3: BMI $\geq$ 30 kg/m² (n=56). Obesity was defined as BMI $\geq$ 30 kg/m². Subjects with BMI between 25 kg/m² and 29.9 kg/m² were accepted as overweight, and subjects with BMI smaller than 25 kg/m² were accepted as normal weight. Diabetes mellitus (DM), impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) were defined according to the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.³³ Subjects taking insulin or oral antidiabetic drugs were considered to have diabetes. The major exclusion criteria were DM, IGT, IFG, asthma/chronic obstructive pulmonary disease, chronic congestive heart failure, rheumatological disease, renal or hepatic dysfunction, cancer, and use of anti-inflammatory therapy or immunosuppressants.

Blood Sampling and Assay

Subjects underwent complete blood count and routine biochemical evaluations including fasting glucose (FG), postprandial glucose (PPG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG). All blood analyses were performed in a central laboratory. Serum TG, TC and HDL-C were measured on Roche Diagnostics Modular Analytics-DP analyzer (Roche Diagnostics, Tokyo, Japan) with the dedicated kits (Roche Diagnostics, Mannheim, Germany). Serum LDL-C concentrations were estimated according to the Friedewald formula at concentrations of triglyceride < 400 mg/dL. The direct LDL-C analysis was performed on a Roche Diagnostics Modular Analytics-DP analyzer at concentrations of triglyceride $\geq$ 400 mg/dL. Fasting venous blood samples (10 mL) were drawn from the antecubital vein into pyrogen-free blood collection tubes without any additives. All blood samples were collected under minimal tourniquet pressure. Blood samples were allowed to clot for 15 to 30 minutes, and were centrifuged at 1500g and 4°C for 10 minutes. The serum was then separated and stored

Table 1. Characteristics of study participants based upon BMI levels.

Characteristic	Group 1	Group 2	Group 3	P
<i>Physical</i>				
Sex, M/F	7/23	15/47	10/46	0.683 ^a
Age, y (mean±SD)	55.00±10.48	53.53±10.07	50.46±8.02	0.070
BMI, kg/m ² (mean±SD)	22.44±1.94	27.62±1.45	35.26±4.23	0.000
Smoking, n (%)	7 (23)	24 (38)	13 (23)	0.128 ^a
HT, n (%)	18 (60)	36 (58)	49 (87)	0.001 ^a
MS, n (%)	6 (20)	30 (48)	51 (91)	0.000 ^b
Waist, cm				
Female (mean±SD)	76.11±6.10	85.99±6.37	99.40±8.95	0.000 ^c
Male (mean±SD)	87.43±5.50	96.43±7.46	110.00±8.15	0.000 ^c
WHR				
Female (mean±SD)	0.82±0.13	0.82±0.05	0.85±0.08	0.008 ^c
Male (mean±SD)	0.89±0.05	0.94±0.05	0.97±0.06	0.010 ^c
<i>Biochemical (mean±SD)</i>				
SBP, mmHg	122.83±13.63	123.79±14.16	128.48±12.54	0.089
DBP, mmHg	78.17±7.37	78.63±7.90	83.04±8.40	0.004
FG, mg/dL	89.83±6.77	91.61±7.41	93.59±7.54	0.070
PPG, mg/dL	98.18±19.81	104.11±16.48	105.98±18.96	0.302
TC, mg/dL	197.93±29.36	203.13±39.62	206.71±38.12	0.589
TG, mg/dL	133.00±137.25	144.50±103.40	158.98±84.21	0.523
LDL-C, mg/dL	113.90±22.40	118.42±34.45	124.79±34.14	0.297
HDL-C, mg/dL	60.87±14.00	56.87±16.95	52.13±15.54	0.045
WBC (×10 ³ /μL)	6.82±1.73	7.11±2.08	7.52±1.88	0.273
Platelets (×10 ³ /μL)	246.07±64.49	263.05±59.32	287.98±75.77	0.022
MPV, fL	8.47±1.13	8.53±1.04	8.51±1.06	0.970
hsCRP, mg/L	1.38±1.07	2.53±2.28	4.61±3.85	0.000
sCD40L, ng/ml	0.38±0.50	0.62±0.53	0.94±1.01	0.003

Group 1, BMI<25 kg/m²; Group 2, BMI 25-29.9 kg/m²; Group 3, BMI>30 kg/m².

BMI: body mass index; HT: hypertension; MS: metabolic syndrome; WHR: waist-to-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; FG: fasting glucose; PPG: postprandial glucose; TC: total cholesterol; TG: triglyceride; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; WBC: white blood cell; MPV: mean platelet volume; hsCRP: high-sensitivity C-reactive protein; sCD40L: soluble CD40 ligand; SD: standard deviation; P: P value.

^aChi-Square; ^bChi-Square for linear trend; ^cKruskal-Wallis.

at -20°C until analysis. Samples were thawed only once. Serum sCD40L concentrations were determined using an enzyme linked immunosorbent assay (ELISA) kit from Biosource (Bender MedSystems Human sCD40L Instant ELISA). The intra-assay and interassay coefficients of variation for sCD40L were 5.00% and 6.20%, respectively, with a sensitivity of 0.062 ng/mL, according to the manufacturer. Analysis of hsCRP was performed on a Cobas Integra 400 Plus analyzer (Roche Diagnostics, Rotkreuz, Switzerland) based on particle-enhanced turbidimetry (CRPLX, Roche Diagnostics, Mannheim, Germany) with a detection limit of 0.085 mg/L and an extended measuring range of 0.085 mg/L to 1600 mg/L (with auto-rerun), according to the manufacturer.

Statistical Analysis

All statistical analyses were performed with an SPSS program for Windows (version 11.0). Means and proportions for baseline variables were compared between groups using Correlation test, multiple linear regression analysis, ANOVA for continuous variables, and nonparametric Kruskal-Wallis with Bonferroni's correction. Differences in categorical variables were measured by Chi-Square test and Chi-Square

for linear trend test. All data of continuous variables were expressed as mean ± SD; P values of 0.05 or less were considered to be statistically significant.

Results

The clinical and biochemical characteristics of the 148 nondiabetic subjects, aged 27 to 80 years, are summarized in table 1. The rate of patients with hypertension in the obese group was significantly higher, statistically, when compared with normal weight and overweight subjects. The means of FG, PPG, TC, TG, LDL and leukocyte count were the highest in obese subjects and the lowest in normal weight subjects, but the difference did not reach statistical significance. The levels of HDL-C were significantly lower in obese subjects compared to normal weight subjects ($P=0.048$). The analysis of platelet count disclosed a statistically significant difference between obese subjects and normal weight subjects ($P=0.028$). However, platelet count in overweight subjects was not significantly different when compared with normal weight ($P=0.831$) and obese subjects ($P=0.157$).

We compared serum concentrations of hsCRP (figure 1) and sCD40L (figure 2) among three BMI groups. Obese patients

Figure 1. Increasing hsCRP values in relation to BMI groups.

had more elevated hsCRP levels than both their normal weight and overweight counterparts ($P=0.000$ and $P=0.000$, respectively). Similarly, serum concentrations of sCD40L were significantly higher, statistically, in obese subjects compared with normal weight subjects ($P=0.003$). In addition, obese subjects had higher values of sCD40L than overweight subjects, but the difference did not reach statistical significance ($P=0.063$).

The levels of BMI were positively correlated with waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), FG, white blood cell count (WBC) and platelets, and negatively correlated with age and HDL-C (table 2). Levels of hsCRP were positively correlated with BMI, waist circumference, SBP, DBP, FG, TC, TG, LDL-C, WBC and platelets. Similarly, soluble CD40L levels were positively correlated with BMI, waist circumference, FG and WBC (table 2). In a multiple linear regression analysis, hsCRP was correlated with BMI, LDL-C and WBC, and sCD40L was correlated with BMI and WBC (table 3).

Discussion

In the present study, we have confirmed significantly higher levels of hsCRP and sCD40L in obese patients compared with normal weight subjects. In the literature, data about the relationship between obesity and sCD40L is limited.³⁰⁻³² Guldiken et al³² reported significantly higher values of sCD40L in patients with severe obesity ($\text{BMI} \geq 35 \text{ kg/m}^2$) compared to obese patients ($\text{BMI} 30\text{--}34.9 \text{ kg/m}^2$) and non-obese subjects ($\text{BMI} < 25 \text{ kg/m}^2$). They did not detect a significant difference between the obese and non-obese groups in terms of mean sCD40L levels. However, in our study we found significantly higher levels of sCD40L in obese patients compared with normal weight subjects. This finding is consistent with the previous studies demonstrating that the sCD40L levels are elevated in obese people and decrease after weight loss.³⁰⁻³¹ In this study, we have demonstrated positive correlations between BMI and both hsCRP and sCD40L levels. In addition, higher waist

circumferences, indicative of a large amount of abdominal visceral fat, were associated with higher hsCRP and sCD40L concentrations. A possible mechanism for the observed association may be the production of several cytokines by adipose tissue. For example, IL-6 production by adipocytes is the main hepatic stimulus for CRP synthesis.⁸ In this study, the levels of the two inflammatory markers correlate directly with the measurements of obesity (BMI and waist circumference). In this context, increased hsCRP and sCD40L levels in obesity may explain further the relationship between obesity and inflammation. Previous studies have reported a positive association between BMI and CRP concentration.^{3,8-12,34-36} Low-grade systemic inflammation has been shown to increase the risk for cardiovascular disease. A number of epidemiological studies have shown that CRP is an important risk factor for atherosclerosis and coronary heart disease.^{12,37-42} Recent studies suggest that sCD40L plays a pathogenetic role in atherothrombotic complications in cardiovascular disease.¹⁶⁻¹⁹ The role of CD40L in atherogenesis is confirmed by the fact that in hyperlipidemic mice anti-CD40L antibodies reduced atherosclerotic lesions.⁴³ Some of the increased risk for cardiovascular disease in obese patients may be explained by our observation that increased CRP and sCD40L concentrations are more prevalent in these patients.

Desideri et al³⁰ reported that sCD40L was not only correlated with BMI in adults, but was also more strongly related to lipid peroxidation. Consistent with this study, we found a significant correlation between the levels of sCD40L and BMI. Lipid peroxidation, a marker of oxidative injury, was not measured in our study, but it is well known that oxidative stress is increased in obesity, which might relate to the dysregulated production of adipocytokines.⁴⁴ Circulating sCD40L was believed to derive predominantly from platelets.¹⁷ It has been reported that obesity is associated with platelet activation and lipid peroxidation.⁴⁵ In this context, the majority of the increased sCD40L in obesity should originate from activated platelets. The mechanism accounting for CD40L expression

Figure 2. Increasing sCD40L values in relation to BMI groups.

Table 2: Pearson correlation test between BMI/hsCRP/sCD40L and all other characteristics evaluated.

Characteristic	BMI		hsCRP		sCD40L	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
<i>Physical</i>						
Age (y)	-0.246	0.003	-0.062	0.458	-0.067	0.421
BMI (kg/m ²)	-	-	0.514	0.000	0.283	0.000
Waist (cm)	0.782	0.000	0.400	0.000	0.239	0.003
WHR	0.086	0.297	0.059	0.479	0.053	0.520
<i>Biochemical</i>						
SBP (mmHg)	0.178	0.030	0.170	0.038	0.067	0.418
DBP (mmHg)	0.259	0.002	0.212	0.010	-0.018	0.830
FG (mg/dL)	0.244	0.003	0.164	0.046	0.168	0.042
PPG (mg/dL)	0.157	0.082	0.171	0.058	-0.047	0.608
TC (mg/dL)	0.086	0.303	0.173	0.037	0.084	0.311
TG (mg/dL)	0.136	0.100	0.194	0.018	0.082	0.321
LDL-C (mg/dL)	0.124	0.133	0.176	0.032	0.085	0.307
HDL-C (mg/dL)	-0.224	0.000	-0.158	0.055	-0.082	0.321
WBC (x10 ³ /μL)	0.185	0.030	0.211	0.013	0.189	0.026
Platelets (x10 ³ /μL)	0.259	0.002	0.217	0.011	0.126	0.141
MPV (fL)	-0.035	0.709	-0.057	0.542	-0.010	0.919
hsCRP (mg/L)	0.514	0.000	-	-	0.090	0.278
sCD40L (ng/ml)	0.283	0.000	0.090	0.278	-	-

r: Pearson's product-moment correlation coefficient; *P*: *P* value; BMI: body mass index; WHR: waist-to-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; FG: fasting glucose; PPG: postprandial glucose; TC: total cholesterol; TG: triglyceride; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; WBC: white blood cell; MPV: mean platelet volume; hsCRP: high-sensitivity C-reactive protein; sCD40L: soluble CD40 ligand.

by activated platelets is still unclear, but Pignatelli et al⁴⁶ showed that superoxide anion production plays a key role in platelet CD40L expression. The fact that oxidative stress also plays a role in platelet CD40L expression suggests that an increase in CD40L expression might be another mechanism through which oxidative stress elicits atherosclerotic damage.⁴⁶

It was reported that obese people had elevated platelet counts.^{7,47} Similarly, we observed significantly increased platelet counts in obese people when compared to normal weight people. This finding suggests that the increased sCD40L level might be a result of the increased platelet count in obese people. Mean platelet volume (MPV), a determinant of platelet function, is a newly emerging risk factor for

atherothrombosis. A positive correlation was reported between the levels of MPV and BMI in obese people.⁴⁸

The levels of WBC, hsCRP, sCD40L were found to correlate with the levels of BMI in all subjects. The mean WBC level, which is another marker of inflammation, was highest in obese people and lowest in normal-weight people. In all subjects, both hsCRP and sCD40L levels were positively correlated to WBC. Our data support the opinion that obesity is a chronic subclinical inflammatory disease as reflected by levels of WBC, hsCRP and sCD40L.^{7,8,49,50} The association of leukocyte count with BMI, hsCRP and sCD40L suggests that the leukocyte count may be of clinical value as a marker of low-grade systemic inflammation in obese patients. The leukocyte count has been reported previously as a marker of

Table 3. Stepwise multiple regression analysis with hsCRP and sCD40L.

Variable	B	SE	Coefficient β	<i>P</i>
<i>hsCRP</i>				
Constant	-7.41	1.57		0.000
BMI	0.24	0.04	0.45	0.000
LDL-C	0.01	0.01	0.14	0.055
WBC	0.22	0.12	0.14	0.066
<i>sCD40L</i>				
Constant	-0.71	0.37		0.055
BMI	0.03	0.01	0.26	0.002
WBC	0.06	0.03	0.14	0.091

B: regression coefficient; SE: Standard error; Coefficient β : standardized regression coefficient; *P*: *P*-value; BMI: body mass index; LDL-C: low density lipoprotein cholesterol; WBC: white blood cell; hsCRP: high-sensitivity C-reactive protein; sCD40L: soluble CD40 ligand.

inflammation and cardiovascular disease risk.^{51,52} Other variables positively correlated to BMI in the present study were SBP, DBP, FG, and negatively with HDL-C. Our data support the previous reports indicating that obesity is a chronic metabolic disorder with increased cardiovascular risk factors such as dyslipidemia, hypertension, inflammatory markers, and the prothrombotic state.⁵³

Conclusion

In our study, obese patients showed a significant increase of hsCRP and sCD40L levels compared with normal weight subjects, which might contribute to the known proinflammatory milieu found in these patients. Because inflammatory markers may be directly involved in atherogenesis, these results suggest an important mechanism through which obesity might affect the risk of cardiovascular disease.

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