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Effects of aging and menopause on serum interleukin-6 levels and peripheral blood mononuclear cell cytokine production in healthy nonobese women

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Abstract Inappropriate interleukin-6 production is thought to play a role in the development of several age-related conditions including atherosclerosis. This study aimed to determine whether aging affects circulating interleukin-6 (IL-6) levels. Healthy, nonobese women (n=208, 44.5±0.70 years, 22.4±0.17 kg/m^2) were categorized into four age groups (22–31, 32–41, 42–51, and 52–63 years; cross-sectional study). Cytokine levels in serum and those produced from peripheral blood mononuclear cell (PBMC) were measured. The oldest group had the highest circulating levels of IL-6 and oxidized low-density lipoprotein (ox-LDL) and higher PBMC production of IL-6, tumor necrosis factor-α (TNF-α), and interleukin-1 alpha (IL-1β). Additionally, significant interactions between age and menopause were found for serum IL-6 (P=0.024), and TNF-α (P=0.011) and IL-1β (P<0.001) produced from PBMCs. Serum IL-6 levels positively correlated with age, waist–hip ratio (WHR), systolic blood pressure, circulating levels of TNF-α, IL-1β, and ox-LDL, and urinary 8-epi-prostaglandin $F_2\alpha$. Multiple stepwise

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regression models identified the following factors for contributing to serum IL-6 levels: serum IL-1β, menopause status, WHR, and serum TNF-α in mode I (R^2=0.302); serum IL-1β, age, serum TNF-α, and WHR (β=0.197; P=0.006) in model II (R^2=0.283). Sub-analysis was performed according to menopausal status. Serum IL-6 levels were positively associated with levels of IL-6, TNF-α, and IL-1β in PBMC supernatants (unstimulated) from postmenopausal women, whereas these were negatively associated in premenopausal women. In conclusion, circulating IL-6 levels may be interactively influenced by age and menopause. Additionally, estrogen deprivation after menopause may enhance PBMC cytokine production in postmenopausal women, resulting in increased IL-6 levels which are closely related to oxidative stress.

Keywords Serum interleukin-6 · Cytokines · Peripheral mononuclear cells · Oxidative stress · Menopause

Introduction

Aging is associated with chronic low-grade increases in the circulating levels of inflammatory mediators including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 alpha (IL-1β; Brüünsgaard and Pedersen 2003). IL-6 is known to exert its major effects at sites distant from its origin, which differs from other cytokines, and these effects depend on the circulating levels of IL-6 (Papanicolaou and Vgontzas 2000). For this reason, IL-6 is referred to as an endocrine cytokine (Papanicolaou and Vgontzas 2000). Inappropriate production of IL-6 is thought to play a role in the development of a number of age-related conditions including atherosclerosis (Ridker et al. 2000; Volpato et al. 2001).

Although IL-6 is the most studied serum cytokine in the elderly, as emphasized by its characterization as "a cytokine for gerontologists" (Ershler 1993), controversy remains regarding whether there is an age-related increase in circulating IL-6 levels. The main consensus is that IL-6 production increases with age; however, previous studies screening participants with strict criteria for good health, including adequate nutrition and absence of disease, failed to detect any significant differences in the production of IL-1 and IL-6 between young, middle-aged, and older populations (Ahluwalia et al. 2001). In addition, Forsey et al. (2003) determined that there was no relationship between age and IL-6 levels in healthy individuals under the age of 60 years.

To determine the effects of aging and menopause on serum IL-6 levels and peripheral blood mononuclear cell (PBMC) cytokine production, we compared the serum levels of bioactive cytokines as well as the levels of TNF-α, IL-6, and IL-1β in PBMC supernatants after lipopolysaccharide (LPS) stimulation for 24 h in 208 healthy, nonobese women aged between 22 and 63 years. In addition, the relationship between IL-6 levels and oxidative stress, including plasma oxidized low-density lipoprotein (ox-LDL) and urinary 8-epi-prostaglandin $F_2\alpha$ (8-epi-$PGF_{2\alpha}$) levels, was examined.

Materials and methods

Participants and study design

Two hundred eight healthy, nonobese women (aged 22–63 years) were recruited during routine check-ups at a health promotion center at Yonsei University Hospital between October 2009 and May 2010. Postmenopausal status (n=94) was defined as an absence of menstruation for at least 12 months and the presence of estrogen deficiency symptoms including "hot flushes," increased sweating, nervousness, irritability, depression, palpitations, insomnia, headaches, dyspareunia, and joint pains. The cessation of ovarian function was confirmed by measurement of serum levels of follicle-stimulating hormone (FSH), which should be above 40 IU/L if loss of ovarian function has occurred (Rachoń et al. 2002). Premenopausal status (n=114) was defined as the presence of regular menses (Rachoń et al. 2002). All participants were clinically healthy and were not taking any medications known to affect their immunological status including oral contraceptives, lipid-lowering agents, anti-hypertension drugs, functional foods, and vitamin and/or mineral supplementation.

Anthropometric parameters, blood pressure, and blood collection

Body mass index (BMI, kg/m^2) was calculated using body height and weight. Waist–hip ratio (WHR) was

calculated using the waist and hip circumferences. Blood pressure was measured in the left arm of seated patients with an automatic blood pressure monitor (TM-2654, A&D, Tokyo, Japan) after a 20-min rest. After a 12-h fasting period, venous blood specimens were collected in ethylenediaminetetraacetic acid (EDTA)-treated or -untreated tubes. The blood specimens collected in the EDTA-treated tubes were used for the isolation of PBMCs or separated into plasma and stored at −70°C until further analysis. The blood samples collected in non-treated tubes were also separated into serum and stored until further analysis.

Serum lipid profile, fasting glucose, and white blood cell count

Fasting total cholesterol and triglyceride levels were measured using commercially available kits on a Hitachi 7150 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). After precipitation of serum chylomicrons with dextran sulfate magnesium, the concentrations of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol in the supernatants were enzymatically measured. Fasting glucose levels were measured using a glucose oxidase method with a Beckman Glucose Analyzer (Beckman Instruments, Irvine, CA, USA). White blood cell count was determined using the HORIBA ABX diagnostic (HORIBA ABX SAS, Parc Euromedicine, France).

Cytokine secretion in stimulated or non-stimulated PBMCs

Whole blood was mixed with the same volume of RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) and gently laid on a histopaque-1077 (Sigma, St. Louis, MO, USA). The sample was then centrifuged at 2,000 rpm for 20 min at 10°C. After the separation, a thin layer of PBMCs was isolated and washed twice with RPMI 1640. The pellet was resuspended in RPMI 1640 with streptomycin. Isolated PBMCs were cultured in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA) and seeded in 12-well plates (1×10^6 cells/ml; Nunc, Roskilde, Denmark) and incubated at 37°C with 5% CO_2 for 24 h. Then, cells were stimulated with increasing concentrations of LPS (*Escherichia coli* 0111: B4, Sigma, St Louis, MO, USA; 0, 5, and 10 ng/mL). After a 24-h incubation, supernatants were collected and stored at −80°C until IL-1β, IL-6, and TNF-α levels were assayed (Rachoń et al. 2002; von Haehling et al. 2003).

Cytokine assay for IL-1β, IL-6, and TNF-α levels in serum and PBMC supernatants

Levels of IL-1β, IL-6, and TNF-α in serum and PBMC supernatants were measured using the Bio-Plex™ Reagent Kit on the Bio-Plex™ (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

Plasma-oxidized LDL and serum hs-C-reactive protein

Plasma LDL was measured using an enzyme immunoassay (Mercodia, Uppsala, Sweden). The resulting color reaction was read at 450 nm with a Wallac Victor^2 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland). Serum high-sensitivity (hs)-C-reactive protein (CRP) levels were measured with an Express Plus™ auto-analyzer (Chiron Diagnostics Co., Walpole, MA, USA) using a commercially available, high-sensitivity CRP-Latex(II) *X*2 kit (Seiken Laboratories Ltd., Tokyo, Japan).

Urinary 8-epi-prostaglandin $F_2\alpha$ levels

Urine was collected in polyethylene bottles containing 1% butylated hydroxytoluene after 12 h of fasting. The bottles were immediately covered with aluminum foil and stored at −70°C until further analysis. The compound 8-epi-$PGF_{2\alpha}$ was measured using an enzyme immunoassay (BIOXYTECH urinary 8-epi-$PGF_{2\alpha}$™ Assay kit, OXIS International Inc., OR, USA). Urinary creatinine levels were determined using the alkaline picrated (Jeffe) reaction. Urinary 8-epi-$PGF_{2\alpha}$ levels are expressed as pmol/mmol creatinine.

FSH and 17ß-estradiol serum levels

Serum levels of FSH and 17ß-estradiol were measured using commercially available kits (ADIVIA Centaur FSH and ADIVIA Centaur Estradiol, respectively, Siemens, USA) on the ADIVIA Centaur (ADIVIA Centur, Siemens).

Data analysis

Statistical analyses were performed using SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). The Student's *t* test was used to compare parameters between the two groups. Frequency was tested with the chi-square test. One-way analysis of variance with the Bonferroni correction was used to test whether there were age group effects (23–32, 33–42, 43–52, and 53–63 years; Taddei et al. 2001).

Pearson correlation coefficients were used to examine relationships between variables. A multiple stepwise regression analysis was performed to examine the potential association between major clinical factors and increased serum IL-6 levels. Age, menopause, WHR, systolic blood pressure, total cholesterol, glucose, ox-LDL, and serum levels of TNF-α and IL-1β were considered independent variables in the model. The interaction between age and menopause were also tested in the multivariate interaction test in a regression model. Each variable was examined for normal distribution, and skewed variables were log-transformed (glucose, ox-LDL, IL-6, TNF-α, IL-1β, and 8-epi-$PGF_{2\alpha}$). For descriptive purposes, mean values are presented using untransformed values. Results are expressed as the mean±standard error. A two-tailed value of $P<0.05$ was considered statistically significant.

Results

Clinical characteristics of study participants

The postmenopausal women had significantly higher WHRs and systolic blood pressures, in addition to higher serum levels of total cholesterol, LDL cholesterol, glucose, and IL-6, compared to the premenopausal women (Table 1). The postmenopausal women had significantly lower serum levels of 17β-estradiol and higher FSH levels than the premenopausal women. These differences confirmed that there was estrogen deficiency in the postmenopausal women. Premenopausal and postmenopausal women did not differ in terms of their BMI, diastolic blood pressure, or serum levels of triglycerides, HDL cholesterol, TNF-α, IL-1β, and hs-CRP. Premenopausal and postmenopausal women also did not differ in terms of their number of circulating leukocytes (Table 1).

Serum levels of cytokines and oxidative stress markers according to age groups

Table 2 provides the serum levels of cytokines and oxidative stress markers for the four age groups. Serum IL-6 levels in the oldest group (group 4, age 52–63 years) were significantly higher than those for groups 1 (22–31 years) and 2 (32–41 years). Serum levels of TNF-α and IL-1β did not significantly differ between the age groups. Plasma ox-LDL levels progressively increased with age (Table 2). The mean value of ox-LDL was highest for the oldest group, and the mean value for group 3 was higher than that for group 1 (22–41 years). On the other hand, BMI, blood pressure, and serum levels of triglyceride and HDL cholesterols did not significantly differ among the four age groups (data not shown). Statistically significant interaction effects of age and menopause were found for serum IL-6 levels ($P=0.024$). Circulating ox-LDL levels seemed influenced by the interaction of age and menopause, but which were not statistically significant ($P=0.065$). In addition, ox-LDL levels positively correlated with age (unadjusted: $r=0.411$, $P<0.001$; menopause-adjusted: $r=0.169$, $P=0.016$), and strongly correlated with LDL cholesterol (unadjusted: $r=0.620$, $P<0.001$; menopause-adjusted: $r=0.516$, $P<0.001$; age and menopause-adjusted: $r=0.504$, $P<0.001$) and total cholesterol (unadjusted: $r=0.579$, $P<0.001$; menopause-adjusted: $r=0.470$, $P<0.001$; age and menopause-adjusted: $r=0.453$, $P<0.001$) levels.

PBMC cytokine production in response to LPS according to age group

Table 3 provides the cytokine levels in cultured PBMC supernatants following LPS stimulation (0, 5, or 10 ng/mL) for the four age groups. Non-stimulated PBMCs from women in the oldest group (group 4, age 52–63 years) secreted significantly higher amounts of IL-6 into the culture media than those from younger groups (age 22–31 and 32–41 years). Production of TNF-α and IL-1β by unstimulated PBMCs was higher for the oldest group (group 4) than for the younger groups (groups 1 and 2); it was also higher for group 3 than for group 1. Production of IL-1β in response to LPS stimulation (5 or 10 ng/mL) was higher for the oldest group than the younger groups (age 22–31 and 32–41 years). However, the production

Table 1 Clinical characteristics of the study participants according to menopausal status

	Premenopausal women (n=114)	Postmenopausal women (n=94)
Age (years)	37.6±0.78	52.8±0.37**
Years since menopause	–	3.06±0.30
BMI (kg/m^2)	22.3±0.25	22.6±0.22
Cigarette smoker, n (%)	1 (0.9)	1 (1.1)
Alcohol drinker, n (%)	54 (47.4)	42 (44.7)
WHR	0.85±0.01	0.88±0.01**
Systolic BP (mmHg)	100.9±1.42	117.2±1.26*
Diastolic BP (mmHg)	73.6±0.99	74.8±0.93
Triglyceride (mg/dL)[a]	92.0±4.46	99.8±4.68
Total cholesterol (mg/dL)	176.3±3.22	211.9±3.31**
LDL cholesterol (mg/dL)	100.9±2.82	133.2±2.98**
HDL cholesterol (mg/dL)	57.0±1.56	59.2±1.57
Glucose (mg/dL)[a]	84.3±0.91	92.2±1.32**
Serum FSH (IU/L)	10.8±1.93	79.9±2.90**
Serum 17ß-estradiol (pg/mL)[a]	135.7±13.7	12.1±0.59**
WBCs (×10^9/L)[a]	5.02±0.11	5.00±0.12
Serum IL-6 (pg/mL)[a]	2.58±0.15	5.16±0.65**
Serum TNF-α (pg/mL)[a]	6.65±0.70	8.08±1.30
Serum IL-1ß (pg/mL)[a]	0.81±0.16	0.73±0.14
hs-CRP (mg/dL)[a]	0.65±0.10	0.57±0.18

Data are mean±SE

BP blood pressure, *WBC* white blood cell

* P<0.01, tested by Student's t test

** P<0.001, tested by Student's t test

[a] Tested by log-transformed

of IL-6 and TNF-α in response to LPS stimulation (5 or 10 ng/mL) did not statistically differ among the age groups (Table 3). In addition, statistically significant interaction effects of age and menopause were found for TNF-α and IL-1β in supernatants from unstimulated PBMCs and IL-1β in supernatants from LPS-stimulated PBMCs. IL-6 in supernatants from unstimulated and LPS (5 ng/ml)-stimulated PBMCs seemed influenced by the interaction of age and menopause, but which were not statistically significant.

Table 2 Serum levels of cytokines and oxidative stress markers in study participants according to age group

	Group 1 22–31 years (n=30)	Group 2 32–41 years (n=48)	Group 3 42–51 years (n=63)	Group 4 52–63 years (n=67)	Difference by group P<0.05	P for interaction
Age (years)	26.7±0.54	37.4±0.40	47.8±0.35	54.5±0.34	–	–
Postmenopause (%)	0	0	49.2	94.0	1, 2 vs. 3, 4;3 vs. 4	–
Serum IL-6 (pg/mL)[a]	2.31±0.30	2.75±0.23	3.87±0.57	5.10±0.79	1, 2 vs. 4	0.024
Serum TNF-α (pg/mL)[a]	8.04±1.39	6.86±1.17	7.67±1.77	6.90±0.91	NS	0.422
Serum IL-1ß (pg/mL)[a]	0.79±0.20	0.58±0.05	0.89±0.27	0.78±0.20	NS	0.443
Plasma ox-LDL(μ/L)[a]	36.4±1.93	43.2±2.23	48.4±2.30	59.9±2.70	1 vs. 3, 4; 2, 3 vs. 4	0.065
Urinary 8-epi-PGF$_{2a}$ (pg/mg creatinine)[a]	1,072.9±57.0	1,167.1±52.2	1,110.9±52.9	1,301.8±58.2	3 vs. 4	0.937

Data are mean±SE or percentage

[a] Tested by log-transformed. Tested by one-way ANOVA with Bonferroni method

P for interaction: p values for the interaction terms between age and menopause were obtained in the multivariate interaction test in a regression model

Table 3 Cytokine production by LPS-stimulated PBMCs according to age group

	Group 1 22–31 years	Group 2 32–41 years	Group 3 42–51 years	Group 4 52–63 years	Difference by group $P<0.05$	P for interaction
Nonstimulated PBMCs						
LPS dose (0 ng/mL)						
IL-6 (pg/mL)[a]	483.4±232.6	365.3±141.8	879.8±430.4	1,516.4±952.9	1, 2 vs. 4	0.090
TNF-α (pg/mL)[a]	13.8±7.27	209.7±183.7	334.2±132.4	721.4±304.5	1 vs. 3, 4/2 vs. 4	0.011
IL-1ß (pg/mL)[a]	13.0±8.78	13.3±5.67	67.2±28.8	112.6±48.1	1 vs. 3, 4/2 vs. 4	<0.001
LPS-stimulated PBMCs						
LPS dose (5 ng/mL)						
IL-6 (pg/mL)[a]	2,308.6±527.2	2,100.7±353.1	2,333.8±575.5	3,502.9±1,015.0	NS	0.068
TNF-α (pg/mL)[a]	242.9±52.4	530.1±326.5	660.7±163.8	994.0±247.9	NS	0.589
IL-1ß (pg/mL)[a]	26.8±8.08	36.7±20.7	171.6±64.9	173.4±55.7	1, 2 vs. 3, 4	0.002
LPS dose (10 ng/mL)						
IL-6 (pg/mL)[a]	2,994.6±705.6	2,338.1±394.9	3,648.7±893.7	3,102.7±725.7	NS	0.123
TNF-α (pg/mL)[a]	323.9±96.1	638.1±378.7	1,202.1±393.0	1,356.6±306.8	NS	0.287
IL-1ß (pg/mL)[a]	28.9±16.5	28.9±10.9	451.1±303.0	251.6±99.2	1, 2 vs. 3, 4	0.002

Data are mean±SE

[a] Tested by log-transformed. Tested by one-way ANOVA with Bonferroni method

P for interaction: *p* values for the interaction terms between age and menopause were obtained in the multivariate interaction test in a regression model

Correlations between levels of IL-6, TNF-α, and IL-1β in PBMC supernatants

Overall, in the PBMC supernatants, IL-6 levels positively correlated with TNF-α ($r=0.809$, $P<0.001$) and IL-1β ($r=0.864$, $P<0.001$) levels, and TNF-α levels positively correlated with IL-1β ($r=0.833$, $P<0.001$). Similarly, levels of IL-6, TNF-α, and IL-1β in the PBMC supernatants following LPS stimulation (5 or 10 ng/mL) were linearly interrelated for all participants (data not shown). In addition, cytokines positively correlated each other in each of age groups or menopause groups, and the correlations were still significant after adjusted for age and menopause, respectively, or together (data not shown).

Relationship between serum IL-6 levels and age, body composition, serum levels of TNF-α and IL-1β, and oxidative stress markers

Overall, serum IL-6 levels positively correlated with age ($r=0.336$, $P<0.001$), WHR ($r=0.251$, $P=0.001$), systolic blood pressures ($r=0.184$, $P=0.012$), serum levels of TNF-α ($r=0.348$, $P<0.001$) and IL-1β ($r=0.346$, $P<0.001$), plasma ox-LDL ($r=0.181$, $P=0.014$), and urinary excretion levels of 8-epi-$PGF_{2\alpha}$ ($r=0.336$, $P<0.001$). Multiple regression analysis was performed with the whole cohort to identify the major sociodemographic and clinical factors associated with the increased serum IL-6 levels (dependent variable). Age, menopause, WHR, systolic blood pressure, serum total cholesterol, serum glucose, plasma ox-LDL, and serum levels of TNF-α and IL-1β were defined as independent variables. In this model I ($R^2=0.302$), serum IL-6 levels were determined to be significantly dependent on serum IL-1β levels ($\beta=0.325$; $P<0.001$), menopause status ($\beta=0.244$; $P<0.001$), WHR ($\beta=0.201$; $P=0.004$), and serum TNF-α levels ($\beta=0.197$; $P=0.007$). Considering the impact of menopause, an additional stepwise regression analysis was performed. In the second model, menopause was excluded and age, WHR, systolic blood pressure, serum total cholesterol, serum glucose, plasma ox-LDL, and serum levels of TNF-α and IL-1β were put as independent variables. In this model II ($R^2=0.283$), serum IL-6 levels were determined to be significantly dependent on serum IL-1β levels ($\beta=0.309$; $P<0.001$), age ($\beta=0.203$; $P=0.005$), serum TNF-α levels ($\beta=0.205$; $P=0.006$) and WHR ($\beta=0.197$; $P=0.006$).

Relationship between serum IL-6 levels and cytokine production by non-stimulated PBMCs and urinary 8-epi-$PGF_{2\alpha}$ levels according to menopausal status

Since menopause was selected as an independent predictor of serum IL-6, we analyzed whether there was a relationship between serum IL-6 levels and cytokine production by non-stimulated PBMCs and oxidative stress according to menopausal status. Serum IL-6 levels negatively correlated with the levels of IL-6 (r=−0.359, P=0.002; Fig. 1), TNF-α (r=−0.207, P=0.077), and IL-1β (r=−0.323, P=0.005) in supernatants from unstimulated PBMCs from premenopausal women, whereas serum IL-6 levels were correlated with the levels of IL-6 (r=0.379, P=0.001), TNF-α (r=0.440, P<0.001), and IL-1β (r=0.306, P=0.008) in supernatants from unstimulated PBMCs from postmenopausal women. However, serum IL-6 levels were correlated with urinary excretion levels of 8-epi-$PGF_{2\alpha}$ in both premenopausal (r=0.323, P=0.002) and postmenopausal (r=0.313, P=0.005) women (Fig. 1).

Discussion

In this study, circulating IL-6 levels were determined to be elevated in healthy, nonobese, older and particularly postmenopausal women without significant changes in the circulating levels of hs-CRP, TNF-α, and IL-1β, as compared to premenopausal women. Furthermore, statistically significant interactions between age and menopause were found for circulating IL-6 levels. These data seem to be related to the phenomena of advancing age together with menopause, rather than inflammation as indicated by the elevated hs-CRP level. An age-related increase in circulating IL-6 levels, but not in TNF-α and IL-1β levels observed in our study is consistent with the findings from previous studies on healthy populations (Ferrucci et al. 2005; Pantsulaia et al. 2002). Many studies also reported an increase in pro-inflammatory serum markers, particularly IL-6 after menopause and suggested that in addition to age, in postmenopausal women, changes of the immune system have been attributed to estrogen depriviation (Cioffi et al. 2002; Kamada et al. 2001; Gameiro et al. 2010; Girasole et

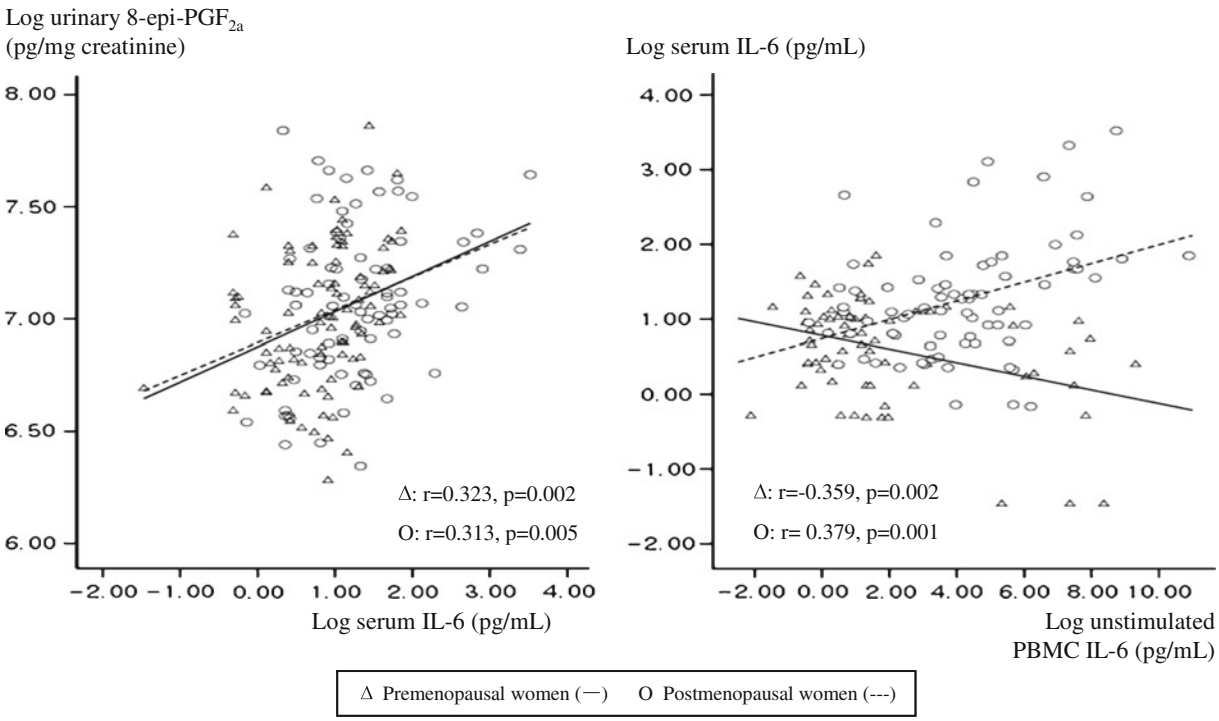

Fig. 1 Relationship between serum IL-6 levels and urinary levels of 8-epi-PGF_{2a} or IL-6 levels in supernatants from unstimulated PBMCs from premenopausal and postmenopausal women. Tested by Pearson correlation analysis; r, correlation coefficient

al. 1990; Yasui et al. 2007). Therefore, circulating IL-6 levels may be a better marker of chronic low-grade inflammatory activity in aging individuals, particularly in older postmenopausal women than systemic levels of TNF-α and IL-1β. The reason for the improved correlation might be attributable to the endocrine role of IL-6, which is not the case for TNF-α and IL-1β (Brüünsgaard and Pedersen 2003; Papanicolaou and Vgontzas 2000). It is possible that locally produced TNF-α and IL-1β do not escape into the circulation, even though they induce a strong systemic IL-6 response.

TNF-α and IL-1β are known to stimulate IL-6 production, while IL-6 influences the synthesis of TNF-α and IL-1β (Brüünsgaard and Pedersen 2003; Barton 1996); thus, it is suggested that the levels of these cytokines may be directly associated. Indeed, our present findings confirm the previous report of a positive correlation between circulating levels of IL-6 and TNF-α in a healthy general population (Pantsulaia et al. 2002). In addition, a strongly positive correlation among levels of IL-6, TNF-α, and IL-1β in unstimulated or LPS-stimulated PBMC supernatants in this study also supports the activation of a myriad of interconnected proinflammatory cytokine networks (Brüünsgaard et al. 2001). We also identified a positive correlation between serum IL-6 levels and cytokine levels in unstimulated PBMC supernatants from postmenopausal women, whereas we identified a negative correlation in PBMCs from premenopausal women. This result might reflect a loss in the regulatory effect of estrogen on PBMC cytokine production in postmenopausal women. This is consistent with the finding of Rachoń et al. (2002), who determined that women with non-stimulated PBMCs that express IL-6 have significantly lower serum levels of estradiol, as compared to women with PBMCs that do not express IL-6. It has also been suggested that increased cytokine production in healthy, older individuals result from reduced levels of sex hormones (Ershler and Keller 2000). Gender and its specific hormones are known to influence the immune system, with estrogens as enhancers of the humoral immunity and androgens and progesterone as natural immunesuppressors. Estradiol acts to inhibit pro-inflammatory cytokine gene expression, NF-κB binding, and production of proinflammatory cytokines (Deshpande et al. 1997; Liu et al. 2005; Ray et al. 1997). Thus, the increased inflammatory reactivity such as increased serum IL-6 and cytokine levels in unstimulated PBMC supernatant among post-menopausal women may be related to lower circulating levels of reproductive hormones. In addition, serum levels of IL-6 and soluble IL-6 receptor (sIL-6R) in humans were highly detected in pathological processes of osteoporosis (Abrahamsen et al. 2000; Giuliani et al. 2001; Scheidt-Nave et al. 2001). IL-6 is a pleiotropic cytokine which has multiple effects on different cell types (Kallen 2002). In bone, IL-6 is produced by osteoblasts, monocytes, and T cells, and induces osteoclastogenesis and osteoclast activity particularly after estrogen loss (Jilka et al. 1992; Tamura et al. 1993). Interestingly, IL-6-deficient mice were protected against osteoporosis after ovariectomy (Poli et al. 1994). These reports may partly explain the association of higher risk of osteoporosis with increased IL-6 levels in post-menopausal women. In our healthy, nonobese women, menopause was selected as one of predictor for serum IL-6 levels in a multiple stepwise regression model.

Oxidative stress occurs when free radical generation exceeds the system's ability to neutralize and eliminate the free radicals (Shoelson et al. 2003; Singh et al. 2005). The free radicals may activate NF-kB and trigger an inflammatory cascade, which induces more free radical production going into a vicious circle (Federico et al. 2007). In our study, serum levels of IL-6 from both premenopausal and postmenopausal women positively correlated with urinary excretion levels of 8-epi-$PGF_{2\alpha}$. This molecule is a biomarker of oxidative stress; it exerts pathophysiological effects such as vasoconstriction (Roberts and Morrow 2002) and has been proposed as an independent cardiovascular risk factor (Kim et al. 2008; Jang et al. 2004). This positive correlation between serum IL-6 and urinary 8-epi-$PGF_{2\alpha}$ levels was independent of smoking cigarettes, alcohol consumption, obesity, menopause, and the presence of medical disorders, because our study population comprised of a homogeneous group of healthy, almost-nonsmoking, and nonobese women. In addition to urinary 8-epi-$PGF_{2\alpha}$ levels, in the future, we may need to measure another oxidative stress markers (i.e., malondialdehyde, oxidative lymphocyte DNA damage etc.) to investigate whether their correlations with IL-6 can be discriminated between pre- menopausal and post-menopausal women. Also, the measurement of sIL-6R needs to be considered in further

study. Circulating IL-6 combines with sIL-6R and the IL-6/sIL6R complex increases the half-life of IL-6 and allows signaling to occur in tissues devoid of membrane IL-6R, thus it may provide more information (Giuliani et al. 2001).

Similar to the previous finding of Oka et al. (2008), serum levels of IL-6 in this study positively correlated with plasma levels of ox-LDL, which is a hallmark of atherosclerosis (Singh et al. 2005). Expression of this molecule was strongly correlated with LDL and total cholesterol levels but not with triglyceride and HDL cholesterol levels. A role for oxidative stress and inflammation has been suggested in the pathogenesis of aging and several age-associated disorders, including atherosclerosis (Brüünsgaard and Pedersen 2003; Singh et al. 2005; Migliaccio et al. 1999; Napoli et al. 2003; Shoelson et al. 2003). Ox-LDL induces inflammatory responses, as it (Brüünsgaard and Pedersen 2003) induces the adhesion and influx of monocytes and (Papanicolaou and Vgontzas 2000) affects cytokine release by monocytes (Singh et al. 2005). In fact, ox-LDL has also been shown to stimulate IL-6 expression (Massy et al. 2000) and IL-1β release (Thomas et al. 1994). Additionally, in vitro studies have shown that monocytes from elderly individuals are in a preactivated state; there is greater initial production of proinflammatory cytokines, but there is no difference in the peak levels of cytokines among elderly and younger subjects (Ahluwalia et al. 2001; Roubenoff et al. 1998).

Production of IL-6, TNF-α, and IL-1β by unstimulated PBMCs in this study was higher in women aged 52–63 years than in younger women (aged 22–41 years). However, unlike age-related increases in cytokine production by PBMCs, there was no effect of age on IL-6 and TNF-α production in LPS-stimulated PBMCs. Only the production of IL-1β was affected by age in the present study. The Framingham Heart study showed an age-related increase in IL-6 production by unstimulated PBMCs, but there were no age-related differences in TNF-α and IL-1β production (Roubenoff et al. 1998). However, von Haehling et al. (2003) determined that a significant correlation exists between age and TNF-α production by PBMCs. Riancho et al. (1994) showed a positive correlation between age and IL-1β levels, but not TNF-α, in supernatants from isolated human monocyte cultures stimulated with LPS for 24 h. On the other hand, Bruunasgaard et al. (1999) reported decreased levels of TNF-α and IL-1β in whole blood supernatants following LPS stimulation from the elderly, as compared to younger individuals. There was no difference with regard to IL-6 levels. In addition, Roubenoff (1998) identified no differences in TNF-α and IL-1β production in the isolated blood mononuclear cells stimulated with LPS for 22 h between older and younger individuals. The reason for these conflicting results is unclear, but they could be attributable to the small sample size, poor selection of subjects, variability in assays, and large interindividual variance.

In summary, our results indicate that circulating IL-6 levels may be a better marker of a chronic low-grade inflammatory activity associated with aging than systemic levels of hs-CRP, TNF-α, and IL-1β in healthy, nonobese women. In addition, the positive correlation identified between serum IL-6 levels and cytokine production by unstimulated PBMCs from postmenopausal women, as compared to the negative correlation in premenopausal women, indicates that estrogen deprivation after menopause may enhance PBMC cytokine production. Thus, this ultimately increases circulating levels of IL-6, which are closely related to oxidative stress. Further studies to confirm these results are warranted as this was a cross-sectional study.

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