PSGL-1-Expressing CD4 T Cells Induce Endothelial Cell Apoptosis in Perimenopausal Women

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Aim: Menopause and subsequent estrogen deficiency correlate with the development of atherosclerosis and cardiovascular diseases in women. However, the relationship between estrogen deficiency and development of atherosclerosis with inflammatory infiltrates is not fully understood. We sought to determine whether perimenopausal women (PMW) exhibited T cell dysfunction related to the expression of adhesion molecules and accelerated endothelial cell (EC) apoptosis.

Methods: Fresh CD4 T cells were isolated from 48 PMW and 54 healthy control women with regular menstrual cycles (CW), and investigated cytotoxicity to ECs by apoptosis assay. The adhesion molecules on CD4 T cells were examined by flow cytometry. CD4 T cell rolling and adhesion on ECs were analyzed by adhesion assay under laminar flow.

Results: CD4 T cells from PMW with low estradiol levels induced significant EC apoptosis (P<0.0152). Furthermore, cytotoxic CD4 T cells from PMW strongly expressed P-selectin glycoprotein ligand-1 (PSGL-1) and integrin β2 (P<0.0001 and P=0.0285, respectively) but not L-selectin or integrin αM when compared to CD4 T cells from CW. Estradiol levels negatively correlated with only PSGL-1 expression (R=-0.781, P=0.0002), and estradiol treatments inhibited both PSGL-1 expression (P=0.0133) and T cell-induced EC apoptosis (P=0.018). An estrogen receptor antagonist inhibited these effects of estradiol (P=0.0355 and P=0.0097, respectively). Moreover, PSGL-1 expression correlated with T cell adhesion to ECs under laminar flow conditions (R=0.636, P=0.0355) and with EC apoptosis (R=0.614, P=0.0196). PSGL-1 specific antibodies effectively suppressed T cell adhesion (P=0.0057) and EC apoptosis (P=0.001) indicating that CD4 T cell-mediated EC apoptosis depended on PSGL-1 adhesion in PMW.

Conclusions: PSGL-1-expressing cytotoxic CD4 T cells are abundant in PMW with low estradiol levels may contribute to T cell-mediated atherosclerotic development.


Key words: PSGL-1, Leukocytes, Endothelial cells, Apoptosis, Perimenopausal women

Introduction

Menopause and subsequent estrogen deficiency accelerate atherosclerosis, which promotes the development of cardiovascular diseases, such as acute coronary syndrome. Women in early menopause have high cardiovascular mortality. On the other hand, acute myocardial infarction in most premenopausal women occurs during the menstrual or early follicular phases when estradiol levels are low. Thus, it appears that endogenous estrogen may protect women from cardiovascular diseases and atherosclerotic development.

Studies of culprit coronary arteries from sudden cardiac death have revealed that the pathology includes plaque rupture (60%) and superficial erosion (40%), which is defined as the occlusion of a plaque...
by a thrombus that is confined to the most luminal portion of a fibrous cap in the absence of a fissure or a rupture. Plaque erosions are more common in younger women (i.e., under 50 years of age), including many premenopausal women. Endothelial cell (EC) apoptosis can cause stable endothelialized plaques to become unstable and prone to thrombotic plaque erosion.

Atherosclerosis is an inflammatory disease wherein inflammatory infiltrates, such as macrophages, T cells, and dendritic cells, are observed in atherosclerotic plaques. Leukocytes roll along activated endothelium and then firmly adhere to the vessel wall, which is a critical first step before their recruitment into the wall. Adherent and transmigrated CD4 T cells are activated in situ and may cause atherosclerotic plaque instability and damage to tissues including endothelial and smooth muscle cells. This process is predominantly mediated by cellular adhesion molecules, which are expressed on both vascular ECs and leukocytes. The expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), L-selectin, P-selectin, and E-selectin, has consistently been observed in human atherosclerotic lesions. Currently, adhesion molecules that are known to be present on T cells include E-selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1), integrin αM/β2 (Mac1), integrin α4/β1 (VLA-4), and integrin αL/β2 (LFA-1). The binding of these T cell adhesion molecules to E-selectin, P-selectin, ICAM-1, and VCAM-1 onto ECs initiates the process of atherosclerotic plaque development.

The protective effects of estrogen on the cardiovascular system are well established. The biological effects of estrogen on ECs and vascular smooth muscle cells (SMCs) are mediated by the binding of estrogen receptors including estrogen receptor (ER)-α, ER-β, and transmembrane G receptor (gpER). CD4 T cells express ER-α and ER-β. Estrogen can elicit rapid biological effects in nongenomic effect-mediated cytoplasmic or cell membrane-bound receptors via mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-OH kinase (PI3K), and tyrosine kinases. Estrogen can increase eNOS levels in endothelial cells and iron channel levels in smooth muscle cells by a nongenomic effect. In contrast, longer-term genomic effects include alterations in the gene expression levels of ligand-activated transcription factors for vasodilator enzymes, such as prostacyclin synthase and nitric oxide synthase. In addition, estradiol-based hormone replacement therapy (HRT) reduces serum levels of MCP-1, ICAM-1, VCAM-1, and E-selectin in postmenopausal women, however, the immunomodulating role of estrogen in women, especially concerning lymphocyte adhesion to ECs in the initial and critical steps of atherosclerosis, has not been fully evaluated.

Therefore, we aimed to investigate whether estradiol levels were associated with PSGL-1 expression and lymphocyte adhesion to ECs in perimenopausal women (PMW) and control women. Furthermore, we examined whether PSGL-1 had pivotal roles in CD4 T cell-induced EC apoptosis in PMW.

Methods

Study Population

We studied 48 PMW (54.5 ± 4.1 years old) who were volunteers or were visiting for health check-ups and who were not taking any medication. PMW were classified as women more than 6 months after their last menstrual period and within 5 years of menopause. A group of 54 healthy women volunteers (31.4 ± 8.3 years old) with regular menstrual cycles who were not in the menstrual or early follicular phases were used as the control group (CW). Exclusion criteria included HRT, cardiovascular disease, hypertension, hypercholesterolemia, obesity, diabetes mellitus, infectious disease, autoimmune disease, or neoplastic disease. Blood samples were drawn at the time of visiting our hospital and were processed immediately. Estradiol levels in plasma were measured by electrochemiluminescence (ECLIA). The investigation conformed to the principles outlined in the Declaration of Helsinki. The Tokyo Women's Medical University Institution Review Board approved all protocols, and all study patients provided written informed consent prior to study enrollment.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood using Ficoll-Hypeaque (Amersham Biosciences, Brown Deer, WI, USA). CD4 T cells were isolated by negative selection (purity ≥ 95%; Rosette Sep CD4 T cell Enrichment Kit; StemCell Technologies, Vancouver, Canada) and cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. THP-1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and grown in the same medium as primary CD4 cells. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville (Walkersville, MD, USA) and were propagated on collagen-coated tissue culture plates in

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EmGM-2 endothelial cell medium (Cambrex, Walkersville, MD, USA), and the HUVECs were used in four to six passages in this study.

**Flow Cytometry**

PBMCs were stained with the following antibodies at a 1:50 dilution for 30 min at 4°C: anti-human-CD4 fluorescein isothiocyanate (FITC) (BD Pharmingen, San Jose, CA, USA), anti-human-CD4 phycoerythrin (PE)-Cy5 (BD Pharmingen), anti-human-CD162 PE (PSGL-1; BD Pharmingen), anti-human-CD62L FITC (L-selectin; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human-CD18 FITC (integrin β2; BioLegend, San Diego, CA, USA), and anti-human-CD11b PE-Cy5 (integrin αM; BioLegend). The expression levels of each adhesion molecule on the T cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

To examine the effect of estradiol on CD4 T cell adhesion molecules, PBMCs were pretreated with or without 10 nM ICI 182780, a pure ER antagonist (ICI; Tocris, Ellisville, MO, USA), for 6 h. The PBMCs were then treated with 10 nM β-estradiol (Sigma-Aldrich, St. Louis, MO, USA) for 48 h at 37°C before staining in several experiments. The data were analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA, USA).

**Apoptosis Assay**

Apoptosis assays using CD4 T cells from CW and PMW were performed as previously described. Briefly, HUVECs were stained with 1 μg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for 30 min. Freshly isolated CD4 T cells were co-cultured on an HUVEC monolayer at an effector : target ratio of 5:1 for 3 h in phenol red-free RPMI medium supplemented with 2% fetal calf serum. Apoptotic HUVECs were identified by alteration of their nuclei by a blinded reader using a fluorescence microscope (200× magnification), and the data were represented as percentages of total HUVEC nuclei. To determine the effect of estradiol on CD4 T cell adhesion molecules, PBMCs were pretreated with or without 10 nM ICI for 6 h before treatment with or without 10 nM β-estradiol for 48 h at 37°C followed by analysis with an apoptosis assay.

**Adhesion Assay under Laminar Flow**

Adhesion assays under laminar flow conditions were performed according to a previously described method. Briefly, HUVECs were stimulated with tumor necrosis factor (TNF)-α (10 μg/ml) for 4 h on coverslips and were then positioned in a flow chamber that was mounted on an inverted microscope (IX80; Olympus, Shinjuku, Tokyo, Japan). The stimulated HUVEC monolayer was immersed in perfusion medium (PBS containing 0.2% FBS) for 3 min. CD4 T cells (1 × 10⁶/ml) were then drawn through the chamber using a syringe pump for 10 min at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². THP-1 cells (1 × 10⁶/ml) were drawn as a control. Rolling and adherent CD4 T cells and THP-1 cells on HUVECs were recorded in 15 different fields for 15 randomly selected seconds using a microscope-attached video camera. The numbers of rolling and adherent CD4 T cells and THP-1 cells on the HUVECs were determined by analyzing video images of the entire period using a personal computer. To examine the effects of PSGL-1 and integrin β2 on CD4 T cell rolling and adhesion, CD4 T cells in several experiments were pretreated with neutralizing anti-human PSGL-1 antibody (10 μg/ml), neutralizing anti-human integrin β2 antibody (10 μg/ml), or IgG (10 μg/ml) as a control for 20 min on ice before utilizing the adhesion assay.

**Statistical Analysis**

Data were analyzed using Student’s t-test for independent samples or the t-test for paired samples. In experiments with skewed distributions, data were analyzed with the Mann-Whitney U test. P < 0.05 was considered significant. The results are shown as the means ± standard deviation (SD) and as box plots with medians and ranges of five percentiles. Correlations between two parameters were analyzed using Pearson’s correlation coefficient.

**Results**

CD4 T Cells Obtained from PMW with Low Estradiol Levels Induce EC Apoptosis

We examined whether estradiol levels differed between PMW and CW. The estradiol levels of PMW (12.6 pg/ml ± 13.8 pg/ml) were significantly lower than those of CW (68.5 pg/ml ± 36.1 pg/ml; P < 0.0001). To assess whether CD4 T cells from PMW with low estradiol levels induced ECs, the cytotoxicity of CD4 T cells was measured by an apoptosis assay. CD4 T cells obtained from PMW more strongly induced HUVEC apoptosis when compared to T cells obtained from CW.
(P=0.0152; Fig. 1A and 1B) suggesting that CD4 T cells from PMW with low estradiol levels were strongly cytotoxic.

**High PSGL-1 Expression on CD4 T Cells Obtained from PMW with Low Estradiol Levels**

Direct contact between CD4 T cells and ECs is important to induce EC apoptosis. Thus, we analyzed the adhesion molecules on CD4 T cells, including L-selectin, PSGL-1, integrin β2, and integrin αM. Expression levels of PSGL-1 and integrin β2 were strongly increased on CD4 T cells from PMW in comparison to CD4 T cells from CW (Fig. 2A and 2B; P<0.0001 and P=0.0285, respectively). In contrast, expression levels of L-selectin and integrin αM on CD4 T cells were not significantly different between these two groups (Fig. 2A and 2B). We also investigated whether adhesion molecule expression on CD4 T cells and estradiol levels in PMW were related. PSGL-1 expression on CD4 T cells negatively correlated with the estradiol levels (R=-0.781 and P=0.0002; Fig. 2C). Moreover, integrin β2, L-selectin, and integrin αM did not show any correlations with the estradiol levels.

**Estradiol Treatment Reduces PSGL-1 Expression on CD4 T Cells and Inhibits CD4 T Cell-Induced EC Apoptosis Via ER**

We investigated whether estradiol treatment reversed the increased PSGL-1 expression and cytotoxic function of CD4 T cells in PMW. The expression of PSGL-1 on CD4 T cells from PMW was reduced by 48 h estradiol treatment (P=0.0133; Fig. 3A). This significant effect of estradiol treatment for PSGL-1 was abolished with ICI pretreatment (P=0.0355; Fig. 3A). Furthermore, estradiol inhibited CD4 T cell-induced HUVEC apoptosis (P=0.018; Fig. 3B), and ICI reversed the effect of estradiol (P=0.0097; Fig. 3B). These data suggest that estradiol may regulate PSGL-1 expression and CD4 T cell cytotoxicity via ER. In contrast, estradiol treatment had no effects on the expression of adhesion molecules and CD4 T cell-induced HUVEC apoptosis in CW (data not shown).

**CD4 T Cell Adhesion onto ECs Depends on PSGL-1 Expression Levels in PMW**

We then analyzed adhesion characteristics of CD4 T cells using activated HUVECs under physiological flow conditions in vitro. When we plotted the number of adherent CD4 T cells and the expression levels of individual adhesion molecules, the expression level of PSGL-1 strongly correlated with the amount of CD4 T cell adhesion to activated HUVECs (R=...
The number of rolling CD4 T cells was not correlated to any of the examined adhesion molecules (Fig. 4A). To confirm whether CD4 T cell adhesion to HUVECs in PMW depended on PSGL-1 or integrin β2, we pretreated CD4 T cells with neutralizing antibodies against PSGL-1 or integrin β2. The anti-PSGL-1 antibody inhibited both CD4 T cell-mediated rolling over and adhesion to HUVECs ($P = 0.0425$ and $P = 0.0057$, respectively; Fig. 4C). Neutralization with the integrin β2 antibody inhibited CD4 T cell adhesion to HUVECs ($P = 0.0084$; Fig. 4D) but not rolling over HUVECs, suggesting a dominant role for PSGL-1 mediating CD4 T cell adhesion to activated HUVEC.

**PSGL-1-Mediated Adhesion of CD4 T Cells to ECs is Important for EC Apoptosis in PMW**

To investigate whether firm adhesion of CD4 T cells to ECs induced EC apoptosis in PMW, CD4 T cells obtained from PMW were simultaneously subjected to adhesion and apoptosis assays. CD4 T cell-induced HUVEC apoptosis strongly correlated with
CD4 T cell rolling over and adhesion to HUVECs (Fig. 5). As shown in Fig. 6A, the expression level of PSGL-1 but not L-selectin or integrin correlated with the amount of EC apoptosis ($R=0.614$ and $P=0.0196$). Furthermore, the anti-PSGL-1 antibody but not the anti-integrin $\beta_2$ antibody inhibited EC apoptosis ($P=0.001$; Fig. 6B) indicating that CD4 T cell-mediated EC apoptosis depended on PSGL-1 in PMW.

**Discussion**

The anti-inflammatory and vasoprotective effects of estrogen have been extensively studied. Estrogen decreases MCP-1 serum levels in postmenopausal women, in addition to soluble E-selectin, ICAM-1, and VCAM-1 in postmenopausal women with coronary artery disease $^{19, 20, 23}$). In autoimmune diseases in women, $17\beta$-estradiol increases the expression of chemokine-like CCR1-5 on CD4 T cells and has been correlated with an increased susceptibility to these diseases and severity of these diseases $^{24}$; however, the immunomodulatory effects of estrogen on T cells in atherosclerosis are poorly understood. In this study, we showed that the expression levels of integrin $\beta_2$ and PSGL-1 on CD4 T cells were significantly increased in PMW with low levels of estradiol. In addition, lower estradiol levels in PMW accentuated the intensity of PSGL-1 expression, and estradiol treatment returned the expression of PSGL-1 to the levels observed in control women with normal estradiol levels. Changes in estradiol levels may directly regulate PSGL-1 expression in PMW.

PSGL-1 is a 240 kDa disulfide-bonded homodimeric mucin-like glycoprotein that binds to P-selectin, E-selectin, and L-selectin. PSGL-1 is an important adhesion molecule for leukocyte tethering and rolling on activated ECs $^{25, 26}$. A neutralizing antibody
Fig. 4. CD4 T cell adhesion to ECs depends on PSGL-1 expression in PMW

CD4 T cells from 13 PMW and control TPH-1 cells (control) were drawn on HUVECs pretreated with TNF-α under a laminar flow for 10 min. The ratios of (A) CD4 T cell rolling to THP-1 cell rolling and (B) CD4 T cell adhesion to TPH-1 cell adhesion onto HUVECs were analyzed. The expression of adhesion molecules on CD4 T cells was analyzed with flow cytometry. (C and D) PMW CD4 T cell rolling over and adhesion to HUVECs was analyzed by an adhesion assay of pretreated CD4 T cells with anti-PSGL-1 antibody (n=7), anti-integrin β2 antibody (n=6), or IgG isotope as a control.

Fig. 5. Rolling and adhesion of CD4 T cells correlate with CD4 T cell-induced EC apoptosis

The rolling over and adhesion to HUVECs of CD4 T cells obtained from PMW were measured under laminar flow (n=10). The rate of HUVEC apoptosis was analyzed with an apoptosis assay as shown.
for PSGL-1 has been demonstrated to reduce neointimal formation and macrophage infiltration in a murine carotid artery wire injury model. Recently, it has been reported that Ly-6C<sup>hi</sup> monocytes, a major subset of monocytes found in atherosclerotic mice, strongly express PSGL-1. PSGL-1<sup>-/-</sup> ApoE<sup>-/-</sup> double-knockout mice have significantly reduced Ly-6C<sup>hi</sup> monocyte infiltrations in atherosclerotic lesions and have impaired development of atherosclerosis; however, the mechanism by which PSGL-1 contributes to the trafficking of CD4 T cells into atherosclerotic lesions is not yet known. Our adhesion assay using the neutralizing PSGL-1 antibody indicated that CD4 T cells derived from PMW strongly suppressed adhesion to and rolling over activated ECs; therefore, PSGL-1 may be an important adhesion molecule for trafficking CD4 T cells to atherosclerotic lesions in PMW.

PSGL-1 is constitutively expressed on T cells and consists of carbohydrate-containing molecules and several glycosyltransferases, including α,1,3-fucosyltransferase-VII (FucT-VII), O-linked branching enzyme core 2 β1,6-glucosaminyltransferase-1 (C2GlcNAcT-I), β1,4-galactosyltransferase-1 (β1,4GalT-I), and at least one sialyl 3-galactosyltransferase (ST3Gal), which are critical for the biosynthesis of selectin ligands. The activation of T cells permits the expression of glycosyltransferases and allows modified PSGL-1 to bind all three selectins (P-selectin, E-selectin, and L-selectin). FucT-VII is upregulated through the T cell receptor (TCR)/RAS/mitogen-activated protein (MAP) kinase and IL-12 receptor (IL12R)/signal transducers and activators of transcription 4 (STAT4) pathways, but is inhibited by the IL-4 receptor (IL4R)/STAT6 pathway. On the other hand, C2GlcNAcT-I expression is upregulated by IL12R/STAT4; thus, FucT-VII and C2GlcNAcT-I may be regulated by different signals during Th1/Th2 differentiation in atherosclerosis. It should be addressed in future studies whether the mechanisms that regulate PSGL-1 expression in PMW CD4 T cells through estrogen/ERs are mediated by FucT-VII or C2GlcNAcT-I.

ECs comprise the inner lining of arteries and exert anticoagulant effects; therefore, EC dysfunction and apoptosis also have key roles in the acceleration of atherosclerosis and plaque erosion. It is known that plaque erosion is observed in 42% of women under 50 years of age with sudden cardiac death, however, in only 17% of men. In addition, it is reported that es-
trogen protects TNF-α-induced or oxidative stress-induced EC apoptosis. PSGL-1 expressing cytotoxic CD4 T cells might have important patho-mechanical roles for plaque instability, especially in women around 50 years. This needs to be addressed in appropriate further studies.

In conclusion, we identified a novel mechanism of endothelial apoptosis in PMW that was induced by PSGL-1-expressing CD4 T cells and depended on CD4 T cell rolling adhesion to ECs. Estrogen treatment abrogated PSGL-1 expression and CD4 T cell-induced endothelial apoptosis via ER, which suggested that PSGL-1 is a key molecule in the development of atherosclerosis and plaque instability in PMW. Blockade of PSGL-1 may be a potential therapeutic approach for preventing cardiovascular disease and mortality in women.

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