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ARTICLE

# Pine Bark Extract Enzogenol Attenuated Tumor Necrosis Factor-α-Induced Endothelial Cell Adhesion and Monocyte Transmigration

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The transmigration and extravasation of leukocytes across the endothelium that lines the vessel wall occurs in distinct multisteps first comprising rolling of the leukocytes over the endothelial cells, resulting in a tightly controlled and very complex system of leukocyte trafficking and transmigration. Vascular endothelial cells are an important target of proinflammatory cytokines modulating many genes involved in cell adhesion, thrombosis, and inflammatory responses. This study examined whether enzogenol blunts transendothelial migration of monocytes through tumor necrosis factor (TNF)-α-activated human umbilical vein endothelial cells (HUVEC). HUVECs were incubated with 10 ng/mL TNF-α for 6 h in the absence and presence of 5–50 $\mu$g/mL enzogenol. Expression of protein and mRNA of adhesion molecules in HUVEC were measured with Western blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) assay. Monocytic THP-1 cell adhesion and transmigration were examined by calcein AM-staining and matrix metalloproteinase-9 (MMP-9) activity measured by gelatin zymography. Intracellular localization of nuclear factor-kappa B (NF-$\kappa$B) p65 revealed involvement of NF-$\kappa$B signaling. TNF-α markedly induced protein expression of cell adhesion molecule and E-selectin with increasing mRNA levels in HUVEC. Nontoxic enzogenol at 5–25 $\mu$g/mL attenuated the expression of all adhesion molecules in a dose-dependent fashion. Consistently, enzogenol suppressed the enhanced THP-1 cell adhesion onto TNF-α-activated HUVEC through diminishing integrin $\beta$2 induction. In TNF-α-activated HUVEC were observed I$\kappa$B dissociation and NF-$\kappa$B nuclear translocation, which was ameliorated by enzogenol. Furthermore, enzogenol hampered the transendothelial migration of THP-1 cells by increasing MMP-9 secretion and activity. Blunting induction of cell adhesion molecules by enzogenol was mediated by their interference with the NF-$\kappa$B-dependent transcription pathways. Thus, enzogenol may have therapeutic potential targeting inflammatory response-associated atherosclerosis.

KEYWORDS: Adhesion molecules; enzogenol; NF-$\kappa$B; transendothelial migration

## INTRODUCTION

Atherosclerosis is a chronic and progressive immune-inflammatory disease leading to advanced cardiovascular diseases such as coronary thrombosis, myocardial infarction, and stroke. Leukocyte transmigration begins with a multistep adhesion process that captures leukocytes from circulating blood and adheres them to the vascular wall. Adhesive selectin molecules initiate the rolling of leukocytes along inflamed endothelium (*1*, *2*). The vascular endothelial cells are an important target of proinflammatory cytokine tumor necrosis factor (TNF)-α, in which it modulates endothelial expression of many genes involved in cell adhesion, thrombosis, and inflammatory responses (*3*, *4*). Adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) have been shown to play an important role in the induction of

inflammation (*5*). Extravasation of circulating leukocytes requires the secretion of matrix-degrading enzymes, matrix metalloproteinases (MMP), which allow leukocytes to penetrate the vessel wall into the extracellular matrix. It has been suggested that MMP-9 is involved in the disruption of basement membrane required for the process of monocytes transmigration (*6*, *7*).

Flavonoids are found as flavonols, flavones, flavanols, flavanones, and proanthocyanidines in a large number of plants. Flavonoids are known to have antioxidant, antitumor, antiangiogenic, anti-inflammatory, and antiallergic activities (*8*–*10*). Flavonoids act as anti-inflammatory agents to inhibit expression of cell adhesion molecules and matrix metalloproteinases (*11*, *12*). In our previous study (*11*), flavones inhibited TNF-α-induced expression of VCAM-1, ICAM-1, and E-selectin at the transcriptional levels. It was shown that grape seed proanthocyanidin extract inhibited agonist-induced VCAM-1 expression not through a nuclear factor-kappa B (NF-$\kappa$B)-dependent pathway (*13*). However, a polyphenol-rich pomegranate fruit extract suppressed

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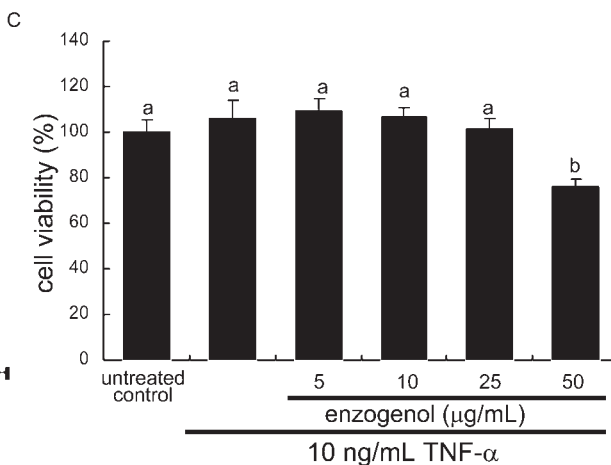| | origin | major components |
|---|---|---|
| enzogenol | Pine bark of New Zealand *Pinus Radiata* trees | a mixture of proanthocyanidins, bioflavonoids (catechin, epicatechin, phenolic acids, quercetin, dihydroquercetin, taxifolin), organic acids |
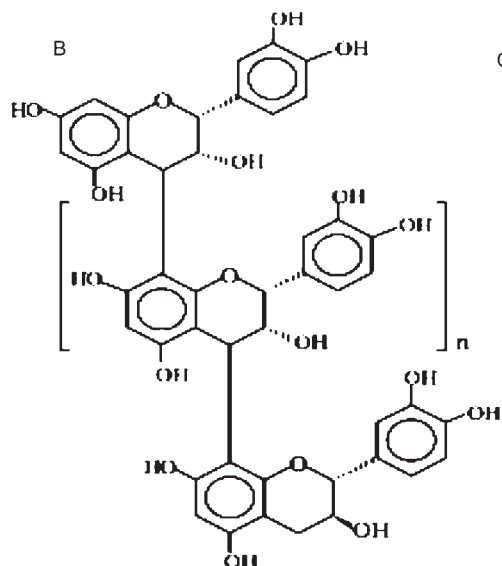


oligo- or poly-proanthocyanidins

**Figure 1.** Major components of enzogenol (A) and structure of oligo- and poly- proanthocyanidins formed from catechin and epicatechin (B). Information for major component profiles of enzogenol was provided from New Directions (Sydney, Australia), where it was shown that enzogenol contained $76-84\%$ proanthocyanidin oligomers/polymers (% mass). HUVEC viability was measured using an 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) assay after 24 h-culture with $5-50\,\mu g/mL$ enzogenol and 10 ng/mL TNF-$\alpha$ (C). Values are means $\pm$ SEM, $n = 3$, expressed as percent cell survival relative to TNF-$\alpha$- and enzogenol-untreated controls (viability = 100%). Values not sharing a letter are different at $P < 0.05$.

expression of proinflammatory cytokines by inhibiting the activation of mitogen-activated protein kinase (MAPK) and NF-$\kappa$B in activated human mast cells and basophils (*14*). Cinnamon polyphenol extract affected immune responses by regulating anti- and proinflammatory and glucose transporter gene expression in mouse macrophages (*15*).

Enzogenol (New Directions, Sydney, Australia) is a commercially available proanthocyanidins-rich bioflavonoid extract derived from the pine bark of New Zealand *Pinus radiate* trees (*16*). Enzogenol is a polyphenolic mixture of natural constituents extracted from the pine bark (**Figure 1A**). In addition, over 30 components have been positively identified as bioactive constituents present in enzogenol (*17*). Major components present in enzogenol are known to be procyanidin dimers, trimers, oligomers, and polymers formed from catechin and epicatechin (**Figure 1B**), flavonoids such as catechin and taxifolin, and phenolic acids (*17*). The superoxide scavenging activity of enzogenol was much more effective as an antioxidant than ascorbic acid under aqueous conditions (*18*). Dietary pine bark extract reduced development of atherosclerotic lesions in male apolipoprotein E (apoE)-deficient mice by lowering the serum cholesterol level, suggesting antiatherogenic effects (*19*). Much research has been conducted on pine bark extract pycnozenol derived European maritime pine (*Pinus maritime*). French maritime pine bark extract pycnogenol exhibited anti-inflammatory and platelet-inhibitory effects by inhibiting activity of cyclooxygenase COX-1 and COX-2 in human plasma (*20*).

On the basis of the existing data on pine bark extract, the present study assessed antiatherogenic properties of enzogenol with respect to the monocyte trafficking on the activated endothelium. This study examined that enzogenol could attenuate expression of cell adhesion molecules in the leukocyte-endothelium interaction system activated by TNF-$\alpha$. It tested the possibility that NF-$\kappa$B-dependent mechanism(s) are involved in enzogenol-mediated down-regulation of expression of cell adhesion molecules in human umbilical vein endothelial cells (HUVEC). Furthermore, the leukocyte extravasation was elucidated by measuring MMP activity and monocyte transmigration of human monocytic THP-1 cells.

**MATERIALS AND METHODS**

**Materials.** M199, RPMI-1640, gelatin, and MTT were obtained from Sigma-Aldrich Chemical (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, human epidermal growth factor, and hydrocortisone were purchased from Cambrex (East Rutherford, NJ). Human monocytic leukemic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA). Tumor necrosis factor (TNF)-$\alpha$ was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Antibodies against human vascular cell adhesion molecule-1 (VCAM-1), human intracellular cell adhesion molecule-1 (ICAM-1), human platelet endothelial cell adhesion molecule-1 (PECAM-1), and human integrin $\beta 2$, human nuclear factor (NF)-$\kappa$B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human I$\kappa$B$\alpha$ and phospho-I$\kappa$B$\alpha$ (ser 32) were obtained from Cell signaling Technology (Beverly, MA). Human $\beta$-actin antibody was purchased from Sigma Chemicals. Horse-radish peroxidase-conjugated goat antirabbit IgG, donkey antigoat IgG and goat antimouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Reverse transcriptase and *Taq* DNA polymerase were purchased from Promega (Madison, WI).

**Preparation and Culture of HUVEC.** HUVEC were isolated from human umbilical cords using collagenase as described elsewhere (*21*). Human umbilical cords were obtained from the Department of Obstetrics and Gynecology, Chuncheon Sacred Heart Hospital (Chuncheon, Korea). Cells were cultured in 25 mM HEPES-buffered M199 containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin supplemented with 0.075 mg/mL human epidermal growth factor and 0.075 mg/mL hydrocortisone. Cultures were maintained at 37 °C humidified atmospheres of 5% $CO_2$ in air. Endothelial cells, confirmed by their cobblestone morphology, were passaged at confluence and used within 10 passages.

HUVEC were plated at 90−95% confluence in all experiments. In experiments for the TNF-$\alpha$-induced expression of ICAM-1 and VCAM-1, cells were incubated for 18 h with 0−25 $\mu$g/mL enzogenol prior to an exposure to 10 ng/mL TNF-$\alpha$ for 6 h. It was previously shown that TNF-$\alpha$-activated HUVEC induced VCAM-1 and ICAM-1 with peak expression at 6 h (*22*).

**Cell Viability Assay.** At the end of the incubation with enzogenol in the absence and presence of TNF-$\alpha$, an MTT assay was carried out to quantify cellular viability. HUVEC were incubated in a fresh medium containing 1 mg/mL MTT for 3 h at 37 °C. The purple formazan product was dissolved in 0.5 mL of isopropanol with gentle shaking. Absorbance of formazan was measured at $\lambda$ = 570 nm using Bio-Rad model 550 microplate reader (Hercules, CA). Nontoxic concentrations of enzogenol were ≤25 $\mu$g/mL in 24 h-culture experiments.

**Culture of THP-1 Monocytes and in Vitro Cell Adhesion Assay.** HUVEC were grown in 25 mM HEPES-buffered M199 at density of $7.0 \times 10^4$ cells on 24-well glass chamber slides. THP-1 cells were labeled with 5 $\mu$M calcein-AM (Molecular Probes, Eugene, OR) in RPMI 1640 medium containing 10% FBS. HUVEC were pretreated with 0−25 $\mu$g/mL enzogenol and exposed to 10 ng/mL TNF-$\alpha$ for 6 h. For the coculture experiments, the calcein-AM-labeled THP-1 cells were seeded at a density of $5.0 \times 10^5$ cells/mL on enzogenol-treated and TNF-$\alpha$-activated HUVEC and incubated for 1 h. After the cocultured cells were thoroughly washed with phosphate buffered saline, photograph images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope (Diagnostic Instrument, Livingston, Scotland).

**Protein Isolation and Western Blot Analysis.** After culture protocols, whole HUVEC lysates were prepared in 1 M Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1% $\beta$-glycerophosphate, 0.1 M $Na_3VO_4$, 0.5 M NaF, and protease inhibitor cocktail. Cell lysates containing equal protein amounts were electrophoresed on 8−10% SDS−PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in TBS-T buffer [0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated for overnight with primary antibodies of antihuman ICAM-1 and antihuman VCAM-1. After three washes with TBS-T buffer, the membrane was then incubated for 1 h with goat antirabbit IgG, donkey antigoat IgG, or goat antimouse IgG horseradish peroxidase. The levels of ICAM-1 and VCAM-1 proteins were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) and AGFA medical X-ray film (Gevaert, Belgium). Incubation with antihuman $\beta$-actin was performed for the comparative control.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis.** Total RNA was isolated from HUVEC using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA). The RNA (5 $\mu$g) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 $\mu$g/$\mu$L oligo-(dT)$_{15}$ primer (Bioneer Co., Korea). The expressions of the mRNA transcripts of ICAM-1 (forward primer: 5′-GGTGACGCT-GAATGGGGTTCC-3′, reverse primer: 5′- GTCCTCATGGTGGGGC-TATGACTC-3′), VCAM-1 (forward primer: 5′-ATGCCTGGGAAGA-TGGTCGTGA- 3′, reverse primer: 5′- TGGAGCTGGTAGACCC TC-GCTG-3′), E-selectin (forward primer: 5′-ATCATCCTGCAACTTCA-CC-3′, reverse primer: 5′-ACACCTCACCAAACCCTTC-3′), and $\beta$-actin (forward primer: 5′-GACTACCTCATGAAG ATC-3′, reverse primer: 5′-GATCCACATCTGCTGGAA-3′) were evaluated by RT-PCR as previously described (*11*). The PCR was performed in 25 $\mu$L of 10 mM Tris-HCl (pH 9.0), 25 mM $MgCl_2$, 10 mM dNTP, 5 units of Taq DNA polymerase, and 10 $\mu$M of each primer and terminated by heating at 94 °C for 10 min. After thermocycling and electrophoresis of the PCR products

(25 $\mu$L) on 1% agarose gel, the bands were visualized using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, France), and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

**Gelatin Zymography.** Gelatin zymography of MMP-9 was performed as previously described with minor modification (*21*). Briefly, the culture supernatants were subject to electrophoresis on 10% SDS−PAGE (0.3 M Tris-HCL, pH 6.8, 4% SDS, 20% glycerol, 0.03% bromophenol blue) copolymerized with 1% gelatin as the substrate. After electrophoresis was terminated, the gel was incubated for 1 h at 37 °C in a 2.5% Triton X-100 solution, washed in 50 mM Tris-HCl buffer (pH 7.5) for 30 min, and incubated for 20 h in incubation buffer containing 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij-35. The gel were stained with 0.1% Coomassie Brilliant Blue G-250, 2% acetic acid, and 45% methanol, and then destained in a solution with 30% methanol and 10% acetic acid.

**Immunocytochemistry.** After HUVEC challenged with 10 ng/mL TNF-$\alpha$ were thoroughly washed with TBS and fixed with 4% formalde-hyde for 15 min, cells were incubated for 1 h with 4% FBS in TBS to block any nonspecific binding. After washing with TBS, polyclonal rabbit antihuman NF-$\kappa$B p65 was added to cells and incubated overnight at 4 °C. Cells were washed with TBS and incubated with a cyanine 3-conjugated goat antirabbit IgG as a secondary antibody. Fluorescent images were obtained by a fluorescence microscope.

**Measurement of THP-1 Monocyte Transmigration.** The experimental models for the monocyte transmigration employed 24-transwell inserts with pore sizes of 8 $\mu$m (Corning Incorporation, Corning, NY). The lower surface of the insert filter was coated with 10 $\mu$L of 1 mg/mL gelatin solution and dried for 1 h. The calcein AM-labeled THP-1 cells ($1 \times 10^6$ cells/mL) in serum-free RPMI 1640 were loaded in the upper compartment of the transwell inserts. These cells were treated with 25 $\mu$g/mL enzogenol and exposed to 10 ng/mL TNF-$\alpha$ for 24 h. Microscopic images were obtained using fluorescence microscopy (three independent experiments). Original magnification, 200×. For the quantification of transendothelial migration, the calcein-AM fluorescent intensity was measured at 485 nm excitation and 538 nm emission by using a Fluoroskan ELISA plate reader (Labsystem Oy, Helsinki, Finland).

**Data Analysis.** The results are presented as mean ± SEM for each treatment group in each experiment. Statistical analyses were conducted using the Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was determined by one-way ANOVA followed by Duncan multiple range test for multiple comparisons. *P* values < 0.05 were considered statistically significant.

## RESULTS

**Effects of Enzogenol on Endothelial Cytotoxicity.** To examine HUVEC toxicity of enzogenol, the MTT analysis was performed. When enzogenol in concentrations between 1 and 50 $\mu$g/mL was added to TNF-$\alpha$-activated HUVEC, the viability was not significantly influenced at ≤25 $\mu$g/mL (**Figure 1C**). Accordingly, nontoxic enzogenol at 5−25 $\mu$g/mL was employed in experiments for the transendothelial migration of monocytes.

**Inhibitory Effects of Enzogenol on Expression of Adhesion Molecules.** Western blot analysis was performed to address whether enzogenol inhibited the induction of ICAM-1, VCAM-1, and E-selectin triggered by TNF-$\alpha$. There was no or relatively weak expression of all three adhesion molecules in quiescent cells (**Figure 2A**). Expression of these adhesion molecule proteins was greatly elevated in TNF-$\alpha$-stimulated HUVEC. When 5−25 $\mu$g/mL enzogenol was added, the enhanced expression of ICAM-1, VCAM-1, and E-selectin was inhibited substantially but yet not fully in a dose-dependent manner. These results imply that enzogenol may block leukocyte trafficking in response to inflammatory mediators by inhibiting induction of endothelial adhesion molecules.

This study attempted to elucidate that enzogenol treatment might inhibit mononuclear leukocyte recruitment on the TNF-$\alpha$-induced vascular endothelium. The in vitro adhesion assay of

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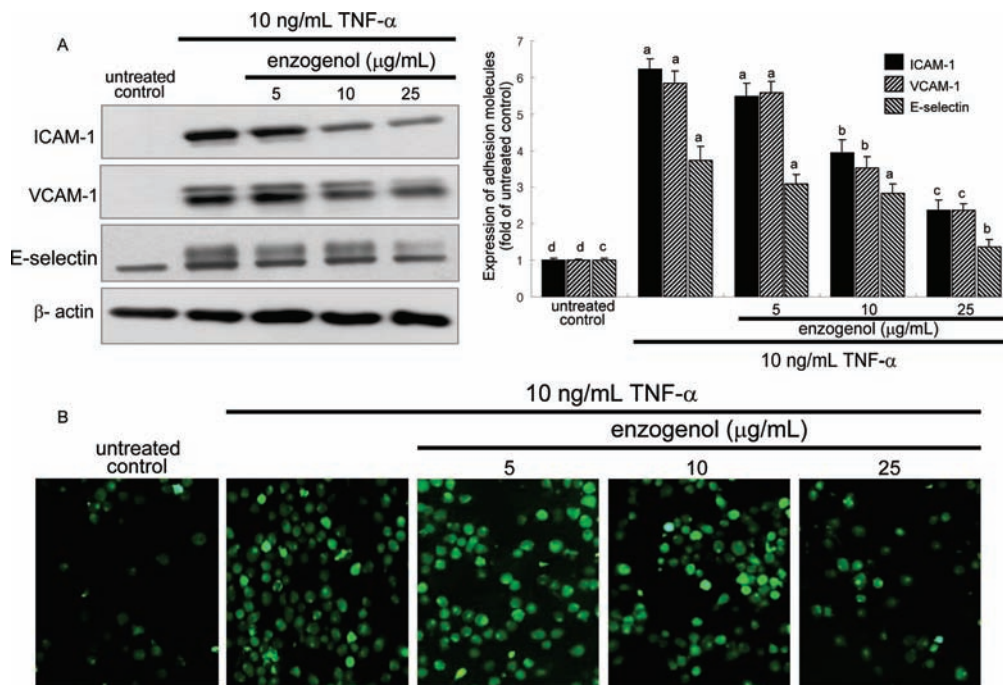
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**Figure 2.** Inhibitory effects of enzogenol on cell adhesion molecules expression on (**A**) and THP-1 monocyte adhesion to TNF-α-activated HUVEC (**B**). Western blot data showing effects of enzogenol on expression levels of ICAM-1, VCAM-1, and E-selectin in TNF-α-stimulated HUVEC. After HUVEC culture protocols, cell lysates were subjected to 8% SDS−PAGE and Western blot analysis with a primary antibody ICAM-1, VCAM-1, and E-selectin (**A**). The β-actin protein was used as an internal control. The bar graphs (means ± SEM, *n* = 3) in the right panel represent quantitative results obtained from a densitometer. Respective values not sharing a letter are different at *P* < 0.05. Monocyte staining showing inhibition of THP-1 monocyte adhesion to the TNF-α-activation HUVEC by enzogenol (**B**). HUVEC were pretreated with and without enzogenol at 5−25 μg/mL for 18 h and then activated with 10 ng/mL TNF-α for 6 h. HUVEC were cocultured with calcein AM-labeled THP-1 monocytes for 1 h. Microphotographs (three independent experiments) were obtained using a fluorescence microscopy with fluorecein blue filter. Magnification: 200×.

monocytes to HUVEC using a calcein-AM staining technique supported this hypothesis. A small number of monocytes adhered to quiescent HUVEC free of TNF-α (**Figure 2B**). There was heavy staining on the HUVEC exposed to TNF-α alone for 6 h, indicative of a marked increase in THP-1 adherence to the activated HUVEC. However, adding enzogenol at nontoxic doses of 5−25 μg/mL to TNF-α-exposed cells attenuated the monocyte adherence markedly in a dose-dependent manner (**Figure 2B**). It should be noted that ≥10 μg/mL enzogenol was effective in inhibiting leukocyte adhesion to TNF-α-activated endothelium. Accordingly, the Western blot data (**Figure 2A**) supported the in vitro adhesion results (**Figure 2B**).

PECAM-1, concentrated in the junctions between endothelial cells, mediates adhesive interactions required during transmigration (*23*). This study investigated whether TNF-α can induce another adhesion molecule PECAM-1 and the induction can be inhibited by treating enzogenol. The PECAM-1 expression was enhanced in TNF-α-stimulated HUVEC, which was considerably retarded by 25 μg/mL enzogenol (**Figure 3A**). It was deemed that enzogenol may block monocyte transmigration following the tight adhesion.

PECAM-1 is also expressed on the surfaces of monocytes and neutrophils (*23*). This study showed that enzogenol at 25 μg/mL reduced the PECAM-1 induction of THP-1 cells exposed to TNF-α (**Figure 3A**), indicating that the THP-1 cell transmigration was inhibited over the intercellular junction. The tight adhesion is known to be mediated by the leukocyte integrins and their endothelial cell counter-receptors (*24*). In this study, enzogenol repressed the integrin β2 induction of THP-1 cells upregulated by TNF-α (**Figure 3B**).

**Suppression of Transcription of Adhesion Molecules by Enzogenol.** There were no signals for the basal mRNA expression of

ICAM-1, VCAM-1, and E-selectin in untreated HUVEC (**Figure 4**). In contrast, RT-PCR data showed that the mRNA levels of VCAM-1, ICAM-1, and E-selectin were greatly enhanced in TNF-α-stimulated cells. The mRNA levels of in enzogenol treated cells were remarkably diminished, suggesting a marked inhibition of these cell adhesion molecules by enzogenol at the transcriptional levels (**Figure 4**). These observations were consistent with a substantial attenuation of protein expression of adhesion molecules by enzogenol (**Figure 2A**).

**Blockade of Cellular Localization and Transactivation of NF-κB by Enzogenol.** We tested whether enzogenol inhibits TNF-α-induced stimulation of expression of adhesion molecules by interfering with the transactivation of NF-κB. Following exposure to 10 μg/mL TNF-α, IκBα phosphorylation and nuclear NF-κB p65 increased (**Figure 5A**). When TNF-α-exposed cells were treated with 50 μg/mL enzogenol, the IκBα phosphorylation decreased. Enzogenol mitigated the nuclear level of NF-κB p65 protein, whereas the NF-κB p65 protein in the cytosolic extract modestly increased. In addition, intracellular localization of NF-κB p65 in HUVEC was evaluated by fluorescent microscopy using specific NF-κB p65 antibody (**Figure 5B**). Cytoplasmic immunofluorescence staining was observed in untreated HUVEC, while heavy nuclear staining in cells exposed to TNF-α alone occurred, indicative of nuclear localization of activated NF-κB p65 at the single cell level. However, enzogenol- and TNF-α-treated cells demonstrated the diminished staining level of nuclear p65.

**Inhibition of MMP-9 Activity by Enzogenol in TNF-α-Exposed THP-1 Monocytes.** This study examined the MMP-9 activity in TNF-α-exposed THP-1 monocytes treated with 5−25 μg/mL enzogenol. In the process of inflammation, MMP-9 plays a crucial role for migration, extravasation, and infiltration of
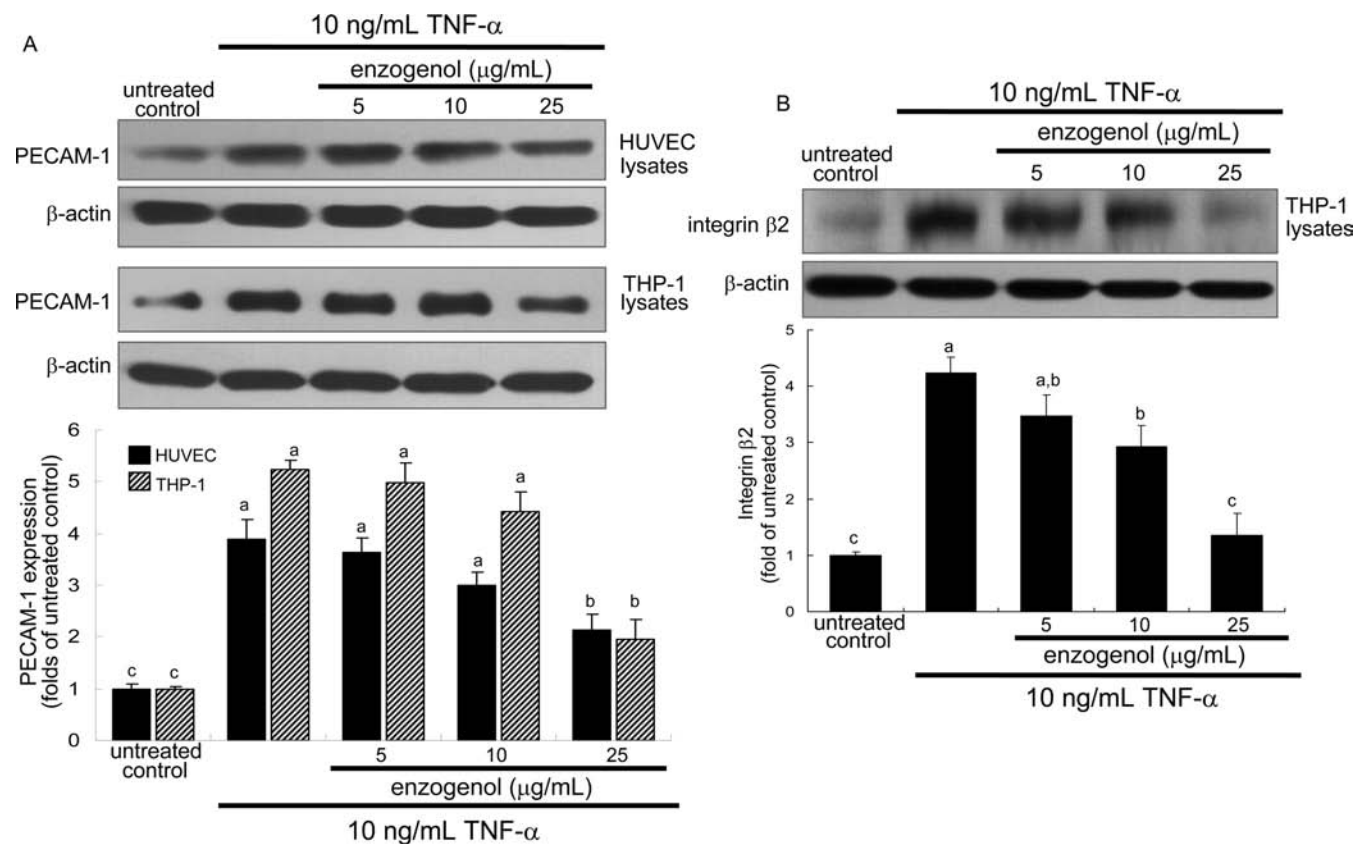
**Figure 3.** Western blot analysis showing suppression of PECAM-1 expression (**A**) and integrin β2 induction (**B**) by enzogenol in HUVEC or THP-1 monocytes exposed to TNF-α. HUVEC and THP-1 cells were pretreated with and without 5−25 μg/mL enzogenol for 18 h and activated with 10 ng/mL TNF-α for 6 h. After cell culture protocols, Western blot analysis was performed with cell lysates using a primary antibody PECAM-1 and integrin β2 (three independent experiments). The β-actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) represent quantitative results obtained from a densitometer. Respective values not sharing a letter are different at $P < 0.05$.

immune cells including monocytes (*25*). In THP-1 monocytes treated with 10 ng/mL TNF-α under serum-free conditions, the MMP-9 secretion and its gelatinolytic activity in culture media within 24 h were augmented, as evidenced by Western blot analysis and gelatin zymography (**Figure 6A,B**). In contrast, enzogenol dose-dependently attenuated MMP-9 secretion and activity up-regulated in TNF-α-exposed THP-1 cells. Additionally, enzogenol at ≥5 μg/mL abrogated the elevated gelatinolytic activity of MMP-9 (**Figure 6B**).

**Targeting TNF-α-Activated Transmigration of THP-1 Monocytes by Enzogenol.** As MMP facilitate the passage of leukocytes across matrix barriers, this study tested the hypothesis that targeting MMP-9 by enzogenol could attenuate transendothelial migration of THP-1 cells. Labeled monocytes that transmigrated across the gelatin layer mimicking basement membranes were collected for 24 h. These monocytes are assumed to have undergone the process of diapedesis in addition to being exposed to MMP-9 and contacting stimulated EC directly. The TNF-α-activated transmigration of THP-1 monocytes was markedly augmented by 2.5-fold (**Figure 6C**). On the contrary, the elevated transmigration was attenuated in 25 μg/mL enzogenol-treated THP-1 cells, indicating that enzogenol reduced cytokine-stimulated monocyte extravasation by suppressing MMP-9 secretion.

## DISCUSSION

Polyphenols are shown to delay LDL oxidation mainly through their antioxidant capacity (*26*, *27*), suggesting that administration of polyphenols may suppress the progression of atherosclerosis. Grape powder polyphenols attenuate atherosclerosis development

in apolipoprotein E deficient mice and reduce macrophage atherogenicity by reducing macrophage-mediated oxidation of LDL and cellular uptake of oxidized LDL (*28*). Polyphenols may inhibit early events in the atherosclerotic process by modulating monocyte adhesion and transmigration. The transmigration and extravasation of leukocytes across the endothelium that lines the vessel wall occur in distinct multisteps first comprising rolling of the leukocytes over the endothelial cells, resulting in a tightly controlled and very complex system of cell trafficking. Although definite mechanisms underlying polyphenol protection against early atherogenic process are not fully understood, they may involve down-regulation of inflammatory cytokines, matrix proteases, and cell adhesion molecules (*29−31*). In this report employing the leukocyte-endothelium interaction system activated by proinflammatory cytokines, the pine bark extract enzogeneol at 10−25 μg/mL attenuated THP-1 monocyte adhesion to TNF-α-activated endothelial cells, at least in part by an inhibition of expression of cell adhesion molecules of ICAM-1, VCAM-1, E-selectin, and PECAM-1, each involved in different successive stages of the process of leukocyte transmigration (*23*).

Dietary pine bark extract rich in oligomeric proanthocyanidins (OPC) exhibited antiatherogenic effects by reducing development of atherosclerotic lesions in male apoE-deficient mice with lowering the serum cholesterol level (*19*). OPC belonging to a special family of flavonoids are found in many common foods including grape seed extract and red wine. Pine bark extracts contain various flavonoids beside the ubiquitous proanthocyanidins (*17*). Pine bark extract enzogenol has been shown to contain a powerful antioxidant that helps protect cells from free radical damage
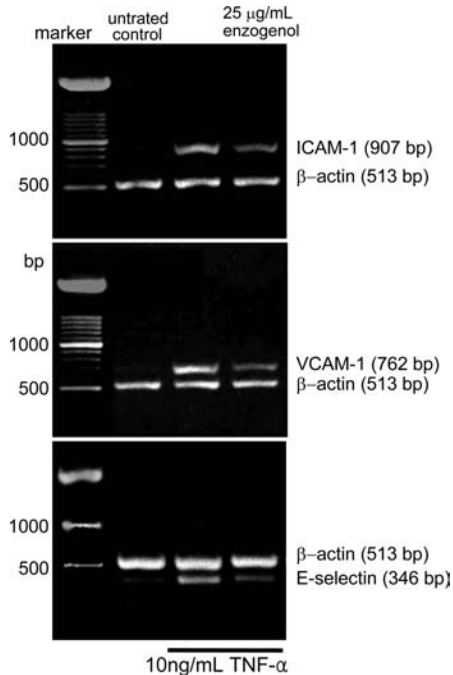
**Figure 4.** Reverse transcription polymerase chain reaction data showing the steady-state mRNA transcriptional levels of ICAM-1, VCAM-1, and E-selectin in enzogenol-treated and TNF-α-stimulated HUVEC. Confluent HUVEC were incubated with 25 $\mu$g/mL enzogenol for 18 h and exposed to 10 ng/mL TNF-α for 6 h. $\beta$-Actin mRNA level was used as an internal control for coamplification with ICAM-1, VCAM-1, and E-selectin (three separate experiments).
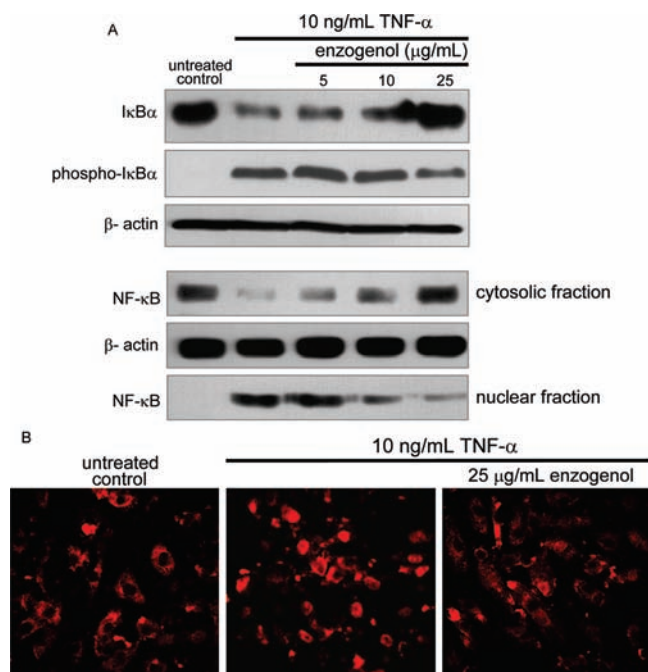


**Figure 5.** Effects of enzogenol on phosphorylation of IκB and translocation of NF-κB p65 in TNF-α-treated HUVEC. HUVEC extracts pretreated with 5–25 $\mu$g/mL enzogenol and exposed to 10 ng/mLTNF-α were electrophoresed on 8% SDS–PAGE gel, followed by Western blot analysis with a primary antibody against human IκBα, phospho-IκBα, and NF-κB p65 (four independent experiments) (**A**). NF-κB localization was visualized by binding with a cyanine 3-conjugated secondary antibody (**B**). Microscopic images were obtained using fluorescence microscopy (three independent experiments). Original magnification, 200×.

and increases the effectiveness of vitamin C (*32*). Much research has been conducted on pine bark extract pycnozenol with anti-inflammatory actions. Pycnogenol exerted viral myocarditis by decreasing myocardial infiltration and by suppressing expression of proinflammatory cytokines and cardiac remodeling genes in mouse hearts (*33*). In addition, pycnogenol supplementation inhibited COX-2 and 5-lipoxygenase gene expression and alleviated leukotriene biosynthesis in human polymorpho-nuclear leukocytes upon proinflammatory stimulation ex vivo (*34*). Similarly, grape seed extract rich in proanthocyanidins inhibited agonist-induced VCAM-1 expression, suggesting therapeutic potential in inflammatory conditions (*13*). Red wine rich in bioflavonoids with OPC has been shown to protect against atherosclerosis by inhibition of the accumulation of oxidized LDL in atherosclerotic lesions, para-oxonase elevation, and removal of atherogenic lesions of apoE-deficient mice (*35*).

The TNF-α-elevated integrin $\beta2$ induction, gelatinolytic MMP-9 activity, and monocyte transmigration of THP-1 were attenuated by the supplementation of enzogenol, which may contribute to its atheroprotective actions with respect to pro-inflammatory cytokine-triggered monocyte extravasation. The MMP-9 release from human monocytes and NF-κB activation were significantly inhibited in human plasma after oral intake of pycnogenol, indicating that it may exert anti-inflammatory effects by inhibition of proinflammatory gene expression (*36*). However, there have been little anti-inflammatory actions of dietary en-zogenol reported in terms of interrupting monocyte extravasa-tion. The findings of this study were consistent with other studies that have reported potential atheroprotective effects following supplementation with proanthocyanidins and bioflavonoids. Treatment of grape seed proanthocyanidin extract down-regulated the expression of urokinase plasminogen activator and type 1 plasminogen activator that play vital roles in various biological processes involving extracellular proteolysis and cell migration (*37*). It was reported that green tea catechin extracts prevent vascular inflammation most likely due to their suppres-sion of leukocyte adhesion to endothelium and subsequent transmigration of inflammatory cells (*38*).

How the cell adhesion molecule genes are selectively modulated in response to proinflammatory cytokines and which signaling pathways are involved in the selective regulation of these genes remain unknown. Clearly, the activation of endothelial expres-sion of ICAM-1, VCAM-1, and E-selectin by TNF-α was blunted possibly by a novel mechanism(s) responsive to enzogenol. Transcriptional expression of cell adhesion molecules involved NF-κB-responsive pathway in the leukocyte-endothelium inter-action system and such expression was mitigated by enzogenol. Pomegranate fruit extract rich in polyphenols dampened expres-sion of proinflammatory cytokines via inhibiting the activation of MAPK and NF-κB in human mast cells and basophils (*14*). In contrast, grape seed proanthocyanidin extract inhibited agonist-induced VCAM-1 expression not through NF-κB-dependent pathway (*13*). In a recent study (*39*), grape seed proanthocyani-dins protect cardiomyocytes from ischemia and reperfusion injury by enhancing nitric oxide (NO) production and increas-ing Akt-endothelial NO synthase signaling. However, definite mechanisms underlying the atheroprotection of enzogenol were not yet fully understood in this study.

In summary, our study demonstrated that the pine bark extract enzogenol block the early atherogenic process involving endothe-lial expression of inducible cell adhesion molecules and monocyte secretion of MMP-9. Consequently, enzogenol may be effective in reducing trafficking and extravasation of circulating leukocytes. Although definite mechanisms underlying the atheroprotection
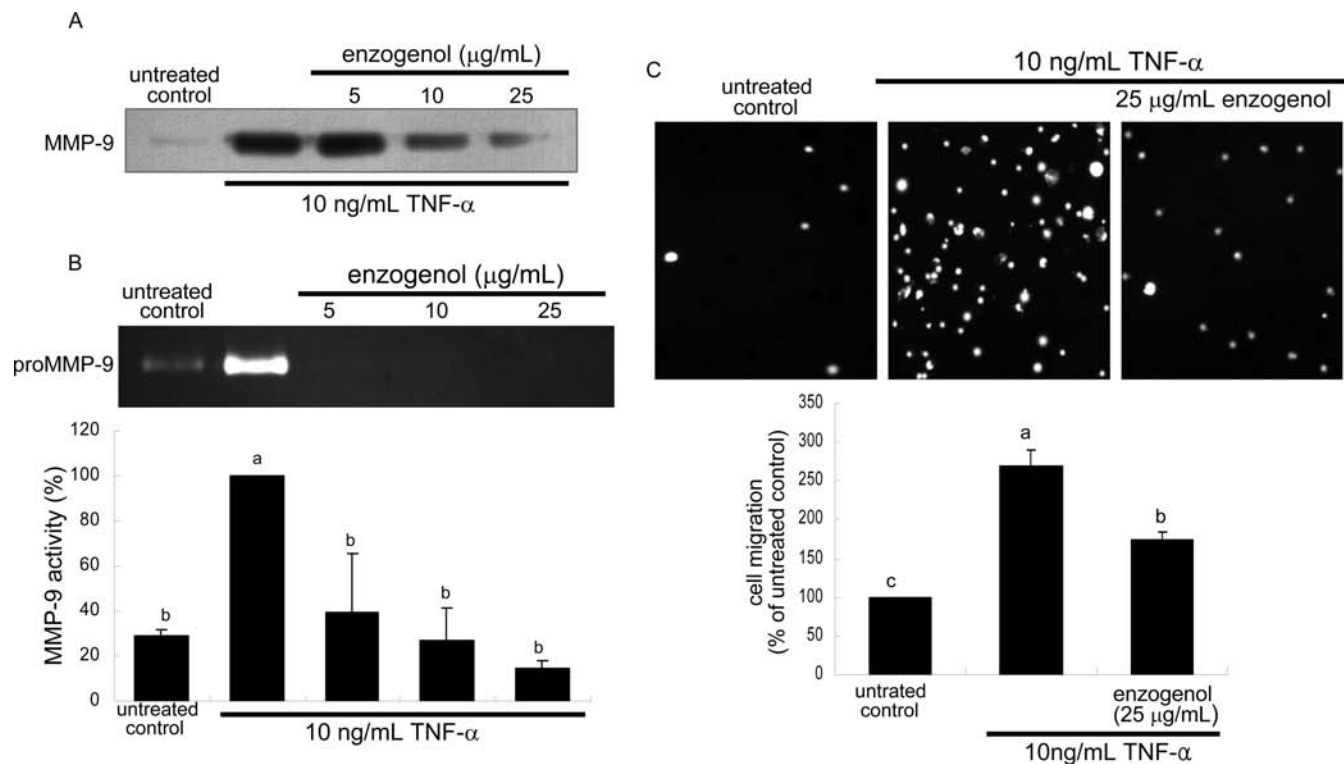
**Figure 6.** Secretion (**A**) and gelatinolytic activity of MMP-9 (**B**) and transendothelial migration (**C**) in TNF-α-exposed THP-1 monocytes. THP-1 monocytes were treated with 5−25 μg/mL enzogenol overnight prior to an addition of 10 ng/mL TNF-α under serum-free conditions, and continuously incubated for 24 h. For the secretion of MMP-9 (**A**), the collected culture media were subject to Western blot analysis with a primary antibody against human MMP-9 (three independent experiments). For the measurement of MMP-9 activity (**B**), the collected culture media were subject to electrophoresis on 10% SDS−PAGE copolymerized by 1% gelatin as the substrate. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Gel photographs (three separate experiments) were obtained, and the bar graphs (means ± SEM, $n = 3$) in the bottom panel represent quantitative results were obtained from a densitometer. Inhibition of TNF-α-induced transendothelial migration of THP-1 monocytes by enzogenol (**C**). The calcein AM-labeled THP-1 monocytes were incubated in serum free-RPMI 1640 on a transwell insert with 1 mg/mL gelatin (10 μL)-coated lower surface. Cells were incubated for 24 h in the presence of 25 μg/mL enzogenol and 10 ng/mL TNF-α. Microphotographs (three independent experiments) were obtained using a fluorescence microscopy with fluorecein blue filter. Magnification: 100×. The bar graphs were obtained by using a Fluoroscan ELISA plate reader at 485 nm excitation and 538 nm emission. Values not sharing a letter are different at $p < 0.05$.

of enzogenol against the early atherogenic process are not fully understood, the expression of cell adhesion molecules in the leukocyte-endothelium interaction system entailed a NF-κB-dependent pathway, which may be a pivotal target of the antiatherogenic actions of enzogenol. Accordingly, these observations might have clinical implications for therapeutic strategies preventing and attenuating inflammatory diseases including atherosclerosis.

## ABBREVIATIONS USED

FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule-1; IκB, inhibitory kappa B; MMP, matrix metalloproteinases; MTT, 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide; NF-κB, nuclear factor-kappa B; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1

## ACKNOWLEDGMENT

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