

Hexane extract of aged black garlic reduces cell proliferation and attenuates the expression of ICAM-1 and VCAM-1 in TNF- α -activated human endometrial stromal cells

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Abstract. Increasing evidence indicates the potentially crucial roles of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the pathological process underlying endometriosis. The present study aimed to investigate the effects of a hexane extract of aged black garlic (HEABG) on the proliferation and expression of ICAM-1 and VCAM-1 in tumor necrosis factor- α (TNF- α)-activated human endometrial stromal cells (HESCs) isolated from patients with endometriosis. HESCs were isolated from endometriotic tissues obtained from women with advanced endometriosis who underwent laparoscopic surgery for ovarian endometrioma (n=18). Cell proliferation and cell cycle analysis were assessed by WST-1 assay and flow cytometry, respectively. The expression of ICAM-1 and VCAM-1 was measured by flow cytometry, immunofluorescence staining, immunoblotting and quantitative reverse transcriptase-PCR. The secretion of interleukin-6 (IL-6) was determined by enzyme-linked immunosorbent assay (ELISA). The activation of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) was detected by electrophoretic mobility shift assay (EMSA) and the activation of c-Jun N-terminal kinase (JNK), extra-

cellular signal-regulated kinase (ERK) and p38 MAPK was analyzed by immunoblotting. Cell proliferation and cell cycle progression were significantly suppressed by HEABG in the TNF- α -induced HESCs through the inhibition of the ERK and JNK signaling pathways. Remarkably, the treatment of the HESCs with HEABG potently suppressed the TNF- α -induced ICAM-1 and VCAM-1 transcript and protein expression by inhibiting the activation of NF- κ B and AP-1 transcription factors. Our results suggest that HEABG may be effective in the prevention and treatment of endometriosis in humans.

Introduction

Endometriosis is a benign, estrogen-dependent, gynecological disorder that is defined as the presence of endometrial glands and stroma outside the uterine cavity. Women with endometriosis may present with chronic pelvic pain, dysmenorrhea, dyspareunia and/or infertility. Retrograde menstruation, peritoneal adhesion and outgrowth of endometrial tissue are major factors in the pathogenesis of endometriosis according to Sampson's classic implantation theory (1). However, the stimuli promoting the attachment and outgrowth of endometrial cells in the peritoneal cavity have remained largely unknown. Among many factors suggested to be involved in the pathogenesis of endometriosis, immunological and inflammatory responses have been suggested to play crucial roles in the development and progression of endometriosis, and thus the disease may be considered an immune-related chronic inflammatory disease (2,3).

Cell adhesion molecules mediate essential cell-cell interactions and play an important role in cell differentiation, the organization of the extracellular matrix and in the recruitment and aggregation of leukocytes from the circulation. Intercellular adhesion molecule-1 (ICAM-1, CD54) mediates various biological processes, including adhesive interactions with integrins, leukocyte extravasation and lymphocyte-mediated

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cytotoxicity (4). The expression of ICAM-1, which is upregulated in response to a number of inflammatory mediators, such as tumor necrosis factor (TNF)- α , is associated with a variety of inflammatory diseases and conditions (4). Similar to ICAM-1, vascular cell adhesion molecule-1 (VCAM-1, CD106) also has a well-characterized role in immunological responses in humans. VCAM-1 plays an important role in mediating mononuclear leukocyte-selective adhesion to vascular endothelial cells, preparing them for transendothelial migration into inflammatory foci. The upregulation of VCAM-1 in endothelial cells is induced by pro-inflammatory cytokines, such as TNF- α , interleukin (IL)-1 β and interferon (IFN)- γ (5). A growing body of evidence suggests that these critical cell adhesion molecules in various immune and inflammatory responses also play pivotal roles in the physiology and pathophysiology of the human endometrium. ICAM-1 is expressed in a regulated pattern by endometrial stromal cells during the menstrual cycle (6). ICAM-1 is expressed in cultured human endometrial stromal cells (HESCs), as well as in endometrial stromal cells in the human endometrium *in situ* (7,8). Furthermore, in patients with endometriosis, ICAM-1 is more strongly expressed in ectopic endometrial stromal cells than in eutopic endometrial stromal cells (9), and the elevated expression of ICAM-1 is induced in endometrial stromal cells by pro-inflammatory cytokines, such as IFN- γ (10). These data suggest that crosstalk may occur between endometrial stromal cells and leukocytes in the endometrium and peritoneal fluid via ICAM-1 and its receptors, contributing to the pathological processes of endometriosis. In addition to ICAM-1, VCAM-1 is expressed in HESCs, but little is known about the expression and role of VCAM-1 in endometriosis (11).

TNF- α , a pleiotropic pro-inflammatory cytokine, is regarded as an important regulatory molecule in eliciting inflammatory immune responses in endometriosis, and its abnormal production may contribute to the pathophysiology of endometriosis, including cell proliferation and adhesion (1,12). The nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) transcription factors play key roles in immune and inflammatory responses, and modulate cell proliferation, apoptosis, adhesion, invasion and angiogenesis in several cell types, including endometrial cells (13,14). The activation of NF- κ B has been implicated in the early development of endometriotic lesions *in vivo* (13). In particular, AP-1 and NF- κ B are the principal transcription factors that regulate the expression of adhesion molecules, such as ICAM-1 and VCAM-1 (15).

Garlic (*Allium sativum*; *A. Savitum*) has long been cultivated as a food and used as a home remedy in Asia. Garlic derivatives have important pharmacological properties, such as anti-inflammatory, anti-microbial, anti-thrombotic, anti-hypertensive, anti-hyperlipidemic, anti-hyperglycemic, immune system enhancement and anti-tumor properties (16). These findings indicate that garlic extracts are clinically effective for the treatment of diverse human diseases. Aged black garlic, intended as a healthy dietary supplement, is a type of fermented garlic used as a food ingredient in Korea, Thailand and Japan. Its unique qualities are the result of the fermentation of whole garlic bulbs under high temperatures for several months. Upon fermentation, regular garlic, which has a strong flavor and pungent odor, turns into soft, sweet, odorless and black garlic.

The aim of the present study was to investigate the effects of a hexane extract of aged black garlic (HEABG) on the cell proliferation and expression of ICAM-1 and VCAM-1 in TNF- α -activated HESCs isolated from patients with endometriosis.

Materials and methods

Plant materials. Garlic (*A. sativum* L.) specimens were collected in January 2008 from Changnyeong, Korea. Aged black garlic was manufactured by Newgreen Food Co. (Changnyeong, Korea) by incubating raw garlic at 75°C and 70% relative humidity for 2 weeks. A voucher specimen (accession no. NBG-PRDR-11) was deposited in the Plant Drug Research Laboratory of Pusan National University (Miryang, Korea).

Preparation of HEABG from aged black garlic. Aged black garlic extracts (500 ml) were purchased directly from Newgreen Food Co. in January 2009. The aged black garlic extracts were successively partitioned with hexane, chloroform and n-butanol. The upper suspension layer was filtered and evaporated under reduced pressure at 45°C and then lyophilized. A yellow, oily residue of hexane extract (17.6 mg) was obtained. The remaining aqueous layer was then sequentially partitioned with chloroform and n-butanol to yield chloroform (348 mg) and n-butanol fractions (11.53 g), respectively. The hexane fraction was used in this study. Hexane, chloroform, ethyl acetate, n-butanol and methanol were purchased from Fisher Scientific, Ltd. (Pittsburgh, PA, USA).

Subjects. Eighteen women (aged 25-45 years; mean age, 35.44 \pm 5.99 years) with advanced endometriosis (III/IV) were enrolled in this study (Table I). These women, who were treated for dysmenorrhea, pelvic pain or leiomyoma, were found to have endometriosis during laparoscopy, had no endometrial hyperplasia or neoplasia, and had not received anti-inflammatory nor any other hormonal treatment in the 3 months prior to surgery (Table I). The menstrual cycle phase of each patient enrolled in this study was recorded (Table I). Ovarian endometrioma specimens were obtained by performing laparoscopic surgery (Table I). Ovarian endometrioma capsules were removed from the normal ovarian cortex for pathologic examination and further culture preparation. Endometriotic biopsy samples were immediately placed in sterile Hanks' balanced salt solution (HBSS) containing 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin and maintained at 4°C. The diagnosis and staging of endometriosis in all cases was performed according to the revised American Society for Reproductive Medicine classification system (17) and confirmed by histopathological analysis. Written informed consent was obtained from all of the patients, and the study protocol was approved by the Institutional Review Board at Pusan National University Hospital.

Cell culture system. Immediately after the tissues were transported to the laboratory, cell culture was performed in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Chemicals and culture media were purchased from Life Technologies (Gaithersburg, MD, USA),

Table I. Clinical characteristics of patients, sources of endometriotic tissue biopsy samples, laparoscopic findings and clinical parameters.

Patient No.	Age (years)	Cell cycle phase	ASRM stage of endometriosis	Location of endometriotic tissue	Clinical parameters	Type of experiment
1	42	Proliferative	IV	Both ovaries	Dysmenorrhea	Cytotoxicity, proliferation
2	38	Secretory	IV	Both ovaries	Menorrhagia, dysmenorrhea	Cytotoxicity, qRT-PCR
3	44	Proliferative	IV	Right ovary	Presence of leiomyoma, intermittent pelvic pain	Proliferation, FACS
4	35	Proliferative	III	Left ovary	Presence of leiomyoma	Western blot, FACS
5	38	Secretory	IV	Right ovary	Dysmenorrhea, pelvic pain	Proliferation, ELISA
6	45	Proliferative	IV	Right ovary	Dysmenorrhea	Cytotoxicity, FACS
7	35	Proliferative	III	Left ovary	Dysmenorrhea	qRT-PCR, western blot
8	31	Secretory	IV	Right ovary	Pelvic pain	Western blot, IF
9	28	Secretory	IV	Left ovary	Dysmenorrhea	Proliferation, EMSA
10	36	Secretory	III	Left ovary	Pelvic pain, presence of leiomyoma	qRT-PCR, IF, FACS
11	30	Proliferative	IV	Both ovaries	Dysmenorrhea, presence of leiomyoma	IF, western blot, EMSA
12	41	Proliferative	IV	Both ovaries	Dysmenorrhea	FACS, EMSA
13	31	Proliferative	IV	Right ovary	Dysmenorrhea	IF, FACS
14	42	Secretory	IV	Right ovary	Dysmenorrhea, pelvic pain	Western blot, ELISA
15	28	Secretory	IV	Both ovaries	Intermittent pelvic pain	FACS, IF, proliferation
16	25	Proliferative	IV	Left ovary	Pelvic pain	FACS, ELISA
17	38	Proliferative	III	Right ovary	Dysmenorrhea	Western blot, qRT-PCR
18	31	Proliferative	IV	Both ovaries	Dysmenorrhea	Western blot, ELISA

ASRM, American Society for Reproductive Medicine; IF, immunofluorescence.

and cytokines were purchased from Chemicon International (Temecula, CA, USA). Culture flasks and dishes were obtained from BD Biosciences Discovery Labware (Bedford, MA, USA). Tissues were gently rinsed and washed in DMEM containing antibiotics/antimycotics (300 IU/ml penicillin G, 300 µg/ml streptomycin and 3 µg/ml fungizone). The tissues were then treated with 0.25% collagenase type I in DMEM at 37°C for 30 min in a shaking water bath. The cells were then passed through a 70-µm sieve (BD Biosciences Discovery Labware). Following sedimentation, the supernatant was transferred to a new tube and the cells were collected by centrifugation. The cells were maintained and proliferated in DMEM supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. The stromal cell-rich supernatant was placed in a culture flask and cells were allowed to adhere for 20 min, and then washed with the medium. Adherent stromal cells were cultured in monolayers in flasks with DMEM/F-12 (1:1) containing antibiotics/antimycotics, 5 µg/ml insulin (Sigma, St. Louis, MO, USA) and 10% FBS. Culture purity was determined morphologically after hematoxylin and eosin (H&E) staining. Stromal cells were fusiform, but more round than fibroblasts. Cells were also evaluated immunohistochemically using antibodies to human cytokeratin, vimentin, von Willebrand factor and CD45. The HESCs expressed vimentin, but not cytokeratin, von Willebrand

factor or CD45. As defined by these criteria, the purity of the HESCs was >97%. After the second subculture, the HESCs were cultured in DMEM/F-12 (1:1) containing antibiotics/antimycotics and 10% FBS. Subsequently, the culture medium was changed to serum-starved (5% FBS) DMEM/F-12 (1:1), and the cells were incubated for an additional 24 h prior to treatment.

Cell cytotoxicity assay. After the HESCs (4x10³ cells/well) in 96-well plates were treated with various doses of HEABG (1, 10, 30, 50 or 70 µg/ml) for 48 h, cell viability was determined using the colorimetric WST-1 conversion assay (Roche Diagnostics, Mannheim, Germany). A total of 10 µl of WST-1 reagent was added to each well, and the cells were incubated for 2 h in a humidified incubator at 37°C under 5% CO₂. Absorbance was measured in a microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm.

Cell proliferation assay. After the HESCs (4x10³ cells/well) in 96-well plates were pre-treated with various doses of HEABG (30 or 50 µg/ml), 50 µM of an extracellular signal-regulated kinase (ERK) inhibitor (PD98059; Tocris Bioscience, Bristol, UK) or 20 µM of a c-Jun N-terminal kinase (JNK) inhibitor (SP600125; Tocris Bioscience) for 1 h, the cells were further treated with 10 ng/ml of TNF-α for 48 h. Cell proliferation

was determined using the colorimetric WST-1 conversion assay as described above.

Cell cycle analysis. Cell cycle analysis was performed using a FACSCanto II with FACSDiva software (both from BD Biosciences, San Jose, CA, USA). Cells were harvested by trypsin-EDTA treatment and washed with phosphate-buffered saline (PBS) containing 1% FBS and 0.1% NaN₃, and fixed with 70% ethanol at 4°C for 20 min. After washing 3 times with ice-cold PBS, the cells were suspended in propidium iodide (PI)-solution (0.001% Triton X-100, 3.4% RNase A, 0.005% PI in PBS). An electronic gate was set on the HESCs using forward and side scatter characteristics. In all the experiments, cells were gated on forward and side scatter to eliminate dead cells and debris.

Flow cytometry. Immunophenotypic analysis of the cells was performed using a FACSCanto II with FACSDiva software (both from BD Biosciences). Cells were harvested by trypsin-EDTA treatment and washed with PBS containing 1% FBS and 0.1% NaN₃. For flow cytometry, the cells were incubated with 2.4G2 (anti-Fc receptor) hybridoma culture supernatant to block non-specific antibody binding and then stained with fluorochrome-conjugated monoclonal antibodies (mAbs). Phycoerythrin (PE)-anti-ICAM-1 (CD54 and MEM-111) and PE-anti-VCAM-1 (CD106 and STA) mAbs were purchased from BioLegend Inc. (San Diego, CA, USA). Background fluorescence was determined by measuring the fluorescent signal from cells stained with fluorochrome-labeled isotype-matched non-reactive mAbs for 30 min at 4°C. An electronic gate was set on the HESCs using forward and side scatter characteristics. In all the experiments, cells were gated on forward and side scatter to eliminate dead cells and debris.

Immunofluorescence staining. For immunofluorescence analysis, the cells were cultured on 12-mm glass coverslips (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and fixed with cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 10 min. Subsequently, the fixative was removed by washing the coverslips 3 times for 5 min each with cold PBS followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. The cells were washed again with cold PBS and then incubated with 1% bovine serum albumin (Sigma) for 60 min at room temperature. Excess solution was shaken off and the cells were incubated for 16-18 h at 4°C with PE-anti-ICAM-1 or PE-anti-VCAM-1 (both from BioLegend) mAbs diluted 1:100. Following incubation with the primary antibodies, the cells were washed 3 times for 5 min each with cold PBS. The cells were then rinsed in cold PBS and mounted on glass slides using Vectashield® (Vector Laboratories, Burlingame, CA, USA). Labeled cells were examined using an Olympus BX50 microscope equipped with fluorescent epi-illumination. Photomicrographs were acquired digitally at 1360x1024 pixel resolution with an Olympus DP70 digital camera.

Preparation of cytosolic and nuclear cell extracts. Cells were resuspended in 70 µl of buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, protease inhibitor cocktail and phosphatase inhibitor cocktail I and II (both from Sigma)] and incubated on ice. After

15 min, 0.5% nonyl phenoxypolyethoxyethanol P (NP)-40 was added to lyse the cells, followed by vortexing for 10 sec. Cytosolic cell extracts were obtained after centrifugation at 6,500 rpm for 60 sec at 4°C. Nuclei were resuspended in 50 ml of buffer C [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM PMSF and protease inhibitor cocktail] and incubated on ice for 20 min with gentle pipetting every 5 min. Nuclear cell extracts were recovered after centrifugation for 10 min at 12,000 rpm at 4°C. Protein concentration was determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA).

Western blot analysis. Equal amounts of protein samples were heated for 10 min at 95°C in sample buffer and separated by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% SDS polyacrylamide gel, using a Mini-Protean III system (Bio-Rad). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (both from Bio-Rad). The blotted PVDF membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) at room temperature for 2 h. The membrane was then incubated overnight at 4°C with anti-ICAM-1 (sc-1511), anti-VCAM-1 (sc-1504), anti-NF-κB p65 (sc-8008), anti-IκBα (sc-203), anti-phospho (p)-IκBα (sc-7977-R) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-p-ERK (p44/42 MAPK) (4370), anti-ERK (p44/42 MAPK) (9102), anti-p-JNK (4668), anti-JNK (9258), anti-p-p38 MAPK (9215) and anti-p38 MAPK mAbs (9212) (Cell Signaling Technology, Danvers, MA, USA). These antibodies, as well as that for α-tubulin (MU121-UC; BioGenex Laboratories, Inc., San Ramon, CA, USA) and β-actin (8226; Abcam, Cambridge, UK), were incubated with the membranes at a dilution of 1:1,000 in TBS containing 2% skim milk. After 3 washes with TBS-T (TBS containing 0.1% Tween-20), the membrane was incubated for 2 h at room temperature with the secondary antibody, rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate (sc-2768), goat anti-mouse IgG HRP conjugate (sc-2031), goat anti-rabbit IgG HRP conjugate (sc-2004) (all from Santa Cruz Biotechnology, Inc.) and goat anti-rabbit IgG HRP (7074; Cell Signaling Technology) diluted 1:2,000, and re-washed 3 times with TBS-T. Immunoreactivity was detected using enhanced chemiluminescence (ECL; SuperSignal West Pico Chemiluminescent Substrate kit; Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Images were acquired and the signals were quantified using a Fujifilm LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Quantitative reverse transcriptase-PCR (qRT-PCR). Total RNA was then isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with DNase (DNA-free kit; Ambion, Austin, TX, USA). Total RNA (500 ng) was converted into cDNA (M-MLV Reverse Transcriptase; Promega, Madison, WI, USA). qRT-PCR amplification was performed using the following qRT-PCR primers: ICAM-1 sense, 5'-CCGTGAATGTGCTCTCCC-3' and antisense, 5'-ATTTCTTGATCTTCCGCTGG-3' (GenBank accession no. NM_000201); and VCAM-1 sense, 5'-CAGGTGGAGCTCTACTCATTCC-3' and antisense, 5'-GAATAGTCTCCCCCTTAAGTAATTC-3' (GenBank accession no. NM_001078). For normalization, the

GAPDH gene was amplified using the following primers: GAPDH sense, 5'-GAAGATGGTGGATGGGATTTTC-3' and antisense, 5'-GAAGGTGAAGGTCGGAGT-3' (GenBank accession no. NM_002046). qRT-PCR amplification was performed in a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) with QuantiTect[®] SYBR[®]-Green PCR Master Mix (Qiagen, Hilden, Germany). qRT-PCR amplification of ICAM-1, VCAM-1 and GAPDH genes were carried out for 50 cycles at 94°C for 10 sec, 56°C for 15 sec and 72°C for 15 sec. Melting curve analysis was conducted for 30 sec at 55-99°C. Amplification products exhibited single, sharp peaks, indicating that the primers amplified only one specific PCR product. All the samples were amplified in triplicate. An amplification reaction containing no template was used as a control to establish non-specific background amplification. Data were analyzed using the Rotor-Gene 6000 program (Corbett Life Science). Gene expression levels were calculated using the formula, $R0 = R_{Ct} \times (1 + E) - Ct$ and were normalized to GAPDH expression levels.

Enzyme-linked immunosorbent assay (ELISA). Cells cultured in 96-well plates (4×10^3 /well) in DMEM/F-12 medium in the presence of 5% FBS were pre-treated with or without HEABG (50 $\mu\text{g/ml}$) for 1 h and stimulated with TNF- α (10 ng/ml) for 15 h, after which supernatants were harvested. The concentrations of IL-6 in the supernatants were measured using standard ELISA kits (R&D Systems, Minneapolis, MN, USA). Diluted supernatant (1:100) and HRP-conjugated anti-IL-6 antibodies were added separately into each antibody-coated well for evaluation. Plate wells were covered with a fresh plastic sealer and incubated at room temperature for 2 h with gentle shaking. The solution was then discarded, and the wells were washed 6 times with wash buffer. Chromogen solution (tetramethylbenzidine) was added to the wells and incubated for 20 min at room temperature, followed by the addition of stop solution. The amount of conjugate bound to each well was quantified photometrically from these colored products by measuring the optical density at 450 nm. A concentration of IL-6 was extrapolated from a standard curve using recombinant IL-6. The sensitivity of the ELISA assay of IL-6 was ≥ 0.7 pg/ml.

Electrophoretic mobility shift assay (EMSA). The cells were pre-treated with HEABG (50 $\mu\text{g/ml}$) for 1 h and then stimulated with TNF- α (10 ng/ml) for 30 min at 37°C prior to the extraction of nuclear protein. The NF- κB oligonucleotide (5'-AGTTGAGGGGACTTTCCAGGC-3') and AP-1 oligonucleotide (5'-CGCTTGATGACTCAGCCGGAA-3') (Bioneer, Inc., Seoul, Korea) were end-labeled with biotin. Nuclear protein extracts (10 μg) were incubated with 20 fmol of biotin-labeled NF- κB oligonucleotide and AP-1 oligonucleotide for 20 min prior to loading onto 4% (for NF- κB) and 6% (for AP-1) non-denaturing polyacrylamide gels. Each gel was run at room temperature in 0.3X TBE buffer at 100 V until the bromophenol blue dye reached the bottom of the gel. When electrophoresis was completed, the gel was blotted onto a Biotinylated B Nylon Membrane (Pierce), and labeled oligonucleotides were detected using the LightShift Chemiluminescent EMSA kit according to the manufacturer's instructions (Pierce). Images were acquired and the signals were quantified using the LAS-3000 imaging system.

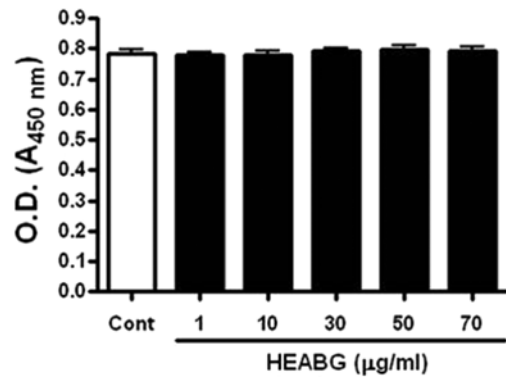


Figure 1. Effect of hexane extract of aged black garlic (HEABG) on the viability of human endometrial stromal cells (HESCs). After the HESCs were cultured for 24 h and then serum-starved (5% FBS) for 24 h, they were treated with various doses of HEABG (1, 10, 30, 50 or 70 $\mu\text{g/ml}$) for 48 h, and cell viability was determined using the WST-1 assay. Data are representative of 3 independent experiments with similar results. Results are the means \pm SD of pentaplicates.

Statistical analysis. The results of the present study are expressed as the means \pm SD under all conditions and statistically analyzed using a two-tailed Student's t-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of HEABG on the viability of HESCs. We first examined the cytotoxic effects of HEABG on the HESCs by WST-1 assay. HEABG did not affect the viability of the HESCs up to a concentration of 70 $\mu\text{g/ml}$ (Fig. 1).

Effect of HEABG on the proliferation of HESCs. Since enhanced endometrial cell proliferation is an important pathological feature of human endometriosis, we evaluated the effect of HEABG on the inhibition of TNF- α -stimulated HESC proliferation by WST-1 assay. The stimulation of HESCs with TNF- α (10 ng/ml) for 48 h significantly enhanced HESC proliferation (Fig. 2). By contrast, HESCs pre-treated with HEABG (30 and 50 $\mu\text{g/ml}$) exhibited a significant reduction in cell proliferation induced by TNF- α , indicating that HEABG used at non-toxic concentrations in HESCs exhibits a significant inhibitory effect on the proliferation of HESCs (Fig. 2). Furthermore, the growth-promoting effect of TNF- α in HESCs was also completely blocked by the treatment with either the ERK inhibitor, PD98059 (50 μM), or the JNK inhibitor, SP600125 (20 μM), for 1 h prior to stimulation with TNF- α (10 ng/ml). This finding led us to investigate the effect of HEABG on TNF- α -induced MAPK activation. Treatment of the HESCs with TNF- α clearly induced the phosphorylation of ERK, JNK and p38 MAPK. Notably, pre-treatment with HEABG resulted in the inhibition of the TNF- α -induced ERK and JNK phosphorylation, but not p38 MAPK phosphorylation (Fig. 3). Taken together, these results indicate that the TNF- α -induced enhancement of the proliferation of HESCs is mediated through the ERK/MAPK and JNK/MAPK signal transduction pathways.

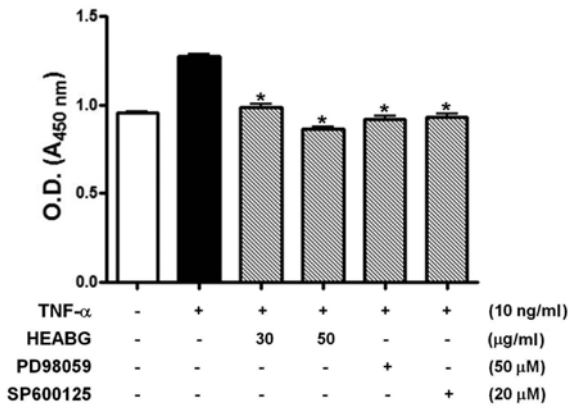


Figure 2. Effect of hexane extract of aged black garlic (HEABG) on the proliferation of TNF- α -stimulated human endometrial stromal cells (HESCs). After the HESCs were cultured for 24 h and then serum-starved (5% FBS) for 24 h, they were treated with various doses of HEABG (30 or 50 μ g/ml), 50 μ M of an ERK inhibitor (PD98059) or 20 μ M of a JNK inhibitor (SP600125) for 1 h, and cell proliferation was determined using the WST-1 assay. Data are representative of 3 independent experiments with similar results. Results are the means \pm SD of pentaplicates. *P<0.001 compared with the TNF- α -treated group as determined by a t-test.

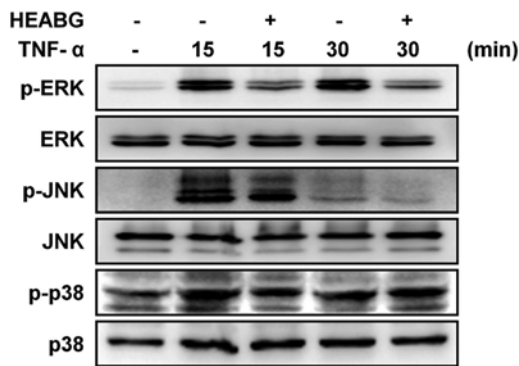


Figure 3. Western blot analysis of the effects of hexane extract of aged black garlic (HEABG) on the activation of p-ERK, ERK, p-JNK, JNK, p-p38 and p38 in TNF- α -treated human endometrial stromal cells (HESCs). Cells were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 30 min. Data are representative of 3 independent experiments with similar results.

Effect of HEABG on the cell cycle in HESCs. The effect of HEABG on the cell cycle of HESCs was determined by flow cytometric analysis. Treatment of the HESCs with TNF- α (10 ng/ml) for 15 h significantly reduced the proportion of cells in the G₀/G₁ phase and increased the number of cells in the S and G₂/M phase (Fig. 4). Pre-treatment with HEABG (30 and 50 μ g/ml) markedly suppressed these cell cycle alterations (Fig. 4). Furthermore, the stimulating effects of TNF- α on cell cycle progression in HESCs were also completely abolished by treatment with either the ERK inhibitor, PD98059 (50 μ M), or the JNK inhibitor, SP600125 (20 μ M), for 1 h prior to stimulation with TNF- α (10 ng/ml), indicating that the TNF- α -induced promotion of the cell cycle progression of HESCs is mediated through the ERK/MAPK and JNK/MAPK signal transduction pathways (Fig. 4).

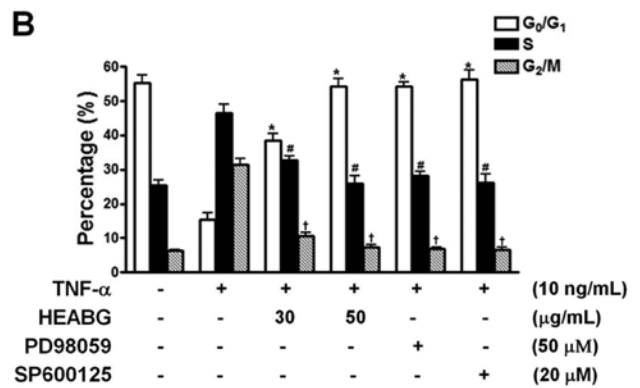
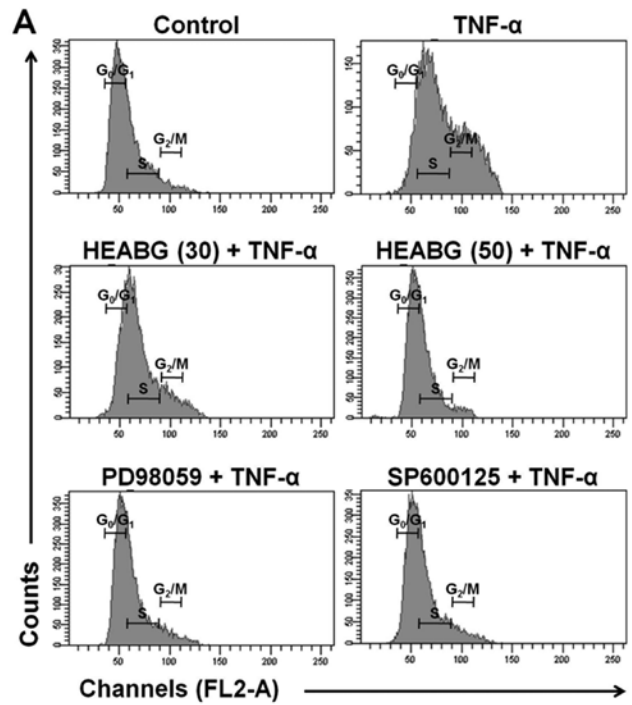


Figure 4. Flow cytometric analysis of the effect of hexane extract of aged black garlic (HEABG) on the cell cycle of human endometrial stromal cells (HESCs). (A) HESCs were pre-treated with HEABG (30 and 50 μ g/ml), 50 μ M of an ERK inhibitor (PD98059) or 20 μ M of a JNK inhibitor (SP600125) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. (B) Data in histograms are also plotted in bar graphs and are expressed as the means \pm SD. Data are representative of 3 independent experiments with similar results. *P<0.01 compared with the TNF- α -treated group as determined by a t-test.

Suppression of ICAM-1 and VCAM-1 transcription by HEABG. To analyze the transcription of cell adhesion molecules in HESCs, the mRNA expression of ICAM-1 and VCAM-1 was evaluated by qRT-PCR analysis. Treatment of the HESCs with TNF- α (10 ng/ml) for 15 h significantly increased the mRNA expression levels of ICAM-1 and VCAM-1 (Fig. 5). Pre-treatment with HEABG (50 μ g/ml) markedly suppressed the TNF- α -induced ICAM-1 and VCAM-1 mRNA expression (42.5 and 44.6% inhibition, respectively; P<0.005) (Fig. 5).

Inhibition of cell surface expression of ICAM-1 and VCAM-1 by HEABG. To analyze cell adhesion molecule expression on endometrial stromal cell surfaces, ICAM-1 and VCAM-1 expression was evaluated by flow cytometry. Treatment of the

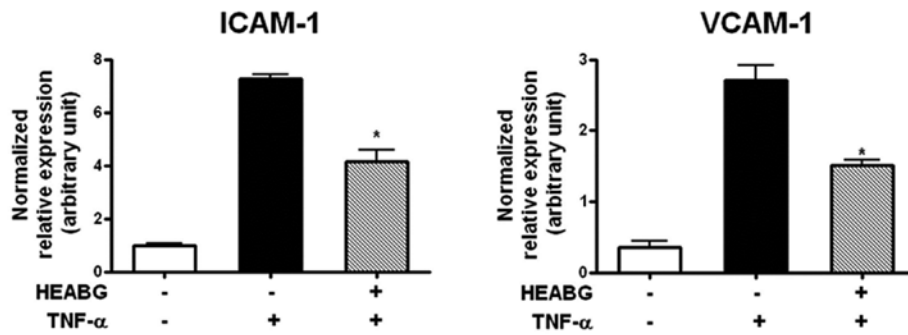


Figure 5. Quantitative real-time RT-PCR analysis of the effect of HEABG on the gene expression of adhesion molecules in TNF- α -activated HESCs. HESCs were pre-treated with HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. Data are representative of 3 independent experiments with similar results. Results are expressed as ratios of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) mRNA normalized to GAPDH mRNA, plotted in bar graphs and expressed as means \pm SD. *P<0.005 compared with the TNF- α -treated group as determined by a t-test.

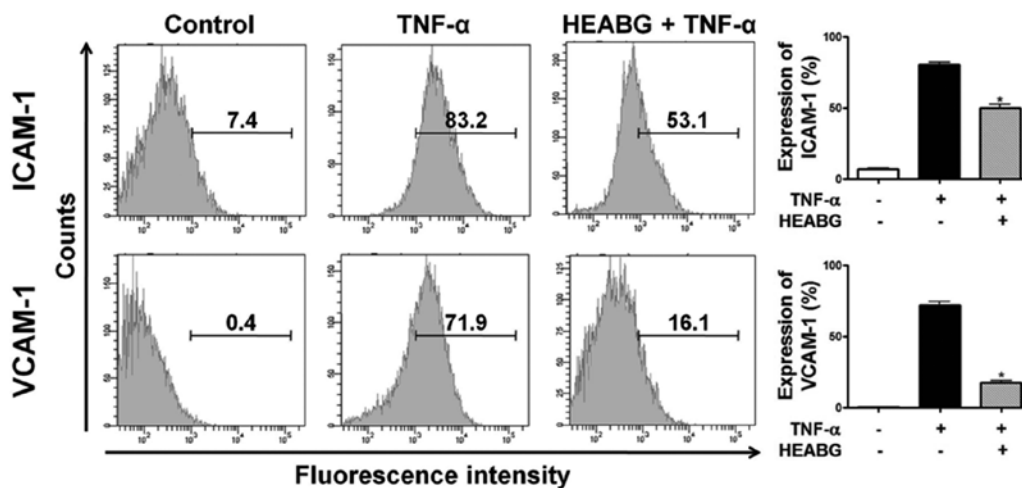


Figure 6. Flow cytometric analysis of the effect of hexane extract of aged black garlic (HEABG) on the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in TNF- α -activated human endometrial stromal cells (HESCs). HESCs were pre-treated with HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. Data in histograms are also plotted in bar graphs and are expressed as the means \pm SD. Data are representative of 3 independent experiments with similar results. *P<0.001 compared with the TNF- α -treated group as determined by a t-test.

HESCs with TNF- α (10 ng/ml) for 15 h markedly induced the cell surface expression of ICAM-1 and VCAM-1 proteins (Fig. 6). Pre-treatment with HEABG (50 μ g/ml) significantly suppressed the TNF- α -induced ICAM-1 and VCAM-1 cell surface expression (37.6 and 75.6% inhibition, respectively; P<0.001) (Fig. 6).

Suppression of total cellular ICAM-1 and VCAM-1 protein expression by HEABG. The incubation of HESCs with TNF- α (10 ng/ml) for 15 h markedly increased the protein levels of total cellular ICAM-1 and VCAM-1, as revealed by immunofluorescence microscopic analysis (Fig. 7). Pre-treatment with HEABG (50 μ g/ml) for 1 h significantly suppressed the total cellular protein expression of ICAM-1 and VCAM-1 in HESCs induced by TNF- α (Fig. 7).

Additionally, western blot analysis revealed a single specific band correlating for ICAM-1 and VCAM-1 in the HESCs treated with TNF- α (10 ng/ml) for 15 h (Fig. 8). Importantly, this effect was effectively inhibited by pre-treatment with HEABG (50 μ g/ml) for 1 h (Fig. 8). The protein expression of ICAM-1

and VCAM-1 in the TNF- α -activated HESCs following treatment with HEABG, as measured by flow cytometric analysis (Fig. 6), immunofluorescence microscopic analysis (Fig. 7) and western blot analysis (Fig. 8), was in accordance with the transcription patterns of ICAM-1 and VCAM-1 shown by qRT-PCR analysis (Fig. 5), clearly indicating that both the cell surface and cytoplasmic protein, as well as the mRNA expression of ICAM-1 and VCAM-1 in the HESCs induced by TNF- α was definitely inhibited by HEABG.

Inhibition of secretion of IL-6 by HEABG. The levels of IL-6 secreted by HESCs were evaluated by ELISA. Treatment of the HESCs with TNF- α (10 ng/ml) for 15 h markedly induced the secretion of IL-6 (Fig. 9). HEABG (50 μ g/ml) potently suppressed the TNF- α -induced secretion of IL-6 from HESCs (60.0% inhibition; P<0.01) (Fig. 9).

Inhibition of the TNF- α -induced translocation of NF- κ B and phosphorylation of I κ B α by HEABG. To determine whether the activation of NF- κ B by TNF- α can be inhibited by

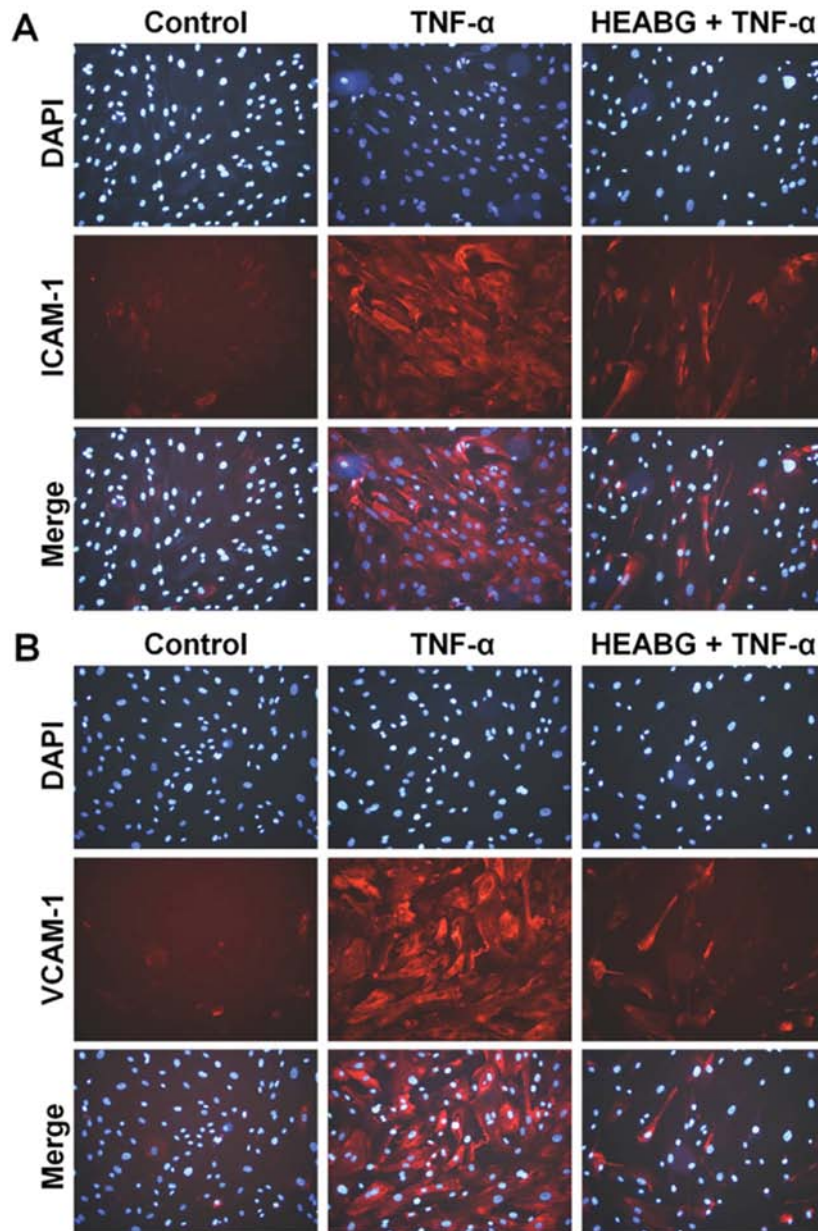


Figure 7. Fluorescence microscopic detection of the effect of hexane extract of aged black garlic (HEABG) on the total cellular expression of (A) intercellular adhesion molecule-1 (ICAM-1) and (B) vascular cell adhesion molecule-1 (VCAM-1) in TNF- α -treated human endometrial stromal cells (HESCs). Cells were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, then stimulated with TNF- α (10 ng/ml) for 15 h. Images were observed under a fluorescence microscope at x200 magnification. Data are representative of 3 independent experiments with similar results.

HEABG, experiments were carried out to measure the major components of the NF- κ B complex. Western blot analysis indicated that TNF- α induced the translocation of NF- κ B p65 from the cytosol to the nucleus (Fig. 10). Additionally, treatment of the HESCs with TNF- α (10 ng/ml) for 1 h induced the phosphorylation and degradation of the NF- κ B inhibitor, I κ B α , in the cytosol (Fig. 10). Pre-treatment with 50 μ g/ml HEABG for 1 h blocked the TNF- α -induced nuclear translocation of NF- κ B p65 from the cytosol to the nucleus and significantly inhibited the TNF- α -induced phosphorylation and degradation of I κ B α (Fig. 10). These results suggest that HEABG inhibits the translocation of NF- κ B from the cytosol to the nucleus in the TNF- α -activated HESCs by the inhibition

of I κ B α degradation, which is due to the inhibition of I κ B α phosphorylation.

Attenuation of TNF- α -induced activation of NF- κ B and AP-1 by HEABG. To further investigate the mechanism responsible for the inhibitory effect of HEABG on ICAM-1 and VCAM-1 expression, we examined the effect of HEABG on NF- κ B and AP-1 activation by EMSA. Under control conditions, the nuclear expression of NF- κ B (Fig. 11) and AP-1 (Fig. 12) in HESCs was barely detectable. However, the stimulation of the HESCs with TNF- α (10 ng/ml) led to the appearance of NF- κ B (Fig. 11) and AP-1 (Fig. 12) as a shifted band. Pre-treatment with HEABG (50 μ g/ml) significantly reduced the density of

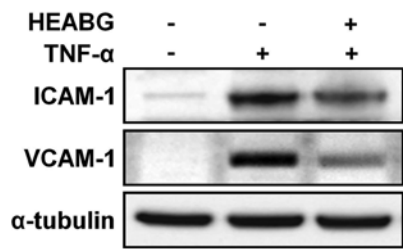


Figure 8. Western blot detection of the effect of hexane extract of aged black garlic (HEABG) on the total cellular expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in TNF- α -activated human endometrial stromal cells (HESCs). Cells were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. Data are representative of 3 independent experiments with similar results.

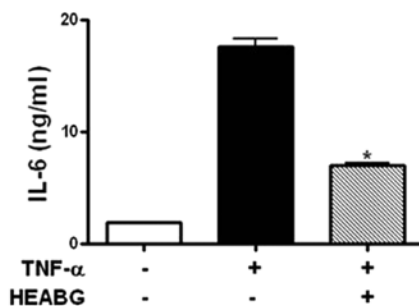


Figure 9. ELISA detection of the effect of hexane extract of aged black garlic (HEABG) on the secretion of IL-6 by TNF- α -activated human endometrial stromal cells (HESCs). Cells were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. Data are representative of 3 independent experiments with similar results. Results in bar graphs are expressed as means \pm SD of triplicates. *P<0.01 compared with the TNF- α -treated group as determined by a t-test.

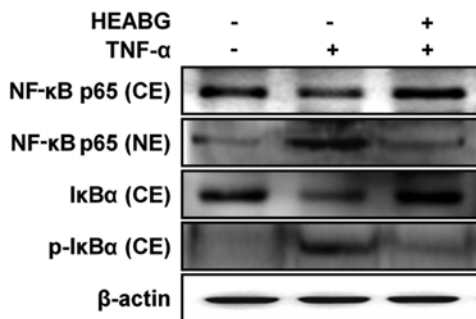


Figure 10. Western blot analysis of the effect of hexane extract of aged black garlic (HEABG) on the nuclear translocation of NF- κ B in TNF- α -activated human endometrial stromal cells (HESCs). Cells were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 1 h. Thereafter, the nuclear fraction was analyzed for NF- κ B p65 translocation from the cytosol into the nucleus by western blot analysis. Levels of I κ B α protein expression and of I κ B α phosphorylation in the cytosol were also analyzed. Data are representative of 3 independent experiments with similar results.

the NF- κ B (Fig. 11) and AP-1 (Fig. 12) shifted band (46.7 and 38.5% inhibition, P<0.01, respectively). These results indicate that HEABG potently inhibits the activation of NF- κ B and AP-1 transcription factors induced by TNF- α in HESCs.

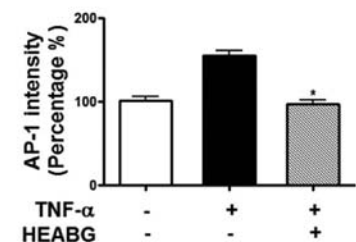
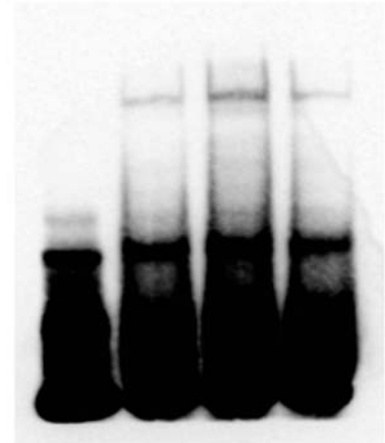
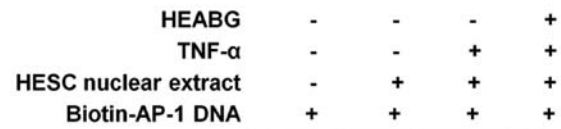


Figure 11. Electrophoretic mobility gel shift assay (EMSA) detection of the effect of hexane extract of aged black garlic (HEABG) on the binding activity of NF- κ B with its target DNA in TNF- α -activated human endometrial stromal cells (HESCs). HESCs were cultured in the presence or absence of HEABG (50 μ g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 30 min. The cells were then fractionated into cytosolic and nuclear extracts. The nuclear fractions were analyzed to determine the binding activity of NF- κ B. Data plotted in the bar graphs are representative of 3 independent experiments with similar results. Results are expressed as the means \pm SD of triplicates. *P<0.01 compared with the TNF- α -treated group as determined by a t-test.

Effects of specific inhibitors of NF- κ B and AP-1 on TNF- α -induced ICAM-1 expression. To determine the role of NF- κ B and AP-1 in TNF- α -induced ICAM-1 expression in HESCs, the specific NF- κ B inhibitor, BAY 11-7085, and the specific AP-1 inhibitor, SR11302, were used. HESCs were incubated with either BAY 11-7085 (5 and 10 μ M) or SR11302 (30 and 50 μ M) for 1 h prior to stimulation with 10 ng/ml TNF- α for 15 h, and the cell surface expression of ICAM-1 was analyzed by flow cytometric analysis. Of note, ICAM-1 expression in the TNF- α -treated HESCs was completely abolished by the NF- κ B inhibitor and partially inhibited by the AP-1 inhibitor in HESCs (Fig. 13). These data indicate that the effects of HEABG on the TNF- α -induced ICAM-1 expression in HESCs are mediated through the NF- κ B signal transduction pathway and partially through the AP-1 pathway.

Discussion

The results of the present study clearly indicate that the treatment of TNF- α -activated HESCs with HEABG suppresses the expression of ICAM-1 and VCAM-1 at the mRNA and protein level, as well as cell proliferation, cell cycle progression and the secretion of IL-6. We demonstrated that TNF- α , a central

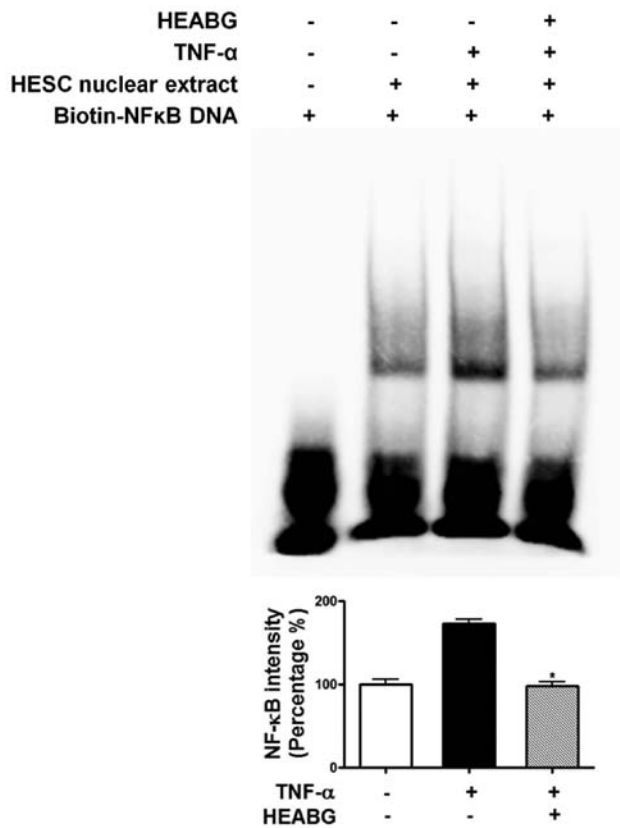


Figure 12. Electrophoretic mobility gel shift assay (EMSA) detection of the effect of hexane extract of aged black garlic (HEABG) on the binding activity of activator protein-1 (AP-1) with its target DNA in TNF- α -activated human endometrial stromal cells (HESCs). HESCs were cultured in the presence or absence of HEABG (50 g/ml) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 30 min. The cells were then fractionated into cytosolic and nuclear extracts. The nuclear fractions were analyzed to determine the binding activity of AP-1. Data plotted in the bar graphs are representative of 3 independent experiments with similar results. Results are expressed as the means \pm SD of triplicates. * P <0.01 compared with the TNF- α -treated group as determined by a *t*-test.

pro-inflammatory cytokine, markedly promotes the proliferation, cell cycle progression and the expression of ICAM-1, VCAM-1 and IL-6, induces the activation of the NF- κ B and AP-1 transcription factors, and activates ERK and JNK and ERK, subgroups of the MAPK family in HESCs. These data suggest that TNF- α plays a crucial role in the pathogenesis of endometriosis through the marked enhancement of cell proliferation, as well as the expression of these critical inflammatory mediators in HESCs, as shown in Fig. 14. It has been shown that the abnormal production of TNF- α contributes to the pathophysiology of endometriosis (18).

The immune system plays an important role in the development of endometriosis (2,3). Cell adhesion molecules, such as ICAM-1 and VCAM-1, play critical roles in various inflammatory and immunological functions, including cell-cell adhesion (4). ICAM-1 and VCAM-1 are strongly expressed in the HESCs of patients with endometriosis, suggesting that ICAM-1 and VCAM-1 play a role in the pathophysiology of this disease (19,20). The pathophysiological importance of ICAM-1 expression in HESCs has attracted much attention. In particular, ICAM-1 expression may be associated with

the defective functions of natural killer (NK) cells in endometriosis (2,6,8). In patients with endometriosis, the elevated expression of ICAM-1 in HESCs gives rise to the shedding of soluble ICAM-1 (sICAM-1) from the cell surface to the peritoneal cavity, which subsequently binds to ICAM-1 receptors on NK cells (6). This interferes with the cytotoxic function of NK cells, and consequently, hinders them from killing ectopic endometrial cells in the peritoneal cavity (2,6,21). This mechanism appears to form the basis for HESCs to escape immune surveillance, thus enabling the ultimate development of endometriosis. This hypothesis is further supported by findings that the endometrial release of sICAM-1 directly correlates with the number and score of endometriotic implants (22), and that women with endometriosis have elevated sICAM-1 levels in serum, demonstrating a correlation between the peritoneal and endometrial sICAM-1 release levels and the extent and severity of the disease (6,23). These facts strongly underline the importance of the identification of agents that inhibit the expression of ICAM-1 for the development of effective inhibitors of the activity of HESCs. Thus, it is of significance that HEABG was found to have an effective and potent suppressive effect on the TNF- α -induced upregulation of ICAM-1 and VCAM-1 expression in HESCs in the present study.

In agreement with the results of previous studies, we also demonstrate that TNF- α stimulation promotes the activation of NF- κ B and AP-1, critical regulators of cell proliferation and inflammatory responses in HESCs, suggesting that these transcription factors are key factors in the pathogenesis of endometriosis (3,14). The canonical pathway of NF- κ B is activated in endometriotic lesions, and modulation of NF- κ B activity and the consequent cell responses, including proliferation, inflammation and adhesion are important in the development of endometriosis (3,13,24,25). Thus, it is noteworthy that the present study demonstrated that HEABG significantly inhibits the TNF- α -induced activation of NF- κ B and AP-1 in HESCs. In particular, the involvement of the NF- κ B and AP-1 pathways in the TNF- α -induced ICAM-1 and VCAM-1 upregulation in HESCs was confirmed in this study, using specific NF- κ B and AP-1 inhibitors. Taken together, these results suggest that HEABG exerts its inhibitory effect on ICAM-1 and VCAM-1 expression via the modulation of the activation and DNA binding activity of NF- κ B and AP-1.

The critical roles of pro-inflammatory cytokines, including IL-6 are well defined in the pathogenesis of endometriosis (2,3). The activation of the NF- κ B and AP-1 transcription factors has been shown to play a crucial role in the enhanced expression of several pro-inflammatory cytokines, including IL-6 in HESCs (14). These findings along with the results of the present study suggest that HEABG blocks the TNF- α -induced IL-6 expression in HESCs through the inhibition of the NF- κ B and AP-1 transcription factors.

Cell proliferation is a fundamental process in the development of endometriotic lesions (2,3). The MAPK cascade plays an important role in the signal transduction pathways that contribute to the regulation of complex cellular functions, including cell proliferation (26). There are at least 3 distinct and parallel MAPK pathways characterized by ERK, JNK and p38 MAPK. These MAPK signaling pathways are also present in endometrial cells, and MAPKs play pathophysiological roles in the development of endometriosis (14,27). It has been shown

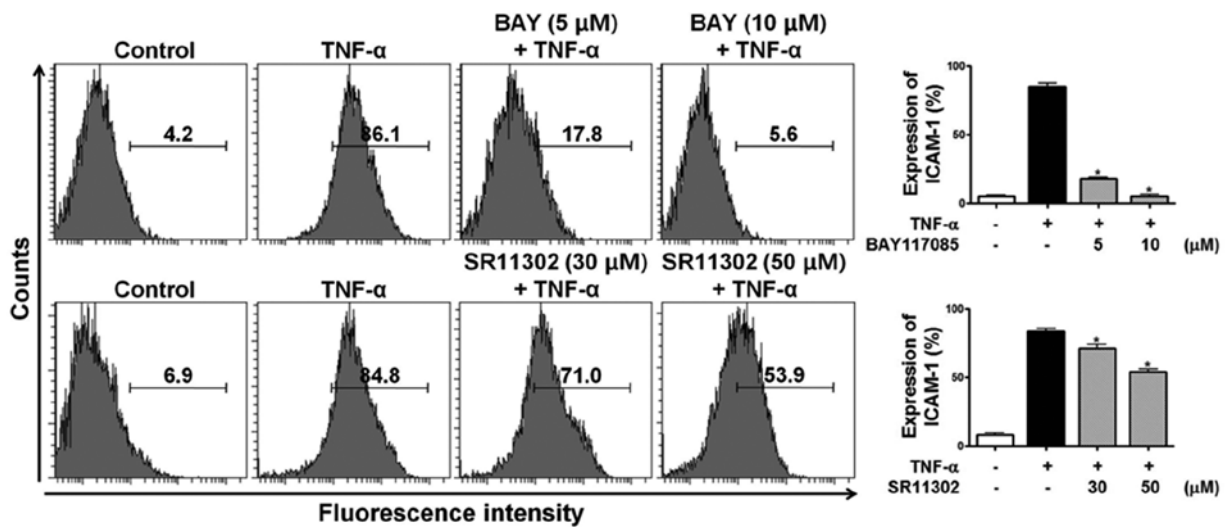


Figure 13. Effect of the specific inhibitors of NF- κ B and activator protein-1 (AP-1) on the expression of intercellular adhesion molecule-1 (ICAM-1) in TNF- α -activated human endometrial stromal cells (HESCs) detected by flow cytometric analysis. HESCs were pre-treated with BAY 11-7085 (specific NF- κ B inhibitor) or SR11302 (specific AP-1 inhibitor) for 1 h, and then stimulated with TNF- α (10 ng/ml) for 15 h. Data in histograms are also plotted in bar graphs and expressed as mean \pm SD. Data are representative of 3 independent experiments with similar results. * P <0.05 compared with the TNF- α -treated group as determined by a t-test.

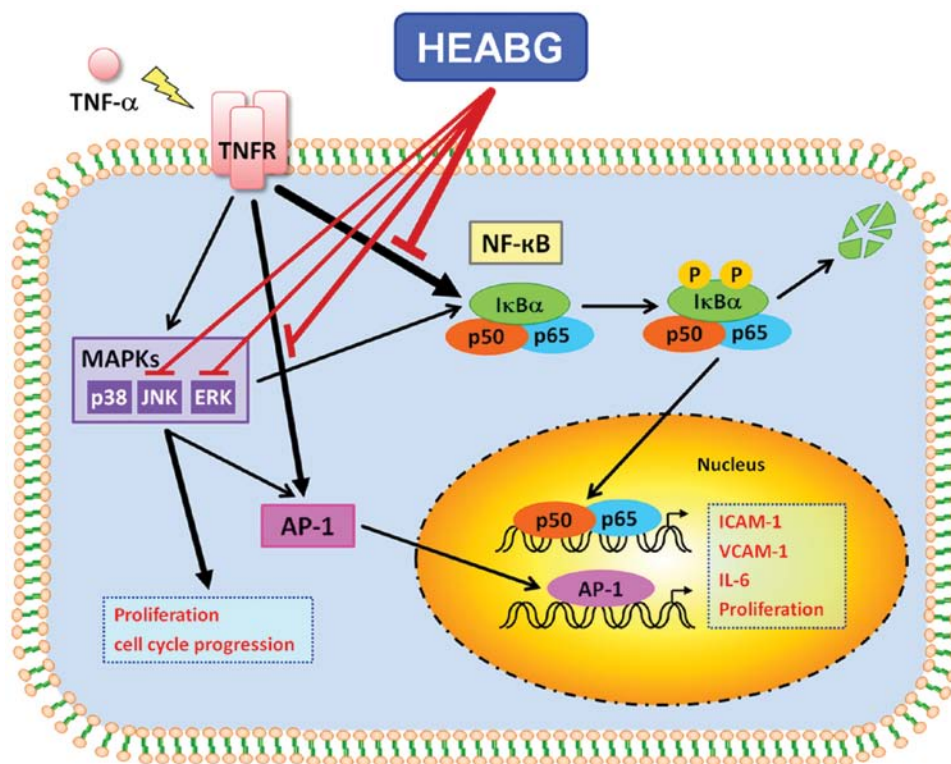


Figure 14. Proposed molecular mechanisms of action for hexane extract of aged black garlic (HEABG) in human endometrial stromal cells (HESCs). HEABG suppressed TNF- α -induced intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression, as well as IL-6 secretion by inhibiting the NF- κ B and AP-1 pathways. In addition, HEABG suppressed the TNF- α -promoted cell proliferation and cell cycle progression via through inhibition of the ERK and JNK signaling pathways.

that MAPK signaling pathways are abnormally activated in HESCs, and that MAPK acts upon the regulation of HESC proliferation (28,29).

In agreement with previous studies, the present study clearly demonstrates that TNF- α induces the expression of all 3 types of MAPKs in HESCs (14,27). Since we observed that

HEABG significantly inhibited the TNF- α -induced activation of ERK and JNK in HESCs, we performed an experiment to define whether MAPKs are involved in the regulation of cell proliferation in TNF- α -activated HESCs using their specific inhibitors. Of note, it was found that the inhibition of ERK and JNK hinders the proliferation and cell cycle progression

of HESCs, suggesting that the TNF- α -induced activation of the ERK/MAPK and JNK/MAPK signaling pathways plays a regulatory role in HESC proliferation. Moreover, HEABG markedly suppressed ERK/MAPK and JNK/MAPK activation in HESCs. Thus, our data indicate that HEABG effectively inhibits the TNF- α -induced proliferation and cell cycle progression of HESCs by suppressing ERK/MAPK and JNK/MAPK activation.

Taken together, our data clearly indicate that HEABG may be effective in the prevention and treatment of endometriosis in humans. Of particular importance, our results indicate that HEABG, as a natural product, provides valuable insight into the development of novel and beneficial therapeutic modalities for combating endometriosis, since traditional hormone-based therapeutic agents have serious adverse effects when used for the long-term management of endometriosis.

Acknowledgements

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