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p38 Mitogen-Activated Protein Kinase is Involved in the Pathogenesis of Endometriosis by Modulating Inflammation, but not Cell Survival

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Abstract

Background: Local pro-inflammatory environment and enhanced cell survival contribute to the endometriosis development. A serine/threonine kinase p38 mitogen-activated protein kinase (MAPK) mediates intracellular signaling of cytokine production, cell proliferation, and apoptosis in different cell types. The current study compares p38 MAPK activity in normal endometrium and endometriosis, and assesses role(s) of p38 MAPK on cytokine production and cell survival in endometriosis. **Methods:** Immunohistochemical levels of total and phosphorylated (active) p38 MAPK as well as its correlation with interleukin 8 (IL-8) expression, and cell proliferation and apoptosis were compared in normal human endometrium and endometriosis. The action of p38 MAPK on pro-inflammatory cytokine-induced IL-8 and monocyte chemotactic protein (MCP)-1 expression in endometriotic cells were assessed by enzyme-linked immunosorbent assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell survival, 5-bromo-2′-deoxyuridine incorporation, and Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assays were used to determine the function of p38 MAPK in cultured human endometriotic stromal cell proliferation and apoptosis. **Results:** p38 MAPK activity was significantly higher in both eutopic and ectopic endometria compared to normal endometria during late proliferative and early secretory phases ($P < .05$). Increased p38 MAPK activity in endometriotic cells correlated with IL-8 expression (Pearson correlation coefficient $r = 0.83$, $P < .01$), but not with apoptosis in vivo. The pro-inflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α induced activation of p38 MAPK. Inhibition of p38 MAPK activity blocked IL-1β and TNF-α-induced IL-8 and MCP-1 secretion in cultured endometriotic stromal cells ($P < .05$), but did not impact on endometriotic cell survival. **Conclusions:** These results suggest that rather than modulating cell survival, increased p38 MAPK activity in endometriotic cells contributes to the pathogenesis of endometriosis by promoting the local inflammatory milieu.

Keywords
endometriosis, p38 MAPK, inflammation, IL-1β, TNF-α

Introduction

Endometriosis is a common gynecological disease defined by the presence of viable endometrial tissue outside the uterine cavity. Increased local estradiol (E_2) production, progesterone resistance, an impaired immune response, and increased levels of cytokines that induce tissue growth, inflammation, and/or angiogenesis as well as stem cell trafficking/differentiation are documented key factors for the pathogenesis of endometriosis.[1-3] The peritoneal environment of women with endometriosis displays increased macrophage number/activity, enhanced T17 cells numbers, dysfunctional natural killer cells, resulting in inflammation around the ectopic endometrium.[4-8] Moreover, a number of cytokines (interleukin [IL]-6, IL-8, glycodelin-A

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and eotaxin) secreted by ectopic endometrial cells also promote survival and growth of endometriotic implants.[7,9,10] In women with endometriosis, the peritoneal fluid has high concentrations of pro-inflammatory cytokines and growth factors, including IL-1β,[11,12] tumor necrosis factor (TNF)-α,[12,13] IL-8,[14,15] monocyte chemotactic protein (MCP)-1,[16] epidermal growth factor,[9] and vascular endothelial growth factor-A.[17] These cytokines induce endometrial cell attachment to the peritoneal surface,[18,19] their invasion into the mesothelium,[20] and growth of ectopic endometrium by stimulating proliferation[21] and angiogenesis.[17,22]

Enhanced cell survival (ie, imbalance between proliferation and apoptosis) in human endometrium is one of the theories proposed to explain the development of endometriosis.[23,24] Compared to disease-free women, the eutopic endometrium in women with endometriosis displays a lower apoptosis index, which is further decreased in ectopic endometrium.[23,25,26] However, the proliferation index of ectopic endometrium was found to be lower compared to eutopic endometrium,[26] indicating increased survival of endometrial cells at ectopic locations.[27]

The p38 mitogen-activated protein kinase (MAPK) is a serine/threonine kinase, which transduces signals from the cell membrane to the nucleus in response to various stimuli including cellular and environmental factors, such as ischemia, inflammatory cytokines, shear stress, UV irradiation, or oxidative stress.[28] Upon activation (phosphorylation), p38 MAPK induces phosphorylation of its substrates at serine and/or threonine residues[29] to activate downstream molecules such as translation/transcription factors, cell cycle molecules, kinases, or scaffold proteins, resulting in a variety of cellular outputs, including cytokine production, proliferation, cycle arrest, migration, differentiation, and apoptosis.[30-33]

Previously, we showed that E_2 induces p38 MAPK phosphorylation (activation) in endometrial stromal cells (ESCs) within minutes,[34] suggesting that local E_2 production in endometriosis may cause constant p38 MAPK activation. Moreover, enhanced secretion of pro-inflammatory cytokines by immune cells in an ectopic site may further increase p38 MAPK activation in endometriosis implants in a paracrine manner. Accordingly, we hypothesize that increased activation of p38 MAPK in ectopic endometrial implants contributes to enhanced expression of chemokines and promotion of cell survival. Therefore, this study evaluated the expression and localization of phosphorylated (active) and total p38 MAPK in endometrial and endometriotic cells. Subsequently, correlation of the p38 MAPK activity with chemotactic cytokine (IL-8) levels and cell survival in endometriosis in vivo and the impact of p38 MAPK signaling in mediating pro-inflammatory cytokine-induced IL-8 and MCP-1 production in ESC cultures were investigated.

Materials and Methods

Tissue Collection

For immunohistochemical and apoptosis detection studies, eutopic and ectopic endometrial samples were obtained from 27 women (mean age, 36.1 years; range, 25–43 years) with endometriosis undergoing laparoscopy for infertility or pelvic pain. As a control group, endometrial tissues were obtained from 30 fertile women (mean age, 35.7 years; range, 28–44 years) with normal menstrual cycles undergoing laparoscopy or hysterectomy for benign gynecological conditions other than endometrial disease. Indications for surgery for the control group were as follows: leiomyoma (n = 18), elective sterilization (n = 10), and benign adnexal mass (n = 2). These women had no visible pelvic inflammation or endometriosis at laparoscopy or laparotomy. The day of the menstrual cycle was established from the women's menstrual history and confirmed by endometrial histology using the criteria of Noyes et al.[35] The presence of ectopic endometrial implants was confirmed by histopathological examination of the biopsies. All endometrial samples were grouped according to the menstrual cycle phase: early proliferative (days 1–7 of the cycle), late proliferative (days 8–14), early secretory (days 14–21), and late secretory (days 22–28). Among eutopic endometrium of women with endometriosis, 6 were in early proliferative, 7 were in late proliferative, 8 in early secretory, and 6 were in late secretory phases. Whereas in the control group, 7 were in early proliferative, 8 were in late proliferative, 8 in early secretory, and 7 were in late secretory phases.

For ESC cultures, eutopic and ectopic endometrial samples (n = 14; 7 from proliferative and 7 from secretory phase of the cycle) were obtained from women with endometriosis (mean age, 33.1 years; range, 24–41 years) undergoing laparoscopy for infertility or pelvic pain. The endometrial samples were placed in Hank's balanced salt solution and transported to the laboratory for ESC isolation and long-term culture. Written informed consent was obtained from each patient using consent forms and protocols approved by the human investigation committee of Yale University.

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were cut into 5-μm sections. After deparaffinization, slides were boiled in 10 mM citrate buffer (pH 6.0) for 15 minutes for antigen retrieval. Then, sections were immersed in 3% hydrogen peroxide (in 50% methanol/50% distilled water) for 15 minutes to block endogenous peroxidase activity. After washing with Tris-buffered saline (TBS) 3 times for 5 minutes, slides were incubated in a humidified chamber with 5% blocking horse or goat serum (LabVision, Fremont, California) in TBS for 30minutes at room temperature (RT). Afterward, excess serum was drained, and serial sections were incubated with primary antibodies (rabbit polyclonal antihuman p38 MAPK antibody, 1:200 dilution in TBS: Cell Signaling Technology, Beverly, Massachusetts; rabbit monoclonal antihuman phospho-specific p38 MAPK [pTpY180/182] antibody, 1:200 dilution in TBS: Cell Signaling Technology; and mouse monoclonal antihuman IL-8 antibody [10 μg/mL] in 1% blocking horse serum in TBS: R&D Systems, Minneapolis, Minnesota) overnight at 4°C in a humidified chamber. For negative controls,

normal rabbit immunoglobin G (IgG) and normal rabbit or mouse IgG isotypes were used at the same primary antibody concentrations. The sections were washed 3 times for 5 minutes with TBS and then biotinylated goat antirabbit or horse antimouse antibody (Vector Laboratories, Burlingame, California) was added at 1:400 dilution for 30 minutes at RT. The antigen–antibody complex was detected by using a strepavidin-biotin-peroxidase kit (Vector Lab) for 30 minutes at RT. 3,3-Diaminobenzidine tetrahydrochloride dihydrate (Vector Lab) was used as the chromogen to visualize immunoreactivity, and sections were counterstained with hematoxylin.

The intensity for IL-8, total and phosphorylated p38 MAPK immunoreactivity was semiquantitatively evaluated using the following intensity categories: 0, no staining; 1+, weak but detectable staining; 2+, moderate or distinct staining; 3+, intense staining. For each tissue, a histological score (HSCORE) value was derived by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the formula HSCORE $= \Sigma$ Pi $(i + 1)$, where i represents the intensity scores, and Pi is the corresponding percentage of the cells as described.[36] In each slide, 5 randomly selected areas were evaluated under a light microscope (×40 magnification), and the percentage of the cells for each intensity within these areas was determined at different times by 2 investigators blinded to the type and source of the tissues. The intra-individual and inter-individual coefficients of variation were 10% and 12%, respectively, for HSCORE evaluation. The average score of 2 investigators was used.

Isolation and Culture of ESCs

Endometrial tissues were minced with a sterile surgical blade and digested in Hank's-balanced salt solution (Sigma-Aldrich, St. Louis, Missouri) containing collagenase B (1 mg/mL, 15 U/mg; Roche, Indianapolis, Indiana), deoxyribonuclease I (0.1 mg/mL, 1500 U/mg; Roche), penicillin (200 U/mL), and streptomycin (200 mg/mL) for 60 minutes at 37°C with agitation. The dispersed endometrial cells were separated by filtration through a wire sieve (73-μm-diameter pore; Sigma-Aldrich) and were cultured in DMEM Ham's F-12 (1:1 vol/vol; Sigma-Aldrich) containing fetal bovine serum (10% vol/vol; Invitrogen, Carlsbad, California). The cultures were maintained in a standard 95% air/5% CO_2 incubator at 37°C[34].

Experimental Setup

After 1 passage, ESCs were grown to pre- or full confluence. Following the first passage, ESCs were evaluated immunocytochemically using specific cell-surface markers. Previously, after the first passage, ESCs were found to contain 0% to 7% epithelial cells, no detectable endothelial cells, and 0.2% macrophages.[37,38] The resulting ESCs were treated with serum-free, phenol red-free media (Sigma-Aldrich) for 24 hours before experimental treatments. Each experiment was repeated at least 3 times using ESCs prepared from endometrial specimens obtained from different patients.

Western Blot Analysis

Confluent ESC cultures were treated with vehicle (control), IL-1β (1 ng/mL; R&D Systems, Inc), or TNF-α (1 ng/mL; R&D Systems) for 10 minutes. Previous studies reported that IL-1β and TNF-α levels can attain 26 and 9.6 ng/mL, respectively, in peritoneal fluid and 7 and 4.3 ng/mL, respectively, in peritoneal macrophage secretions in women with endometriosis.[39-42] Thus, 1 ng/mL was chosen for both proinflammatory cytokines as representative of the in situ level present in peritoneal fluid and/or in peritoneal macrophage secretions in women with endometriosis. To evaluate the effect of a p38 MAPK inhibitor, the cultures were preincubated with SB203580 (10 μM; EMD Biosciences, San Diego, California) for 30 minutes before treatment with IL-1β or TNF-α. After experimental treatments, total protein from treated ESCs was extracted with cell extraction buffer (BioSource International, Camarillo, California) containing 3 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was determined by a detergent-compatible protein assay (Bio-Rad, Hercules, California). Samples (40 μg) were loaded on 10% Tris–HCl Ready Gels (Bio-Rad), electrophoretically separated, and electroblotted onto nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in TBS-containing 0.1% Tween 20 (TBS-T) for 1 hour to reduce nonspecific binding. Subsequently, the membrane was incubated for 2 hours with primary antibodies against total- and phospho-p38 MAPK (polyclonal rabbit antihuman p38 MAPK and monoclonal rabbit antihuman phospho-specific p38 MAPK (pTpY180/182), both at 1:1000 dilution, in 5% nonfat dry milk in TBS-T (Cell Signaling Technology)]. The membrane was washed with TBS-T for 1 hour and incubated with horseradish peroxidase-conjugated antirabbit secondary antibody (Vector Laboratories) diluted at 1:10 000 in TBS-T. The protein was visualized by light emission on film (Amersham Biosciences, Buckinghamshire, UK) with enhanced chemiluminescence substrate (Amersham Biosciences). Immunoblot bands for total and phospho-p38 MAPK were quantified using a laser densitometer.

Enzyme-Linked Immunosorbent Assay

Confluent ESC cultures were treated with vehicle (control), or IL-1β (1 ng/mL; R&D Systems, Inc), or TNF-α (1 ng/mL; R&D Systems) for 24 hours. To evaluate the effect of a p38 MAPK inhibitor, the cultures were preincubated with SB203580 (10 μM) for 30 minutes before the treatment with IL-1β or TNF-α. Concentrations of immunoreactive IL-8 and MCP-1 in conditioned media were measured using an enzyme-linked immunosorbent assay (ELISA) according to instructions provided by the manufacturer (R&D Systems). The sensitivity of IL-8 ELISA was 1.5 pg/mL. The intra- and interassay coefficients of variation of IL-8 ELISA were 4.6% and 6.8%,

respectively. The sensitivity of MCP-1 ELISA was 5 pg/mL. The intra- and interassay coefficients of variation of MCP-1 ELISA were 4.2% and 5.9%, respectively. According to the manufacturer, there is no significant cross-reactivity or interference with other known cytokines in these assays.

Levels of IL-8 and MCP-1 were normalized to the total cell culture protein content as determined by Bradford assay (Bio-Rad). Briefly, after collecting culture supernatants and washing the monolayers with Hanks' balanced salt solution, the cells were harvested using a cell scraper in cold PBS. After centrifugation, the cell extraction buffer (20 mM Tris–HCl buffer with 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and complete protease inhibitor cocktail [Roche, Indianapolis, Indiana]) was added to the cell pellets and sonicated for 5 seconds. After the final centrifugation, the supernatant was collected, and protein content was measured at 650 nm using a multiwell plate reader.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End Labeling

Apoptosis in vivo and at ESCs plated on tissue chamber slides was detected by labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. The TUNEL assay was performed using a cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Formalin-fixed paraffin-embedded endometrial samples from normal, eutopic and ectopic endometrium were deparaffinized and washed 3 times in PBS. Moreover, preconfluent eutopic and ectopic ESCs in chamber slides were treated with vehicle (dimethyl sulfoxide [DMSO)] or a specific MAPK inhibitor, SB203580 (1 and 10μM; EMD Biosciences) for 24 and 72 hours. Chamber slides were fixed in 4% paraformaldehyde for 20 minutes at 4°C, washed 3 times in PBS. All slides were then treated with permeablization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 5 minutes on ice. Afterward, the labeling reaction was performed for 1 hour at 37°C using TUNEL reagent for each sample, except the negative control, in which reagent without enzyme was added. After PBS washing, slides were incubated with converter reagent for 30minutes at 37°C. After an additional wash, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the chambers with Fast Red substrate solution for 10 minutes. Quantitation of apoptotic cells was accomplished by counting the number of cells stained by TUNEL assay per a total of 100 cells, and labeling index was calculated (labeled cells per 100 cells).

5-Bromo-2′-deoxyuridine (BrdU) incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays: Cell proliferation was assessed by colorimetric assays using BrdU and MTT. Preconfluent eutopic and ectopic ESCs in 96-well plates were treated with vehicle (DMSO) or a specific MAPK inhibitor, SB203580 (1 and 10 μM; EMD Biosciences) for 24 and 72 hours. Each experiment involved replicates of 8 wells per treatment.

This MTT assay detects the formation of dark blue formazan product from MTT in active mitochondria was performed as described previously.[43] Four hours before the end of each experiment, 10 μL of MTT solution was added onto each well of 96-well plates. The optical absorbance at 570 nm was read within 30 minutes. The last column of each 96-well plate did not contain cells and was used as a blank. Data are expressed in optic density units.

A BrdU cell proliferation assay kit (Chemicon International, Temecula, California) assessed BrdU incorporation levels into newly synthesized DNA. Briefly, BrdU solution was added to each well 12 hours before termination of each experiment and then cells were washed in PBS and fixed. Cells were then incubated with anti-BrdU antibody for 1 hour, washed and incubated with peroxidase-conjugated secondary antibody for 30 minutes. After washing, cells were exposed to tetramethyl benzidine peroxidase substrate. Plates were read with a multiwell plate reader. Data are expressed in optical density units, with higher values indicating greater BrdU incorporation.

Statistical Analysis

Because the data from immunohistochemistry, TUNEL assay, MTT, and BrdU cell proliferation assays, and Western blot analysis were normally distributed (as determined by Kolmogorov-Smirnov test), comparisons of samples were analyzed with Student t test or 1-way analysis of variance (ANOVA) followed by post hoc Holm-Sidak test. In contrast, the data from ELISA were not normally distributed and therefore analyzed with nonparametric ANOVA on ranks (Kruskal-Wallis test) followed by post hoc Student-Newman-Keuls test. Statistical calculations were performed using SigmaStat for Windows (Jandel Scientific Corp, San Rafael, California). Statistical significance was defined as $P < .05$.

Results

Increased p38 MAPK Activity in Endometriosis

Total p38 MAPK immunoreactivity was localized in both cytoplasm and nucleus and its expression did not change throughout the menstrual cycle in both endometrial and endometriotic cells. However, phosho-p38 MAPK staining was mostly nuclear (Figure 1). Expression of phospho-p38 MAPK did not change throughout the menstrual cycle in normal endometrial cells. On the other hand, both stromal and epithelial cells of late proliferative and early secretory samples displayed significantly higher phospho-p38 MAPK levels compared to those of late secretory and early proliferative samples in eutopic and ectopic endometrial tissues of women with endometriosis ($P < .01$; Figure 1). Both stromal and epithelial cells of eutopic and superficial ectopic endometrium exhibited significantly higher phosho-p38 MAPK staining compared to normal endometrium during late proliferative and early secretory phases ($P < .05$; Figure 1). Moreover, phosho-p38 MAPK levels in epithelial cells of ectopic endometrium of endometriosis patients were

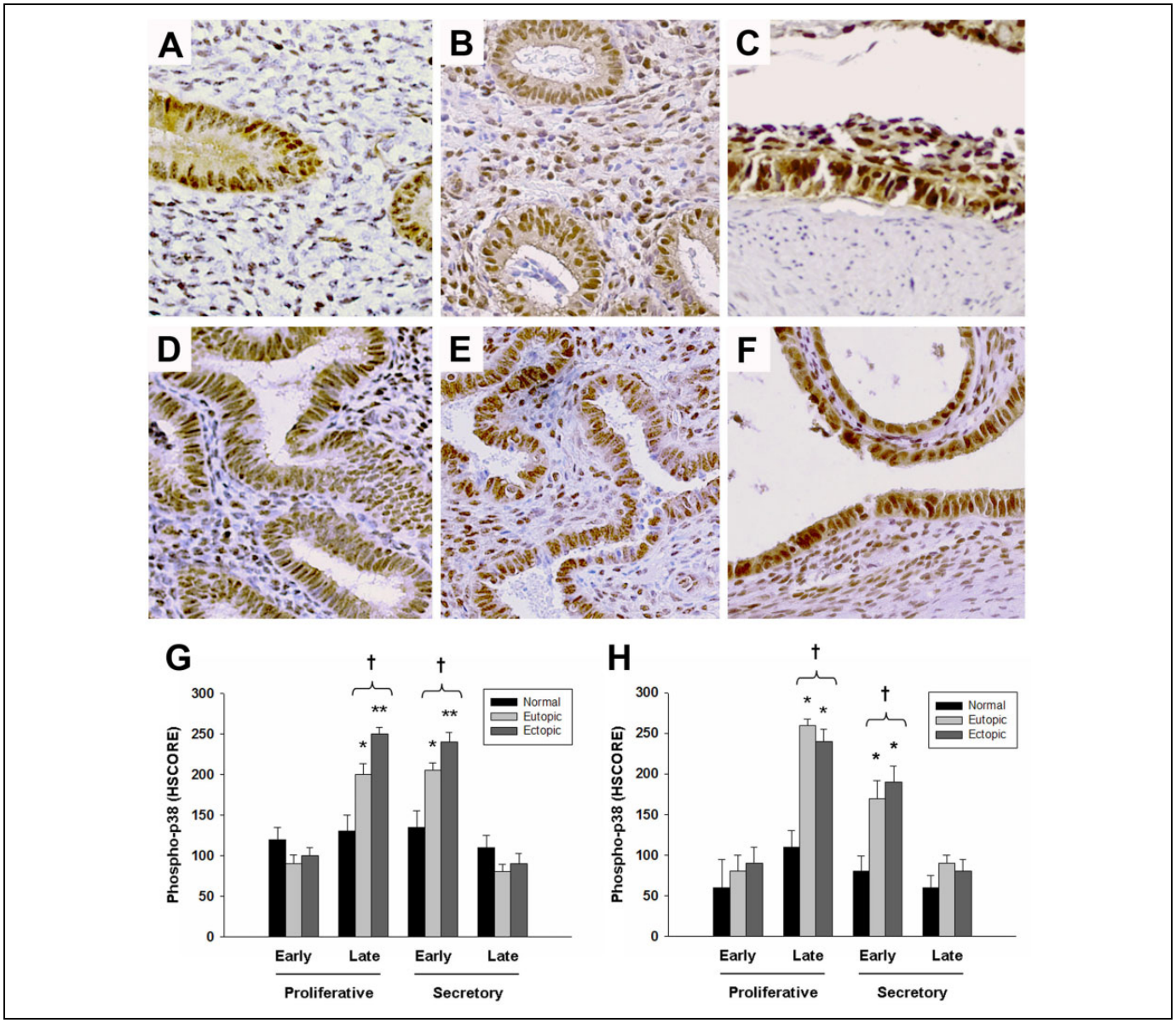

Figure 1. Phospho-p38 MAPK immunoreactivity in normal, eutopic, and ectopic endometrium. Representative micrographs of immunohistochemical staining for phospho-p38 MAPK in normal (A and D), eutopic (B and E) and ectopic (C and F) endometrium from late proliferative (A, B and C) and early secretory phases (D, E, and F). HSCORE analysis of phospho-p38 MAPK immunostaining in endometrial epithelial (G) and stromal (H) cells from normal, eutopic, and ectopic endometrium. Both stromal and epithelial cells of eutopic and superficial ectopic endometrium showed significantly higher phosho-p38 MAPK staining compared to those of normal endometrium during late proliferative and early secretory phases. Moreover, phosho-p38 MAPK levels in epithelial cells of ectopic endometrium of endometriosis patients were significantly higher than those of the same patient's eutopic endometrium. *$P < .05$ versus normal endometrium of the same menstrual phase. **$P < .05$ versus eutopic endometrium of the same menstrual phase. †$P < .01$ versus eutopic and ectopic endometrium from early proliferative and late secretory phases. HSCORE indicates histological score; MAPK, mitogen-activated protein kinase.

significantly higher than those of eutopic endometrium of the same patient ($P < .05$; Figure 1). Superficial ectopic endometrial epithelial and stromal cells revealed the highest phosho-p38 MAPK immunoreactivity (Figure 1). Interestingly, phospho-p38 MAPK expression was significantly higher in both epithelial and stromal cells of the superficial ectopic endometrial implants compared to those of deeper implants of the same sample ($P < .05$; Figure 2).

Increased p38 MAPK Activity Correlates With IL-8 Expression, but not With Apoptosis

To evaluate the correlation between p38 MAPK activity, IL-8 expression and apoptosis; serial ectopic endometrial sections were immunostained with phospho-p38 MAPK and IL-8 antibodies, and TUNEL assay was performed. The IL-8 was highly expressed in endometriosis implants, and its expression was higher in ectopic endometriotic cells with high immunoreactive

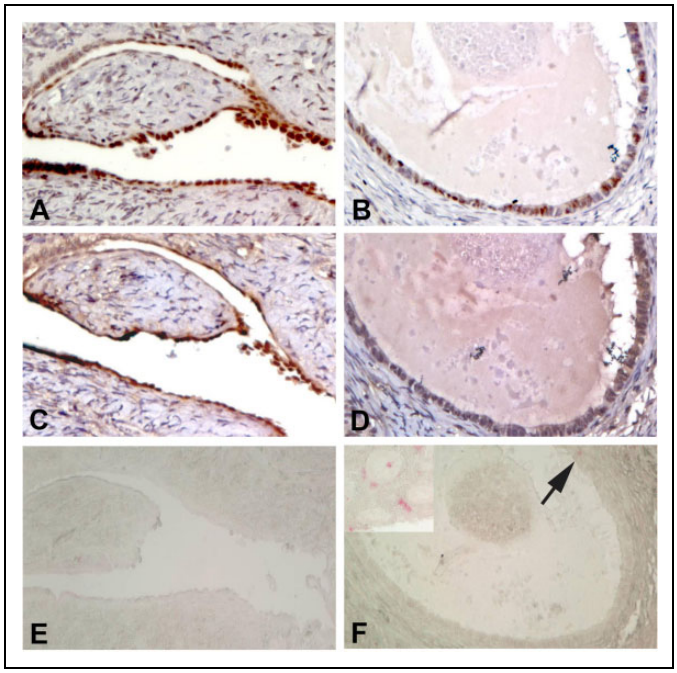

Figure 2. Correlation of p38 MAPK immunoreactivity with IL-8 expression and apoptosis in ectopic endometriosis implants. Representative micrographs of immunohistochemical staining for phospho-p38 MAPK (A and B) and IL-8 (C and D), and TUNEL (E and F) in superficial (A, C, and E) and deep (B, D, and F) ectopic endometrium in serial sections. The IL-8 expression was higher in ectopic endometriotic cells with high phospho-p38 MAPK immunoreactivity and lower in those with low p38 MAPK activity. However, there was no correlation between phospho-p38 MAPK levels and percentage of TUNEL-positive cells. Arrow demonstrates a TUNEL (+) cell in endometriosis implant. Inset picture shows TUNEL (+) cells in the eutopic endometrium of the same patient. MAPK indicates mitogen-activated protein kinase; IL-8, interleukin 8; TUNEL, Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

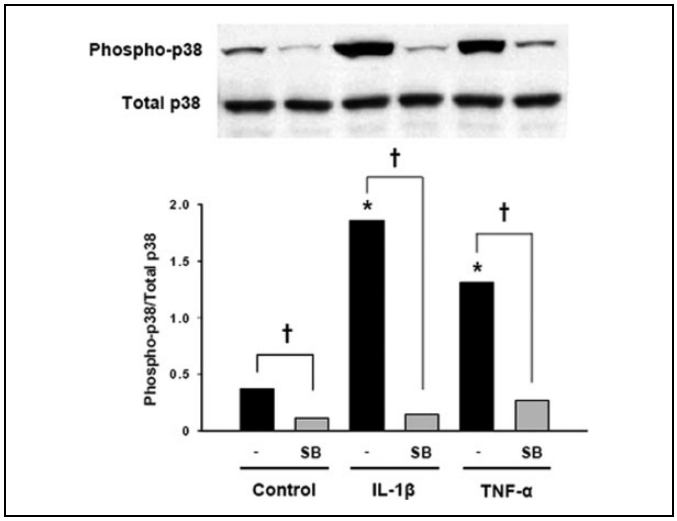

Figure 3. Western blot analysis demonstrating the effect of inflammatory cytokines and p38 MAPK inhibitor (SB203580) on total and phospho-p38 MAPK levels in ESCs. Confluent ESC cultures were treated with vehicle (control), IL-1β (1 ng/mL), or TNF-α (1 ng/mL) for 10 minutes. To evaluate the effect of a p38 MAPK inhibitor, the cultures were preincubated with SB203580 (10 μM) for 30 minutes before the treatment with IL-1β or TNF-α. *$P < .05$ versus control. †$P < .05$ between individual treatments with and without SB203580 in pairwise comparison. Data are representative of 3 independent experiments. MAPK indicates mitogen-activated protein kinase; ESCs, endometrial stromal cells; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α.

phospho-p38 and lower in those with low immunoreactive p38 MAPK (Figure 2). Similar to phospho-p38 MAPK immunostaining, IL-8 expression was higher in both epithelial and stromal cells of the superficial ectopic endometrial implants compared to those of deeper implants of the same sample. Moreover, IL-8 expression correlated positively with phospho-p38 MAPK levels (Pearson correlation coefficient $r = 0.83$, $P < .01$). On the other hand, there were only few TUNEL-positive cells (1.5% of total cell number) in endometriosis implants with no correlation between phospho-p38 MAPK levels and percentage of TUNEL-positive cells (Figure 2).

Pro-inflammatory Cytokines Induce p38 MAPK Phosphorylation in Endometriotic Stromal Cells

To investigate the effects of pro-inflammatory cytokines on p38 MAPK activation, after pretreatment with either vehicle or SB203580 (10 μM, specific p38 MAPK inhibitor) for 30 minutes, cultured endometriotic stromal cells were incubated with vehicle (control), or IL-1β (1 ng/mL) or TNF-α (1 ng/mL) for 10 minutes. The IL-1β and TNF-α significantly induced p38 MAPK phosphorylation in endometriotic cells ($P < .05$; Figure 3), and this effect was blocked by SB203580. However, total p38 MAPK levels did not change with any of the treatments.

Pro-inflammatory Cytokine Induced IL-8 and MCP-1 Expression in Endometriotic Stromal Cells is Regulated by p38 MAPK

To investigate the role of p38 MAPK in the regulation of pro-inflammatory cytokine-induced IL-8 and MCP-1 expression after pretreatment with either vehicle or SB203580 (10 μM) for 30 minutes, cultured endometriotic stromal cells were incubated with vehicle (control), IL-1β (1 ng/mL) or TNF-α (1 ng/mL) for 24 hours. The IL-1β and TNF-α treatments increased IL-8 levels by 120 ± 11 (mean ± standard error of the mean [SEM]) and 37 ± 7-fold, and MCP-1 levels by 93 ± 12 and 68 ± 9-fold in endometriotic stromal cells, respectively ($P < .01$; Figure 4). However, SB203580 significantly suppressed the effects of IL-1β and TNF-α on IL-8 levels by 53% ± 6% and 75% ± 9%, and MCP-1 levels by 33% ± 3% and 39% ± 5%, respectively ($P < .05$; Figure 4). Moreover, SB203580 also significantly decreased the basal secretion of IL-8 and MCP-1 by 46% ± 3% and 38% ± 4% in endometriotic stromal cells, respectively ($P < .05$; Figure 4).

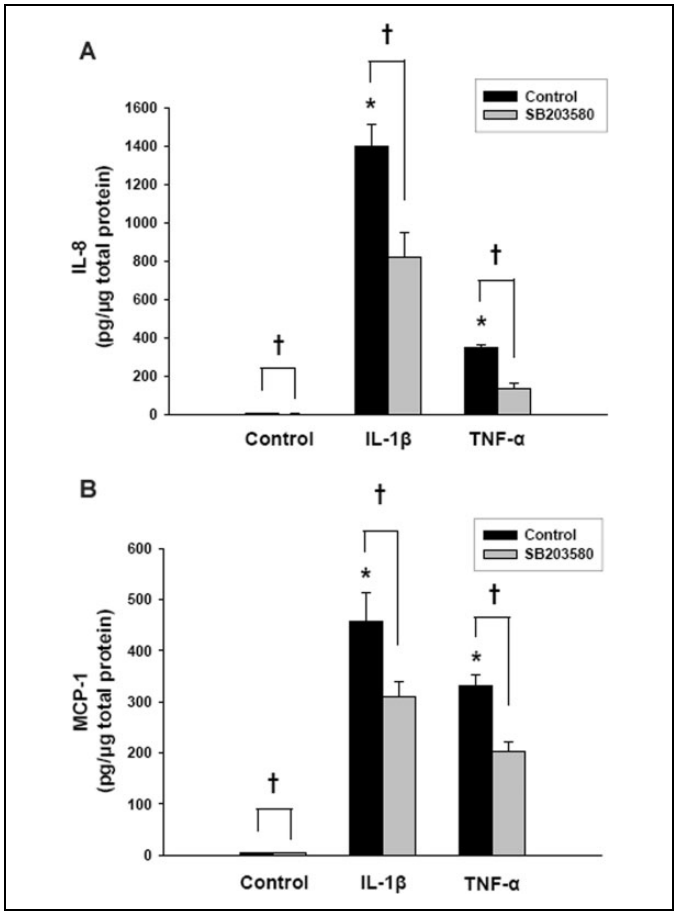

Figure 4. Effects of IL-1β, TNF-α, and p38 MAPK inhibitor (SB203580) on IL-8 (A) and MCP-1 (B) secretions in cultured ESC. Confluent ESC cultures were treated with vehicle (control), IL-1β (1 ng/mL), or TNF-α (1 ng/mL) for 24 hours. To evaluate the effect of a p38 MAPK inhibitor, the cultures were preincubated with SB203580 (10 μM) for 30 minutes before the treatment with IL-1β or TNF-α. IL-8 and MCP-1 levels were quantified by ELISA in culture media and normalized to total cell protein (mean ± standard error of mean [SEM]; n = 3). *$P < .01$ versus Control. †$P < .05$ between individual treatments with and without SB203580 in pairwise comparison. IL-1β indicates interleukin 1β; TNF-α, tumor necrosis factor α; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein 1; ESC, endometrial stromal cell; ELISA, enzyme-linked immunosorbent assay.

p38 MAPK Does not Directly Regulate Cell Survival in Endometriotic Stromal Cells In Vitro

In order to determine the impact of p38 MAPK on the apoptotic index of cultured endometriotic stromal cells, TUNEL assay was used to assess DNA breaks indicative of early-stage apoptosis under basal and SB203580 (1 and 10 μM)-treated conditions. The TUNEL assay showed that treatment of endometriotic stromal cells with either SB203580 concentration did not change the percentage of TUNEL-positive endometriotic stromal cells following 24- and 72-hour treatments (Figure 5A). Moreover, to determine whether p38 MAPK has a role in the proliferation of endometriotic cells, BrdU and MTT colorimetric assays were performed following 24- and 72-hour treatments with p38 MAPK inhibitor. Neither 1 μM nor 10 μM SB203580 treatment altered the endometriotic cell proliferation index compared to controls (Figure 5B and C). On the other hand, the MTT assay revealed that compared to the control group, SB203580 at 10 μM concentration significantly reduced survival rate of normal ESCs (control vs SB203580: mean ± standard error of mean: 0.99 ± 0.11 vs 0.69 ± 0.04 arbitrary units, respectively; $P < .05$, n = 3), indicating that p38 inhibition contributes to suppression of normal stromal cell survival/proliferation, whereas endometriotic stromal cells are resistant to this inhibition of p38 MAPK signaling.

Discussion

High concentrations of chemokines, growth factors, and inflammatory cytokines in the peritoneal fluid of women with endometriosis are derived from the lesions and secretory products of macrophages and other immune cells.[12,14,44,45] These factors induce attachment and further exacerbate the inflammatory response as well as promote growth and survival of endometriotic implants by activating different signaling pathways.[46,47]

This study demonstrated that although total p38 MAPK expression levels are similar in normal endometrium and endometriotic tissues, activated (phosphorylated) levels of p38 MAPK are significantly higher in ectopic implants compared to normal endometrium in late proliferative and early secretory phases. Ovarian endometrioma, superficial peritoneal endometriosis as well as deep infiltrating endometriosis are the most frequently occurring phenotypes. Studies indicate that these phenotypes may have different pathogenetic mechanism.[48] Deep infiltrating endometriosis suggested as the most severe form due to their similarity in expression profiles/levels of molecules (myostatin, myostatin receptors, matrix metalloproteinases, and activins, etc) to endometrial cancer cells.[48,49] Interestingly, superficial ectopic endometriosis implants displayed higher p38 MAPK activity compared to those of deeper implants from the same patient in our study. We previously reported significantly higher levels of phospho-p38 MAPK immunostaining in stromal and epithelial cells in the functional layer compared to the basal layer of normal endometrium.[34] Similarly, higher p38 MAPK phosphorylation levels in superficial versus deep ectopic endometriotic cells may mimic the phospho-p38 MAPK expression pattern seen between the layers of normal endometrium. Alternatively, this discrepancy in phospho-p38 MAPK expression can result from direct exposure of superficial implants to the inflammatory peritoneal environment dominated by peritoneal fluid and/or macrophages. Moreover, a correlation between IL-8 expression and p38 MAPK activity in ectopic endometrial implants suggests that increased IL-8 levels is at least partially regulated by the p38 MAPK pathway. Our in vitro findings showing induction of p38 MAPK phosphorylation and, in turn, stimulation of MCP-1 and IL-8 expression in cultured endometriotic stromal cells by IL-1β or TNF-α complement this suggestion. Also,

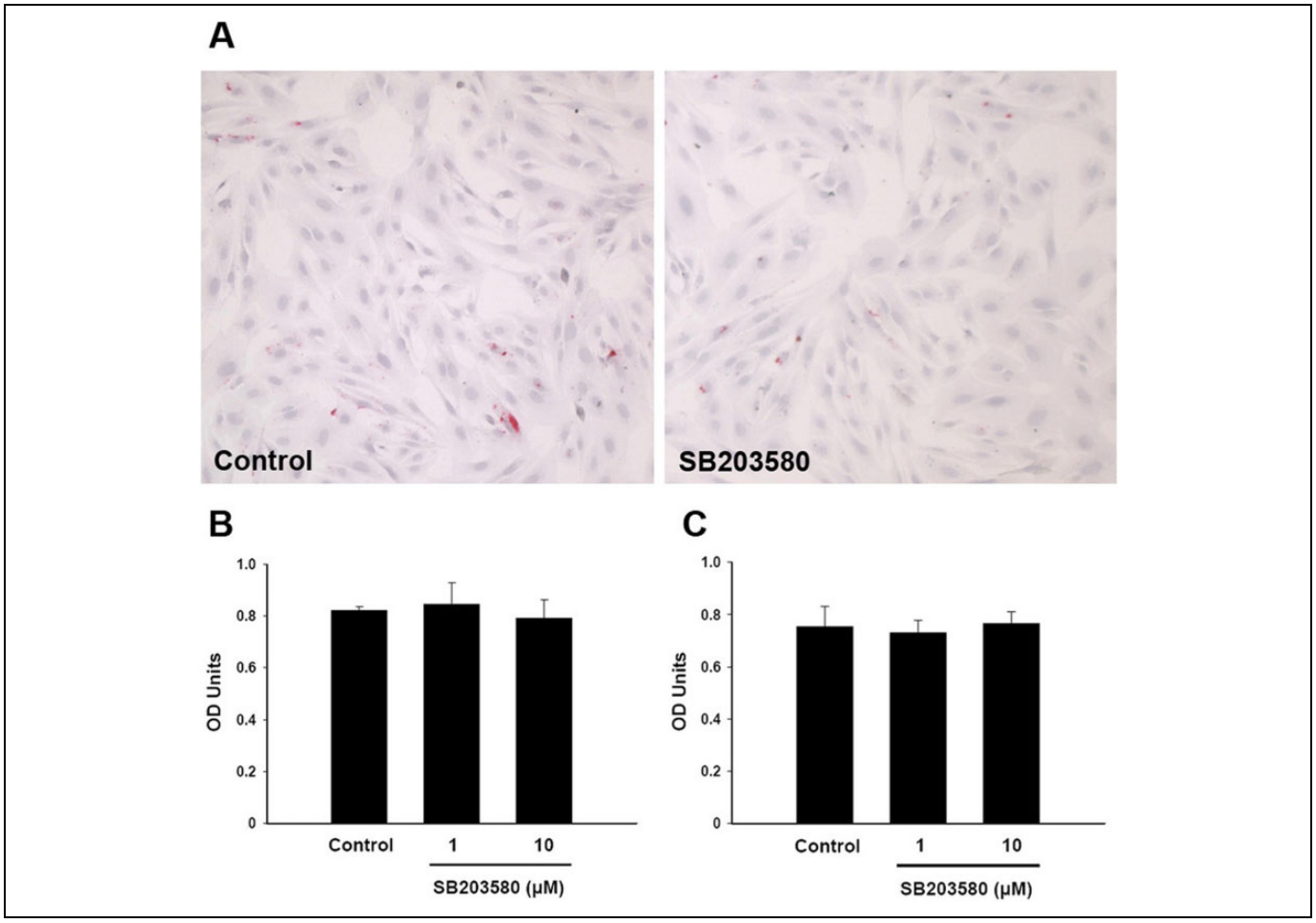

Figure 5. Evaluation of the role of p38 MAPK in apoptosis and cell proliferation in endometrial stromal cells (ESC) in vitro. TUNEL assay (A) was used to detect ESCs with DNA strand breaks indicative of apoptosis. TUNEL assay showed that SB203580 (10 μM) did not change the percentage of TUNEL-positive endometriotic stromal cell after 24 hours. BrdU incorporation (B) and MTT proliferation assays (C) were performed following treatments with SB203580 (1 and 10 μM) for 24 hours. Inhibition of p38 MAPK did not result in change in the proliferation of endometriotic cells compared to controls at any concentration used. MAPK indicates mitogen-activated protein kinase; ESC, endometrial stromal cell; TUNEL, Deoxyuridine Triphosphate Nick End Labeling; BrdU, 5-bromo-2′-deoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

previous studies that described high MCP-1 and IL-8 concentrations in peritoneal fluid of patients with endometriosis strongly support this premise.[14,16,50] In endometriotic cells, a specific p38 MAPK inhibitor completely blocked IL-1β- or TNF-α-induced p38 MAPK activation, whereas this inhibitory effect only partially occurred on IL-1β- and TNF-α-induced MCP-1 and IL-8 expression, indicating involvement of other signaling pathways in IL-1β or TNF-α-mediated production of these cytokines.[50]

Another interesting finding is the temporal regulation of the p38 MAPK activity in endometriotic cells but not in normal endometrial cells throughout the menstrual cycle. In eutopic and ectopic endometriosis tissues, higher p38 MAPK activity in late proliferative and early secretory samples compared to the remainder of the cycle may be associated with high estrogenic dominance of the environment of endometriotic tissues. In this regard, we previously demonstrated that E_2 induces the phosphorylation of p38 MAPK in normal endometrium.[34] Alternatively, increased E_2 production may exaggerate cytokine-induced activation of p38 MAPK in endometriotic cells since the peak of serum E_2 levels coincides with higher p38 MAPK activity in endometriosis. Moreover, estrogen receptor (ER) switching (increased ERβ/ERα) in ectopic versus normal endometrium may be associated with changes in activation of p38 MAPK observed in endometriosis since augmentation of ERβ expression in ESCs inhibits E_2-induced p38 MAPK phosphorylation.[51]

Resistance to apoptosis in ectopic endometriosis implants likely contributes to enhanced survival of endometriotic cells.[52,53] Abundant evidence exists for the involvement of p38 MAPK signaling in apoptosis/cell survival based on concomitant activation of p38 MAPK and apoptosis by a variety of influences such as neuronal growth factor withdrawal and Fas ligation.[54-56] Cysteine proteases (caspases)

are central to the apoptotic pathway, and p38 MAPK may function as both upstream and downstream modulators of caspases in apoptosis.[57-60] However, the role of p38 MAPK in apoptosis is cell type and stimulus-dependent. Although p38 MAPK signaling has been shown to promote cell death in some cell lines, it can also enhance survival, cell growth, and differentiation in different cell lines.[61-64] Our in situ observations revealing low rate of apoptosis in ectopic endometrial implants is consistent with other studies.[23,26] Moreover, the absence of any in situ correlation between apoptosis rate and p38 MAPK activity in endometriosis implants, and lack of effect of a p38 MAPK inhibitor on cultured endometriotic cell proliferation/apoptosis observed in the current study suggest that increased p38 MAPK signaling is not involved in endometriotic cell proliferation or apoptosis. Worth noting is that previous studies suggested the role of activation of extracellular signal regulated kinases 1 and 2 and protein kinase B signaling cascades in endometriotic cell proliferation and resistance to apoptotic cell death.[60,65] However, further studies are required to investigate the role of p38 MAPK in other endometriosis-related cellular mechanisms, such as reactive oxygen species–induced impaired immune response[1] and/or epithelial–mesenchymal transition that induce fibrotic tissue formation, which Zheng et al have recently demonstrated to be a hallmark of deep infiltrating endometriotic lesions.[66,67]

Due to restrictions in isolation and growing pure culture of endometrial epithelial cells as well as their limitation in passaging in culture, especially from ectopic sites, has prevented us in performing in vitro comparisons of the role p38 MPAK signaling in regulating proliferation, apoptosis, and cytokine secretion in normal versus endometriotic epithelial cells. Another limitation of the study was that the information about stage and severity of endometriosis was not available in some cases and, therefore, stage and severity of endometriosis were not reported in this study.

In summary, we have demonstrated that p38 MAPK activity is higher in both eutopic and ectopic epithelial and stromal cells of patients with endometriosis compared to normal endometrium during late proliferative and early secretory phases. Moreover, increased MAPK activity in endometriotic cells was correlated with inflammatory cytokine production but not with cell survival. Therefore, IL-1β and TNF-α induction of the p38 MAPK signaling pathway may contribute to the inflammatory peritoneal milieu in women with endometriosis. Although the p38 MAPK pathway is not directly involved in the survival of ectopic endometriosis implants, p38 MAPK-mediated expression of pro-inflammatory cytokines such as IL-8 and MCP-1 may enhance cell survival thereby contributing to endometriosis pathogenesis. Thus, the blockage of this pathway by using specific p38 MAPK inhibitors that have been proven to lack side effects may alleviate the inflammatory endometriotic environment and, in turn, impede the establishment and progression of endometriosis as previously shown in a murine endometriosis model.[68] Finally, in a BALB/c mouse with surgically induced endometriosis, increased p38 activation is reported in sensory nerve cells of the rostral–ventromedulla,[51] supporting a potential role for this pathway in inflammation-mediated endometriotic pain.[69] Thus, the inhibition of the p38 MAPK pathway may also provide a treatment option for endometriosis-related pain or infertility in the future since inflammatory environment may also cause decreased oocyte quality and implantation.[70] The molecular mechanism responsible for endometriotic resistance to p38 MAPK inhibitor-mediated inhibition of cell survival is an important subject for future investigation.

Authors' Note

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Declaration of Conflicting Interests

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References

1. Cakmak H, Guzeloglu-Kayisli O, Kayisli UA, Arici A. Immune-endocrine interactions in endometriosis. *Front Biosci (*Elite *Ed)*. 2009;1:429-443.
2. Othman ER, Meligy FY, Sayed AA, El-Mokhtar MA, Refaiy AM. Stem cell markers describe a transition from somatic to pluripotent cell states in a rat model of endometriosis [Published online ahead of print]. *Reprod Sci*. 2017:doi: 10.1177/1933719117697124.
3. Pluchino N, Taylor HS. Endometriosis and stem cell trafficking. *Reprod Sci*. 2016;23(12):1616-1619.
4. Gogacz M, Winkler I, Bojarska-Junak A, et al. Increased percentage of Th17 cells in peritoneal fluid is associated with severity of endometriosis. *J Reprod Immunol*. 2016;117:39-44.
5. Jeung I, Cheon K, Kim MR. Decreased cytotoxicity of peripheral and peritoneal natural killer cell in endometriosis. *Biomed Res Int*. 2016;2016:2916070.
6. Schatz F, Guzeloglu-Kayisli O, Arlier S, Kayisli UA, Lockwood CJ. The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding. *Hum Reprod Update*. 2016;22(4):497-515.
7. Ulukus M, Cakmak H, Arici A. The role of endometrium in endometriosis. *J Soc Gynecol Investig*. 2006;13(7):467-476.
8. Wickstrom K, Stavreus-Evers A, Vercauteren O, Olovsson M, Edelstam G. Effect of lignocaine on IL-6, IL-8, and MCP-1 in peritoneal macrophages and endometriotic stromal cells. *Reprod Sci*. 2017;24(3):382-392.
9. Rakhila H, Al-Akoum M, Bergeron ME, et al. Promotion of angiogenesis and proliferation cytokines patterns in peritoneal fluid from women with endometriosis. *J Reprod Immunol*. 2016;116:1-6.

10. Kocbek V, Vouk K, Bersinger NA, Mueller MD, Lanisnik Rizner T. Panels of cytokines and other secretory proteins as potential biomarkers of ovarian endometriosis. *J Mol Diagn*. 2015;17(3): 325-334.
11. Fakih H, Baggett B, Holtz G, Tsang KY, Lee JC, Williamson HO. Interleukin-1: a possible role in the infertility associated with endometriosis. *Fertil Steril*. 1987;47(2):213-217.
12. Mori H, Sawairi M, Nakagawa M, Itoh N, Wada K, Tamaya T. Peritoneal fluid interleukin-1 beta and tumor necrosis factor in patients with benign gynecologic disease. *Am J Reprod Immunol*. 1991;26(2):62-67.
13. Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohi J, Simon C. The follicular and endocrine environment in women with endometriosis: local and systemic cytokine production. *Fertil Steril*. 1998;70(3):425-431.
14. Arici A, Tazuke SI, Attar E, Kliman HJ, Olive DL. Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. *Mol Hum Reprod*. 1996;2(1):40-45.
15. Ryan IP, Tseng JF, Schriock ED, Khorram O, Landers DV, Taylor RN. Interleukin-8 concentrations are elevated in peritoneal fluid of women with endometriosis. *Fertil Steril*. 1995; 63(4):929-932.
16. Arici A, Oral E, Attar E, Tazuke SI, Olive DL. Monocyte chemotactic protein-1 concentration in peritoneal fluid of women with endometriosis and its modulation of expression in mesothelial cells. *Fertil Steril*. 1997;67(6):1065-1072.
17. Young VJ, Ahmad SF, Brown JK, Duncan WC, Horne AW. Peritoneal VEGF-A expression is regulated by TGF-beta1 through an ID1 pathway in women with endometriosis. *Sci Rep*. 2015;5: 16859.
18. Garcia-Velasco JA, Arici A. Interleukin-8 stimulates the adhesion of endometrial stromal cells to fibronectin. *Fertil Steril*. 1999; 72(2):336-340.
19. Zhang RJ, Wild RA, Ojago JM. Effect of tumor necrosis factor-alpha on adhesion of human endometrial stromal cells to peritoneal mesothelial cells: an in vitro system. *Fertil Steril*. 1993; 59(6):1196-1201.
20. Arici A. Local cytokines in endometrial tissue: the role of interleukin-8 in the pathogenesis of endometriosis. *Ann N Y Acad Sci*. 2002;955:101-109; discussion 118, 396-406.
21. Arici A, Seli E, Zeyneloglu HB, Senturk LM, Oral E, Olive DL. Interleukin-8 induces proliferation of endometrial stromal cells: a potential autocrine growth factor. *J Clin Endocrinol Metab*. 1998; 83(4):1201-1205.
22. Lebovic DI, Bentzien F, Chao VA, Garrett EN, Meng YG, Taylor RN. Induction of an angiogenic phenotype in endometriotic stromal cell cultures by interleukin-1beta. *Mol Hum Reprod*. 2000; 6(3):269-275.
23. Gebel HM, Braun DP, Tambur A, Frame D, Rana N, Dmowski WP. Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. *Fertil Steril*. 1998;69(6):1042-1047.
24. Song Y, Fu J, Zhou M, et al. Activated hippo/yes-associated protein pathway promotes cell proliferation and anti-apoptosis in endometrial stromal cells of endometriosis. *J Clin Endocrinol Metab*. 2016;101(4):1552-1561.
25. Dmowski WP, Ding J, Shen J, Rana N, Fernandez BB, Braun DP. Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. *Hum Reprod*. 2001;16(9): 1802-1808.
26. Beliard A, Noel A, Foidart JM. Reduction of apoptosis and proliferation in endometriosis. *Fertil Steril*. 2004;82(1):80-85.
27. Harada T, Taniguchi F, Izawa M, et al. Apoptosis and endometriosis. *Front Biosci*. 2007;12:3140-3151.
28. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev*. 2001;81(2):807-869.
29. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002;298(5600):1911-1912.
30. Olson JM, Hallahan AR. p38 MAP kinase: a convergence point in cancer therapy. *Trends Mol Med*. 2004;10(3):125-129.
31. Delaney AA, Khan Z, Zheng Y, et al. KLF10 mediated epigenetic dysregulation of epithelial CD40/CD154 promotes endometriosis. *Biol Reprod*. 2016;95(3):62.
32. Yang RQ, Teng H, Xu XH, et al. Microarray analysis of microRNA deregulation and angiogenesis-related proteins in endometriosis [Published online ahead of print]. *Genet Mol Res*. 2016; 15(2). doi: 10.4238/gmr.15027826.
33. Au HK, Chang JH, Wu YC, et al. TGF-betaI regulates cell migration through pluripotent transcription factor OCT4 in endometriosis. *PLoS One*. 2015;10(12):e0145256.
34. Seval Y, Cakmak H, Kayisli UA, Arici A. Estrogen-mediated regulation of p38 mitogen-activated protein kinase in human endometrium. *J Clin Endocrinol Metab*. 2006;91(6):2349-2357.
35. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol*. 1975;122(2):262-263.
36. Yen CF, Liao SK, Huang SJ, et al. Decreased endometrial expression of leukemia inhibitory factor receptor disrupts the STAT3 signaling in adenomyosis during the implantation window. *Reprod Sci*. 2017;24(8):1176-1186.
37. Arici A, Head JR, MacDonald PC, Casey ML. Regulation of interleukin-8 gene expression in human endometrial cells in culture. *Mol Cell Endocrinol*. 1993;94(2):195-204.
38. Kayisli UA, Selam B, Guzeloglu-Kayisli O, Demir R, Arici A. Human chorionic gonadotropin contributes to maternal immunotolerance and endometrial apoptosis by regulating Fas-Fas ligand system. *J Immunol*. 2003;171(5):2305-2313.
39. Sikora J, Mielczarek-Palacz A, Kondera-Anasz Z. Association of the precursor of interleukin-1beta and peritoneal inflammation-role in pathogenesis of endometriosis. *J Clin Lab Anal*. 2016; 30(6):831-837.
40. Tao Y, Zhang Q, Huang W, Zhu H, Zhang D, Luo W. The peritoneal leptin, MCP-1 and TNF-alpha in the pathogenesis of endometriosis-associated infertility. *Am J Reprod Immunol*. 2011;65(4):403-406.
41. Sukhikh GT, Sotnikova NY, Antsiferova YS, Posiseeva LV, Veryasov VN, Van'ko LV. Cytokine production by immunocompetent cells of peritoneal fluid in women with external genital endometriosis. *Bull Exp Biol Med*. 2004;137(6):568-571.
42. Rana N, Braun DP, House R, Gebel H, Rotman C, Dmowski WP. Basal and stimulated secretion of cytokines by peritoneal

macrophages in women with endometriosis. *Fertil Steril*. 1996; 65(5):925-930.
43. Seli E, Guzeloglu-Kayisli O, Cakmak H, Kayisli UA, Selam B, Arici A. Estradiol increases apoptosis in human coronary artery endothelial cells by up-regulating Fas and Fas ligand expression. *J Clin Endocrinol Metab*. 2006;91(12):4995-5001.
44. Yeol SG, Won YS, Kim YI, Lee JW, Choi YJ, Park DC. Decreased Bcl-6 and increased Blimp-1 in the peritoneal cavity of patients with endometriosis. *Clin Exp Obstet Gynecol*. 2015; 42(2):156-160.
45. Borrelli GM, Kaufmann AM, Abrao MS, Mechsner S. Addition of MCP-1 and MIP-3beta to the IL-8 appraisal in peritoneal fluid enhances the probability of identifying women with endometriosis. *J Reprod Immunol*. 2015;109:66-73.
46. Zhang L, Xiong W, Xiong Y, Liu H, Liu Y. 17 beta-Estradiol promotes vascular endothelial growth factor expression via the Wnt/beta-catenin pathway during the pathogenesis of endometriosis. *Mol Hum Reprod*. 2016;22(7):526-535.
47. Ahn JH, Choi YS, Choi JH. Leptin promotes human endometriotic cell migration and invasion by up-regulating MMP-2 through the JAK2/STAT3 signaling pathway. *Mol Hum Reprod*. 2015; 21(10):792-802.
48. Tosti C, Pinzauti S, Santulli P, Chapron C, Petraglia F. Pathogenetic mechanisms of deep infiltrating endometriosis. *Reprod Sci*. 2015;22(9):1053-1059.
49. Carrarelli P, Funghi L, Ciarmela P, et al. Deep infiltrating endometriosis and endometrial adenocarcinoma express high levels of myostatin and its receptors messenger RNAs [Published online ahead of print]. *Reprod Sci*. 2017. doi: 10.1177/1933719117698579.
50. McKinnon BD, Kocbek V, Nirgianakis K, Bersinger NA, Mueller MD. Kinase signalling pathways in endometriosis: potential targets for non-hormonal therapeutics. *Hum Reprod Update*. 2016; 22(3): 382-403.
51. Chen S, Wu RF, Su L, Zhou WD, Zhu MB, Chen QH. Lipoxin A4 regulates expression of the estrogen receptor and inhibits 17beta-estradiol induced p38 mitogen-activated protein kinase phosphorylation in human endometriotic stromal cells. *Fertil Steril*. 2014; 102(1):264-271.
52. Hapangama DK, Turner MA, Drury JA, et al. Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length. *Hum Reprod*. 2008;23(7): 1511-1519.
53. Watanabe A, Taniguchi F, Izawa M, et al. The role of survivin in the resistance of endometriotic stromal cells to drug-induced apoptosis. *Hum Reprod*. 2009;24(12):3172-3179.
54. Henkart PA. ICE family proteases: mediators of all apoptotic cell death? *Immunity*. 1996;4(3):195-201.
55. Juo P, Kuo CJ, Reynolds SE, et al. Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. *Mol Cell Biol*. 1997;17(1): 24-35.
56. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*. 1995;270(5240):1326-1331.
57. Huang S, Jiang Y, Li Z, et al. Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity*. 1997;6(6):739-749.
58. Natale DR, Paliga AJ, Beier F, D'Souza SJ, Watson AJ. p38 MAPK signaling during murine preimplantation development. *Dev Biol*. 2004;268(1):76-88.
59. Leconte M, Santulli P, Chouzenoux S, et al. Inhibition of MAPK and VEGFR by Sorafenib Controls the Progression of Endometriosis. *Reprod Sci*. 2015;22(9):1171-1180.
60. Li MQ, Shao J, Meng YH, et al. NME1 suppression promotes growth, adhesion and implantation of endometrial stromal cells via Akt and MAPK/Erk1/2 signal pathways in the endometriotic milieu. *Hum Reprod*. 2013;28(10):2822-2831.
61. Mao Y, Wang J, Yu F, et al. Ghrelin protects against palmitic acid or lipopolysaccharide-induced hepatocyte apoptosis through inhibition of MAPKs/iNOS and restoration of Akt/eNOS pathways. *Biomed Pharmacother*. 2016;84:305-313.
62. Chen NN, Wei F, Wang L, Cui S, Wan Y, Liu S. Tumor necrosis factor alpha induces neural stem cell apoptosis through activating p38 MAPK pathway. *Neurochem Res*. 2016;41(11):3052-3062.
63. Santulli P, Marcellin L, Tosti C, et al. MAP kinases and the inflammatory signaling cascade as targets for the treatment of endometriosis? *Expert Opin Ther Targets*. 2015;19(11): 1465-1483.
64. Huang Y, Li X, Wang Y, Wang H, Huang C, Li J. Endoplasmic reticulum stress-induced hepatic stellate cell apoptosis through calcium-mediated JNK/P38 MAPK and Calpain/Caspase-12 pathways. *Mol Cell Biochem*. 2014;394(1-2):1-12.
65. Cinar O, Seval Y, Uz YH, et al. Differential regulation of Akt phosphorylation in endometriosis. *Reprod Biomed Online*. 2009; 19(6):864-871.
66. Zheng Y, Khan Z, Zanfagnin V, Correa LF, Delaney AA, Daftary GS. Epigenetic modulation of collagen 1A1: therapeutic implications in fibrosis and endometriosis. *Biol Reprod*. 2016; 94(4):87.
67. Richards EG, Zheng Y, Shenoy CC, et al. KLF11 is an epigenetic mediator of DRD2/dopaminergic signaling in endometriosis. *Reprod Sci*. 2017:24(8):1129-1138.
68. Yoshino O, Osuga Y, Koga K, et al. FR 167653, a p38 mitogen-activated protein kinase inhibitor, suppresses the development of endometriosis in a murine model. *J Reprod Immunol*. 2006;72(1-2): 85-93.
69. McKinnon BD, Bertschi D, Bersinger NA, Mueller MD. Inflammation and nerve fiber interaction in endometriotic pain. *Trends Endocrinol Metab*. 2015;26(1):1-10.
70. Donaghay M, Lessey BA. Uterine receptivity: alterations associated with benign gynecological disease. *Semin Reprod Med*. 2007;25(6):461-475.