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ORIGINAL ARTICLE

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Inhibition of IAP (inhibitor of apoptosis) proteins represses inflammatory status *via* nuclear factor-kappa B pathway in murine endometriosis lesions

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Problem: How is the role of inhibitor of apoptosis proteins (IAPs) in the development of murine endometriosis lesions?

Method of study: BALB/c female mice (n = 36) were used for the murine endometriosis model. Endometriotic lesions were surgically induced in mice by transplanting mouse uterine tissue. After 4 weeks of IAP antagonist (BV6) treatment, the expression of inflammatory factors in the implants was evaluated using real-time RT-PCR. Inflammatory state, angiogenic activity, and nuclear factor-kappa B (NF-κB) activation were assessed by immunohistochemical staining.

Results: The number, size, and level of inflammatory cytokines (*Vegf*, *Il-6*, *Ccl-2*, *Lif*) gene expression in the murine endometriosis-like lesions were reduced by BV6 treatment. BV6 repressed the intensity and rate of positive cells of CD3, F4/80, and PECAM immunostaining; in addition, the expression of NF-κB p65 and phospho-NF-κB p65 was also attenuated.

Conclusion: Inhibitor of apoptosis proteins antagonist represses the inflammation status of murine endometriosis-like lesions *via* NF-κB pathway. IAPs may be a novel therapeutic target for endometriosis.

KEYWORDS
endometriosis, IAP antagonist, inflammation, murine model, NF-κB pathway

1 | INTRODUCTION

Endometriosis is defined as ectopic growth of endometrial tissue outside the uterine cavity. This benign gynecologic disorder, which affects 10% to 15% of reproductive age women,[1,2] causes dysmenorrhea, infertility, and chronic pelvic pain. Local inflammatory reaction in the peritoneal environment is one of the contributing factors in the pathogenesis of endometriosis. In women with endometriosis, the peritoneal fluid (PF) is remarkable for an increased number of activated macrophages and inflammatory factors. For instance, we demonstrated the presence of increased levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) α, interleukin (IL)-6, and IL-8 in the PF of patients with endometriosis.[3,4]

Many factors implicated in an endometriosis-associated inflammatory environment are upregulated by the nuclear factor-kappa B (NF-κB), which is a pleiotropic transcription factor favoring cell proliferation and inhibiting apoptosis in human endometriotic stromal cells.[5,6] The pro-inflammatory cytokines, including TNFα and IL-8 released by peritoneal macrophages and endometriotic stromal cells, are upregulated by NF-κB[5]. The main source of these cytokines is thought to be macrophages in the peritoneal cavity; in addition, we showed that ESCs themselves produce cytokines independent of macrophages.[7]

The inhibitors of apoptosis proteins (IAPs) are a family of proteins that are implicated in multiple ways in cell death regulation, ranging from the inhibition of apoptosis to the regulation of cell cycle and inflammation. The IAP family is composed of eight proteins: neuronal

apoptosis inhibitory protein (*birc1*), cellular IAP-1 (cIAP-1, *birc2*), cIAP-2 (*birc3*), X-chromosome-linked IAP (XIAP, *birc4*), survivin (*birc5*), apollon (*birc6*), ML-IAP (*birc7*), and IAP-like protein 2 (*birc8*). The members of this protein family are characterized by the presence of one to three baculoviral IAP repeats (BIR) domains.[8] IAPs have widespread tissue protein expression and suppress mitochondria-dependent and mitochondria-independent apoptosis by binding to and inhibiting caspases through their BIR domains.[9] IAPs have emerged as modulators in an evolutionarily conserved step in apoptosis. Overexpression of IAPs confers protection against a number of proapoptotic stimuli in malignant diseases.[10,11]

We previously proved that cIAP-1, XIAP, and survivin mRNAs in human endometriotic stromal cells derived from ovarian endometriomas were highly expressed compared with those in endometrial stromal cells.[12] We also found that the expression levels of four IAPs (cIAP-1, cIAP-2, XIAP, and survivin) in human endometriotic tissues were higher than those in endometrial tissues,[13] indicating that aberrant expression of IAPs in endometriotic tissues may sustain their abnormal survival in ectopic sites. An IAP antagonist, BV6, is the small-molecule IAP antagonist that binds and inhibits mainly c-IAP1, c-IAP2, and XIAP.[14] BV6 can neutralize IAPs action in the various human and mouse cells.[15,16,17] IAP inhibition may be reasonable as a treatment for endometriosis because these three IAPs expressions were enhanced in the endometriotic cells. We therefore focused on the efficacy of IAP antagonist with emphasis on its potential as a new treatment for endometriosis.

We established the homologous murine endometriosis model by transplanting uterine tissue.[13,18] In the previous study, we proved that the murine endometriosis-like lesions grew in the abdominal cavities of all the mice. IAPs, including cIAP-1, cIAP-2, XIAP, and survivin, were expressed in the murine endometriosis-like implants, especially in the epithelial cells.[13] After 4 weeks of the BV6 treatment, the growth of endometriosis-like lesions was significantly suppressed.[13] In this study, we sought to investigate the efficacy of IAP antagonist and its corresponding pathway on the inflammatory state using a murine endometriosis model. Our results suggest that IAPs and their associated NF-κB pathway would be the key factors as a novel treatment for endometriosis.

2 | MATERIALS AND METHODS

2.1 | Animal care and treatment

All animal handling protocols and surgical procedures were approved by our Institutional Animal Care and Use Committee. Female mice (6 weeks of age, BALB/c) were purchased from Japan SLC (Shizuoka, Japan). Before initiating the experiments, animals were allowed to acclimate for 7 days. All 36 mice included were ovariectomized through a 1 cm longitudinal skin incision then injected subcutaneously with estradiol valerate (E_2: 0.5 μg/mouse/week; Fuji Pharma, Tokyo, Japan) for 6 weeks until the experimental endometriosis induction and kill. 2 weeks after ovariectomy, the uteri of donor mice (n = 12) were removed en bloc after euthanasia and cleaned of excess tissue in sterile saline. Each uterus was cut to include the uterine horns in each half with a linear incision longitudinally and minced (approximately 0.5 mm in diameter) with dissecting scissors. The ovariectomized recipient mice (n = 24) were anesthetized using pentobarbital sodium. A 0.5 cm subabdominal midline incision was made. Each recipient received half of the donor uterus (1:2 donor uterus to host ratio) minced and added to 500 μL saline, and injected into the peritoneal cavity, and the peritoneum sutured. Injected uterine tissue weighed approximately 50 mg per mouse. For the next 4 weeks, recipient mice were treated with a single intraperitoneal injection of BV6, an IAP antagonist provided by Dr. Vucic (n = 8; 10 mg/kg; Genentech, Inc., San Francisco, CA, USA).[19] Parthenolide, a NF-κB inhibitor (10 mg/kg, n = 8; Tocris Bioscience, Bristol, United Kingdom),[18] or vehicle (n = 8; 1% DMSO) twice weekly. After 24 hours from the injection of uterine tissue, the administration of these drugs was started, because it would be harmful for the recipient mice to be treated on the same day of surgical treatment. In considering the results that Dr. D. Vucic (Genentech, Inc.) and his coauthors presented in their papers and his advice to us,[14] we examined this concentration of BV6 (10 mg/kg) for these model mice. The peritoneal cavities of the mice were thoroughly inspected. Endometriosis-like lesions were carefully removed and photographed to document in situ images of the lesions by microscope. The images were transferred to ImageJ software (NIH, Bethesda, MD, USA) for measurement. Using this ImageJ, the surface area of each lesion could be calculated by the manual measurement of perimeter. Lesions were fixed in 10% formalin and stored at −80°C in RNA later™ solution (Life Technologies, Tokyo, Japan).

2.2 | Real-time RT-PCR analysis

Total RNA was extracted from the endometriosis-like tissues using RNeasy Mini Kit (Qiagen, Tokyo, Japan). Reverse transcription (RT) of RNA (1 μg) from the tissues into complementary DNA was performed. The mRNA levels were quantified using the ABI 7900 HT real-time PCR system (Applied Biosystems, Tokyo, Japan). Gene expression of the major inflammatory cytokines, murine vascular endothelial growth factor (*Vegf*), IL-6 *(Il-6)*, monocyte chemotactic protein-1 (*Ccl-2*), and leukemia inhibitory factor (*lif)* in the endometriosis-like lesions was evaluated by real-time RT-PCR. The ABI TaqMan probes for each gene and TaqMan mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagents (Applied Biosystems) were used. The absolute values were normalized to that for *GAPDH*, and the relative values compared with the control were shown. All samples were tested in triplicate, and each run included no-template and no-RT controls.

2.3 | Immunohistochemical staining for murine tissues

Formalin-fixed specimens were paraffin-embedded, cut into 5 μm sections, and immunohistochemical staining was performed using a standard protocol. Sections were deparaffinized, blocked in methanol/0.3% H_2O_2, and then in 10% normal goat serum. The primary

antibody for CD3 (T-cell marker; Abcam, Tokyo, Japan), F4/80 (macrophage cell marker; Abcam), PECAM (CD31, endothelial cell marker, Abcam), NF-κB p65 (Abcam), and phospho-NF-κB p65 (phospho-S536, Abcam) was used. Sections were incubated with biotinylated goat anti-rabbit IgG (1:200) for 1 hour, with streptavidin-peroxidase complex for 30 minutes, with diaminobenzidine for 10 minutes, and then counterstained with hematoxylin. H-score was calculated by a semi-quantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong) and the percentage of positive cells. H-score is assigned using the following formula: (1 × [% cells 1 +] + 2 × [% cells 2 +] + 3 × [% cells 3 +]). The range of scores was from 0 to 300. Negative control slides were incubated similarly, but the primary antibody was replaced with PBS.

2.4 | Statistical analysis

All experiments were repeated a minimum of three times. Results were analyzed using one-way ANOVA, followed by Fisher's protected least significant differences post hoc test. All data sets are presented as means with SEMs. All statistical analyses were carried out using JMP Software (SAS Institute, Cary, NC, USA). $P < .05$ was considered to be statistically significant.

3 | RESULTS

Endometriosis-like cystic implants were found in the abdominal cavities of all recipient mice. Most of the lesions were located around the peritoneal incision and the intestinal membrane. After the treatment of IAP antagonist, BV6 or NF-κB inhibitor, parthenolide (Part), for 4 weeks, the total number (Veh: 5.0 ± 1.6 vs BV6: 2.5 ± 0.6: $P < .01$, and Part: 3.4 ± 0.5: $P < .05$), the average weight (Veh: 73.3 ± 27.8 mg vs BV6: 25.5 ± 10.7 mg: $P < .01$, and Part: 32.6 ± 4.2 mg: $P < .01$), and the surface area (Veh: 43.3 ± 17.1 mm^2 vs BV6: 20.4 ± 6.2 mm^2: $P < .01$, and Part: 22.2 ± 5.8 mm^2: $P < .01$) of lesions per mouse were significantly less than in the vehicle group (Figure 1A and B). BV6 also decreased the *Vegf*, *Il-6*, *Ccl-2*, *and Lif* mRNA expression in the murine endometriosis-like lesions compared with the vehicle group ($P < .05$) (Figure 2). Similarly, parthenolide diminished these inflammation-related cytokines mRNA levels in the lesions (Figure 2).

To evaluate the inflammatory and angiogenic status of the endometriosis-like lesions, the intensity of CD3, F4/80, and PECAM after BV6 or parthenolide treatment was assessed by the immunohistochemical analysis (Figure 3A). By H-score analysis in these implants, BV6 significantly attenuated the intensity and rate of positive cells of CD3 in T cells (Veh: 7.0 ± 2.6, vs BV6: 4.7 ± 1.7: $P < .05$, and Part: 4.2 ± 1.9: $P < .05$), F4/80 in macrophages (Veh: 10.2 ± 3.3, vs BV6: 5.0 ± 1.4: $P < .01$, and Part: 4.8 ± 2.3: $P < .01$), or PECAM in endothelial cells (Veh: 13.7 ± 4.9, vs BV6: 8.0 ± 3.3: $P < .05$, and Part: 7.8 ± 3.2: $P < .05$), indicating both IAP antagonist and NF-κB inhibitor could repress the inflammatory and angiogenic activity (Figure 3B).

Expression of NF-κB p65 detected in the cytoplasm of epithelial and stromal cells in endometriosis-like lesions was significantly repressed by BV6 and parthenolide treatment (Veh: 30.8 ± 3.1, vs BV6: 15.3 ± 2.9: $P < .01$, and Part: 14.2 ± 3.1: $P < .01$). With regard to phospho-p65 NF-κB expression, BV6 also inhibited the expression in the nuclei of epithelial and stromal cells in the implants (Veh: 31.3 ± 5.4, vs BV6: 15.5 ± 3.3: $P < .01$, and Part: 13.4 ± 3.5: $P < .01$) (Figure 4A and B). These expressions regarding the repression of NF-κB pathway were found, especially in epithelial cells of implants.

4 | DISCUSSION

Involvement of inflammation in the pathogenesis of endometriosis is an intriguing subject. An important general concept concerning endometriosis is a local pelvic inflammatory process with altered function of immune-related cells and molecular anomalies and inherent defects

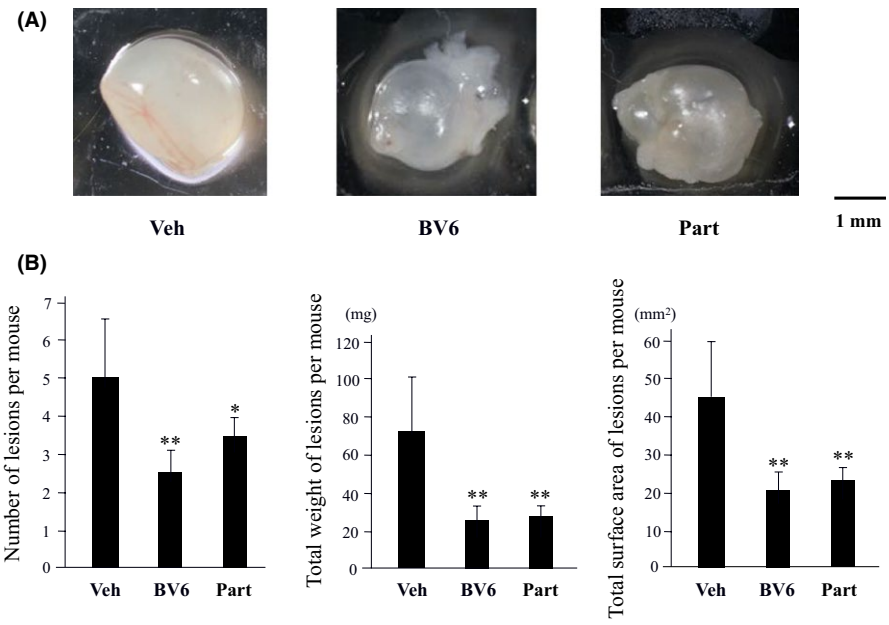

FIGURE 1 Effects of inhibitor of apoptosis proteins (IAP) antagonist (BV6) on the development of the murine endometriosis-like lesions. Veh: vehicle (n = 8). BV6: (n = 8), Part: parthenolide (n = 8). (A) Representative of the excised implants. (B) Total number, weight, and surface area of endometriosis-like lesions were assessed. Bars represent the average ± SE. *$P < .05$ or **$P < .01$ vs vehicle

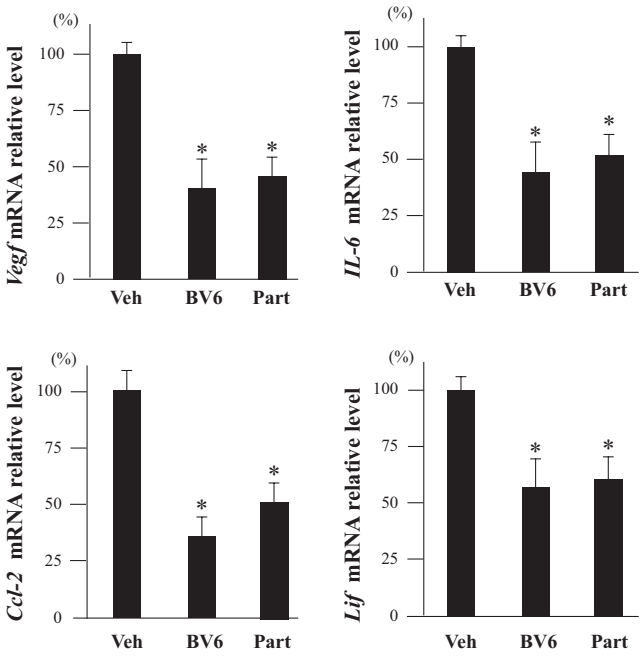

FIGURE 2 Effects of inhibitor of apoptosis proteins (IAP) antagonist (BV6) on the inflammation-associated gene expression in the murine endometriosis-like lesions. The mRNA levels of *Vegf*, *Ccl-2*, *Il-6*, and *Lif* were evaluated by real-time RT-PCR. Veh: vehicle (n = 8). BV6: (n = 8), Part: parthenolide (n = 8). Results are expressed as a percentage of vehicle values. Bars represent the average ± SE. $^{*}P < .05$ vs vehicle

in the immune system of the peritoneal environment. Endometriosis-associated intraperitoneal inflammation is thought to promote propagation of disease and to impair fecundity.[20]

Inhibitor of apoptosis proteins are able to regulate the numerous cell functions, including proliferation, differentiation, and migration, as well as pro-inflammatory and immune responses. Using human endometriotic stromal cells, we previously demonstrated that a potent inflammatory mediator, TNFα, and its downstream NF-κB pathway contribute to being the critical regulator of highly expressed cIAP-2.[21] Moreover, BV6 abrogated innate cIAP-1 and cIAP-2 expression, inhibited IκB phosphorylation, and consequently exhibited a decrease in human endometriotic stromal cells proliferation.[21] These previous and current data indicate that BV6 can mainly suppress NFκB pathway, although there is the possibility that BV6 attenuates other signaling pathways.

A murine model of endometriosis has provided insight into the pathogenesis of peritoneal endometriosis.[18,22,23] As Figure 1 indicated, we previously showed the growth of murine endometriosis-like implants was inhibited by the administration of IAP antagonist, BV6.[13] In this study, we verified that BV6 represses the pro-inflammatory environment in the murine endometriosis-like lesions by inactivation of NF-κB pathway. We presented that IAPs antagonist inhibited the mRNAs levels of major pro-inflammatory cytokines (Figure 2), the cyst formation, the aggregation of T cell or macrophages, and the growth of endothelial cells in the implants (Figure 3). Recently, Jorgensen et al identified that MCP-1 (Ccl-2 in mouse), LIF, and other cytokines to illuminate potential differences in immune profiles and reported these were more characteristic of endometriosis than non-endometriosis patients.[24] In addition, this phenomenon is due to the attenuation of NF-κB (mainly p65) phosphorylation (Figure 4). Our present and previous results strongly proved that IAPs derived from endometriotic tissues are implicated in their progression and inflammation in ectopic sites.

Resistance toward apoptosis is a hallmark of cancer cells, and overexpression of IAPs can contribute to the development of cancer through inhibiting apoptosis. A number of studies have demonstrated that elevated expression levels of IAPs, particularly cIAP-1, cIAP-2, and XIAP, in many tumor types correlates with a poor prognosis.[25] For instance, the higher expression of IAPs might contribute to colon cancer and poor prognosis of colorectal cancer patients.[26] Based on the positive results from pre-clinical studies, several IAP antagonists (LCL161, TL32711, AT406, and HGS1029) have entered the phase I or II clinical trials.[14,27] The first IAP antagonist to enter human clinical trials was compound GDC-0152, a potent inhibitor of cIAP-1, cIAP-2, XIAP, and ML-IAP.[28] GDC-0152 showed linear pharmacokinetics over a wide range of doses in humans without any sign of significant toxicity. LCL161, an orally bioavailable IAP antagonist, was well tolerated in patients with cancer. No dose-limiting toxicities were reported. The bivalent IAP antagonists, TL32711 and HGS1029, were well tolerated with grade 2 transient lymphopenia and neutrophilia reported in some patients.[14] Future clinical trials will explore the safety and efficacy of IAPs antagonists for treating human diseases. The role of IAPs might be areas of future exploration for therapeutic intervention in endometriosis. Several groups showed that proapoptotic activity of IAPs antagonists led to cell death.[15,29,30] Existing major pharmacological treatments for endometriosis, such as GnRH agonists and progestins, have adverse effects. In contrast, BV6 is a therapeutic agent with few side-effects because it has no hormonal action.[28] In this experiment, we checked the body weight and behavior of mice to assess the toxicity of BV6. Loss of body weight and abnormal behavior after treatment were not seen. No difference between treated and untreated mice was found.

NF-κB plays various roles in a pleiotropic signaling pathway involved in a diverse range of biological processes, particularly in innate and adaptive immunity, proliferation, and inflammatory response, in the pathogenesis of endometriosis. More evidence suggests that NF-κB activity stimulates inflammation and cell proliferation, but inhibits apoptosis in human endometriotic stromal cells.[5,31] The medical herb feverfew, including the parthenolide, has long been used as a folk remedy for the treatment of fevers, migraine, rheumatoid arthritis, and dysmenorrhea. Parthenolide, an inhibitor of NF-κB activity, is considered the primary bioactive compound in feverfew having anti-tumor and anti-inflammatory properties. The biological activity of parthenolide is due to the inhibition of IκB kinase and/or direct modification of the p65 protein. IκB polyubiquitination and degradation result in nuclear translocation of the active p50/p65 NF-κB that eventually regulates the expression of target genes, such as IAPs. Parthenolide may be a candidate for treating endometriosis

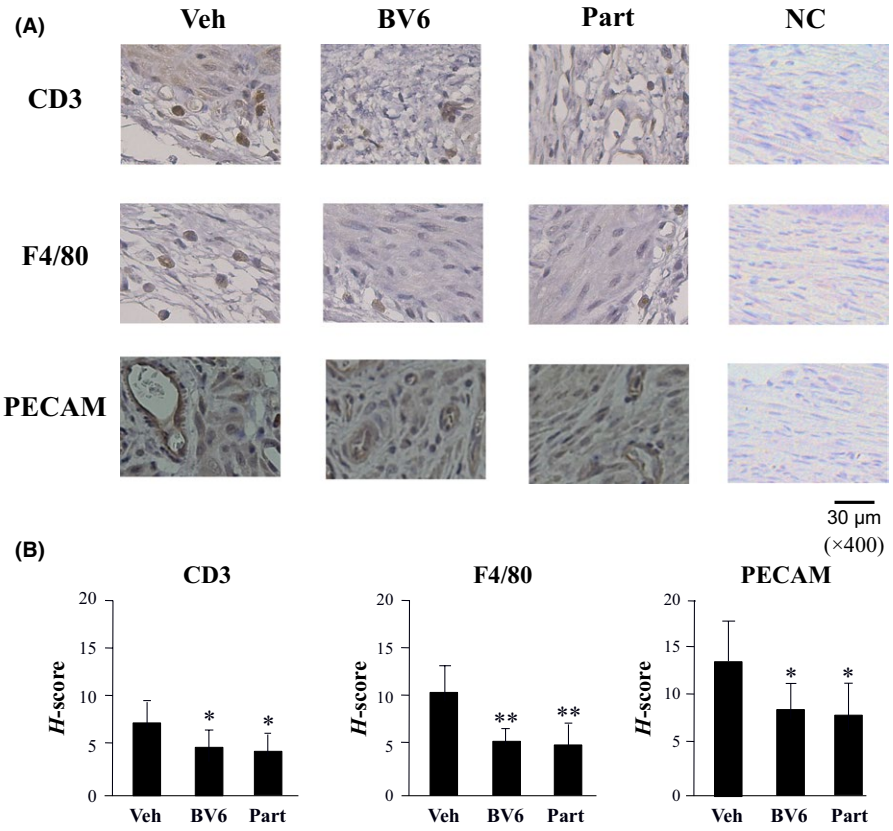

FIGURE 3 Effects of inhibitor of apoptosis proteins (IAP) antagonist (BV6) on the inflammatory status were assessed by immunohistochemical analysis of CD3, F4/80, and PECAM in the murine endometriosis-like lesions. (A) epresentatives of immunostained slides and (B) semi-quantitative analysis (H-score) were shown. Veh: vehicle (n = 8), BV6: (n = 8), Part: parthenolide (n = 8), NC: negative control. For each case, slides of tissues with an absence of the primary antibody were included as NC. Original magnification: ×400. Bars represent the average ± SE. *P < .05 or **P < .01 vs vehicle

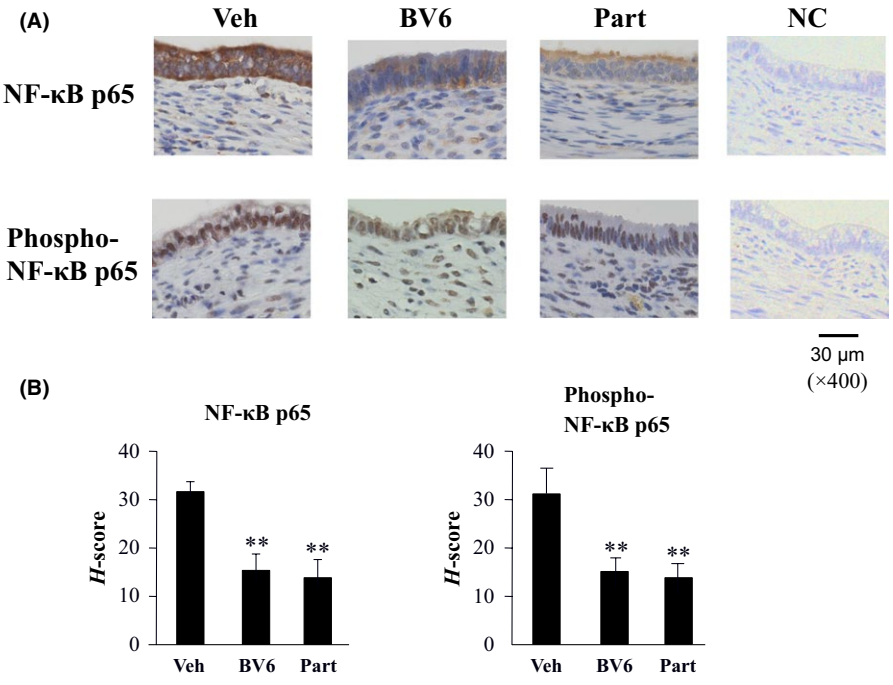

FIGURE 4 Effects of inhibitor of apoptosis proteins (IAP) antagonist (BV6) on NF-κB p65 and phospho-NF-κB p65 expression in the murine endometriosis-like lesions were evaluated by immunohistochemical analysis. (A) Representatives of immunostained slides and (B) semi-quantitative analysis (H-score) were shown. Veh: vehicle (n = 8), BV6: (n = 8), Part: parthenolide (n = 8), NC: negative control. For each case, slides of tissues with an absence of the primary antibody were included as NC. Original magnification: ×400. Bars represent the average ± SE. *P < .05 or **P < .01 vs vehicle

because it exerts a potent inhibitory effect on the NF-κB pathway with low toxicity in vivo.[18]

The inflammatory mediators, such as bacterial endotoxin or LPS (lipopolysaccharide), the gram-negative bacterial cell component, can activate the innate immune cells, such as macrophages, and trigger the secretion of various cytokines, chemokines, and growth factors. We previously exhibited that LPS promoted proliferation and invasion of human endometriotic stromal cells *via* upregulation of cyclooxygenase-2 (COX-2) and prostaglandin E2. Thus, an inflammatory reaction may trigger the development of endometriosis. Recently, we showed

that LPS-induced pelvic inflammation status enhanced the development of murine endometriosis-like lesions *via* NF-κB pathway.[23] In contrast to BV6, LPS promoted the rate of Ki67-positive cells, the aggregation of T cells (CD3) and macrophages (F4/80), the angiogenic activity of endothelial cells (PECAM), and p65 phosphorylation in endometriosis-like implants, suggesting that pelvic inflammation in patients with endometriosis consequently would promote disease and impair fecundity *via* NF-κB pathway. NF-κB is basically activated in peritoneal endometriotic lesions, showing higher p65 activity in red (more inflammatory) endometriotic lesions than in black lesions.[32]

In summary, our current data indicate that the IAP antagonist repressed inflammatory status through the attenuation of NF-κB pathway. The main cell processes that NF-κB is a main regulator of cell proliferation and inflammatory responses. Further study will be needed to analyze the pharmacological potential of this agent to treat endometriosis. IAPs are attractive for developing a novel treatment for endometriosis.

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CONFLICT OF INTERESTS

The authors have no conflict of interests to disclose relative to this work.

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