Effectiveness of NLRP3 Inhibitor as a Non-Hormonal Treatment for Ovarian Endometriosis

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Abstract

Background

Endometriosis is a complex syndrome characterized by an estrogen-dependent chronic inflammatory process that affects 10% of women of reproductive age. Ovarian endometriosis (OE) is the most common lesion in endometriosis and may cause infertility in addition to dysmenorrhea. Hormonal treatments for endometriosis suppress ovulation; hence, they are not compatible with fertility. The inflammasome is a complex that includes Nod-like receptor (NLR) family proteins that sense pathogen-/danger-associated molecular patterns and homeostasis-altering molecular processes. It has been reported that the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain-containing (NLRP) 3 inflammasome, which contributes to the activation of interleukin-1 beta (IL-1β), might be related to the progression of endometriosis. Therefore, the aim of the present study was to evaluate non-hormonal therapies for OE, such as the inhibitors of the NLRP3 inflammasome.

Methods

The expression of NLRP3 was measured in the eutopic endometrium (EM) of patients with/without endometriosis and OE and stromal cells derived from the endometrium of patients with endometriosis and OE (endometrial stromal cells [ESCs] and cyst-derived stromal cells [CSCs]). The effect of an NLRP3 inhibitor (MCC950) on ESC and CSC survival and IL-1β production was evaluated. We then administered MCC950 to a murine model of OE to evaluate its effects on OE lesions and ovarian function.

Results

NLRP3 gene and protein expression levels were higher in OE and CSCs than in EM and ESCs, respectively. MCC950 treatment significantly reduced the survival of CSCs but not that of ESCs. Moreover, MCC950 treatment reduced the co-localization of NLRP3 and IL-1β in CSCs and IL-1β concentrations in CSC supernatants. In the murine model, MCC950 treatment reduced OE lesion size compared to phosphate-buffered saline treatment (89 ± 15 vs. 49 ± 9.3 mm³ per ovary; P < 0.05). In addition, IL-1β and Ki67 levels in the OE-associated epithelia and oxidative stress markers of granulosa cells were reduced in the MCC950-treated group.

Conclusions

These results indicate that NLRP3/IL-1β is involved in the pathogenesis of endometriosis and that NLRP3 inhibitors may be useful for suppressing OE and improving the function of ovaries with endometriosis.

Background
Endometriosis is a complex syndrome characterized by an estrogen-dependent chronic inflammatory process that affects 10% of women of reproductive age [1, 2]. Ovarian endometriosis (OE) is the most common lesion in endometriosis [3] and causes infertility in addition to dysmenorrhea [4]. Currently, the most common treatments for endometriosis are surgery and pharmacotherapy, including hormone therapy and non-steroidal anti-inflammatory drugs for pain relief. The first line of hormone therapy is oral contraceptives/low-dose estrogen progestin and progestins with a gonadotropin-releasing hormone (GnRH) analog as an option, although its use is limited because of the side effects that it causes [5]. Recently, GnRH-antagonists have also been used [6]. Although the surgical excision of endometriosis improves pain and enhances fertility [7, 8], a recent systematic review of the literature estimated the recurrence rate of endometriosis to be 21.5% at 2 years and 40–50% at 5 years [9], and recurrence and repeated surgery can further exacerbate pain and reduce fertility, respectively [10]. Therefore, regular and prolonged medication is highly recommended to prevent postoperative recurrence of endometriosis [11]. However, because estrogen is involved in the development of endometriosis, these hormone therapies suppress follicular development and ovulation. For this reason, the treatment of endometriosis in women who wish to become pregnant is very challenging, and non-hormonal drugs for endometriosis are desired.

The development of endometriosis involves the interaction of endocrine, immunologic, proinflammatory, and proangiogenic processes [12]. Sampson’s theory has been accepted as a strong hypothesis [13], but other factors must determine the ability of endometrial cells to adhere to peritoneal or ovarian surfaces, proliferate, and develop into endometriotic lesions, because retrograde menstruation is common in all women of reproductive age [14, 15]. Chronic inflammation is one of the distinguishing features of endometriosis, and a relationship between cell adhesion and inflammation has been reported [16, 17].

The inflammasome is a complex that can contain the Nod-like receptor (NLR) family proteins, leucine-rich repeat, and pyrin domain-containing (NLRP) 1b, NLRP3, NLRP6, NLRP9b, and NLR family caspase recruitment domain (CARD)-containing protein (NLRC) 4, which senses pathogen-associated molecular patterns, danger-associated molecular patterns, and homeostasis-altering molecular processes [18, 19]. Previous studies have reported that the NLRP3 inflammasome may be related to the progression of endometriosis [14, 20].

NLRP3 is involved in hereditary Cryopyrin-associated periodic syndromes, such as Muckle-Wells syndrome, diabetic retinopathy, colorectal inflammatory disease, and gouty arthritis, and treatment with NLRP3 inhibitors has been reported in these diseases [21–24]. We focused our attention on MCC950, a small-molecule compound with high specificity for NLRP3 [21]. We hypothesized that inhibiting NLRP3 with MCC950 would suppress interleukin-1 beta (IL-1β) and alleviate endometriosis.

In this study, we first examined the expression of NLRP3 in the eutopic endometrium (EM) with and without endometriosis and OE. We then confirmed that MCC950 treatment inhibits the secretion of IL-1β in primary human endometrial stromal cells (ESCs). Finally, we examined the effect of MCC950 on endometriotic lesions in a murine model of OE, which has been established previously [25].
Materials And Methods

Patients and sample collection

Patients diagnosed with endometriomas, uterine leiomyomas, carcinoma in situ (CIS), and early-stage cervical cancer who were referred to the Nagoya University Hospital and Toyota Kosei Hospital between July 2018 and March 2021 were enrolled in this study. The ethics committee of the Nagoya University Graduate School of Medicine (2014-0134) and Toyota Kosei Hospital (2018-ST01) approved the experiments. Written informed consent was obtained from each patient prior to participation in this study.

OE samples and endometrial tissues from patients with endometriosis (eEM) who had been resected for therapeutic purposes were used for quantitative reverse transcription polymerase chain reaction (qRT-PCR), western immunoblotting, and stromal cell isolation. Normal endometrial tissues (nEMs) were obtained from patients with uterine leiomyomas, CIS, and stage IB1 cervical cancer without endometriosis.

Primary human ESC isolation

Primary human ESCs with endometriosis and endometriotic cyst-derived stromal cells (CSCs) were isolated from human endometrial biopsies or resected endometriomas. Tissue biopsies were finely chopped in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan). Chopped tissues were incubated with the collagenase solution (1 mg/mL; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30 min at 37°C and the cell suspension was filtered through 70 μm filter membranes, followed by centrifugation to obtain a stromal cell pellet. ESCs and CSCs were then resuspended in fresh DMEM containing 10% fetal bovine serum (Cosmo Bio, Tokyo, Japan), 100 IU/mL of penicillin, 100 mg/L of streptomycin, and 25 mg/L of amphotericin B. The next day, media containing unattached cells were transferred to a second dish before the media were removed and discarded. The cells were routinely maintained at 37°C until they reached 90% confluence and were then seeded for experimental purposes as detailed below.

Real-time quantitative polymerase chain reaction

Gene expression was analyzed by SYBR green based qRT-PCR of cDNA that was synthesized from mRNA extracted from the samples. Tissues or ESCs/CSCs were washed with phosphate-buffered saline (PBS), and total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was measured using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies, USA). Next, 2 μg of total RNA from each sample was used for reverse transcription with 5× RT Master Mix (TOYOBO, Osaka, Japan) to generate first-strand cDNA in a 20 μL reaction mixture. The cDNA was diluted at a ratio of 1:10, and qRT-PCR was performed in a 96-well, 0.2 mL thin-wall PCR tubes using the LightCycler 96 (Roche, Basel, Switzerland). The real-time PCR mixture contained KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) (10 μL), primers (2 μM), and cDNA template (2 μg) in a total volume of 20 μL. Quantitative RT-PCR was
performed to measure mRNA expression with the following primers: human NLRP3 (forward, 5′-GCACCTGTTGTGCAATCTGAA-3′; reverse, 5′-TCCTGACAACATGCTGATGTGA-3′), human NLRP1 (forward, 5′-CCACAACCTCTGTCTACATTAC-3′; reverse, 5′-GCCCATCTAACCCATGCTTC-3′), human NLRC4 (forward, 5′-GGAAAGTGCAAGGCTCTGAC-3′; reverse, 5′-TGTCCTGCTTGATTGTC-3′), and human absent in melanoma 2 (AIM2) (forward, 5′-CTGCAGTGATGAACCATTCGTA-3′; reverse, 5′-GGTGCAGCACGTTGCTTTG-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-CAGCCTCAAGATCATCAGCA-3′; reverse, 5′-GTCTTCTGGGTGGCAGTGAT-3′). The PCR protocol was as follows: initial incubation at 98°C for 2 min, denaturation at 98°C for 10 s, annealing at 60°C for 10 s (55 cycles), and extension at 68°C for 30 s. Quantitative RT-PCR was performed in triplicate for all samples. Quantification was performed by calculating the ratio of the gene of interest to GAPDH using the comparative C_t method.

**Western immunoblotting**

Tissues or cells were lysed using RIPA lysis buffer, 10× (Millipore, Burlington, Massachusetts, USA) containing 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, and 10 mM EDTA in Milli-Q H_2O (Roche, Basel, Switzerland).

Lysates were clarified by centrifugation at 8000 × g for 10 min at 4°C, and the supernatants were collected.

Protein concentration was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature with 5% (v/v) nonfat dry milk. After three washes with PBS with Tween (PBST), the membrane was incubated in PBST at 4°C overnight with anti-NLRP3 (No. 19771-1-AP, 1:1000, AdipoGen Life Sciences, Liestal, Switzerland) and anti-IL-1β (No. 12242, 1:100, Cell Signaling Technology, Danvers, MA). The membrane was again washed with PBST and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody. Signals were developed using a standard ECL western blot detection reagent (Amersham Biosciences, Arlington Heights, IL, USA). A densitometric analysis was performed using ImageJ software version 2.2.0 (https://imagej.net/).

**Cell viability assay**

The effect of MCC950 on cell viability was determined using cell counting. ESCs/CSCs (1.5 × 10^5 cells) were seeded into each well of a 6-well plate and allowed to adhere overnight. Cells were cultured in serum-free media (SFM) to starve them for 24 h. Then, the cells were treated with different concentrations of MCC950 (0.1, 1, 10, and 100 μM; AG-CR1-3615-M005, AdipoGen Life Sciences, Liestal, Switzerland) for 24 h. Finally, 1 mL of 0.25% trypsin was added to each well, followed by incubation for 5 min at 37°C and the cells were then collected, centrifuged, and the number of viable cells was counted.

**Immunocytochemistry**
ESCs and CSCs were cultured on coverslips, and the medium was replaced with SFM containing MCC950 (0.1, 1, 10, 100 μM). After incubation for 16 h, the cells were fixed in methanol for 2 min at room temperature and permeabilized with 0.5% Triton X-100 for 1 min. After blocking with 1% bovine serum albumin for 1 h at room temperature, the cells were incubated with the primary antibodies (NLRP3 [No. 19771-1-AP, 1:200, Proteintech, Chicago, IL] and IL-1β [sc-32294, 1:100, Santa Cruz Biotechnology Inc., Heidelberg, Germany]) for 2 h at room temperature. Then, the cells were incubated with goat anti-rabbit (Alexa Fluor 568, 1:500, Thermo Fisher Scientific for anti-NLRP3 antibodies) and goat anti-mouse (Alexa Fluor 488, 1:500, Thermo Fisher Scientific for anti-IL-1β antibody) secondary antibodies for 1 h at room temperature. 4',6-diamidino-2-phenylindole (#4083, 1:1000, Cell Signaling Technology, Danvers, MA, USA) was used for nucleus staining. Visualization was performed using a confocal laser scanning microscope (BZ9000, Keyence, Osaka, Japan).

**Enzyme-linked immunosorbent assay (ELISA)**

ESCs/CSCs were seeded at a density of 1×10^6 cells/well in 6-well plates. The following day, the medium was replaced with SFM containing MCC950 (0.1, 1, 10, and 100 μM). After incubation for 16 h, cell culture supernatants were collected. ELISAs were conducted on the culture media collected after the treatment. Media samples were immediately centrifuged for 5 min at 8000 × g to collect the conditioned culture supernatant, which was stored at −80°C until use. IL-1β released by ESCs and CSCs was measured using an ELISA kit (DuoSet, R&D System, Minneapolis, MN, USA) according to the manufacturer’s protocol.

**Animal model of endometriosis and MCC950 treatment**

All animal experiments were approved by the Animal Experimental Committee of the Nagoya University Graduate School of Medicine (31452). We used a murine OE model, as described by Hayashi (2020) [25]. C57BL/6N female mice (8 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Before starting the experiments, the animals were acclimatized for 7 days and maintained at 23–25°C in a 12-h dark/light cycle and given standard chow (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water in a pathogen-free environment. The cages were changed weekly.

Donor female mice (9 weeks of age; n = 16) were euthanized to obtain uterine tissue, which was cleaned of supplementary fibroadipose tissues with PBS. The uterus was cut longitudinally with a linear incision and minced (approximately 0.5 mm in diameter) with scissors. The uterus was incubated with the collagenase solution (1 mg/mL; FUJIFILM Wako Pure Chemical Corporation) and centrifuged to remove the supernatant containing collagenase. The pellet of the uterine tissue was immediately used for transplantation. Sixteen female mice were used as the recipients of uterine pellets for OE. The mice underwent uterine transplantation after the week of acclimatization. Induction and maintenance of systemic anesthesia were achieved with isoflurane (3% for induction and 2.5% for maintenance). The incisions of 5–7 mm were performed on the bilateral back skin and muscle layers to search for ovaries. The half of the uterine tissue pellet prepared from one donor female mouse was placed equally over each
surface of the bilateral ovaries. Ovaries with attached uterine pellets were then pushed back into the peritoneal cavity, and the incisions were closed.

Over the next four weeks, the recipient mice were treated with a single intraperitoneal injection of MCC950 (n = 8; 20 mg/kg) or PBS (n = 8) three times a week. The first injection of MCC950 was administered 1 h before the inoculation of the donor's uterine tissues.

**Histological analysis and measurement of murine endometriotic cysts**

The endometriotic cysts were excised at 13 weeks of age as a single mass with the ovaries and the cyst diameters were measured. The endometriotic cysts with the ovaries were fixed with 10% phosphate-buffered formalin, embedded in paraffin, cut into sections of 4 μm thickness, and examined by routine immunohistochemical analysis as described below. Immunohistochemical staining was performed as described previously [16]. For heat-induced epitope retrieval, deparaffinized sections in 0.01 mM citrate buffer were heated for 20 min at 95°C using a microwave oven. Immunohistochemical staining was performed according to the avidin-biotin immunoperoxidase method using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in methanol for 20 min, and nonspecific Ig binding was blocked by incubation for 10 min in PBS with 10% normal serum and the corresponding secondary antibody. The sections were incubated at 4°C overnight with the following primary antibodies: IL-1β (1:100, Cell Signaling Technology, Danvers, Massachusetts), Ki67 (AB9260, 1:300, Merck KGaA, Darmstadt, Germany), and 4-hydroxynonenal (4-HNE) (BS-6313R, 1:400; Bioss Antibodies Inc., Woburn, MA). The sections were then rinsed and incubated with biotinylated secondary antibodies for 10 min. After washing with PBS, the sections were further incubated with horseradish peroxidase-conjugated streptavidin for 5 min and finally treated with diaminobenzidine in 0.01% H₂O₂ for 5 min. The slides were counterstained with Meyer's hematoxylin, and the stained sections were observed under a microscope (Axio Imager 2, Zeiss, Oberkochen, Germany).

Stained areas inside the endometrial cyst epithelium were quantitated using Image J, threshold 235 (IL-1β)/180 (Ki67). The average values were calculated based on the stained area ratio of randomly selected fields of view for at least two cysts (OE) or magnified images (EM) in each of the four mice, which were compared between the two groups. The quantification of 4-HNE positive ovarian follicles was evaluated by measuring the ratio of immunopositive follicles in each follicular area with Image J (threshold 165).

**Statistical analyses**

Statistical analyses were performed using Student's t-test and one-way analysis of variance with GraphPad Prism 8 software (San Diego, CA, USA). Differences were considered significant at \( P < 0.05 \). The data are expressed as the mean ± standard error of the mean (SEM) unless otherwise specified.

**Results**

**NLRP3 inflammasomes were upregulated in OE**
To identify whether NLRP3 is involved in endometriosis, we detected the expression of NLRP3 in e/nEM and OE using qRT-PCR and western blot analysis. As shown in Figure 1A–C, NLRP3 gene expression and protein levels were significantly increased in OE compared with those in e/nEM. There was no significant difference between nEM and eEM; therefore, eEM and OE were selected for subsequent experiments.

Other inflammasomes involved in the caspase1-mediated pathway that activate IL-1β were also upregulated in OE compared to in EM. However, NLRP3 was clearly more highly expressed in EM than in OE (Figure 1D).

We then examined whether the same trend was observed in the cells isolated from patients. As shown in Figure 2A–D, NLRP3 mRNA and protein levels were also significantly increased in CSCs compared with those in ESCs. In contrast, the expression of NLRP1 and NLRC4 was not significantly different between CSCs and ESCs.

**MCC950 decreases viability of CSCs**

Because the expression of NLRP3 was increased in CSCs, MCC950 was added to the cultured cells to evaluate the effect of inhibition of IL-1β. When ESCs and CSCs were treated with 0.01, 1, and 100 μM MCC950, the survival fractions of CSCs at 24 h were 89 ± 4.1%, 78 ± 3.4%, and 73 ± 5.8%, respectively, compared to that of CSCs treated with 0 μM MCC950 (P = 0.14, 0.011, and 0.0065, respectively), whereas the viability of ESCs did not change significantly (Figure 3A).

**Attenuation of IL-1β secretion by MCC950 pretreatment in CSCs**

The effect of MCC950 on ESCs and CSCs was evaluated by using immunofluorescence. Immunofluorescence experiments indicated that the number of NLRP3 and IL-1β double-labeled cells in CSCs was higher than that in ESCs. Furthermore, when CSCs were treated with MCC950, the expression of IL-1β was downregulated and the number of double-labeled cells was reduced (Figure 3B).

Next, IL-1β secretion in the cell supernatant was examined, and we found that the concentration of IL-1β was higher in the supernatant of CSCs than in that of ESCs, although this did not reach statistical significance. The level of IL-1β in CSC supernatant was significantly reduced after 16 h of treatment with MCC950 (Figure 3C).

**MCC950 prevents progression of ovarian endometriotic cysts in murine models**

The experimental protocol using a murine model of OE is summarized in Figure 4A. Both the MCC950-treated and PBS-treated groups were euthanized four weeks after the operation (at the age of 13 weeks), and endometriotic lesions were evaluated. Single or multiple cystic lesions were recognized in association with the bilateral ovaries (Figure 4B). After treatment with MCC950 for four weeks following implantation with minced murine uterine tissues, the volume of lesions was significantly reduced compared to that in the PBS-treated group (89 ± 15 vs. 49 ± 9.3 mm³ per ovary; P < 0.05, Figure 4C).
evaluate the proliferative activity of the endometriotic lesions, the ratio of Ki67-positive cells was calculated. The number of Ki67-positive epithelial cells in the endometriotic lesions significantly decreased after MCC950 treatment (40.8 ± 5.3% vs. 28.1 ± 3.2%; \( P < 0.05 \); Figure 4D).

We then evaluated the effect of MCC950 on NLRP3 and IL-1\( \beta \) in murine endometriotic cysts. The levels of IL-1\( \beta \) in endometriotic cysts after MCC950 treatment were assessed by the immunohistochemical analysis. The IL-1\( \beta \)-positive area in the epithelial cells was higher in the ovarian endometriotic cysts than in EM in same animals after PBS treatment, which significantly decreased after MCC950 treatment (OE-PBS [29.3 ± 3.6%] vs. OE-MCC950 [19.1 ± 2.6%], \( P < 0.05 \); Figure 4E).

**MCC950 reduced oxidative stress in granulosa cells induced by endometriosis in murine model**

We evaluated oxidative stress in granulosa cells of ovarian follicles as it has been reported that iron and oxidative stress are major factors in impaired fertility in OE and that the level of oxidative stress marker 4-HNE is significantly increased in the primordial, preantral, and antral follicles of the murine model of OE [25]. 4-HNE is one of the most specific lipid peroxidation products in the iron-catalyzed Fenton reaction. The representative data of the different maturation stages of 4-HNE-stained follicles are summarized in Figure 4F. In the primordial, pre-antral, and antral follicles, 4-HNE levels in granulosa cells were lower in the MCC950-treated group than in the PBS-treated group (Figure 4G–K).

**Discussion**

In this study, we investigated the NLRP3 inflammasome, which contributes to IL-1\( \beta \) activation, as a target for the non-hormonal treatment of OE.

The involvement of IL-1\( \beta \) in endometriosis has been studied [17, 26, 27], and research on the treatment of endometriosis using IL-1\( \beta \) receptor antibodies has been reported [28]. However, among patients who received a monoclonal antibody against IL-1\( \beta \) frequently reported adverse events, such as infection [29]. Inflammasomes facilitate the cleavage and activation of caspase-1, which leads to the maturation of IL-1\( \beta \) [30], and these inflammasomes consists of NLR, the adaptor protein apoptosis-associated speck-like protein containing a CARD, and the effector molecule pro-caspase-1. The NLR family of proteins is a group of pattern recognition receptors (PRRs) [31], and some PRRs assemble the inflammasome complex after sensing their respective stimuli. The NLRP3 inflammasome is activated by various pathogen-derived ligands and physiological aberrations resulting in danger-associated molecular patterns (DAMPs). The NLRP1 inflammasome senses *Bacillus anthracis* toxin, pathogen-associated proteins released by pathogenic bacteria cause NLRC4 to assemble the inflammasome complex. DNA viruses and intracellular bacteria release DNA during infection, which activates the AIM2 inflammasome [32].

In the present study, NLRP3 expression levels were elevated in cultured CSCs compared with those in ESCs, although there were no differences in NLRP1 and NLRC4 levels, indicating that NLRP3 might be involved in the pathogenesis of endometriosis. We detected notably increased NLRP3 levels in the OE tissue from surgical specimens compared with those in EM, which is consistent with other studies [33]. In
contrast, the expression of other NLRs was upregulated in OE compared to that in EM, although it was to a lesser extent than NLRP3. Considering the heterogeneity in clinical conditions, it is possible that other NLRs were also elevated in ovarian endometrial cysts in response to stimuli, such as infection of the cyst or bacteria in the abdominal cavity. NLRP3, which is also highly expressed in cultured cells, is continuously activated in clinical conditions due to continued exposure to the contents of endometrial cysts, which can progress to become DAMPs. In this regard, NLRP3 is more highly activated in the stressful environment of exposure to the contents of endometrial cysts than in that of cultured cells, and the therapeutic strategy of inhibiting NLRP3 may be more effective in vivo than in vitro. Additionally, MCC950 does not block the major antimicrobial inflamasomes NLRC4 and NLRP1 [21]. The NLRP3 level was significantly higher than those of other NLRs in OE and CSCs; therefore, the specific inhibition of NLRP3 by MCC950 could suppress IL-1β production in endometriosis, while essential responses against bacterial infections may remain intact.

In previous reports, several peritoneal models have been reported as the murine models of endometriosis [34–36], and in this study, we used an OE model that was recently established [25]. Therefore, we were able to assess the effects on the ovaries, especially follicles. Regarding the effects of MCC950 on the ovary, a previous report suggested that the administration of MCC950 inhibits ovarian aging and improves fertility in mice [37]. Increased oxidative stress in follicles corresponding with decreased fertility has been reported in the mouse models of endometriosis [25]. Our results indicate that the administration of MCC950 reduced the oxidative stress of granulosa cells in follicles and further increased the number of small follicles and antral follicles (Supplemental Figure 1A,B). Taken together, these effects of MCC950 may improve fertility in murine models and possibly in patients with OE.

The limiting factor of this study is that there are variabilities in OE samples; consequently, there may be individual differences in the effectiveness of NLRP3 inhibition. NLRP3 has been reported to vary with the menstrual cycle [38]; however, this has not been fully investigated. Additionally, MCC950 has not been used in clinical settings. Tranilast, an NLRP3 inhibitor that is clinically applied as an antiallergic drug but is less specific than MCC950 [24, 39], may have positive effects on the treatment of endometriosis.

**Conclusions**

The expression of NLRP3 is upregulated in OE compared with that in EM. The treatment of CSCs with MCC950 suppressed IL-1β production and cell proliferation. The administration of MCC950 reduced endometriotic lesions in a murine model of OE and improved the functions of ovaries with endometriosis, suggesting that MCC950 is a potential non-hormonal treatment for endometriosis.

**Abbreviations**

OE
Ovarian endometriosis

NLRP
Nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain

**IL-1β**
Interleukin-1 beta

**EM**
Eutopic endometrium

**CSC**
Primary human endometrial stromal cell, with endometriosis

**ESC**
Primary human endometriotic cyst-derived stromal cell

**PBS**
Phosphate-buffered saline

**GnRH**
Gonadotropin releasing hormone

**NLR**
Nucleotide-binding oligomerization domain-like receptor

**NLRC**
NLR family CARD (Caspase recruitment domain)-containing protein

**CIS**
Carcinoma in situ

**eEM**
Endometrial tissues from patients with endometriosis

**nEM**
Endometrial tissues from patients without endometriosis

**qRT-PCR**
Quantitative reverse transcription polymerase chain reaction

**DMEM**
Dulbecco's modified Eagle's medium

**AIM2**
Absent in melanoma 2

**GAPDH**
Glyceraldehyde-3-phosphate dehydrogenase

**PBST**
Phosphate-buffered saline with Tween

**SFM**
Serum-free media

**ELISA**
Enzyme-Linked Immunosorbent Assay

**4-HNE**
4-Hydroxynonenal

**ANOVA**
Declarations

Ethics approval and consent to participate

The ethics committee of the Nagoya University Graduate School of Medicine (2014-0134) and Toyota Kosei Hospital (2018-ST01) approved the experiments. Written informed consent was obtained from each patient before participating in this study. All animal experiments were approved by the Animal Experimental Committee of the Nagoya University Graduate School of Medicine (31452).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declared that they have no competing interests.

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Not applicable.

Authors' contributions

SO designed the study. MM, AM, SH, BS, YK, and SY performed the experiments. YH, KS, AM, RS, NM, and MM collected the human samples. HT and NN helped with the animal experiments. MM wrote the initial manuscript. SO supervised and supported the entire project and edited the manuscript. TN, MG, and HK supervised the entire project. All authors have read and agreed on the final version of the manuscript.

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Figures

Figure 1

Expression of inflammasomes in n/eEM and OE tissues. (A) Relative mRNA expression level of NLRP3 was quantified by qRT-PCR. Expression levels are shown relative to GAPDH. Data are shown as the mean ± SEM of samples assayed in duplicate obtained from at least 10 patients. (B) The NLRP3 protein levels
in n/eEM and OE tissues assessed by western blotting. β-actin was used as a loading control. These results are representative of three separate experiments. (C) Relative protein levels of NLRP3 were quantified. Protein levels are shown relative to those of β-actin. Data are shown as the mean ± SEM obtained from at least three patients. (D) Relative mRNA expression levels of NLRP3, NLRP1, NLRC4, and AIM2 were quantified. Expression levels are shown relative to those of GAPDH. Data are shown as the mean ± SEM of assays conducted in duplicate obtained from at least five patients. Statistical analyses were conducted using a one-way ANOVA followed by Dunnett’s multiple comparison test. *P < 0.05; **P < 0.01; n.s., not significant; n/eEM = eutopic endometrium without/with endometriosis; OE = ovarian endometriosis.

Expression of NLRP3 in ESCs and CSCs. (A) Relative mRNA expression level of NLRP3 was quantified by qRT-PCR. Expression levels are shown relative to those of GAPDH. Data are shown as the mean ± SEM of assays conducted in duplicate obtained from at least six patients. (B) The protein level of NLRP3 in ESCs and CSCs was assessed by western blotting. β-actin was used as a protein loading control. These results are the representative of three independent experiments. (C) The relative protein levels of NLRP3 were quantified. Protein levels are shown relative to those of β-actin in each group. Data are shown as the mean ± SEM from at least five patients. (D) Relative mRNA expression levels of NLRP3, NLRP1, and NLRC4 were quantified. Expression levels are shown relative to those of GAPDH. Data are shown as the mean ± SEM of assays conducted in duplicate obtained from at least four patients. Statistical significances were calculated using Student’s t-test (A and C) and one-way ANOVA followed by Dunnett’s multiple comparison test (D). *P < 0.05; ESCs = eutopic endometrium derived stromal cells; CSCs = ovarian endometriosis (chocolate cyst) derived stromal cells.
Figure 3

Effects of MCC950 on ESCs and CSCs. (A) ESCs and CSCs were treated with MCC950 (0.01, 1, and 100 μM) and the surviving fraction was measured after a 24 h incubation. Data are shown as the mean ± SEM of triplicate samples obtained from four patients. (B) Immunofluorescence analysis of the expression of NLRP3 and IL-1β in ESCs and CSCs. Representative immunostaining images (upper) and quantitative analysis (lower) of the ratio of NLRP3/IL-1β co-labeled cells to total cells after incubating with or without MCC950 (100 μM) for 16 h. Data are shown as mean ± SEM obtained from three different primary cell cultures; Bars, 50 μm. (C) Ratio of IL-1β levels in CSC culture supernatant in untreated and MCC950-treated (100 μM) cultures for 16 h. Data are shown as the mean ± SEM of duplicates obtained from at least seven different primary cell cultures. Statistical significances were calculated using one-way ANOVA followed by Dunnett's multiple comparison test (A and B) and Student's t-test (C). *P < 0.05; **P < 0.01.

Figure 4
Effects of MCC950 on the endometriosis lesions and ovaries of the murine endometriosis model. (A) Experimental design of the role of MCC950 in a murine ovarian endometriosis model. (B) Hematoxylin and eosin staining of lesions; Bars, 500 μm (left), 50 μm (right). (C) Macroscopic view of lesions (left) and volumes of lesions per ovary were assessed (right). Data are presented as the mean ± SEM; PBS (n = 8), MCC950 (n = 7). (D and E) Histological analysis of murine endometriosis lesions treated with vehicle and MCC950 stained for Ki67 (D) and IL-1β (E). Representative immunohistochemical localization (left, bar = 50 μm), and quantitative analysis of positive epithelial area ratio of the endometriotic cyst wall or eutopic endometrium (right). (F) Representative 4-HNE immunohistochemical results of ovarian follicles in primordial, primary, secondary, pre-antral and antral stages; bar = 20 μm. (G–K) Quantitative analysis of each follicular stage of the ovary. Data are shown as the mean ± SEM of at least eight follicles from four mice. Statistical significances were calculated using Student's t-test (C, D, and G–K) and one-way ANOVA followed by Dunnett’s multiple comparison test (E). *P < 0.05; **P < 0.01; n.s., not significant; IP = intraperitoneal; OE = ovarian endometriosis, EM = eutopic endometrium; 4-HNE = 4-Hydroxynonenal.

Supplementary Files

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