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症例報告

腸閉塞を発症した回腸子宮内膜症の1例

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内容要旨

症例は34歳, 女性. 開腹歴はなく, 30歳時に子宮内膜症の診断を受けたことがあった. 腹痛を主訴に前医を受診し, 腸閉塞の診断で入院となり, イレウス管による保存的治療が施行された. 小腸造影検査で回腸末端に狭窄像を認め, 骨盤MRI検査では腸管子宮内膜症による小腸狭窄が強く疑われた. 腸閉塞症状が軽快した後, 精査加療目的で当院に紹介となった. 回腸子宮内膜症に対してホルモン療法後に, 腹腔鏡下手術を施行した. 腹腔内を観察すると後腟円蓋と直腸前壁に子宮内膜症による軽度の癒着を認めた. また, 回腸末端部より約20cmの回腸に漿膜の引きつれと硬化を認め, 小腸部分切除術を施行した. 病理組織検査では, 狭窄を認めた回腸の粘膜下層から漿膜下層に子宮内膜腺および間質細胞が分布しており, 回腸子宮内膜症と診断した. 術後経過は良好で第9病日に退院となった.

索引用語:腸閉塞, 腸管子宮内膜症, 腹腔鏡下手術

はじめに

腸管子宮内膜症は, 全子宮内膜症の約10%に認められる疾患である[1]. 小腸に発生する症例は稀で, 腸閉塞の症状で発症し手術後に判明することが多い. 今回, 腸閉塞を発症した回腸子宮内膜症に対して腹腔鏡下手術を施行した症例を経験したので, 文献的考察を加え報告する.

症　例

症　例:34歳, 女性.

主　訴:腹痛.

家族歴:特記すべきことなし.

既往歴:30歳時に子宮内膜症の診断を受ける.

現病歴:平成20年8月に腹痛のため前医を受診し, 腸閉塞の診断で入院となり, イレウス管による保存的治療が施行された. 骨盤MRI検査では回腸子宮内膜症が疑われた. イレウス症状が軽快後, 精査加療目的で当科紹介受診となった.

入院時現症:身長156cm, 体重52kg. 腹部は平坦, 軟で, 腫瘤は触知しなかった. また, 右側腹部に軽度の圧痛を認めた.

入院時血液検査所見:血液生化学検査では異常がみられなかったが, 腫瘍マーカーではCA125が38μg/mlと軽度上昇していた.

腹部単純X線検査:前医受診時の腹部単純X線では, 鏡面形成像を伴う小腸の拡張を呈していた.

腹部造影CT検査:広範な小腸の拡張と回腸に腫瘤状に造影効果を伴った腸管壁の肥厚を認めた(**Fig. 1**).

骨盤MRI検査:CT検査でみられた肥厚した回腸に一致して, T1強調像で高信号, T2強調像で低信号を示す腫瘤を認めた(**Fig. 2a, b**).

イレウス管造影検査:回腸末端部近傍に強度の狭窄を認めた(**Fig. 3**).

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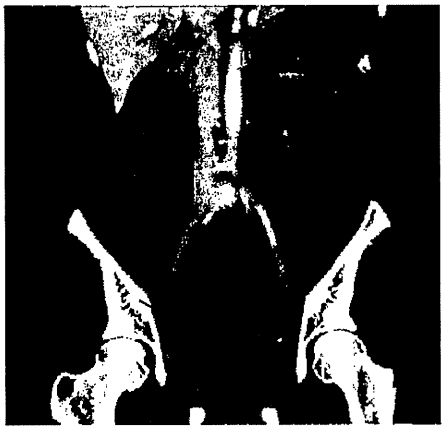

Fig. 1　A coronal contrast-enhanced CT image revealed the presence of an enhanced tumoral lesion (arrow) and dilatation of the small bowel.

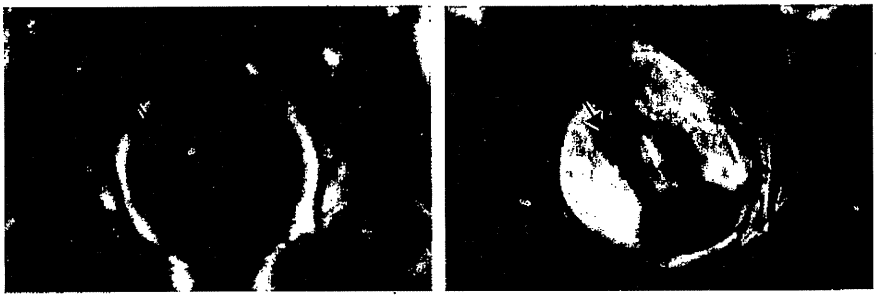

Fig. 2　a: Pelvic MRI (T1 weighted image) showed a high intensity mass.
b: Pelvic MRI (T2 weighted image) showed a low intensity mass.　　a|b

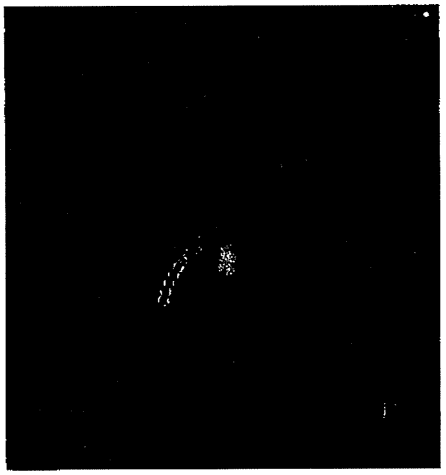

Fig. 3　Enterography showed a stenosis at the end of the small intestine (arrow).

以上より，回腸子宮内膜症による腸閉塞を強く疑った．婦人科で3カ月間のホルモン治療を行った後に，腹腔鏡下小腸部分切除術を施行した．

手術所見：臍に第1ポートを挿入して気腹後，左右の上・下腹部にポートを追加して計5ポートで行った．腹腔鏡で腹腔内を観察すると，Douglas窩に少量の淡黄色透明の腹水を認めた．癒着は後膣円蓋と直腸前壁，虫垂と後腹膜に軽度みられた．また，Blue berry spotは膀胱子宮窩，Douglas窩，子宮後壁から仙骨子宮靭帯にかけて散在し，直腸S状結腸部にBlue berry spotは認められなかった（**Fig. 4a-c**）．

観察範囲内に認められたBlue berry spotは全て焼灼した．回腸末端部より約20cm口側に腸管子宮内膜症が原因と思われる狭窄部位を認めた

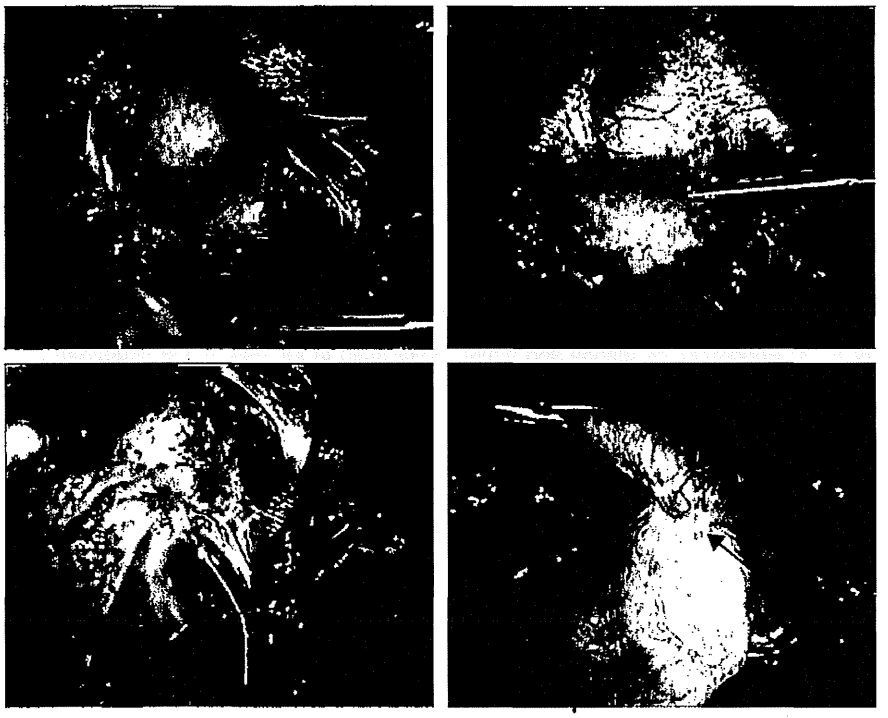

Fig. 4 Laparoscopic Flindings.
a-c: There were adhesions within the peritoneal cavity in the posterior fornix of the vagina, anterior rectal wall and appendix. Blue berry spots were diffusely seen along douglas' pouch, vesicouterine pouch, uterosacral ligament, and posterior wall of uterus.
d: The obstruction had the gross appearance of stenosis with fibrosis, 20cm proximal to the ileocecal valve (arrow).

a|b
c|d

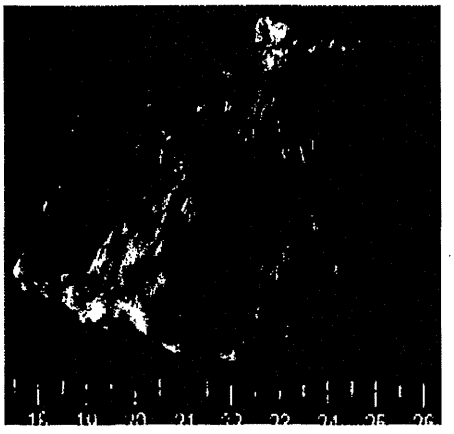

Fig. 5 A stenosis lesion with fibrosis (arrow) was seen in the ileum, 20cm proximal to the ileocecal valve.

(**Fig. 4d**). 回盲部を臍の小切開創より創外に引き出し，小腸部分切除術を施行した．手術時間は3時間6分で，出血量は5gであった．

切除標本肉眼的所見：検体は6.0×8.5cm大で中央部に結節性隆起を認めた（**Fig. 5**）．狭窄部の粘膜面は保たれており，漿膜面には線状瘢痕を認めた．

病理組織所見：粘膜下層から漿膜下層にかけて子宮内膜腺および間質細胞の島状分布を認め，回腸子宮内膜症と診断した．また狭窄部の漿膜下層には線維化を認めた（**Fig. 6a, b**）．

術後経過：術後経過は良好で，第9病日に退院となった．

考　察

子宮内膜症は子宮内膜組織が異所性に増殖する良性疾患である．腸管壁に異所性に子宮内膜が増

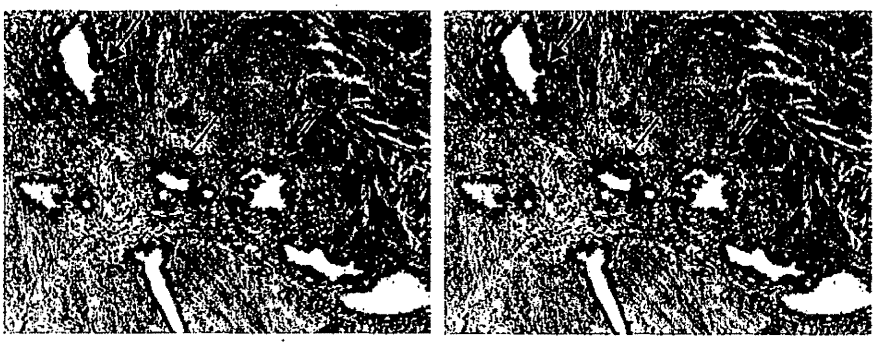

Fig. 6　a: Endometriotic glands and stroma were seen in the wall of the ileumfrom the submucosal to subserosal level (hematoxylin and eosin, ×3.5).
b: The magnified detail showed an area of endometriosis within the wall of the ileum (H&E, ×50).　a|b

殖した状態を腸管子宮内膜症と定義し，Macafeeら[1]は腸管子宮内膜症の発症頻度を全子宮内膜症の12％と報告している．発生部位はS状結腸・直腸が約70～90％と多く，次いで小腸７％，盲腸3.6％となっている[2]．近年，子宮内膜症は増加しており，それに伴い本症の報告例も増加している[3]．臨床症状では月経周期に一致して周期的に出現する腹痛や下血，排便困難が特徴的とされ，腸管狭窄が高度になると腸閉塞症状を併発する．

診断は病歴，臨床症状から本症を疑うことが第一である．特異的な検査法はないが，MRI検査においてT1強調像で高信号，T2強調像で低信号を示すことが有用という報告がある[4]．一般的に，周期的出血を繰り返した病変では，新旧の出血が混在するため，T1強調像で均一な高信号を示す腫瘤が主体となり，T2で低信号が出現すれば診断は確実とされる．本例でも，T1強調像で高信号，T2強調像で低信号を示す腫瘤を認めており，診断に有用であった．

内視鏡検査では粘膜下腫瘍の形態を取りやすく，生検の診断率は６％以下とされるが[5]，最近では超音波ガイド下穿刺吸引法（EUS-FNA）を用いた生検で組織診断率が40％との報告もみられる[6]．

自験例では過去に子宮内膜症の診断があったことと画像検査から腸管子宮内膜症が強く疑われた．一般的には術前に確定診断を得ることなく手術に至る症例も少なくない．実際に腸閉塞をきたした回腸子宮内膜症で，術前診断が得られた症例は約28％と報告されており[4]，術後の病理診断で確定診断に至ることが多い[7]．鑑別診断としては，消化管腫瘍，平滑筋腫，悪性リンパ腫，平滑筋肉腫，GIST，クローン病などの炎症性疾患が挙げられ[8][9]，治療法が大きく異なるため慎重な鑑別を要する．

本症の治療としては，GnRHアナログ療法，ダナゾール，低用量ピル，ジェノゲストなどの保存的治療と外科的治療に分けられるが，腸管の通過障害をきたしている場合には，腸管切除やバイパス術が選択される．回腸子宮内膜症では，内膜組織の過形成と線維化により腸閉塞で発症することが多く，外科的治療が選択されることがほとんどである．実際に医中誌Webで「回腸子宮内膜症」「腸閉塞」をキーワードとして会議録を除き，1983年から2013年までの文献を検索したところ，52例の報告がみられ，１例[10]を除いた51例で腸管切除が行われていた．ホルモン療法は異所性の子宮内膜組織の増生自体が病態の原因となる症例では有効であるが，腸閉塞を発症するような腸管の高度狭窄例では，線維化や過形成のために抵抗性であることが報告されている[11]．一方で腹腔内の内膜症組織を軟化，縮小させるので手術手技を容易にするためには術前の投与が有効であるとする報告もみられる[12][13]．したがって，症状，臨床経過，検査所見，妊孕性の温存などを考慮し，治療方針を検討する必要がある[14]．本症例では，腸管子宮内膜症に対しては，近年，腹腔鏡下手術が増えてきており，小腸と直腸の２カ所に同時発生した腸管

子宮内膜症に対する同時手術[15]や，単孔式手術の報告もある[16]．そして，腸管子宮内膜症に対しては海外での無作為比較試験により開腹手術に対する非劣性も証明されてきている[17][18]．今後も腸管子宮内膜症に対して腹腔鏡下手術は増加すると考えられるが，腹腔鏡から開腹へのconversionに関しては0～10%と報告があり[19]～[22]，回腸子宮内膜症に限った報告でもconversionは1例もなかったと報告され[23]，開腹移行の割合は少ないと考えられる．そのため術式の第一選択として腹腔鏡手術を選択することは問題ないと考えられる．しかし，腹腔鏡手術による膀胱損傷，尿管損傷，直腸膣瘻，縫合不全，骨盤内膿瘍などの合併症が約10%に発症すると報告されており[24][25]，腹腔内の所見次第では躊躇せず開腹手術に移行して，安全な手術を心がけるべきであると考える．

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A Case of Endometriosis of Ileum Presenting with Intestinal Obstruction

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We report a case of small bowel obstruction that occurred secondary to endometriosis of the terminal ileum. A 34-year-old woman presented to our hospital with purpose of the operation for ileal endometriosis. Except indicated intestinal endometriosis in 30-years-old, she had no background history. She presented at the former hospital with complain of abdominal pain. Abdominal X-ray and CT examination showed dilated loops of the small intestine, and a contrast-enhanced tumoral lesion located in the right ileocecal area. She was diagnosed with intestinal obstruction by suspected small intestinal endometriosis and had been treated with endocrine therapy before surgery.

We performed laparoscopic partial small bowel resection. The obstruction had the gross appearance of stenosis with fibrosis, 20cm proximal to the ileocecal valve. Intraoperative findings showed there were adhesions within the peritoneal cavity in the posterior fornix of the vagina, anterior rectal wall and appendix and were dissected. Blue berry spots were diffusely seen along douglas'pouch, vesicouterine pouch, uterosacral ligament, and posterior wall of uterus and was mostly ablated. Partial small bowel resection with end-to-end anastomosis was performed, as well as appendectomy. Pathologically, ileal endometriosis was diagnosed. The patient's recovery was uneventful and she was discharged home feeling well on postoperative day 9.

Key words: intestinal obstruction, intestinal endometriosis, laparoscopic surgery

G protein–coupled estrogen receptor 1 agonist G-1 induces cell cycle arrest in the mitotic phase, leading to apoptosis in endometriosis

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Objective: To demonstrate the effects of the selective G protein–coupled estrogen receptor 1 (GPER) agonist G-1 in human ovarian endometriotic stromal cells (ESCs).
Design: Experimental in vitro study.
Setting: University hospital.
Patient(s): A total of 33 patients with ovarian endometrioma.
Intervention(s): Endometriotic stromal cells from ovarian chocolate cysts were treated with the GPER agonist G-1.
Main Outcome Measure(s): The primary outcomes were cell proliferation, measured using the WST-8 assay; cell cycle, as analyzed using flow cytometry, fluorescent immunocytochemistry, and cytotoxicity; caspase activity, as measured by fluorescent and luminescent enzyme assays; and protein expression levels, as determined by Western blot analysis.
Result(s): G-1 suppressed ESC proliferation in a concentration-dependent manner. The inhibitory effect was not blocked when GPER signaling pathways, including the GPER itself, were inhibited. G-1 induced cell cycle arrest and accumulation in the sub-G_1 phase in ESCs. Immunofluorescence analysis demonstrated that G-1 interrupted microtubule assembly at the mitotic phase. G-1 also induced caspase-3–dependent apoptosis without significant cytotoxicity.
Conclusion(s): G-1 suppressed proliferation and induced apoptosis in ESCs, suggesting the potential use of this compound as a therapeutic drug for the treatment of endometriosis. (Fertil Steril® 2015;103:1228–35. ©2015 by American Society for Reproductive Medicine.)
Key Words: GPER, G-1, endometriosis, cell cycle arrest, apoptosis

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Endometriosis affects approximately 10% of women of reproductive age and is defined as the extrauterine presence of endometrium-like tissues. This disorder causes various clinical symptoms, such as pelvic pain, dysmenorrhea, dyspareunia, and infertility (1). Because there is substantial improvement in these symptoms once menopause begins, estrogens (Es) are thought to have indispensable roles in the development and maintenance of endometriosis. The classic biological effects of Es are mediated by the estrogen receptors (ERs) ERα and ERβ, which regulate target protein expression by binding to their promoter regions in target genes (2). G protein–coupled estrogen receptor 1 (GPER), a novel transmembrane ER, mediates the balance between nongenomic (rapid) cell signaling mechanisms and genomic (slow) transcriptional activity in response to Es (3). Tissue E responsiveness depends on the balance of ER expression and function. Indeed, the expression, distribution, and function of ER proteins are different in endometriotic tissues and normal endometrium (4, 5), contributing to the pathologic

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characteristics of endometriosis. Although few studies have been carried out on GPER in endometriosis, recent studies have demonstrated that GPER exhibits relatively high expression in endometriotic tissues compared with the normal endometrium (6) and in the endometrium of patients with endometriosis compared with that in healthy individuals (7). Moreover, the aberrant expression of GPER in E-dependent diseases suggests the potential involvement of GPER in the pathogenesis of endometriosis (8–12). Thus, understanding the functional mechanisms of GPER in endometriosis may facilitate the development of novel E-targeted therapies for endometriosis.

The most common method for determining receptor function is stimulation by ligands. Therefore, establishing a selective agonist or antagonist for a receptor is fundamental to understanding the pathways that are mediated by the specific receptor. However, GPER-specific stimulation is difficult because of the cross-reactivity of ERs and GPER. To overcome this problem, a selective GPER agonist, named G-1 (13), was developed; this GPER agonist does not exhibit significant effects on ER expression, E response element transcriptional activity over a concentration range of 1–10 μM (14), or 25 other GPCRs (15). Indeed, stimulation with G-1 has been commonly adopted in experiments targeting GPER and has facilitated the elucidation of GPER function in cell proliferation, cell death, and signaling in many E-dependent diseases (8, 11, 12, 16–20). Conversely, recent reports have demonstrated that G-1 suppresses ERs and GPER-negative ovarian and breast cancer cell proliferation (21) and inhibited ovarian cancer cell tubulin formation (22), suggesting that G-1 may also have GPER-independent effects. With regard to endometriosis, GPER was found to stimulate proliferation of endometriotic cells by using immortal endometriotic epithelial cells (23). However, little is known about the functional role of GPER in endometriosis, and no studies have used primary cultured endometriotic stromal cells (ESCs) or verified G-1 as a selective GPER agonist in endometriosis.

In this study we investigated the effects of G-1 on cell proliferation in endometriotic cells to verify the suitability of G-1 as a selective agonist for GPER in endometriosis, using ESCs derived from ovarian endometrioma.

MATERIALS AND METHODS

Patients and Samples

We obtained ovarian endometrioma samples from 33 women of reproductive age (mean age 35.5 years; range 29–45 years) who had undergone surgery for therapeutic reasons. The endometriosis stages were III (n = 18) and IV (n = 15), according to the American Society for Reproductive Medicine classification of endometriosis. None of the patients had received hormone treatment for at least 6 months before surgery, and all specimens were collected at the proliferative phase of the regular menstrual cycle. This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the institutional review board of Kyoto Prefectural University of Medicine (ERB-C-108). Informed consent was obtained from all patients.

Cell Preparation and Ribonucleic Acid Transfection

We used the following procedures for the isolation and culture of ESCs derived from ovarian endometrioma, as described previously (24, 25). Tissue digestion was carried out with 2.5% collagenase (Nacalai Tesque) and 15 IU/mL of DNase I (Takara Shuzo). After the resulting cell suspension was filtered through a nylon cell strainer, the cell debris was centrifuged using lymphocyte separation solution (Nacalai Tesque) to remove the red blood cells. Stromal cell preparations (>95% purity) were confirmed by positive staining for CD10 and vimentin and negative staining for cytokeratin, CD31, and CD45. We collected and subcultured the primary cultured stromal cells after they became subconfluent, using 0.1% trypsin, and the cells were then resuspended in phenol red-free DMEM/Ham's F12 (Nacalai Tesque) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum and 1% penicillin and streptomycin (100 μg/mL) for subsequent experiments. Knockdown of GPER was performed when the cells were subcultured using RNAiMAX (Invitrogen) and validated with short interfering RNA (siRNA) for GPER (Applied Biosystems), according to the manufacturer's instructions.

Cell Proliferation Assay and Assessment of Cell Morphology

Cells with or without GPER knockdown were seeded into 96-well plates containing normal growth medium at a density of 5×10^3 cells per well. After 24 hours the cells were treated with or without G-1 and/or the GPER antagonist G15 (10 μM) (26), mitogen-activated protein kinase kinase (MEK1/2)-selective inhibitor U0126 (10 μM) (27), and phosphoinositol 3-kinase (PI3K)-selective inhibitor LY294002 (10 μM) (28) (Sigma-Aldrich) dissolved in dimethyl sulfoxide (Nacalai Tesque). Proliferation was determined by SF reagent (Nacalai Tesque) containing WST-8 after a 96-hour incubation, using a multiwell spectrophotometer multiplex 9120 (Bio-Rad) at a wavelength of 450 nm. The cell morphology was examined by phase contrast microscopy after a 96-hour incubation with or without G-1 treatment in a six-well culture plate.

Cell Cycle and Apoptosis Analysis

Cells were treated with or without G-1 (10 μM) for 7 days and permeabilized with 0.1% Triton-X100. The nuclei were then stained with propidium iodide (PI), and DNA content was measured using a FACSCalibur (Becton-Dickinson). Data were analyzed using ModFit LT software (Verity Software House) and the CellQuest software package (Becton-Dickinson).

Measurement of Cytotoxicity and Caspase-3/7 Activity

CytoTox-Fluor cytotoxicity assays and Caspase-Glo 3/7 assays (Promega) used fluorescent substrates for dead-cell proteases and luminescent substrates for luciferase reactions, with activating caspase-3/7 as markers for cell cytotoxicity and caspase activity, respectively. These assays were performed after 7 days

of treatment with or without G-1 (10 μM). Fluorescence was measured at 485Ex/520Em using a SpectraMax M2e instrument (Molecular Devices), and luminescence was measured using a GloMax 20/20 Luminometer (Promega).

Immunofluorescence Cytochemistry

Cells were treated with or without G-1 (10 μM) for 96 hours on covered glass and were fixed with 4% paraformaldehyde in Tris-buffered saline (TBS) at room temperature for 15 minutes. The cells were blocked with 5% normal goat serum in TBS containing 0.3% Triton X-100 for 1 hour and were incubated overnight at 4°C with primary antibodies (Cell Signaling Technology) against phospho-histone H3 (Ser10) and α-tubulin in TBS containing 1% bovine serum albumin and 0.3% Triton X-100. The next day the cells were incubated with Alexa-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 1 hour and were mounted using Vectashield Mounting Medium with PI (Vector Laboratories). An FV1000 confocal laser scanning microscope and FLUOVIEW software (Olympus) were used for image capturing and cell cycle distribution analysis.

Western Blot Analysis

Cell protein extracts were prepared at the indicated times using RIPA buffer (Nacalai Tesque) and loaded onto polyacrylamide gels (ATTO) for electrophoresis. Proteins were then transferred to polyvinylidene fluoride membranes. The membranes were blocked with blocking buffer (5% skimmed milk in TBS containing 0.1% Tween-20) for 1 hour at room temperature and incubated overnight at 4°C with the appropriate primary antibody diluted in blocking buffer. The next day the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) at room temperature for 1 hour, and signals were detected using Chemi-Lumi One (Nacalai Tesque) and ChemiDoc XRS+ systems (Bio-Rad). Primary antibodies against phospho-cdc2 (Tyr15), cdc2, phospho-cdc25c (Ser216), cdc25c, phospho-wee1 (Ser642), wee1, phospho-histone H3 (Ser10), cyclin B1, caspase 3, survivin, XIAP, p21, Bcl-2, GAPDH (Cell Signaling Technology), and GPER (Abcam) were used.

Statistical Analysis

Statistical analysis was performed to determine differences in cell proliferation, cell cycle distribution, apoptosis, cytotoxicity, and caspase activity. Cell cycle distribution was analyzed using one-factor analysis of variance followed by multiple comparisons using Dunnett's procedure or Welch's t test. Each assay was performed in triplicate. Data are presented as means $\pm$ SEM. Differences with P values of $<.05$ were considered statistically significant.

RESULTS

G-1 Suppressed the Proliferation of ESCs

First, we examined the effects of G-1 on ESC proliferation. Interestingly, G-1 inhibited the proliferation of ESCs in a concentration-dependent manner (Fig. 1A). With respect to morphologic changes, ESCs were small and exhibited an immature structure with an increased number of dead detached cells (Fig. 1B). This effect was not blocked by GPER antagonism, MEK1/2 inhibition, PI3K inhibition (Fig. 1C), or GPER knockdown (Fig. 1D), even though treatment with G15 (23), U0126 (29, 30), or LY294002 and knockdown of GPER had suppressive effects on cell proliferation. Although G-1 did not inhibit the expression of GPER in ESCs, we achieved successful knockdown of GPER by siRNA transfection, as shown by Western blot analysis (Fig. 1E). Furthermore, our Western blot analysis showed that G-1 neither induced nor inhibited the expression of GPER (Fig. 1F). These results indicated that G-1 had suppressive effects on the proliferation of ESCs, implying that this effect was independent of the GPER signaling pathway, including GPER itself.

G-1 Caused Cell Cycle Arrest and Sub-G_1-phase Accumulation in ESCs

Flow cytometry analysis was used to estimate the effects of G-1 on the cell cycle of ESCs. From this analysis, we observed cell cycle arrest at the G_2/M phase (Fig. 2A), with an increase in the sub-G_1 population (Fig. 2B) after 7 days of G-1 treatment. To determine the mechanism through which the sub-G_1 population was increased, we performed cytotoxicity and caspase-3/7 activity assays. Interestingly, although G-1 caused an increase in caspase activity, there was no increase in cytotoxicity observed in this experiment (Fig. 2C), suggesting that G-1 did not have cytotoxic effects on ESCs. These results indicated that ESCs were arrested at the G_2/M phase of the cell cycle, resulting in apoptosis, without any significant necrotic effects after exposure to G-1.

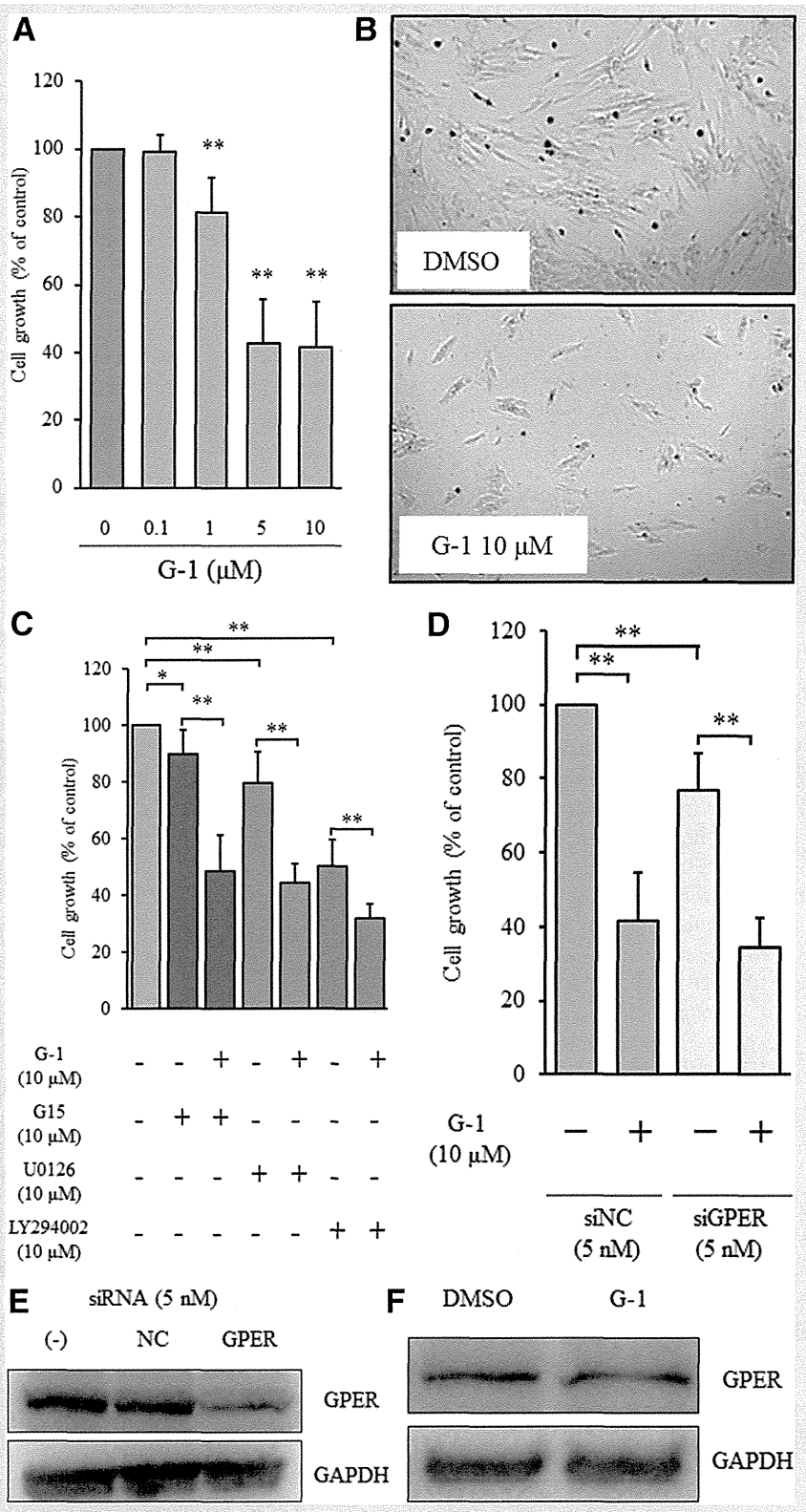
G-1 Induced Cell Cycle Arrest at the Prometaphase of Mitosis

To elucidate the mechanisms mediating the observed cell cycle arrest in ESCs, we used immunofluorescence cytochemistry using PI and antibodies against phospho-histone H3 or α-tubulin as markers of the cell cycle stage. We observed an increase in the proportion of cells at the prometaphase of mitosis and a decrease in the proportion of cells at interphase, accompanied by the absence of cells in metaphase (cytokinesis) (Fig. 3A). Representative images of the cell stages are shown in Figure 3B. These results indicated that cell cycle arrest might occur as a result of abnormalities in the mitotic stage.

G-1 Affected the Expression Patterns of Various Signaling Proteins in ESCs

Next we performed Western blot analysis to examine the mechanisms mediating cell cycle arrest during mitosis and the activation of caspase-3/7 induced by G-1 in ESCs. We found elevated phospho-histone H3 protein levels and unchanged cyclin B1 expression but decreases in phosho-cdc2 (Tyr15), cdc2, phospho-cdc25c (Ser216), cdc25c, phospho-wee1 (Ser642), and wee1 levels (Fig. 4A), in addition to decreases in caspase-3 and survivin (Fig 4B). No changes were observed in XIAP, p21, and Bcl-2 expression. These results,

FIGURE 1



(**A**) Effects of G-1 on the proliferation of ESCs. (**B**) Representative morphologic images of ESCs treated with the indicated concentrations of G-1. (**C**) Effects of GPER antagonism, MEK inhibition, and PI3K pathway inhibition or (**D**) GPER knockdown on the effects of G-1. (**E**, **F**) Expression of GPER protein in ESCs at (**E**) 48 hours after transfection with siRNA or (**F**) 96 hours after treatment with G-1 (10 μM). The results are expressed as a percentage of the control values and presented as means ± SEMs for at least six independent experiments. * $P<.05$, ** $P<.01$ versus the control.

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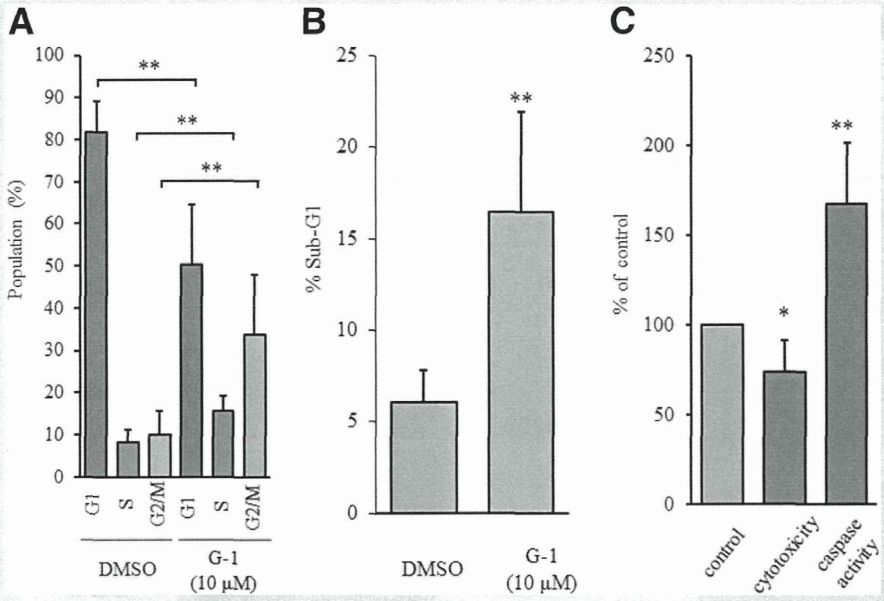
FIGURE 2



Effects of G-1 on the cell cycle and survival in ESCs. (**A**, **B**) Flow cytometry analysis of the effects of G-1 on (**A**) the ESC cell cycle distribution and (**B**) the sub-G_1 population. (**C**) The cytotoxic and caspase activation effects of G-1 (10 μM) in ESCs. Values are presented as means ± SEMs for at least six independent experiments. * $P<.05$, ** $P<.01$ versus the control. DMSO = dimethyl sulfoxide.

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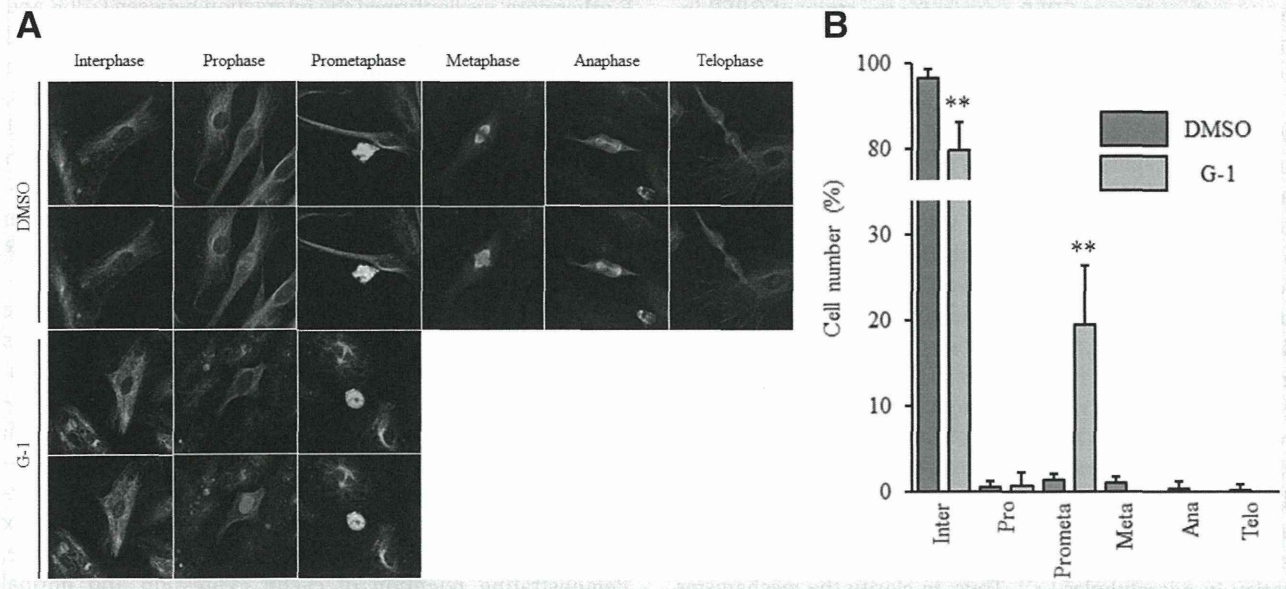
which demonstrated protein expression patterns corresponding to cell cycle arrest at the G_2/M phase and a proapoptotic status, were consistent with those obtained in the cell cycle and apoptosis examination.

DISCUSSION

In this study we found that G-1 inhibited ESC proliferation by inducing apoptosis. Furthermore, our results indicated that these inhibitory effects were independent of GPER. From

FIGURE 3



Endometriotic stromal cell analysis by immunofluorescence cytochemistry. (**A**, **B**) Representative images of (**A**) the cell cycle phases and (**B**) their proportion with or without G-1 treatment. Endometriotic stromal cells were stained with antibodies against α-tubulin (*green*) or phosphohistone H3 (Ser10) (*magenta; pseudocolor*) after a 96-hour incubation with G-1 (10 μM). The nuclei were counterstained with PI (*red*). Data are presented as means ± SEMs for five independent experiments. * $P<.05$, ** $P<.01$ versus the control.

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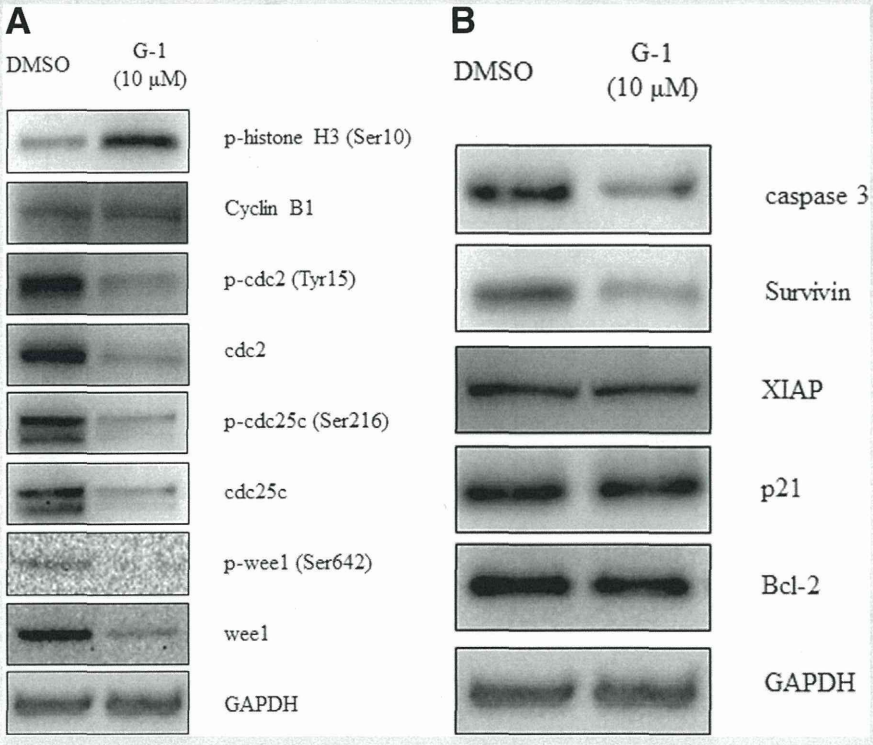
FIGURE 4



(**A**, **B**) Effect of G-1 on protein expression patterns in (**A**) the cell cycle and (**B**) survival of endometriotic stromal cells. After a 96-hour treatment with G-1 (10 μM), the levels of cell M-phase promoting factor-related proteins (phospho-histone H3, cyclin B1, phospho-cdc2, cdc2, phospho-cdc25c, cdc25c, phospho-wee1, and wee1) and apoptosis-related proteins (caspase 3, survivin, XIAP, p21, and Bcl-2) were examined by Western blot analysis. Representative results of at least six independent experiments are shown.

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these findings we propose that G-1, which may no longer be considered a selective GPER agonist for the study of GPER in endometriosis, may have therapeutic potential in the treatment of endometriosis.

Previous studies have shown that G-1 affects the cell cycle; however, the results of these studies have not been consistent. G_1-phase arrest has been observed in breast cancer, in which G-1 induces p21 expression through dysregulation of p53 (16). Moreover, G-1 was shown to induce S/G_2-phase arrest in endothelial cells, inhibiting DNA synthesis (31). Additionally, G_2/M-phase arrest was found in prostate cancer, suggesting the involvement of the mitogen-activated protein kinase pathway (20). These previous studies have implicitly presumed the specificity of G-1 as a GPER ligand, and the differences in the effects of G-1 are assumed to have resulted from differences in the expression levels or distributions of GPER. However, recent reports have suggested that G-1 may also have GPER-independent effects (21) or may be dependent on the putative ER isoform ERα36 (32). In addition, one report showed that G-1 has an inhibitory effect on tubulin polymerization in microtubules (22). Thus, to clarify the mechanisms through which G-1 affects ESCs, we used a GPER antagonist, G-15, which inhibits the GPER signaling pathway, as well as siRNA to knockdown GPER and G-1 for comparison of the effects of these treatments on cell proliferation. Our results showed that G-1 acted independently of GPER in this context. Furthermore, we confirmed the interaction between GPER and G-1 in ESCs by examining phosphorylation and the total amount of ERK or Akt and pivotal kinases in the activation of PI3K and mitogen-activated protein kinase pathways, which act downstream of GPER. No significant effect of G-1 was observed (Supplemental Fig. 1, available online), which may be attributed to the constitutive activation of these pathways (27, 28), making it difficult to detect changes in phosphorylation levels and to completely exclude GPER involvement in the G-1 effect.

To investigate the GPER-independent effects of G-1 in the present study, we examined the expression of proteins involved in the cell cycle in addition to apoptosis regulation. Elevation of phospho-histone H3 levels indicated the accumulation of cycle-arrested cells at the mitotic phase, which supported our flow cytometry and immunofluorescence cytochemistry results. Alternatively, G-1 did not affect the activity of the M-phase promoting factor, a key protein complex promoting entry into the mitotic phase from the G_2 phase, demonstrating retention of cyclin expression and normal activation status of kinases and phosphatases involved in modulating M-phase promoting factor activity. These findings were in agreement with those of a previous analysis in ovarian cancer cells (22), suggesting that the effects of G-1

on cell cycle arrest were not directly regulated by proteins involved in cell cycle modulation.

Regarding the apoptotic pathway, endometriotic cells show decreased spontaneous apoptosis (33) and resistance to apoptotic stimuli, such as cytokines (34). Moreover, impaired apoptotic mechanisms contribute to their abnormal survival in ectopic sites. In this study we found a decrease in survivin, a representative protein from the inhibitor of apoptosis protein (IAP) family, which inhibits activated caspase-3 (35). Survivin is expressed during the G_2/M phase of the cell cycle associated with microtubules of the mitotic spindle during mitosis, suppressing caspase-3 activity (36). Importantly, the involvement of survivin in the resistance to apoptosis induction has been previously reported in ESCs (37). In contrast, X chromosome–linked IAP, another member of the IAP family, did not exhibit altered protein expression. A previous report describing the effects of G-1 on ovarian cancer also showed that survivin was upregulated, which may be attributed to the compensatory increase in stress in cells undergoing cell cycle arrest (22). Our data were inconsistent with the results of this previous study but suggested the possibility of interactions among microtubules, survivin, and caspase-3. Therefore, the effects of G-1 may promote apoptotic features through the repression of survivin expression in endometriosis. Furthermore, we also examined the relationship between caspase activation and its pivotal mitochondrial pathway by examining Bcl-2, which functions in an antiapoptotic manner by inhibiting mitochondrial cytochrome c release (38), and p21, which serves to inhibit kinase activity, regulates cell cycle progression from G_1 to S phase, and is involved in cell cycle arrest caused by G-1 (16, 20). However, we did not observe significant changes in Bcl-2 or p21 protein expression. Taken together, our initial findings for survivin expression implied that microtubules might be involved in cell cycle arrest and the apoptotic process. However, further studies are needed, including observations of cell cycle progression at different times, to describe the involvement of this protein in more detail.

The pharmacologic features of G-1 observed in ESCs in this study (i.e., antiproliferative and proapoptotic effects) led us to explore the possibility of using G-1 as a therapeutic drug for endometriosis. However, although no cytotoxicity was observed in vitro at 10 μM of G-1, future studies will be required to examine the potential clinical applicability of G-1 in detailed dose-related analyses. We also confirmed that G-1 suppressed cell proliferation and induced G_2/M-phase cycle arrest in stromal cells derived from normal endometrium, endometriotic endometrium, and immortalized ESCs (39) (data not shown). Therefore, the potential therapeutic applications of G-1 in the treatment of endometriosis may be limited to the treatment of postmenopausal or posthysterectomy residual endometriotic lesions due to the effects of G-1 on the endometrium.

In conclusion, G-1 induced cell cycle arrest in ESCs in the mitotic phase and led to apoptosis of ESCs without significant cytotoxicity. Furthermore, these effects may be independent of GPER, and G-1 and microtubules may interact to elicit the observed effects. Our findings imply that G-1 may be applicable as a therapeutic drug for endometriosis.

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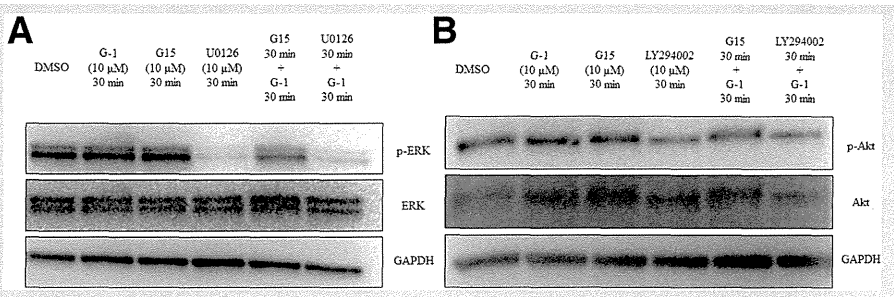
SUPPLEMENTAL FIGURE 1



Western blot analysis of the effects of G-1 on the proteins involved in G protein-coupled estrogen receptor signaling. (**A**) Phosphorylated and total protein expression of ERK in ESCs treated with G-1 (10 μM) for 30 minutes, G-15 (10 μM) for 30 minutes, U0126 (10 μM) for 30 minutes, or a combination of 10 μM G-1 and 10 μM G-15 (G-1 added 30 minutes after G-15) or 10 μM G-1 and 10 μM U0126 (G-1 added 30 minutes after U0126). (**B**) Phosphorylated and total Akt levels in ESCs treated with G-1 (10 μM) for 30 minutes, G-15 (10 μM) for 30 minutes, LY294002 (10 μM) for 30 minutes, or a combination of 10 μM G-1 and 10 μM G-15 (G-1 added 30 minutes after G-15) or 10 μM G-1 and 10 μM LY294002 (G-1 added 30 minutes after LY294002). Representative results of at least six separate experiments are shown.

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Dienogest reduces HSD17β1 expression and activity in endometriosis

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Abstract

Endometriosis is an estrogen-dependent disease. Abnormally biosynthesized estrogens in endometriotic tissues induce the growth of the lesion and worsen endometriosis-associated pelvic pain. Dienogest (DNG), a selective progesterone receptor agonist, is widely used to treat endometriosis and efficiently relieves the symptoms. However, its pharmacological action remains unknown. In this study, we elucidated the effect of DNG on enzymes involved in local estrogen metabolism in endometriosis. Surgically obtained specimens of 23 ovarian endometriomas (OE) and their homologous endometrium (EE), ten OE treated with DNG (OE w/D), and 19 normal endometria without endometriosis (NE) were analyzed. Spheroid cultures of stromal cells (SCs) were treated with DNG and progesterone. The expression of aromatase, 17β-hydroxysteroid dehydrogenase 1 (HSD17β1), HSD17β2, HSD17β7, HSD17β12, steroid sulfatase (STS), and estrogen sulfotransferase (EST) was evaluated by real-time quantitative PCR. The activity and protein level of HSD17β1 were measured with an enzyme assay using radiolabeled estrogens and immunohistochemistry respectively. OESCs showed increased expression of aromatase, HSD17β1, STS, and EST, along with decreased HSD17β2 expression, when compared with stromal cells from normal endometria without endometriosis (NESCs) ($P<0.01$) or stromal cells from homologous endometrium (EESCs) ($P<0.01$). In OESCs, DNG inhibited HSD17β1 expression and enzyme activity at 10^{-7} M ($P<0.01$). Results of immunohistochemical analysis displayed reduced HSD17β1 staining intensity in OE w/D ($P<0.05$). In conclusion, DNG exerts comprehensive inhibition of abnormal estrogen production through inhibition of aromatase and HSD17β1, contributing to a therapeutic effect of DNG on endometriosis.

Key Words
- 17β-hydroxysteroid dehydrogenase 1
- dienogest
- endometriosis
- ovarian endometrioma
- spheroid culture

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Introduction

Endometriosis is defined as the presence of endometrium-like tissues at extra-uterine sites. Clinical symptoms associated with endometriosis include pelvic pain, dysmenorrhea, dyspareunia, and infertility (Giudice 2010). There is marked relief of symptoms after menopause, clearly demonstrating the dependency of endometriosis on estrogens. Besides systemic circulating estrogens secreted from the ovaries, abnormally biosynthesized estrogens in endometriotic tissues also contribute to the growth of the lesion and worsening symptoms (Bulun 2009). In the eutopic and ectopic endometria of women with endometriosis, overexpressed aromatase (also known as estrogen synthase) biosynthesizes estrogens, namely estrone and estradiol, from the androgens,

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androstenedione, and testosterone respectively (Noble *et al.* 1996, Kitawaki *et al.* 1997, Matsuzaki *et al.* 2006, Dassen *et al.* 2007, Smuc *et al.* 2007). In these tissues, estradiol, the most potent estrogen, is predominantly synthesized from less potent estrone by 17β-hydroxysteroid dehydrogenase 1 (HSD17β1), and the reverse reaction is catalyzed mainly by HSD17β2. In endometriotic tissues, the expression of HSD17β1 is higher than that of HSD17β2; thus, the reaction is tilted in favor of producing estradiol (Zeitoun *et al.* 1998, Dassen *et al.* 2007). The other major source of estrogens is estrone sulfate, an inactive conjugated form abundant in the circulation. Estrone sulfate is desulfated to estrone by steroid sulfatase (STS) and estrone is inactivated by estrogen sulfotransferase (EST) (Utsunomiya *et al.* 2004, Colette *et al.* 2013). Understanding how the aberrant expression of these enzymes in endometriosis contributes to local estrogen production and metabolism will allow the development of improved therapeutic agents.

Dienogest (DNG), a selective progesterone (P_4) receptor (PR) agonist, is widely used to treat endometriosis (McCormack 2010) and efficiently relieves endometriosis-associated pelvic pain (Harada *et al.* 2009, Momoeda *et al.* 2009, Strowitzki *et al.* 2010, Petraglia *et al.* 2012). DNG directly inhibits PR-mediated cell proliferation (Okada *et al.* 2001, Fu *et al.* 2008, Shimizu *et al.* 2009) and production of the inflammatory factors involved in the pathology of endometriosis, such as prostaglandin estradiol (E_2) (Shimizu *et al.* 2011, Yamanaka *et al.* 2012), inflammatory cytokines (Horie *et al.* 2005), Toll-like receptor 4 (Mita *et al.* 2011), and nerve growth factor (Mita *et al.* 2014). Supprerssion of these inflammatory factors is considered to contribute, in part, to the improvement of pain symptoms. DNG restores the antigen-presenting ability of peritoneal fluid macrophages by increasing human leukocyte antigen-DR expression (Maeda *et al.* 2014). DNG also suppresses aromatase expression in human immortalized endometrial epithelial cells (Shimizu *et al.* 2011) and primary cultured stromal cells (SCs) derived from ovarian endometrioma (OE) (Yamanaka *et al.* 2012). However, the effect of DNG on other estrogen-metabolizing enzymes in endometriotic cells remains unknown, and a more detailed analysis is needed to understand its clinical effectiveness and pharmacological function.

The purpose of this study was to investigate the effect of DNG on enzymes involved in estrogen metabolism using spheroid cultures of primary cultured SCs derived from OE, endometrium with endometriosis (EE), and normal endometrium without endometriosis (NE).

Patients and methods

Patients and samples

Patient characteristics are given in Table 1. OE tissues from patients ($n=23$) who did not receive any hormonal treatment and their homologous EE specimens ($n=10$), in addition to OE specimens from patients treated with DNG at a dose of 1 mg twice daily for 3–5 months (OE treated with DNG (OE w/D)) ($n=11$), were obtained from women undergoing surgery for OE. NE specimens were obtained from women undergoing surgery for uterine fibroids ($n=19$). All women were of reproductive age, and all specimens, with the exception of OE w/D, were collected at the proliferative phase of the regular menstrual cycle. Women who had undergone hormonal treatments within 6 months before surgery were excluded. OE w/D specimens were not used for *in vitro* experiments to avoid the effect of previous DNG exposure on the results. The endometriosis stages were evaluated according to the American Society for Reproductive Medicine classification of endometriosis. This study was conducted in accordance with the guidelines of the Declaration of

Table 1 Clinical characteristics of study patients. Values are presented as means±S.E.M.

	NE ($n=19$)	EE ($n=10$)	OE ($n=23$)	OE w/D ($n=11$)
Age (years)	41.8±4.3	41.4±3.9	32.5±7.0*,†	37.5±4.2
CA-125 (U/ml)	NA	68.9±29.1	82.5±83.4	68.0±55.7
r-ASRM stage (%)				
III	NA	6 (60)	14 (61)	6 (55)
IV		4 (40)	9 (39)	5 (45)
Duration of drug administration (weeks)	NA	NA	NA	13.4±6.0

P values were obtained by Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure or χ^2 test. NE, normal endometrium; EE, endometrium with endometriosis; OE, ovarian endometrioma; OE w/D, OE treated with dienogest; r-ASRM, revised American Society for Reproductive Medicine; DNG, dienogest. *$P<0.01$ versus NE and †$P<0.05$ versus EE.

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Helsinki and was approved by the institutional review board of the Kyoto Prefectural University of Medicine. Informed consent was obtained from all patients.

Isolation and culture of SCs

The isolation and culture of SCs was conducted as described previously (Yamanaka *et al.* 2012). Briefly, tissue digestion was performed with 2.5% collagenase (Nacalai Tesque, Kyoto, Japan) and 15 IU/ml of DNase I (Takara Shuzo, Tokyo, Japan). After filtering through a nylon cell strainer, the digested cells were centrifuged in lymphocyte separation solution (Nacalai Tesque) to remove the red blood cells. The >95% purity of SC preparations was confirmed by positive staining for CD10 and vimentin and negative staining for cytokeratin, CD31, and CD45. The cells were cultured in DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (100 μg/ml), under a humidified atmosphere at 37 °C in 5% CO_2. The cells that reached subconfluence were dispersed using 0.1% trypsin (Nacalai Tesque) and resuspended in phenol-red-free DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 10% dextran-coated charcoal-treated FBS and 1% penicillin and streptomycin (100 μg/ml). For mRNA analysis, SCs were subcultured in U-bottom 96-well culture plates (Sumilon) at a density of 4×10^4 cells/well to form spheroids. For HSD17β1 activity assays, SCs were plated into six-well culture plates at a density of 4×10^5 cells/well to form monolayers. The OESC spheroid expression of estrogen receptor α (ERα (ESR1)), ERβ (ESR2), PR, aromatase, cyclooxygenase-2 (COX2), and nuclear factor-κ B (NFκB) p50 subunit nuclear localization was validated by immunocytochemistry as described previously (Yamanaka *et al.* 2012).

Treatment of cultured SCs with DNG or P_4

The culture medium was replaced either after 72 h (spheroid culture for RNA extraction) or when cells reached subconfluence (HSD17β1 activity assay) by medium with or without DNG (10^{-8}, 10^{-7}, and 10^{-6} M; Bayer Schering Pharma, Berlin, Germany) or P_4 (10^{-8}, 10^{-7}, and 10^{-6} M; Sigma–Aldrich), and the cells were incubated for a further 48 h.

RNA extraction, cDNA preparation, and real-time PCR

Total RNA was extracted from cultured SCs using the RNeasy Mini Kit (Qiagen). After quantification and determination of the quality of the RNA by u.v. absorption (OD 260 nm/280 nm) using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA), cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen) and a GeneAmp PCR 9700 machine (Applied Biosystems). Quantitative real-time PCR was conducted using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and a StepOne Real-Time PCR System (Applied Biosystems) with TaqMan assay primer/probe sets (Applied Biosystems) for the target genes: aromatase (*CYP19A1*) (Hs00240671_m1), *HSD17β1* (Hs00166219_g1), *HSD17β2* (Hs00157993_m1), *HSD17β7* (Hs00367686_m1), *HSD17β12* (Hs00275054_m1), *STS* (Hs00996676_m1), *EST* (*SULT1E1*) (Hs00960941_m1), and endogenous control *GAPDH* (Hs03929097_g1). Real-time quantitative PCR was performed under the following thermal cycling conditions: denaturing at 95 °C for 60 s; 3 s at 95 °C; 40 cycles of 30 s at 60 °C. Threshold cycle (Ct) values were calculated using the ΔΔCt method.

HSD17β1 enzyme assays

HSD17β1 activity was measured using thin layer chromatography, as described previously (Kitawaki *et al.* 2000). Briefly, cells were washed twice with phenol-red-free DMEM/Ham's F-12, and then incubated at 37 °C/5% CO_2 for 6 h with 0.5 ml of serum-free medium containing [6,7-^{3}H]estrone (Perkin Elmer, Waltham, MA, USA) (1.8×10^6 dpm, 37 μM). The reaction was stopped by transferring the medium to the test tubes containing 2 ml chloroform and the corresponding carrier steroids: [4-^{14}C]estradiol (Perkin Elmer) (1.3×10^4 dpm) and non-radioactive estrone and estradiol (0.2 mg each). The steroids were isolated by thin-layer chromatography using Silicagel 60 F254 (0.25 mm; Merck) in a system of chloroform:ethyl acetate (4:1, v/v). The aliquot was mixed with Clear-sol I (Nacalai Tesque), and radioactivity was measured using a scintillation counter (Beckman Coulter, Fullerton, CA, USA). Enzyme activity was calculated and normalized according to the ratios of the estradiol formed. Protein concentration (pmol/mg protein per h) was measured by the Bradford method.

Immunohistochemistry

Specimens from OE, OE w/D, EE, and NE were stained immunohistochemically as described previously (Yamanaka *et al.* 2012) using an anti-HSD17β1 antibody (200 μg/ml; Abcam, Cambridge, UK). Normal term placenta tissue was used as a positive control. Because the cell components

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of epithelial cells and SCs were considerably different between eutopic endometrium and OE, we compared the immunostaining intensity in SCs using the *H*-score, a semi-quantitative index involving an algorithm described previously (Yamanaka *et al.* 2012). Briefly, two independent observers evaluated approximately 500 cells/slide and scored them as follows: 3×percentage of strongly staining cells+2×percentage of moderately staining cells+percentage of weakly staining cells. The *H*-score was calculated as the mean of the two scores.

Statistical analyses

The mRNA expression levels of enzymes in the three types of SCs were analyzed by Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure because of the unequal variances in the results. Results of real-time PCR and the HSD17β1 activity assay measuring the drugs' effects on mRNA expression and enzyme activity levels in OESCs were assessed by repeated measures ANOVA followed by multiple comparisons using Dunnett's procedure. Statistical analysis of the immunohistochemical results was performed using an unpaired *t*-test. Each assay for individual experiments was performed in triplicate. Data are presented as means± S.E.M. *P* values of <0.05 were considered statistically significant.

Results

mRNA expression of enzymes in spheroid-cultured SCs

In OESCs, the mRNA expression levels of aromatase ($P<0.01$), *HSD17β1* ($P<0.01$), *STS* ($P<0.01$), and *EST* ($P<0.01$) were greater compared with those in NESCs and EESCs. In NESCs or EESCs, we detected neither aromatase nor *EST* mRNA expression and an extremely low level of *HSD17β1* mRNA expression. In contrast, *HSD17β2* mRNA expression was lower in OESCs compared with that in NESCs ($P<0.01$) and EESCs ($P<0.01$) (Fig. 1A).

Effects of drugs on enzymes in OESCs

Incubating the spheroids for 48 h with DNG (10^{-7} M ($P<0.01$) and 10^{-6} M ($P<0.01$)) and P_4 (10^{-7} M ($P<0.05$) and 10^{-6} M ($P<0.01$)) significantly decreased

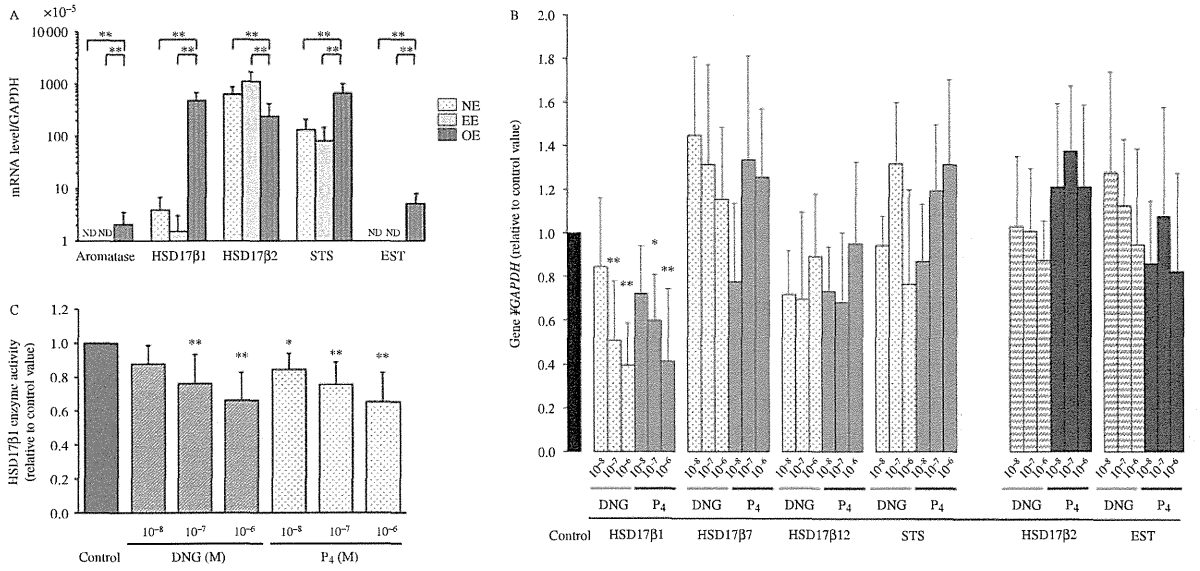

Figure 1
(A) The baseline mRNA expression levels of estrogen-metabolizing enzymes in spheroid-cultured stromal cells (SCs) derived from normal endometrium (NE), endometrium with endometriosis (EE), and OE. (B) Effects of dienogest (DNG) and progesterone (P_4) on mRNA expression levels of enzymes in OESCs (at least seven separate experiments respectively) and on (C) 17β-hydroxysteroid dehydrogenase 1 (HSD17β1) activity in OESCs ($n=8$). All assays were performed in triplicate, and data are presented as means±S.E.M. *P* values for the statistical analysis of the mRNA expression of enzymes in SCs in spheroid culture are based on Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure, and effects of drugs on OESC enzyme mRNA expression and activity are based on repeated measures ANOVA followed by multiple comparisons using Dunnett's procedure. ND, not detectable; *$P<0.05$ and **$P<0.01$ versus control.

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