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Bradykinin system is involved in endometriosis-related pain through endothelin-1 production

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Conflicts of interest
None declared.

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Abstract

Background: Endometriosis is a gynaecological disease exhibiting severe pelvic pain, but the mechanism of pain production remains unknown. Bradykinin (BK) is known as an inflammatory mediator, and shows elevated levels in inflammatory diseases such as rheumatoid arthritis. In the present study, we evaluated whether BK is involved in endometriosis-related pain.
Methods: Endometriotic lesions were used for immunohistochemistry. Primary cultures of endometriotic stromal cells (ESC) were stimulated with IL-1β and/or BK. Quantitative RT-PCR was used to evaluate the mRNA expressions of BK receptors (BKR) and endothelin-1 in ESC. The concentration of endothelin-1 in cystic fluid of endometrioma or non-endometrioma was measured with ELISA. The conditioned medium of ESC stimulated with IL-1β and/or BK was injected intraplantarly in mice, and evaluated whether pain-related licking behaviour was elicited.
Results: The expressions of BK and BKR in endometriotic lesions were observed by immunohistochemistry. *In vitro* experiments showed that IL-1β induced BKR-B1 and B2 on ESC. Activation of these receptors by BK significantly induced endothelin-1 expression in ESC, which was negated completely by HOE-140, a BKR-B2 antagonist. The cystic fluid of endometrioma contained higher amount of endothelin-1 compared to non-endometrioma. Intraplantar injection of the conditioned medium of ESC treated with IL-1β and BK significantly induced licking behaviour, which was suppressed with BQ-123, an endothelin type-A receptor antagonist.
Conclusions: The present study demonstrated the presence and the function of the BK axis in endometriosis, and established a potential new therapy target for endometriosis-related pain.
Significance: The present study demonstrated (1) the presence and the function of the BK system in endometriosis, (2) activation of BKR induced endothelin-1 in endometriotic lesion and (3) blocking endothelin-1 was effective to decrease pain.

1. Introduction

Endometriosis is a gynaecological disease of which the primary symptoms are infertility and severe pelvic pain, affecting about 10% of women of childbearing age (Giudice and Kao, 2004). Although enormous efforts have been made to unravel its pathological process, the mechanism underlying its pain production remains unknown.

Inflammation has been suggested to play a pivotal role in the pathogenesis and progression of the disease (Harada et al., 2001; Lebovic et al., 2001; Gonzalez-Ramos et al., 2010; Kajihara et al., 2010). Aberrant expression of inflammatory mediators in endometriotic tissue, such as interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1 and prostaglandins (PGs), acts as modulators of the disease (Yoshino et al., 2004, 2011; Hirata et al., 2008). The prevailing medications for pain control are nonsteroidal anti-inflammatory drugs (NSAIDs) and hormone therapy to regulate these inflammatory mediators (Giudice and Kao, 2004; Kajihara et al., 2010). However, NSAIDs have limited response rate and hormone therapy cannot be prescribed to women who desire to get pregnant. Therefore, further exploration on dynamics of pain-producing substances is essential.

Bradykinin (BK), a derivative of kininogen, is one of the most algogenic substances known (Steranka et al., 1988; Linhart et al., 2003). When the tissue is impaired by noxious stimuli, BK is released from the tissue and binds to one of the nociceptive receptors at the nerve terminal (Mizumura et al., 2009). The half-life of BK is known to be very short, 15 s in plasma (Marceau et al., 1998). The activation of BK receptors induces pain through both direct transduction and sensitization of the neuron to the other stimuli (Burgess et al., 2000). BK is also known as an inflammatory mediator, and its receptors show elevated levels in regions of severe injury and inflammatory diseases such as rheumatoid arthritis and asthma (Proud and Kaplan, 1988; Lee et al., 2008). BK acts close to the site of production on a wide variety of cell types, with effects that include smooth muscle contraction, vasodilatation and increased vascular permeability, glandular secretion, and immune cell stimulation (Burgess et al., 2000). Two distinct receptors for kinins, BK receptor B1 and B2 (BKR-B1 and BKR-B2), have been defined, based initially on pharmacological criteria (Farmer and Burch, 1992). Recently, BKR antagonists, such as HOE-140, have been developed as anti-inflammatory agents or inhibitors of the vascular effects of kinins (Cicardi et al., 2010; Charignon et al., 2012). Therefore, BK attracts much attention as a therapeutic target in various diseases (Proud and Kaplan, 1988; Cicardi et al., 2010). Nevertheless, as far as we know, there have been no reports regarding BK and endometriosis using human samples. Further exploration of the role of BK in pain production of endometriosis may give rise to broader therapeutic choices. As a first step towards understanding a possible role of BK in endometriosis, we investigated the presence and the regulation of BK and its receptors in human endometriotic tissues. Especially, we examined the relationship between BK and endothelin-1, which causes neuropathic pain (De-Melo et al., 1998; Jarvis et al., 2000), using *in vivo* and *in vitro* study.

2. Materials and methods

2.1 Agents

Type I collagenase, bradykinin (BK) and antibiotics (mixture of penicillin, streptomycin and amphotericin B) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM/Ham's F12 (DMEM/F12) medium was from Life Technologies (Rockville, MD, USA). HOE-140, a BKR-B2 antagonist, was from Tocris Bioscience (Bristol, UK), and BQ-123, an endothelin-receptor type A antagonist, was from Phoenix Pharmaceutical Inc. (St. Joseph, MO, USA).

2.2 Collection of endometriotic tissues

Tissue specimens were obtained from patients undergoing laparoscopic surgery after obtaining written informed consent under a study protocol approved by the Institutional Review Board of the University of Toyama. The visual analogue scale (VAS) score (0–100) of menstrual pain had been obtained before operation. The patients had not received any hormones or GnRH agonist for at least 3 months before surgery. During laparoscopic surgery, cystic fluid of endometrioma or non-endometriosis cystadenoma was collected from 22 patients (endometriosis; $N = 15$, non-endometriosis; $N = 7$ △ in Fig. 5 and Supporting Information Fig. S1, in detail, three cases of dermoid cyst, two cases of mucinous cyst and two cases of serous cyst) by needle aspiration. Among endometriosis group, five patients harboured exclusively endometrioma ($N = 5$, ○ in Fig. 5 and Supporting Information Fig. S1), while 10 patients possessed endometrioma plus peritoneal or Douglas pouch lesions group ($N = 10$, ● in Fig 5 and Supporting Information Fig. S1). Fluid samples were centrifuged at 400 × *g* for 10 min, and aliquots of the supernatants were stored at −80 °C until assay. Endometriotic tissue samples were obtained from the cyst wall of ovarian endometrioma under sterile conditions and transported to the laboratory in DMEM/F12.

2.3 Isolation and purification of endometriotic stromal cells (ESC)

Primary culture of ESC was conducted as described (Yoshino et al., 2004). Briefly, endometriotic tissue was dissected free of underlying parenchyma, minced into small pieces, incubated in DMEM/F12 with type I collagenase (2.5 mg/mL) and DNase I (15 U/mL) for 1–2 h at 37 °C, and separated using serial filtration. Debris was removed using a 100 μm nylon cell strainer (Becton Dickinson, Lincoln Park, NJ, USA), and dispersed epithelial glands were eliminated with a 70 μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, re-suspended in DMEM/F12 with 10% charcoal-stripped FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (250 ng/mL), plated onto 100-mm dishes (Iwaki, Chiba, Japan). When the cells became confluent after 2 days, they were dissociated with 0.25% trypsin, harvested by centrifugation and replanted in six-well plates at 2×10^5 cells/well. They were kept at 37 °C in a humidified 5% CO_2/95% air environment, until they were grown to confluence over the course of 2 days. Purification of the stromal cell population was confirmed by immunocytochemical staining for the following antibodies: vimentin (stromal cells), cytokeratin (epithelial cells) and CD45 (monocytes and other leukocytes). The purity of the stromal cell was more than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45.

2.4 Treatment of cultured ESC

To evaluate BK receptors mRNA levels, cultured ESC were treated with IL-1β (5 ng/mL Genzyme, Cambridge, MA, USA) or TNF-α (10 ng/mL Genzyme) for 8 h. To evaluate the effects of BK, after pre-treatment of IL-1β for 24 h, BK (0.1–10 μmol/L) was added for up to 24 h. In a pilot study, the optimal concentration of BK was 1 μmol/L. In some experiments, a long-acting BKR-B2 antagonist, HOE-140 (5 μmol/L) was added 1 h before stimulating with BK.

2.5 Reverse transcription (RT) and quantitative real-time PCR analysis

Total RNA was extracted from ESC, using the RNeasy minikit (QIAGEN, Hilden, Germany). RT was performed using Rever Tra Dash (TOYOBO, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time PCR was performed using LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. The PCR primers were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences were as follows: BKR-B1 (NM_000710.3: 326–347 and 495–473), BKR-B2 (NM_000623.3: 432–453 and 605–584), endothelin-1 (NM_000963.3: 614–639 and 918–895) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_002046: 628–648 and 1079–1060). PCR conditions were described elsewhere (Yoshino et al., 2004, 2011; Hirata et al., 2008). After amplification, melting curve analysis was performed. Relative expressions of mRNA were normalized by GAPDH mRNA.

2.6 Animal model of pain assessment

2.6.1 Preparation of conditioning medium

Supernatants obtained from cultured ESC for 48 h were tested for their potential nociceptive effects. ESC were cultured in the various conditions as follows; control: control medium for 48 h, IL-1β: IL-1β (5 ng/mL) for 48 h, BK: control medium for 24 h followed by BK (1 μmol/L) for 24 h, IL-1β+ BK: IL-1β for 24 h followed by adding BK for 24 h (Fig. 4A). As a control, four conditions above mentioned without ESC (cell free) were also prepared.

2.6.2 Behavioural assessment of pain

All experiments were performed using male ICR mice (5-week old). They were kept under controlled temperature (22 ± 1 °C) and humidity (55 ± 10%). The room was lighted from 7:00 a.m. to 7:00 p.m. Food and water were freely available. Procedures for animal experiments were approved by the Committee for Animal Experiments at the University of Toyama. A mouse model was utilized to evaluate pain (Fujita et al., 2008). Briefly, prior to the test, mice were placed in the observation chamber of a transparent acrylic cage for at least 1 h. The test reagents in a volume of 20 μL were injected under the dorsal surface of the plantar surface of the right hind paw. The mice were placed back into the chamber, and were videotaped from underneath for another 1 h. No one stayed in the observation room during this period. Licking of the injected paw is known to be one of the main behavioural responses induced by stimuli (Fujita et al., 2008). The cumulative time spent licking the injected paw was measured by observers without any information concerning the reagents tested. We

assessed and compared the pain induced behaviour by applying the supernatants noted above (Fig. 4A). In some experiments, a selective endothelin-receptor type A (ETR-A) antagonist, BQ-123 (10 nmol/site and in 10 μL) or saline were injected intraplantarly 5 min prior to injection of cultured media into the same paw (Fujita et al., 2008).

2.7 Immunohistochemistry

Tissue specimens of human endometriotic lesions were obtained under signed informed consent from six women who underwent surgery. Tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 6 μm sections were prepared. Antigen retrieval was performed using sodium citrate buffer (10 mmol/L, pH 6.0) (Shi et al., 1993). The sections were stained with BK antibody (1:200, LSBio, Seattle, WA, USA), BKR-B1(1:200, LSBio,), BKR-B2 (1:200, LSBio) or CD 14 (1:50, Becton Dickinson, Tokyo, Japan) using an Envision+ System/HRP rabbit (DAB+) kit (Dako, Tokyo, Japan). Control IgGs were used as a negative control, and specific blocking peptides for each antibody (LSBio, five times of volume of antibody) were used to block BK, BKR-B1 or BKR-B2 antibodies, respectively.

2.8 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of endothelin-1 in the contents of ovarian cysts were measured using ELISA (R&D, Minneapolis, MN, USA). The limit of sensitivity was 0.3 pg/mL. The intra- and inter-assay coefficients of variation were <5%, in the assays.

2.9 Statistical analysis

All experiments were repeated at least three times. Non-normally distributed data were analysed by nonparametric tests (Kruskal–Wallis one-way analysis of variance and Mann–Whitney test) using JMP software (SAS Institute Inc., Cary, NC, USA). A p-value of <0.05 was considered statistically significant.

3. Results

3.1 Localization of BK and its receptors in endometriotic lesions

The expression of BK and its receptors in ovarian endometrioma was examined via immunohistochemistry. In ovarian endometrioma (Fig. 1A) and peritoneal endometriotic lesions (Fig. 1B), BK was strongly detected at cytoplasm of both epithelial and stromal cells. BK receptors, B1 and B2 (BKR-B1 and B2), were also detected in both epithelial and stromal cells of endometriotic lesions. The localization of BKR-B1 was nucleus mainly, whereas BKR-B2 was detected at cytoplasm. These immune-reactive signals were abolished with antibodies which were pre-incubated with each blocking peptide (Fig 1A-b, d, f). The localization of macrophage, dominant immune cells in endometriotic lesions (Cirkel et al., 1993), was shown by staining CD14 (Fig 1A-g, arrow).

3.2 The regulation of BK receptors mRNAs in ESC by IL-1β or TNF-α

IL-1β (5 ng/mL) or TNF-α (10 ng/mL) significantly increased both BKR-B1 and B2 mRNAs (Fig. 2). As IL-1β induced receptor expression more robustly than TNF-α, IL-1β was utilized for subsequent experiments to induce BKR-B1 and B2.

As shown in Fig. 3, neither IL-1β nor BK solely induced endothelin-1 mRNA expression, while with pre-treatment of IL-1β, BK did induce endothelin-1 mRNA expression significantly compared to controls ($p < 0.01$). Same tendency was observed at protein levels in cultured supernatant (Fig. 4B). The up-regulation of endothelin-1 mRNA by IL-1β with BK was completely abrogated by HOE-140, a BKR-B2 antagonist (Fig. 3, $p < 0.01$).

3.3 Assessment of pain induced by conditioned medium of ESC

The writhing behaviour for the evaluation of visceral pain in animals is often used. However, writhing behaviours cannot be developed without strong stimulation, such as acetic acid (>1%). In this study, therefore, we evaluated whether the conditioned medium of ESC elicited pain as the index of pain-related licking behaviour induced by intraplantar injection of algogenic substances (Fujita et al., 2008). The conditioned medium of ESC was inoculated to mouse paw, and the cumulative time spent licking the injected paw was measured for 1 h. The conditions were as follows: control, IL-1β, BK and IL-1β with BK (Fig. 4A). The protein levels of endothelin-1 in conditioned medium are shown in Fig. 4B. Consistent with the mRNA data, IL-1β with BK significantly induced endothelin-1 protein levels compared to control. As shown in Fig. 4C, neither the conditioned medium of ESC stimulated with IL-1β alone nor BK alone resulted in significant increased cumulative time of licking compared to the control. With the conditioned medium obtained from IL-1β and BK combination, significantly increased pain behaviour was observed compared to the control

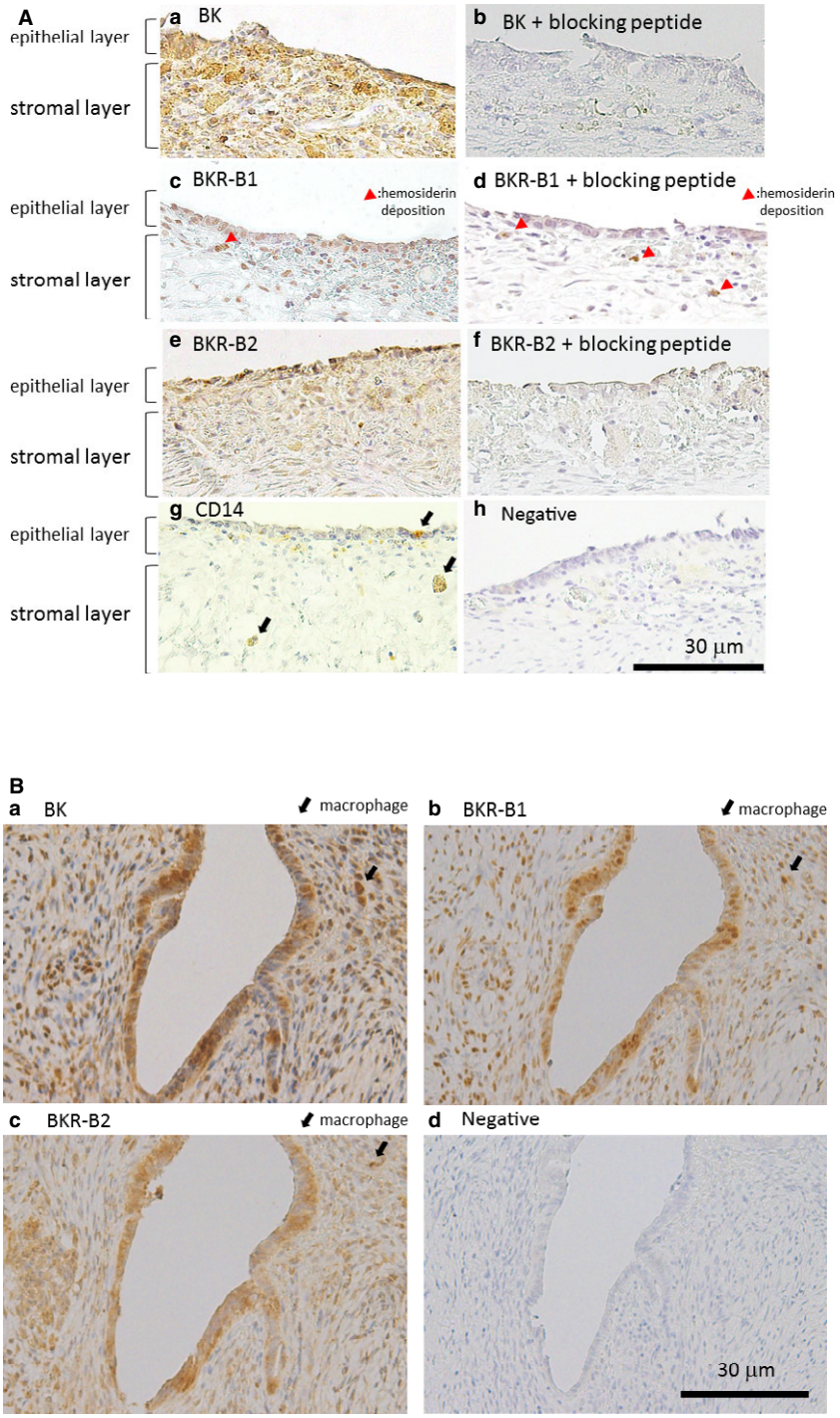

Figure 1 Immunohistochemistry of bradykinin and its receptors in endometriotic lesions. The expression of bradykinin (BK) and its receptors (BKR-B1 and BKR-B2) in endometrioma (A) or peritoneal endometriotic lesions (B) were examined by immunohistochemistory. (A): (a) BK, (c) BKR-B1 and (e) BKR-B2 expression in endometrioma. (b,d,f) Specific blocking peptides for each antibody were used to block first antibodies. Arrow heads indicated hemosiderin deposition. (g) CD14 was used to detect macrophages. Arrow indicated macrophages. (h) negative; control IgG was used. (B): (a) BK, (b) BKR-B1 and (c) BKR-B2 expression in peritoneal endometriotic lesions. (d) negative; control IgG was used. Arrow indicated macrophages. Representative data from six patients were shown.

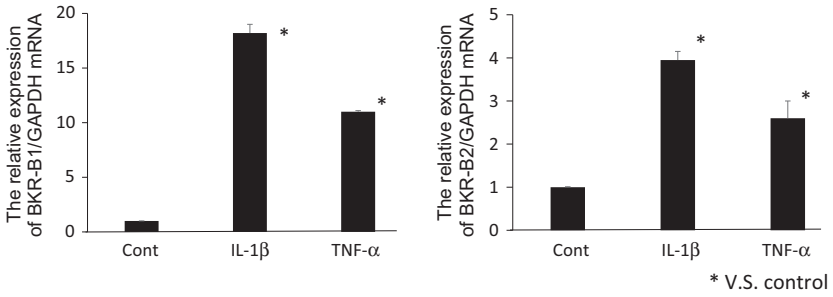

Figure 2 The modulator of bradykinin receptor in endometriotic stromal cells (ESC). After 8 h stimuli of IL-1β (5 ng/mL) or TNF-α (10 ng/mL), the expression of bradykinin receptors (BKR-B1 and BKR-B2) in ESC was examined with real-time RT-PCR. The data were normalized by GAPDH mRNA levels to show the relative abundance. Representative data from five different experiments were shown as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the each control. *$p < 0.01$ (vs. control).

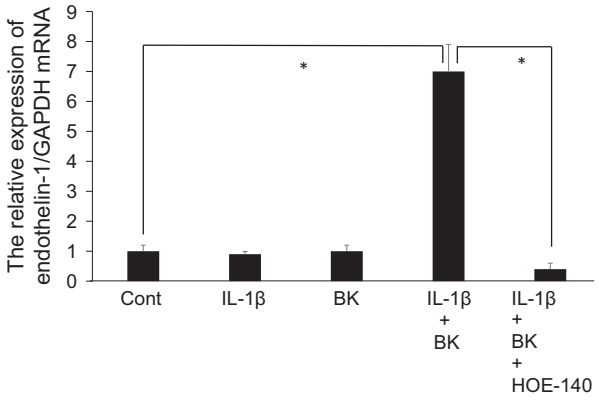

Figure 3 The activation of endometriotic stromal cells by bradykinin. After pre-treatment of IL-1β (5 ng/mL) for 24 h, bradykinin (BK, 1 μmol/L) was added for another 8 h followed by real-time RT-PCR for endothelin-1 mRNA. One hour before adding BK, HOE-140 (5 μmol/L), an antagonist for BK receptor B2 or control (DMSO) were added. The endothelin-1 mRNA data were normalized by GAPDH mRNA levels to show the relative abundance. Representative data from three different experiments were shown as the mean ± SD relative to an adjusted value of 1.0 for the mean value of the control. *$p < 0.01$.

($p < 0.01$), which was significantly reduced when the mice were pre-treated with a selective endothelin type-A receptor antagonist, BQ-123, at a dose of 10 nmol/site ($p < 0.05$). When cell-free conditioned media, which had been subjected to the same culture conditions described above without ESC, were tested, there was no difference in licking time between groups and the median licking time was 33 s (data not shown).

3.4 The concentration of endothelin-1 in cystic fluid of endometriomas

Endothelin-1 concentrations in cystic fluid of endometriomas and non-endometriomas were measured using ELISA (Fig. 5). In non-endometriomas (△ in Fig. 5), only one case out of seven patients showed detectable levels of endothelin-1, while 14 out of 15 samples of endometrioma exhibited detectable levels. The medial concentrations were 0 pg/mL in non-endometrioma (range; <0.3–10 pg/mL) and 22 pg/mL in endometrioma (range; <0.3–324 pg/mL), which is significantly different ($p < 0.01$). When endometrioma groups were divided into exclusively endometrioma case group ($N = 5$, ○ in Fig. 5) and endometrioma plus peritoneal or Douglas pouch lesions group ($N = 10$, ● in Fig. 5), there was no difference in endothelin-1 concentration.

We investigated the relationship between pain score and endothelin-1 concentration in cystic fluid of patients. The visual analogue scale (VAS; range 0–100) of menstrual pain of 15 endometrioma patients (median 53 (15–90)) was significantly higher than that of seven non-endometriosis cystadenoma patients (median 20 (0–60), $p < 0.05$). The relevance between VAS score of menstrual pain and endothelin-1 concentration among all subjects (△: non-non-endometrioma, ○: exclusively endometrioma case and ●: endometrioma plus peritoneal or Douglas pouch lesions) were examined in Supporting Information Fig. S1. There was no correlation between VAS score and endothelin-1 concentration in ovarian cystic fluid ($p = 0.98$).

4. Discussion

We reported for the first time that endometriotic lesions contained BK, which is one of the most algogenic substances (Linhart et al., 2003). As a high density of nerve fibre infiltration is observed in endometriotic lesions, including adenomyotic

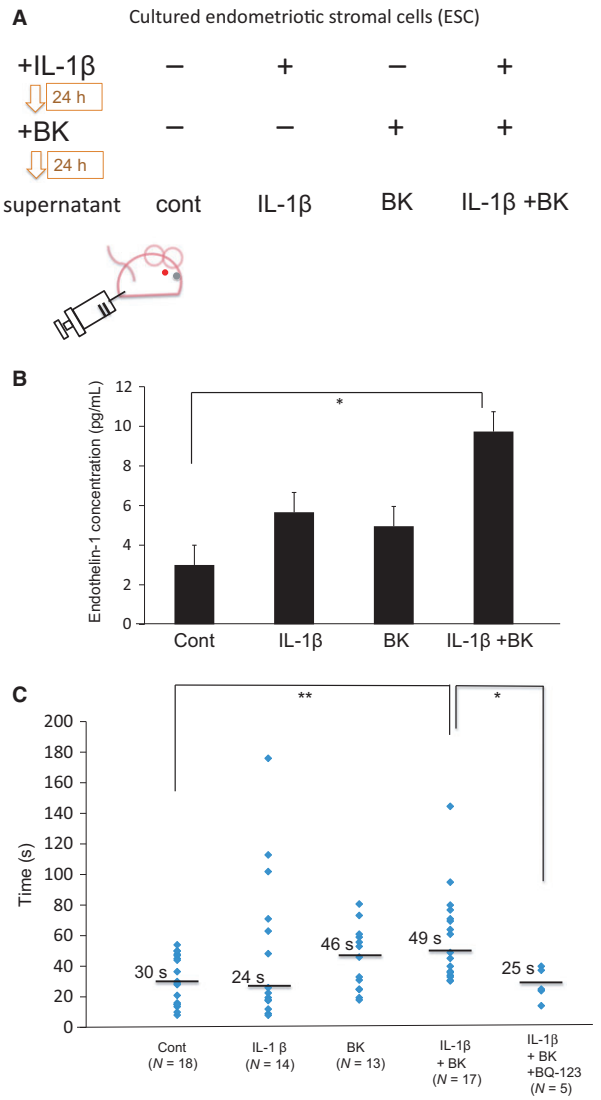

Figure 4 Assessment of pain induced by supernatant of endometriotic stromal cells (ESC). (A) ESC were cultured for 48 h in the various conditions as follows: control: control medium for 48 h, IL-1β: IL-1β (5 ng/mL) for 48 h, bradykinin (BK): control medium for 24 h followed by BK (1 μmol/L) for 24 h, IL-1β+ BK: IL-1β for 24 h followed by adding BK for 24 h. The conditioned medium was inoculated to mouse paw to assess pain-related behaviour. (B) The concentrations of endothelin-1 in cultured supernatant were measured by ELISA. Representative data from four different experiments were shown as the mean ± SD. (C) The supernatant of cultured ESC were inoculated to mouse paw, the cumulative time spent for licking the injected paw was measured for 1 h. BQ-123, a selective endothelin-receptor type A antagonist (10 nmol/site), or control (saline) were injected intraplantarly 5 min before inoculation of ESC conditioned medium. Median data (seconds) were shown, and the numbers in parenthesis are mice tested. *$p < 0.05$, **$p < 0.01$.

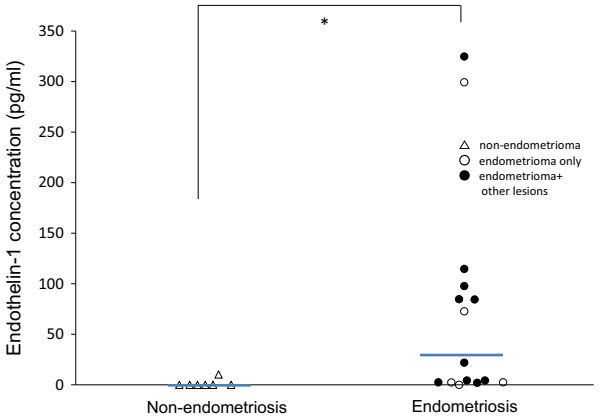

Figure 5 The concentration of endothelin-1 in human ovarian cysts. Endothelin-1 concentrations in content fluid of 15 endometriomas (○: $N = 5$, exclusively endometrioma cases, ●: $N = 10$, endometrioma plus peritoneal or Douglas pouch lesions group) and seven non-endometriomas (△) were measured using ELISA. *$p < 0.01$ between two groups. Bar indicates median data, 0 pg/mL in non-endometrioma, and 22 pg/mL in endometrioma.

nodules, peritoneal and ovarian endometriosis (Anaf et al., 2002; Zhang et al., 2010), BK derived from endometriotic lesions may provoke activation of neurons directly as an algogenic substance.

BK is also known as a chemical mediator via activation of BK receptors, BKR-B1 and BKR-B2. BKR-B1 has higher affinity for the metabolites desArg^{9}bradykinin and desArg^{10}kallidin, whereas BKR-B2 has higher affinity for the ligands BK and kallidin (Burgess et al., 2000). The expression levels of these receptors were elevated in regions of severe injury and inflammatory diseases such as rheumatoid arthritis, asthma and hereditary angioedema (Proud and Kaplan, 1988). Jingwei et al. (2015) reported that using mouse endometriosis model, the control of bradykinin by herbal medicine leads to inhibit the development of endometriosis and relieve dysmenorrhea. As far as we searched, there have been no studies regarding bradykinin system in human endometriosis. Although macrophages, which are dominant immune cells in endometriotic lesion (Cirkel et al., 1993) were detected (Fig. 1A-g), the proportion of macrophages was less than that of endometriotic epithelial and stromal cells. Therefore, we concluded that endometriotic epithelial and stromal cells expressed BK, BKR-B1 and BKR-B2. In the present study, we demonstrated that BKR-B1 was found at nucleus mainly, whereas BKR-B2 was detected at cytoplasm in endometriotic lesions in patients. Recently, bradykinin receptor is also

expressed in the nucleus (Takano and Matsuyama, 2014). It is also reported that BKR-B2, which is activated by BK, is constitutively expressed. In contrast, the BKR-B1 is normally absent but is induced under inflammatory conditions (Marceau and Regoli, 2004). In robust inflammatory condition, such as ovarian abscess, BKR-B1 might be also induced at cytoplasm strongly in endometrioma. Further study is needed to prove this notion.

Additionally, we evaluated the regulation and function of these receptors in ESC. We found for the first time that IL-1β or TNF-α, which are involved in the pathophysiology of endometriosis (Harada et al., 2001; Lebovic et al., 2001), increased both BKR-B1 and BKR-B2. After pre-treatment of IL-1β for 24 h, induced BK receptor was proved to be functional, as the findings that upon addition of BK, endothelin-1 was up-regulated, and the increase in endothelin-1 was completely abrogated with HOE-140, a BKR-B2 antagonist. BK solely failed to induce endothelin-1 *in vitro* without priming of IL-1β, (Figs 3 and 4B), indicating the importance of up-regulation of BK receptor in endothelin-1 production. In fact, we confirmed that endometriomas, in which BK and BK receptors were expressed, contained much higher levels of endothelin-1 compared to non-endometriomas (Fig. 5), suggesting that BK might induce endothelin-1 production in endometriotic lesions.

Endothelin-1, a potent vasoconstrictor, has high binding affinity for two receptor subtypes, endothelin receptor (ETR)-A and ETR-B (Yanagisawa et al., 1988), and exerts various biological actions including inflammatory and neuropathic pain (De-Melo et al., 1998; Jarvis et al., 2000). Notably, activation of ETR-A by endothelin-1 is involved in inducing allodynia and spontaneous pain in neuropathy or cancer (Jarvis et al., 2000; Fujita et al., 2008). Although it is reported that endometriotic lesions express endothelin-1 (Chen et al., 2009), the regulation and its function have not been investigated. As far as we searched, our present study tried to reveal the involvement of endothelin-1 in endometriosis-related pain for the first time. To investigate whether endothelin-1, which were induced with BK, provoke pain *in vivo*, the conditioned medium of ESC was inoculated to mouse paw. A combination of IL-1β and BK resulted in the highest levels of endothelin-1 production in ESC, and its supernatant significantly potentiated the pain behaviour compared to controls (Fig. 4C). Importantly, BQ-123, an ETR-A antagonist suppressed the prolonged pain behaviour time induced with the conditioned medium of IL-1β and BK combination, suggesting the importance of endothelin-1 in inducing pain. As the half-life of BK is very short, 15 s in plasma (Marceau et al., 1998), our present study did not examine the direct effect of BK in provoking pain, but examined the role of BK as a chemical mediator. In this study, the intraplantar injection of the conditioned medium (20 μL) of ESC treated with

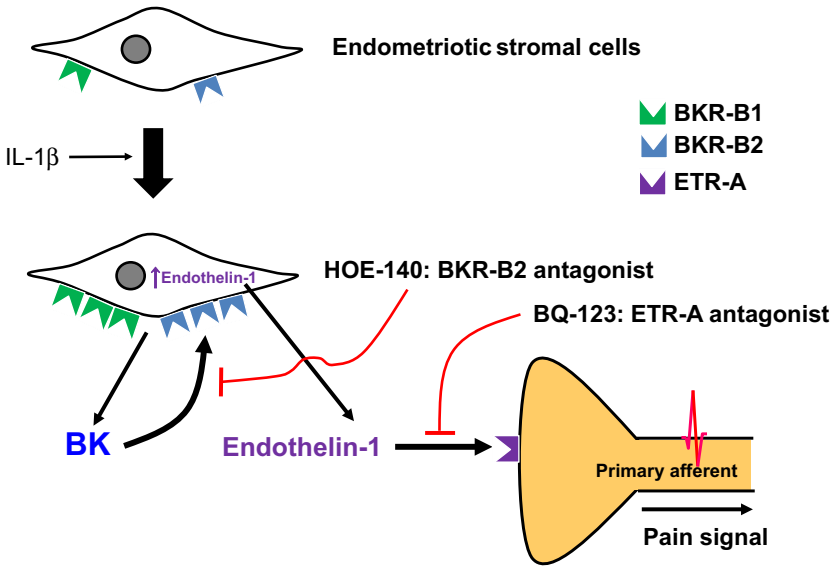

Figure 6 Schema of the hypothesis. IL-1β induced bradykinin receptors (BKR) in endometriotic lesions. The activation of BKR by bradykinin (BK) augmented the production of endothelin-1, which potentiated pain signals in neurons. HOE-140, a BKR-B2 antagonist suppressed endothelin-1 production in endometriotic stromal cells. BQ-123, an endothelin type A receptor (ETR-A) antagonist, suppressed pain by antagonizing endothelin-1 in neurons.

BK (1 μmol/L = 1 nmol/20 μL) did not induce licking in mice. An intraplantar injection of BK (1 nmol/20 μL) also does not elicit licking in mice (Fujita et al., 2008). Therefore, at least, BK in the conditioned medium may not be involved in the induction of pain under the condition of this study.

In the present study, endometriosis patient group showed higher VAS score of menstrual pain and endotheolin-1 concentration in ovarian cysts compared to non-endometriosis group (Fig. 5 and Supporting Information Fig. S1). As endothelin-1 can induce allodynia and spontaneous pain (Jarvis et al., 2000; Fujita et al., 2008), endothelin-1 derived from endometrioma might cause pain. When endometrioma groups were divided into exclusively endometrioma case group ($N = 5$, ○ in Fig. 5 and Supporting Information Fig. S1) and endometrioma plus peritoneal or Douglas pouch lesions group ($N = 10$, ●), the latter (●) showed higher levels of VAS score (median 60 (20–90)) compared to exclusively endometrioma case group (○, median 30 (15–66)) significantly ($p < 0.05$, Supporting Information Fig. S1). But there was no difference in endothelin-1 concentration in endometrioma between groups (Fig. 5 and Supporting Information Fig. S1). These data suggested that pelvic pain is stemmed from not only endometrioma, but also from other lesions including peritoneal or Douglas pouch endometriotic lesion and uterus. Previously, the concentration of IL-6 or TNF-α in peritoneal fluid is correlated to endometriosis-related pain (Velasco et al., 2010, Scholl et al., 2009). Further study is needed to evaluate endothelin-1 concentration in peritoneal fluid at menstrual period.

In summary (shown in Fig. 6), we demonstrated for the first time the role of the BK system in endometriotic lesions to induce endothelin-1, which caused pain. Moreover, we showed that BK receptor antagonist and endothelin-1 receptor antagonist could be candidates for the management of endometriosis-related pain. In fact, both drugs are in clinical use for hereditary angioedema (Cicardi et al., 2010) and pulmonary arterial hypertension (Kuntz et al., 2016), respectively. Further study is warranted to examine the effects and side-effects of these drugs for women exhibiting endometriosis-related pain.

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Authors contribution

Osamu Yoshino; study design, execution, analysis, manuscript drafting and critical discussion. Kaori Yamada-Nomoto: execution. Mutsumi Kobayashi: execution. Tsugunobu Andoh: execution, critical discussion. Manami Hongo: execution. Yosuke Ono: execution. Akiko Hasegawa-Idemitsu: execution. Akiho Sakai: execution. Yutaka Osuga: critical discussion. Shigeru Saito: manuscript drafting, critical discussion.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. The relationship between endothelin-1 concentration in human ovarian cysts and pain score.