ORIGINAL

Intraperitoneal administration of activin A promotes development of endometriotic lesions in a mouse model of endometriosis

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Abstract : PURPOSE : This study aimed to investigate the effect of intraperitoneal administration of activin on the occurrence of endometriosis using a mouse model of endometriosis. METHODS : A mouse model of endometriosis was prepared by intraperitoneally administering endometrial tissue and blood collected from donor mice to C57BL/6J 7-8- week-old recipient mice. A total of 400 μg of activin A was intraperitoneally administered to model mice in the activin group for 5 days. Intraperitoneal endometriotic lesions were confirmed macroscopically and IL-6 and TNF-α levels in washed ascites were measured by ELISA. RESULTS : Endometriotic lesions were observed in all mice. In the activin group, the maximum diameter of endometriotic lesions was significantly larger than that in control group (4.7± 1.3 vs 2.9± 0.9 mm, $p< 0.01$). The total area of the lesion was also significantly higher in the activin group than in the control group (21.1± 9.9 vs 8.8± 5.4 mm^2, $p< 0.01$). Furthermore, IL-6 and TNF-α levels in ascites were significantly higher in the activin group than in the control group (IL-6 : 85.8± 15.3 vs 75.1± 19.3 pg/ml, $p< 0.05$; TNF-α : 629.8± 15.4 vs 605.9± 11.4 pg/ml, $p< 0.05$). CONCLUSION : Activin promotes occurrence of endometriosis. Inflammatory cytokines are also elevated by activin administration, suggesting that they may contribute to progression of endometriosis J. Med. Invest. 66 : 123-127, February, 2019

Keywords **:** *Endometriosis, Activin A, Mouse model, Inflammatory cytokine*

INTRODUCTION

Endometriosis affects 6%-10% of reproductive age women (1). The main symptoms of endometriosis are dysmenorrhea and infertility, and both significantly impair a woman's quality of life. Endometriosis is characterized by the presence of tissue that is similar to the normal endometrium at sites outside of the uterus. Although the mechanism of pathogenesis has not been identified for endometriosis, transplantation of the endometrium due to menstrual blood reflux (2-4) and metaplasia are proposed (5, 6). In particular, the idea that inflammation in the peritoneal cavity is involved in the onset of proliferation of endometriosis is most influential in the transplantation theory (4,7).

Activins are members of the transforming growth factor-β (TGF-β) superfamily, forming a subfamily of dimer. Activin is found in follicular fluid as a factor promoting secretion of follicle-stimulating hormone in the pituitary (8). However, studies have reported various effects of activin, such as embryogenesis (9), neuroprotection (10), cell apoptosis (11, 12) and fibrosis (13). Activin A is produced, while immune system cells, such as monocytes, macrophages, and lymphocytes, are activated, and it is an important mediator in the process of inflammation (14-16).

Therefore, activin is likely to participate in the occurrence and proliferation of endometriosis. In this study, we investigated whether administration of activin A in a mouse model of endometriosis promotes occurrence and proliferation of endometriosis. We examined inflammatory cytokine levels in ascitic fluid. We investigated the mechanism of occurrence of endometriosis by confirming the difference and presence or absence of signal transduction.

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MATERIALS AND METHODS

1. Animals

Eight-week-old female C57BL/6J mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and CLEA Japan, Inc. (Tokyo, Japan). The mice were fed on a mouse diet and water ad libitum and kept in a light/dark cycle of 12/12h under controlled conditions. Before any invasive procedure, the mice were anesthetized with sevoflurane.

The surgical technique was performed under sterile conditions. This study was approved by the Committee of the Institute of Animal Experimentation of Tokushima Graduate School.

2. Treatment

Donor female C57BL/6J mice were ovariectomized on day -7 and injected subcutaneously with estrogen (β-estoradiol ; Sigma-Aldrich, MO, USA) in peanut oil (0.2 μg/mouse/day) daily. On day 0, donor mice were euthanized and their uterus was removed. The endometrium was gently peeled from the uterine muscle and cut into small pieces (approximately 1 mm in diameter) in 0.2 ml of phosphate-buffered saline (PBS) with small scissors. Endometrial fragments in PBS and 0.1 ml of blood from donor mice were injected into the peritoneal cavity of recipient mice. For 5 days, recipient mice were treated with an intraperitoneal injection of activin A (R&D, MN, USA, n=10 ; 400 ng/mouse) or vehicle (n=10, PBS) every day.

3. Evaluation of murine endometriotic lesions

On day 5, recipient mice were euthanized. The peritoneal cavity of

each mouse was inspected. Endometriotic lesions were measured with a max diameter and the total area of lesions per mouse. Lesions were removed and fixed in 4% paraformaldehyde and embedded in paraffin.

4. Measurement of interluekin-6 and tumor necrosis factor-α levels

Peritoneal lavage was performed upon infusion of 1 ml PBS into the peritoneal cavity of the mice. The fluid was removed and centrifuged at 1000×g for 20 min, and aliquots of the supernatants were stored at -20℃ until assay. Concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by a mouse IL-6 ELISA Kit (Cloud-Clone, TX, USA) and a mouse TNF-α ELISA Ki t(Cloud-Clone) .

5. Immunohistochemical staining

Briefly, after deparaffinization and rehydration, antigen retrieval was performed by Antigen Unmasking Solution (VEC, CA, USA) with application of a pressure cooker for 5 min. Endogenous peroxidase activity was inhibited with 3% H_2O_2 for 15 min and nonspecific biding was blocked with 10% normal horse serum for 20 min. All sections were incubated with primary antibodies for estrogen receptor alpha (1 : 2000, Abcam, Cambridge, UK) and pSmad3L (Thr 179) / pSmad2L (Thr220) (1 : 20, IBL, Takasaki, Japan) for 30 min at room temperature. Sections were then incubated with ImmPRESS Reagent Anti-Rabbit Ig (VEC) for 30 min. Visualization of the antigens was achieved by diaminobenzidine for 2.5 min. Finally, the slides were counterstained with hematoxylin QS (VEC) for 30 s, dehydrated, and mounted. Negative control slides were incubated similarly, but the primary antibody was replaced with PBS.

6. Statistical analysis

Statistical analysis for comparing treatment groups was performed by the non-parametric Mann Whitney U test. A p value of less than 0.05 was considered to indicate statistical significance. All statistical analysis was carried out using R version 3.4.2 (R Core Team (2017). R : A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http : // www.r-project.org/).

RESULTS

Endometriotic lesions grew in the abdominal cavities of all mice (Fig. 1A, B). Most lesions occurred around the peritoneal incision and the intestinal membrane. We found I found erythrocytes and macrophages inside the cyst. Expression of estrogen receptor was confirmed in epithelial and stromal cells in the lesions (Fig. 1C).

The maximum diameter of lesions in the activin group (4.7± 1.3 mm) was significantly larger than that in the control group (2.9± 0.9 mm, $p< 0.01$) (Fig. 2A). The total area of lesions in the activin group (21.1± 9.9 mm^2) was significantly larger that in the control group (8.8± 5.4 mm^2, $p< 0.01$) (Fig. 2B).

IL-6 levels in the activin group (85.8± 15.3 pg/ml) were significantly higher than those in the control group (75.1± 19.3 pg/ml, $p< 0.05$) (Fig. 3A). TNF-α levels in the activin group (629.8± 15.4 pg/ml) were also significantly higher than those in the control group (605.9± 11.4 pg/ml, $p< 0.05$) (Fig. 3B).

In immunohistochemical analysis, expression of pSmad2/3 was observed in epithelial and stromal cells in lesions of in the activin group (Fig. 4).

DISCUSSION

The menstrual blood reflux theory is widely supported as the mechanism of the pathogenesis of endometriosis (2-4). However, 75%-99% of women have reflux of menstrual blood, but not all women develop endometriosis (4). Therefore, in addition to regurgitation of menstrual blood, some abnormalities in the abdominal cavity and the endometrium may contribute to development of

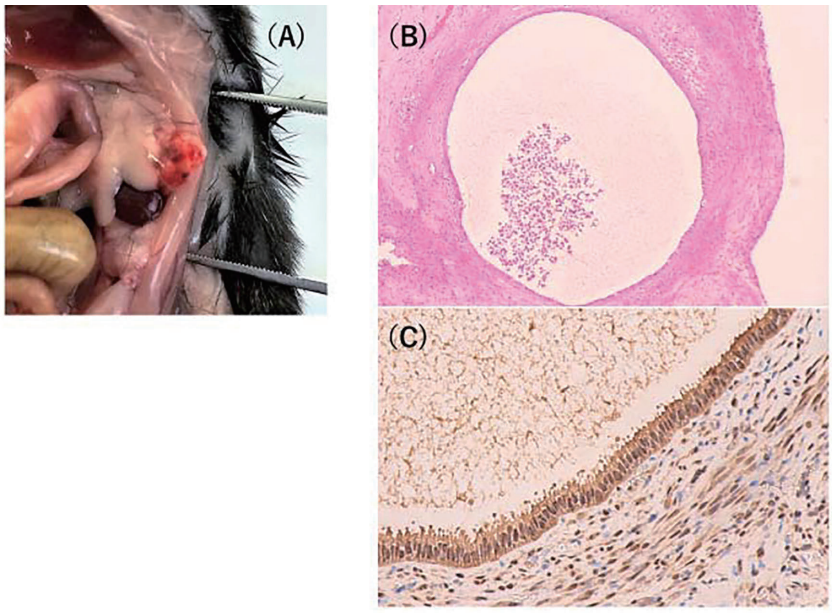

Fig. 1
Photograph of an endometriotic lesion (A). A lesion was stained with hematoxylin-eosin.
Original magnification, ×200 (B). An endometriotic lesion as assessed by immunohistochemical analysis with estrogen receptor alpha. Original magnification, ×400 (C).

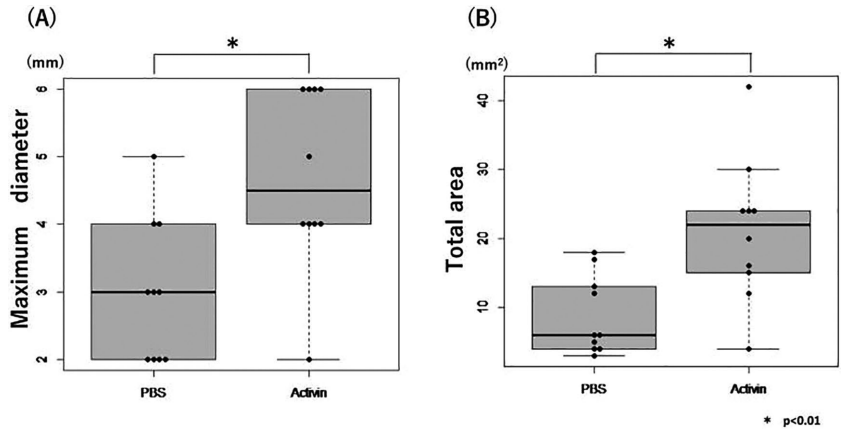

Fig. 2
Maximum diameter (A) and area (B) of lesions in a mouse model of endometriosis.

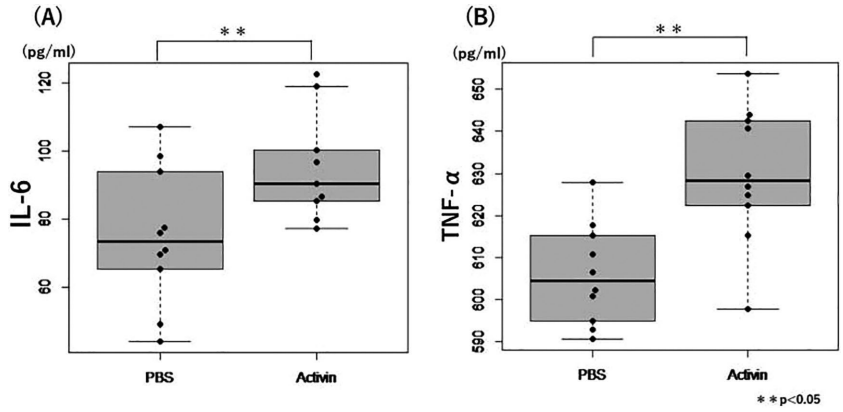

Fig.3
IL-6 levels (A), and TNF-α levels (B) in peritoneal lavage

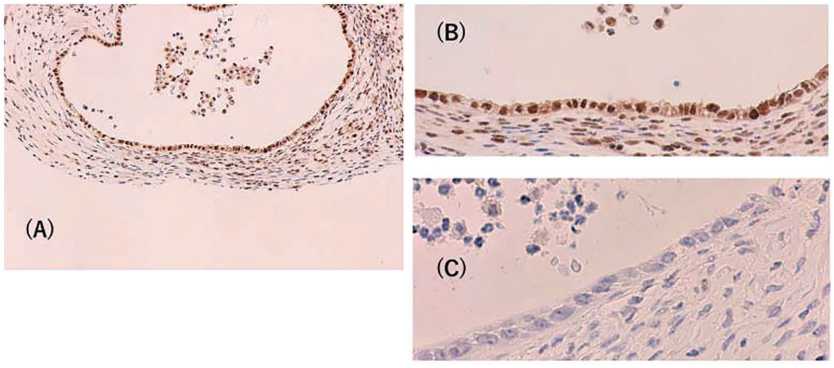

Fig.4
An endometriotic lesion as assessed by immunohistochemical analysis with pSmad 2/3 (A, B) and negative control (C). Original magnification, ×200 (A) and ×400 (B, C).

endometriosis. Inflammation in the abdominal cavity is also important. There have been numerous reports on the relationship between endometriosis and the inflammatory response. Hills *et al.* reported that leukocytes, macrophages, helper T cells, and natural killer cells were markedly increased in the ascites of patients with endometriosis (17). Furthermore, many inflammatory cytokines, such as IL-8 (18), IL-6 (19), and TGF-β (20), were found in the ascites of patients with endometriosis.

We investigated the occurrence and proliferation of the inflammatory environment and endometriosis in the abdominal cavity.

Azuma *et al*. and we showed that intraperitoneal administration of lipopolysaccharide (LPS) promoted proliferation of endometriotic lesions in a model mouse of endometriosis (21, 22). Our mouse model was created by implanting intrauterine tissue pieces and blood into the peritoneal cavity for mimicking the reflux of menstrual blood (23). Our study showed that LPS induced an inflammatory response in the peritoneal cavity, thereby promoting proliferation of endometriotic lesion (22).

Activin A was found in follicular fluid as a factor that promotes follicle-stimulating hormone secretion from the pituitary gland in 1986 (8). Expression of activin A was later found in all cells of the body, and its action is diverse. Activin A has mesenchymal-inducing activity, it induces differentiation of various cells, and has proliferative abilities in cells, and many physiological activities (9-13). Activin A is expressed in follicular granulosa cells and corpus luteum in the reproductive area, and regulation of follicular development is thought to be regulated by autocrine and paracrine effects (24).

Many roles of activin in inflammation have been reported. Experiments in a rat model of embryonic fibrosis using bleomycin suggested that activin acts to promote inflammation and fibrosis (25). Activin A is also produced during activation of immune system cells, such as monocytes, macrophages, and lymphocytes, and is an important mediator in the process of inflammation (14-16).

Several reports have suggested that activin is involved endometriosis. Mabuchi *et al*. reported that activin A is produced in normal endometrium and endometrial cysts and that a signal transduction system is present (26). Rombauts *et al*. also reported that secretion of activin A in the eutopic endometrium is increased in patients with endometriosis compared with non-endometriosis patients (27). Yoshino *et al*. reported that addition of activin A to endometrial stromal cells increased IL-6 and PAR 2 mRNA expression and promoted cellular endometrial stromal cell proliferation (28). In this study, we found that intraperitoneal administration of activin A increased endometriotic lesions in mice. For the mechanism of this process, initially, activin triggers inflammation, which results in promotion of proliferation of endometriotic lesions. Furthermore, more inflammatory cytokines are present in the ascites of patients with endometriosis (18, 19). Our finding that IL-6 and TNF-α levels in ascites in the activin group were elevated supports previous findings. Activin induces inflammatory cytokines, and thus inflammation is caused by administration of activin (16, 28). Our experimental results also support this finding.

As a different pathway from inflammation, differentiation-inducing action of activin may be related to occurrence of endometriosis. We found phosphorylated Smad 2/3 expression, which is a signaling factor of activin, in endometriotic lesions in a model mouse of endometriosis. This finding indicated that activin activated this signaling pathway. Activin may be directly involved in differentiation and proliferation of endometriotic tissue, but its mechanism has not been proved.

Our study shows that activin A promotes proliferation of endometriosis. Because there is a large amount of activin in follicular fluid, activin is scattered near the uterus and ovaries by ovulation and It is suggested that it may be related to proliferation of endometriosis.

CONFLICT OF INTERESTS

None of the authors have any conflicts of interest associated with this study.

REFERENCE

1. Eskenazi B, Warner ML : Epidemiology of endometriosis. Obstet Gynecol Clin North Am 24 : 235-258, 1997
2. Sampson J : Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 14 : 433-69, 1927
3. Bartosik D, Jacobs SL, Kelly LJ : Endometrial tissue in peritoneal fluid. Fertil Steril 46 : 796-800, 1986
4. D'Hooghe TM, Debrock S : Endometriosis, retrograde menstruation and peritoneal inflammation in women and in baboons. Hum Reprod Update 8 : 84-88, 2002
5. Lauchlan SC : The secondary Müllerian system. Obstet Gynecol Surv 27 : 133-146, 1972
6. Taketani Y, Mizuno M : Evidence for direct regulation of epidermal growth factor receptors by steroid hormones in human endometrial cells. Hum Reprod 6 : 1365-1369, 1991
7. Malutan AM, Drugan T, Costin N, Ciortea R, Bucuri C, Rada MP, Mihu D : Pro-inflammatory cytokines for evaluation of inflammatory status in endometriosis. Centr Eur J Immunol. 40 : 96-102, 2015
8. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R : Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. Nature 321 : 779-782, 1986
9. Hemmati-Brivanlou A, Melton DA : A truncated activin receptor inhibits mesoderm induction and formation of axial structures in Xenopus embryos. Nature 15 : 609-614, 1992
10. Tretter YP, Munz B, Hübner G, Bruggencate G, Werner S, Alzheimer C : Strong induction of activin expression after hippocampal lesion. Neuroreport 29 : 1819-1823, 1996.
11. Chen W, Woodruff TK, Mayo KE : Activin A-induced HepG2 liver cell apoptosis : involvement of activin receptors and smad proteins. Endocrinology 141 : 1263-1272, 2000
12. Carey JL, Sasur LM, Kawakubo H, Gupta V, Christian B, Bailey PM, Maheswaran S : Mutually antagonistic effects of androgen and activin in the regulation of prostate cancer cell growth. Mol Endocrinol 18 : 696-707, 2004
13. Werner S, Alzheimer C : Roles of activin in tissue repair, fibrosis, and inflammatory disease. Cytokine Growth Factor Rev 17 : 157-171, 2006
14. Eramaa M, Hurme M, Stenman UH, Ritvos O : Activin A/erythroid differentiation factor is induced during human monocyte activation. J Exp Med 176 : 1449-1452, 1992
15. Ebert S, Zeretzke M, Nau R, Michel U : Microglial cells and peritoneal macrophages release activin A upon stimulation with Toll-like receptor agonists. Neurosci Lett 21 : 241-244, 2007
16. Jones KL, Mansell A, Patella S, Scott BJ, Hedger MP, Kretser DM, Phillips DJ : Activin A is a critical component of the inflammatory response, and its binding protein, follistatin, reduces mortality in endotoxemia. PNAS 104 : 16239-1644, 2007
17. Hill JA, Faris HM, Schiff I, Anderson DJ : Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. Fertil Steril 50 : 216-222, 1988
18. Harada T, Iwabe T, Terakawa N : Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. Am J Obstet Gynecol 176 : 593-597, 1997
19. Iwabe T, Harada T, Tsudo T, Tanikawa M, Onohara Y, Terakawa N : Pathogenetic significance of increased level of interleukin-8 in peritoneal fluid of patients with endometriosis. Fretil Steril 69 : 924-930, 1998
20. Oosterlynck DJ, Meuleman C, Waer M, Koninckx PR : Transforming growth factor-beta activity is increased in peritoneal fluid from women with endometriosis. Obstet

Gynecol 83 : 287-292, 1994
21. Azuma Y, Taniguchi F, Nakamura K, Nagira K, Yin Mon Khine, Kiyama T, Uegaki T, Izawa M, Harada T : Lipopolysaccharide promotes the development of murine endometriosis-like lesions via the nuclear factor-kappa B pathway. Am J Reprod Immunol 77 : e12631, 2017
22. Keyama K, Kato T, Kadota Y, Otgontsetseg Erdenebayar, Kasai K, Kawakita T, Tani A, Matsui S, Iwasa T, Yoshida K, Maegawa M, Kuwahara A, Matsuzaki T, Irahara M : Lipopolysaccharide promotes early endometrial-peritoneal interactions in a mouse model of endometriosis. J Med Invest 66 : 2019
23. Kobayashi A, Maegawa M, Yamamoto S, Ugumori N, Kasai Y, Tani A, Uemura H, Kuwahara A, Matsuzaki T, Yasui T, Furumoto H, Kamada M, Irahara M : The role of blood in early endometrial-peritoneal interactions in a syngeneic mouse model of endometriosis. Reprod Med Biol 10 : 15-20, 2010
24. Miró F, Hillier SG : Relative effects of activin and inhibin on steroid hormone synthesis in primate granulosa cells. J Clin Endocrinol Metab 75 : 1556-1561, 1992
25. Matsuse T, Ikegami A, Ohga E, Hosoi T, Oka T, Kida K, Fukayama M, Inoue A, Nagase T, Ouchi Y, Fukuchi Y : Expression of immunoreactive activin A protein in remodeling lesion associated with interstitial pulmonary fibrosis. Am J Pathol 148 : 707-713, 1996
26. Mabuchi Y, Yamamoto M, Minami S, Umesaki N : Immunohistochemical localization of inhibin and activin subunits, activin receptors and Smads in ovarian endometriosis. Int J Mol Med 25 : 17-23, 2010
27. Rombauts L, Donoghue J, Cann L, Jones RL, Healy DL : Activin-A secretion is increased in the eutopic endometrium from women with endometriosis. Aust N Z J Obstet Gynaecol 46 : 148-153, 2006
28. Yoshino O, Izumi G, Shi J, Osuga Y, Hirota Y, Hirata T, Harada M, Nishii O, Koga K, Taketani Y : Activin-A is induced by interleukin-1β and tumor necrosis factor-αand enhances the mRNA expression of interleukin-6 and protease-activated receptor-2 and proliferation of stromal cells from endometrioma. Fertil Steril 96 : 118-121, 2011