Suppression of the Development of Experimentally Induced Uterine Adenomyosis by a Novel Matrix Metalloproteinase Inhibitor, ONO-4817, in Mice

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The inhibitory effects of a novel, orally active matrix metalloproteinase (MMP) inhibitor, ONO-4817, on the development of uterine adenomyosis induced experimentally by pituitary grafting were examined in mice. Mice were given transplants of isologous anterior pituitary glands (PGs) into the right uterine lumen at 7 weeks of age and were fed chow containing 0.1% to 1.0% ONO-4817 from 8 to 14 weeks of age. Mice treated with 0.3% or 1.0% ONO-4817 showed a significantly lower incidence of the development of adenomyosis than vehicle-treated mice. To evaluate the inhibitory effects of ONO-4817 on the progression of the invasion of the adenomyotic tissues, mice receiving PG grafts at 7 weeks of age were treated with 1.0% ONO-4817 from 13 to 17 weeks of age. The degree of pathological progression of adenomyosis was graded from 1 to 5 in increments of 1. The degree of the progression of the lesion was less in the uteri exposed to ONO-4817 (2.71 ± 0.93) than in the uteri not exposed to the inhibitor (4.33 ± 0.75). Finally, the invasiveness of endometrial stromal cells obtained from adenomyotic uteri into Matrigel consisting mainly of type IV collagen and laminin was examined using an invasion assay. The assay showed that the treatment with ONO-4817 markedly suppressed the invasion of the stromal cells of the adenomyotic uteri into the gel. These results indicate that ONO-4817 may be an effective inhibitor of the development of adenomyosis.

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Key words: MMP inhibitor; extracellular matrix; adenomyosis; mice

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Uterine adenomyosis is a benign lesion that occurs spontaneously in humans and animals (1–3). While adenomyosis is a serious disease for women because of abnormal bleeding, pain, cramps, and sterility, the mechanisms of its development have not been fully elucidated (1). We have developed an efficient method to induce adenomyosis in which mice are given transplants of isologous anterior pituitary glands (PGs) into the uterine lumen (4–6). In the development of adenomyosis induced by PG grafting, an early sign of the disorder is the invasion of stromal fibroblasts into the myometrium along the branches of blood vessels (7–10). Disintegration of muscular bundles of the inner myometrium due to derangement and involution of muscle cells associated with widening of intercellular spaces appears to facilitate the invasion of stromal cells (9, 11).

Generally, the process of cell migration or invasion is connected with the degradation and reconstruction of the extracellular matrix (ECM), which consists of a number of substances, including collagen, fibronectin, and proteoglycan. Meanwhile, matrix metalloproteinases (MMPs) are known to be important enzymes in the degradation and reconstruction of the ECM (12). Recently, we have found that the stromal cells obtained from adenomyotic uteri show a high ECM-degrading activity, and that an inhibitor of MMPs, 4-Abz-Gly-Pro-D-Leu-D-Ala-NHOH, suppresses the invasiveness of these stromal cells in an invasion assay (13). Therefore, degradation of the ECM by MMPs is thought to be responsible for the development of adenomyosis.

On the other hand, an inhibitor of MMPs, ONO-4817, which shows a broad inhibitory activity against MMPs, has been developed (14). Since ONO-4817 suppresses the release of proteoglycan from the cartilage of the knee joints in

guinea pigs, its potential therapeutic utility for MMP-related disease was proposed (15). In this study, therefore, the inhibitory activity of ONO-4817 on the development of uterine adenomyosis was examined using a mouse model. We also tested whether ONO-4817 can retard the progress of the lesions at an early stage of adenomyotic changes that have already developed. To examine the direct inhibitory activity of ONO-4817 on the invasiveness of endometrial cells of adenomyotic uteri, an invasion assay was carried out using Matrigel constituted using the components of basement membrane.

Materials and Methods

Animals. Virgin female mice of the SHN strain maintained by brother-to-sister mating in the Department of Biological Sciences, The University of Tokyo, were used. They were housed under controlled conditions of light (12 hr of light, 12 hr of darkness; lights on at 6:00 hr) and temperature (25° ± 0.5°C) with free access to chow and tap water. All experiments were in accordance with the principles outlined in the *Guide for Animal Care and Use of the Committee of the Graduate School of Science*, The University of Tokyo, and in the regulations described in the National Institutes of Health Guide to the Care and Use of Laboratory Animals.

Experiment 1. Forty-eight SHN mice each received a transplant of a single isologous PG in the right uterine lumen at 7 weeks of age, since PG grafting quickly induces the development of uterine adenomyosis (4–6). The grafts were obtained from age-matched male littermates. These mice were divided into four groups consisting of 12 mice each. Three groups of mice with PG grafts were fed three different kinds of laboratory chow (CRF-1; Charles River, Tokyo, Japan): chow containing ONO-4817 ($C_{22}H_{28}N_2O_6$: [2*S*,4*S*]-*N*-Hydroxy-5-ethoxymethyloxy-2-methyl-4-[4-phenoxybenzoyl] aminopentanamide; ONO Pharmaceutical Co., Osaka, Japan) (14) at a dosage of 0.1%, 0.3%, or 1.0%, starting at 8 weeks of age and continuing until the end of the experiment. The remaining group was maintained as a PG control group without ONO-4817 treatment. All groups of mice were killed at 14 weeks of age by cervical dislocation.

Food intake and body weights in all groups of mice were measured weekly from 8 weeks of age, except that only food intake was determined at 9 weeks. Immediately after autopsy, uteri and ovaries were removed and fixed in Bouin's solution overnight. The tissues were embedded in paraffin and prepared as 7-μm serial sections. Sections were stained with Mayer's hematoxylin and eosin. Uteri were examined histologically for the development of adenomyosis. In addition, the presence of PGs was checked in the serial sections, which revealed the success of PG grafting. To compare the development of adenomyosis, the degree of progression of the lesion was graded from 1 to 5 in increments of 1 (Fig. 1) (4). In this study, only data from the most severe adenomyotic change in the right uterine horn were used, since development of adenomyosis was more severe

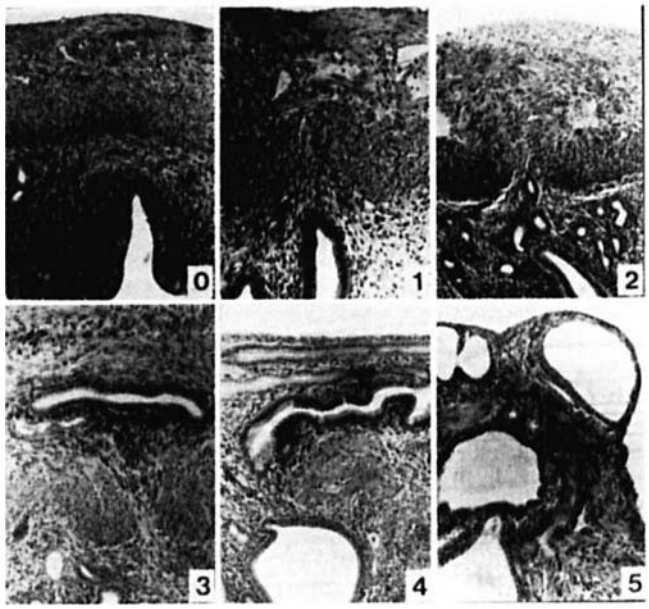

Figure 1. Classification of progression of adenomyosis in mice. Grade 0, normal uterus; Grade 1, uterus with an invasion of endometrial stromal cells into the inner layer of myometrium; Grade 2, uterus with an invasion of endometrial stromal and gland cells into the inner layer of myometrium; Grade 3, uterus with an invasion of endometrial stromal and gland cells in the connective tissue space between the inner and outer myometrial layers; Grade 4, uterus with cystic hyperplasia of the endometrial glands and small nodules beneath the serosa; Grade 5, uterus with cystic hyperplasia of the endometrial glands and a large number of subserosal nodules. Uteri were obtained from 17-week-old SHN mice that received PG grafts at 7 weeks of age, except that uterus for Grade 0 was obtained from an intact 7-week-old mouse and uteri for Grade 1 and 2 were obtained from 13-week-old mice receiving PG grafts at 7 weeks of age. Grades 0, 2, 3, 4, and 5, ×120; Grade 1, ×180.

in the right horn than in the left one, as reported previously (4–6). Ovaries were checked to determine whether they contained corpora lutea.

Experiment 2. To test the suppressive activity of ONO-4817 against the progress of the invasion of endometrial tissues into the myometrium, mice receiving a single isologous PG graft in the right uterine lumen at 7 weeks of age were divided into three groups consisting of 10 mice each. The first group of mice was killed at 13 weeks of age to check the development of adenomyosis at the commencement of ONO-4817 treatment. The second group of mice was fed chow containing 1.0% ONO-4817 from 13 weeks of age. The third group of mice was fed normal chow without ONO-4817 during whole period of the experiment. These two groups of mice were killed at 17 weeks of age.

At autopsy, uteri were removed and examined histologically for the graded levels of cell invasiveness and the success of PG grafting as described above.

Experiment 3. Six female mice each received a transplant of a single isologous PG in the right uterine lumen at 7 weeks of age. Six months after PG grafting the mice were killed by cervical dislocation. All mice developed severe adenomyosis with subserosal nodules, an advanced state of this lesion (4–6). The uteri were removed and used in an invasion assay.

Isolation of uterine endometrial stromal cells was performed by a procedure described previously (16). Uteri were minced, then washed with Hank's balanced salt solution (HBSS) and digested with 0.02% of collagenase (Sigma, St. Louis, MO) in HBSS for 2 hr at 37°C. The tissue sample was centrifuged at 500*g* for 7 min and the supernatant was discarded. The pellet was washed twice with HBSS by centrifuging at 100*g* for 30 sec and the supernatants were pooled. The pooled supernatant was centrifuged at 200*g* for 3 min and the pellet was used as the cell sample. The pellet was suspended in 10% heat-inactivated fetal calf serum (FCS) in Dulbecco's modified Eagles medium (DMEM)/F12 and plated in a 10-cm dish. The dishes were incubated in a CO_2 incubator for 1 hr at 36.6°C in an atmosphere of 5% CO_2 and 95% air. The dishes were then washed with 10% FCS in DMEM/F12 to remove the unattached cells, and the attached cells were used for following assay. Our previous experiments confirmed that thus-prepared cell samples were mainly composed of endometrial stromal cells (13).

In the invasion assay, the attached cells were removed, suspended in DMEM containing 0.1% bovine serum albumin (BSA), and seeded onto filters with 8-μm pores in Transwell chambers (Corning Costar Co., Cambridge, MA) at the concentration of 2×10^3 cells/well. The filters were coated in advance with 0.1 ml of a 2 mg/ml solution of Matrigel (solubilized basement membrane preparation extract from the Engelbreth-Holm-Swarm mouse sarcoma, of which the major components are laminin, collagen type IV, and heparin sulfate proteoglycan; Sigma). NIH3T3 cell-conditioned medium obtained by the incubation of the cells overnight in serum-free DMEM supplemented with 0.1% BSA was used as the bottom culture medium (17). After 6 hr of incubation, the cells invading the lower surface of the filters were fixed with 70% ethanol for 30 min and stained with hematoxylin-eosin. All invading cells were counted in each well obtained from six mice. The number of invading cells divided by the number of seeded cells was determined, and the value was represented as a percentage and was used to express the invasion rate. To assess the effect of ONO-4817 on the cell invasion, the inhibitor was added to the culture system at the concentration of 10 nM/ml or 1 μM/ml.

Statistics. The statistical significance of the differences in the incidence of adenomyosis between groups was evaluated by the chi-square test with Yate's correction. The differences in the degree of the invasion of uterine tissues and the invasiveness of endometrial stromal cells in the invasion assay were evaluated by Student's *t* test.

Results

Experiment 1. Food intake per mouse per day was not significantly different among all groups of mice with or without ONO-4817 treatment. The average food intakes in all groups were 3.4 ± 0.1 g, 3.7 ± 0.1 g, 3.9 ± 0.3 g, 3.7 ± 0.2 g, 3.8 ± 0.1 g, and 3.6 ± 0.1 g during each week from 8 to 14 weeks of age, respectively. If the daily intakes of ONO-4817 are computed roughly from the average food intakes, the maximum estimate is 39 mg in 1.0% group and the minimum estimate is 3.4 mg in 0.1% group. There were also no differences of body weights among the four groups (data not shown). In addition, almost all mice contained corpora lutea in their ovaries (Table I).

The effect of ONO-4817 on the development of uterine adenomyosis is shown in Table I. Almost all mice with PG grafts and without ONO-4817 treatment exhibited invasion of endometrial tissues into the inner myometrium, and thus met the criteria for uterine adenomyosis. On the other hand, the incidence of adenomyosis in the mice given ONO-4817 for 6 weeks was decreased. The incidences of adenomyosis in mice fed chow containing 0.3% ONO (42%) and 1.0% ONO (8%) were significantly lower than the incidence in age-matched control mice (92%). No differences were found in the grade of adenomyotic progression among all groups of mice developing the lesion, which was due to lower grade of the progression in all groups (Table I).

Experiment 2. Mice that received PG grafts at 7 weeks of age and were killed at 13 weeks of age developed adenomyosis showing an invasion of endometrial tissues into the inner layer of myometrium (Fig. 1 and Table II). The grade of progression of the lesion was low (Table II). The mice that received PG grafts at 7 weeks of age and were killed at 17 weeks of age had more progressed lesions where a large amount of endometrial tissues reached the serosa and sometimes formed subserosal nodules (Fig. 1). The difference between the PG-bearing mice killed at 13 and 17 weeks of age was significant at the 0.02 level. On the other hand, the mice receiving PG at 7 weeks and 1.0% ONO-4817 from 13 to 17 weeks of age also showed 100% incidence of adenomyosis, but had a significantly lower grade of progression of the lesion than age-matched control mice not treated with the inhibitor at the 0.05 level (Table II).

Table I. Incidence of Adenomyosis in 14-Week-Old Mice With or Without ONO-4817 Treatment

Group	Number of mice examined	Number of mice with adenomyosis (graded levels of cell invasiveness in adenomyotic uteri)	Number of mice with corpora luea
0.1% ONO-4817	12	6 (2.17 ± 0.40)	11
0.3% ONO-4817	12	5[a] (2.20 ± 0.49)	12
1.0% ONO-4817	12	1[b] (1.00)	12
Control	12	11[a,b] (2.18 ± 0.23)	12

Note. [a] $P < 0.05$.
[b] $P < 0.01$.

Table II. Degree of Progression of the Adenomyosis in Mice With or Without ONO-4817 Treatment

Group		Number of mice examined	Graded levels of cell invasiveness
Age at autopsy	1.0% ONO-4817 treatment		
13	None	10	$1,83 \pm 0.88^{a}$
17	None	10	$4.33 \pm 0.75^{a,b}$
17	13–17 weeks	10	2.71 ± 0.93^{b}

Note. ^{a} $P < 0.02$.
^{b} $P < 0.05$.

Experiment 3. In the invasion assay using the Matrigel, adenomyotic stromal cells treated with ONO-4817 showed significantly less invasiveness than the cells not treated with ONO-4817, although there was no difference between the invasiveness of cells treated with 10 nM and 1 μM ONO (Fig. 2).

Discussion

We have established a mouse model for the development of uterine adenomyosis; in this model the incidence of adenomyosis reaches more than 90% about 50 days after PG grafting (4–6). This was confirmed in the present Experiment 2 in which the incidence reached 100% 7 weeks after PG grafting. The present study clearly demonstrated that administration of ONO-4817 significantly suppressed the initiation of the development of adenomyosis in mice subjected to PG grafting to induce the genesis of adenomyosis. In addition, the inhibitor retarded the progression of the pathological grade of adenomyosis that had probably developed at the commencement of the inhibitor treatment.

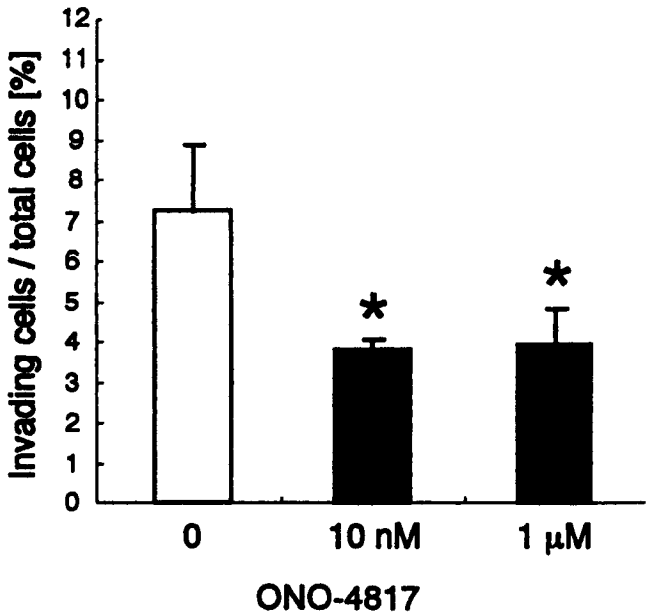

Figure 2. The effect of ONO-4817 on cells in an invasion assay using Matrigel. Endometrial stromal cells were seeded into chambers coated with Matrigel at a density of 2×10^3 cells/well. ONO-4817 was added to the cells at 10 nM or 1 μM. The assay was performed in six mice each. The mean values of invasiveness were significantly different between the stromal cells treated or not treated with ONO-4817 (*$P < 0.05$). Vertical bars represent SEM.

Uterine adenomyosis is characterized by an abnormal growth of stroma and glands into and beyond the myometrial layers of which the pathological change is certainly associated with the degradation and reconstruction of the ECM. In the normal uteri, the intrusion of uterine stromal cells is blocked by the musculature consisting of smooth muscle cell layers with ECM. The degradation and reconstruction of the ECM are, in general, known to be related to the expression of MMPs, whose expression in normal uteri is under the control of physiological factors such as the estrous cycle, and which are involved in blastocyst implantation and trophoblast invasion (18, 19). Our previous experiments revealed that uterine stromal cells obtained from adenomyotic uteri had higher invasiveness than those from normal uteri into Matrigel containing type IV collagen, and that the invasiveness was suppressed by an MMP inhibitor (13). However, the stromal cells of adenomyotic uteri did not invade gels consisting of type I, type III, or type V collagen (13). It is known that in the musculature of the uterus there are various types of collagen such as type I, type III, type IV, and type V (20, 21). Type IV collagen, which is present in the basement membrane of blood vessels in humans and animals (22), is degraded by gelatinases, MMP-2, and -9. We have found that adenomyotic stromal cells invade the myometrium along the branches of blood vessels, which suggests that the stromal cells may possess the ability to invade perivascular ECM, including the basement membrane (23). In addition, a higher expression of mRNAs of MMP-2 and -9 was detected in the adenomyotic uteri (13). It has been reported that ONO-4817 shows a high inhibitory activity against MMP-2 and -9 and that this inhibition is specific for MMPs because ONO-4817 showed almost no inhibitory activity against other proteases (14, 15). Therefore, the present invasion assay was carried out using reconstituted basement membrane, Matrigel-containing type IV collagen. As a result, a direct inhibitory activity of ONO-4817 on the invasion of stromal cells into Matrigel was detected.

The present experiments showed that there were no differences during the observation period in food intake and body weight among groups of mice treated with different doses of ONO-4817 and mice not treated with ONO-4817. In addition, all mice treated with no more than 1% ONO-4817 contained corpora lutea along with a large number of follicles with various sizes in the ovaries, suggesting a lack of obvious effects on the estrous cycle and ovulation. Notably, ONO-4817 could retard the progress of the pathogenesis of adenomyosis already initiated at the commencement of the treatment. Thus, short-term usage of this inhibitor appears to be a promising treatment to prevent severe progression of this kind of invasion.

In conclusion, the present results demonstrated that ONO-4817 inhibits the development and progression of

uterine adenomyosis in mice, which suggests its possible therapeutic utility for human adenomyosis.

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