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Dienogest reduces proliferation, NGF expression and nerve fiber density in human adenomyosis

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ABSTRACT

Objectives: To evaluate the *in vivo* effect of dienogest on proliferation, apoptosis, aromatase expression, vascular density, nerve growth factor (NGF) expression and nerve fiber density in human adenomyosis tissue.

Study design: Twelve women who underwent hysterectomy for adenomyosis were enrolled. Six patients received dienogest treatment prior to hysterectomy (dienogest group), and age-matched six patients who had not received any hormonal treatment for ≥3 months before surgery (control group). Cell proliferation, vascular and nerve fiber density in adenomyosis tissue were evaluated by staining for Ki67, von Willebrand factor and PGP9.5, respectively. Apoptosis was detected using the TUNEL assay. The expression aromatase and NGF were evaluated by staining for corresponding antibodies.

Results: The proportion of Ki67 positive epithelial cells was significantly lower in samples from dienogest-treated patients in comparison with controls ($p < 0.05$). The density of blood vessels in adenomyosis was marginally lower in the dienogest group in comparison with controls but statistical significance was not reached ($p = 0.07$). The intensity of NGF expression and the density of nerve fibers were significantly lower in the dienogest group compared with controls ($p < 0.05$ for both).

Conclusion: This study demonstrates that adenomyosis, taken from patients treated with dienogest, shows remarkable histological features, such as reductions in proliferation, NGF expression and nerve fiber density. These findings indicate the impact of dienogest on local histological events, and explains its therapeutic effect on adenomyosis.

Introduction

Adenomyosis is a benign uterine disorder defined by the presence of endometrial tissue within the myometrium [1]. Clinically, adenomyosis is characterized by abnormal uterine bleeding, menorrhagia, dysmenorrhea, infertility and miscarriages. The strategy for controlling adenomyosis basically follows the principle of the management, that is, reducing the production of endogenous estrogen or inducing endometrial differentiation by progestins [2]. The most commonly used therapeutics are progestins and gonadotropin-releasing hormone (GnRH) agonists.

Dienogest (17-hydoroxy-3-oxo-19-nor-17α-pregna-4,9-diene-21-nitrile), a derivative of 19-norsteroid, is a novel progestin that is highly selective for progesterone receptors. In addition to its therapeutic effects on endometriosis, our group [3] and others [4] have demonstrated that dienogest is effective in controlling pain symptoms associated with adenomyosis. Despite these clinical evidence indicating that dienogest controls adenomyosis, the mechanistic understating of the effect is very limited, only a single study demonstrated that dienogest promotes apoptosis in primary cultured human adenomyotic stromal cells [5].

The aim of this study was to evaluate the *in vivo* effect of dienogest on adenomyosis tissue. We therefore collected adenomyosis tissues from patients treated with dienogest, or not treated, and conducted histological analyses for cell proliferation and apoptosis, aromatase expression, densities of blood vessels and nerve fibers, and NGF expression.

Materials and methods

Collection of adenomyosis tissues and preparation of tissue sections

The experimental procedures were approved by the institutional review board of the University of Tokyo (the registration number is

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0324-4). After obtaining written informed consents, adenomyosis tissues were obtained from patients undergoing laparoscopic or abdominal hysterectomy between 2013 and 2015. The decision whether or not administer dienogest in the pre-operative period was made by patient's preferences; balance between the benefit (pain control) and adverse effects (uterine bleeding *etc.*). During this period, a total of six patients chose to take dienogest (2 mg per day) in the pre-operative period (1–15 months, mean: 5.5 months, dienogest group) and all these six patients were allocated to the 'dienogest group'. Age-matched six patients who had not received any hormonal treatment for ≥3 months in the pre-operative period were allocated to the 'control group'. Adenomyosis tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks, and 5 μm serial sections were prepared. Paraffin-embedded sections were deparaffinized and then rehydrated in decreasing concentrations of ethanol.

Diagnosis of adenomyosis

The diagnosis of adenomyosis had been done with a histological examination performed by certified pathologists. Further, subtype classification was made using the criteria proposed by Kishi et al. [6].

Patients characteristics

Patients characteristics are shown in Table 1. There was no significant difference between control and dienogest group in patients' age, gravidity and parity, the prevalence of dysmenorrhea and hypermenorrhea, and the distribution of adenomyosis subtypes.

Immunohistochemistry

Immunohistochemistry was conducted for Ki67 (to detect proliferating cells), aromatase, von Willebrand factor (to detect endothelial cells for blood vessels), nerve growth factor (NGF) and PGP9.5 (to detect nerve fibers). The following antibodies were used: monoclonal mouse anti-human Ki67 antibody (M7240) (Dako Cytomation, Carpinteria, CA, USA) at a 1:50 dilution, polyclonal rabbit anti-human aromatase antibody (kindly provided by Dr. Harada) at a 1:500 dilution, monoclonal mouse anti-human von Willebrand factor antibody (M0616) (Dako Cytomation) at a 1:25 dilution, polyclonal rabbit anti-human NGF antibody (Sc548) (Santa Cruz Biotechnology) at a 1:500 dilution and polyclonal rabbit anti-human PGP9.5 antibody (Z5116) (Dako Cytomation) at a 1:100 dilution. Rehydrated sections were treated with 0.3% hydrogen peroxide (H_2O_2) for 5 min for neutralizing endogenous peroxidases, and rinsed for 5 min with distilled water. Antigen retrieval was performed using Target Retrieval (Dako Cytomation) for Ki67, aromatase, NGF and PGP9.5 staining. Antigen retrieval was not performed for von Willebrand factor staining. After washing with phosphate buffered saline, slides were incubated with antibodies in a moist-chamber with primary antibody at 4 °C for overnight. All sections were visualized using DAB (Dako Cytomation) for substrate, followed by hematoxylin counterstaining and analysis under a light microscope.

Table 1
Comparison of patients' characteristics between control and dienogest group.

	Control group	Dienogest group	
Age[a]	43.5 ± 3.6	48 ± 2.3	N.S.
Gravidity[a]	1.0 ± 0.30	1.0 ± 0.30	N.S.
Parity[a]	1.0 ± 0.80	1.0 ± 0.30	N.S.
Dysmenorrhea (%)	50	100	N.S.
Hypermenorrhea (%)	100	100	N.S.
Adenomyosis subtypes			
Type1 (%)	0	33.3	N.S.
Type2 (%)	83.3	33.3	N.S.
Type3 (%)	16.7	33.3	N.S.
Type4 (%)	0	0	N.S.

[a] Median ± SD.

TUNEL assay

Apoptotic cells were detected using a TUNEL colormetric staining (*in situ* cell death detection kit, POD 11684817910 Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions.

Statistical analysis

For Ki67 and aromatase staining evaluation, adenomyosis lesions larger than 1 mm^2 (25 lesions in total) in six patients' specimen were observed. The number of positively-stained epithelial cells was counted and the proportion of positively-stained epithelial cells over total epithelial cells was calculated. For TUNEL assay, the number of positively stained stromal cells of adenomyosis lesion/0.25 mm^2 were counted in three randomly-selected fields and averaged. For von Willebrand factor and PGP9.5 staining evaluation, the number of von Willebrand factor-stained blood vessels/mm^2 and the number of PGP9.5-stained nerve fibers/mm^2 were counted in three randomly-selected fields and averaged. NGF staining was evaluated by the scoring of immunoreactivity (IR score). The IR score quantifies the immunoreactivity by multiplication of the staining intensity (graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of positively-stained cells (0 = 0%, 1 = ≤10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells and 4 = ≥81% of the cells). The staining of NGF in both the epithelium and the stroma of adenomyosis lesion/0.25 mm^2 was scored in three randomly-selected fields and averaged.

Data analysis was conducted using Jmp software (version 11.0, SAS Institute Inc., Cary, NC). The differences between groups were calculated using non-parametric analysis (Mann–Whitney *U* test). The correlation between the level of staining and the duration of dienogest therapy was analyzed using Spearman's rank Correlation Coefficient. A p value of <0.05 was considered as statistically significant.

Results

Expression of Ki67

Positive staining for Ki67 was observed in the nuclei of both epithelial and stromal cells, although percentage of positive staining cells was higher in the epithelium than in the stroma. When compared the percentage of positive staining cells in epithelial cells between the dienogest group and controls, there were less positive staining of epithelial cells in the dienogest group than in controls (Fig. 1A and B). Statistical analysis supported this observation since the proportion of Ki67 positive epithelial cells was significantly lower in the dienogest group than in controls (9.2 ± 2.6% versus 52.8 ± 5.0%, mean ± SEM% respectively, $p < 0.05$, Fig. 2A).

Expression of aromatase

Aromatase expression was observed in both the epithelium and the stroma of adenomyosis, although positive cells appeared more frequently in the epithelium than in the stroma (Fig. 1C and D). Thus, the proportion of positive cells in the epithelium was analyzed. The proportion of aromatase positive epithelial cells was