Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy

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BACKGROUND: Information is limited regarding the multifunctional role of GnRH agonist (GnRHa) therapy in reproductive diseases. We investigated the pattern of changes in inflammatory reaction, micro-vessel density and apoptosis in the tissues collected from women with endometriosis, adenomyosis and uterine myoma who were treated with or without GnRHa therapy.

METHODS: Biopsy specimens were collected from lesions, myometria and corresponding endometria of 45 women with ovarian endometrioma, 35 women with adenomyosis and 56 women with uterine myoma. A fraction of these women were treated with GnRHa therapy for a variable period of 3–6 months before surgery. We performed immunohistochemical analysis of CD68, a macrophage (Mφ) marker and von Willebrand factor (VWF), a vessel marker, using respective antibodies. Changes in apoptosis were examined using TdT-mediated dUTP-biotin nick end-labeling assay and by the immunoexpression of activated caspase-3 in tissues after GnRHa therapy.

RESULTS: The infiltration of CD68-positive Mφ and VWF-positive micro-vessel density were significantly decreased in the endometria of women with endometriosis, adenomyosis and uterine myoma in the GnRHa-treated group when compared with that in the non-treated group. Marked decreases in inflammatory and angiogenic responses were observed in lesions and myometria of these diseases. When compared with the non-treated group, a significant increase in apoptotic index (apoptotic cells per 10 mm² area) and quantitative-histogram scores of activated caspase-3 after GnRHa therapy were observed in the eutopic endometria, lesions and myometria of these diseases.

CONCLUSIONS: GnRHa was able to markedly reduce the inflammatory reaction and angiogenesis and to significantly induce apoptosis in tissues derived from women with endometriosis, adenomyosis and uterine myoma. These multiple biological effects at the tissue level may be involved in the regression of these reproductive diseases.

Key words: reproductive diseases / macrophages / micro-vessels / apoptosis / GnRH agonist

Introduction

Endometriosis, adenomyosis and uterine myoma mostly affect women of reproductive age, manifesting a variable degree of symptoms and causing reproductive failure in a percentage of patients. The development of endometriosis, adenomyosis and uterine myoma is commonly considered to be estrogen-dependent. Besides hormonal regulation, a number of other factors such as inflammatory processes, genetic factors and environmental factors has been reported to regulate these diseases (Lebovic et al., 2001; ASRM, 2004; Giudice and Kao, 2004; Kitajima et al., 2004a, b; Benagiano and Brosens, 2006; Levy et al., 2007; Khan et al., 2007, 2008, 2009). We previously reported an endocrine-immune cross talk in the regulation of endometriosis and the occurrence of a variable amount of inflammatory reactions.
in the different types of uterine myoma (Khan et al., 2004, 2008, 2009; Miura et al., 2006). A strong inflammatory reaction in endometriosis and uterine myoma was reported to be associated with the detrimental effect on fertility (Khan et al., 2004; Miura et al., 2006).

In general practice, gonadotrophin-releasing hormone (GnRH) agonist has been commonly used for the treatment of endometriosis, adenomyosis and uterine myoma. GnRH agonist (GnRHa) therapy aims for the resolution of pain symptoms and regression of these diseases by causing a state of hypo-estrogenemia. The response of this hormonal medication to reproductive diseases is variable depending on the type of the medication, the patient’s background and the GnRH receptor-ligand binding affinity for individual cells or tissues (Qayum et al., 1990; Emons et al., 1993; Borroni et al., 2000). Tissue variation in the inflammatory response among these diseases has been described (Khan et al., 2004; Miura et al., 2006). However, information regarding the tissue effect of GnRHa on the inflammatory response, angiogenic response and apoptosis in women with endometriosis, adenomyosis and uterine myoma is unclear.

We investigated the changes in inflammatory reaction, as demonstrated by tissue infiltration of macrophages and angiogenic response, as measured by micro-vessel density, in the tissue specimens derived from women with endometriosis, adenomyosis and uterine myoma who were treated with GnRHa therapy for a variable period of time. As a potential chemo-attractant of monocytes, we also measured tissue levels of monocyte chemotactic protein 1 (MCP-1) in the endometrium. Finally, we examined changes in the degree of apoptosis in tissues derived from women with these reproductive diseases.

Materials and Methods

Subjects
The subjects in this study were women of reproductive age. From February 2004 to June 2009, biopsy specimens were collected from a total of 45 women with ovarian endometrioma, 35 women with adenomyosis and 56 women with uterine myomas who underwent hysterectomy, laparoscopy or laparotomy during this period. All these women were admitted to our hospital with the complaint of abnormal genital bleeding, hypermenorrhea or amenorrhea with or without associated complaint of dysmenorrhea or pelvic pain. A number of patients in these study groups also had variable lesions of pelvic endometriosis. The ovarian endometrioma, adenomyosis and uterine fibroids in all these women were diagnosed by ultrasonography and magnetic resonance imaging before operation. The diagnosis of ovarian endometrioma was confirmed by laparoscopy. There were 20 women with ovarian endometrioma, 15 women with adenomyosis and 20 women with uterine myoma who were treated with GnRHa (leuprolide acetate) for a variable period of 3–6 months before operation. About a third of women with GnRHa treatment withdrew their treatment after 3–4 months due to side effects and other women continued treatment until 6 months. Groups of women without GnRHa treatment did not receive oral contraceptives or any other therapy within 12 months prior to surgery. This was a prospective non-randomized follow-up study with retrospective analysis of samples derived from GnRHa-treated and non-treated women after surgery. The reasons for pre-operative GnRHa therapy in these groups of women were either due to reproductive desire, anemia or recurrence of disease and/or pain or to reduce excessive blood loss during operation. Surgical therapy was assigned to all these groups for diagnostic confirmation of disease and to reduce the burden of complaints or recurrence of disease.

The phases of the menstrual cycle in women without hormonal therapy were determined by histological dating of eutopic endometria samples taken simultaneously with endometrioma, adenomyoma and nodules and are shown in Table I. All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval of the Nagasaki University Institutional Review Board. Informed consent was obtained from all women.

Table I  Clinical profiles of women with ovarian endometrioma, adenomyosis and uterine myoma

<table>
<thead>
<tr>
<th></th>
<th>GnRHa (−)</th>
<th>GnRHa (+)</th>
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<tbody>
<tr>
<td>Ovarian endometrioma (n = 45)</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Age in years (range)</td>
<td>26–39</td>
<td>28–38</td>
</tr>
<tr>
<td>Age in years (mean ± SD)</td>
<td>30.4 ± 3.7</td>
<td>31.5 ± 2.4</td>
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<tr>
<td>Size in cm (range)</td>
<td>4–10</td>
<td>6–10</td>
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<tr>
<td>Size in cm (mean ± SD)</td>
<td>6.0 ± 2.1</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>Unilateral/bilateral</td>
<td>18/7</td>
<td>12/8</td>
</tr>
<tr>
<td>Menstrual cycle: P/S/M/A</td>
<td>8/15/2/0</td>
<td>0/0/0/20</td>
</tr>
<tr>
<td>Duration of therapy (month)</td>
<td>4–6</td>
<td></td>
</tr>
<tr>
<td>Adenomyosis (n = 35)</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>Age in years (range)</td>
<td>36–48</td>
<td>35–47</td>
</tr>
<tr>
<td>Age in years (mean ± SD)</td>
<td>41.9 ± 4.0</td>
<td>42.1 ± 2.6</td>
</tr>
<tr>
<td>Anterior wall/posterior wall/both</td>
<td>3/7/10</td>
<td>2/8/5</td>
</tr>
<tr>
<td>Menstrual cycle: P/S/M/A</td>
<td>8/12/0/0</td>
<td>0/0/0/15</td>
</tr>
<tr>
<td>Duration of therapy (month)</td>
<td>3–6</td>
<td></td>
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<tr>
<td>Uterine myoma (n = 56)</td>
<td>36</td>
<td>20</td>
</tr>
<tr>
<td>Age in years (range)</td>
<td>23–50</td>
<td>27–48</td>
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<tr>
<td>Age in years (mean ± SD)</td>
<td>39.1 ± 6.2</td>
<td>36.1 ± 5.8</td>
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<td>Size in cm (range)</td>
<td>2.5–12</td>
<td>2.2–10</td>
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<tr>
<td>Size in cm (mean ± SD)</td>
<td>5.0 ± 2.1</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>SMM/IMM/SSM</td>
<td>18/16/2</td>
<td>10/10/0</td>
</tr>
<tr>
<td>Menstrual cycle: P/S/M/A</td>
<td>12/18/6/0</td>
<td>0/0/0/20</td>
</tr>
<tr>
<td>Duration of therapy (month)</td>
<td>3–6</td>
<td></td>
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</table>

The results are expressed as mean ± SD. GnRHa (−), without GnRH agonist therapy; GnRHa (+), with GnRH agonist therapy; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; SMM, submucosal myoma; IMM, intramural myoma; SSM, subserosal myoma. Women with endometrioma and uterine myoma were significantly younger than women with adenomyosis (P < 0.001 and P < 0.01, respectively).
endometria were collected from all the women mentioned above during the operation. Biopsy specimens obtained after reduction surgery and hysterectomy were analyzed for the histological diagnosis of adenomyosis. A total of three to four biopsy specimens from different anatomical locations of the eutopic endometrium were also studied for women with adenomyosis and myoma who underwent hysterectomy. Samples of the endometrioma and eutopic endometrium were collected from all women with ovarian endometrioma. All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study and also for terminal deoxy-UTP nick end-labeling (TUNEL) assay.

A fraction of biopsy specimens from the corresponding endometria of the three groups of women with or without GnRHa therapy were homogenized in homogenizing buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland) (Miura et al., 2006). The respective tissue suspension was centrifuged at 400 g for 5 min to obtain the supernatant and stored at −80°C for the subsequent measurement of MCP-1.

Antibodies used
We performed immunohistochemical studies to investigate the immunoreaction of CD68 for macrophages (Mφ) in intact tissues. CD68 (KP1), a mouse monoclonal antibody was derived from Dako, Denmark. A 1:50 dilution was used. CD68 antigen (clone KP1), which we used for our current study as a marker of matured and activated Mφ, is a glycosylated trans-membrane glycoprotein that is mainly located in lysosomes. It belongs to a family of lysosomal granules (Holness and Simmons, 1993). We also used anti-human von Willebrand factor (VWF) antibody (clone F8/86, code M0616; Dako, Denmark), a mouse monoclonal antibody, to investigate immunoreaction to micro-vessels. A 1:25 dilution was used. Affinity-purified anti-human activated caspase-3 (AF835, R&D system, Minneapolis, MN, USA), a rabbit polyclonal antibody, was used (1:100 dilution) to distinguish apoptotic cells from non-apoptotic cells in tissue specimens. Rabbit anti-mouse IgG mAb conjugated with avidin–biotin complex was used as the secondary antibody (1:50 dilution, Dako, Denmark). Non-immune mouse immunoglobulin (Ig) G1 antibody (Dako, Denmark) in a 1:50 dilution was used as a negative control. Lymphoid tissue was used as a positive control for CD68 and VWF, and dermal tissue was used as a positive control for apoptosis.

Immunohistochemistry
The details of immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004; Ishimaru et al., 2004). Briefly, 5-μm thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H2O2/methanol to block endogenous peroxidase activity, sections were pre-incubated with 10% normal goat serum to prevent non-specific binding and then

Figure I The immunohistochemical localization of Mφ as indicated by the CD68-immunoreactive spots in the biopsy specimens derived from the cyst wall, adenomyotic lesion, myoma nodule and autologous endometria and myometria of women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C) who were treated with GnRH agonist [GnRHa (+)], lower column of block A, B and C] and without GnRH agonist [GnRHa (−), upper column of block A, B and C]. Final magnification was adjusted at ×200 using a light microscope.
incubated overnight at 4°C with anti-CD68 antibody, anti-VWF antibody or anti caspase-3 antibody. The slides were subsequently incubated with biotinylated secondary antibody for 10 min, followed by incubation with avidin–peroxidase for 10 min and visualized with diaminobenzidine. Finally, the tissue sections were counterstained with Mayer’s hematoxyline, dehydrated with serial alcohols, cleared in xylene and mounted.

The immunoreactive CD68 spots were counted in five different fields of one section (×200 magnification) by light microscopy and expressed as the mean Mϕ number per field in one specimen. Micro-vessel density as measured by total micro-vessel number and as immunoreactive to VWF was counted by light microscopy of those areas that contained the highest number of capillaries and venules. We used a combination of a ×20 objective and a ×10 ocular (0.785 mm²/field). The number of Mϕ and micro-vessels per field in each biopsy specimen was recounted and confirmed by a second observer who did not know the history of the patients.

The immunostaining of activated caspase-3 was quantified by a modified method of quantitative-histogram score (Q-H score) as described recently (Khan et al., 2003; Ishimaru et al., 2004). The Q-H score was calculated using the following equation: Q-H score = ΣPi (i + 1), where i = 1, 2 or 3 and Pi is the percentage of stained cells for each intensity. The staining intensity was graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong. We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification (×200).

**Cytokine assays**

The tissue concentrations of MCP-1 in the homogenized supernatant of endometria derived from women with or without GnRHa therapy were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbent assay (Quantikine; R&D System, Minneapolis, MN, USA) according to the manufacturer’s instructions and as described recently (Khan et al., 2004). The protein concentration of samples was measured by the method of Bradford (1976) to standardize MCP-1 level. The antibodies used in MCP-1 determination do not cross-react with other cytokines. The limit of detection was <5.0 pg/ml for MCP-1. Both the intra-assay and inter-assay coefficients of variation were <10% for this assay. The tissue concentration of MCP-1 was expressed as pg/μg protein.

**Terminal deoxy-UTP nick end-labeling assay**

Tissue sections (5 μm thick) were stained with TUNEL assay as described previously (Dmowski et al., 2001; Okazaki et al., 2005) with minimal modification to identify the apoptotic cells. Briefly, paraffin-embedded tissues were deparaffinized and the nuclei with fragmented DNA were detected using a TUNEL detection kit (Wako, Osaka, Japan). Tissue sections were digested with a protease for 5 min at 37°C. After washing with 0.01 M PBS for 15 min, the slides were incubated with the terminal deoxynucleotidyl transferase (TdT) reaction mixture in a humidified chamber at 37°C for 1 h. The slides were subsequently incubated with biotinylated secondary antibody for 10 min, followed by incubation with avidin–peroxidase for 10 min and visualized with diaminobenzidine. Finally, the tissue sections were counterstained with Mayer’s hematoxyline, dehydrated with serial alcohols, cleared in xylene and mounted.

**Figure 2** Tissue infiltration of macrophages (Mϕ) in the endometria (A), in different pathological lesions such as cyst wall, adenomyotic lesion or myoma nodule (B) and in the myometria (C) derived from GnRH-a-treated (hatched box) and GnRH-a-non-treated (white box) women with ovarian endometrioma, adenomyosis and uterine myoma. The significance in Mϕ infiltration in the respective tissue specimens between GnRH (−) group and GnRH (+) group is shown in this figure. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

**Figure 3** Levels of MCP-1 in the homogenized tissue samples derived from the eutopic endometria of GnRH-a-treated (hatched box) and GnRH-a-non-treated (white box) women with endometrioma, adenomyosis and uterine myoma. A significant decrease in tissue levels of MCP-1 was found in the endometria derived from women with ovarian endometrioma, adenomyosis and uterine leiomyoma (P < 0.05, P < 0.01 and P < 0.01, respectively) after GnRH-a treatment. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.
37°C for 1 h. The specimens were immersed in 3% H₂O₂ with 0.01 M PBS for 5 min at room temperature to reduce the endogenous peroxidase activity, and were washed again with 0.01 M PBS for 10 min. Then, the slides were treated with the peroxidase-conjugated antibody for 10 min at 37°C. After washing with 0.01 M PBS for 15 min, the immunoreaction was visualized with diaminobenzidine and H₂O₂. Counterstaining was done with methyl green dye.

Nuclear morphological features of an apoptotic cell were considered as TUNEL-positive stained nucleus, i.e. shrinkage of the nucleus with condensed chromatin and/or densely aggregated marginal chromatin or dot-like or drop-like condensed nuclear fragments. TUNEL stained swollen nuclei were considered as degenerated necrotic cells and were excluded from the apoptotic cell population. Quantitative analysis of the apoptotic cells was performed with a cytometer under ×200 magnification using Olympus (model DP20) microscope. Each area of respective tissue varied from 10 to 50 mm² depending on the size of the tissue sample. The number of apoptotic cells in the endometria, lesions and myometria were counted separately. The apoptotic index was defined as the number of apoptotic cells per 10 mm² unit area. In the cases of endometria and adenomyotic lesions, the combined apoptotic index of glands and stroma was expressed.

Statistical analysis

All results are expressed as either mean ± SD or medians. The clinical characteristics of the subjects were compared with one-way analysis of variance and the $\chi^2$ test for any difference between two groups. Logistic conversion analysis was performed to normalize the distribution of our samples and results. Since our samples and results were still not normally distributed, differences in Mφ number, micro-vessel number, cytokine concentration, apoptotic index or Q-H scores of activated caspase between two groups were analyzed by the non-parametric Mann–Whitney U-test. For comparisons among groups, the Kruskal–Wallis test was used to assess the differences. A box plot analysis of our all results was performed using the medians and inter-quartile range. A value of $P < 0.05$ was considered statistically significant.

Results

The detail clinical profiles of women with and without GnRHa therapy among ovarian endometrioma, adenomyosis and uterine myoma are shown in Table I. The women with ovarian endometrioma and uterine myoma were significantly younger than women with adenomyosis ($P < 0.001$ and $P < 0.01$, respectively). When we distributed all clinical parameters between women with GnRHa therapy and women without GnRHa therapy, we did not find any difference in age or mean size of ovarian endometrioma or uterine myoma and anatomical location of adenomyosis between these two groups of women as shown in Table I. The duration of GnRHa therapy was also comparable among these three study groups, 4–6 months for women with ovarian endometrioma.

![Figure 4](image-url) The immunohistochemical staining of micro-vessel marker, VWF in the biopsy specimens derived from women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C) derived from GnRHa-treated (+) and GnRHa-non-treated (−) women. Final magnification was adjusted at ×200 using a light microscope.
There was no difference in the number of women presenting with pain, anemia, reproductive desire or body mass index between GnRHa users and non-users (data not shown). When we distributed tissue infiltration of Mφ, tissue levels of MCP-1, micro-vessel density and markers of apoptosis in samples derived from different phases of menstrual cycle in GnRHa-non-treated women, no significant differences in all these markers were found among different menstrual phases. Therefore, we represented our data irrespective of phases of menstrual cycle.

CD68-positive Mφ infiltration in endometrioma, adenomyosis and uterine myoma

Mφ infiltration, as shown by CD68-positive brown spots, are shown in the endometria and cyst wall of women with ovarian endometrioma (Fig. 1A) and in the endometria, pathologic lesions and myometria derived from women with adenomyosis and uterine myoma (Fig. 1B and C) in GnRHa-treated and -non-treated groups. No obvious differences in Mφ infiltration was observed in the adenomyotic lesion or in the autologous myometria of women with uterine myoma between GnRHa treated and non-treated groups (Fig. 1B and C).

Quantitative analysis of Mφ infiltration in endometrioma, adenomyosis and uterine myoma

The mean Mφ number (± SEM) per field in the respective tissue specimens of GnRHa-treated and -non-treated groups of women with ovarian endometrioma, adenomyosis and uterine myoma are shown in Fig. 2. We found that tissue infiltration of Mφ in the endometria derived from women with endometrioma, adenomyosis and uterine myoma was significantly decreased after GnRHa therapy when compared with that of similar tissues derived from GnRHa-non-treated women (P < 0.001, P < 0.01 and P < 0.01, respectively, upper panel, A). A significant difference in Mφ infiltration was also observed in the cyst wall and in the myoma nodule, but not in adenomyotic lesions, between GnRHa (−) and GnRHa (+) groups (P < 0.01 for cyst wall and P < 0.05 for myoma nodule, middle panel, B). The autologous myometrial tissues derived from GnRHa-treated women with adenomyosis showed a significant decrease in Mφ infiltration (P < 0.01), but this was not so for women with uterine myoma (Fig. 2C, lower panel).

Tissue levels of MCP-1 in GnRHa-treated and -non-treated endometria

As a chemokine for the recruitment of monocytes, tissue levels of MCP-1 were measured in the endometria of women with endometrioma, adenomyosis and uterine myoma. Tissue levels of MCP-1 in the endometria derived from women with ovarian endometrioma, adenomyosis and uterine myoma after GnRHa treatment were significantly decreased (P < 0.05, P < 0.01 and P < 0.01, respectively) when compared with that of endometrial samples derived from GnRHa-non-treated women (Fig. 3).

Immunoreaction of VWF in endometrioma, adenomyosis and uterine myoma

Figure 4 shows the immunohistochemical staining of VWF in the biopsy specimens derived from GnRHa-treated and -non-treated women with ovarian endometrioma (upper left block, A), adenomyosis (upper right block, B) and uterine myoma (lower block, C). The immunoreaction of VWF appeared to be lower in the endometria derived from women with these diseases after GnRHa treatment (Fig. 4A–C).

Figure 5 Shows micro-vessel numbers in the endometria (A), in different pathological lesions such as cyst wall, adenomyotic lesion or myoma nodule (B) and in the myometria (C) derived from GnRHa-treated (hatched box) and GnRHa-non-treated (white box) women with ovarian endometrioma, adenomyosis and uterine myoma. The significance in the mean micro-vessel number in the respective tissue specimens derived from women with GnRHa (−) group and GnRHa (+) group is shown in this figure. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.
Quantitative analysis of micro-vessel density in endometrioma, adenomyosis and uterine myoma

We calculated micro-vessel density after counting the number of VWF-immunoreactive vessels in the biopsy specimens of women with or without GnRHa therapy (Fig. 5). A significantly decrease in micro-vessel density was found in the endometria derived from women with endometrioma, adenomyosis and uterine myoma after GnRHa treatment ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively, Fig. 5A, upper panel). A similar significant reduction in VWF-positive micro-vessel number was also found in the cyst wall ($P < 0.05$, Fig. 5B, middle panel), myoma nodule ($P < 0.001$, Fig. 5B, middle panel) and in the myometria derived from women with adenomyosis and uterine myoma ($P < 0.05$ for each, Fig. 5C, lower panel) after GnRHa treatment when compared with that of similar tissues derived from GnRHa-non-treated women. No difference in micro-vessel density was found in the adenomyotic lesions between GnRHa (−) and GnRHa (+) groups.

TUNEL-positive cells in endometrioma, adenomyosis and uterine myoma

Figure 6 shows TUNEL stained nuclei in the different tissue specimens derived from GnRHa-treated and GnRHa-non-treated women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C). Apoptotic cells were identifiable after TUNEL staining in endometria, lesions and autologous myometria.

Apoptotic index in tissues derived from women with endometrioma, adenomyosis and uterine myoma

As shown in Fig. 7, apoptotic indices were significantly higher in the endometria derived from women with endometrioma, adenomyosis and uterine myoma after GnRHa treatment when compared with that of similar tissues derived from non-treated groups ($P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively, Fig. 7A, upper panel). The apoptotic index was also found to be markedly higher in the cyst wall ($P < 0.001$) and myoma nodule ($P < 0.001$) after GnRHa treatment, but not in adenomyotic lesions (Fig. 7B, middle panel). When numbers of apoptotic cells were examined in the autologous myometria, a significantly increased apoptotic index was found in the surrounding myometrial tissues derived from GnRHa-treated women with adenomyosis ($P < 0.01$) and uterine myoma ($P < 0.05$) comparing to that in tissues derived from GnRHa-non-treated women (Fig. 7C, lower panel).
Immunoreaction of activated caspase-3 in endometrioma, adenomyosis and uterine myoma

In order to confirm our findings by TUNEL assay, we examined changes in apoptotic cells in tissues derived from GnRHa-treated and -non-treated women with endometrioma (A), adenomyosis (B) and uterine myoma (C) by immunoreaction to activated caspase-3 (Fig. 8). We found an apparent increase of caspase-3 immunostained cells in the endometria, lesions and myometria derived from women with these reproductive diseases after GnRHa treatment.

Q-H scores of activated caspase-3 in endometrioma, adenomyosis and uterine myoma

We quantified the immunostaining of caspase-3 in each tissue specimen by Q-H scores (Fig. 9). We found that Q-H scores of activated caspase-3 were significantly increased in the endometria derived from women with endometrioma, adenomyosis and uterine myoma (P < 0.01, P < 0.05 and P < 0.01, respectively) after GnRHa treatment (Fig. 9A, upper panel). The cyst wall (P < 0.001) and myoma nodules (P < 0.01) but not the adenomyotic lesions (Fig. 9B, middle panel) showed a marked increase in the Q-H scores after GnRHa treatment. The surrounding myometria derived from GnRHa-treated women with adenomyosis (P < 0.001) and myoma (P < 0.05) also showed a significantly increased Q-H scores of caspase-3 staining when compared with that of similar tissues derived from women without GnRHa treatment (Fig. 9C, lower panel).

Discussion

We demonstrated for the first time that in addition to hypo-estrogenic effect, GnRHa therapy for a variable period of time retains multifunctional roles in the peripheral tissues of women with endometriosis, adenomyosis and uterine myoma. We report here that GnRHa therapy significantly reduces inflammatory reaction and angiogenic response and induces a remarkable degree of apoptosis in different tissues derived from women with these reproductive diseases.

The reduction in inflammatory reaction and angiogenic response was determined by a significant decrease in the amount of Mφ infiltration and micro-vessel density in the biopsy specimens derived from GnRHa-treated women when compared with that of similar samples derived from GnRHa-non-treated women. Our demonstrated results in the decrease of Mφ infiltration and micro-vessel density could be due to the direct effect of GnRHa at the tissue level or indirect effect of decreased estrogen level. Ovarian steroids have a profound effect on the establishment of infections. Although progesterone suppresses uterine immune function, estradiol may play a role in the recruitment of immune cells such as Mφ (Beagley and Gockel, 2003). Therefore, it is reasonable to speculate that to a variable degree, the reduction of inflammatory reaction at the tissue level could be due to the decrease in endogenous estrogen levels among GnRHa users. The decrease in inflammatory response among GnRHa users might be the dual effect of systemic and local suppression of estrogen. GnRHa has been reported to decrease the expression of aromatase cytochrome P450 in the eutopic endometrium from women with endometriosis, adenomyosis or leiomyoma (Ishihara et al. 2003). Further studies are needed to evaluate the direct effect of GnRHa in decreasing the tissue accumulation of macrophages and their association with chemotactic proteins.

Our findings of decreased inflammatory reaction among GnRHa users do not agree with some results published previously (Sozen et al., 2001). Using myoma tissue, this study found that there is an increase in the MCP-1 protein expression in the myometrium of women receiving GnRHa treatment. On the other hand, they did
not observe any difference in Mφ infiltration in tissues derived from women between GnRHa users and non-users. In this study, we found a significant difference in the tissue levels of MCP-1 in the endometria derived from GnRHa-treated women when compared with that of similar tissues derived from GnRHa-non-treated women in endometrioma, adenomyosis and uterine myoma. The authors of the previous study (Sozen et al., 2001) have speculated that the GnRHa exposed uterus has reduced arterial blood flow and this may prevent the accumulation of monocytes in the myometrium in response to the elevated MCP-1 expression. The discrepancy between these results and ours could be due to the difference in tissue type, tissue specificity and number of samples. The hypo-estrogenic response at the tissue level or at the vasculature in GnRHa users compared with non-users among women with endometriosis, adenomyosis and different types of uterine myoma may explain our current findings.

To our knowledge, there is no report describing the pattern of changes in micro-vessel density in the tissue specimens derived from women with different reproductive diseases after GnRHa treatment. Although micro-vessels in the adenomyotic lesions did not show a significant effect of GnRHa treatment, a significant decrease in micro-vessel density was observed in the endometria and autologous myometria derived from women with adenomyosis. Women with endometrioma and uterine myoma showed a similar significant decrease in micro-vessel density in the endometria, pathologic lesions and autologous myometria after GnRHa treatment. These findings indicate that reduction in the size of ovarian endometrioma, adenomyosis and myoma nodule after GnRHa treatment may be caused by reduction in blood flow in the pathologic lesions or in surrounding myometrial tissues. Although data were not shown, we also found decreases in the size of endometriotic cysts, uterine size of women with adenomyosis and myoma nodules in most of the cases after GnRHa treatment as evaluated by MRI-image. The capacity of GnRHa to reduce both inflammatory reaction and blood flow in endometria, pathologic lesions and corresponding myometria may explain effective alleviation of pain symptoms of women suffering from these reproductive diseases. In clinical practice, several GnRHa treatments, including leuprolide acetate, have been used for the treatment of pain symptoms with an effective alleviation rate of at least 50% in endometriosis and possibly in adenomyosis and uterine myoma as well (Crosignani et al., 2006).

It has been reported that exogenous treatment of human endometrial cells with estradiol dose-dependently increases the expression of vascular endothelial cell growth factor (VEGF) both at the gene and protein levels (Shifren et al., 1996). In fact, VEGF is an endothelial cell-specific angiogenic protein that appears to play an important role in

Figure 8 Shows the immunohistochemical staining of activated caspase-3 in the biopsy specimens derived from GnRHa-treated (+) and GnRHa-non-treated (−) women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C). Final magnification was adjusted at ×200 using a light microscope.
both physiological and pathological neovascularization. The antiangiogenic response at the tissue level among GnRHa users could be due to low estrogen levels, low VEGF levels or decreases in endothelial cell proliferation. Further experiments relating to the expression of GnRH receptors in vascular endothelial cells and the effect of GnRHa on these cells may clarify the anti-angiogenic response of GnRHa.

Apoptosis is an important regulator of eutopic endometrial function. A number of studies have reported that increased cellular proliferation and decreased apoptosis of endometrial cells shed during menstruation facilitate their ectopic survival and implantation in women with endometriosis (Dmowski et al., 2001; Béliard et al., 2004). Goumerou et al. (2004) have demonstrated that apoptosis occurs in ovarian endometriotic lesions at significantly higher levels than in control tissues (serous or mucinous cyst adenoma) and the apoptotic rate in ovarian endometriotic cells is not affected by the stage of endometriosis or the phase of the menstrual cycle. A study by Tesone et al. (2008) describes the effect of leuprolide acetate on apoptosis and angiogenesis on endometrial cells derived from women with and without endometriosis. The effect of GnRHa on cell proliferation, apoptosis and angiogenesis in endometrial cell cultures has also been described (Meresman et al., 2003; Bilotas et al., 2007). Most of these studies of apoptosis were done only in women with endometriosis and information on apoptosis in women with adenomyosis and uterine myoma is less readily available. Besides endometriosis, we also studied changes in apoptotic cells in tissues derived from women with adenomyosis and uterine myoma after GnRHa treatment. Although the samples were not analyzed according to different stages of endometriosis, we did not find any difference in inflammatory reaction, angiogenic response or in apoptosis depending on the phases of the menstrual cycle, when we analyzed data of women without GnRHa treatment.

We report here that GnRHa treatment was able to significantly induce a variable degree of apoptosis in the cyst wall, myoma nodule, autologous myometria and corresponding endometria of women with ovarian endometrioma, adenomyosis and uterine myoma except in adenomyotic lesions. This was confirmed by a marked increase in apoptotic index as measured by TUNEL assay as well as by increased Q-H scores of activated caspase-3 in the biopsy specimens derived from women with these diseases after GnRHa treatment. There is a debate about the TUNEL assay as to whether it reflects truly the apoptotic cells or necrotic cells. To rule out this bias, addition of a specific apoptotic marker to justify the findings of TUNEL assay is recommended (Bozec et al., 2005; Kumar, 2007). Accordingly, we extended our experiment with an effector caspase molecule such as caspase-3 and found a parallel increase in the activation of caspase-3 in these samples after GnRHa treatment. These results strengthen our current findings on the apoptotic effect of GnRHa treatment in these diseases.

Finally, we conclude that in addition to, or because of, a hypo-estrogenic effect, GnRHa has a multifunctional role in the peripheral tissues of women with endometriosis, adenomyosis and uterine myoma. Our current study demonstrated that GnRHa was able to significantly decrease the inflammatory reaction and angiogenic response and at the same time, induce a remarkable degree of apoptosis in tissues derived from women with these diseases. These biological effects of GnRHa at the tissue level were not influenced by different treatment periods. In fact, we did not find any significant difference in any of markers between samples derived from women who were treated with GnRHa for 3–4 months and samples of women who were treated for 6 months.

Our current study suggests that multiple local biological effects of GnRHa therapy may be involved in the regression of these reproductive diseases with consequent resolution of symptoms suffering from these hazardous diseases. A recent study demonstrated that leuprolide acetate is effective in reducing the growth of endometrial cells, not only due to their classical pituitary endocrine effects, but also

![Figure 9](image-url) The Q-H scores (see details in Materials and Methods) of activated caspase-3 immunoreaction in the endometria (A), in different pathological lesions such as cyst wall, adenomyotic lesion or myoma nodule (B) and in the myometria (C) derived from GnRHa-treated (hatched box) and GnRHa-non-treated (white box) women with ovarian endometrioma, adenomyosis and uterine myoma. The significance in the change of Q-H scores in the respective tissue specimens between GnRHa (−) group and GnRHa (+) group is shown in this figure. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

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via a direct effect on the endometrial cells themselves (Tesone et al., 2008). Our ongoing study on the local tissue expression of GnRH receptors and direct cell proliferation effect of GnRHa in different reproductive diseases may further explain the exact mechanism of action of this hypo-estrogenic agent in peripheral tissues.

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