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

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Running Title: Effect of GnRHa therapy on inflammation, angiogenesis and apoptosis.

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Abstract

Background: Information is limited regarding multifunctional role of GnRH agonist (GnRHa) therapy in reproductive diseases. We investigated 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 (M ϕ) marker, and von Willebrand factor (VWF), a vessel marker, using respective antibodies. The changes in apoptosis were examined using TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay and by the immunoexpression of activated caspase-3 in tissues after GnRHa therapy.

Results: The infiltration of CD68-positive M ϕ 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. A marked decrease in inflammatory and angiogenic responses were observed in lesions and myometria of these diseases. When compared with non-treated group, a significantly increase in apoptotic index (apoptotic cells per 10 mm^2 area) and quantitative-histogram (Q-H) scores of activated caspase-3 after GnRHa therapy were observed in the eutopic endometria, lesions and myometria of these diseases.

Conclusion: GnRH agonist was able to markedly reduce the inflammatory reaction and angiogenesis and 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 as estrogen-dependent. Besides hormonal regulation, a number of other factors such as inflammatory process, genetic factor, environmental factor has been reported to regulate these diseases (Lebovic et al., 2001; Giudice et al., 2004; ASRM 2004; Kitajima et al., 2004a, 2004b; Benagiano et al., 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 occurrence of a variable amount of inflammatory reaction 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, gonadotropin-releasing hormone (GnRH) agonist has been commonly used for the treatment of endometriosis, adenomyosis and uterine myoma. GnRHa therapy is involved in 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, patients background and 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 GnRH agonist 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 angiogenenic response as measured by micro-vessel density in the tissue specimens derived from women with endometriosis, adenomyosis and uterine myoma who were treated with GnRH agonist 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.

Material 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 hysteroscopy, laparoscopy or laparotomy during this period. All these women were admitted to our hospital with the complaint of abnormal genital bleeding, hypermenorrhoea or anemia with or without associated complaint of dysmenorrhea or pelvic pain. A fraction of these study groups was coexistent with variable lesions of pelvic endometriosis. The ovarian endometrioma, adenomyosis and uterine fibroids in all these women were diagnosed by ultrasonograghapy and magnetic resonance image before operation. The diagnosis of ovarian endometrioma was confirmed by laparoscopy. Twenty women with ovarian endometrioma, 15 women with adenomyosis and 20 women with uterine myoma were treated with GnRH agonist (leuprolide acetate) for a variable period of three to six 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 continue 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 is a prospective non-randomized follow-up study with retrospective analysis of samples derived from GnRHa treated and non-treated women after surgery. The reasons of pre-operative GnRHa therapy in these groups of women were either recurrence of disease and/or pain, anemia, reproductive desire or to reduce excessive blood loss during operation. Surgical therapy was assigned to all these groups either 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 was determined by histological dating of eutopic endometria samples taken simultaneously with endometrioma, adenomyoma and nodules and are shown in Table 1. 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. An informed consent was obtained from all women.

Biopsy specimens. Twenty-five women with adenomyosis (10 women with GnRHa therapy and 15 women without GnRHa therapy) and 27 women with uterine

myoma (12 women with GnRHa therapy and 15 women without GnRHa therapy) underwent hysterectomy. Therefore, we could collect myometrial samples only from these women. The other 10 women with adenomyosis were operated by reduction surgery (thin slice excision of myometrium containing adenomyotic lesions to reduce uterine size) and 29 women with myoma were operated by either trans-cervical resection (TCR) or laparoscopic myomectomy (LM). Therefore, instead of studying myometrial samples, we could study only pathologic lesions and endometria derived from these women who underwent reduction surgery, TCR or LM. Biopsy specimens from the respective cyst wall, adenomyosis, myoma nodule, autologous myometria or endometria were collected from all these women during 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. 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 specimen from the corresponding endometria of these 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 1500 rpm for 5 minutes to obtain the supernatant and stored at -80℃ for the subsequent measurement of monocyte chemotactic protein 1 (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), 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 1:50 dilution was used as a negative control. Lymphoid tissue was used as a positive control for CD68 and VWF and dermal tissue were 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, five-micrometer thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H_20_2/methanol to block endogenous peroxidase activity, sections were pre-incubated with 10% normal goat serum to prevent nonspecific binding and then incubated overnight at 4℃ with anti-CD68 antibody, anti-VWF antibody or anti caspase-3 antibody. The slides were subsequently incubated with biotinylated second antibody for 10 minutes, followed by incubation with avidin-peroxidase for 10 minutes and visualized with diaminobenzidine. Finally, the

tissue sections were counterstained with Mayer's hematoxylene, dehydrated with serial alcohols, cleared in xylene, and mounted.

The immunoreactive CD68 spots were counted in five different fields of one section (x200 magnification) by light microscopy and expressed as the mean M ϕ 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 x20 objective and a x10 ocular (0.785mm2/field). The number of M ϕ and micro-vessels per field in each biopsy specimen was recounted and confirmed by a second observer who did not know the history of these 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= Σ Pi (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 (x200).

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) 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 less than 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/ μ g protein.

Terminal Deoxy-UTP Nick End-Labeling (TUNEL) assay: Tissue sections (5 μ m 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℃. After washing with 0.01M PBS for 15 min, the slides were incubated with the terminal deoxynucleotidyl transferase (TdT) reaction mixture in a humidified chamber at 37℃ for 1 hr. The specimens were immersed in 3% H_2O_2 with 0.01M PBS for 5 min at room temperature to reduce the endogenous peroxidase activity, and were washed again with 0.01M PBS for 10 min. Then, the slides were treated with the peroxidase-conjugated antibody for 10 min at 37℃. After washing with 0.01M PBS for 15 min, the immunoreaction was visualized with diaminobenzidine and H_2O_2. 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 x200 magnification using Olympus (model DP20) microscope. Each area of respective tissue varied from 10 to 50 mm^2 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 $10mm^2$ unit area. In the cases of endometria and adenomyotic lesions, 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 X^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 not normally distributed, therefore, differences in M ϕ number, micro-vessel number, cytokine concentration, apoptotic index or Q-H scores 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 (IQR). A value of $p<0.05$ was considered statistically significant.

Results:

The detail clinical profiles of women with and without GnRH agonist (GnRHa) therapy among ovarian endometrioma, adenomyosis and uterine myoma are shown in Table 1. 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 1. The duration of GnRHa therapy was also comparable among these three study groups, 4-6 months for women with ovarian endometrioma, 3-6 months each for women with adenomyosis and uterine myoma. 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 M ϕ, 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 M ϕ infiltration in endometrioma, adenomyosis and uterine myoma. M ϕ infiltration, as shown by CD68-positive brown spots, are shown in the endometria and cyst wall of women with ovarian endometrioma (Figure 1, A) and in the endometria, pathologic lesions and myometria derived from women with adenomyosis and uterine myoma (Figure 1, B and C). in GnRHa-treated and –non-treated groups. No apparent difference in M ϕ infiltration was observed in the adenomyotic lesion or in the autologous myometria of women with uterine myoma between GnRHa treated and non-treated groups (Figure 1, B and C).

Quantitative analysis of M ϕ infiltration in endometrioma, adenomyosis and uterine myoma. The mean M ϕ 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 Figure 2. We found that tissue infiltration of M ϕ 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 or in the myoma nodule except that 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 not for women with uterine myoma (Figure 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 (Figure 3).

Immunoreaction of VWF in endometrioma, adenomyosis and uterine myoma. Figure 4 shows the immunohistochemical staining of von Willebrand factor (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 (Figure 4 A, B and C).

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 (Figure 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, Figure 5A, upper panel). A similar significant reduction in VWF-positive micro-vessel number was also found in the cyst wall ($p<0.05$, Figure 5B, middle panel), myoma nodule ($p<0.001$, Figure 5B, middle panel) and in the myometria derived from women with adenomyosis

and uterine myoma ($p<0.05$ for each, Figure 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 Figure 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, 7A, upper panel). Except adenomyotic lesions, 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 (7B, middle panel). When changes 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 (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 (Figure 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 (Figure 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 ($p<0.01$, $p<0.05$, $p<0.01$, respectively) after GnRHa treatment (9A, upper panel). Except adenomyotic lesions, the cyst wall ($p<0.001$) and myoma nodules ($p<0.01$) (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 (9C, lower panel).

Discussion

We demonstrated for the first time that in addition to hypo-estrogenic effect, GnRH agonist therapy for a variable period of time retains multifunctional roles in the peripheral tissues of women with endometriosis. adenomyosis and uterine myoma. We reported 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 GnRH agonist at the tissue level or indirect effect of decreased estrogen level. Ovarian steroids have a profound effect on the establishment of infections. While progesterone suppresses uterine immune function, estradiol may play a role in the recruitment of immune cells such as Mϕ (Beagley et al., 2003). Therefore, it is

reasonable to speculate that a variable degree in the reduction of inflammatory reaction at the tissue level could be due to a variation in the decrease of 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. GnRH agonist 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 the 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 M ϕ 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 (Sozen et al., 2001) speculated that GnRHa exposed uterus to have 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 different hypo-estrogenic response at the tissue level or at the vasculature between GnRHa users and 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 were resistant to the 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 shown not, we found decrease 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 ability of GnRHa in reducing 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 including leuprolide acetate has 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 (Crosignani et al., 2006).

It has been reported that exogenous treatment of human endometrial cells with estradiol dose-dependently increased 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

both physiological and pathological neovascularization. The anti-angiogenic response at the tissue level among GnRHa users could be due to low estrogen level, low VEGF level or decrease 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 direct anti-angiogenic response of GnRH agonist.

Apoptosis is an important regulator of eutopic endometrial function. A number of literatures reported that increased cellular proliferation and decreased apoptosis of endometrial cells shed during menstruation facilitate their ectopic survival and implantation in women with endometriosis (Béliard et al., 2004; Dmowski et al., 2001). Goumenou et al. (2004) demonstrated that apoptosis occurs in ovarian endometriotic lesions at significantly higher levels than that of controls (serous or mucinous cyst adenoma) and apoptotic rate in ovarian endometriotic cells were not affected by the stage of endometriosis or the phase of the menstrual cycle. The study by Tesone et al., (2008) also 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

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 scanty. Besides endometriosis, we also studied changes in apoptotic cells in tissues derived from women with adenomyosis and uterine myoma after GnRHa treatment. Although not analyzed according to different endometriosis stage, 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 reported 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 on TUNEL assay 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 hypo-estrogenic effect, GnRHa retains multifunctional role in the peripheral tissues of women with endometriosis, adenomyosis and uterine myoma. Our current study demonstrated that GnRH agonist was able to significantly decrease the inflammatory reaction and angiogenic response and at the same time, induced 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 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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Figure legends

Figure 1. Shows 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 x200 using a light microscope.

Figure 2. Shows tissue infiltration of macrophages (Mϕ) in the endometria (**A**), 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 Mϕ infiltration in the respective tissue specimens between GnRH (-) 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.

Figure 3. Shows levels of monocyte chemotactic protein-1 (MCP-1) in the homogenized tissue samples derived from the eutopic endometria of GnRHa-treated (hatched box) and GnRHa-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 GnRHa treatment. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

Figure 4. Shows the immunohistochemical staining of micro-vessel marker, von Willebrand factor (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 x200 using a light microscope.

Figure 5. Shows micro-vessel numbers in the endometria (**A**), 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.

Figure 6. Shows the terminal deoxy-UTP nick end-labeling (TUNEL) -positive cells in the biopsy specimens derived from GnRHa-treated (+) and GnRHa-non-treated (-) women with ovarian endometrioma (**A**), adenomyosis (**B**) and uterine myoma (**C**). A variable distribution of apoptotic cells was identifiable after TUNEL staining in endometria, pathologic lesions and autologous myometria.

Figure 7. Shows the apoptotic index as measured by the number of TUNEL-positive apoptotic cells per 10 mm^2 area in the endometria (**A**), 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 apoptotic index 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.

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 x200 using a light microscope.

Figure 9. Shows the quantitative-histogram (Q-H) scores (see details in methods) of activated caspase-3 immunoreaction in the endometria (**A**), 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.

Figure 1.

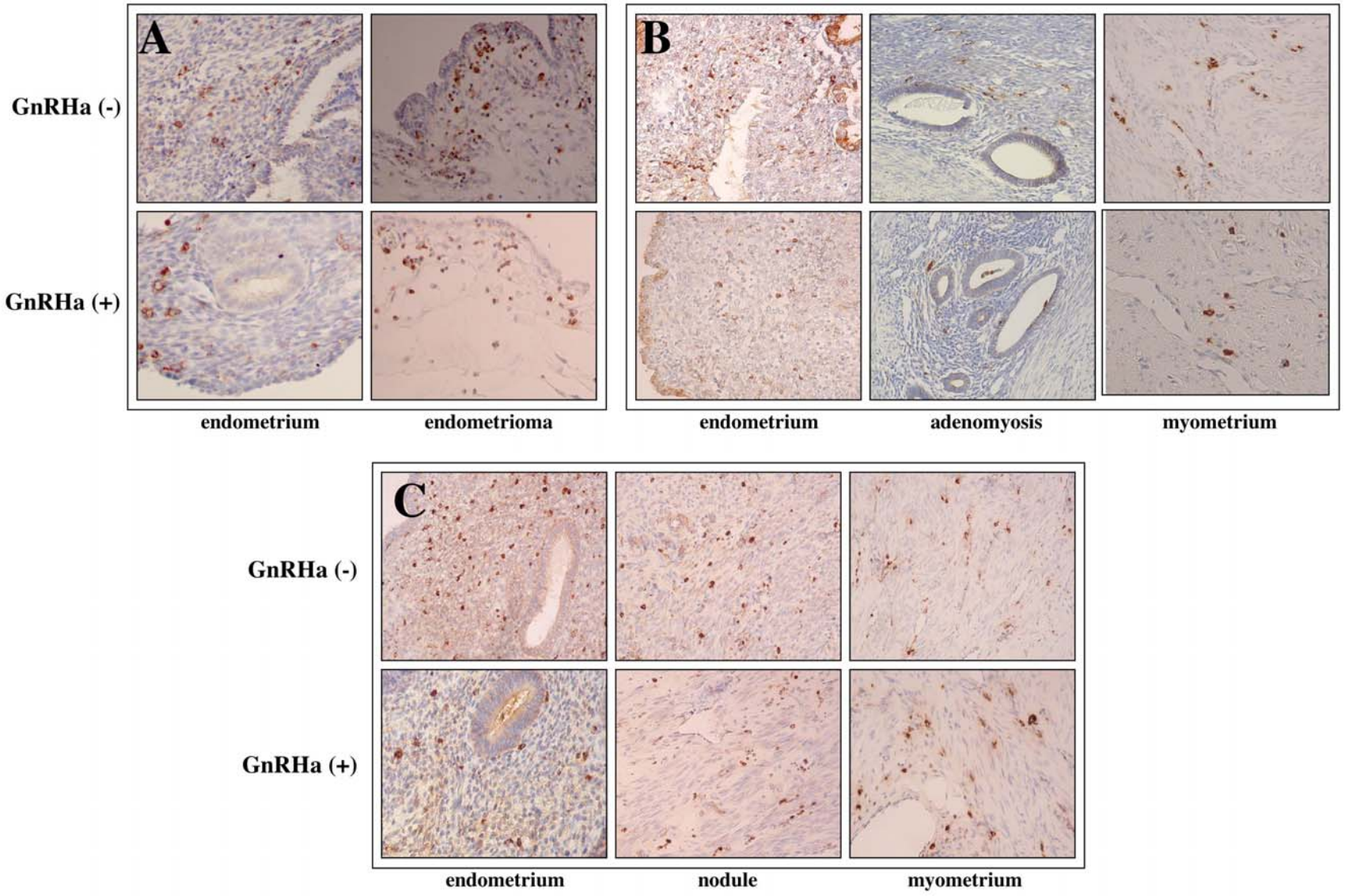
A
B
C
GnRHa (-)
GnRHa (+)
endometrium
endometrioma
endometrium
adenomyosis
myometrium
GnRHa (-)
GnRHa (+)
endometrium
nodule
myometrium

Figure 2.

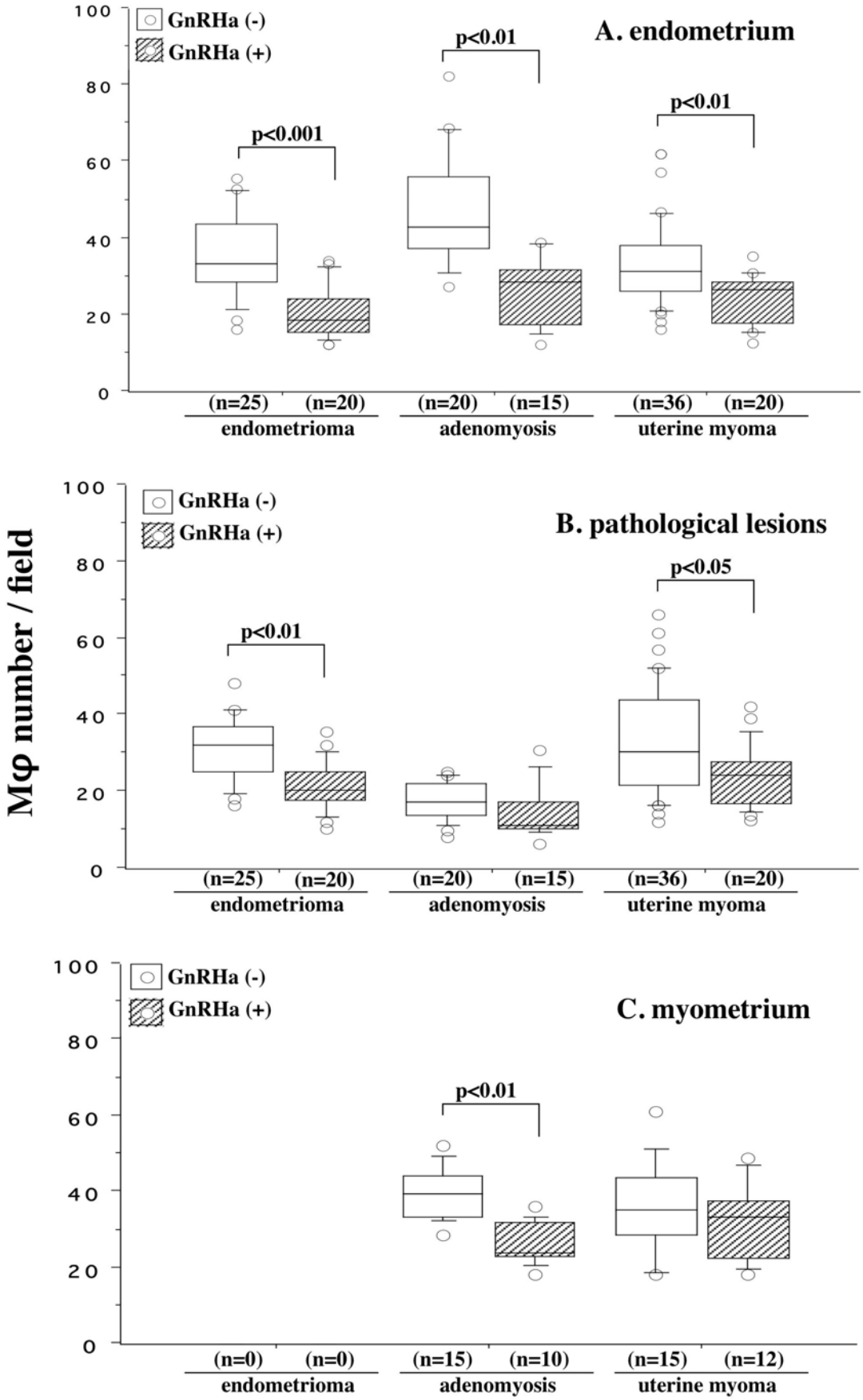
A. endometrium
GnRHa (-)
GnRHa (+)
p<0.001
p<0.01
p<0.01
100
80
60
40
20
0
(n=25) (n=20)
(n=20) (n=15)
(n=36) (n=20)
endometrioma
adenomyosis
uterine myoma
Mφ number / field
B. pathological lesions
p<0.01
p<0.05
C. myometrium
p<0.01
(n=0) (n=0)
(n=15) (n=10)
(n=15) (n=12)

Figure 3.

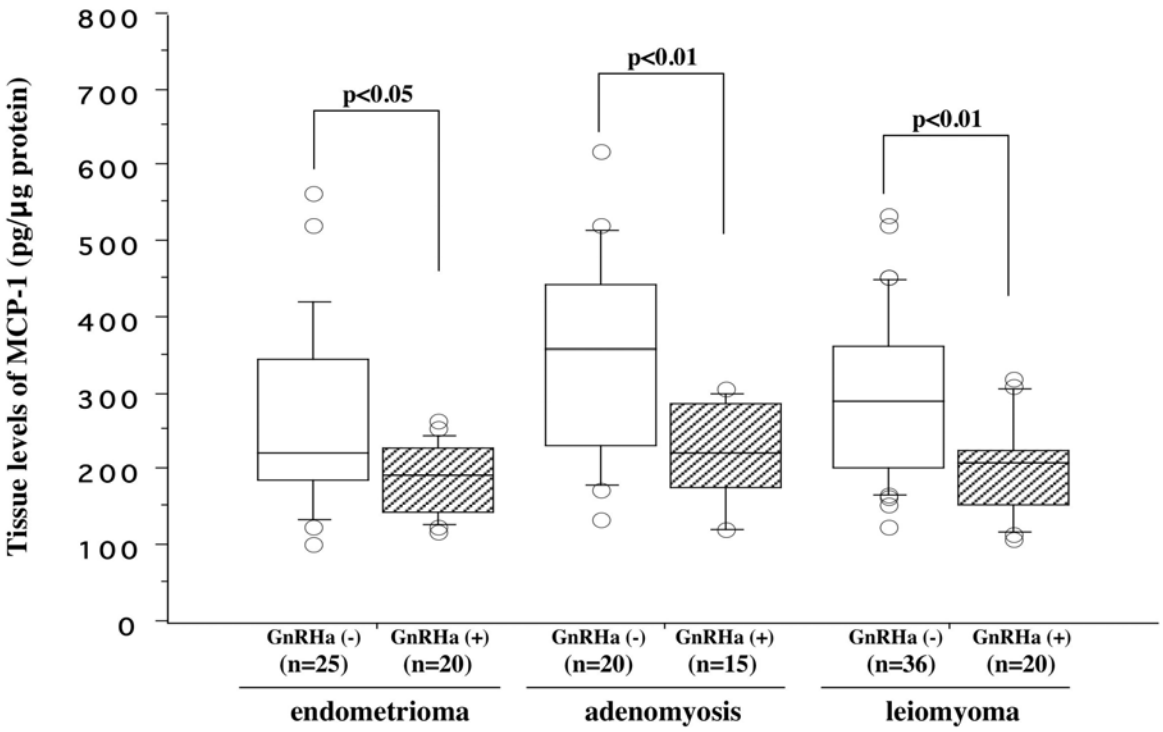
Tissue levels of MCP-1 (pg/µg protein)
800
700
600
500
400
300
200
100
0
p<0.05
p<0.01
p<0.01
GnRHa (-) (n=25)
GnRHa (+) (n=20)
GnRHa (-) (n=20)
GnRHa (+) (n=15)
GnRHa (-) (n=36)
GnRHa (+) (n=20)
endometrioma
adenomyosis
leiomyoma

Figure 4.

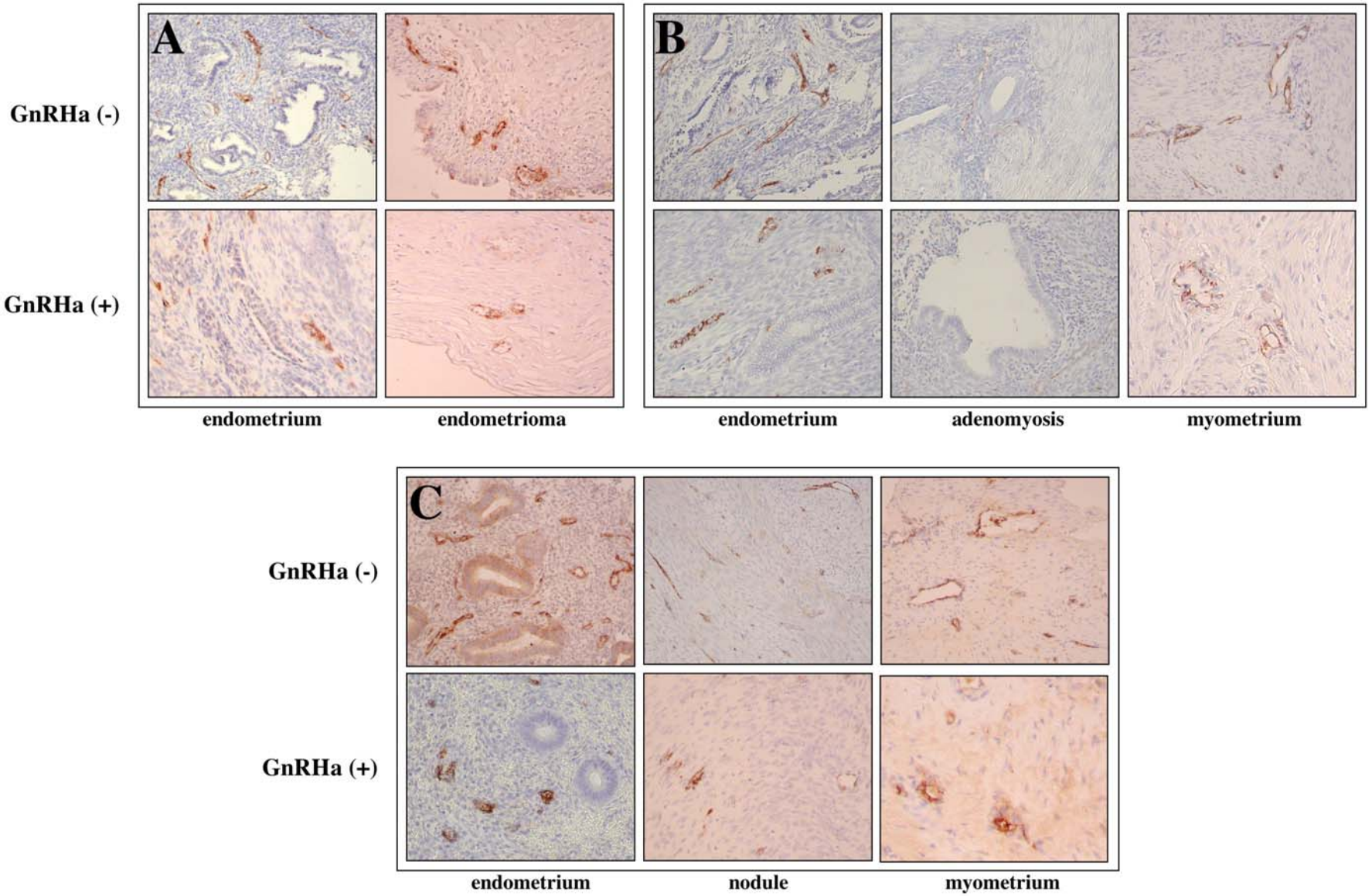
A
B
C
GnRHa (-)
GnRHa (+)
endometrium
endometrioma
endometrium
adenomyosis
myometrium
GnRHa (-)
GnRHa (+)
endometrium
nodule
myometrium

Figure 5.

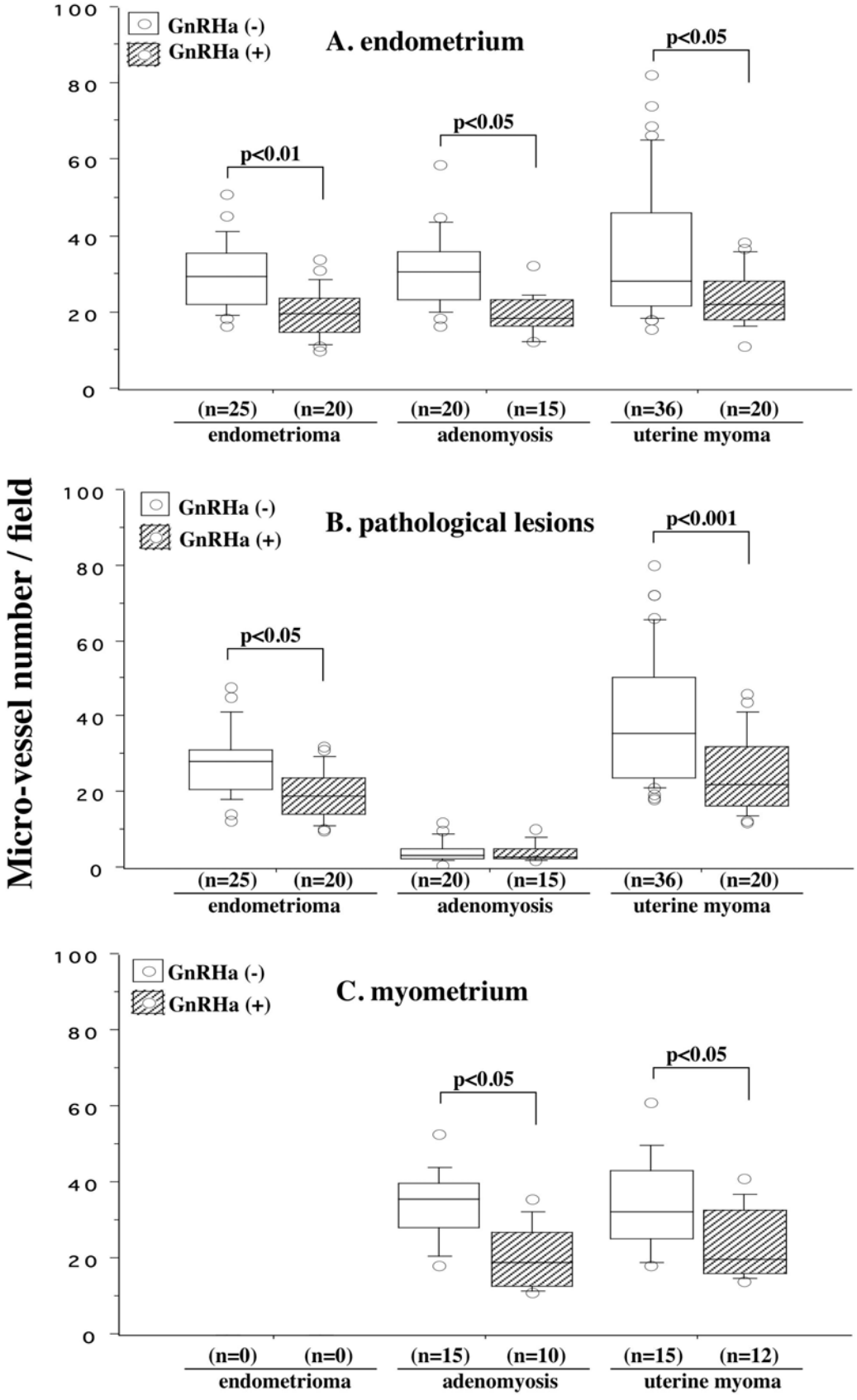
Micro-vessel number / field
GnRHa (-)
GnRHa (+)
A. endometrium
p<0.01
p<0.05
p<0.05
B. pathological lesions
p<0.05
p<0.001
C. myometrium
p<0.05
p<0.05
100
80
60
40
20
0
(n=25) (n=20)
(n=20) (n=15)
(n=36) (n=20)
endometrioma
adenomyosis
uterine myoma
(n=0) (n=0)
(n=15) (n=10)
(n=15) (n=12)

Figure 6.

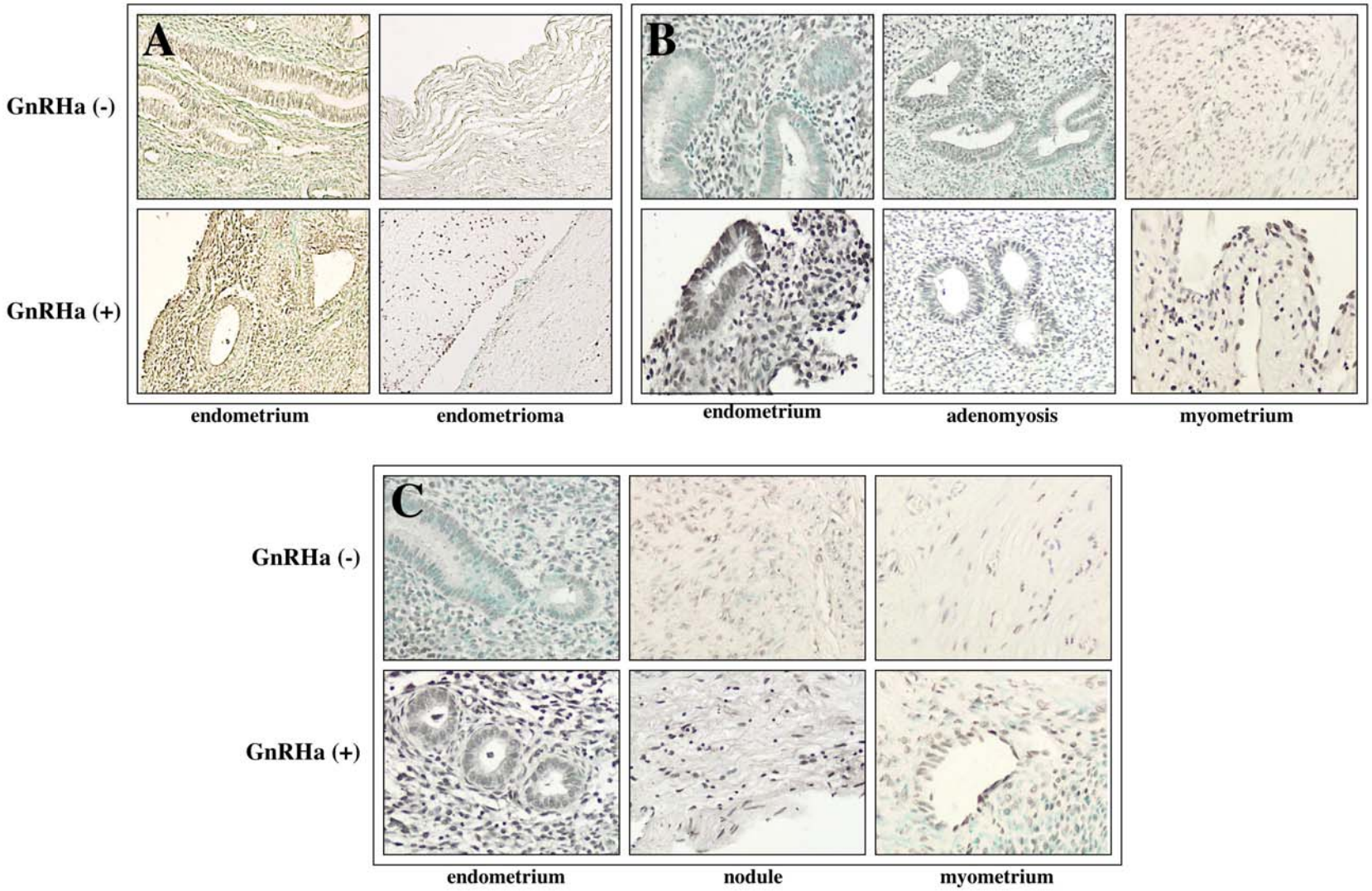
A
B
C
GnRHa (-)
GnRHa (+)
endometrium
endometrioma
endometrium
adenomyosis
myometrium
GnRHa (-)
GnRHa (+)
endometrium
nodule
myometrium

Figure 7.

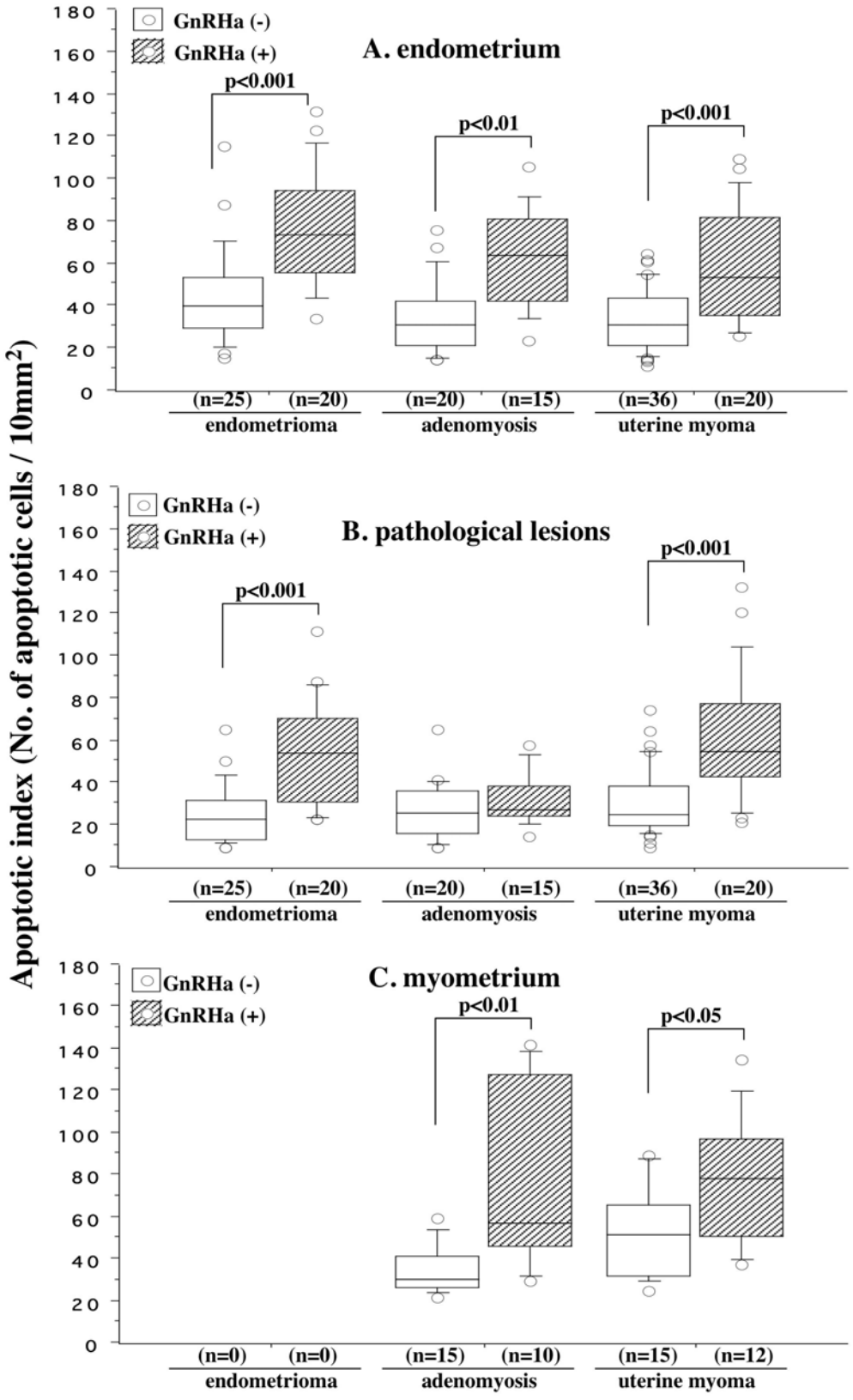
Apoptotic index (No. of apoptotic cells / 10mm²)
GnRHa (-)
GnRHa (+)
A. endometrium
p<0.001
p<0.01
p<0.001
(n=25) (n=20) (n=20) (n=15) (n=36) (n=20)
endometrioma adenomyosis uterine myoma
B. pathological lesions
p<0.001
p<0.001
C. myometrium
p<0.01
p<0.05
(n=0) (n=0) (n=15) (n=10) (n=15) (n=12)

Figure 8.

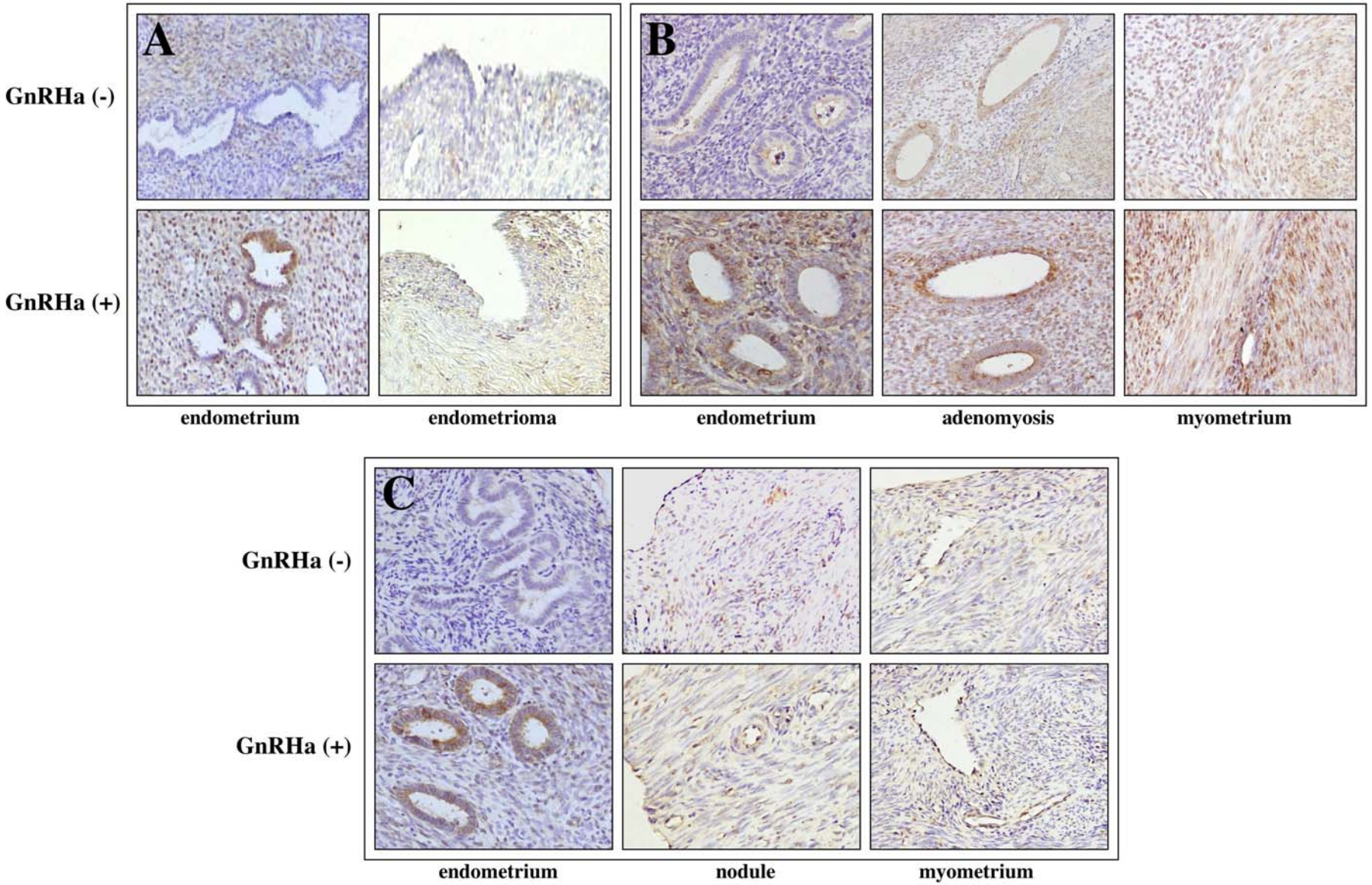
A
B
C
GnRHa (-)
GnRHa (+)
endometrium
endometrioma
endometrium
adenomyosis
myometrium
GnRHa (-)
GnRHa (+)
endometrium
nodule
myometrium

Figure 9.

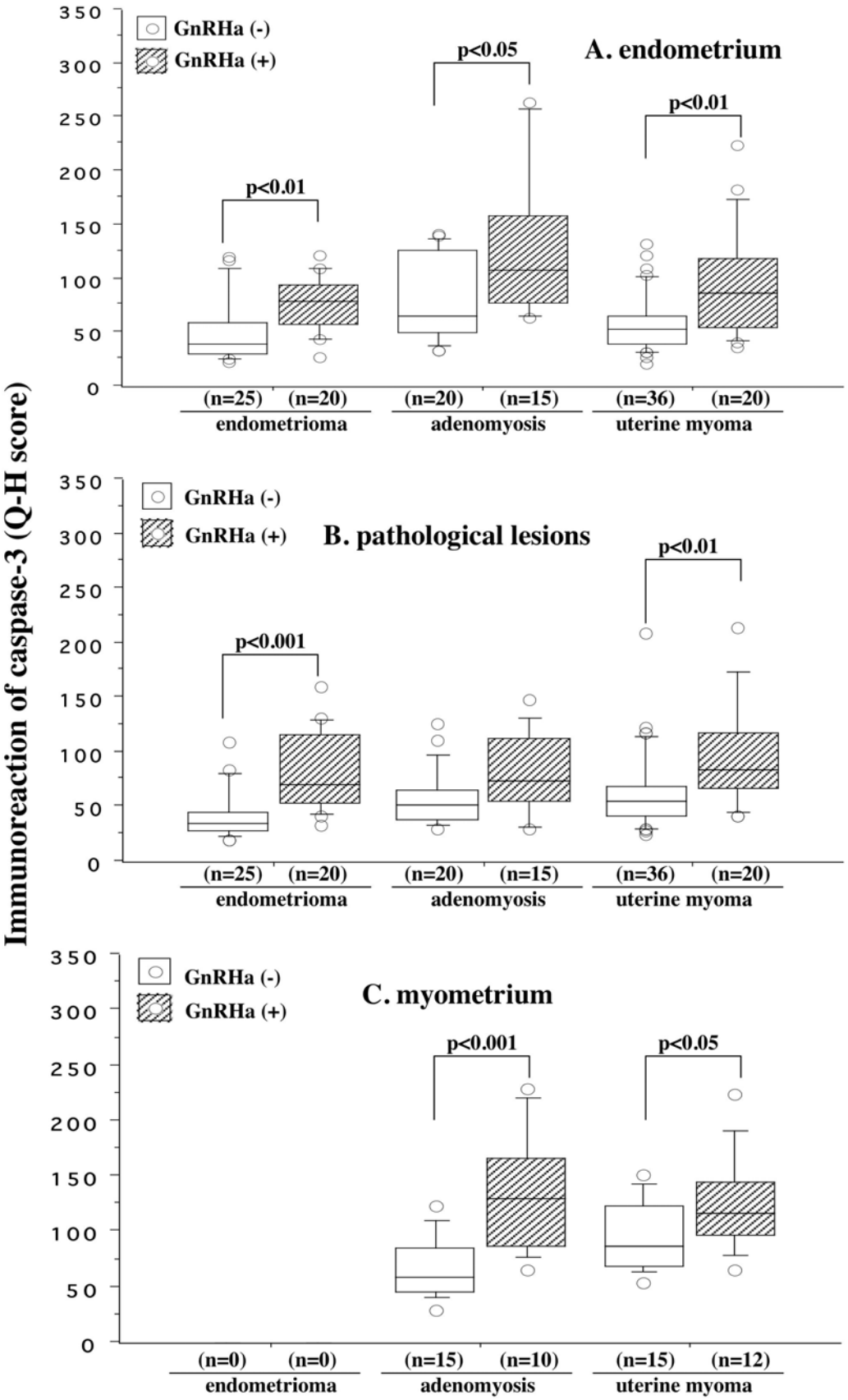
Immunoreaction of caspase-3 (Q-H score)
GnRHa (-)
GnRHa (+)
A. endometrium
p<0.01
p<0.05
p<0.01
B. pathological lesions
p<0.001
p<0.01
C. myometrium
p<0.001
p<0.05
(n=25) (n=20) (n=20) (n=15) (n=36) (n=20)
endometrioma adenomyosis uterine myoma
(n=0) (n=0) (n=15) (n=10) (n=15) (n=12)
endometrioma adenomyosis uterine myoma

Table 1. Clinical profiles of women with ovarian endometrioma, adenomyosis and uterine myoma.

	GnRHa (-)	GnRHa (+)
Ovarian endometrioma (n=45)	25	20
age in yrs (range)	26-39	28-38
age in yrs (mean ± SD)	30.4 ± 3.7	31.5 ± 2.4
size in cm (range)	4-10	6-10
size in cm (mean ± SD)	6.0 ± 2.1	7.5 ± 1.4
unilateral / bilateral	18 / 7	12 / 8
menstrual cycle: P/S/M/A	8/15/2/0	0/0/0/20
duration of therapy (month)		4-6
Adenomyosis (n=35)	20	15
age in yrs (range)	36-48	35-47
age in yrs (mean ± SD)	41.9 ± 4.0	42.1 ± 2.6
anterior wall /posterior wall/both	3 / 7 / 10	2 / 8 / 5
menstrual cycle: P/S/M/A	8/12/0/0	0/0/0/15
duration of therapy (month)		3-6
Uterine myoma (n=56)	36	20
age in yrs (range)	23-50	27-48
age in yrs (mean ± SD)	39.1 ± 6.2	36.1 ± 5.8
size in cm (range)	2.5-12	2.2-10
size in cm (mean ± SD)	5.0 ± 2.1	6.1 ± 1.3
SMM/IMM/SSM	18/16/2	10/10/0
menstrual cycle: P/S/M/A	12/18/6/0	0/0/0/20
duration of therapy (month)		3-6

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).