Cell proliferation effect of GnRH agonist on pathological lesions of women with endometriosis, adenomyosis and uterine myoma

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Submitted on March 23, 2010; resubmitted on July 24, 2010; accepted on August 9, 2010

Introduction

With the advent of isolation and synthesis of the gonadotrophin-releasing hormone (GnRH) by Schally et al. (1971) in the early 1970s, interest in the clinical application of GnRH agonist (GnRHa) has grown. Now in clinical practice, GnRHa has been used for the medical treatment of prostate cancer, precocious puberty, endometriosis, adenomyosis and uterine myoma. Traditionally, the effect of GnRHa is mediated by competitive down-regulation of pituitary GnRH receptors (GnRHRs), causing a state of hypo-estrogenemia resulting in the resolution of pain symptoms and regression of diseases.

Endogenous GnRH (GnRH I and GnRH II) as well as exogenous GnRHa have been demonstrated to exert anti-proliferative and apoptotic effects on cultured endometriotic cells and some cancer cells derived from reproductive organs (Borroni et al., 2000; Imai et al.,...
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 30 control women, 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, hypermenorrhoea or anaemia with or without associated complaints of dysmenorrhoea or pelvic pain. A fraction of these study groups had variable coexistent lesions of pelvic endometriosis. We also studied 35 women with biopsy-proven pelvic endometriosis. All women with pelvic and ovarian endometriosis, adenomyosis and uterine fibroids were diagnosed by ultrasonography and magnetic resonance image before operation, or by laparoscopy, and this was subsequently confirmed by histology. The phases of the menstrual cycle in women without hormonal therapy were determined by histological dating of eutopic endometria samples taken simultaneously with peritoneal lesions, endometrioma, adenomyoma and nodules.

The staging and morphological distribution of peritoneal lesions of endometriosis were based on the revised classification of the American Society for Reproductive Medicine (r-ASRM) (1997). As we described recently (Khan et al., 2004a), peritoneal lesions were categorized according to the colour appearance of pelvic endometriosis. Biopsy specimens from each of these peritoneal lesions were collected for subsequent experimental analysis. 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.

Biopsy specimens

Biopsy specimens were collected from the cyst wall, adenomyotic lesion, myoma nodule, autologous myometria and respective endometria from women with endometrioma, adenomyosis and uterine myoma during operation. Endometrial samples were collected from all women with and without GnRHa therapy. We also collected biopsy samples from the eutopic endometria, ectopic endometria and adjacent peritoneum of women with pelvic endometriosis and control women. All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study.

Isolation of endometrial cells

Isolation and culture of endometrial stromal cells and epithelial cells derived from the eutopic/ectopic endometria of women with endometriosis, adenomyosis and uterine myoma were processed as described previously (Koga et al., 2001; Khan et al., 2005a,b,c; 2008). Briefly, the tissues were minced into small pieces and incubated in Dulbecco’s modified Eagle’s medium (DMEM/F-12) containing collagenase Type I (2.5 mg/ml) (Sigma, St Louis, MO, USA) and deoxyribonuclease I (15 U/ml) (Takara, Tokyo, Japan) for 2−3 h at 37°C. The resultant dispersed endometrial cells were separated by filtration through a 60-μm nylon cell strainer (Becton Dickinson and Co., Franklin lakes, NJ, USA). Endometrial

| Table I Clinical profiles of women in the study group and control group. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | GnRHa (−)       | GnRHa (+)       |
| Ovarian endometrioma (n = 45) | 25 ± 3.7        | 31.5 ± 2.4      |
| Age in years (mean ± SD) | 30.4 ± 3.7      | 31.5 ± 2.4      |
| Size in cm (mean ± SD) | 6.0 ± 2.1       | 7.5 ± 1.4       |
| Menstrual cycle; P/S/M/A | 8/15/2/0        | 0/0/0/20       |
| Duration of therapy (month) | 4–6             |                 |
| r-ASRM Stages I and II | 25              | 20              |
| Adenomyosis (n = 35) | 20 ± 0.0        | 20 ± 0.0        |
| Age in years (mean ± SD) | 41.9 ± 4.0      | 42.1 ± 2.6      |
| Menstrual cycle; P/S/M/A | 8/12/0/0        | 0/0/0/15       |
| Duration of therapy (month) | 3–6             |                 |
| Hysterectomy done (n = 25) | 15              | 10              |
| Uterine myoma (n = 56) | 36 ± 2.0        | 36 ± 2.0        |
| Age in years (mean ± SD) | 39.1 ± 6.2      | 36.1 ± 5.8      |
| Size in cm (mean ± SD) | 5.0 ± 2.1       | 6.1 ± 1.3       |
| Menstrual cycle; P/S/M/A | 12/18/6/0       | 0/0/0/20       |
| Duration of therapy (month) | 3–6             |                 |
| Hysterectomy done (n = 27) | 15              | 12              |
| Pelvic endometriosis (n = 35) | 35              |                 |
| Age in years (mean ± SD) | 28.6 ± 4.2      |                 |
| Menstrual cycle; P/S/M | 10/20/5        |                 |
| r-ASRM Stages I and II | 35              |                 |
| Opaque/non-opaque/ | 20/26/30       |                 |
| black+white |                 |                 |
| Control group (n = 30) | 30              |                 |
| Age in years (mean ± SD) | 28.4 ± 3.9      |                 |
| Menstrual cycle; P/S/M | 10/15/5        |                 |
| Dermoid/serous or mucinous cyst | 22/8          |                 |

The results are expressed as mean ± SD. GnRHa (−), without GnRHa therapy; GnRHa (+), with GnRHa therapy; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhoea; r-ASRM, revised staging of American Society of Reproductive Medicine.
epithelial glands that remained intact were retained by the strainer whereas dispersed stromal cells passed through the strainer into the filtrate.

Endometrial stromal cells in the filtrate were collected by centrifugation and re-suspended in phenol-red free DMEM/F-12 containing 10% charcoal-stripped FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (all from Sigma). Stromal cells were seeded in a 100-mm culture plate and kept at 37°C in a humidified 5% CO₂–95% air atmosphere. At the first passage, cells were plated at a density of 2×10⁵ cells/ml. The cells reached confluence in 2 or 3 days and then were used for experiments.

The chocolate cyst linings of the ovaries were collected as the source of endometriotic tissues. Stromal cells from cyst walls were collected according to the method described in detail previously (Iwabe et al., 1998). We used stromal cells in a monolayer culture after the first passage.

Endometrial epithelial cells were collected by back washing the strainer with DMEM/F-12 containing 10% charcoal-stripped FBS, seeded in a 100-mm plate and incubated at 37°C for 60 min to allow contaminated stromal cells to attach to the plate wall. The non-attached epithelial cells were recovered and cultured in the medium at a density of 2×10⁵ cells/ml. The cells, which reached confluence in 2 or 3 days, were used for experiments. The purity of stromal and epithelial cell preparation was more than 95%, as judged by positive cellular staining for vimentin and cytokeratin respectively and negative cellular staining for CD45 (pan-leukocytes) and von Willebrand factor (vWF; endothelial cells).

Isolation of smooth muscle cells

Biopsy specimens from each of the adenomyotic lesions and myoma nodules of GnRHα-treated and -non-treated women with adenomyosis and uterine myoma were collected and smooth muscle cells were isolated in primary culture as described previously (Rossi et al., 1992).

Briefly, fresh biopsy specimens collected in sterile medium were rinsed with PBS to remove blood cells. The tissues were cut into small pieces and digested in 2% collagenase (Sigma, St Louis, MO, USA) with DMEM and incubated in a shaking water bath at 37°C for 3–6 h. Isolated smooth muscle cells were collected by centrifugation at 460g for 5–10 min, washed several times with DMEM containing 1% antibiotic-antimycotic solution (Sigma) and cultured in 75 cm² flasks in different aliquots. The isolated smooth muscle cells were grown as monolayers in 75 cm² flasks at a density of 2 × 10⁵ cells per flask. The cells were maintained in pheno-red free DMEM supplemented with charcoal-stripped 10% FBS and 1% antibiotic-antimycotic solution for 6 days at 37°C in a humidified culture atmosphere.

**Figure 1** Standard RT–PCR analysis of the expression of Type I GnRHR and Type II GnRHR mRNA in the eutopic endometrial tissues of a control woman (Lane 1) and a woman with pelvic endometriosis (Lane 2), and in isolated endometriotic epithelial (Lane 3) and stromal cells (Lane 4) are shown in the upper panel (A). The lower panel (B) shows the mRNA expression of Types I and II GnRHR in the eutopic endometrium (Lane 1) and cyst wall (Lane 2) of a woman with endometrioma, in the eutopic endometrium (Lane 3) and adenomyotic tissues (Lane 4) of a woman with adenomyosis, and in the eutopic endometrium (Lane 5) and nodule (Lane 6) of a woman with uterine myoma. The PCR products show the predicted sizes on the basis of cDNA sequence. M represents molecular marker; RT (–) indicates negative control with no reverse transcription. The PCR band of each lane is representative of six different samples with similar results.
Gene expression of GnRHR

Total RNA was isolated using the ISOGEN method (Molecular Research Center, Tokyo) according to the manufacturer’s protocol. RNA (1 μg) was added to the reverse transcription reaction (RT–PCR), and cDNA (1 μl) was subjected to real-time qPCR using a Light Cycler (Roche Diagnostics, Mannheim, Germany). All primers and probes and amplification conditions for Type I GnRHR and Type II GnRHR were designed and described previously (Koga et al., 2000; Morimoto et al., 2005). Reaction parameters were as follows: for GnRHR, 40 cycles of denaturing (95°C, 15 s), annealing (62°C, 10 s) and extension (72°C, 12 s); for β-actin, 30 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. All PCR conditions were followed by melting curve analysis.

The gene expression levels of Type I GnRHR and Type II GnRHR were calculated and normalized by dividing the corresponding values of the housekeeping gene β-actin (GB accession number NM001101). Each PCR product was purified with a QIAEX II gel extraction kit, and their identities were confirmed using an ABI PRISMTM 310 genetic analyser (Applied Biosystems, Foster city, CA, USA).

Antibodies used

We performed immunohistochemical studies to investigate the immunoreaction of GnRHR in intact tissues. GnRHR (AT2.G7:sc-57176), a mouse monoclonal antibody against both Type I and Type II receptor (1:25 dilution), was used to immunolocalize GnRHR expression in tissues (Santa Cruz Biotechnology, Inc., CA, USA). Ki-67 (MIB-1, Immunotech, Marseille, France), and a mouse monoclonal antibody (1:100) was used to immunolocalize proliferating cells in biopsy specimens. Non-immune mouse immunoglobulin (Ig) G1 antibody at a 1:50 dilution was used as a negative control.

Immunohistochemistry

The details of immunocytochemical or immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004b, 2008; Ishimaru et al., 2004). We used at least two biopsies per patient and three slides per biopsy for immunohistochemical analysis.

The immunoreactivity of GnRHR in biopsy specimens was quantified by a modified method of quantitative-histogram score (Q-H score) as described recently (Khan et al., 2003, 2005c; 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 = none, 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). The cell proliferation index (Ki-67 index) in each tissue section was calculated by measuring the mean percentage of Ki-67-positive nuclei among total cells in four different microscopic fields (×200).

Cell proliferation assays

The 5-bromo-2-deoxyuridine (BrdU) labelling and detection kit measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993). The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak, Nagasaki University Library on February 29, 2012 http://humrep.oxfordjournals.org/ Downloaded from
Amersham Pharmacia Biotech Ltd, UK) using monoclonal antibodies directed against BrdU. It offers a non-radioactive alternative to the [3H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993). We examined the proliferation of epithelial cells, stromal cells and smooth muscle cells in response to variable doses (10⁻² – 10⁻⁵ M) of GnRHa (Leuplin: leuprolide acetate, Takeda, Tokyo, Japan).

The detailed procedure of BrdU incorporation assay has been described previously (Khan et al., 2005a,b,c). Briefly, desired cells (endometrial cells and smooth muscle cells) were cultured in 96-well microtiter plate (10⁴ cell/well). After a 24 h pre-incubation period without serum, respective cells were treated with or without GnRHa in a serum-free medium and incubated for an additional 24 h. After that, the cells were labelled with 10⁻⁶ M of BrdU (100 µl/well) and incubated for 4 h at 37°C. The cells were fixed and genomic DNA was denatured by adding 200 µl/well of blocking reagent (1:10) for 30 min at room temperature. Peroxidase-labelled anti-BrdU antibody (1:100) was added (100 µl/well) and incubated for 90 min at room temperature. After washing three times, TMB (3,3′,5′,5′-tetramethylbenzidine) substrate solution was added (100 µl/well) and incubated for 15 min at room temperature for colour appearance and finally optical density was measured using a microplate reader at an absorbance of 450 nm. The intra- and inter-assay coefficients of variation were <10% for this assay.

The values of BrdU-incorporated cells in response to the GnRHa were expressed as the percentage of controls (non-treated cells). The cells treated with BrdU diluent (PBS solution) were used as negative control. The absorbance values correlated directly to the amount of DNA synthesis and to the number of proliferating cells in culture.

**Statistical analysis**

All results are expressed as either mean ± SEM or median and inter-quartile range (IQR). The clinical characteristics of the subjects were compared with one-way analysis of variance and the X² test for any difference between two groups. Mann–Whitney U-test or Student’s t-test was used to analyse any difference in cell proliferation or protein expression between two groups. For comparisons among groups, the Kruskal–Wallis test was used. A value of P < 0.05 was considered to be statistically significant.

**Results**

The clinical features of women with ovarian endometrioma, adenomyosis and uterine myoma between the GnRHa-treated group and GnRHa-non-treated group and of women with pelvic endometriosis and of control women are 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, 3–6 months each for women with adenomyosis and uterine myoma. Ten women with ovarian endometriosis, 10 with adenomyosis and 8 with uterine myoma had coexisting peritoneal lesions.

Gene expression of GnRHR in endometriosis, adenomyosis and uterine myoma

As shown in Fig. 1, standard RT–PCR analysis demonstrated amplified products of Type I GnRHR and Type II GnRHR in the eutopic endometria of control women and women with pelvic endometriosis as well as in cultured endometriotic epithelial cells and stromal cells (Fig. 1A). Both Type I and Type II GnRHR were also detected in tissues derived from cyst wall, adenomyotic tissue, myoma nodule and their corresponding eutopic endometria (Fig. 1B). Each PCR product was sequenced and confirmed to be identical to the sequence of Type I GnRHR (Chi et al., 1993) and Type II GnRHR (Grundker et al., 2002). The relative gene levels of GnRHR in the eutopic and ectopic endometria are shown in Table II. An almost similar level of GnRHR gene expression was observed in all studied cells and tissues.

Immunohistochemical staining of GnRHR (A) and (Q-H) scores of GnRHR immunoreactivity (B) in different peritoneal lesions (white bar) based on colour appearance and in their adjacent peritoneum (black bar) derived from 35 women with pelvic endometriosis. The left upper column of (A) shows HE staining of respective peritoneal lesions, the middle column shows immunostaining of GnRHR in peritoneal lesions and the right column shows immunostaining of GnRHR in adjacent peritoneum of respective peritoneal lesions. The Q-H scores of GnRHR expression were significantly higher in the blood-filled opaque red lesions and in their adjacent peritoneum (*P < 0.05 for each) when compared with that in non-opaque red lesions or black and white (blueberry spot) lesions. 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 Immunohistochemical staining of GnRHR (A) and (Q-H) scores of GnRHR immunoreactivity (B) in different peritoneal lesions (white bar) based on colour appearance and in their adjacent peritoneum (black bar) derived from 35 women with pelvic endometriosis. The left upper column of (A) shows HE staining of respective peritoneal lesions, the middle column shows immunostaining of GnRHR in peritoneal lesions and the right column shows immunostaining of GnRHR in adjacent peritoneum of respective peritoneal lesions. The Q-H scores of GnRHR expression were significantly higher in the blood-filled opaque red lesions and in their adjacent peritoneum (*P < 0.05 for each) when compared with that in non-opaque red lesions or black and white (blueberry spot) lesions. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

GnRHa and cell proliferation in reproductive diseases

The immunostaining of GnRHR was found to be weak during the proliferative phase (Fig. 2A, upper column), moderate during the secretory phase (Fig. 2A, middle column) and strong during the menstrual phase (Fig. 2A, lower column). The immunoreactivity of GnRHR as measured by Q-H scores showed similar results (Fig. 2B). The tissue localization of GnRHR was found in both gland cells and stromal cells. Compared with control samples, GnRHR expression was found to be higher in samples derived from the secretory phase or menstrual phases of women with endometriosis (Fig. 2B). We did not find any significant difference in GnRHR expression between samples derived from 35 women with Stages I and II endometriosis and 25 women with Stages III and IV endometriosis (Fig. 2A and B). We also found GnRHR expression in the endometria, pathological lesions and myometria derived from women with endometrioma, adenomyosis and uterine myoma (data not shown).
**Immunoexpression of GnRHR in peritoneal lesions and adjacent peritoneum**

When we distributed immunoexpression of GnRHR according to the different peritoneal lesions and their adjacent peritoneum of pelvic endometriosis, we found a significantly stronger immunoreaction and higher Q-H scores of GnRHR expression in the blood-filled opaque red lesions and their adjacent peritoneum (\(P < 0.05\) for both) when compared with that in non-opaque red lesions, or black and white lesions and their adjacent peritoneum (Fig. 3A and B).

**Immunoexpression of GnRHR in GnRHa-treated and -non-treated samples**

As shown in Fig. 4, we did not find any significant difference in GnRHR immunoexpression and Q-H scores in the endometria (upper column), cyst wall (middle column) and coexisting peritoneal lesions (lower column) between GnRHa-treated and GnRHa-non-treated samples derived from women with ovarian endometrioma. Non-immune mouse IgG-stained slides are shown against each specimen. We did not find any significant difference in the Q-H scores of GnRHR expression in samples between GnRHa-non-treated \((n = 25)\) (white bar) and GnRHa-treated \((n = 20)\) (black bar) women with endometrioma \((B)\). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

**Effect of GnRHa on the proliferation of endometrial cells**

After an initial time-dependent study from Day 1 to Day 3, we found a significant inhibitory response of GnRHa on cell proliferation from Day 2 to Day 3 without any variation among these days. Therefore, all the following dose-dependent studies were performed with an incubation period of 48 h. We examined the direct cell proliferation effect of a variable dose of GnRHa on epithelial cells and stromal cells derived from the endometria of 25 women with ovarian endometrioma, 20 women with adenomyosis and 36 women with uterine myoma. All these women had no GnRHa treatment before surgery.

As shown in Fig. 5, GnRHa, at a concentration between \(10^{-9}\) and \(10^{-5}\) M, caused a significant and a dose-dependent inhibition of BrdU incorporation into DNA of endometrial epithelial cells and stromal cells derived from women with ovarian endometrioma \((A)\), adenomyosis \((B)\) and uterine myoma \((C)\). The maximal anti-proliferative...
An effect (30–32% decrease below the control) was observed at $10^{-5}$ and $10^{-6}$ M concentration of leuprolide acetate.

Although lower ($10^{-9}$ and $10^{-8}$ M) to higher doses ($10^{-7}$–$10^{-5}$ M) of GnRHa were able to inhibit BrdU incorporation into endometrial cells derived from women with ovarian endometrioma (A), adenomyosis (B) or uterine myoma (C), both GnRHa-treated and -non-treated endometrial epithelial cells and stromal cells were incubated for 48 h. Results are shown as the mean percentage of the untreated cells ($\pm$ SEM) of triplicate experiments using cells derived from different women who were responsive to GnRHa. Black and white bars at far right indicate negative control of cells treated with BrdU diluent. *$P < 0.05$ versus non-treated cells for (A–C).

Figure 5 Effects of GnRHa (leuprolin acetate) on BrdU incorporation into epithelial cells (black bar) or stromal cells (open bar) derived from the eutopic endometria of women with ovarian endometrioma (A), adenomyosis (B) or uterine myoma (C). Both GnRHa-treated and -non-treated endometrial epithelial cells and stromal cells were incubated for 48 h. Results are shown as the mean percentage of the untreated cells ($\pm$ SEM) of triplicate experiments using cells derived from different women who were responsive to GnRHa. Black and white bars at far right indicate negative control of cells treated with BrdU diluent. *$P < 0.05$ versus non-treated cells for (A–C).

**Figure 6** Effects of GnRHa (leuprolin acetate) on BrdU incorporation into stromal cells (A) derived from cyst wall of women with ovarian endometrioma or smooth muscle cells derived from the pathological lesions of women with adenomyosis (B) or uterine myoma (C). Cyst wall stromal cells and respective smooth muscle cells were treated with GnRHa at the indicated concentration for 48 h. Results are shown as the mean percentage of the untreated cells ($\pm$ SEM) of triplicate experiments using cells derived from different women who were responsive to GnRHa. The white bar indicates negative control of cells treated with BrdU diluent. *$P < 0.05$ versus non-treated cells for (A–C).

and dead cells were not different between GnRHa-non-treated and GnRHa-treated cells (data not shown).

**Effect of GnRHa on the proliferation of cells derived from pathological lesions of women with endometrioma, adenomyosis and uterine myoma**

We examined direct cell proliferation effect of GnRHa on cyst wall stromal cells derived from women with ovarian endometrioma and
on smooth muscle cells derived from adenomyotic lesions or myoma nodules (Fig. 6).

We found that GnRHa (leuprolide acetate) was able to significantly suppress BrdU incorporation into cyst wall stromal cells at a dose of $10^{-7} - 10^{-5}$ M (Fig. 6A, $P < 0.05$ versus non-treated cells), into smooth muscle cells derived from adenomyotic lesions at a dose of $10^{-6}$ and $10^{-5}$ M (Fig. 6B, $P < 0.05$ versus non-treated cells) and into smooth muscle cells derived from myoma nodules at a dose of $10^{-8} - 10^{-5}$ M (Fig. 6C, $P < 0.05$ versus non-treated cells).

We did not find any significant anti-proliferative effect of GnRHa (leuprolide acetate) on cells derived from endometria (data not shown) or pathological lesions in 8/25 (32%) of women with endometrioma, in 6/20 (30%) of women with adenomyosis and in 10/36 (28%) of women with uterine myoma (Fig. 7).

**Effect of GnRHa on the proliferation of cells derived from the eutopic and ectopic endometria of women with pelvic endometriosis**

In addition to its effect on cells derived from the endometria and pathological lesions of women with ovarian endometrioma, adenomyosis and uterine myoma, we also found that GnRHa retains its direct cell proliferation effect on cells derived from women with pelvic endometriosis.

As shown in Fig. 8, GnRHa was able to significantly suppress BrdU incorporation into epithelial cells and stromal cells derived from the eutopic endometria (A) and ectopic endometria (B) of women with pelvic endometriosis. Both GnRHa-treated and non-treated epithelial cells and stromal cells were incubated for 48 h. Results are shown as the mean percentage of the untreated cells (± SEM) of triplicate experiments using cells derived from women who were responsive to GnRHa. $*P < 0.05$ versus non-treated cells for (A) and (B).
not find any significant anti-proliferative effect of GnRHa on cells derived from 8/35 (23%) of women with pelvic endometriosis (data not shown).

**Immunoeexpression of Ki-67 in endometria and pathological lesions**

In order to examine the cell proliferation effect of GnRHa in intact tissue, we investigated immunoreaction of Ki-67, a cell proliferation marker, biopsy specimens derived from the endometria and respective pathological lesions of women, with ovarian endometrioma, adenomyosis and uterine myoma, who were either treated or not treated with GnRHa.

Figure 9 shows the tissue localization of Ki-67 in the endometria and pathological lesions of GnRHa-treated and -non-treated biopsy specimens derived from women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C). GnRHa (–), GnRHa-non-treated samples; GnRHa (+), GnRHa-treated samples.

**Discussion**

Although the anti-proliferative effect of exogenous GnRHa or endogenous GnRH II on endometrial cells derived from women with endometriosis is known (Borroni *et al.*., 2000; Morimoto *et al.*, 2005), we further demonstrated the direct anti-proliferative effect of GnRHa on the cells derived from endometria and pathological lesions of four groups of women with ovarian endometriosis, pelvic endometriosis, adenomyosis or uterine myoma. Based on the limited information on the presence of GnRHR expression in peripheral tissues, we could detect both gene and protein expression of GnRHR in cells and tissues derived from these women.

We found that exogenous treatment with a variable concentration of GnRHa was able to significantly suppress the proliferation of cells derived from the endometria and pathological lesions of women not only with endometriosis but also of women with adenomyosis and uterine myoma. These direct anti-proliferative effects of GnRHa *in vitro* correspond to *in vivo* results with Ki-67, a cell proliferation marker, in intact tissues. In fact, Ki-67 index was significantly lower in both endometria and pathological lesions derived from GnRHa-treated women than in samples from GnRHa-non-treated women. Although we could not exclude the hypo-estrogenic effect of GnRHa on the changes in Ki-67 index, our *in vitro* cell proliferation study confirmed a direct anti-proliferative effect of GnRHa on pathological lesions.

Additional interesting findings of our current study are that both eutopic and ectopic endometrial cells derived from women with
pelvic endometriosis equally express GnRHR at both the gene and protein level and that GnRHa also retains its direct anti-proliferation effect on both eutopic and ectopic endometrial cells derived from women with pelvic endometriosis. This indicates that when women with ovarian endometrioma, adenomyoma or uterine myoma are treated with GnRHa, this estrogen suppressing agent is equally effective in reducing the growth of peritoneal lesions coexisting with these reproductive diseases.

Although we did not find any significant difference in GnRHR protein expression between early (Stages I and II) and advanced endometriosis (Stages III and IV), we found increased GnRHR expression at different phases of the menstrual cycle. Our results are consistent with previous results that demonstrated protein expression of GnRHR throughout all phases of the menstrual cycle with a significant increase in the secretory phase when compared with the proliferative phase (Casañ et al., 1998; Raga et al., 1998). Again, we did not find any significant difference in GnRHR protein expression in biopsy samples between GnRHa-treated and GnRHa-non-treated women with endometrioma, adenomyosis and uterine myoma. This indicates that unlike the central pituitary action, the anti-proliferative effect of GnRHa on each cell type may not depend on the down-regulation of GnRHR but rather on the amount and binding affinity of GnRHa for its receptor in peripheral tissues. Further studies are needed to clarify our current findings.

We also found higher immunoexpression of GnRHR in the blood-filled opaque red lesions and their adjacent peritoneal lesions than in non-opaque or other less active peritoneal lesions and their adjacent peritoneum derived from women with pelvic endometriosis. This could be due to increased production of different pro-inflammatory cytokines and growth factors by opaque red lesions than by other peritoneal lesions (Khan et al., 2004a). These results are biologically significant. GnRHa treatment may not only suppress the growth of peritoneal lesions coexisting with these reproductive diseases, this treatment may also reduce blood loss or inflammatory reactions in the pelvic environment. In fact, we recently demonstrated that GnRHa was able to suppress the inflammatory response and the angiogenic response and induce apoptosis in women with endometriosis, adenomyosis and uterine myoma (Khan et al., 2010).

One important finding of our current study is that exogenous treatment with similar doses of GnRHa did not affect cell growth in 23% of women with pelvic endometriosis, 32% of women with endometrioma, 30% of women with adenomyosis and 28% of women with uterine myoma regardless of the expression of GnRHR. Our results are supported by the study of Borroni et al. (2000) who also found no anti-proliferative effect of GnRHa (leuprolide acetate) on endometriotic cells derived from 60% of cases with ovarian endometriosis. In addition, we found a differential cellular response of GnRHa in different diseases, more in cells from endometriosis and myoma and less in cells from adenomyosis. This cellular variation in growth inhibition in response to GnRHa treatment can be explained by a difference in cellular histogenesis or a difference in GnRHR—ligand binding affinity in cells derived from women with different reproductive diseases. In fact, previous studies investigating the presence of GnRHR in different tissues revealed the presence of high affinity/low capacity and low affinity/high capacity binding sites (Qayum et al., 1990; Limonta et al., 1992; Emons et al., 1993a,b) that may coexist in the same tissue.

Leuprolide acetate, the type of GnRHa we used in our current study, has been found to interact with Type I GnRHR, where Type II GnRHR functions as a co-receptor (Borroni et al., 2000; Imai et al., 2000). In our present study, concentrations of GnRHa used are within the pharmacological range in the peripheral circulation. In humans, serum leuprolide acetate concentration after subcutaneous injection of 1.88 and 3.75 mg may range from $10^{-8}$ to $10^{-7}$ M and $0.5 \times 10^{-6}$ to $10^{-5}$ M, respectively, over a 5-week period (Mazzei et al., 1990). Thus, theoretically, this compound may affect endometrial cell proliferation also in vivo.

Finally we conclude that in addition to ovarian endometriosis, peripheral tissues derived from women with pelvic endometriosis, adenomyosis and uterine myoma equally express GnRHR at the gene and protein levels. In addition to the hypo-estrogenic effect and other multifunctional roles of GnRHa in peripheral tissues that we described recently (Khan et al., 2010), GnRHa may also exert a direct anti-proliferative effect on different cells derived from women with these reproductive diseases. All these biological functions of GnRHa may
be involved in the regression of these diseases with consequent remission of symptoms. Further studies are needed to strengthen our current findings.

Acknowledgements
We thank Dr Tetsuro Samejima, the Japanese Red Cross Nagasaki Atomic Bomb Hospital, Nagasaki; Miss Kazumi Hayashida and Miss Kyoko Ishida, Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences for their excellent technical assistance.

Funding
This work was supported by Grants-in-Aid for Scientific Research (Grant No. 16591671 and 18591837) from the Ministry of Education, Sports, Culture, Science and Technology of Japan (to K.N.K.).

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