Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis

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The objective of this study was to determine the distribution of cyclooxygenase-2 (COX-2) in eutopic and ectopic endometria in endometriosis and adenomyosis. The subjects were 35 patients with endometriosis diagnosed by laparoscopy, 33 patients with histologically confirmed adenomyosis and 50 female controls with normal fecundity. Expression of COX-2 was immunohistochemically investigated in tissues from eutopic endometrium and myometrium and ectopic endometrium of the wall of ovarian chocolate cysts using polyclonal antibody. Surface epithelial cells, endometrial glandular epithelial cells or stromal cells were assessed. Cells were semi-quantitatively assessed on a scale of 1 to 5 using a nomogram created from positive cell count and the degree of staining. COX-2 expression in surface and glandular epithelia of the control group varied markedly during the menstrual cycle. It was lowest in the early proliferative phase and gradually increased thereafter. It remained high throughout the secretory phase. However, in patients with endometriosis, expression of COX-2 in glandular epithelium was higher than that in the control group, though it varied throughout the menstrual cycle. On the other hand, there was no variation in expression of COX-2 in the adenomyosis group during the menstrual cycle, and it was lower than that in the endometriosis group in all phases. Pronounced COX-2 expression was observed in glandular cells from ectopic endometrial tissue of ovarian chocolate cyst walls in all cases regardless of the menstrual phase. In summary, increased COX-2 expression in eutopic and ectopic endometria was believed to be strongly correlated with pathological abnormalities in these disorders.

Key words: adenomyosis/cyclooxygenase/endometriosis/endometrium/prostaglandin

Introduction

Cyclooxygenase-2 (COX-2) has prostaglandin (PG) hydroperoxidase activity to synthesize PGH2 from PGG2. COX-2 exists in two isoforms, COX-1 and COX-2. The human COX-1 gene is localized on chromosome 9 and the COX-2 gene is localized on chromosome 1 (Hla and Neilson, 1992; Appleby et al., 1994). They are governed by completely different genes, but their protein structures are very similar. COX-1 is constitutively present in almost all tissues. It acts to maintain cell homeostasis (Zweifel et al., 1995). COX-2 is almost never expressed under normal conditions. It is induced during inflammation and cell proliferation and differentiation. It is expressed in macrophages, fibroblasts, vascular endothelial cells, neurons and chondrocytes as a result of interleukin-1 (IL-1) (O’Banion et al., 1992), human chorionic gonadotrophin (Sirois and Richards, 1992) or serum (Xie et al., 1991) stimulation. COX-2 is not only induced by the site of inflammation, but also produced in colon cancer tissues (Sheng et al., 1998). COX-2 is also intimately involved in reproductive functions. It has been proven that COX-2 knock-out mice were infertile, the corpus luteum was missing and the uterus was small (Dinchuk et al., 1995).

It is known that human endometrium produces PGE2 and PGF2α (Smith and Kelly, 1988). It has been reported (Jones et al., 1997) that COX-2 is distributed in endometrial glandular epithelia and vascular endothelia and that it varies during the menstrual cycle. Other studies have also found that COX-2 is distributed in the human placenta (Wetzka et al., 1997) and decidual tissue (Ishihara et al., 1995). A significant amount of prostaglandins are produced from the endometrium and endometriotic tissues (Lumsden et al., 1984). It is interesting to note that low concentrations of arachidonic acid are only metabolized by COX-2 (Morita et al., 1995). Accordingly, COX-2 expression may be abnormal in endometriosis, but there appear to be no reports of investigations of COX-2 kinetics in this disorder. Therefore, in this study, we investigated the expression of COX-2 in eutopic and ectopic endometria in endometriosis.
Materials and methods

Patients

The subjects consisted of 118 women who were treated at the Department of Obstetrics and Gynecology at Akita University Hospital. They were divided into three groups: (i) fertile controls; (ii) the endometriosis group (n = 35) which included women diagnosed by laparoscopy; and (iii) the adenomyosis group (n = 33), who had undergone hysterectomy. Endometriosis patients associated with adenomyosis were excluded from the present study and vice versa. The controls consisted of 50 fertile women with regular and biphasic menstrual cycles, 20 in the proliferative phase and 30 in the secretory phase. All of the controls were parous women with clear male factor infertility (mild oligozoospermia or azoospermia). None of controls had identifiable endometriosis confirmed by laparoscopy and adenomyosis by serum CA-125, ultrasonography and/or magnetic resonance imaging.

These women conceived after artificial insemination using husband’s or donor’s semen within three treatment cycles and delivered full-term babies. The mean ages in the fertile control group, the endometriosis group, and the adenomyosis group were 28.9 years (range 22–36 years), 32.1 years (range 23–41 years), and 43.1 years (range 34–49 years) respectively.

Before starting any medication in the control group, or just after laparoscopy in the patients with endometriosis or after hysterectomy in the patients with adenomyosis, endometrial tissue was obtained when it became available during any phase of the menstrual cycle. Ectopic endometrial tissues in ovarian chocolate cysts in endometriosis (n = 10) or in the myometrium in adenomyosis (n = 9) were studied in the identical patients at the same time. In the control and the endometriosis group, endometrial specimens were obtained by curettage. The tissues were fixed in neutral-buffered 10% formalin solution. The menstrual cycle of the patients was estimated by endometrial dating according to previously described criteria (Noyes et al., 1950). Informed consent was obtained in each case, and approval for the study was granted by the Institutional Review Board.

Reagents

The goat polyclonal antibody for human COX-2 (sc-1745) raised against a peptide mapping at the carboxy terminus of COX-2 of human origin was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The second antibody [rabbit anti-sheep immunoglobulins (Ig) H+L horseradish peroxidase conjugate; 6155–04] was obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA).

Staining

The endometrial tissue samples were cut into blocks (~1 cm³). Serial 3 mm sections of tissue were cut, deparaffinized, and rehydrated through ethanol, as in routine histology. The sections were stained using the indirect method. Before the staining, a microwave antigen retrieval technique was utilized, whereby the sections were heated in sodium citrate buffer (pH 6.0) for 5 min at high power (500 W), and 0.05 was considered to be statistically significant.

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Statistical analyses

The results are expressed as the mean ± SEM where applicable. Statistical analysis was performed by the Kruskal–Wallis test. P < 0.05 was considered to be statistically significant.

Results

Variation in COX-2 expression during the menstrual cycle in eutopic endometrium

Expression of COX-2 in the surface epithelium in the control group varied markedly during the menstrual cycle (Figure 1, Table I). It was lowest in the early proliferative phase and gradually increased thereafter. It remained high throughout the

Table I. Evaluation of nomogram scores of cyclooxygenase-2 in the surface epithelium during the phases of the menstrual cycle in endometriosis and adenomyosis. Values are presented as mean ± SEM. Values in parentheses indicate the number of patients examined for the antigen

<table>
<thead>
<tr>
<th>Phase of menstrual cycle</th>
<th>Study group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control⁴</td>
</tr>
<tr>
<td>Progesterone phase</td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>1.0 ± 0.0 (3)</td>
</tr>
<tr>
<td>Middle</td>
<td>1.8 ± 0.2 (8)</td>
</tr>
<tr>
<td>Late</td>
<td>2.1 ± 0.1 (9)</td>
</tr>
<tr>
<td>Secretory phase</td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>2.8 ± 0.2 (12)</td>
</tr>
<tr>
<td>Middle</td>
<td>2.8 ± 0.2 (10)</td>
</tr>
<tr>
<td>Late</td>
<td>2.9 ± 0.3 (8)</td>
</tr>
</tbody>
</table>

⁴P < 0.0001 between the six phases in the control group by the Kruskal–Wallis test.
⁵P < 0.05 between the six phases in the endometriosis group by the Kruskal–Wallis test.

P < 0.05 among the three groups by the Kruskal–Wallis test.

tetrahydrochloride containing 0.005% hydrogen peroxide in PBS. Finally, the sections were counterstained with Carazzi’s haematoxylin. Negative controls for immunostaining were prepared by substituting the first antibody with non-immune rabbit serum IgG. In each run, a section of placenta with strong COX-2 staining was routinely included as a positive control.

Evaluation of staining

Ten non-overlapping fields of view were examined per biopsy in a systematic random sampling pattern (magnification ×400). Surface and glandular epithelia and stromal cells in eutopic and ectopic endometria were evaluated for COX-2 staining. Staining was evaluated using an evaluation nomogram as previously reported (Ota and Igarashi, 1993). Briefly, each section was graded according to the frequency of positive cells and intensity of staining in endometrium. The frequency was defined as 1+, 2+ or 3+ when the number of positive cells in the endometrium in each section was <10%, 10–50% or >50% respectively. Intensity was defined as 3+ when staining of the cells was as strong as that observed in the positive controls, as 1+ when staining was weakly positive but distinct from the negative controls, and as 2+ when the staining was between 1+ and 3+. The sections were ranked from 1 to 5 according to the evaluation nomogram. Sampling and grading of each specimen were done by two different observers blinded as to the specimen source. Sections were assigned a score by a first observer, and confirmed by a second observer.
secretory phase. In patients with endometriosis, however, it varied throughout the menstrual cycle, but the range of the variation was narrower than that in the control group. In the late proliferative phase, expression of COX-2 was significantly more pronounced than that in the control group. On the other hand, there was no variation in expression of COX-2 in the adenomyosis group during the menstrual cycle.

COX-2 expression in the glandular epithelium in the control group showed a similar tendency to the shift in expression in the surface epithelium (Figure 2, Table II). In other words, it was lowest in the early proliferative phase and gradually increased thereafter. It peaked at the early secretory phase, and the same amount of expression was maintained up to the late secretory phase. In patients with endometriosis, however, there was a significant variation in COX-2 expression during the menstrual cycle, but, like the surface epithelium, the difference in expression was reduced. In the mid- to late proliferative phases, expression of COX-2 was significantly more pronounced than that in the control group. On the other hand, there was no variation in expression of COX-2 in the adenomyosis group during the menstrual cycle, and COX-2 expression was lower than that in the endometriosis group in all phases.

Expression of COX-2 in stromal cells in the control group was weaker than that in the surface and glandular epithelia, and there was no variation during the menstrual cycle (Table III). In patients with endometriosis, unlike that in the surface and glandular epithelia, there was no variation during the menstrual cycle, but COX-2 expression tended to be slightly higher than that in the control group. In patients with adenomyosis, there was no difference from the control group.
**Figure 3.** Cells stained for cyclooxygenase-2 antigen. Eutopic endometrium in (A) the late proliferative phase in the control group (evaluation nomogram score, 2) and (B) in the early secretory phase in the control group (evaluation nomogram score, 3). Eutopic endometrium in (C) the early secretory phase in the endometriosis group (evaluation nomogram score, 4) and (D) surface epithelium in the late secretory phase in the adenomyosis group (evaluation nomogram score, 4). (E) Ectopic endometrial tissue in the late secretory phase in the endometriosis group (evaluation nomogram score, 3) and (F) ectopic endometrial tissue in the late secretory phase in the adenomyosis group (evaluation nomogram score, 3). (A–D) ×100 magnification; (E and F) ×50 magnification.

**COX-2 expression in ectopic endometrium**

Expression of COX-2 was observed in ectopic endometrial tissue in all cases. Mean evaluation nomogram levels in glandular epithelium and stromal cells in ovarian chocolate cyst wall were 3.0 ± 0.2 and 1.3 ± 0.2 respectively. They were higher than that in the secretory phase of the control group. On the other hand, evaluation nomogram scores in glandular epithelium and stromal cells in adenomyosis tissue were 2.2 ± 0.1 and 1.1 ± 0.2 respectively, approximately the same as that in the early to mid-proliferative phases in the eutopic endometrium.

**Histological findings**

COX-2 formed a granular pattern or texture in the cytoplasm of glandular cells and cells were stained almost diffusely (Figure 3). Pronounced localization was not found in cell
membranes. Polarity in the basal and apical sides was not particularly pronounced. No localization was seen in nuclei. Cytoplasm of interstitial cells was slightly positive.

Discussion

Expression of COX-2 varied with menstrual cycle. In a previous report (Jones et al., 1997), the expression of COX-2 in endometrial glandular epithelium peaked at the menstrual phase, bottomed out in the ovulatory phase and gradually began to increase again in the secretory phase. These results, however, differed slightly from theirs. In this study, it was low in the early proliferative phase and gradually increased thereafter. It peaked at the early secretory phase, and the same amount of expression was maintained into the late secretory phase. Possible reasons for this difference include a variation in controls (women with underlying disease versus healthy women with fecundity), parameters of assessing staining (intensity only versus evaluation nomogram score), difference in the immunized animal for the first antibody (rabbit versus goat) and staining method. In addition, the volume of prostaglandins secreted from endometrium differs during the menstrual cycle. The volume of PGE$_2$ and PGF$_{2\alpha}$ secreted from endometrial glandular epithelial cells was investigated in vitro (Lumsden et al., 1984). According to their findings, PGE$_2$ in the proliferative phase was 1.5 times that in the secretory phase, whereas PGF$_{2\alpha}$ was 3.4 times higher in the secretory phase. Consequently, increased expression of COX-2 in the secretory phase may contribute mainly to production of PGF$_{2\alpha}$.

This is the first study to look at the expression of COX-2 in eutopic and ectopic endometria in endometriosis. Expression of COX-2 in eutopic endometrium varied significantly during the menstrual cycle. Expression was higher in the secretory phase than in the proliferative phase, but it was higher than that in the control throughout the menstrual cycle. Over-expression of COX-2 leads to increased production of prostaglandins. The mechanism of over-expression of COX-2 in eutopic endometrium in endometriosis is unclear. It has been indicated that an increased autoimmune response occurs in the endometrium in endometriosis. For instance, T cells and B cells are increased (Witz et al., 1994; Ota et al., 1996a), macrophage is activated (Haney et al., 1981), and immunoglobulin and complements are deposited in endometrial glandular epithelial cells (Weed and Arquembourg, 1980; Ota and Maki, 1990). Furthermore, human leukocyte antigen expression in glandular epithelial cells is increased (Ota and Igarashi, 1993) and adhesion molecules are abnormally expressed (Lessey et al., 1992; Ota et al., 1996b). In addition, expression of free radical-related enzymes such as nitric oxide synthase (Ota et al., 1998), superoxide dismutase (Ota et al., 1999a), and glutathione peroxidase is increased (Ota et al., 1999b; Ota et al., 2000). Consequently, a variety of cytokines are secreted from these immune cells and macrophages, including IL-1 and IL-2. These cytokines may very well initiate COX-2 expression. Over-expression of COX-2 and the accompanying increase in prostaglandin production likely initiate an abnormal state in the uterus. Endometriosis is often accompanied by dysmenorrhoea. Abnormal uterine contraction is also observed in endometriosis.

Myometrial movement in patients with endometriosis was observed using ultrasonography (Leyendecker et al., 1996) and they found that hyperperistalsis and dysperistalsis are frequently observed in patients with endometriosis, and speculated that abnormal peristalsis may inhibit normal sperm transport in the uterus. Secondly, excessive prostaglandins may be impairing implantation of fertilized oocytes. The implantation rate was said to have decreased significantly in an experimental endometriotic model using rabbits that were subsequently artificially inseminated (Hahn et al., 1986).

COX-2 expression was found in endometriotic tissues of the ovarian chocolate cyst wall in all cases, and expression of COX-2 in glandular epithelial cells was almost as pronounced as that in the secretory phase in the eutopic endometrium. In fact, there have been many reports that large amounts of PGs are produced in ascites and endometrial tissues in endometriosis (Badawy et al., 1985; De Leon et al., 1986; Moon et al., 1981). Over-expression of COX-2 in ectopic endometrial tissue, and prostaglandins that is produced as a result, may be involved in the proliferation and differentiation of cells and malignant transformation. For example, it was reported that when COX-2 was over-expressed in epithelial cells of mouse small intestine, expression of adhesion molecules such as laminin increased, while apoptosis was suppressed (Tsujii and DeBois, 1995). It has also been reported that the incidence of colon cancer and the number and size of colorectal polyps decreased in people who took aspirin for an extended length of time (Giovannucci et al., 1995; Marcus, 1995). It is said that apoptosis is reduced in the eutopic and ectopic endometria in endometriosis compared with normal women (Dmowski et al., 1998; Imai et al., 2000). Furthermore, apoptosis is further reduced in the ectopic endometrium compared with the eutopic endometrium (Gebel et al., 1998). In fact, loss of heterozygosity was frequently observed on chromosomes in endometriotic tissue from ovarian chocolate cysts (Jiang et al., 1996). It is concluded that COX-2 expression in endometriosis appears to be intimately involved in its pathology. Further investigations into the involvement of COX-2 are required.

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References


