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ORIGINAL ARTICLE *Gynaecology*

Expression and possible role of non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) in the human endometrium and endometriosis

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BACKGROUND: Non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) is involved in cellular processes such as inflammation, apoptosis and tumorigenesis. However, little is known about the expression and function of NAG-1 in the endometrium. This study aimed to evaluate the expression of NAG-1 in the endometrium and in the absence or presence of endometriosis and to investigate the effect of celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, on NAG-1 mRNA levels and apoptosis in human endometrial stromal cells (HESCs).

METHODS: Eutopic endometrial samples were obtained during surgery from 40 patients with, and 40 patients without, endometriosis. Real-time PCR was used to quantify NAG-1 mRNA levels and immunohistochemistry was used to localize NAG-1 protein in the endometrium. To investigate the effects of celecoxib, HESCs were isolated and cultured with different concentrations of celecoxib or with 100 μM celecoxib at different times. Apoptosis was assessed by flow cytometry.

RESULTS: NAG-1 mRNA levels and immunoreactivity showed cyclical changes through the menstrual cycle, increasing during the late secretory and menstrual phases. NAG-1 mRNA and protein levels were significantly lower in patients with endometriosis, compared with the control group. Celecoxib induced NAG-1 mRNA levels and apoptosis in cultured HESCs, with the effects dependent on drug concentrations and duration of treatment. Celecoxib treatment had no effect on prostaglandin E_2 levels in the culture supernatants.

CONCLUSIONS: NAG-1 may be important in maintaining homeostasis in the normal endometrium and alterations in NAG-1 expression may be associated with the establishment of endometriosis. NAG-1 might be a therapeutic target for endometriosis.

Key words: NAG-1 / endometrium / endometriosis / apoptosis

Introduction

The human endometrium is a dynamic tissue that undergoes monthly cyclic changes, including proliferation, differentiation and degeneration. These sequential changes are regulated by ovarian steroid hormones and apoptosis appears to play an important role in cyclic remodeling of the endometrium (Hopwood and Levison, 1976; Kokawa *et al.*, 1996). Evidence suggests that apoptosis mainly occurs in the human endometrium through the late secretory to menstrual phases and the BCL-2 family and Fas/FasL system are involved in the regulation of apoptosis (Kokawa *et al.*, 1996; Harada *et al.*, 2004).

Endometriosis, which is the presence of endometrial glands and stroma at extrauterine locations, is a benign gynecological disease that may cause infertility and pelvic pain. It is a common disease that affects about 10% of reproductive-age women and 20–50% of infertile women (Eskenazi and Warner, 1997; Pritts and Taylor, 2003). Various theories have been proposed about the pathogenesis of endometriosis, but a definitive theory remains obscure. The most

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widely accepted theory presumes that endometriosis is caused by implantation and growth of refluxed endometrial cells into the peritoneal cavity, and several studies have asserted that reduced apoptosis could contribute to the survival of refluxed endometrial cells and the establishment of endometriosis (Gebel et al., 1998; Dmowski et al., 2001).

Non-steroidal anti-inflammatory drugs (NSAIDs) are clinically used as therapeutic agents for pain and inflammation. Recently, the use of NSAIDs was extended to chemoprevention of colorectal cancer (Cuzick et al., 2009). Experimental evidence suggests that NSAIDs exert their chemopreventive effects by induction of apoptosis, cell growth suppression, inhibition of angiogenesis and inhibition of metastasis (Gupta and Dubois, 2001; Kismet et al., 2004). Previously, inhibition of cyclooxygenase (COX)-1 and/or COX-2 activity by NSAIDs was thought to be the sole explanation for their chemopreventive effect. However, COX-independent effects may contribute to their anti-cancer activity (Hanif et al., 1996; Piazza et al., 1997; Minter et al., 2003; Pang et al., 2007). More recently, a selective COX-2 inhibitor was found to enhance apoptosis in human endometrial epithelial cells (HEECs) in women with and without endometriosis (Olivares et al., 2008). However, little is known about the genes involved in the intracellular regulatory events that determine the susceptibility of endometrial cells to NSAID-induced apoptosis.

Non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) is a member of the transforming growth factor (TGF)-β superfamily, which is associated with cell growth, differentiation and apoptosis (Li et al., 2000; Baek et al., 2001). NAG-1 is activated by various NSAIDs, including selective COX-2 inhibitors, but its precise biological functions are not fully understood. Previous studies demonstrated that NAG-1 had pro-apoptotic and anti-tumorigenic activities. The over-expression of NAG-1 by COX-2 inhibitors in various cancer cells results in apoptosis induction and growth arrest (Baek et al., 2004; Kim et al., 2005; Kim et al., 2008; Yoshioka et al., 2008). Thus, NAG-1 seems to be a link between NSAIDs and their pro-apoptotic activity and might explain NSAID-induced apoptosis. However, the expression of NAG-1 in the endometrium and its relationship to endometriosis has not been reported.

Therefore, we evaluated NAG-1 expression in the endometrium and investigated whether altered NAG-1 expression is associated with endometriosis. In addition, we assessed the effect of celecoxib, a selective COX-2 inhibitor, on NAG-1 mRNA levels and apoptosis in human endometrial cells.

Materials and Methods

Participants

Patients of reproductive age who were treated for benign gynecological conditions at Gangnam Severance Hospital in Seoul, Korea participated in this study. All patients had regular menstrual cycles (range: 26–32 days) and had not undertaken hormonal therapy with agents such as GnRH agonsists or steriods, for at least 6 months before endometrial sampling. Patients with gynecological cancer or endometrial pathologies, such as endometrial hyperplasia or endometrial polyps, were excluded.

Eutopic endometrial samples were obtained from 40 patients with endometriosis (mean age: 32.6 years; range: 22–45 years) and 40 patients without endometriosis (mean age: 34.6 years; range: 21–47 years) by endometrial curettage before surgical procedures. All 40 patients with endometriosis were at revised American Society for Reproductive Medicine (ASRM) stage III ($n = 24$) or revised ASRM stage IV ($n = 16$), as diagnosed by histological examination of the ectopic endometrial specimens taken at the time of laparoscopy. The control group included 21 patients with benign ovarian cysts, 6 patients with fibroids, 11 patients with cervical intraepithelial neoplasia and 2 patients without visible pathology in the pelvis. Benign ovarian cysts included functional cysts, simple cysts, paratubal cysts and dermoid cysts.

Endometrial dating was determined by menstrual history and endometrial histological features (Noyes et al., 1950) and divided into early proliferative, late proliferative, early secretory, mid-secretory, late secretory and menstrual phases. The 40 endometriosis patients were distributed as menstrual ($n = 8$), early proliferative ($n = 8$), late proliferative ($n = 8$), early secretory ($n = 6$), mid-secretory ($n = 5$) and late secretory ($n = 5$), and the 40 control women were distributed as menstrual ($n = 8$), early proliferative ($n = 8$), late proliferative ($n = 8$), early secretory ($n = 5$), mid-secretory ($n = 5$) and late secretory ($n = 6$).

This study was approved by the Institutional Review Board of Gangnam Severance Hospital and written informed consent was obtained from participants.

RNA extraction and SYBR green real-time PCR

Total RNA was extracted from endometrial tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and 2 μg was reverse transcribed into cDNA by SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. NAG-1 mRNA levels were measured by SYBR green real-time PCR using an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). β-actin, a housekeeping gene, was used as a normalization control. Specific primers for NAG-1 (Hs 00171132) and β-actin (Hs 99999903) were obtained from Applied Biosystems. PCR amplification was performed in 20 μL with 2 μL of cDNA, 5 ρM of each primer, and power SYBR green PCR master mix (Applied Biosystems). Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative NAG-1 mRNA levels were calculated by the ΔΔCt method using the normalization formula (Livak and Schmittgen, 2001): target amount $= 2^{-\Delta\Delta Ct}$, where ΔΔCt = [Ct (NAG-1 sample) − Ct (β-actin sample)] − [Ct (NAG-1 calibrator) − Ct (β-actin calibrator)].

Immunohistochemistry

The Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used for immunohistochemistry (IHC). Paraffin embedded sections were deparaffinized with xylene, rehydrated in a graded ethanol series and immersed in 3% H_2O_2 for 10 min to block endogenous peroxidase. Sections were incubated with the anti-NAG-1 rabbit polyclonal IgG (1:200 dilution; Upstate, Lake Placid, NY, USA) overnight at 4°C, rinsed with a washing buffer, and incubated with biotinylated second antibody (rabbit IgG) solution for 30 min at room temperature. After 5-min washes in buffer, Vecstatin ABC Reagent was applied for 30 min. Chromogen solution was added for 2 min and slides were counterstained with Meyer's hematoxylin. Negative controls used a secondary antibody without the primary antibody.

For H-scores, five randomly selected areas were evaluated under a light microscope at 400× magnification. NAG-1 intensity was evaluated as: 0 (no staining), 1+ (weak staining), 2+ (moderate staining) and 3+ (strong staining). For each slide, an H-score was calculated as follows: H-score = (% of cells that stained at intensity category 1 × 1) + (% of cells that stained at intensity category 2 × 2) + (% of cells that stained at intensity category 3 × 3). Slides were scored at different times by

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two investigators blinded to the clinical data. The average of the two evaluations was used for the final analysis.

Isolation and culture of endometrial stromal cells

Human endometrial stromal cells (HESCs) were isolated from endometrial tissue of 15 patients with fibroids undergoing hysterectomy and cultured as described previously (Lee and Nowak, 2001; Park *et al*., 2003). Briefly, endometrial tissues were minced into small pieces and placed in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) with 5 ml of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco-BRL). Endometrial tissue was digested for 30 min in a 37°C incubator with gentle shaking. Dispersed cells were centrifuged at 2000 g for 5 min and the cell pellet was re-suspended in DMEM/F12 plus 10% FBS. The stromal-enriched fraction was cultured in 75-ml culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO_2. We used HESCs in a monolayer culture after the first passage. The purity of the HESCs was assessed by IHC for vimentin and cytokeratin. The IHC results demonstrated that the purity of HESCs was >95%.

Effect of celecoxib on NAG-1 mRNA levels and prostaglandin E_2 production in cultured HESCs

HESCs were plated at 1×10^5 cells per well in six-well plates for real-time PCR. After 48 h in 10% FBS medium, HESCs were washed and cultured with 0% FBS medium and treated with celecoxib at 0, 25, 50 or 100 μM for 24 h or with 100 μM celecoxib for 0, 12, 24 or 48 h. The total RNA was extracted from cultured HESCs using the RNeasy Mini Kit (Qiagen) and NAG-1 mRNA levels were measured as described above.

The culture supernatants were collected for measuring levels of prostaglandin E_2 (PGE_2) using an ELISA kit (Cusabio Biotech, China), according to the manufacturer's protocols. The minimum detectable concentration was 20 pg/ml.

Measurement of apoptototic HESCs by flow cytometry

1×10^6 HESCs per well were plated in 10-cm culture dishes. After 48 h in 10% FBS medium, HESCs were washed and cultured with 0% FBS medium, and treated with celecoxib at the concentrations and times described above before collection for the apoptosis assay. HESCs were stained with propidium iodide, according to the manufacturer's protocol. Apoptotic cells were assessed by flow cytometry (FC 500, Beckman Coulter, Fullerton, CA, USA) and data were analyzed using WinMDI version 2.9 software (The Scripps Research Institute, San Diego, CA, USA).

Statistical analysis

Data were expressed as the mean ± SEM. The Mann–Whitney *U*-test was used to compare the NAG-1 mRNA levels and H-scores between the two groups. Differences in the NAG-1 mRNA levels and H-scores over the menstrual cycle within each group were compared using the Kruskal–Wallis test followed by Dunn's procedure. NAG-1 mRNA levels and apoptotic index after different COX-2 inhibitor treatment concentrations and times were compared with the control using the Mann–Whitney *U*-test. All statistical analyses were performed using SPSS version 15.0 software (SPSS, Inc., Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant.

Results

NAG-1 endometrial mRNA levels

NAG-1 mRNA levels showed cyclical changes through the menstrual cycle and significantly increased during the late secretory and menstrual phases in both endometriosis patients and controls. However, the variability of NAG-1 mRNA levels appeared to be lower in the endometriosis group. When the NAG-1 mRNA levels were compared by menstrual cycle, NAG-1 mRNA levels were significantly lower during the late secretory and menstrual phases in the endometriosis group than in the control group (Fig. 1).

Immunohistochemistry

NAG-1 was expressed in glandular epithelial and stromal cells throughout the menstrual cycle, localized within the cytoplasm. NAG-1 immunostaining varied over the menstrual cycle in both groups. In the proliferative, early secretory and mid-secretory phases, NAG-1 staining in glandular epithelial cells was weak, and stromal cells were negative or very weakly stained. Although the immunoreactivity of NAG-1 increased during the late secretory and menstrual phases in both groups, NAG-1 expression was significantly lower in both glandular epithelial cells and stromal cells of the endometriosis group, when compared with the control group (Fig. 2).

Induction of NAG-1 mRNA levels and apoptosis by celecoxib in cultured HESCs

Treatment with celecoxib significantly increased both NAG-1 mRNA levels and HESC apoptosis, and both of these effects depended on celecoxib concentration (Fig. 3). In addition, NAG-1 mRNA levels and apoptosis also increased with the duration of treatment with 100 μM celecoxib (Fig. 4).

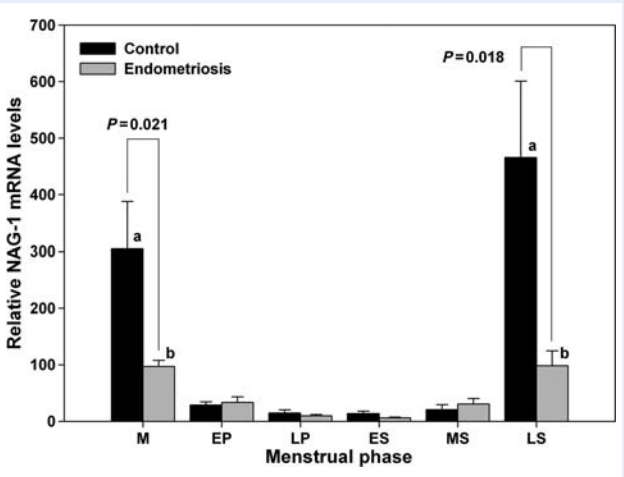

Figure 1 Comparison of NAG-1 mRNA levels in endometriosis and control group through the menstrual cycle. M, menstrual phase; EP, early proliferative phase; LP, late-proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late-secretory phase. Data are expressed as mean ± SEM. [a]Statistically significant versus LP, ES and MS in the Dunn procedure. [b]Statistically significant versus LP and ES in the Dunn procedure.

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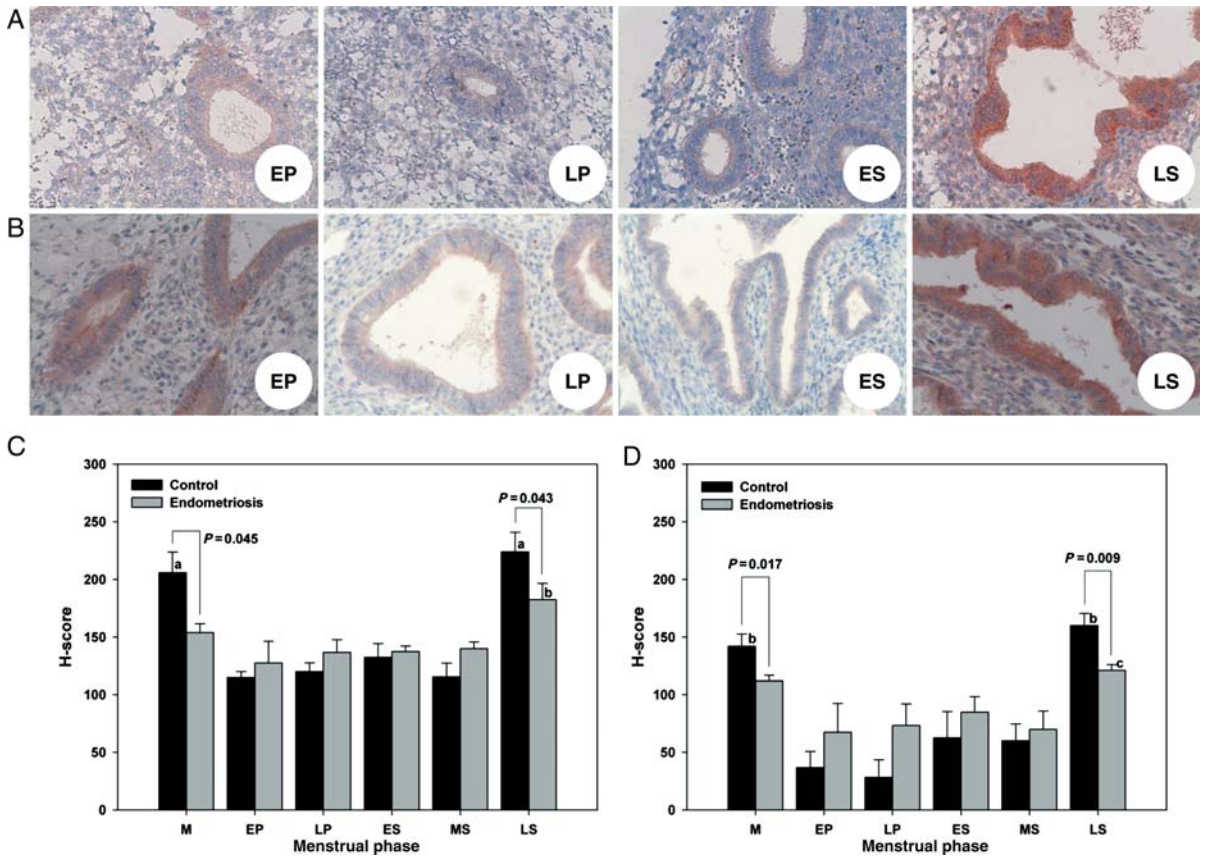

Figure 2 Expression of NAG-1 in the endometrium (×400). Representative micrographs of NAG-1 immunostaining in the endometrium of the control (**A**) and endometriosis group (**B**), and H-score in endometrial glandular (**C**) and stromal cells (**D**) throughout the menstrual cycle. M, menstrual phase; EP, early proliferative phase; LP, late-proliferative phase; ES, early secretory phase; MS, mid-secretory phase; LS, late-secretory phase. Data are expressed as mean ± SEM. [a]Statistically significant versus EP, LP, and ES in the Dunn procedure. [b]Statistically significant versus EP and LP in the Dunn procedure. [c]Statistically significant versus LP in the Dunn procedure.

Effect of celecoxib on PGE_2 production

We investigated COX-2 activity by measuring levels of PGE_2 released into the conditioned media of cultured HESCs treated for 24 h with different concentrations of celecoxib. In HESCs treated with celecoxib up to 100 μM, no significant reduction of PGE_2 levels was observed (Fig. 5).

Discussion

We found cyclic expression of NAG-1 in the human endometrium over the menstrual cycle and demonstrated altered NAG-1 expression in the eutopic endometrium from women with endometriosis. We also found that celecoxib increased NAG-1 mRNA levels and apoptosis in cultured HESCs.

TGF-β superfamily members are stage-specifically expressed in the human endometrium, consistent with their general role in tissue homeostasis (Jones *et al.*, 2006). NAG-1, a member of the TGF-β superfamily, was cyclically expressed in endometrial epithelial and stromal cells, peaking during the late secretory and menstrual phases. When comparing patients with endometriosis with a control group, NAG-1 expression was significantly decreased in both endometrial glandular and stroma cells of patients with endometriosis during the late secretory and menstrual phases. The cyclic variability of NAG-1 expression was also reduced in the endometriosis group. The present study further demonstrated that NAG-1 was associated with apoptosis in cultured HESCs. Previous studies have shown that in patients with endometriosis, the number of apoptotic cells is significantly lower during the late secretory and menstrual phases in both endometrial glandular and stroma cells and apoptotic cyclic variability is not seen (Johnson *et al.*, 2005; Szymanowski, 2007). Both endometrial glandular and stroma cells have a higher proliferative capacity in women with endometriosis, when compared with normal controls (Wingfield *et al.*, 1995; Johnson *et al.*, 2005). Moreover, aberrant expression of apoptosis-related molecules in patients with endometriosis may account for the individual susceptibility to endometriosis (Harada *et al.*, 2004). Therefore, altered expression of NAG-1 may affect apoptosis of endometrial cells and be involved in the pathogenesis of endometriosis.

In the animal model, celecoxib prevents the implantation of endometrial tissue and induces the regression of endometrial implants at ectopic sites by suppressing angiogenesis and inducing apoptosis

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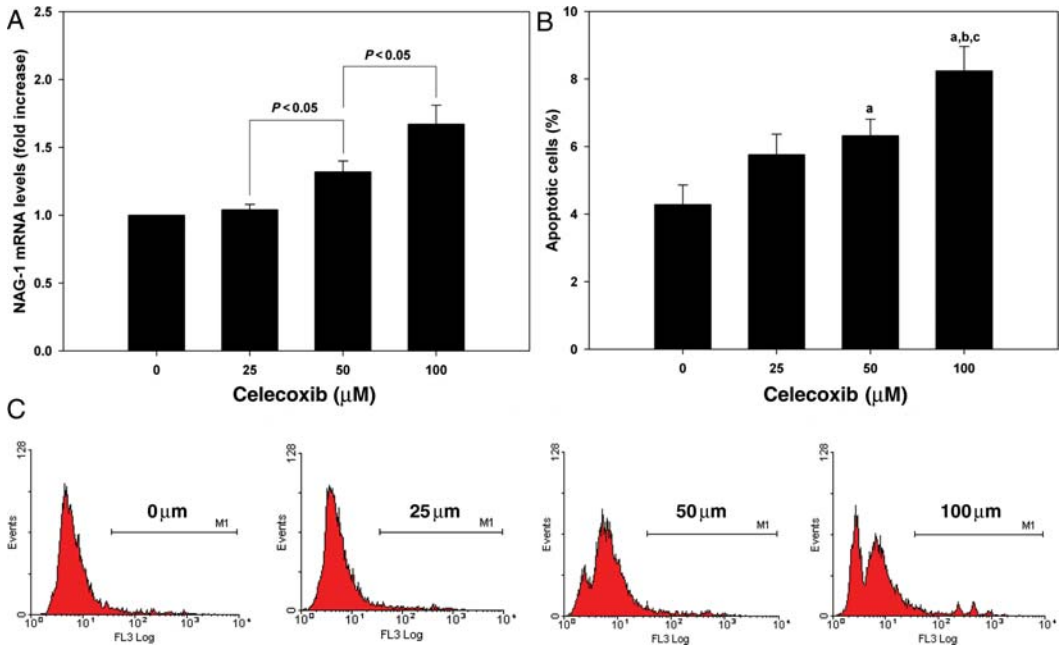

Figure 3 Dose-dependent NAG-1 expression (**A**) and apoptosis (**B**) in cultured HESCs. HESCs were treated with celecoxib as indicated for 24 h. Apoptotic HESCs were assessed by flow cytometry (**C**). Data are expressed as mean ± SEM of five independent experiments. $^{a}P < 0.05$ versus 0 μM, $^{b}P < 0.05$ versus 25 μM, $^{c}P < 0.05$ versus 50 μM.

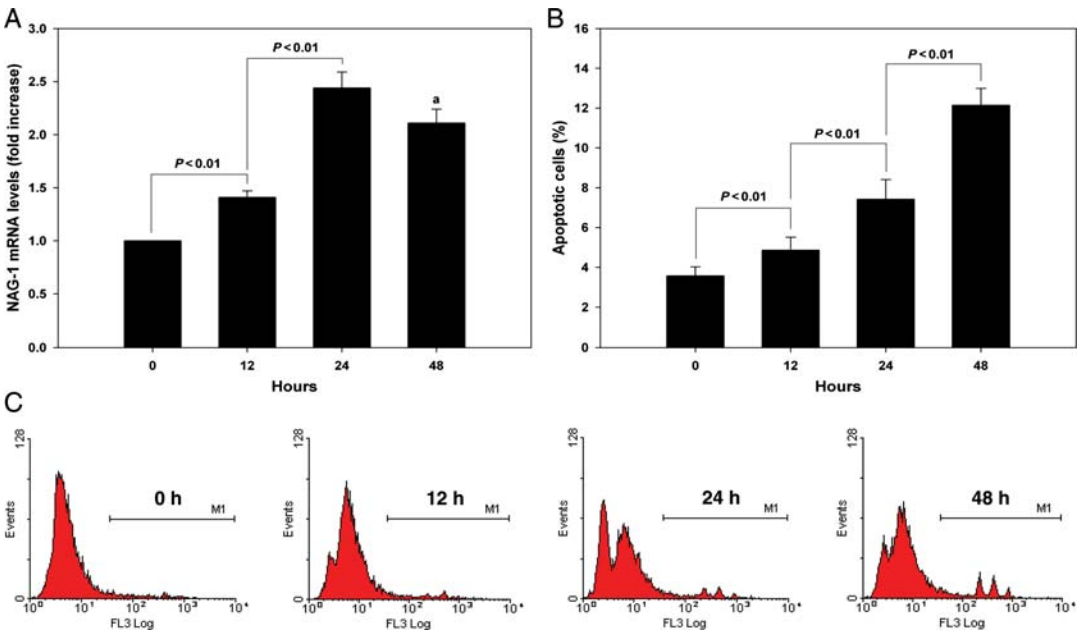

Figure 4 Time-dependent NAG-1 expression (**A**) and apoptosis (**B**) in cultured HESCs. HESCs were treated with 100 μM celecoxib as indicated. Apoptotic HESCs were assessed by flow cytometry (**C**). Data are expressed as mean ± SEM of five independent experiments. $^{a}P < 0.01$ versus 0 and 12 h.

(Matsuzaki *et al*., 2004). Recently, celecoxib was found to inhibit proliferation and induce apoptosis of HEECs. Treatment with ≥50 μM celecoxib showed a significant effect on endometrial growth and enhanced apoptosis in cultured HEECs compared with controls (Olivares *et al*., 2008). Our study also found that ≥50 μM celecoxib significantly increased apoptosis in cultured HESCs. These results

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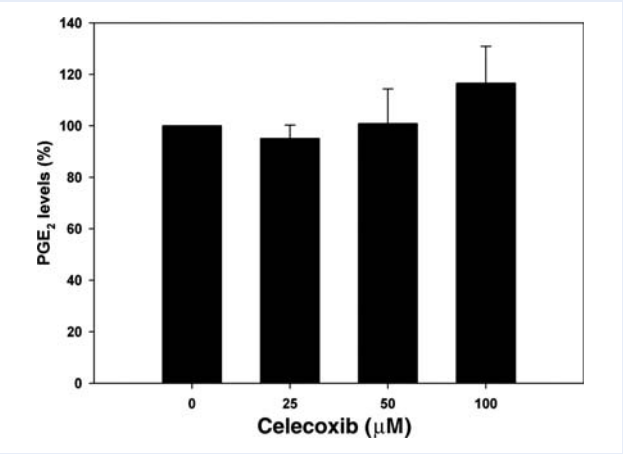

Figure 5 Effects of celecoxib on COX-2 activity in cultured HESCs. COX-2 activity was measured as PGE_2 levels in culture supernatants after treatment with celecoxib as indicated. Control PGE_2 levels were set as 100%. Data are expressed as mean ± SEM of five independent experiments.

suggest that selective COX-2 inhibitors may be effective for the prevention and treatment of endometriosis. Selective COX-2 inhibitors also provide pain relief with fewer gastrointestinal side effects than non-selective COX inhibitors. However, selective COX-2 inhibitors may increase the risk of cardiovascular thrombotic disease (Mukherjee *et al.*, 2001).

It is generally accepted that COX-2 inhibitors induce apoptosis through inhibition of COX-2 expression and subsequent PGE_2 production. However, accumulating evidence suggests that COX-independent pathways may be involved in the effects of COX-2 inhibitors. Significantly higher concentrations of NSAIDs are necessary to inhibit cell growth and to induce apoptosis than those required for the inhibition of PGE_2 production (Hanif *et al.*, 1996; Piazza *et al.*, 1997; Minter *et al.*, 2003) and exogenous addition of PGE_2 cannot reverse the apoptosis-inducing effects of NSAIDs (Hanif *et al.*, 1996; Piazza *et al.*, 1997). Furthermore, NSAIDs induce apoptosis in COX-2-deficient cell lines (Elder *et al.*, 1997; Pang *et al.*, 2007). This study showed that celecoxib treatment had no effect on PGE_2 levels in culture supernatants, whereas ≥50 μM celecoxib significantly increased NAG-1 mRNA levels and apoptosis in cultured HESCs. These results strongly support the hypothesis that celecoxib-induced apoptosis in endometrial stromal cells is independent of COX-2.

NAG-1 is a pro-apoptotic and anti-tumorigenic gene induced by various NSAIDs, and its expression is lower in colon and prostate cancers compared with adjacent normal tissue (Thomas *et al.*, 2001; Kim *et al.*, 2002). In addition, overexpression of NAG-1 by NSAIDs results in growth arrest and increases apoptosis in various cancer cells (Baek *et al.*, 2004; Kim *et al.*, 2005; Kim *et al.*, 2008; Yoshioka *et al.*, 2008). Here, we demonstrated that NAG-1 mRNA levels are up-regulated by celecoxib and that increased NAG-1 mRNA levels are associated with apoptosis induction. We also demonstrated that celecoxib increases NAG-1 mRNA levels and apoptosis in a dose- and time-dependent manner. Consequently, the effects of COX-2 inhibitors on apoptosis in endometrial cells may be mediated, at least in part, by NAG-1, and NAG-1 may be a potential target for the prevention and treatment of endometriosis as well as for various cancers.

To our knowledge, this is the first report to examine NAG-1 expression in the human endometrium. Furthermore, we are also the first to report alterations in NAG-1 expression in women with endometriosis and provide a partial explanation for a novel signaling pathway to induce apoptosis of endometrial cells by COX-2 inhibitors.

In conclusion, we reported cyclic changes in NAG-1 expression in the human endometrium through the menstrual cycle and showed a significant decrease in NAG-1 expression in patients with endometriosis compared with controls during the late secretory and menstrual phases. We also demonstrated that a COX-2 inhibitor increased NAG-1 mRNA levels and apoptosis in human endometrial cells. These results suggest that NAG-1 plays an important role in maintaining homeostasis of the normal endometrium by regulating apoptosis, and that changes in NAG-1 expression may contribute to the establishment of endometriosis. Therefore, NAG-1 can be a therapeutic target for endometriosis.

Authors' roles

S.K.S. designed the study, researched data, analyzed data and wrote the manuscript. A.N. researched data. Y.E.J. analyzed data. S.C. and Y.S.C. contributed to the discussion. B.S.L. contributed to the discussion and reviewed/edited the manuscript.

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