Possible implication of midkine in the development of endometriosis

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BACKGROUND: The present study was conducted to assess whether midkine (MK), a multifunctional molecule known to stimulate tumor growth, may be involved in the development of endometriosis. METHODS: The mitogenic activity of MK on cultured endometriotic stromal cells was examined by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation. Concentrations of MK in the peritoneal fluid (PF) of women without or with endometriosis and those under GnRH agonist treatment were measured using a specific enzyme immunoassay. The expression of MK mRNA in peritoneal bone marrow-derived cells, peritoneum and endometriotic tissues was evaluated by RT–PCR. RESULTS: MK significantly increased BrdU incorporation into the DNA of cultured endometriotic stromal cells. The MK concentrations in the PF of the women with advanced endometriosis (stages II, III and IV) Median: 1.21 ng/ml; interquartile range 0.80–2.27 were significantly higher than those of the women without endometriosis and with stage I endometriosis (0.06 ng/ml, 0.67–1.26, \( P < 0.05 \)). As for the menstrual phase, the MK concentration in PF in the interal phase (1.32 ng/ml, 0.72–2.21) were significantly higher than those in the follicular phase (0.95 ng/ml, 0.68–1.24, \( P < 0.05 \)). In addition, women with adnexal adhesions had higher concentrations of MK in PF than those without adhesions (\( P < 0.05 \)). The MK concentrations of the women under GnRH agonist treatment were significantly lower than those of the other groups (\( P < 0.001 \)). The expression of MK mRNA was detected in peritoneal bone marrow-derived cells, peritoneum and endometriotic tissues. CONCLUSIONS: The present findings suggest that MK may play roles, such as stimulation of endometriotic cell proliferation, in the development of endometriosis.

Key words: adhesions/endometriosis.midkine/peritoneal fluid/cell proliferation

Introduction

Endometriosis is an enigmatic disease that deteriorates the health of women of reproductive age (Momoea et al., 2002; Osuga et al., 2002). A widely believed etiology is that endometrial debris in retrograde menstrual implants, survives and grows in the peritoneal cavity. However, this hypothesis leads to the question of why only some women develop endometriosis while retrograde menstruation is observed in most women (Halme et al., 1984). First, accumulating evidence suggests that eutopic endometrium from women with endometriosis has aberrant properties as compared to that from women without the disease, and the aberrance may play central roles in the pathogenesis and pathophysiology associated with the disease (Sharpe-Timms, 2001). Secondly, alteration of the peritoneal environment observed in women with endometriosis is suggested to be distinctively important in the development of the disease (Harada et al., 2001; Lebovic et al., 2001). A number of substances, such as growth factors and cytokines, have been shown to change in their concentrations in the eutopic endometrium and peritoneal fluid (PF) of women with endometriosis, as compared to in those of women without the disease (Osuga et al., 1999; Koga et al., 2000; Osuga et al., 2000; Yoshino et al., 2003). These substances are suggested to play various roles in cell survival, cell proliferation and angiogenesis, stimulating the progress of endometriosis.

Midkine (MK) is a basic, low molecular weight nonglycosylated protein, composed of two domains held by disulfide bridges. MK belongs to a member of the heparin-binding growth factor family and has been identified as the product of a retinoic acid-responsive gene. MK plays important roles in development and is strongly expressed during midgestation. Although its expression is restricted to certain tissues in the adult, it is strongly induced during oncogenesis, inflammation and tissue repair. MK has pleiotropic activities, such as cell proliferation, cell migration, angiogenesis and fibrino-
Midkine in endometriosis

Endometriosis, and its possible sources. MK has been detected in the endometrium, the levels being high in women with endometriosis as compared to those in women without the disease (Chung et al., 2002). In view of high levels of MK in a number of malignant tumors, suggesting its involvement in cancer development (Tsutsui et al., 1993; Garver et al., 1994; Aridome et al., 1995; Nakagawara et al., 1995; O’Brien et al., 1996; Koide et al., 1999; Konishi et al., 1999; Kato et al., 2000), increased MK in the endometrium of women with endometriosis is speculated to subserve proliferation of refluxed endometrial cells and to promote the formation of endometriotic foci on the peritoneum in an autocrine/paracrine fashion. In addition, a recent study demonstrating that MK plays key roles in intraperitoneal adhesions imply its possible relevance to endometriosis-associated intraperitoneal adhesion (Inoh et al., 2004). In the present study, to address the possible implication of MK in endometriosis, we first evaluated whether MK has a proliferative effect on cultured endometriotic stromal cells (ESC). Secondly, considering the importance of peritoneal environment for the development of endometriosis, we examined the concentrations of MK in PF of women with or without endometriosis, and its possible sources.

Materials and methods

Sample collection

A total of 139 women with (n = 106) and without (n = 33) endometriosis aged 20–48 years who were undergoing laparoscopy for pain and/or infertility were included in this study. Endometriosis was diagnosed both laparoscopically and histologically. The extent of the disease was scored according to the revised American Society for Reproductive Medicine (r-ASRM) Classification System (American Society for Reproductive Medicine, 1997). The distribution of the stages of endometriosis is shown in Table I. The endometriosis group included 12 women treated with GnRH agonist for more than 3 months until laparoscopy (GnRHa group). All of the women except those in the GnRHa group had regular menstrual cycles. In the endometriosis group without GnRHa therapy, the status of endometriosis (r-ASRM score, 1–5, n = 25) or advanced endometriosis (r-ASRM score, ≥6, n = 69), on the assumption that minimal endometriosis is the initiation stage of the disease. The age of the women in the endometriosis group was 32.7±4.1 years (mean±SD), which was essentially the same as that of the women in the non-endometriosis group (31.1±5.6 years). This study was approved by the Institutional Review Board of the University of Tokyo, and signed informed consent for sample collection was obtained from each woman.

PF was collected via a laparoscopic cannula introduced into the cul-de-sac before any manipulative procedure. The fluid was centrifuged at 400 g for 10 min, and the supernatants were frozen and stored at −80°C until assay. Peritoneum and endometriotic tissues were also collected. Peritoneal bone marrow-derived cells were collected as previously described (Yoshino et al., 2003). Briefly, the collected PF was centrifuged at 200 g for 5 min, and the supernatants were removed. The cell pellet was resuspended in phosphate-buffered saline (PBS), layered onto Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and centrifuged at 150 g for 30 min. Peritoneal bone marrow-derived cells were recovered from the interface.

Endometriotic tissues were collected from cyst walls of ovarian endometriomas of three patients without GnRHa therapy. Likewise, normal peritonea that did not include endometriotic lesions were collected under laparoscopy. The tissues for RNA extraction were snap-frozen in liquid nitrogen, and stored at −80°C until assay.

Table I. Characteristics of the subjects

<table>
<thead>
<tr>
<th>No. of women</th>
<th>Total</th>
<th>follicular phase</th>
<th>luteal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-endometriosis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Endometriosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I (1–5)</td>
<td>25</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Stage II (6–15)</td>
<td>15</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Stage III (16–40)</td>
<td>25</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>Stage IV (41+)</td>
<td>29</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>GnRH agonist</td>
<td>12</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Parenthesis shows revised American Society for Reproductive Medicine (r-ASRM) score.

Isolation, purification and culture of ESC

Primary cultures of ESC were prepared from the obtained endometriotic tissues according to the method described by Ryan et al. (1994) with minor modifications. Endometriotic tissue was dissected free from underlying parenchyma, minced into small pieces, incubated in Dulbecco’s modified Eagle’s medium (DMEM)/F12 with 0.25% type I collagenase (Sigma, St Louis, MO) and SU/m1 deoxyribonuclease I (Takara, Tokyo, Japan) for 1–2 h at 37°C, and separated using serial filtration. Debris and some epithelial glands were removed with a 100-μm and a 70-μm nylon cell strainer (Becton Dickinson, Lincoln Park, NJ), and some epithelial glands were removed with a 70-μm nylon cell strainer (Becton Dickinson). Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12, and plated onto 100-mm dishes and allowed to adhere at 37°C for 30 min, after which nonadhering epithelial cells and blood cells were removed with PBS rinses. The cells were cultured in DMEM/F12 reconstituted with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and antibiotics (Sigma). When the cells become confluent, they were dissociated with 0.25% trypsin, harvested by centrifugation, replated in Falcon 96-multitwell plates (Becton Dickinson) at 1 × 10^6 cells/well, and kept at 37°C in a humidified 5% CO_2/95% air environment. After 24 h, purification of the stromal cell population was confirmed by immunocytochemical staining for the following antibodies (Dako, Kyoto, Japan): vimentin (stromal cells), cytokeratin (epithelial cells) and CD45 (monocytes and other leukocytes). The purity of the stromal cells was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45.

Cell proliferation assay

The cell proliferation assay was performed as we have reported previously (Tang et al., 2002). The effect of MK on the proliferation of ESC was examined by measuring 5-bromo-2′-deoxyuridine (BrDU) incorporation into DNA using the Biotrak cell proliferation enzyme-linked immunosorbent assay (ELISA) system (Amersham...
Biosciences) according to the manufacturer’s instructions. Briefly, ESC were seeded into Falcon 96-microwell plates at a density of 1 × 10^4 cells per well in 100 μl of the culture medium with MK at different concentrations (0, 10, 100 and 1000 ng/ml) or with 20% PF from patients of endometriosis (r-ASRM score ≥ 6) (Braun et al., 2002). After 24 h, 100 μl of BrdU solution was added and incubated at 37°C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of 200 μl/well of fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in the newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

**Measurement of MK**

Concentrations of MK in PF were determined using an ELISA kindly provided by Cell Signals (Yokohama, Japan). The sensitivity limit of this assay was 0.1 ng/ml per sample.

**RNA extraction and RT–PCR of MK mRNA**

Total RNA was extracted from peritoneal bone marrow-derived cells, peritoneum and endometriotic tissues using an RNase Mini Kit (Qiagen, Hilden, Germany). RT–PCR was performed using Rever Tra Dash (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse-transcribed in a total volume of 20 μl, and complementary DNA (cDNA) was amplified using oligonucleotide primers based on the human MK sequence. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Toyobo) were used to ensure RNA quality and amounts. MK primers (sense, 5'-CCTGCAACTGGAAGAGGAG-3'; antisense, 5'-AGCAGACAGAAAGGACTGGT-3') were chosen to amplify a 320 bp fragment. PCR conditions for the amplifications of MK and GAPDH were 30 cycles at 98°C for 10 s, 60°C for 2 s, and 74°C for 20 s. PCR products were analysed by agarose gel electrophoresis with ethidium bromide. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). RT–PCR was performed using oligonucleotide primers based on the human MK sequence. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Toyobo) were used to ensure RNA quality and amounts. MK primers (sense, 5'-CCTGCAACTGGAAGAGGAG-3'; antisense, 5'-AGCAGACAGAAAGGACTGGT-3') were chosen to amplify a 320 bp fragment. PCR conditions for the amplifications of MK and GAPDH were 30 cycles at 98°C for 10 s, 60°C for 2 s, and 74°C for 20 s. PCR products were analysed by agarose gel electrophoresis with ethidium bromide. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen), and their identities were confirmed using an ABI PRISM™ 310 genetic analyzer (Applied Biosystems, Foster City, CA).

**Statistical analysis**

Analysis of variance was used to compare the BrdU incorporation of the cultured cells. The data of MK in PF were described as median and interquartile range (IQR). Mann–Whitney test was used to compare concentrations of MK in PF. *P < 0.05 was considered significant.

**Results**

The effects of MK on DNA synthesis in ESC are shown in Figure 1. BrdU incorporation into DNA was significantly increased by MK at 100 (P < 0.05) and 1000 ng/ml (P < 0.001). MK at 1000 ng/ml enhanced the level of BrdU incorporation to 125% of that of the control. Addition of PF, which was used as a positive control, increased BrdU incorporation to 242% of the control.

All of the PF samples examined contained detectable concentrations of MK above the assay limit. The MK concentrations in the PF of the women with minimal endometriosis (r-ASRM score ≥6) were significantly higher than those of the women with no or minimal endometriosis (0.93 ng/ml, 0.87–1.17; median, IQR) were virtually the same as those of the women without endometriosis (0.97 ng/ml, 0.67–1.27). Thus, we combined the data of the women with no or minimal endometriosis to compare with those of the women with advanced endometriosis (r-ASRM score ≥6). As shown in Figure 2, the MK concentrations in the PF of the women with advanced endometriosis (1.21 ng/ml, 0.80–2.27) were significantly higher than those of the women with no or minimal endometriosis (0.96 ng/ml, 0.67–1.26, P < 0.05). The MK concentrations of the GnRHa group were significantly lower than those of the other two groups (P < 0.001). When MK concentrations in PF of all...
women except GnRHa group were compared according to the menstrual phase, those in the luteal phase (1.32 ng/ml, 0.68–2.24) were significantly higher than those in the follicular phase (0.95 ng/ml, 0.68–1.24, P < 0.05). Therefore, the MK concentrations in PF were further analysed, stratified by the menstrual phases. Accordingly, the significant increase in the MK concentrations in advanced endometriosis was definite in the follicular phase (P < 0.01; Table II).

To evaluate whether peritoneal adhesions have any relevance to the elevation of MK in PF, we divided the subject into two groups, excepting the iRHA group, with regard to adnexal adhesions and cul-de-sac obliteration (Table III). PF from women with adnexal adhesions (n = 64) had significantly higher MK concentrations (1.21 ng/ml, 0.87–2.24) than that from women without adhesions (0.94 ng/ml, 0.63–1.49, n = 63; P < 0.05). On the other hand, the concentrations of MK in PF were not different, irrespective of the presence of cul-de-sac obliteration.

The concentration of MK in follicular fluids was 265 ng/ml (199–359), over 200-fold higher than that in PF.

As demonstrated in Figure 3, the expression of MK mRNA was detected as a clear band at 320 bp in peritoneal bone marrow-derived cells, peritoneum and endometriotic tissues.

**Discussion**

In the present study, we showed that MK stimulates the proliferation of ESC. In addition, MK levels in PF were increased in the women with advanced endometriosis as compared to those in the women with minimal or no endometriosis.

MK has pleiotropic functions not only for promoting cell proliferation, as demonstrated in the endometriotic cells in the present study, but also for inducing angiogenesis and inflammation (Takada et al., 1997; Horiba et al., 2000). All these functions are suggested to be important for developing tumors and tumor-like lesions such as endometriosis. Indeed, MK expression has been shown to increase in a number of malignant tumors compared to that in the adjacent non-cancerous tissue (Aridome et al., 1995; Koide et al., 1999; Konishi et al., 1999), suggesting its relevance in tumor formation. Interestingly, eutopic endometrial cells of women with endometriosis expressed increased levels of MK as compared to those of women without endometriosis (Chung et al., 2002). In the light of the proliferative effect of MK on ESC, the increased levels of MK in eutopic endometrial cells may contribute, in part, to the formation of endometriotic foci when they are refluxed into the peritoneal cavity.

Consistent with the notion that peritoneal environment is important for the development of endometriosis, the concentration of MK in PF of women with advanced endometriosis was increased. As shown in this study, in addition to endometriotic tissues, peritoneal bone marrow-derived cells and peritoneal tissues produce MK. Given that locally produced MK stimulates the proliferation of ESC, it may be plausible that the concentrations of MK in PF are relatively low, compared to those effective in vitro, possibly due to dilution. These findings also imply that MK may stimulate the growth of endometriotic lesions in an autocrine and paracrine fashion, although ectopic endometriotic lesions express lower levels of MK than eutopic endometrium (Chung et al., 2002).

A recent report has shown that MK stimulates intraperitoneal adhesion in a mouse model of peritoneal injury (Inoh et al., 2004). The present study demonstrated that the concentrations of MK in women with adnexal adhesions are increased as compared with those without adhesions. In view of the propensity of endometriosis to form peritoneal adhesions along the progress of the disease, MK might also contribute to the adhesion formation.

The sources of MK in PF appear to be widely distributed. Our RT–PCR analysis demonstrated that the concentration of MK in women with adnexal adhesions are increased as compared with those without adhesions. In view of the propensity of endometriosis to form peritoneal adhesions along the progress of the disease, MK might also contribute to the adhesion formation.

The sources of MK in PF appear to be widely distributed. Our RT–PCR analysis demonstrated that the concentration of MK in women with adnexal adhesions are increased as compared with those without adhesions. In view of the propensity of endometriosis to form peritoneal adhesions along the progress of the disease, MK might also contribute to the adhesion formation.

**Table II.** Midkine (MK) concentrations in peritoneal fluid (PF) of women classified by r-ASRM score with stratification by the menstrual phases

<table>
<thead>
<tr>
<th>r-ASRM score</th>
<th>Follicular phase</th>
<th>Luteal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–5</td>
<td>0.80 ng/ml [0.59–0.99] (n = 29)</td>
<td>1.21 ng/ml [0.82–1.99] (n = 29)</td>
</tr>
<tr>
<td>≥6</td>
<td>1.12 ng/ml [0.84–1.90] (n = 34)</td>
<td>1.63 ng/ml [0.72–2.33] (n = 35)</td>
</tr>
</tbody>
</table>

Median [IQR] (sample numbers). NS: not significant.

**Table III.** MK concentrations in PF of women classified by the presence of adnexal adhesions or cul-de-sac obliteration

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adnexal adhesions</td>
<td>1.21 ng/ml [0.87–2.24] (n = 64)</td>
<td>0.94 ng/ml [0.63–1.49] (n = 63)</td>
</tr>
<tr>
<td>Cul-de-sac obliteration</td>
<td>1.21 ng/ml [0.71–1.90] (n = 36)</td>
<td>1.01 ng/ml [0.71–1.83] (n = 91)</td>
</tr>
</tbody>
</table>

Median [IQR] (sample numbers). NS: not significant.
centrations of MK in human ovarian follicles, over 200-fold higher than those in PF. The high concentration of MK in follicular fluid may partly explain the finding that MK in PF was increased in the luteal phase as compared to that in the follicular phase. On the other hand, it might be partially due to the lack of follicular fluid influx to PF that the difference of the MK concentrations between advanced endometriosis and no/minimal endometriosis was more evident in the follicular phase than in the luteal phase.

A remarkable finding in this study is that MK levels in the women undergoing GnRHa treatment were significantly lower as compared to those in the women with advanced endometriosis and those with minimal or no endometriosis. It has been reported that estradiol (E2) induced MK mRNA expression in endometrial epithelial cells (Zhang et al., 1995). The several estrogen responsive element half-palindromic motifs (Kato et al., 1992) residing in the promoter region of the MK gene (Uehara et al., 1992) have been suggested to cause this effect of E2 (Zhang et al., 1995). Hypoestrogenic status induced by GnRHa treatment may suppress gene transcription of MK in various cells. In addition, anovulation caused by GnRHa treatment could contribute to the suppression of MK levels in PF, hindering the flux of follicular fluid into the peritoneal cavity.

MK has been shown to proliferate in several cells, including fibroblasts, tumor cells and keratinocytes (Muramatsu and Muramatsu, 1991; Muramatsu et al., 1993; Inazumi et al., 1997). The signaling pathways mediating the MK effects are only partially revealed at present. While receptor complexes containing PTPz, LRP, ALK and syndecans are suggested to be at work for MK functions (Muramatsu, 2002; Sakaguchi et al., 2003; Deepa et al., 2004), LRP and syndecan-1 have been shown to be present in the endometrium (Sayegh et al., 1995; Inki, 1997; Foca et al., 2000). In view of these findings, it may be interesting to explore the therapeutic potential of MK inhibitors for endometriosis. In mouse rectal carcinoma cells, antisense oligonucleotides to MK have been shown to suppress tumor formation (Takei et al., 2001).

In summary, the present study demonstrated that MK stimulates the proliferation of ESC and that MK levels are increased in the PF of women with advanced endometriosis, suggesting that MK may play a role in the development of endometriosis.

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References

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