Altered expression of claudin-3 and claudin-4 in ectopic endometrium of women with endometriosis

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Objective: To investigate the expression of claudin-3 and claudin-4 in the eutopic and ectopic endometrium of women with endometriosis and to evaluate the role of claudin-3 and claudin-4 in the pathogenesis of endometriosis.

Design: Cross-sectional measurement of gene expression levels of claudin-3 and claudin-4 on endometriotic tissue.

Setting: Academic.

Patient(s): Thirty-five patients with endometriosis and 35 healthy women who were free of endometriosis were recruited for the study.

Intervention(s): Expression of claudin-3 and claudin-4 were investigated with immunohistochemical analysis, Western blot, and real-time polymerase chain reaction. Morphologic change of tight junction was also observed in different kinds of endometria.

Main Outcome Measure(s): The expression levels of claudin-3 and claudin-4 in epithelial cells from 35 ectopic endometrial tissues, 27 eutopic endometrial tissues from women with endometriosis, and 35 normal endometrial tissues from women without endometriosis.

Result(s): Expression of claudin-3 and claudin-4 was significantly lower in the ectopic endometriotic tissue than in the eutopic endometrium from women with endometriosis and normal controls at both the messenger RNA and protein levels. No significant difference was found between eutopic endometrium from women with endometriosis and normal endometrium from women without endometriosis.

Conclusion(s): Down-regulated expression of claudin-3 and claudin-4 in ectopic endometrium suggests that claudin-3 and claudin-4 might play a pathogenic role in the formation of endometriosis. (Fertil Steril 2009;91:1692–9.

Key Words: Endometriosis, tight junction, claudin-3, claudin-4

Endometriosis is a common benign gynecologic disease that affects approximately 10% of women of reproductive age (1). It occurs at many intra-abdominal sites, including the ovaries, fallopian tubes, broad ligaments, cervix, rectovaginal septum, pouch of Douglas, small and large intestines, appendix, and anus. Rarely, it may be found in the kidneys, lungs, and pleura (2). Different theories as to the origin and pathogenesis of this disease have been developed throughout the years, including retrograde menstruation (3, 4), migration through the vascular or lymphatic system (5, 6), or neoplastic dedifferentiation from mesothelial cells (7, 8). To date, Sampson’s theory of retrograde menstruation that postulates reflux of shed endometrial tissue through the fallopian tube with implantation on the peritoneal surface is widely accepted (4). According to the theory, retrograde menstruation, peritoneal adhesion of shed endometrial tissue, degradation of extracellular matrix, and outgrowth of these endometrial cells are essential steps (9, 10).

Although endometriosis is a “benign” disease, it has shown many characteristics of neoplasia: unrestrained growth, increased vascularization, and even features classically associated with malignancy, such as a high risk of recurrence, tissue invasion, and metastasis. However, only a few investigations have been carried out to study its progressive character (11, 12).

The Claudin family of proteins, the main transmembrane proteins of tight junctions, has crucial roles in the control of paracellular transport and in the maintenance of cell polarity (13). Gene expression profiling analyses have shown that claudin gene expression is frequently altered in various cancers. Abnormal expression of claudin molecules, such as claudin-1, claudin-3, claudin-4, and claudin-7, by neoplastic cells is likely to be an important determinant of local invasion and dissemination, and claudin represents a promising target for cancer detection, diagnosis, and therapy (14, 15). Recently we have found that the expression of claudin-3 and claudin-4 in endometrial tissue was downregulated.
significantly higher in patients with atypical hyperplasia and endometrioid adenocarcinoma than in women with normal cyclic endometrium. This demonstrates that claudin-3 and claudin-4 are involved in the malignant transformation of normal endometrium (16). We hypothesized that claudin-3 and claudin-4 might also be associated with the pathogenesis of endometriosis and that expression levels of claudin-3 and claudin-4 might be different in the ectopic endometrium of patients with endometriosis and normal women. In the present study we also tried to determine the expression of claudin-3 and claudin-4 in the eutopic and ectopic endometrium of patients with endometriosis.

MATERIALS AND METHODS

Tissue Samples

The study was approved by the local institutional review board, and written informed consent for participation in the study was obtained from each subject. The presence of endometriosis was suspected either clinically or by ultrasound examination and confirmed by the surgical findings and the postoperative pathological study. Endometriosis was scored and staged according to the revised classification system of the American Fertility Society (17). Patients with irregular menstruation, steroid treatment, or who had received hormonal treatment for at least 3 months before the study were excluded. All endometriotic tissue specimens were obtained from endometriotic ovarian cysts (n = 35; mean age 36 years, range 23–47 years; stages III and IV). Eutopic endometrium was obtained from 27 of these 35 women with endometriosis. Of the 27 women with endometriosis, 10 were in the proliferative phase of the menstrual cycle, 17 in the secretory phase. Thirty-five control endometrial tissues from women without endometriosis (mean age 39 years, range 29–49 years) were obtained. Fifteen were in the proliferative phase, and 20 were in the secretory phase.

Immunohistochemistry

Tissue sections were incubated with polyclonal rabbit anti-claudin-3 and monoclonal mouse anti-claudin-4 (Zymed, San Francisco, CA) after antigen retrieval. Antigen-bound primary antibody was detected with standard avidin-biotin immunoperoxidase complex (DAKO, Carpinteria, CA). Normal colonic mucosa was used as positive control. For negative control slides, the addition of primary antibody was omitted. Immunostaining intensity was classified as follows: –, no immunostaining present; +, weak staining; ++, medium staining; and ++++, intense staining.

Western Blot

Protein samples (25 μg per lane) were separated by 12% sodium dodecyl sulphate–polyacrylamide gels under denaturing conditions and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed with the primary antibody (anti-claudin-3, 1:200; anti-claudin-4, 1:250; Zymed), then incubated in horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse IgG, 1:5000; ZhongShan Biotech, Beijing, China). For detection, enhanced chemiluminescence was carried out with the enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). An internal reference sample (same on each blot) was included as the standard for quantification. The standard was set to 100. The signal from each band was correlated to the standard, and this relative number was used for statistical analysis.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed as previously described (16). Five micrograms of total RNA from each sample was reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI). Typical real-time PCR reaction was conducted with 2 μL complementary DNA template (from 50 μL of total volume), 0.8 μL forward primer (10 μmol/L), 0.8 μL reverse primer (10 μmol/L), 6.4 μL RNase-free water, and 10 μL SYBR Green I mixture (Takara, DaLian, China). A four-step experimental run protocol was used: [1] denaturation program (10 minutes at 95°C); [2] amplification and quantification program repeated 40 times (10 seconds at 95°C; 5 seconds at 57°C for claudin-3 and -4 or 5 seconds at 55°C for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; 10 seconds at 72°C for claudin-3 and -4 or 15 seconds at 72°C for GAPDH with a single fluorescence measurement); [3] melting curve program (65°C–95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement); [4] cooling program down to 40°C. The relative expression is based on the expression ratio of a target gene vs. a reference gene (GAPDH).

Transmission Electron Microscopy

Normal secretory-phase endometrium, eutopic secretory-phase endometrium of woman with endometriosis, and ovarian endometriotic tissue were fixed and embedded in Epon-812. The ultrathin sections were stained and observed with an electron microscope (1200EX; JEOL, Tokyo, Japan) at 80 kV.

Statistical Analysis

Statistical differences in claudin-3 and claudin-4 expression were tested by χ² test and one-way analysis of variance. P<.05 indicated statistical significance.

RESULTS

Morphologic Change of Tight Junction

Intact tight junction appears at the apical end of adjacent glandular epithelial cells in normal secretory endometrium. As a series of fusion points, tight junction completely eliminated the intercellular space (Fig. 1A). No obvious change of tight junction was found in the eutopic secretory-phase
endometrium of woman with endometriosis (Fig. 1B). Conversely, the ovarian endometriotic tissue appeared to have a lack of tight junction. The morphology of the junctional complex was disrupted, and collagen bundles could be easily detected between adjacent ectopic endometrial cells (Fig. 1C).

Expression of Claudin-3 and Claudin-4 Protein in Ovarian Endometrioma Vs. Endometrial Tissue from Women with Endometriosis

The immunohistochemical analysis of claudin-3 and claudin-4 showed a specific brownish immunostaining localized to the glandular epithelial cell membrane. There was no signal
detected in the stromal cell. Claudin-3 and claudin-4 expressed at the apical border of the epithelium in a segmental linear to dot-like membranous pattern, respectively, in 12 and 15 cases of endometrium from women with endometriosis. Of 17 cases of secretory eutopic endometrium glandular epithelial cells, 2 exhibited a moderate circumferential pattern of staining for claudin-3 and claudin-4. Strong epithelial staining was not found in any of the 27 uterine endometria. In ovarian endometriotic tissues, 6 and 7 cases showed weak staining for claudin-3 and claudin-4, respectively. Claudin-3 was absent in 29 cases, and no expression of claudin-4 was detected in 28 samples (Table 1, Fig. 2).

Semiquantitative immunoblotting was used to compare and estimate statistical differences in the expression of claudins in eutopic and ectopic endometrial tissue. Claudin-3 and claudin-4 were readily detectable at approximately 22 kd in approximately half of the eutopic endometria, whereas weak bands were found in only 10 cases of ectopic endometrium (Fig. 3). The expression extent of claudin-3 and claudin-4 in eutopic endometrium was significantly higher than that in ectopic endometrium in both proliferative and secretory phases. When the 27 eutopic endometria were compared with their matched ectopic endometria, ovarian endometriomas also showed significantly lower levels than eutopic endometria.

Expression of Claudin-3 and Claudin-4 Protein in Ovarian Endometrioma Vs. Endometrium from Women without Endometriosis

According to Western blot the relative expression levels of claudin-3 and claudin-4 in ovarian endometrioma were 20.7 ± 11.2 and 26.8 ± 13.6. In endometrium from women without endometriosis, the expression levels of claudin-3 and claudin-4 were 85.5 ± 16.9 and 89.4 ± 24.1, respectively. Ovarian endometriotic tissue showed a significant decrease in protein levels of claudin-3 and claudin-4 compared with endometrium from women without endometriosis.

Expression of Claudins Messenger RNA

As shown in Table 2, much lower levels of claudin-3 and claudin-4 were found in ovarian endometrioma. The analysis of claudin-3 and claudin-4 messenger RNA (mRNA) expression showed no statistically significant difference between normal endometrium and eutopic endometrium of women with endometriosis, consistent with the result obtained by Western blot. The expression level of endometrial claudin-4 mRNA at the secretory phase of control women seemed slightly higher than at the proliferative phase, but there was no significant statistical difference.

DISCUSSION

Sampson’s theory (4) implies that endometrial cells must have the capability to detach from the endometrium, re-attach after passive transport into the peritoneum, and invade the

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Claudin-3</th>
<th></th>
<th></th>
<th></th>
<th>Claudin-4</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7 (46.6)</td>
<td>8 (53.3)</td>
<td>0</td>
<td>0</td>
<td>6 (40)</td>
<td>9 (60)</td>
<td>0</td>
</tr>
<tr>
<td>SE</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 (50)</td>
<td>8 (40)</td>
<td>2 (10)</td>
<td>0</td>
<td>7 (35)</td>
<td>9 (45)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>Eutopic</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE</td>
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<td>0</td>
<td>0</td>
<td>4 (40)</td>
<td>6 (60)</td>
<td>0</td>
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<tr>
<td>SE</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 (47.1)</td>
<td>7 (41.2)</td>
<td>2 (11.7)</td>
<td>0</td>
<td>6 (35.3)</td>
<td>9 (52.9)</td>
<td>2 (11.8)</td>
</tr>
<tr>
<td>Ectopic</td>
<td>35</td>
<td>29 (82.9)</td>
<td>6 (17.1)</td>
<td>0</td>
<td>0</td>
<td>28 (80)</td>
<td>7 (20)</td>
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</table>

Note: Values are number (percentage). PE = proliferative-phase endometrium; SE = secretory-phase endometrium.<sup>a</sup>P < .05 compared with ectopic endometrium.

host tissues. Theoretically, the occurrence of alteration in claudin expression may be of particular interest. Claudin is the major component of tight junction, which is essential in the control of paracellular ion flux and the maintenance of cell polarity. Changes in the expression of claudin have been described in several human tumors and diseases. The imbalance of tight junction component might have caused morphologic change, as we saw by transmission electron microscopy and a further loosening of cell–cell contacts, allowing the liberation of individual malignant cells from the primary tumor. Another aspect is change of permeability: loss of claudin protein may be important to allow the diffusion of nutrients and other factors necessary for the survival and growth of tumor cells (18). In the present study,
Claudin-3 and claudin-4 protein expression presented with representative immunoblots. 1: Normal endometrium, proliferative phase; 2: normal endometrium, secretory phase; 3: eutopic endometrium with endometriosis, proliferative phase; 4: eutopic endometrium with endometriosis, secretory phase; 5, 6: ectopic endometrium.


expression of claudin-3 and claudin-4 in the ectopic and eutopic endometrium of women with endometriosis has been investigated. We found that expression of claudin-3 and claudin-4 was significantly lower in the ectopic endometriotic tissue than in the eutopic endometrium from women with endometriosis and normal controls at both the mRNA and protein levels. Therefore, our results suggest that the loss of the tight junction property may play a role in the shedding of endometrial cells during menstruation and in the attachment of endometrial tissue fragments to the peritoneum. The decrease or lack of claudin-3 and claudin-4 expression seems to be crucial in the invasive phenotype of endometriotic cells.

Eutopic endometrium of patients with endometriosis is histologically similar to normal endometrium of women without endometriosis, although biochemical differences exist between them (19, 20). In the present study we found that expression of claudin-3 and claudin-4 in eutopic endometrium from women with endometriosis was lower compared with normal endometrium from women without endometriosis, but the difference was not statistically significant. This result agrees with that from a previous study, in which no difference was found in integrin expression between endometrium from women with and without endometriosis (21). In contrast, some evidence has suggested that the eutopic endometrium from women with endometriosis has altered expression of several components of the junctional complexes, such as connexin, cadherin, and catenin (22, 23). It is difficult to explain the reasons for this discrepancy. However, to our knowledge, this is the first study to evaluate tight junction in relation to endometriosis. Our finding of decreased levels of claudin-3 and claudin-4 in ovarian endometrioma suggests that down-regulated expression of claudin-3 and claudin-4 is correlated with the formation of endometriosis.

It is well known that the development and progression of endometriosis depends on the presence of systemic sex steroids (24, 25). Endometriotic lesions grow in an estrogenic environment and tend to regress when local sex steroid concentrations are low (26). However, the present study shows that expression of claudin-3 and claudin-4 is not dependent on the phase of the menstrual cycle and thus indicates a minor role for estrogen and progesterone in the regulation of cell tight junction molecules. Because of our small sample size, it is difficult to verify whether the glandular expression of claudin-3 and claudin-4 was not influenced by the cycle phase. The result of amplified sample number in each menstrual phase needs further investigation.

Recent research has shown that claudin-3 and claudin-4 are significantly elevated in several malignancies, such as breast (27, 28), pancreas (29), prostate (30), and uterine serous papillary carcinoma (31, 32). In our previous study claudin-3 and claudin-4 were also found to be up-regulated in endometrial atypical hyperplasia and endometrioid adenocarcinoma compared with normal endometrium (16). This suggests that claudin-3 and claudin-4 involve in malignant transformation of normal endometrium and may be associated with carcinogenesis and tumor progression. Endometriosis, albeit considered a “benign” disease, resembles the biologic behavior of malignant disease in several ways. Paradoxically, not similar to the case in malignant endometrium, ectopic endometrium showed significantly down-regulated expression of claudin-3 and claudin-4. In addition, although the histogenesis of ovarian endometriotic cyst is different from that of other ovarian tumors, it is interesting to note that endometriotic lesions share some clinical features with carcinomas of the ovary, such as recurrence and peritoneal spreading. Furthermore, endometriosis may progress to invasive endometrioid adenocarcinoma, particularly in the ovary (33, 34). However, a high increase in claudin-3 and claudin-4

TABLE 2

Expression of claudin-3 and -4 in normal, eutopic and ectopic endometrium according to real-time PCR.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Claudin-3</th>
<th>Claudin-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controla</td>
<td>35</td>
<td>12.9 ± 6.4</td>
<td>14.5 ± 6.8</td>
</tr>
<tr>
<td>PEa</td>
<td>15</td>
<td>11.6 ± 3.9</td>
<td>13.4 ± 6.2</td>
</tr>
<tr>
<td>SEa</td>
<td>20</td>
<td>14.8 ± 5.7</td>
<td>16.1 ± 7.6</td>
</tr>
<tr>
<td>Eutopica</td>
<td>27</td>
<td>12.4 ± 6.3</td>
<td>13.8 ± 9.5</td>
</tr>
<tr>
<td>PEa</td>
<td>10</td>
<td>10.8 ± 4.5</td>
<td>12.3 ± 5.9</td>
</tr>
<tr>
<td>SEa</td>
<td>17</td>
<td>13.7 ± 7.0</td>
<td>15.6 ± 8.3</td>
</tr>
<tr>
<td>Ectopic</td>
<td>35</td>
<td>2.0 ± 1.4</td>
<td>2.6 ± 2.5</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SD. PE = proliferative-phase endometrium; SE = secretory-phase endometrium.

aP < .01 compared with ectopic endometrium.

expression was noted in ovarian carcinomas (35, 36). These results are rather conflicting and are further complicated by our incomplete knowledge regarding the role of claudins in the pathogenesis of endometriosis. It is generally accepted that tight junction is a crucial structure for normal epithelial cell homeostasis (37). Down-regulation of claudin protein has been suggested as a mechanism for tight junction dismantling and to be an early event in the process of metastasis. In fact, claudin-1 has been found to be reduced in breast cancer as well as in colon cancer (38, 39). Claudin-7 has also been found down-regulated in invasive breast cancer and in head-and-neck cancer (40, 41). Therefore, claudins’ function may be highly tissue specific and may depend on the exact molecular circuitry of the cell (42).

In conclusion, the down-regulation of claudin-3 and claudin-4 observed in ectopic endometrium from women with endometriosis might be an important clue to the invasive potential and the growth of endometrial tissue outside the uterus. This process would lead to the formation of endometriotic lesions. In this study, we investigated only two claudin family members. Because of the high specificity of claudin expression patterns, further study is needed to clarify the effects of other claudin proteins on the pathogenesis of endometriosis.

Acknowledgment: The authors thank Professor Guo-Xiang Zou for help with the transmission electron microscopy experiments.

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ovarian surface epithelium as compared to epithelia in inclusion cysts and epithelial ovarian tumours. Int J Cancer 2006;118:1884–91.


