Morphometric evaluation of stromal vascularization in the endometrium in adenomyosis

Hirotaka Ota1,3, Shinichi Igarashi2 and Toshinobu Tanaka1

1Departments of Obstetrics and Gynecology, Akita University School of Medicine, Akita-city, Akita-ken 010 and 2Koto General Hospital, Hachirogata, Akita-ken 018–16, Japan
3To whom correspondence should be addressed

A computerized morphometric investigation of stromal vascularization in the endometrium during adenomyosis was performed retrospectively. Using a polyclonal antibody and a peroxidase–antiperoxidase method, formalin-fixed paraffin sections of the tissue were stained for von Willebrand factor, a marker for endothelium. The subjects were divided into two groups: 42 patients with histologically proven adenomyosis and 29 fertile control subjects, 12 in the proliferative phase and 17 in the secretory phase. Objective quantitative colour image analysis was used to assess the staining intensity and hence the degree of vascularization. In the control group, stromal vascularization increased in the secretory phase. In adenomyosis, vascularization increased markedly, up to 11.6 times that of the controls, in terms of the in total surface area of capillaries per mm2 in the endometrium in the proliferative phase. These findings suggest clinical relevance to severe functional disturbances such as hypermenorrhoea or iron deficiency anaemia.

Key words: adenomyosis/endometrium/endothelial cell/vascularization/uterus

Introduction

Adenomyosis typically affects parous women in their forties (Parazzini et al., 1997). The disease is characterized by the ectopic growth of endometrial tissues into the myometrium as well as by a diffuse enlargement of the uterus. Adenomyosis causes distressing and often serious functional disturbances such as hypermenorrhoea, dysmenorrhoea, among others (Benson et al., 1958; Emge, 1962; Molitor, 1971). We often encounter patients with severe iron deficiency anaemia requiring blood transfusion and/or major surgery. However, the reason patients with adenomyosis develop hypermenorrhoea remains unclear.

It is well known that peritoneal vascularization is a characteristic finding in the active lesions of endometriosis (Donnez et al., 1995). It is interesting to note that the major histological difference between adenomyosis and endometriosis is the site of the endometriotic tissues, i.e. inside or outside the uterus. We have reported that adenomyosis patients exhibit marked vascularization of the surface endometrium under hysteroscopy (Ota, 1992). To our knowledge, however, no reports are available that indicate to what extent stromal vascularization is developed in the endometrium in adenomyosis. To determine whether there is any relation between hypermenorrhoea and stromal vascularization, we examined the distribution of capillaries in the endometrium in adenomyosis using histochemical methods and computerized colour image analysis.

Materials and methods

Patients

The subjects consisted of 71 women who were treated at the Department of Obstetrics and Gynecology at Akita University Hospital. They were divided into two groups: the fertile controls (n = 29), and the adenomyosis group (n = 42), who had undergone hysterectomy. The controls consisted of fertile women with regular and biphasic menstrual cycles, 12 in the proliferative phase and 17 in the secretory phase. All of the controls were parous women with clear male factor infertility (mild oligozoospermia orazoospermia).

None of the controls had identifiable endometriosis or adenomyosis. These women conceived after artificial insemination of husband’s or donor’s semen within three treatment cycles and delivered full-term babies. The mean age in the fertile control and the adenomyosis group was 29.1 years (range 23–36 years) or 42.5 years (range 33–49 years), respectively. When the patients in the adenomyosis group consulted our outpatient clinic for treatment, blood sampling was performed for peripheral haematological analysis, CA125 and the level of serum iron before treatment. All adenomyosis patients did not take any medication except for a ferric medicine to help their dysmenorrhoea and hypermenorrhoea for at least 1 month prior to the hysterectomy, and diagnoses were confirmed histologically after the surgery. Adenomyosis patients with submucous leiomyoma or giant intramural/subserosal leiomyoma, having a size occupying more than one-third of the corpus uteri, were excluded from the present study.

Before starting any medication in the control group, or just after the hysterectomy in the adenomyosis patients, endometrial tissue was obtained during each optional phase. In the control group, endometrial specimens were obtained by curettage. The tissues were fixed in neutral buffered 10% formalin solution (Wako, Osaka, Japan). The menstrual cycle of the patients was estimated by endometrial dating according to the described method (Noyes et al., 1950). Informed consent was obtained in each case, and approval for the study was granted by the Institutional Review Board.

Reagents

A rabbit polyclonal antibody against human von Willebrand Factor (A-082; vWf) was obtained from Dakopatts (Glostrup, Denmark). This antibody has been proven to bind specifically on the endothelial cells of vessels in the endometrium (Au and Rogers, 1993).
secondary antibody [goat F(ab')2 anti-rabbit Ig (H+L) horseradish peroxidase conjugate; 458] was obtained from MBL (Nagoya, Japan).

Staining
The endometrial tissue samples were cut into blocks (~1 cm³) for standard histology. Serial 3 µm sections of tissue were cut, deparaffinized, and rehydrated through ethanol. The sections were stained using the peroxidase–antiperoxidase (PAP) method. First, the sections were incubated in 1% hydrogen peroxide in phosphate buffer saline solution (PBS; 0.1 mol/l) for 15 min to block endogenous peroxidase activity. Non-specific background staining was reduced by treating the sections with non-immune 10% swine serum (809; Cosin Bio, Sakato, Japan) in PBS. Then the polyclonal antibody (×40 dilution) was added and the sections were incubated for one h at 37°C. After washing them with PBS, the second antibody (×2500 dilution) was added and they were incubated for 1 h at 37°C; then the PAP complex was layered on the slides. After washing the sections with PBS, they were stained with 0.2 mg/ml 3,3′-diaminobenzidine (DAB) tetrahydrochloride containing 0.005% hydrogen peroxide in PBS. Finally, the sections were counterstained with Harris haematoxylin. Negative controls for immunostaining were prepared by substituting the first antibody with non-immune rabbit serum IgG. In each run, a section of early secretory endometrium with strong vWF staining was routinely included as a positive control.

Evaluation of staining
Tissue sections were viewed through a ×10 objective and a ×10 eyepiece on an Olympus light microscope (Olympus, Tokyo, Japan). In the present study, only capillaries in the functional layers of the endometrium were evaluated, since the nature of specimens was different between the control and adenomyosis groups. Ten fields of each biopsy specimen were captured by a CoolScan scanner (Nikon, Tokyo, Japan) on an LC575 Apple computer (Apple Computer Japan, Tokyo, Japan), and the data were saved as a PICT file (1200 dpi) in Adobe Photoshop (Version 3.0; Systemsoft Co., Fukuoka, Japan). After correcting for uneven illumination and background colour, the image features were displayed on an RGB monitor and stored for processing by the image analysis program. A software-generated blue filter was placed over the image to enhance the positive red–brown staining of the DAB. The pictures were processed using an NIH image (Version 1.6; Wayne Rasband, NIH, USA) and the area of capillaries somewhat >40 µm² was measured. The grey-level images were converted to binary images. The interactive measurements of the selected parameters were appended and stored in a database. Assessment was based on positive staining associated with the wall of capillaries. The parameters of capillaries evaluated were: the mean surface area, mean circumference, mean major axis, mean minor axis or mean number of capillaries per field, and total surface area or total number of capillaries per mm². The morphometric analysis of each specimen was done by two different observers blinded as to the specimen source.

Statistical analyses
The results are expressed as the mean ± SE where applicable. Statistical analysis was performed by the unpaired t-test (Welch) test. Differences were regarded as significant if P < 0.05.

Results
Clinical parameters are shown in Table I. The most frequent symptom in the adenomyosis group was hypermenorrhoea, followed by dysmenorrhoea. In 95% of all patients the weight of the uterus was <400 g. Iron deficiency anaemia was noted in 71.4% of the cases, therefore only 28.6% of the cases were within the normal range before surgery.

In the control group, the mean surface area, mean major axis, mean minor axis, total surface area and total number of capillaries, all increased significantly in the secretory phase compared with to the proliferative phase (Table II). Of these parameters, the total surface area of capillaries per mm² increased by 2.4 times that of the proliferative phase. In contrast, all parameters increased in the adenomyosis group in both the proliferative and secretory phase compared to the control group. In particular, the total surface area of capillaries per mm² markedly rose, by 11.6 times, compared to that of the proliferative phase in the control group.

Histologically, positive staining was observed in all specimens in the control and adenomyosis groups (Figure 1). All negative controls were unstained and strong staining was consistently seen in the positive controls. Specific staining was confined to the endothelium lining the blood capillaries. No staining was found in the endometrial glands or stroma.

Discussion
In adenomyosis, the two most frequent symptoms are hypermenorrhoea and dysmenorrhoea. According to several reports, the incidence of hypermenorrhoea ranges from 36 to 70% (Benson et al., 1958; Dougherty and Anderson, 1964; Molitor, 1971). Hypermenorrhoea often becomes increasingly severe and develops into a major functional nuisance leading to iron deficiency anaemia. In the present study, the incidence of hypermenorrhoea and iron deficiency anaemia was high, 88.1 and 71.4%, respectively.

Vascularization is a normal physiological process, during folliculogenesis, the development of the endometrium, implantation, placentation and in embryonic development. However, vascularization occurs in various pathological processes, e.g. wound healing, rheumatoid arthritis and tumours. Vascularization is also found in endometriosis, particularly in the active lesions of peritoneal endometriosis (Donnez et al., 1995). As far as we know, however, there are no reports focused on the stromal vascularization of the endometrium in adenomyosis. Various substances have been cited as possible
### Table II. Morphometric parameters of capillaries in the endometrium in the control and adenomyosis groups (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Mean surface area/field (µm²)</th>
<th>Mean circumference/field (µm²)</th>
<th>Mean major axis/field (µm)</th>
<th>Mean minor axis/field (µm)</th>
<th>Mean number/field</th>
<th>Total surface area (×10³/mm²)</th>
<th>Total number (l/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative phase</td>
<td>496 ± 54</td>
<td>382 ± 20</td>
<td>47.8 ± 2.2</td>
<td>23.0 ± 0.8</td>
<td>22 ± 4</td>
<td>8 ± 2c</td>
<td>33 ± 3c</td>
</tr>
<tr>
<td>Secretory phase</td>
<td>730 ± 70</td>
<td>426 ± 24</td>
<td>54.8 ± 1.8</td>
<td>26.8 ± 1.0</td>
<td>29 ± 4</td>
<td>19 ± 2c</td>
<td>53 ± 8c</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>(n = 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01 NS</td>
<td>0.05</td>
<td>0.01 NS</td>
<td>0.01 NS</td>
<td>NS</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Adenomyosis group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative phase</td>
<td>1924 ± 308</td>
<td>768 ± 60</td>
<td>63.6 ± 2.2</td>
<td>32.0 ± 1.0</td>
<td>98 ± 9</td>
<td>93 ± 11</td>
<td>110 ± 12</td>
</tr>
<tr>
<td>(n = 26)</td>
<td>(n = 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Secretory phase</td>
<td>1220 ± 124</td>
<td>686 ± 48</td>
<td>60.0 ± 1.6</td>
<td>29.2 ± 0.8</td>
<td>110 ± 5</td>
<td>83 ± 10</td>
<td>131 ± 6</td>
</tr>
<tr>
<td>(n = 16)</td>
<td>(n = 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.001</td>
<td>0.05</td>
<td>0.05</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Figures in parentheses indicate the percentage increase for each value in the proliferative phase in the control group. The two phases were analysed statistically.*

*Figures in parentheses indicate the percentage increase for each value in the proliferative or secretory phase in the control group, respectively. The two groups were analysed statistically.*

*Although it is clear that there were more capillaries in the secretory than the proliferative phase, Figure 1A and B appears to show the reverse effect. This was because those particular fields were chosen for greater visual clarity of capillaries and histology. It should be noted that the mean circumference of capillaries was indeed larger in Figure 1B (secretory) than 1A (proliferative), in keeping with the data presented in this table. NS = not significant.*

---

**Figure 1.** Endometrial endothelial cells stained for von Willebrand factor. (A) Late proliferative phase in a control patient; (B) midsecretory phase in a control patient; (C) late proliferative phase in an adenomyosis patient; (D) in the midsecretory phase in an adenomyosis patient. Original magnification ×100. Bar = 115 µm. See footnote ‘c’ to Table II.
FACTORS IN VASCULARIZATION: GROWTH FACTORS, CYTOKINES, ADHESION MOLECULES AND EXTRACELLULAR MATRICES. WITH REGARD TO GROWTH FACTORS AND CYTOKINES, PLATELET-DERIVED GROWTH FACTOR (Pierce et al., 1992), VASCULAR ENDOTHELIAL GROWTH FACTOR (Aiello et al., 1994), TUMOUR GROWTH FACTOR-β (Pertovaara et al., 1994) AND TUMOUR NECROSIS FACTOR-α (Leibovich et al., 1987) HAVE BEEN SUGGESTED TO HAVE A ROLE IN VASCULARIZATION. IT IS NOTEWORTHY THAT THE LEVELS OF SEVERAL OF THESE SUBSTANCES ARE ALSO INCREASED IN ENDOMETRIOSIS (Eisermann et al., 1988; Oosterlynck and Koninckx, 1995; McLaren et al., 1996). IT HAS BEEN REPORTED THAT ABNORMAL IMMUNE PHENOMENA ARE FREQUENTLY OBSERVED NOT ONLY IN ENDOMETRIOSIS (Haney et al., 1981; Fakih et al., 1987; El-Roeiy et al., 1988), BUT ALSO IN ADENOMYSIS (Ota et al., 1992, 1996a). INTERESTINGLY, FEW DIFFERENCES IN THE IMMUNOLOGICAL PARAMETERS WERE NOTED BETWEEN THE TWO DISEASES (Ota et al., 1993, 1996b). THEREFORE, IT IS PLAUSIBLE THAT THESE SUBSTANCES INCREASE IN ADENOMYSIS, ALTHOUGH THE DETAILS ARE STILL UNKNOWN.

IN THE PRESENT STUDY, IT BECAME APPARENT THAT IN ADENOMYSIS THE MEAN SURFACE AREA OF EACH CAPILLARY EXPANDED UP TO 3.9 TIMES MORE THAN THOSE IN THE PROLIFERATIVE PHASE IN THE CONTROL GROUP. SEVERAL VASODILATORS SUCH AS STEROIDS, PROSTAGLANDINS, CATECHOLAMINES AND ENDOTHELIN (Åkerlund, 1995) HAVE BEEN SUGGESTED AS THE CAUSE. FIRST, OESTRADIOL IS KNOWN TO BE A POTENT VASODILATOR IN MAMMALS, WHILE PROGESTERONE HAS NO SUCH ROLE (Greiss and Rose, 1989). SECOND, THERE IS INCREASING EVIDENCE THAT A MAJORITY OF PROSTAGLANDINS CAUSE SIGNIFICANT VASODILATION (Greiss and Rose, 1989). KOIKE ET AL. (1992) FOUND THAT IN ADENOMYSIS, THE PRODUCTION OF PROSTAGLANDIN I2, A POTENT VASODILATOR AND INHIBITOR OF PLATELET AGGREGATION, WAS DOMINANT. THUS, IT IS LIKELY THAT PROSTAGLANDINS ARE RESPONSIBLE FOR VASODILATATION IN ADENOMYSIS.


THESE FACTS MIGHT EXPLAIN, AT LEAST IN PART, THE REASON FOR HYPERMENORRHOEA IN ADENOMYSIS. THAT IS, BLOOD FLOW IN THE SPIRAL ARTERIES AND CAPILLARIES IN THE SECRETORY PHASE BEGINS TO DECREASE AS MENSTRUATION APPROACHES (Fraser et al., 1987; Greiss and Rose, 1989). SPIRAL ARTERIES BEGIN TO EXHIBIT INTENSE AND PROLONGED VASOCONSTRICTION SHORTLY BEFORE THE ONSET OF MENSTRUATION, WITH RESULTING ISCHAEMIA IN THE ENDOMETRIUM (Rogers, 1996). WHEN MENSTRUATION BEGINS, FUNCTIONAL LAYERS OF THE ENDOMETRIUM ARE DESQUAMATED, PREVENTING AN EXCESS LOSS OF BLOOD. IN CONTRAST, IN ADENOMYSIS THE TOTAL SURFACE AREA OF CAPILLARIES AS WELL AS THE NUMBER OF CAPILLARIES IN THE ENDOMETRIUM INCREASES UP TO 11.6 TIMES THAT OF THE CONTROLS. ONCE MENSTRUATION STARTS, THESE STATES COULD CAUSE THE ARTERIES TO CONTRACT INSUFFICIENTLY, RESULTING IN INCREASED BLOOD LOSS, AND LEADING TO HYPERMENORRHOEA. FURTHER STUDIES ARE NEEDED TO ECLUCIDATE THE PROCESS OF VASCULARIZATION IN THE ENDOMETRIUM IN ORDER TO RELIEVE CLINICAL SYMPTOMS IN ADENOMYSIS PATIENTS.

REFERENCES

Vascularization in endometrium in adenomyosis


Received on August 22, 1997; accepted on December 3, 1997