Flow cytometric DNA analysis in endometriotic tissue compared to normal uterine endometrium

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Endometriotic tissue sometimes shows an invasive pattern, but the growth regulation of the tissue is insufficiently characterized. In a research programme on factors regulating endometriotic growth, the DNA ploidy status and S-phase fraction (SPF) were studied. Fresh-frozen endometriotic tissue from 14 women and endometrium from 11 of them were studied using flow cytometry. A clear diploid pattern was seen in most cases of endometriotic (8/14) and endometrial (8/11) samples. In the remaining cases the G₀/G₁ peak was broad or skewed, which might indicate a near-diploid cell population. To clarify this, a second group was studied, consisting of 29 formalin-fixed endometriotic samples from 22 women and endometrium from five of them. All these samples were diploid, with one having a broad G₀/G₁ peak. No convincing difference in SPF between endometrium and endometriotic tissue was found, as the calculations had to be handled with caution because of debris in many samples. Although the study of fresh-frozen samples gave some indications of differences in DNA ploidy status, flow cytometric DNA analysis of formalin fixed samples of endometriosis showed a diploid DNA pattern in all samples. In conclusion, DNA flow cytometry did not show a convincing aneuploid DNA pattern in endometriotic tissue.

Key words: endometriosis/flow cytometry/growth regulation/ploidy/S-phase

Introduction

Endometriotic tissue has a structure similar to uterine endometrium, but the cellular pattern often shows a varying degree of disorganization and polymorphism compared to the more regular pattern in endometrium (Czernobilsky and Morris, 1979; Bergqvist et al., 1984). In endometriotic tissue, the glands are often irregularly outlined and varying in size and the epithelium shows varying degrees of cytological polymorphism. Atypical cellular structures with irregular shape, size, polarity and density of epithelial nuclei are not quite common. The morphology and chromatin distribution of the nuclei often vary within the same gland (Bergqvist et al., 1984; Schewpe and Wynn, 1984). The histological phase patterns of the two tissue types are often similar, although both histological and biochemical findings indicate differences in hormonal regulation (Bergqvist et al., 1984; Carlstrom et al., 1988; Schewpe, 1989; Bergqvist, 1991).

Endometriotic tissue, usually regarded as ectopically implanted uterine endometrium, often exhibits a growth pattern different from eutopic endometrium. Contrary to uterine endometrium, endometriotic tissue sometimes shows an invasive growth into the surroundings without regard to structure or organ limits. Mechanisms regulating the growth of endometriotic tissue have been focused only to a limited extent. Some growth regulating factors, such as oncoproteins and epidermal growth factor (EGF), have been studied (Bergqvist et al., 1991, Haining et al., 1991; Prentice et al., 1992), results indicating a difference in growth regulation of the two tissue types. Similar alterations in growth regulation have been observed in malignant tumours, associated with abnormal DNA content (aneuploidy) as measured by flow cytometry (Hedley et al., 1983). The question has been raised whether this pattern also exists in endometriotic tissue, i.e. are the discrepancies in cellular features and growth pattern associated with DNA aneuploidy? In various types of tumours the DNA pattern is strongly related to the tumour growth pattern and metastatic capacity (Barlogue et al., 1983; Seckinger et al., 1989; Ambros and Kurman, 1992; Coleman et al., 1993).

Flow cytometry (FCM) enables rapid quantification of DNA content of individual cells, and the cellular DNA content provides useful information about the ploidy, expressing the modal DNA value, and the proliferative activity in a tissue.

As a part of studies on growth regulating factors in endometriotic tissue, the purpose of this pilot study was to estimate the DNA ploidy status and the fraction of DNA-synthesizing cells, i.e. S-phase fraction (SPF), in endometriotic tissue compared with uterine endometrium.

Materials and methods

Tissue samples

Two groups of tissue samples were used. We initially studied fresh-frozen endometriotic tissue, obtained from 14 women at laparotomy (group A). Samples were obtained from the inside of endometriomas (n = 5), ovarian lesions (n = 5), the peritoneal surface (n = 3), including one from the tubal serosa, and from the proximal vaginal pouch (n = 1). Endometriosis was obtained at curettage from 11 of the women. The work was approved by the local medical ethics committee and the subjects gave their informed consent. The tissue samples were immediately rinsed in cold saline at 4°C, freed by knife from inappropriate tissue, and divided into two parts. One part was fixed in 4% formalin, embedded in paraffin and stained with haematoxylin–eosin for histological examination. The other part was
Suspension was suctioned five times through a needle (21 gauge) and filtered through a 50 μm mesh. The definition of ploidy status and Nonidet P40 was added. After 30 min of incubation the nuclear phosphate buffered saline with calcium and 50 μm/ml propidium then resuspended in a DNA-staining solution (Dulbecco's isotonic g). for 10 min and thereafter incubated with a trypsin solution (0.25% iodide (PI), (Sigma P-5264), 0.1 mg/ml RNAse and 0.6 % (v/v) detergent [Nomdet P40 0.6% (v/v), Sigma N-6507] and filtered through a 50 μm nylon filter. Propidium iodide intercalates into further to dissolve the cellular aggregates and to obtain a suspension of separate nuclei free from double-stranded RNA and cytoplasm. The nuclei were stained with propidium iodide in solution with detergent [Nonidet P40 0.6% (v/v), Sigma N-6507] and filtered through a 50 μm nylon filter. Propidium iodide intercalates into double-stranded DNA. Normal human female lymphocytes were prepared in the same way, and used as an external standard control which was run before and at regular intervals between the samples. All samples were run twice.

Definition of ploidy status
Samples with one symmetrical G0/G1 peak were considered as diploid and those with two or more G0/G1 peaks as non-diploid (Hiddeman et al., 1984).

Calculation of the DNA index (DI)
Frozen tissue
DI was defined as the ratio between the modal value of the G0/G1 peaks in a study cell population and that of the external standard lymphocytes (Ewers et al., 1984).

Paraffin-embedded material
The G0/G1 peak with the lowest DNA value was considered to contain diploid cells and served as an internal diploid reference (DI = 1.00).

Calculation of the SPF
SPF was calculated with a planimetric method, assuming the fluorescence intensity values between G0/G1 and G2 peaks to represent DNA synthesizing cells being rectangularly distributed (Baisch et al., 1975). In cases where the distribution pattern of debris obviously contaminated the S-phase region, SPF was not calculated. SPF was also not calculated in cases where the histogram contained <3000 analysed cells.

No statistical calculations were made because of the small number of samples.

Results
Histological examination of sections from the fresh-frozen samples showed pure endometriotic tissue in the samples from minor endometriotic lesions. The formalin-fixed samples contained 10-15% of inappropriate connective tissue.

The DNA histograms from the 14 endometriotic fresh-frozen samples showed a diploid pattern (DI 0.99 ± 0.05, mean ± SD) (Figure 1A), but four of them (27%), one each from tubal serosa, vaginal mucosa, peritoneum and ovary, also showed a shoulder on the diploid G0/G1 peak, or even a second peak, which might correspond to a near non-diploid cell population with DI around 1.1 (Figure IB). All 11 fresh-frozen endometrial samples showed a diploid pattern (DI 1.00 ± 0.05), but it was noted that three of them (27%) also showed a distinct shoulder on the G0/G1 peak (Figure 1C). All four endometriotic samples with a skewed G0/G1 peak had a large amount of debris and, to a lesser extent, so did the endometrial samples with a skewed top.

SPF in fresh-frozen endometrium and endometriotic samples, respectively, are presented in Table I. In order not to take the risk of including SPF in debris, the samples with a skewed G0/G1 peak were excluded from calculations. No convincing difference was found between the remaining endometriotic lesions from different sites or between endometriotic samples and uterine endometrium.

All formalin fixed samples were diploid showing symmetrical G0/G1 peaks (Figure 1D). One sample, however, showed a G0/G1 peak broader than normal (Figure 1E). The median SPF is presented in Table II. Although the SPF was lower in endometriotic tissue than in endometrium, the range was wide and the significance of the difference was not tested.
Figure 1 DNA histogram from (A) a fresh-frozen endometriotic tissue sample showing a symmetrical and narrow G0/G1 peak; (B) a fresh-frozen endometriotic tissue sample showing a possible near diploid G0/G1 peak; (C) a fresh-frozen endometrial tissue sample showing a shoulder on the G0/G1 peak; (D) a paraffin embedded endometriotic tissue sample showing a symmetrical and narrow G0/G1 peak; (E) a paraffin embedded endometriotic tissue sample showing a symmetric but broad (CV = 9.3%) G0/G1 peak.

Table I. The S-phase fraction of cells in fresh-frozen uterine endometrium and endometriotic samples

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>No of samples</th>
<th>SPF (%) median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>10*</td>
<td>6.7 (3.5-14)</td>
</tr>
<tr>
<td>Endometriomas</td>
<td>5</td>
<td>5.9 (3.4-9.1)</td>
</tr>
<tr>
<td>Minor ovarian lesions</td>
<td>2</td>
<td>11.7 (8.3-15.0)</td>
</tr>
<tr>
<td>Peritoneal and vaginal lesions</td>
<td>2*</td>
<td>11.8 (6.6-17.0)</td>
</tr>
</tbody>
</table>

*One sample excluded because of debris
*Three samples excluded because of debris
*Two samples excluded because of debris

Table II. The S-phase fraction (SPF) of cells in formalin-fixed uterine endometrium and endometriotic tissue

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>No of samples</th>
<th>SPF (%) median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>5</td>
<td>2.3 (2.1-4.1)</td>
</tr>
<tr>
<td>Endometriomas</td>
<td>11</td>
<td>1.4 (0.9-1.8)</td>
</tr>
<tr>
<td>Minor ovarian lesions</td>
<td>3</td>
<td>1.7 (1.6-1.7)</td>
</tr>
<tr>
<td>Peritoneal and vaginal lesions</td>
<td>7</td>
<td>1.3 (0.5-7.2)</td>
</tr>
<tr>
<td>Tubal serosa</td>
<td>2*</td>
<td>1.8 (1.6-1.9)</td>
</tr>
<tr>
<td>Appendix endometrosis</td>
<td>5</td>
<td>1.4 (1.2-2.5)</td>
</tr>
<tr>
<td>Bowel endometrosis</td>
<td>1</td>
<td>2.5</td>
</tr>
</tbody>
</table>
There was not thought to be any difference in SPF between samples from different sites of endometriosis from the same patient but the significance was not tested.

Discussion

For this study we had the rather unique opportunity to obtain endometrium and endometriotic tissue simultaneously from the same woman in 16 cases for paired comparison. In the fresh-frozen series there was a possible near-diploid DNA pattern in four out of 14 endometriotic samples indicated by a right-sided shoulder of the diploid 2C-peak. Moreover, three out of 11 uterine endometrial samples also showed the same DNA pattern as above. The latter finding might reveal cases with a true non-diploid cell population, but also suggests that 'false' near-diploid cell populations may occur, possibly due to methodological pitfalls such as low cell density or excessive debris. However, this was not true of all samples (Figure 1).

The occurrence of near-diploid samples in histologically normal endometrium means that any conclusions concerning endometriotic tissue must be cautious. Knowing that cells from different normal tissue material can have different DNA stainability, we prefer to interpret data obtained about abnormal DNA content with caution. A so-called false aneuploidy with DI up to 1.2 has been reported in a couple of studies (Alanen et al., 1989). Technical pitfalls have previously been demonstrated in the case of cervical carcinoma, where a 'false' near-diploid cell population was abolished after HCl treatment (Ferno et al., 1990). The two-peak appearance in cervical carcinoma was suggested to be caused by different diploid cell types with a different stainability for propidium iodide. A similar situation cannot be excluded in endometriotic tissue. Therefore different techniques should be used in different tissues (Shankey et al., 1993). Control experiments using another method for frozen tissue (Thormthwaite et al., 1980; Lee et al., 1984), including 23 of the samples in the present series (five with an indication of abnormal DNA content), could not demonstrate any abnormal DNA content (data not shown). Moreover, no abnormal DNA content was demonstrated when using formalin-fixed tissue. These consistent findings suggest that the abnormal DNA content found in certain samples from endometriotic tissue and from endometrium, can probably be explained by debris. Another factor known for inducing DNA staining artefacts resulting in false non-diploidy is autolysis of the cells, which takes place in degenerated tissue kept at room temperature (Alanen et al., 1989). Our tissue samples were processed quickly (fixed in formalin or placed in -70°C) within 30 min after removal; therefore autolysis does not explain the detected shoulders of the G0/G1 peaks. In some samples we found more debris than in others, which may have been caused by either the presence of apoptotic activity and/or necrosis reflected by signals below the diploid G0/G1 peak, or by preparation artefacts.

Confirmation of an abnormal DNA content using other techniques such as cytogenic karyotyping, fluorescent in-situ hybridization (FISH) or comparative genomic hybridization (CGH) is desirable. The occurrence of a near-diploid peak also in three out of 11 endometrial samples from endometriotic women is surprising if it is not the result of technical pitfalls, and indicates that a larger number of endometrial samples from normal, non-endometriotic compared to endometriotic women should be studied.

It was not possible to calculate the SPF in one endometrial and five endometriotic fresh-frozen samples because of excessive debris. Although there seemed to be a difference in the S-phase in fresh-frozen endometriotic samples according to Table I, this could not be relied on because of a wide range of figures and the small numbers of samples. The larger number of formalin-fixed samples confirmed that there was no difference either between endometriotic samples from different locations or between endometrium and endometriotic tissue.

The occurrence of debris was more pronounced than in other types of tissue, for example breast tumours, and suggests that FCM is not the optimal technique for studies of cell proliferation in endometrium and endometriotic tissue. Computed DNA image cytometry (ICM) might be superior to FCM in certain cases. In a recent study, we have shown the usefulness of ICM as a complementary technique to DNA FCM in breast cancer when debris and non-relevant cells dominate the sample (Baldetorp et al., 1992). Other techniques, such as immunohistochemistry of markers of proliferation (e.g. Ki-67 and proliferating cell nuclear antigen (PCNA)), might also be preferred. These data might be compared to data on oestrogen receptors (ER) and progesterone receptors (PR) in endometriotic tissue from different locations. ER levels were found to be significantly lower in endometriomas and minor ovarian lesions than in endometrium but there was no difference between biopsies from peritoneal endometriotic lesions and endometrium (Bergqvist and Ferno, 1993). However, the steroid receptor level may also be influenced by other regulatory mechanisms. For further clarification of this relationship, immunohistochemical studies on cell-cycle related factors such as PCNA, Ki-67 or thymidine labelling index might be valuable.

Other indications for a different genetic growth regulation of endometriotic tissue are the expressions of the proto-oncogene c-erbB-2 and epidermal growth factor receptor (EGF-R) that were found to be somewhat more pronounced in endometriotic tissue than in endometrium (Bergqvist et al., 1991).

The invasive growth sometimes exhibited by endometriotic tissue may also be regulated by other growth factors such as proteolytic enzymes, produced in endometriotic tissue. The content of the lysosomal proteolytic enzyme cathepsin D was significantly higher in endometriotic tissue than in endometrium obtained simultaneously from the same woman (Bergqvist and Ferno, 1993).

However, factors in the surrounding tissues might also influence the regulation of endometriotic growth. In an experimental study, where endometrium and endometriotic tissue from the same woman were transplanted simultaneously to nude mice, all the grafts were encapsulated and showed no tendency to invasive growth (Bergqvist et al., 1985).

In conclusion, abnormal DNA content could not be demon-
strated with certainty using FCM. A near-diploid DNA pattern in frozen samples is possibly explained by debris.

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References
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