Tracing cellular and molecular mechanisms involved in endometriosis

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Introduction

Endometriosis, one of the most common gynaecological diseases, is characterized by the presence of ectopic endometrial-like tissue outside the uterine cavity. Today, the most favoured theory of the pathogenesis of endometriosis is the implantation theory (Sampson, 1940), which postulates that viable endometrial cells are transported to the peritoneal cavity by retrograde menstruation. Subsequently, these cells adhere to the peritoneal wall and proliferate. There is still an ongoing discussion as to whether endometriosis might also be established by hormone-dependent transformation of peritoneum into Mullerian-type epithelium (metaplasia theory; Meyer, 1919; Novak, 1931; Fujii, 1991). Regardless of the aetiology of endometriosis, it seems clear from clinical observations that it is an invasive disease (Spuijbroek et al., 1992; Foidart et al., 1993). This would mean that endometriotic cells are able to penetrate organs of the pelvic cavity or, more distant organs after haematogenic and/or lymphatic spread. Thus, the invasiveness of endometriotic cells might contribute to the pathogenesis of endometriosis. In order to increase our understanding of the mechanisms of this invasiveness we investigated the following questions: (i) can we demonstrate the invasiveness of endometriotic cells in an in-vitro assay?; (ii) is it possible to establish an endometriotic cell line for molecular studies in vitro; (iii) does the peritoneal fluid contain factor(s) which might contribute to the invasiveness of endometriotic cells?; and aspects with tumour metastasis, but might also have unique mechanisms.

Key words: E-cadherin/endometriotic cell culture/invasion/peritoneal fluid

The aetiology and pathogenesis of endometriosis, defined as the presence of endometrium-like tissue outside the uterine cavity, is largely unknown. In this paper we present and discuss possibilities to study the putative pathogenic properties of endometriotic cells in vitro. The current focus of our investigations is on the invasive phenotype of the disease, assuming that this might contribute to the pathogenesis of endometriosis. So far, we have shown that: (i) cytokeratin-positive and E-cadherin-negative endometriotic cells have an invasive phenotype in a collagen invasion assay in vitro similar to metastatic carcinoma cells; (ii) the invasiveness of endometriotic but not of eutopic endometrial cells can be stimulated by a heat-stable protein present in peritoneal fluid; and (iii) the endometriotic cell line EEC145T, which we established, may be a useful tool for the identification of gene products which are, positively or negatively, invasion-related. Finally, our studies suggest that the invasive phenotype in endometriosis shares aspects with tumour metastasis, but might also have unique mechanisms.

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(iv) are we able to identify gene products which might be related to the invasive (or other) properties of endometriotic cells?

Invasiveness of endometriotic cells in vitro

Analysis of cadherins, cytokeratins and peritoneal fluid

Initially, we investigated whether the postulated invasive properties of endometriotic cells in vivo could also be shown in cell culture. In order to do this, we examined the invasiveness of cells from endometriotic and endometrial biopsies in a collagen invasion assay (Gaetje et al., 1995). The human bladder carcinoma cell lines RT112 (non-metastatic) and EJ28 (metastatic) were used as non-invasive and invasive controls, respectively. The invasion indices of endometriotic cells were similar to those of the metastatic carcinoma cell line EJ28 (Figure 1). In contrast to this, cells from eutopic endometrium and non-metastatic RT112 carcinoma cells were not invasive in the collagen invasion assay (Gaetje et al., 1995).

Previous findings have shown that the metastatic capacity of carcinoma cells correlates with the absence, mutation or functional inactivation of the calcium-dependent cell adhesion molecule, E-cadherin (Frixen et al., 1991; Umbas et al., 1992; Becker et al., 1994; Risinger et al., 1994; Yoshiura et al., 1995). Therefore, we characterized the invasive cell type with regard to the expression of cytokeratins and E-cadherin. The results of these experiments revealed that the invasive cells from endometriotic biopsies were epithelial cells which did not express E-cadherin. Cells from eutopic endometrium and RT112 cells, both expressing E-cadherin, did not exhibit invasive properties (Gaetje et al., 1997). Analysis of sections of endometriotic biopsies demonstrated that an epithelial cell type corresponding to the invasive cell type in culture could also be found in vivo. This cell type was cytokeratin-positive but E-cadherin-negative, and might be discussed as a potentially invasive cell in endometriosis (Figure 2; Gaetje et al., 1997).

Establishment of an endometriotic cell line

Rationale of the approach

One problem which severely restricts the reproducible performance of molecular and cellular investigations of the invasiveness of endometriosis is the limited amount of biological material available for cell cultures. In addition, primary cell cultures are usually not composed of a homogeneous cell type and die after three to four passages in culture. This might be overcome, at least in part, by the establishment of endometriotic cell lines. However, these must be carefully validated for the features which appear to be relevant or associated with the invasive phenotype of the primary endometriotic cells. Thus, cell lines for studies of invasiveness should be at least E-cadherin-negative.
epithelial cells (expression of cytokeratin), exhibit invasive properties in a collagen invasion assay (Gaetje et al., 1997), and retain the expression of oestrogen and progesterone receptors which are present in endometriotic cells (Segars, 1997).

One way to prolong the lifespan of primary human cells is by the expression of SV40 T antigen, a viral oncogene. Usually, those cell lines can be kept in culture for up to 40 passages before they become senescent. In rare cases, immortalized cell lines arise from those cultures after undergoing crisis (Pantel et al., 1995; Brosens et al., 1996).

Transformation of endometriotic cells by SV40 T antigen

In order to establish an endometriotic cell line, cells from freshly prepared peritoneal endometriotic biopsies, treated as described in Gaetje et al. (1995, 1997) were taken into culture. The biopsies were obtained from patients undergoing laparoscopy for unexplained infertility, known endometriosis or lower abdominal pain, and were taken during the proliferative phase of the menstrual cycle. The patients gave their informed consent, the study having been approved by the local ethics committee.

For transformation, the primary endometriotic cells, of which ≥ 90% expressed cytokeratin, were microinjected with a plasmid expressing SV40 T antigen. Three biopsies were used for the injection of about 300 up to 1000 cells per sample. From these injections, one endometriotic cell line, EEC145T, has been obtained to date. In all cases, selection was performed by allowing SV40 T antigen-positive cells to overgrow the cells not expressing SV40 T antigen. When the cell populations were 100% SV40 T antigen-positive, as confirmed by immunocytochemical stainings against T antigen, they were expanded and frozen in aliquots. As shown in Table I, the endometriotic cell line EEC145T was a cytokeratin-positive and E-cadherin-negative cell type which exhibited an invasive phenotype and was able to grow in cell culture for about 35 passages. The mean invasion index (for definition, see legend to Figure 1) ranged between 5 and 6. Thus, for the parameters tested, EEC145T cells had features similar to the primary invasive endometriotic cells, except that they could be kept in culture and used for ten times more passages than primary cells. Finally, it should be emphasized that EEC145T cells expressed both the progesterone and the oestrogen receptor as revealed by immunocytochemical analyses which were performed as described (Imam et al., 1995; Gaetje et al., 1997).

At approximately passage 25, EEC145T cells began to lose expression of cytokeratin, the invasive features and expression of Frag-1 mRNA (for description, see below) without changing the proliferative potential. Thus, EEC145T cells retained their initial characteristics for as long as 25 passages.
Table I. Characterization of the endometriotic cell line EEC 145

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<th>Characterization of the endometriotic cell line EEC 145</th>
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<td>Primary invasive endometriotic cells</td>
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<td>Cytokeratin</td>
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<td>Invasiveness</td>
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<td>No. of passages</td>
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<td>Steroid receptors</td>
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ND = Not detected.

**Influence of factors in the peritoneal fluid on the invasiveness of endometriotic cells**

Many clinical investigators suspected that factors in the peritoneal fluid, possibly secreted by cells of the immune system, might stimulate the progression of endometriosis (Haney *et al.*, 1981; Halme *et al.*, 1982; Fakih *et al.*, 1987; Dunselmann *et al.*, 1988; Eisermann *et al.*, 1988; Koutsilieris *et al.*, 1993). Therefore, we investigated the hypothesis whether factor(s) in the peritoneal fluid might contribute to the pathogenesis of endometriosis, for example by influencing the invasive capacities of endometriotic cells.

The putative invasion-promoting activity of peritoneal fluid was tested in the collagen invasion assay. Addition of 10% (v/v) of peritoneal fluid into the culture medium of collagen invasion assays of EEC 145T cells enhanced the invasive capacity of endometriotic cells considerably (Figure 3). Depleting the peritoneal fluid of steroids and growth factors did not abolish the invasion-promoting activity. Furthermore, treatment of the peritoneal fluid at 95°C had no effect on the invasion-promoting activity. In conclusion, the invasion-promoting activity in the peritoneal fluid appeared to be a protein (defined by its sensitivity to proteinase K) which did not seem to stimulate proliferation of endometriotic cells and was heat-resistant. Finally, we found that this peritoneal protein factor was unable to stimulate or promote the invasion of eutopic endometrial cells in the collagen invasion assay (Gaetje *et al.*, 1996).

**Molecular analysis of endometriosis: the search for genes regulated in endometriotic cells**

One possibility of identifying gene products which are associated with the invasive properties of endometriotic cells, would be the comparison of mRNA populations from endometriosis and eutopic endometrium. It would be difficult, however, to attribute the identified genes to specific properties of, and molecular events in, endometriosis. Therefore, invasive and non-invasive variants of an endometriotic cell clone would be more suitable tools for the identification of genes regulated in endometriotic cells.

By chance, our endometriotic cell line EEC145T developed a non-invasive variant after several passages in culture without changing its proliferative potential. Based on the assumption that the loss of the invasive phenotype is also reflected at the level of gene expression, we used these cells for the analysis of differentially expressed gene products which were associated with either the invasive or non-invasive phenotype. The identification of differentially expressed gene products in EEC145T (invasive) and
Figure 4. Experimental design for the search of invasion-related genes expressed in the invasive and non-invasive variant of the endometriotic cell line EEC145T.

EEC 145T\textsuperscript{ni} (non-invasive) cells was done by differential display reverse transcriptase polymerase chain reaction (DDRT–PCR; Liang and Pardee, 1992). The scheme of this experimental approach is outlined in Figure 4. Gene products amplified by DDRT–PCR were separated on polyacrylamide gels. Differentially expressed PCR-cDNAs were cut out, reamplified and sequenced. Several of the cDNAs identified hybridized to differentially expressed mRNAs.

Figure 5 shows an example of the expression pattern of one mRNA identified by DDRT-PCR. This 4 kb RNA, detected by DD-fragment 1, was expressed in the invasive variant of EEC145T cells but not in the non-invasive variant, nor in metastatic EJ28 or non-metastatic RT112 carcinoma cells. It was also undetectable in a SV40T-transformed peritoneal cell line, suggesting that this mRNA is not induced by the SV40 T antigen. RNA from eutopic endometrium contained low amounts of fragment-1 mRNA which could be detected by RT–PCR (not shown) but not by Northern blot analysis (Figure 5).

In addition, we found other cDNAs corresponding to differentially expressed mRNAs (data not shown). Currently, we are isolating full-length cDNAs in order to obtain information on the structural and functional nature of these gene products, as well as of their pattern of expression.

Conclusions

We have presented evidence that it is possible to study putative pathogenic properties of endometriotic cells \textit{in vitro}. The current focus of our investigations is on the invasive phenotype of the disease, assuming that this contributes in many cases to the pathogenesis in endometriosis. In summary, we have shown that: (i) endometriotic cells have an invasive phenotype similar to that of metastatic carcinoma cells; (ii) the invasiveness of endometriotic cells, but not of eutopic endometrial cells, can be stimulated by a heat-stable protein present in peritoneal fluid; and (iii) the endometriotic cell line
EC145T may be a useful tool for the identification of gene products which are differentially associated with the invasive or non-invasive phenotype.

Finally, our studies have suggested that the invasive phenotype in endometriosis shares aspects with metastasis of carcinomas, but may also have unique mechanisms.

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References


