Reduced proliferation and cell adhesion in endometriosis

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Endometriosis is defined as endometriotic tissues growing outside the uterine cavity. The cell biological processes responsible for the pathogenesis of this disease are not well understood. In order to detect differences in proliferative activity between endometria and endometriotic lesions, Ki67 staining was analysed. In addition, expression of epidermal growth factor (EGF) and its receptor was examined using immunohistochemistry. For dedifferentiation processes pointing to invasive properties of the uterine epithelium, the presence of the adhesion complex E-cadherin with the associated α- and β-catenin was investigated.

Specimens of endometrium in the proliferative phase of 36 patients without, and 79 patients with, endometriosis together with endometriotic lesions were studied. The study revealed a significantly reduced proliferation activity in uterine epithelium within the ectopic lesions but no differences between eutopic endometria of non-affected and affected patients. Furthermore, a lower expression of both EGF and its receptor in the epithelial cells of the ectopic glands was observed. The adhesion complex E-cadherin, together with α-, and β-catenin, was slightly reduced in uterine epithelial cells of women with endometriosis and less expressed in endometriotic lesions. The results indicate that epithelial cells of endometriotic lesions are not hyperproliferative, but do appear to dedifferentiate, displaying an invasive character.

Key words: cell adhesion/endometriosis/endometrium/epidermal growth factor/proliferation

Introduction

Endometriosis is a common disease in ~10% of women while of reproductive age. The definition of this disorder is the presence of endometrial glands and stroma outside the uterine cavity. The presence of endometriotic tissues is associated with pelvic pain and to a high incidence with infertility (Schweppe et al., 1990). The origin of these endometriotic lesions is not completely clear, but the widely accepted theory is the implantation of endometrium on the peritoneal surfaces after retrograde menstruation. Although retrograde menstruation is a common event in women (Liu and Hitchcock, 1986), it does not necessarily lead to a development of lesions. In >60% of the cases, reappearance of endometriotic lesions occurs within 1 year after apparently successful hormonal and/or surgical treatment (Regidor et al., 1996). Hormonal responsiveness of this disease is found in the presence of both oestrogen and progesterone receptors of epithelial and stromal components which are expressed to a different extent in these lesions (Prentice et al., 1992; Bergqvist and Ferno, 1993; Bergqvist, 1995, Jones et al., 1995). Proliferation capability of these ectopic implants seems to be a precondition for persistence, however, proliferation activity of endometriotic lesions is described with contradictory results. Several authors have shown increased proliferation in endometriotic lesions (Li et al., 1993) and in the endometrium of patients with endometriosis (Wingfield et al., 1995). In addition, apoptotic events seem to be reduced in endometriotic lesions as well as in corresponding endometria (Gebel et al., 1998). These synergistic events (enhanced proliferation combined with reduced apoptosis) could explain the mechanisms of growing and maintaining the ectopic endometrial tissue. Other authors, however, have found a reduced proliferation in ectopic endometrial implants (Jones et al., 1995) or almost no difference between eutopic and ectopic endometria (Nisolle et al., 1997). Invasive properties of the ectopic endometrial fragments seem to be needed to establish a stable contact to the environment. One study (Gaetje et al., 1995) demonstrated that epithelial cells of ectopic glands reveal an increased invasion capacity using a collagen invasion assay. Concomitantly, the cell adhesion molecule E-cadherin decreases in eutopic endometrium in endometriosis and is lost in epithelial cells of the ectopic glands (Gaetje et al., 1997). In contrast to these publications, other studies (Beliard et al., 1997) found no differences in the E-cadherin expression pattern between black peritoneal endometriotic implants and the endometria of healthy women.

The high divergence in the appearance of the cell biological characteristics of endometriosis is a great challenge for scientists to gain more insight into the basics of the pathogenesis of this disorder. Different abilities of the endometrial fragments to adhere to the peritoneal surface and an enhanced invasive capacity to penetrate the environment at the site of adhesion could be one reason. In addition, proliferation and neoangiogenesis is needed for growth and persistence of the lesions.
In this study we tried to focus on proliferative activity and expression of the epidermal growth factor (EGF) and its receptor (EGF-R) involved in endometrial proliferation (Haining et al., 1991). We focused on EGF and EGF-R among other growth factors since it has been shown that both are localized in the epithelial as well as in the stromal compartment of human endometrium (Smith, 1994). In contrast to insulin-like growth factor-I receptor (IGF-I-R), EGF-R shows an increase in the endometrium during the proliferative phase (Konopka et al., 1998) and in oestrogen-exposed endometria (McBean et al., 1997). In addition, EGF is able to stimulate proliferative activity in human stromal cells in vitro (Chegini et al., 1992). On the other hand, we investigated the cell adhesion molecule E-cadherin and its associated proteins, α- and β-catenin, in endometriosis to gain information on the invasive character of this disease. To discriminate between dedifferentiation processes which occur in the endometriotic lesions due to the influence of an ectopic environment and those primary events which cause the pathogenesis, we compared endometriotic lesions with endometria from patients with and without endometriosis from the same cyclic phase.

Dedifferentiation of the uterine epithelium at ectopic sites seems to be accompanied by a loss of cell–cell adhesion molecules which enables the cells to escape from their tissue organization and migrate into the neighbouring organs as a precondition for the persistence of ectopic endometriotic lesions. Growth of endometriotic lesions, however, seems to be regulated, since uncontrolled proliferation of the ectopic tissues is rarely observed in endometriosis. To test the hypothesis that this disease has a more invasive than proliferative character, we investigated markers for invasion properties as well as proliferative activity.

Materials and methods

Patients

Endometrial biopsies and endometriotic tissues were obtained from patients in the proliferative phase of the menstrual cycle who were undergoing laparoscopy or being hysterectomized at the Department of Gynaecology of the University of Essen, Germany. Endometrial tissues were recruited from women who were undergoing hysterectomy because of leiomyomata or hysteroscopy for infertility reasons. These endometrial tissues were compared with biopsies taken from women diagnosed for endometriosis by hysteroscopy and laparoscopy. The peritoneal endometriotic lesions were taken from the vesicouterine pouch, the rectouterine pouch and the ligamenta sacro–uterina of the uterus.

The women were aged 21–44 years. Blood of all patients was collected 24–48 h before surgery and sera were tested for 17β-oestradiol and progesterone concentrations with a commercially available radioimmunoassay (Sorin Biomedica, Italy). For immunostaining, the endometria from 36 patients without endometriosis and 79 endometria and/or endometriotic lesions of affected patients were investigated. However, due to methodical problems, the complete set of marker genes could not be analysed from every patient. The precise data are given in the figure legends.

Immunohistochemistry

Sources of the different antibodies and concentrations used are summarized in Table I. For cryostat sections, the biopsies were

<table>
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<th>Table I. Primary antibodies</th>
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<tr>
<td>Antibody Source Concentration (µg/ml)</td>
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<tr>
<td>E-cadherin Dianova, Hamburg, Germany 7</td>
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<tr>
<td>α-catenin Santa Cruz Biotechnology, Heidelberg, Germany 2</td>
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<tr>
<td>β-catenin Sigma-Aldrich Chemie, Deisenhofen, Germany 30</td>
</tr>
<tr>
<td>EGF Dianova, Hamburg, Germany 8</td>
</tr>
<tr>
<td>EGF-R Dako Diagnostik GmbH, Hamburg, Germany 30</td>
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<tr>
<td>Ki67 Medac GmbH, Hamburg, Germany 0.5</td>
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EGF = epidermal growth factor; EGF-R = epidermal growth factor receptor.

Figure 1. Immunolocalization of Ki67 in the endometrium of (a) women without endometriosis, (b) patients with endometriosis and (c, d) in endometriotic lesions. (d) Scale bar = 220 µm.
immediately frozen in liquid nitrogen and kept at -80°C. For staining of antigens to EGF, EGF-R, E-cadherin-, α-catenin-, and β-catenin, 7 μm thick cryostat sections were processed routinely. Briefly, sections were fixed for 10 min in 96% ethanol and after rinsing with phosphate-buffered saline (PBS) covered with 1% bovine serum albumin solution (BSA; Sigma, Germany) to block non-specific binding sites. Incubation with the primary antibody for 1 h was followed by the appropriate secondary fluorescein isothiocyanate (FITC)-conjugated antibody for 45 min. Sections were covered with Vectashield (Vector Laboratories, Peterbourgh, UK) and analysed using an Axiophot immunofluorescence microscope (Zeiss, Oberkochen, Germany). The intensity of the staining was analysed twice on different days. The intensity of immunostaining was semiquantitatively evaluated as: - = no staining; + = weak staining; ++ = moderate staining; and +++ = strong staining.

Ki67 antigen expression was determined using a streptavidin–biotin peroxidase complex immunohistochemical technique (Dako LSAB + Kit Peroxidase; Dako, Hamburg, Germany) on cryostat sections as described above. After incubation with the primary antibody and washing with PBS, sections were incubated with a biotinylated anti-mouse immunoglobulin G (IgG; Dako LSAB + Kit Peroxidase) for 30 min and washed twice with PBS. Sections were treated with streptavidin-conjugated horseradish peroxidase (Dako LSAB + Kit Peroxidase) for 30 min. The reaction was developed with 3,3'-diaminobenzidine (DAB; Dako) containing 0.02% hydrogen peroxide. The sections were mounted in Eukitt (Kindler GmbH & Co, Freiburg, Germany). Controls were performed by replacing the primary antibody by preimmune serum to allow for the assessment of the non-specific binding of the secondary antibody.

Quantification of labelled cells

Cells stained for Ki67 were investigated at a primary magnification of ×40. A minimum of 400 stromal and 100 epithelial cells of endometrial and endometriotic tissue were evaluated per section. Two sections of each sample have been investigated. The ratio of nuclear stained cells to non-stained cells gives the percentage of Ki67-positive cells.

The significance of the differences in proliferative activity of glandular epithelium in eutopic and ectopic endometria was determined by a generalized linear model assuming a binomial distribution with overdispersion. Overdispersion (McCullagh and Nelder, 1989) between replicates of binary distribution occurs when the variation between the observation is higher than could be explained by a sample of independent binomial variates. Modelling overdispersion allows a more realistic estimation of P values and confidence intervals in the context of generalized linear models.

Results

Immunohistochemistry was performed in eutopic as well as ectopic endometrial tissues during the early to late proliferative phase on days 6–14 of the menstrual cycle.

Proliferation

Ki67, a marker of cell proliferation, is a nuclear protein expressed in human cells during the late G1, S, M and G2 phases of the cell cycle. Both the glandular epithelium of eutopic endometrium of patients with and without endometriosis showed high amounts of stained nuclei (Figure 1a, b) and an average in proliferative activity of ~38% (Figure 3a). In comparison with eutopic endometrium this proliferation rate was significantly (P < 10⁻¹¹) reduced in the glandular epithelium of ectopic endometrium (Figure 3a). In 18 of 24 endometriotic biopsies no Ki67 antigen was detectable (Figure 1c). In the remaining samples only some glandular epithelial cells revealed Ki67 staining (Figure 1d). Evaluation of the stained cells leads to an average proliferation rate of 1.2% in ectopic lesions (Figure 3a).

Of stromal cells in the endometrium of both women with and without endometriosis, ~10% were proliferatively active (data not shown), whereas no staining for Ki67 was detected.
Proliferation and cell adhesion in endometriosis

nearly identical. The intensity of the immunoreaction was moderate (Figure 3b). The EGF was localized mainly close to the apical portion and showed a weak expression in the basal region and the lateral membranes of the glandular epithelium (Figure 2a,b).

In contrast to eutopic endometria, endometriotic biopsies revealed only weak EGF immunostaining in the same distribution pattern. In 34% of the samples, EGF was undetectable (Figures 2c, 3b).

In endometria of women with and without endometriosis the immunoreaction against EGF-R was similar (Figure 3c). Most of the samples of these eutopic endometria showed a moderate immunofluorescence staining (Figure 3c). EGF-R was localized at the basal, and to some extent at the lateral, portion of the epithelial cell membranes (Figure 2d,e).

In contrast to the eutopic endometria, endometriotic biopsies (48%) revealed a low concentration of EGF-R protein (Figure 2f) and even in 32% of the lesions EGF-R expression was not detectable (Figure 3c). Comparison of EGF with its receptor revealed approximately the same expression intensity, but they were differently distributed at the membrane compartments with a remarkable decrease in both marker genes in ectopic endometrial lesions (Figure 3b,c). A moderate staining of EGF and EGF-R could also be detected in stromal cells of eutopic endometrium but not in the ectopic samples.

E-cadherin

Most of the endometria of the not affected women showed a strong to moderate E-cadherin expression (Figure 4a), only 20% revealed a weak reactivity to E-cadherin (Figure 5a). The adhesion molecule E-cadherin was found along the complete lateral cell membranes of the glandular epithelium (Figure 4a,b).

The endometrium of patients with endometriosis showed a shift to a more moderate expression, in the same distribution pattern along the complete lateral membrane (Figures 4b, 5a). In the remaining biopsies only a low expression of α-catenin was found within the surrounding stroma of the ectopic endometrial lesions (Figure 1c,d).

**EGF and EGF-R**

Expression of EGF in the uterine epithelium of eutopic endometrium of patients with and without endometriosis was in the surrounding stroma of the ectopic endometrial lesions.

**E-cadherin**

Most of the endometria of the not affected women showed a strong to moderate E-cadherin expression (Figure 4a), only 20% revealed a weak reactivity to E-cadherin (Figure 5a). The adhesion molecule E-cadherin was found along the complete lateral cell membranes of the glandular epithelium (Figure 4a,b).

α- and β-catenin

The investigation of α-catenin and β-catenin expression in eutopic endometrium of women and patients with and without endometriosis in the proliferative phase of the menstrual cycle resulted in a positive immunoreaction for both E-cadherin associated molecules in the uterine glandular epithelium. The catenins were located at the apical portion of the lateral membrane of the uterine epithelial cells of non-affected patients (Figure 4d–e, g–h).

Of endometrial sections of normal cycling women, 61% exhibited a strong and 39% a moderate staining of α-catenin whereas only 52% of the samples of endometriosis patients were strong and 29% moderately stained. In the remaining biopsies only a low expression of α-catenin was found within the glandular epithelium (Figure 5b).

In contrast to α-catenin, the β-catenin concentration differed more distinctly between the eutopic endometrial tissues of
patients with and without endometriosis. In the endometrium of normal cycling women, staining for $\beta$-catenin was strong in 21% of the samples and in 61% a moderate immunofluorescence was observed (Figures 4g, 5c). Endometrial biopsies of patients with endometriosis showed a strong (20%) and moderate (24%) or low (44%) expression of $\beta$-catenin (Figures 4h, 5c). 12% were $\beta$-catenin immunonegative (Figure 5c).

The $\alpha$-catenin and $\beta$-catenin immunoreactivity differed clearly between eutopic and ectopic endometria. A strong expression of $\alpha$-catenin could be shown only in 12% of the ectopic lesions, a moderate immunoreaction in 27%. Of ectopic glands, 46% expressed $\alpha$-catenin at a low intensity and in an inhomogeneous distribution pattern (Figure 4f). In 15% of the samples staining for $\alpha$-catenin was negative (Figure 5b).

Immunoreaction to $\beta$-catenin was mainly very weak in the glandular epithelium of the lesions and was completely missing in a third of all samples (Figures 4i, 5c).

**Correlation of differentiation markers to serum oestrogen concentrations**

Since in our study the serum oestradiol concentrations demonstrated variations of 17–290 pg/ml, we tested whether these different concentrations influence the expression pattern of the differentiation markers. For this reason we divided the patients into three groups, based on the serum concentration of oestradiol (<30; 30–100; and ≥100 pg/ml) and correlated these concentrations of oestradiol with the expression intensity of EGF, EGF-R, E-cadherin, $\alpha$- and $\beta$-catenin within the three different tissue groups, endometria from normal cycling patients, from patients with endometriosis and ectopic lesions. As shown for EGF-R in Figure 6, there was also no variation in the expression intensity of all other marker genes investigated related to the different oestrogen concentrations during the proliferative phase. The progesterone serum concentrations varied only moderately during this phase and were <1.0 ng/ml. For this reason a correlation with progesterone serum concentrations was not performed.

**Discussion**

The molecular mechanisms underlying the pathogenesis of endometriosis are far from being understood. Since most of the observations described in the literature concerning different marker molecules characteristic for this disease are contradictory, it is difficult to draw a clear line about the cell biological processes involved. In this study we tried to define if generation and persistence of endometriotic lesions are due to a more proliferative or more invasive character. For this reason we investigated marker molecules involved in invasion and markers discussed for increased proliferation of endometriotic lesions within the same group of patients. In our study, we found no differences in proliferation activity between eutopic endometria of patients with and without endometriosis, but a remarkably reduced proliferative activity of the ectopic lesions was shown. These observations are in agreement with previously published studies (Jones et al., 1995) and seem quite reasonable because all lesions investigated have been very small in extent. The reduced proliferative activity is correlated with a reduction in EGF and EGF-R in the epithelial cells of the ectopic glands.
EGF which acts through its receptor via paracrine or autocrine mechanisms is known to be regulated during the menstrual cycle (Bonaccorsi et al., 1989; Troche et al., 1991). In animal models, oestrogen is able to enhance the amount of EGF-R (Mukku and Stancel, 1985) and EGF is able to replace the action of oestrogen in the mouse genital tract with respect to growth and differentiation (Nelson et al., 1991). Our observations of a reduction of EGF-R in endometriotic lesions, but not in eutopic endometria, is in agreement with a previous study (Huang and Yeh, 1994). These authors showed that eutopic endometria have a higher relative abundance of EGF-R mRNA than endometriotic lesions. However, using immunohistochemistry, no differences were found in the staining intensity of eutopic and ectopic endometria. We would postulate that this disease is less responsive to oestrogens by reduction of the EGF-R and this may cause the reduction in proliferation of the ectopic glands. A further indication is that, although the serum concentrations of oestrogen differed among patients, no differences in proliferation activity could be observed. However, other growth factors which may compensate for EGF, e.g. IGF-I (Roy et al., 1999) or transforming growth factor α (TGFα) (Imai et al., 1995, Niikura et al., 1996), which have been shown to be expressed in human endometria and endometrial carcinoma, have not been investigated in this study. In conclusion, some proliferation probably responsible
for persisting and slow growing of the lesions, seems to be needed. However, in our study there is no evidence for enhanced proliferative activity as a mechanism for pathogenesis and progression of this disease.

The transmembrane cell adhesion protein E-cadherin is thought to be involved as tumour suppressor gene in carcinogenesis (Vlemmixx et al., 1991; Birchmeier et al., 1995). Loss of intercellular adhesion mediated by the cadherin/catenin complex plays a key role in the precondition for loss of cell polarity and the onset of cell invasion leading to metastasis (for reviews, see Birchmeier and Behrens, 1994; Aberle et al., 1996). Our findings indicate that the whole adhesion complex (E-cadherin, α- and β-catenin) is reduced in the endometriotic lesions and to some extent in the endometria of patients with endometriosis. Similar results have been obtained previously (van der Linden et al., 1994; Gaetje et al., 1997); these studies showed a reduced immunofluorescence of E-cadherin staining in epithelial glands of ectopic endometria and a strong (Gaetje et al., 1997) expression in eutopic glandular epithelium. In addition, endometriotic cells were shown to have an invasive potential similar to a tumour cell line derived from bladder epithelium (Gaetje et al., 1995). However, contradictory results were given by Beliardi et al. who could find no differences in the E-cadherin expression pattern of endometriotic and endometrial samples (Beliard et al., 1997).

β-Catenin plays a dual role as a major component of adherent junctions linking E-cadherin via α-catenin with the actin molecule and forming a complex with the transcriptional factor, leukaemia enhancer factor-1 (LEF-1). Translocation of β-catenin into the nucleus and binding to LEF-1 leads to transcriptional activation of a number of genes which are related to the transformation from the epithelial to a mesenchymal type of cells (Huber et al., 1996; Novak et al., 1998) and induces or enhances cyclin D1 gene expression (Shutman et al., 1999). However, in our immunohistochemical studies β-catenin was only detected at apical portions of the lateral membranes of the glandular epithelium, but no obvious immunostaining for β-catenin was observed in the nucleus of epithelial cells of eutopic and ectopic endometria.

The results described on reduced proliferation in addition to dedifferentiation of the epithelial phenotype of the uterine glands have to be interpreted cautiously in regard to the different growth conditions between eutopic and ectopic endometria. The different results described in literature concerning the dedifferentiation pattern of the ectopic glands could simply be due to different growth conditions, e.g. extent of neoangiogenesis and inflammatory reaction of the local environment.

Taken together, despite the clear results in the endometrial tissue samples investigated, the loss of the adhesion complex could not be generalized for all endometriotic tissues and is not a common marker for the dedifferentiation process of the endometriotic tissues. It can be speculated that the lesions dedifferentiate, depending upon their environment (Konincx et al., 1998) and develop different mechanisms for adhesion and invasion. The different extent of those invasion processes into organs could be due to the degree of changes in integrin chains and the loss of E-cadherin adhesion complex. This idea that invasion of endometriotic lesions is influenced by the local environment is supported by the other experiments in which the invasive capacity of an endometriotic cell line could be enhanced by adding peritoneal fluid in a collagen invasion assay (Starzinski-Powitz et al., 1998). In conclusion, our studies suggest that ectopic endometrium undergoes a dedifferentiation process along the invasive pathway but shows no evidence for enhanced proliferation properties.

Acknowledgements

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Proliferation and cell adhesion in endometriosis


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