Aberrant expression pattern of gap junction connexins in endometriotic tissues

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The expression of gap junction connexins (Cx) in the female reproductive tract of rodents and in the human endometrium is highly regulated by steroid hormones. Here we have investigated the distribution and regulation properties of Cx43, Cx26 and Cx32 in the human ectopic endometrium of 41 patients, using immunohistochemistry. The biopsies were obtained during the early or late follicular phase (26 cases), during the corpus luteum phase (five cases) and after a 6 month treatment with a gonadotrophin-releasing hormone (GnRH) agonist (three cases) or progestin (seven cases). Aberrant expression of Cx43 was found in the epithelium of nearly all endometriotic glands whereas Cx26, typical for human uterine epithelium cells, was only detected in 18 cases; in 17 it was co-expressed with Cx43. The stromal compartment of the tissues did not express any connexins investigated. Staining for Cx32 was absent in all endometriotic tissues. Strong expression of Cx43 was correlated with a high serum value of 17β-oestradiol, whereas a strong expression of Cx26 was found with high values of progesterone mainly in patients after progestin treatment. The epithelium of endometriotic implants collected after GnRH agonist treatment expressed Cx26 and Cx43 only moderately. The patterns described demonstrate an aberrant connexin expression and a different hormonal regulation pattern in endometriotic tissues compared to the normal cyclic uterine endometrium, thus indicating a high dedifferentiation from the normal situation. However, endometriosis still remains a hormonally-dependent benign disease, and hence, can be treated hormonally.

Key words: connexins/endometriosis/gap junctions/proliferative benign disease

Introduction

Although endometriosis is a well documented disease, first described in 1861 by Rokitansky, it remains a complex and unsolved issue in medical research. The phenomenon of ectopic endometrial tissue has led to many questions concerning the pathophysiology, aetiology, hormonal regulation properties and, last but not least, appropriate therapy. The complexity of the different types of endometriotic lesion makes it difficult to compare different studies dealing with the therapeutic approaches to this disease. Therefore, the applied therapies are in part contradictionary (for review see Wingfield and Healy, 1993). Endometriosis is found in ~8–12% of sexually mature women and is combined with dysmenorrhoea, with cyclic or acyclic chronic pain and/or dyspareunia (Barbieri, 1988; Schwepe et al., 1990; Schindler, 1995). In ~30–40% of cases, endometriosis seems to be responsible for sterility (Kistner, 1979). Different theories on the origin and pathogenesis of this disease have been developed throughout the years, including retrograde menstruation (Sampson, 1921), migration via the vascular or lymphatic system (Halban, 1925; Staffeld, 1968) or remnants of the Mullerian ducts as well as neoplastic dedifferentiation from mesothelial cells (Meyer, 1903; Geipel, 1927). The most supported concept, however, is that endometriosis is derived from a retrograde menstruation in combination with a defect in the immune system (Leyendercker et al., 1995). Though endometriosis is probably a hormone-dependent, proliferative but benign disease, only a few investigations have been carried out to study its progressive character (Dizerga et al., 1980; Walz et al., 1983). Also the mechanisms involved in the development of endometriosis-associated pain symptoms are still under investigation. Zahradnik (1995) postulated that a local burst of prostaglandins, induced by macrophages and mesothelial cells surrounding endometriotic implants, seems to be involved in this process.

The management of women with endometriosis is complex, due to the fact that this disorder is not well defined and lacks appropriate cell biological markers. In normal endometrial tissue several markers including integrins (Lessey et al., 1994) and gap junctions (Jahn et al., 1995) have been described for an appropriate differentiation related to the menstrual cycle. In this study we have focused on the expression pattern and hormonal regulation properties of different gap junction proteins, connexins (Cx), in endometriotic tissues. Connexins belong to a multigene family, of which 13 members in the murine genome have already been cloned (Willecke, 1993). All of these different channels seem to exhibit different physiological properties including voltage gating and conductance, as well as the possibility of a different dye transfer (for review see Waltzmann and Spray, 1993). Direct cell–cell communication seems to be involved in coordinating proliferation and differentiation processes, probably mediated through exchange of second messengers such as cAMP, inositol 1,4,5-...
trisphosphate (IP$_3$) as well as Ca$^{2+}$ (Saez et al., 1989). Connexin expression is normally found in adult tissue in a steady state situation. In the target organs of steroid hormones, connexins seem to be highly regulated. This has been extensively studied in the myometrial compartment of the uterus of several species (Garfield et al., 1980, 1981; Sakai et al., 1992; Petrocelli and Lye, 1993; Piersanti and Lye, 1995). In the myometrium, the increase of oestrogen and the decrease of progesterone serum concentrations seems to be responsible for the induction of gap junctions prior to term to synchronize muscle contraction at birth (Garfield et al., 1988).

A similar regulation of connexins by 17β-oestradiol and progesterone could be shown in the endometrium of several species during the pre- and peri-implantation phase (Winterhager et al., 1988, 1991; Grümmer et al., 1994). In humans, the spatial and temporal pattern of gap junctional proteins is precisely regulated in relation to the hormonal status of the cycle (Jahn et al., 1995). Cx26, which is expressed in the uterine epithelium, and Cx43 which is seen between the stromal cells, are down-regulated during the luteal phase and dramatically increase during the oestrogen-dominated phase of the menstrual cycle.

The loss of cell–cell communication as well as an inappropriate expression of connexins may be responsible for dedifferentiation processes leading to tumorigenesis (Yamasaki, 1991). This is supported by several investigations (Wilgenbus et al., 1992; Holder et al., 1993) which have shown that tumorigenesis is correlated with aberrant or low connexin expression or a chemically-induced suppression of cell–cell communication (Yamasaki et al., 1995).
The characterization of cell biological markers specific for endometrial differentiation may elucidate the mechanisms involved in the pathogenesis of endometriosis and could give some help for an appropriate medical treatment. Here we studied the expression pattern and hormonal regulation properties of connexins in the ectopic glandular uterine epithelium to gain an insight into the extent of dedifferentiation and hormonal regulation of endometriotic tissue.

**Material and methods**

**Patients and therapies**

Samples from human endometriotic lesions located in the peritoneum were collected from 41 women at the Department of Gynaecology at the University of Essen, Germany, between 1994 and 1995. In all, 17 patients suffered from infertility, 16 from endometriosis-associated pain symptoms, including dysmenorrhoea, dyspareunia and chronic pelvic pain, and eight from both. The improvement of the endometriosis-associated pain symptoms was evaluated after gonadotrophin-releasing hormone (GnRH) agonist or progesterin therapy using a specific questionnaire that was given to the patients.

Tissue samples were obtained by laparoscopy, snap frozen in liquid nitrogen and stored at −20°C. Biopsies were taken during the early and the late proliferative phase (n = 26) or during the luteal phase (n = 5) of the menstrual cycle. In three cases samples were collected after a 6 month treatment with a GnRH agonist (Leuprorelinacetate; Takeda Pharma, Aachen, Germany) given in a dosage of 3.75 mg i.m. every 4 weeks and in seven cases after 6 months of treatment with the progestin dienogest (Jenapharm, Jena, Germany) given in a dosage of 2 mg orally daily.

**Hormone analyses**

For all patients, serum concentrations were evaluated for 17β-oestradiol, progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) shortly before surgery. 17β-oestradiol and progesterone were measured using a commercially available radioimmunoassay (Sorin Biomedica, Saluggia, Vercelli, Italy). FSH and LH were determined by an enzyme-linked immunosorbent assay (Johnson and Johnson, Norderstedt, Germany).

**Immunohistochemistry**

Cryostat sections (4–7 µm) were fixed in ice-cold absolute ethanol for 5–10 min. An affinity purified rabbit monoclonal antibody to Cx26 from mouse liver gap junctions (Traub et al., 1989), a rat monoclonal antibody against Cx32 of mouse liver gap junctions (Janssen-Timmen et al., 1986) and a monoclonal mouse antibody to Cx43 (Affinity, Cambridge, UK) were used as primary antibodies. Immunohistochemical staining was performed as described previously (Winterhager et al., 1991).

Briefly, slides were rinsed in a phosphate-buffered saline/bovine serum albumin (PBS/BSA) buffer for 10 min before incubation with the primary antibodies in a dilution of 1:40 for 90 min at room temperature in a humidity chamber. Incubation with a secondary anti-mouse and anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) at a dilution of 1:40 was then carried out for 45 min. After washing, the slides were covered with glycerol containing 0.1% paraphenylenediamine to avoid photobleaching.

Negative controls were obtained by substituting the primary antibody with preimmune rat serum or mouse immunoglobulin (IgG). For positive controls, samples of human tissues were used which had a known strong expression (human liver for Cx32 and Cx26 and human heart tissue for Cx43). Because of the small diameter of the endometriotic lesions (~2 mm), the complete slice of tissue was examined and microscopically evaluated. Two slides were analysed for each class of connexin molecule and each type of endometriotic tissue.

The intensity of connexin staining was semiquantitative: strong (+ + +), moderate (+ +), low (+) or negative (−). This semiquantitative evaluation of the slides was determined using the IR score of Remmele and Stegner (1987) which takes the staining intensity (SI) and the percentage of positive cells or cell membranes (PP) into consideration. The staining intensity is subdivided into: 0 = no staining intensity; 1 = low staining intensity; 2 = moderate staining intensity; 3 = strong staining intensity.

The percentage of cells expressing connexin was subdivided as follows: 1 = 1–10% positive cells; 2 = 11–50% positive cells; 3 = 51–80% positive cells; 4 = 81–100% positive cells.

The multiplication of the points given for staining intensity and percentage of positive cells yields the IR score. We used a score of 2 points as a cut-off point between connexin-positive and negative cells, i.e. IRS values of 0 and 1 point were considered as connexin negative, values between 2 and 4 were considered as low positive.

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**Figure 1.** Immunohistochemical staining of connexin43 (Cx43) of an endometriotic implant of a non-treated patient. The biopsy was obtained during the follicular phase of the menstrual cycle. The endothelial-like shape of the glandular epithelial cells reveals moderate staining for Cx43 (arrows); original magnification ×1600.

**Figure 2.** Immunohistochemical staining of connexin43 (Cx43) of an endometriotic implant of a non-treated patient. The biopsy was obtained during the follicular phase of the menstrual cycle. The endothelial-like shape of the glandular epithelial cells shows strong staining for Cx43 (arrow); original magnification ×1500. L = lumen.

**Figure 3.** Immunohistochemical staining of connexin26 (Cx26) of an endometriotic implant of a patient treated with the progestin dienogest. The cylindrical epithelium of the glandular epithelial cells demonstrates strong immunolabelling for Cx26; original magnification ×1500. L = lumen; S = stroma.

**Figure 4.** Immunohistochemical staining of connexin32 (Cx32) of an endometriotic implant of a non-treated patient. The biopsy was obtained during the follicular phase of the menstrual cycle. No specific immunohistochemical staining can be detected; original magnification ×370. S = stroma; E = epithelium.

**Figure 5.** Stromal region of an endometriotic implant. No immunohistochemical connexin43 (Cx43) staining can be detected, original magnification ×1500.

**Figure 6.** Strong immunohistochemical staining of connexin43 (Cx43) in the stromal compartment of human endometrium; original magnification ×370. S = stroma; E = epithelium.
Table I. Individual clinical and immunohistochemical data of 41 patients

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Cx = connexin; GnRH = gonadotrophin-releasing hormone.

(+) between 5 and 8 as moderate positive (++) and between 9 and 12 as strong positive (+++).

Statistical analyses
Statistical analyses were performed using the SAS program (SAS Institute, 1985) for the correlation of the 17β-oestradiol and progesterone values with the expression of Cx43 and Cx26 respectively. The χ², the likelihood ratio χ², the Mantel–Haenszel χ², the π coefficient, the contingency coefficient and Cramer’s V test were used. The level of significance was set at P < 0.05.

Results
Modulation of the connexin expression pattern of the ectopic implants was found to be dependent on the oestrogen and progesterone serum concentrations of the individual patients, regardless of whether they were hormonally treated or not.

In general, Cx43 was preferentially expressed in the glandular epithelium of endometriotic tissues of nearly all patients (40 from 41) independent of either the hormonal phase or treatment; Figure 1 shows a low Cx43 expression (+) and Figure 2 a strong (+++) Cx43 expression. Cx26 could be detected in the ectopic epithelium of 18 samples (Figure 3). Except for one case, Cx26 was found co-expressed with Cx43. The expression of Cx26 was usually lower than the expression of Cx43 as shown in Table I. The epithelial cells which expressed Cx26 in addition to Cx43 were morphologically characterized by a cylindrical shape indicating a more differentiated feature. Cx32 staining was not found in any endometriotic tissue, epithelium or stroma, respectively (Figure 4). Surprisingly, in all the samples, none of the stromal cells surrounding the glands of the endometriotic implants expressed any of the connexins investigated (Figure 5).

In contrast, the uterine epithelial cells of normal cyclic human endometrium expressed Cx26 during the oestrogen-dominated phase and, to some extent, Cx32 but never Cx43, which is exclusively found in the stromal compartment (Jahn et al., 1995) (Figure 6). Table I shows the distribution pattern of Cx43, Cx26 and Cx32 of the 41 patients, their serum concentrations of 17β-oestradiol and progesterone and the relationship with hormonal treatment in 10 cases.
Gap junction connexins in endometriotic tissues

Figure 7. Connexin43 expression correlated with 17β-oestradiol concentrations; n = 41. $\chi^2$ value = 13 253; $P < 0.05$.

Hormonal regulation of connexin expression in endometriosis

17β-oestradiol

As summarized in Figure 7, high concentrations of 17β-oestradiol (>100 pg/ml) at the time of surgery were correlated with a high level of expression of Cx43. The three patients with a strong expression (++) for Cx43 revealed 17β-oestradiol concentrations of 150, 152 and 110 pg/ml respectively. In patients with 17β-oestradiol concentrations of 50–100 pg/ml the Cx43 expression in the ectopic glandular epithelium was low (+) in 12 cases and moderate (+++) in two cases. Patients with 17β-oestradiol concentrations <50 pg/ml (13 cases) showed in only one case a moderate (++) expression of Cx43, whereas 11 patients showed only a low (+) expression of Cx43. In the single case where no Cx43 expression was detected, the 17β-oestradiol concentration was very low (12 pg/ml).

The three patients treated for 6 months with the GnRH agonist leuprorelinacetate had a significant suppression of the ovarian function (17β-oestradiol concentration <30 pg/ml and progesterone concentration <0.3 ng/ml) and were included in the group with 17β-oestradiol <50 pg/ml. The endometriotic implants showed not only a regressive morphology and an endothelial like glandular epithelium, but expressed Cx43 only in a low (+) amount. Cx26 could only be found in one of these three patients, also in a low (+) amount, thus suggesting that suppression of ovarian function leads to a minimal expression of connexins. These three patients showed no endometriosis associated pain symptoms after therapy and had only minimal endometriotic residuals [post-treatment revised American Fertility Society (AFS, 1985) score of I].

All the correlations between the 17β-oestradiol concentrations and Cx43 expression were statistically significant ($\chi^2$ 13 253; $P < 0.05$).

Progesterone

As shown in Figure 8 the intensity of Cx26 immunostaining in the ectopic glandular epithelium was observed. As shown in Figure 9, only four of the 18 patients with a positive Cx26 immunostaining had endometriosis-associated pain symptoms. The remaining 14 patients had no endometriosis-specific pain symptoms.

Figure 8. Connexin26 expression correlated with progesterone concentrations; n = 41. $\chi^2$ value = 10 192; $P < 0.05$.

Figure 9. Patients with positive immunohistochemical staining for connexin26 (see Table I) and their endometriosis-associated pain symptoms in relation to progesterone concentrations and gestagen therapy; n = 18.
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Discussion

The present study deals with the expression pattern of gap junction connexins in endometriotic implants of patients under different hormonal situations. While the uterine epithelium of normal cycling patients expresses Cx26 and to a low extent, Cx32, the epithelium of endometriotic glands demonstrates an aberrant expression of Cx43. Only more differentiated ectopic endometrial glands with a cylindrical epithelium show the appropriate Cx26 protein in addition to Cx43 (Winterhager et al., 1993; Jahn et al., 1995). The stromal cells, normally expressing Cx43, lacked the connexins studied in the endometriotic implants. It has been shown in several reports that gap junctional connexins are hormonally regulated in the endometrium during preimplantation in rats (Grümmer et al., 1994) as well as during the menstrual cycle in humans (Jahn et al., 1995).

Although the connexin expression in the endometriotic implants was aberrant, this work suggests that it is still under hormonal control. As in normal endometrium, Cx43 expression in the epithelium is enhanced in the presence of elevated oestrogen serum concentrations. Cx26 regulation, however, does not seem to follow the same rules as described for normal cycling endometrium. Cx26 expression in endometriotic gland epithelium is correlated with higher progesterone serum values even after progesterin treatment which normally suppresses this gene (Grümmer et al., 1994; Jahn et al., 1995). These different regulation properties of Cx26 between normal and endometriotic tissue could be caused by the abnormal expression of oestrogen and progesterone receptors in ectopic glands. In contrast to the uterine endometrium, a lower concentration of receptor types was found in the epithelium as well as in the stromal compartment (Regidor et al., 1994). The aberrant connexin expression also could explain the findings of Schweppe and Wynn (1981), which showed that endometriotic implants did not undergo cyclical changes compared with the corresponding endometrium.

Interestingly, the patients with higher progesterone serum concentrations showed no clinical endometriosis-associated pain symptoms. One can speculate that the progesterin-induced differentiation of the endometriotic implants leads to a consecutive loss of the ability to induce prostaglandin secretion, and hence pain, by the surrounding macrophages and mesothelial cells.

Patients treated with GnRH agonists showed a complete down-regulation of the connexins studied and also showed a significant improvement in their pain symptomatology.

The hormonally induced hypo-oestrogenic and hypoprogesterogenic status is similar to the situation a few days before menstruation in the normal cycling human endometrium. The down-regulation of connexins with the subsequent degenerative processes in the human endometrium could explain why, in almost all the cases of GnRH agonist-treated patients, pain relief was observed even if endometriotic implants were still found after the GnRH agonist treatment (Mettler et al., 1991; Regidor et al., 1993; Schindler et al., 1994a;b; Regidor et al., 1995). Oestrogen-dependent expression of Cx43 in the epithelium of the ectopic glands seems to indicate a dedifferentiation process and is correlated with the development of pain.

It has been shown that dedifferentiation processes mainly involved in the multistep processes of carcinogenesis are correlated with aberrant or low expression of connexins (Yamasaki et al., 1995). The proof that appropriate tissue-specific intercellular communication is involved in regulation of cell growth and control of differentiation has been provided by experiments using tumour and neoplastic cell lines transfected with different connexin genes. Mesnil et al. (1995) demonstrated a significantly reduced growth of HeLa cells in culture as well as in nude mice when transfected with Cx26. Chen et al. (1995) found that neoplastic kidney cells transfected with Cx43 had reduced proliferation accompanied by a decrease in cell cycle regulatory elements including cyclins A, D1 and D2. This points to the fact that a tissue-specific connexin expression is important for regulation of coordinated proliferation. In this context the aberrant expression of Cx43 and of Cx26 could probably reflect the progressive extension of endometriosis.

Thus the pain relief correlated with endometriotic tissue characterized by Cx26 expression could explain the positive subjective development of endometriosis-associated pain symptoms in patients treated with progestins in controlled studies (Kouridis and Kistner, 1968; Moghissi, 1976). Due to the loss of cell–cell communication in the stroma as well as an inappropriate expression of connexins, endometriosis can also be described as a disease which undergoes both proliferative and dedifferentiation processes. Therefore, our data confirm the clinical observations of Malinak et al. (1992) and Walz et al. (1983) who showed that endometriosis exhibits a progressive character. That means that endometriosis should not be underestimated and considered as a disease that has to be treated either surgically and/or hormonally to avoid its progressive development.

Nevertheless, additional markers specific for appropriate differentiation of human endometrium such as integrins (Lessey et al., 1994), CD44 (Regidor et al., 1996) or E-cadherin (van der Linden et al., 1994) have to be studied in endometriotic implants under therapy to understand the complex dedifferentiation process as well as differentiation properties of endometriosis. This could help to develop individual therapeutic concepts in the treatment of endometriosis.

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References


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Gap junction connexins in endometriotic tissues