Expression of Cytokeratin 8 (CK8) in Human Endometrium and Endometriosis

Untersuchung der Expression von Zytokeratin 8 (CK8) im Endometrium und Endometriose

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Abstract

Cytokeratins are a multigene family of polypeptides expressed in distinct combinations along the various routes of epithelial differentiation. In endometrial cancer and cervical intraepithelial neoplasia/cervical squamous cell carcinoma, the expression of cytokeratins correlates with aggressive tumor features, lesion grade, and the International Federation of Gynecology and Obstetrics (FIGO) stage. Although the central elements involved in the pathogenesis of endometriosis are as yet unexplained, it is generally accepted that invasive mechanisms and hormonal effects influence endometriotic disease. On the basis of a preliminary microarray analysis showing increased mRNA expression of cytokeratin 8 in peritoneal endometriosis in comparison with eutopic endometrium, the present study therefore investigated the expression of CK8 in human eutopic and ectopic endometrium, using immunohistochemical staining. The results of our analyses points to a posttranscriptional regulation of cytokeratin 8 in endometriotic lesions possibly through noncoding miRNAs.

Zusammenfassung

Die Proteine der Zytokeratin-Multigenfamilie werden im Rahmen der epithelialen Differenzierung in verschiedenen Kombinationen exprimiert. Bei Endometrium- und Zervixkarzinomen korreliert die Expression von Zytokeratinen mit aggressiven Eigenschaften des Tumors, dem Grad der Läsion und dem FIGO-Stadium. Obwohl die zentralen Elemente der Pathogenese von Endometriose noch immer umstritten sind, so ist doch allgemein akzeptiert, dass die Krankheit durch invasive Mechanismen und hormonelle Einflüsse beeinflusst wird. Aufgrund von Microarray-Analysen, die eine erhöhte mRNA-Expression von Zytokeratin 8 (CK8) bei peritonealer Endometriose im Vergleich zu eutopem Endometrium vermuten ließen, untersuchte die vorliegende Studie die Expression von Zytokeratin 8 in eutopem und ektopem Endometrium mittels Immunhistochemie. Die Ergebnisse dieser Analysen deuten auf eine posttranskriptionelle Regulation von Zytokeratin 8 in Endometrioseläsionen z.B. durch miRNAs hin.

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Introduction

Cytokeratins are cytoskeletal intermediate filament proteins that are thought to be involved in the regulation of tissue homeostasis. At present, there are 20 known subtypes, which are expressed in various types of human epithelial cell. The cytokeratin (CK) isotypes depend on the cell type and localization in the cytoplasm [1]. Although cytokeratin expression does not appear to be influenced by the natural menstrual cycle, synthetic progestogens appear to reduce cytokeratin expression in human endometrium [2,3]. In endometrial cancer and cervical intraepithelial neoplasia/cervical squamous cell carcinoma, the expression of cytokeratins correlates with aggressive tumor features, lesion grade, and the International Federation of Gynecology and Obstetrics (FIGO) stage [4,5].

Although the central elements involved in the pathogenesis of endometriosis are as yet unexplained, it is generally accepted that invasive mechanisms and hormonal effects influence endometriotic disease. On the basis of a preliminary microarray analysis showing increased mRNA expression of cytokeratin 8 in peritoneal endometriosis in comparison with eutopic endometrium, the present study therefore investigated the ex-

Table 1 Patients characteristics.

Group 1		
Peritoneal endometriosis		
Age	33,7 ±	5,7
Diagnosis	AFS I/II	10
	AFS III/IV	2
Menstrual phase	proliferative phase	9
	secretory phase	1
	hormone therapy	2
Group 2		
Adenomyosis		
Age	45,1±	4,8
Diagnosis	adenomyosis	13
Menstrual phase	proliferative phase	7
	secretrory phase	3
	hormone therapy	3
Group 3		
Control (no evidence of endometriosis or adenomyosis)		
Age	45,9 ±	4,6
Diagnosis	Ut my	16
	CIN	4
Menstrual phase	proliferative phase	8
	secretory phase	9
	hormone therapy	3

pression of CK8 in human eutopic and ectopic endometrium, using immunohistochemical staining.

Methods

Materials

Endometrial biopsies were taken from consecutive patients undergoing hysterectomy, dilation and curettage, and other procedures for benign gynecological diseases. All peritoneal biopsy specimens were taken from the lateral abdominal wall. Characteristics of the patients are given in **Table 1**. The monoclonal antibody directed against cytokeratin 8 was obtained from Medac (Hamburg, Germany). Secondary goat anti-mouse antibody (FAST-RED) was purchased from Dianova, Hamburg (Germany). The study was approved by the local ethics committee.

Microarray analysis

RNA preparation and microarray analysis using the Affymetrix Human Genome U133A GeneChip platform (Affymetrix, Inc., Santa Clara, California, USA) containing 22 283 probes was performed as described elsewhere [6,7]. Hybridization intensity data were automatically acquired and processed using the Affymetrix Microarray Suite 5.0 program. The expression level of each gene was determined by calculating the average differences in intensity (perfect match–mismatch) between its probe pairs. Scans were rejected if the scaling factor exceeded 2 or a "chip surface scan" revealed scratches, specks, or gradients affecting the overall data quality (Refiner, GeneData, Inc., Basle, Switzerland).

The expression data were subsequently analyzed using the Cluster and Treeview software package [8]. Prior to cluster analysis, gene chip expression values were adjusted by log transformation and median centering of the gene chips. Hierarchical gene clustering was carried out using the Pearson correlation as distance metric and average linkage clustering.

Analyses of published datasets

Affymetrix gene expression data of endometrial tissues from the study of Burney et al. [9] (n = 37, GEO database entry GSE6364) were downloaded from the NCBI Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo/. These independent data sets were used for CK8 expression during the different phases of the endometrial cycle.

Immunohistochemistry

Paraffin sections (2 µm) were mounted on Superfrost Plus slides, dewaxed in xylene and rehydrated through graduated ethanol to water. Antigens were retrieved by microwaving sections in 10 mM citrate buffer (pH 6.0) for 20 min at 800 W. Blocking was performed using antibody dilution buffer (DCS-Diagnostics, Hamburg, Germany) at room temperature for 15 min. Subsequently, antibodies were diluted 1 : 100 individually in this buffer. Sections were incubated with antibodies 1 h at room temperature. For negative controls, the primary antibodies were replaced with PBS. For secondary antibody incubations and detection the Dako REAL Detection System Alkaline Phosphatase/RED (Dako, Danmark) was used following the protocol of the supplier and sections were counterstained with Mayer's hematoxylin.

Statistical analyses

All reported p values are two-sided. P values of less than 0.05 were considered to indicate a significant result. The Mann-Whitney U-test was used to compare expression values between different tissues. All analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

Immunohistochemistry

Immunostaining positive for cytokeratin 8 was observed in almost all eutopic and ectopic endometrial epithelial cells. Expression of cytokeratin 8 showed no dependence on the phase of menstrual cycle, the intake of oral contraceptives or the patient's diagnosis within each group (data not shown).

In ectopic endometrium of peritoneal endometriotic lesions, a tendency toward weaker staining of cytokeratin 8 can be identified (**Table 2**) which is in contrast to the data from microarray analysis suggesting stronger expression of cytokeratin 8 in ectopic endometrium. In contrast, the endometrial glands of adenomyotic foci revealed stronger staining compared to the corresponding eutopic endometrial epithelial cells (see **Fig. 1**). The staining pattern within the specimen is inhomogeneous in 3 of 12 of the peritoneal endometriosis lesions and 3 of 20 of endometrial specimens – weakly as well as strongly staining glands may be all present simultaneously.

Discussion

Cytokeratins are a multigene family of polypeptides expressed in distinct combinations along the various routes of epithelial differentiation. Currently, at least 20 different cytokeratins have been distinguished, and these have been divided into specific subgroups according to their migration in gel electrophoresis [1]. Cytokeratin 8 is expressed in several simple epithelia, and also in early embryonic epithelia. Relatively little is yet known about the functioning of the cytokeratins beyond their cyto-

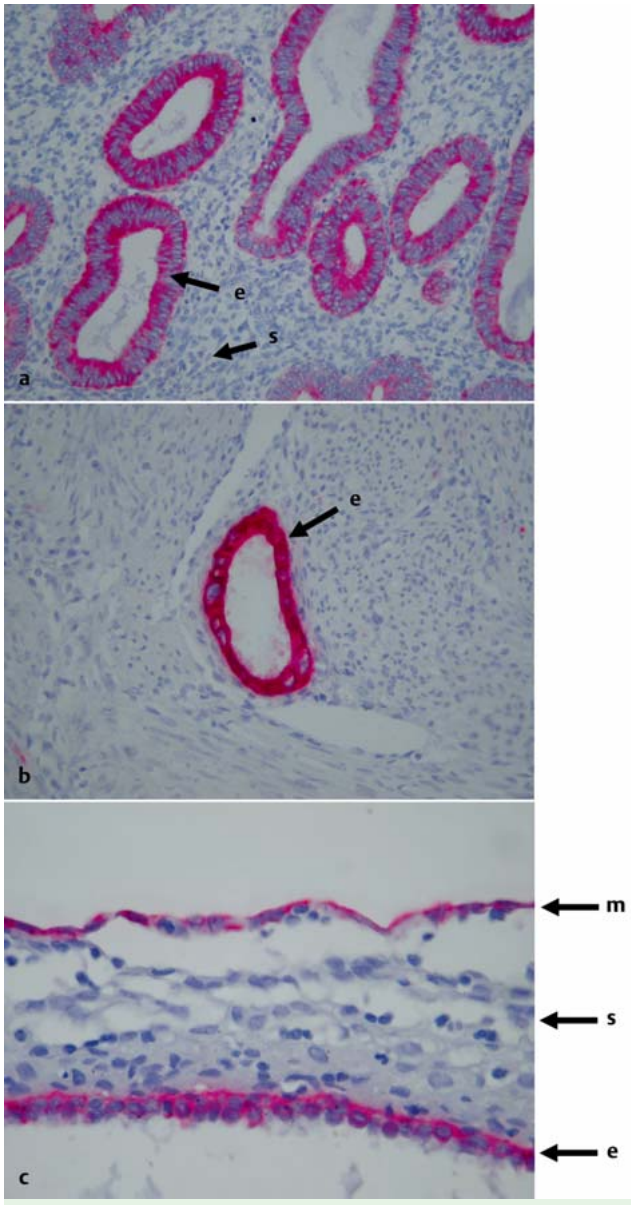

Fig. 1 a to **c** Immunostaining of cytokeratin 8 in eutopic and ectopic endometrium. Representative examples of the results from immunohistochemistry using an CK8 antibody (red staining) are shown for samples from eutopic endometrium (**a**), adenomyosis uteris (**b**) and peritoneal endometriosis (**c**). Arrows depict the mesothelial (m), stromal (s), and epithelial (e) cell layer (Magnification 20×).

skeletal tasks. For a number of tumors, a correlation has been demonstrated between cytokeratin and carcinogenesis, prognosis, and other factors in tumor biology [4,5,10,11]. For example, loss of CK5/6 in endometrial carcinoma is associated with increased Ki-67 expression, tumor necrosis, and a higher FIGO stage and grading [5]. On the one hand, this can be explained by the dependency of cytokeratin expression on tissue type, cell differentiation, and also dedifferentiation. On the other hand, it is also known for example that the keratin 8/18 dimer has a key function in tumor necrosis factor (TNF)-mediated apoptosis [12]. Previously published data concerning cytokeratin expression in eutopic endometrium, like the results of the present study, have not demonstrated any dependency on the phase of the menstrual cycle [2,3]. The reduction of cytokeratin expression by levonorgestrel reported by Wonodirekso et al. is not necessarily in contradiction with this. The more complex activity profile of the synthetic gestagen levonorgestrel – as a nortestosterone derivative that also has a partial androgenic effect in comparison with natural progesterone – may be the reason for the different effect on cytokeratin expression [3]. On the other hand, the sufficiently well-known discrepancy in hormone receptor expression between eutopic endometrium, adenomyosis, and peritoneal endometriosis may explain the differences in cytokeratin expression observed in this study. Although there is no dependency on the menstrual cycle in "normal" endometrium, an influence of cytokeratin via the effect of sex steroid receptors is possible, as the study by Wonodirekso et al. [3] shows.

Although the results of the microarrays indicate stronger expression of CK8 in ectopic endometrium in comparison with eutopic endometrium, this was not confirmed by immunohistochemistry (IHC). It is unlikely that the different proportion of epithelial cells in eu- and ectopic samples can account for these discrepancies, because the number of glandular cells is higher in normal endometrium. Therefore higher expression values of CK8 were expected for eutopic samples. A rational explanation for the differences between IHC and chip analyses is the assumption of a posttranscriptional regulation of CK8 potentially by microRNAs. Those small noncoding RNA have been shown to be involved in various human pathologies. Further studies are needed in order to clarify a contribution of miRNA in the generation of endometriotic lesions.

Adenomyosis uteri consistently shows more intense immunostaining with CK8 than the corresponding endometrium. This is in contrast to the reduced immunostaining seen in ectopic peritoneal endometriotic lesions. It has been debated for many years whether adenomyosis uteri and the typical forms of endometriosis in the lesser pelvis are different disease entities with a differing pathophysiology. The difference in CK8 expression between ectopic peritoneal locations and adenomyosis might pro-

Table 2 Analysis of cytokeratin 8 expression in glandular cells of peritoneal endometriosis, adenomyosis, eutopic endometrium of patients with adeomyosis and controls.

	Eutopic endometrium		Ectopic endometrium	
Staining pattern*	control	patients with adenomyosis	adenomyosis	peritoneal endometriosis
Weak	2/20	0/13	0/13	2/12
Moderate	7/20	6/13	3/13	5/12
Strong	11/20	7/13	10/13	5/12

* the number of specimen out of the total number of samples analyzed

vide support for this hypothesis; on the other hand, however – as discussed above – differences in the surrounding conditions between the uterus and the peritoneal cavity might be the cause.
Recent studies have provided evidence for the key role of CK8/CK18 in modulating TNF signaling. In vivo, the level of CK8/CK18 correlates with the sensitivity for TNF-mediated apoptosis. CK8/18 protects cells from apoptosis through binding of the cytoplasmic domain of TNFR2 [12]. Numerous studies in the literature have already drawn attention to altered or reduced apoptosis in both ectopic endometrium and adenomyosis [13 – 15]. It may be speculated that the increased expression of CK8 observed in adenomyosis plays a role in the pathogenesis of endometriosis.

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