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RESEARCH ARTICLE

Immunohistochemical analysis of steroid receptors, proliferation markers, apoptosis related molecules, and gelatinases in non-neoplastic and neoplastic endometrium

Cornelia Amalinei, Corina Cianga, Raluca Balan, Petru Cianga, Simona Giusca, Irina-Draga Caruntu *

University of Medicine and Pharmacy "Gr. T. Popa" Iasi, 16 Universitatii Street, Iasi 700115, Romania

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ABSTRACT

Endometrioid endometrial carcinoma developed from endometrial hyperplasia is associated with anomalies of proliferation, apoptosis, and matrix metalloproteinase (MMP) expression. Our study was designed to investigate steroid receptor (ER, PR) expression and its correlation with proliferative activity (PCNA), apoptosis (Fas, FasL, Bcl-2, Bax, and p53), gelatinases (MMP-2 and MMP-9) and their tissue specific inhibitor (TIMP-1 and TIMP-2) immunoexpression in endometrial carcinogenesis. A total of 38 cases were investigated, 10 non-neoplastic, 11 hyperplastic, and 17 carcinomatous endometria. Immunolabeling showed a higher expression of steroid receptors in hyperplasia and carcinoma than in non-neoplastic endometria and an ER/PR imbalance in carcinoma. The epithelial component of endometrial carcinomas had the highest proliferative index. Bcl-2 had a stronger expression in hyperplasia and carcinoma compared to non-neoplastic endometria and stromal tissue. The Bcl-2/Bax ratio was lower in endometrial carcinoma. Fas and FasL expression was stronger in hyperplasia and furthermore in carcinoma. p53 expression was progressively stronger along the sequence non-neoplastic endometrial to hyperplasia-carcinoma. Both types of investigated MMPs showed an increased expression in neoplastic endometria reaching a maximum level in carcinomas. MMP-9 immunostaining could be correlated to myometrial invasion. TIMP-1 decreased and TIMP-2 increased in expression from non-neoplastic endometria to hyperplastic and carcinomatous endometrial, respectively. Our study demonstrates that coordinated anomalies of steroid receptors, apoptosis and invasiveness factors are already present in hyperplasia as cumulative steps along the way to malignant transformation and that a complex MMP-2, MMP-9, TIMP-2/TIMP-1 imbalance seems to be responsible for the endometrial proliferation.

1. Introduction

Endometrial carcinomas have the highest incidence among the malignancies of the feminine genital tract (Bockman, 1983). According to knowledge of epidemiologic, histopathologic, and molecular events endometrial carcinogenesis follows two different pathways. In the dualistic model of endometrial carcinogenesis the first pathogenetic type (endometrioid endometrial carcinoma, type I), representing 65–80% of all endometrial cancers, is characterized by the following features: obesity, hyperlipidemia, hyperestrogenism (revealed by endometrial and ovarian stromal hyperplasia), highly and moderately differentiated tumors (G1 and G2 in 82.3%), superficial invasion of the myometrium (69.4%), high sensitivity to progestogens (80.2%), and favorable prognosis (5-year survival rate of 85.6%) (Bockman, 1983). The pathogenic correlation between G1 endometrial endometrioid carcinoma and atypical hyperplasia is currently sustained by numerous immunohistochemical studies that demonstrate analogous molecular markers expression (Mutter, 2000). The pathogenetic proposal of endometrial carcinogenesis is that an accumulation of gene mutation is associated with morphologic features (Ronnett et al., 2002). A multi-step endometrial carcinogenesis involving coordinated intervention of hormonal regulation, gene mutation, adhesion molecules, apoptosis, imbalance between metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) is currently accepted (Graesslin et al., 2006). The current research objectives in endometrial carcinogenesis are the identification of characteristic molecular markers and the analysis of the correlation between their expression and progression of precursors or aggressiveness of carcinomas.

Estrogens are direct promoters of endometrial carcinogenesis through stimulation of rapid proliferation of epithelial cells, estro-

* Corresponding author.
E-mail addresses: cornelia.amalinei@histo-m.umfiasi.ro, irina_caruntu@yahoo.com (I.-D. Caruntu).

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gen receptors (ERs) being overexpressed both in hyperplasia and carcinoma, in epithelial and stromal cell populations (Jazaeri et al., 1999; Isaka et al., 2003; Atasoy and Bozdogan, 2006; Giuffre et al., 2006). Progesterone receptors (PRs) and ERs show a generally concordant expression as a result of their interrelations and their functional status may influence the development of endometrial carcinoma (Ito et al., 2006, 2007).

Proliferating cell nuclear antigen (PCNA) also has an increased expression both in endometrial hyperplasias and carcinomas (Mitselou et al., 2003).

Apoptosis is crucial in human endometrium turnover. Alterations of apoptosis play an important role in endometrial carcinogenesis being revealed by Bcl-2/Bax, Fas/Fas ligand (FasL) and p53 protein systems functionality (Mitselou et al., 2003; Zhang et al., 2004).

Invasive capacities defining any malignancy result from the intervention of proteases that degrade the extracellular matrix (Puente et al., 2003). The matrix metalloproteinases (MMPs) are involved both in the endometrial physiology (characterized by variable MMPs expression along the endometrial cycle) (Freitas et al., 1999; Henriet et al., 2002; Cornet et al., 2005) and pathology (Goffin et al., 2003). The endometrium expresses a large spectrum of MMPs: MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-23, MMP-26, and MT1-MMP (membranar type 1-MMP) (Velasco et al., 1999; Henriet et al., 2002; Chegini et al., 2003; Curry and Osteen, 2003; Li et al., 2004), being produced both by stromal and epithelial carcinomatous cells (Turpeenniemi-Hujanen, 2005). The interpretation of MMPs expression is difficult as remodelling of the extracellular matrix (ECM) is an essential feature in the menstrual turnover of the human endometrium (Cornet et al., 2005). Various research methods have detected MMP-9 in endometrium with a significant increase in menstruation. The stromal fibroblasts, the glandular epithelium, the endothelium, the leukocytes, and the macrophages localised in areas of collagen fibers disruption had been identified as the cells responsible of its production (Cornet et al., 2005; Curry and Osteen, 2003). Both MMP-2 (A gelatinase) and MMP-9 (B gelatinase) are correlated to tumor growth and invasion in endometrial and cervical neoplasia (Egeblad and Werb, 2002; Hojilla et al., 2003; Isaka et al., 2003; Lambert et al., 2003; Rubin, 2003). However, an opposite effect of gelatinases is obtained by *in vitro* generation of antiangiogenic polypeptides (Pozzi et al., 2000) by cleaving plasminogen to an angiostatin fragment (Lucas et al., 1998) and collagen type XVIII to an endostatin-containing fragment (Heljasvaara et al., 2005).

The tissue inhibitors of metalloproteinases (TIMPs) are four types of natural inhibitors of MMPs: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. TIMPs partially regulate MMP activity by forming complexes with their active forms and with their precursors (progelatinases) (TIMP-1 with proMMP-9 and TIMP-2 with proMMP-2). TIMP-1 and TIMP-2 can form complexes with active MMP-9 and with both forms of MMP-2 (active and latent) (Määtä et al., 2000). High levels of TIMPs 1–3 presumably maintain tissue integrity and ECM homeostasis in non-malignant endometrium. Estrogen and progesterone modulate MMP and TIMP expression in human cycling endometrium. The progesterone effect is the down-regulation of gelatinases expression. Previous research has reported a high MMP-2, MMP-9, and TIMP-2 co-expression in endometrial adenocarcinoma (Määtä et al., 2000).

Our research is designed as a correlative investigation of several types of markers characteristic for the transition from non-neoplastic to neoplastic endometrium. Our study was based on the main molecular events along the pathway of endometrial carcinogenesis being focused on the immunohistochemical profiles of ER-PR, PCNA, Bcl-2/Bax, Fas/FasL, p53, MMP-2/TIMP-2, and MMP-9/TIMP-1, and has attempted to demonstrate that several malignant anomalies are already detectable in hyperplasia.

2. Materials and methods

A total of 38 cases selected from the files of the IIIrd Obstetrics and Gynecology University Clinic of Iasi, Romania were included in our study, with the approval of the Ethics Committee of the University of Medicine and Pharmacy "Gr. T. Popa": 10 non-neoplastic endometria, with patient's ages ranging from 33 to 55 years (mean 45), 11 endometrial hyperplasias, with patient's ages ranging from 29 to 47 years (mean 43), and 17 endometrial carcinomas, with patient's ages ranging from 34 to 64 years (mean 50).

The endometrial fragments were routinely processed, paraffin-embedded, and sectioned at 4 μm. After deparaffinization antigen retrieval was performed by heating the sections for 20 min, in Antigen Retrieval Solution (Dako, Denmark), pH 6.10, at 96 °C, in a water-bath. This was followed by incubation in 3% H_2O_2, for 5 min, at room temperature (RT) to quench endogenous peroxidase. Non-specific binding sites were blocked by incubating the slides for 1 h, in 50 mM Tris–HCl (pH 7.4), containing 10% normal goat serum and 1% BSA. The histological sections were incubated overnight at 4 °C with the primary antibodies that were used to reveal in human endometrial tissues several molecules (Table 1): estrogen receptor alpha (ERα), PR, PCNA, Bcl-2/Bax, Fas/FasL systems, p53, MMP-2, MMP-9, TIMP-1, and TIMP-2. Specific reactions were detected by successive incubations with a second biotinylated antibody (LSAB kit, Dako, Denmark) 30 min, at RT with enzymatic complex streptavidin-peroxidase (30 min, at RT) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) extemporaneously activated with 3% H_2O_2, for 30 min, at RT. The following step was the counterstaining with Meyer's haematoxylin. Negative controls were represented by sections from the same tissues incubated with a mixture of mouse isotypes (Universal Negative Control for N-Series Mouse Primary Antibodies-N1698, Dako, Denmark). Positive reactions were visualized as brown precipitates with nuclear pattern for ERα, PR, PCNA, and p53, nuclear or cytoplasmic labeling for Bcl-2 and cytoplasmic or membrane labeling for Bax, Fas, FasL, MMP-2, MMP-9, TIMP-1, and TIMP-2.

The IHC score was determined by analysing two variables: the percentage of immunostained cells and the average staining intensity, considering the amount of positive cells as the most important criterion.

2.1. Semi-quantitative analysis

ERα and PR expressions were quantified as the percentage of positive cells per 1000 cells in each section (Shiozawa et al., 1996) and results were recorded as the mean percentage of positive cells ± standard deviation (SD) in each histological group.

PCNA estimation of the proliferative activity was performed by choosing ten fields with maximum staining intensity, 100 epithelial cells being selected in each field. The PCNA index was expressed as percent of positive cells (Yokoyama et al., 1998).

Table 1
Antibodies used for immunohistochemical staining.

Marker	Clone
Mo-a-hu ERα	1D5, Dako, Denmark
Mo-a-hu PR	PgR636, Dako, Denmark
Mo-a-hu PCNA	PC10, Dako, Denmark
Mo-a-hu Bcl-2	124, Dako, Denmark
Ra-a-hu Bax	A3533, Dako North America, USA
Mo-a-hu Fas	DX2, Dako, Denmark
Mo-a-hu FasL	NOK1, BD Biosciences Pharmingen
Mo-a-hu P53	DO-7, Dako, USA
Mo-a-hu MMP-2	D2705, Santa Cruz, Biotechnology
Mo-a-hu MMP-9	K1504 Santa Cruz, Biotechnology
Mo-a-hu TIMP-1	63515, R&D Systems, Minneapolis, USA
Mo-a-hu TIMP-2	89025.11, R&D Systems, Minneapolis, USA