Heparanase expression increases throughout the endometrial hyperplasia–cancer sequence

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Abstract

Objective: To assess the expression of heparanase in the different stages leading to endometrial cancer. Methods: The 38 examined specimens included adenocarcinoma, hyperplasia, and normal endometrium specimens. Heparanase, estrogen, and progesterone receptor expressions were analyzed immunohistochemically and the intensity was scored. Results: Secretory normal endometrium and simple hyperplasia specimens expressed the lowest mean values of expression (1.00 and 0.63, respectively); the complex hyperplasia specimens and G2 endometrioid adenocarcinoma showed the highest values of expression (2.33 and 2.71, respectively). A linear trend (P=0.005) of heparanase expression was observed when comparing the normal endometrium and simple hyperplasia group with the complex hyperplasia + G1 carcinoma group and the G2 + G3 carcinoma group. Evaluation of atrophic and inactive endometrium compared with papillary serous carcinomas yielded no significant differences. We found no significant correlation between heparanase expression and estrogen receptor or progesterone receptor expression. Conclusion: Heparanase expression was tightly regulated in endometrial tumorigenesis.

Key words: Endometrial cancer; Endometrial hyperplasia; Estrogen receptor; Heparanase; Progesterone receptor

1. Introduction

Endometrial carcinoma is the fourth most common malignancy in women of North America and Europe [1]. Endometrial carcinomas are classified histologically and biologically into 2 types. Type I endometrial cancers are associated with long-term unopposed estrogen exposure which gives rise to well-differentiated...
tumors through a sequence of hyperplastic lesions. These tumors tend to have a favorable prognosis [2]. Earlier studies have shown a progressive propensity of hyperplastic lesions to advance to malignancy, dependent upon specific histopathological characteristics [3]. In contrast, type II tumors are not estrogen dependent and are associated with atrophic endometrium, their histology is nonendometrioid (i.e., serous papillary), and the tumors have aggressive biology [4]. The molecular mechanisms of carcinogenesis are still obscure, but abnormalities in KRAS2, PTEN, and β-catenin genes have been linked to type I tumors, while abnormalities in TP53 and ERBB2 (HER-2/neu) genes were associated with type II tumors [5].

Heparan sulfate proteoglycans (HSPGs) are macromolecules present on the cell surface and extracellular matrix of every eukaryotic cell. Heparan sulfate (HS) chains bind a wide variety of bioactive molecules, such as chemokines, lipoproteins, enzymes, and growth factors, thus influencing many physiological and pathological cellular and matrix processes, such as cell-cell, cell-matrix adhesion, cell proliferation, angiogenesis, wound healing, tumorigenesis, and metastasis [6]. Human heparanase is an endo-β-glucuronidase that cleaves specific heparan sulfate side chains of HSPGs in a variety of normal and malignant cells [7]. It has been implicated in a multitude of biological processes, such as cell tumor metastasis, angiogenesis, inflammation, and wound healing by releasing HS-bound growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [8]. Increased expression of heparanase has been linked to progressive dysplasia and a worse prognosis in a myriad of cancers [9,10]. Heparanase expression was found to be increased in correlation with clinical stage, tumor grade, lymph node metastasis, and decreased overall survival in endometrial cancer [11,12]. One recent study suggested a possible role for estrogen in inducing heparanase overexpression in breast carcinoma [13].

In the present study we assessed the expression of heparanase in the different stages leading to the 2 types of endometrial cancer. In addition, we evaluated a possible correlation between the expression of estrogen receptor (ER) and progesterone receptor (PR) and heparanase expression in the endometrial carcinogenesis.

2. Materials and methods

2.1. Tissue samples

The files of patients with endometrial cancer who were operated on at the Tel-Aviv Sourasky Medical Center, Israel, during 1999–2005 were obtained from the Pathology Institute database. All patients had surgical staging according to the FIGO classification [14]. The specimens were reviewed by a dedicated gynecological pathologist (S.B.) and only the ones with good histological architecture and sufficient tissue for analysis were chosen. Specimens from patients with endometrial hyperplasia and normal endometrium were used as controls. A total of 38 formalin-fixed and paraffin-embedded specimens (patients’ median age 57 years; range, 35–94 years) were selected, and they included 13 endometrioid adenocarcinomas, 3 papillary serous carcinomas, 11 hyperplasias (8 simple and 3 complex), and 11 normal endometrium specimens (atrophic, inactive, and secretory). Endometrioid adenocarcinoma specimens included 11 endometrioid cancers in stage I, and 2 in stage III. All studies were approved by the institutional review board.

2.2. Immunohistochemistry

Paraffin-embedded tissue sections (2 μm thick) were deparaffinized and rehydrated. The tissue was then denatured for 20 min in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0). Blocking steps included successive incubations in 0.2% glycine, 3% hydrogen peroxide in methanol, and 10% goat serum. The first 2 steps were followed by 2 washes in phosphate-buffered saline (PBS). Sections were incubated overnight at 4°C with a polyclonal mAb (733) antihuman heparanase antibody diluted 1:100 in PBS followed by incubation with horseradish peroxidase-conjugated goat–anti-mouse IgG+IgM antibody (Jackson Laboratory, Bar Harbor, ME, USA). mAb 733 is directed against the c-terminus region of the 50-kDa heparanase subunit. The preparation and specificity of this mAb were previously described and demonstrated [15]. Color was developed using either Sigma Fast 3,3′-diaminobenzidine tablet sets (Sigma Chemical Co, St. Louis, MO, USA) or a Zymed aminoethyl carbazole (AEC) substrate kit (Zymed, South San Francisco, CA, USA) for 10 min followed by counterstain with Mayer’s hematoxylin.

ER and PR expression were analyzed using the monoclonal antibody 6F11 and monoclonal antibody 312, respectively (Novocastra, Newcastle, UK). After deparaffinization, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in absolute methanol. Microwave antigen retrieval was performed in a 10 mmol/L citrate buffer for 10 min at high power.

Antibody labeling was performed with the avidin–biotin complex method and the 3′, 3′-diaminobenzidine detection kit on a semi-automated instrument (Ventana ES; Ventana Medical Systems, Tucson, AZ, USA). Antibody labeling took place at 37 °C with the sequential addition of the following reagents: inhibitor for endogenous peroxidase, primary antibody, avidin and biotin blockers, biotinylated secondary antibody, avidin–biotin complex with horseradish peroxidase, 3′-diaminobenzidine-hydrogen peroxide, copper enhancer, and hematoxylin. The primary antibodies that were used were anti-PR (1:20 dilution with microwave antigen retrieval; Novocastra Laboratories, Newcastle, UK) and anti-ER (Ventana Medical Systems, Tucson, AZ, USA) with the procedure carried out as specified by the manufacturer.

![Figure 1](image-url) Heparanase expression increases throughout the endometrial hyperplasia–cancer sequence (mean values). Note the increase in heparanase expression with the progression from normal to hyperplasia and finally to cancer. Abbreviations: NE, normal endometrium; SH, simple hyperplasia; CH, complex hyperplasia; G1, well-differentiated adenocarcinoma; G2, intermediate differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma.
2.3. Scoring methodology

Heparanase immunoreactivity in glandular tissue was determined by categorizing staining intensity into 4 groups: (0) no staining; (1) weak staining, barely observed over nonspecific background staining; (2) moderate staining observed as coarse aggregates, partially obscuring cytoplasm and counterstained chromatin; and (3) strong staining, observed as coarse membranous bands obscuring the cell boundaries. ER and PR immunoreactivity was scored as 0 (absent), +1 (5%–25%), +2 (25%–50%), and +3 (>50%).

2.4. Statistical analysis

Differences between the study groups were evaluated using the Kruskal–Wallis test. The correlation between heparanase expression and ER and PR expression was determined using a Spearman’s test. A one-way analysis of variance (ANOVA) was used to determine a linear trend of heparanase expression between the different groups. A *P* value < 0.05 was taken as the level of significance for all tests.

3. Results

3.1. Heparanase expression in the endometrioid pathway

Heparanase expression was found to be significantly different (*P* = 0.009) among the various stages in the endometrial pathway to cancer (Fig. 1, Table 1). Secretory normal endometrium and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean values of heparanase, estrogen receptor, and progesterone receptor expression</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
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<tr>
<td>Normal</td>
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<tr>
<td>Inactive</td>
<td>4</td>
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<tr>
<td>Atrophic</td>
<td>3</td>
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<tr>
<td>Secretory</td>
<td>4</td>
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<tr>
<td>Hyperplasia</td>
<td>(n=11)</td>
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<tr>
<td>Simple</td>
<td>8</td>
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<tr>
<td>Complex</td>
<td>3</td>
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<tr>
<td>Endometrial carcinoma</td>
<td>(n=13)</td>
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<tr>
<td>G1</td>
<td>4</td>
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<tr>
<td>G2</td>
<td>7</td>
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<tr>
<td>G3</td>
<td>2</td>
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<td>Papillary serous carcinoma</td>
<td>(n=3)</td>
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Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

Figure 2  Immunohistochemical staining of heparanase, estrogen receptor (ER), and progesterone receptor (PR) (rows 2–4, respectively) in a panel of normal endometrium tissue samples (magnification ×20). Atrophic endometrium (column A); Proliferative endometrium (column B); Secretory endometrium (column C). HE staining for tissue definition is shown for samples as outlined above (first row) (magnification ×10). Note that heparanase is highly expressed in atrophic tissue and absent in secretory and proliferative specimens.
simple hyperplasia specimens expressed the lowest mean values of expression (1.00 and 0.63, respectively) (Fig. 2), while the complex hyperplasia specimens and G2 specimens showed the highest values of expression (2.33 and 2.71, respectively) (Figs. 3 and 4, Table 1).

Using the one-way ANOVA, we found a linear trend (P=0.005) of heparanase expression when comparing the normal secretory endometrium and simple hyperplasia group with the complex hyperplasia +G1 carcinoma group and the G2+G3 carcinoma group (Fig. 1).

A comparison of endometrioid carcinomas (all grades) and complex hyperplasias yielded no difference among the groups, with high mean expression values of 2.23 and 2.33, respectively.

3.2. Heparanase expression in the papillary serous pathway

Evaluation of atrophic and inactive endometrium compared with papillary serous carcinomas yielded no significant difference between the groups, with high mean expression values of 2.14 and 2.33, respectively (Table 1). In addition, we found no significant difference between heparanase expression in papillary serous carcinomas and its expression in G2+G3 endometrioid cancers, although both groups expressed high mean values (2.33 and 2.44, respectively).

3.3. Correlation between heparanase expression and ER and PR expressions

ER and PR expressions were found among the various stages in the endometrial pathway to cancer (Figs. 3 and 4, Table 1). A Spearman’s correlation revealed no significant correlation between heparanase expression and ER expression (correlation coefficient = −0.232; P=0.16) nor any significant correlation between heparanase expression and PR expression (correlation coefficient = −0.198; P=0.23).

4. Discussion

The present study is the first to assess heparanase expression in the premalignant stages preceding endometrial carcinoma. We observed significantly different heparanase expression throughout the various stages, and most importantly, a pattern of increased expression throughout the hyperplasia–cancer continuum.

Previous studies have shown that increased heparanase expression is correlated to tumor aggressiveness and metastatic potential of many human tumors, including bladder [16], colon [9], pancreas [10], and breast [17]. Only a few studies investigated heparanase’s expression in premalignant lesions and, more specifically, its role in the hyperplasia–dysplasia sequence. Similar to our results, increased levels of heparanase mRNA were found in prostate cancer tissues compared with benign prostate hyperplasia (BPH) samples [18]. In colon cancer, heparanase expression gradually increased along the normal to the mild and severe dysplasia to the malignant stages [9]. One earlier study postulated a possible role for heparanase in mouse mammary tumorigenesis through induction of generalized mammary hyperplasia [19]. Heparanase mRNA and protein expression were increased progressively in sequential premalignant lesions in a mouse model of multistage pancreatic islet carcinogenesis [20]. In contrast, Stadlmann et al. [21] found that heparanase expression was decreased in malignant prostatic tissue compared with BPH tissue. Using the same anti-heparanase polyclonal antibody used by us in order to study heparanase expression in colorectal cancer, Doviner et al. [22] observed heparanase upregulation in the progression from normal epithelia to moderate dysplasia, although expression was almost nonexistent in carcinomatous tissue. They suggested that heparanase may play different roles throughout the carcinogenesis process and thus may have a preferential expression pattern during that process.

Previous studies have shown a progression paradigm describing the natural history of endometrial hyperplasia in a limited number of patients [3]. Complementary molecular research has indicated a sequential progression model of genetic mutations involving PTEN and Kras mutations early in the process, i.e., simple hyperplasia, with β-catenin mutations and microsatellite instability presenting at a later stage, atypical hyperplasia, and finally E-cadherin alteration with p53 mutations appearing in already neoplastic tissue [23]. Considering that even the most neoplastically prone lesion (i.e., atypical complex
hyperplasia) will progress to frank carcinoma in only 29% of cases, there remains the question as to which lesions will indeed undergo malignant transformation. Our results suggest a potential novel marker to address this issue.

Classic diagnostic gyneco-pathology relies on subjective assessment of architectural and cytologic alterations to stratify patients into high risk and low risk groups according to the WHO 1994 classification system for endometrial hyperplasia. There is, however, considerable lack of reproducibility when using that classification, particularly in the diagnosis of cytologic atypia and, as a consequence, this has presumably led to overtreatment in terms of performing hysterectomies. As a result, an alternative classification system, endometrial intraepithelial neoplasia (EIN), was introduced in 2000 and is based on computerized analysis of morphometric features that reflect glandular volume, architectural complexity, and cytologic (nuclear) abnormality. Recent studies suggest that it has higher accuracy in the detection of premalignant lesions [24]. EIN, however, requires selection of representative areas of focal lesions at the risk of overlooking early lesions that have not yet transformed morphologically but have already undergone pre-neoplastic stages. Accordingly, we propose that heparanase expression levels could be used as a novel diagnostic tool, complementary to EIN, in risk stratification of endometrial hyperplasia.

Since the putative precursor lesion of papillary serous carcinoma (SPC) is endometrial intraepithelial carcinoma (EIC), which has been known to develop in atrophic or resting endometrium [4], we tried to assess the expression pattern of heparanase in atrophic endometrium and serous papillary carcinoma. Our results suggest that heparanase expression is upregulated in both atrophic endometrium and SPC, thus supporting a poor prognosis in SPC and possibly implying that atrophic endometrium is, by definition, more prone to carcinogenesis. In contrast, a recent study by Kodama et al. [12] showed heparanase expression to be absent. The differences between our study and theirs might be accounted for by the small number of samples tested by both groups.

ER expression had been previously shown to be decreased in high-grade endometrial tumors compared with low-grade tumors [25]. Elkin et al. [13] suggested that estrogen may induce heparanase overexpression in breast cancer via stimulation of transcriptional activity of the heparanase promoter. Our results, however, did not show ER expression to be correlated with heparanase expression and thus they do not support this potential crosstalk between estrogen and heparanase in endometrial cancer. Estrogen mediates its biological effects in target tissues by binding to specific intracellular receptors, estrogen receptor-α (ERα) and ERβ. Although the uterus is thought to predominantly express ERα, increased cell

Figure 4  Immunohistochemical staining of heparanase, estrogen receptor (ER), and progesterone (PR) (rows 2–4 respectively) in a panel of endometrial cancer tissue samples (magnification ×20). G1 endometrial carcinoma (A); G2 endometrial carcinoma (B, C); Papillary serous endometrial cancer (D). H&E staining for tissue definition is shown for samples as outlined above (first row) (magnification ×10). Note the weak heparanase staining in the G1 specimen compared with the intense staining pattern in the G2 and papillary cancer specimens.
proliferation and exaggerated response to estrogen in ER $\beta$ knockout mice indicates that ER $\beta$ might modulate ER $\alpha$ function in the uterus and have an antiproliferative function. Therefore, an imbalance in ER $\alpha$ and ER $\beta$ expression could be a crucial step in estrogen-dependent tumorigenesis. Tamoxifen is the most prescribed antineoplastic drug worldwide, and it is used to treat patients with all stages of hormone-responsive breast cancer. Despite having a robust anti-estrogenic function in the breast, tamoxifen shows only partial estrogenic effects in other target tissues. These partial estrogenic actions have beneficial effects on bones and the cardiovascular system in postmenopausal women, but they are also associated with an increased incidence of cancer in the endometrium. This may explain the different correlation of heparanase and ER expressions between the breast and endometrium tissues.

Atypical hyperplasia and endometrial cancer are usually treated with hysterectomy, however 2%–14% of these patients are under 40 years of age and wish to maintain fertility. These women are currently treated with progestin therapy and have good response rates in atypical complex hyperplasia (83%–94%) and moderate response rates in endometrial cancer (57%–75%). Nevertheless, there is a 13% recurrence rate in atypical hyperplasia and an up to 50% recurrence rate in endometrial cancer patients [26], indicating that additional therapeutic modalities should be considered. Consequently, heparanase inhibition therapy with agents such as PI-88 might serve as future adjunct therapy for endometrial cancer and hyperplasia.

In summary, the results of the present study show heparanase expression to be tightly regulated in endometrial tumorigenesis and indicate that heparanase may prove to be a novel marker and prognostic factor in the management of patients with endometrial hyperplasia and cancer. Future studies may demonstrate a therapeutic role for heparanase using targeted biologic therapy.

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