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ORIGINAL ARTICLE

Serum and tissue angiogenin in patients with endometrial hyperplasia

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Abstract *Objective:* To evaluate angiogenin levels in both tissue and serum of patients with endometrial hyperplasia with and without atypia.
Methods: Sixty women were classified according to the histopathological findings of endometrium into three groups. The control group consisted of 20 women with normal non-hyper plastic endometrium. The second group included 20 women diagnosed as complex endometrial hyperplasia without atypia. The third group included 20 women diagnosed as complex endometrial hyperplasia with atypia. Serum and tissue angiogenin were measured by enzyme immunoassay (EIA) technique and confirmed in tissues with Western Blotting (WB) technique.
Results: There was a statistically significant increase in serum and tissue angiogenin levels of endometrial hyperplasia groups compared to those of control group ($P < 0.001$). Serum and tissue angiogenin levels were with a statistically significant higher ($P < 0.001$) in group III compared to group II. The sensitivity of serum angiogenin to detect the potential possibility of endometrial hyperplasia with atypia in endometrial hyperplasia patients was 100%, specificity 85%, positive predictive value 86.9%, negative predictive value 100%, positive likelihood ratio 6.6%, negative likelihood ratio 0% and accuracy 91.7%.
Conclusion: Elevated levels of serum angiogenin in endometrial hyperplasia could assist in determining which patients are at high risk for atypical change requiring aggressive treatment.
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1. Introduction

Endometrial hyperplasia (EH) is a non-invasive proliferation of the lining of the uterus that results in a spectrum of glandular alteration (1). EH is classified primarily as either simple or complex, based on the degree of architectural complexity as seen by glandular crowding (with back-to-back crowding in the case of complex hyperplasia), and with or without cytological atypia; that is, nuclear irregularity, such as the loss of axial

polarity; rounded, stratified nuclei and prominent nucleoli (2). The wide range of histomorphological presentation of endometrial hyperplasia is accompanied by high intra and inter observer variability in diagnostic classification (3). Previous studies have shown that only 10–20% of EH progress to carcinoma when left untreated (4).

The lack of criteria that could accurately predict the disease outcome may have been an important cause of over and under treatment and hence the need of establishment of a new classification composed of three groups: endometrial hyperplasia (EH), endometrial intraepithelial neoplasm (EIN) and endometrial carcinoma (3). EIN is defined as neoplastic focal lesion with cytological features of crowded gland architecture and a volume percentage less than 55%, with a minimum size of 1 mm and careful exclusion of mimics (5,6). It's important to characterize high or low risk groups before initiation of therapy, because about 1–28% of hyperplasia progress to carcinoma, depending on the degree of severity (7).

Angiogenesis, the process of new blood vessel growth, plays an essential role in normal physiological processes, such as development and reproduction. However, pathological angiogenesis occurs in many angiogenesis dependent diseases such as tumours and other non-neoplastic diseases (8). Angiogenin (ANG) is the first human tumour cell-derived protein with *in vivo* angiogenic activity. ANG, a heparin binding 14.1 kDa single chain polypeptide, was initially isolated from supernatants of colon cancer cells and was found to be a member of the pancreatic ribonuclease superfamily (9).

Endometrial adrenomedullin, microvessel density and area of venules, which are tissue markers of angiogenesis, increase in a stepwise manner from normal, simple or complex hyperplasia with or without atypia to grade I adenocarcinoma (10).

Treatment of EH depends on the patient's age, fertility desire and the type of hyperplasia. Progestagens are still the most commonly used medical treatment modality in these patients. Response rates are higher for cases without atypia. In selected cases, hysterectomy may be performed as a definitive treatment modality (11).

The aim of the present study was to measure ANG levels in both tissue and serum of patients with endometrial hyperplasia with or without atypia.

2. Materials and methods

2.1. Patients

This case control study was performed on sera and endometrial biopsies from 60 women attending the University Hospital in Benha between January 2006 and January 2009.The study was approved by the local institutional review board and all women gave informed consent before enrollment in this study. They were classified according to the histopathological findings of the endometrium into three groups. The control group consisted of 20 women with normal non-hyperplastic endometrium. They were selected from perimenopausal women attending the out patient clinic for gynecological consultation. The second group included 20 women who complained of perimenopausal bleeding and diagnosed as complex endometrial hyperplasia without atypia. The third group included 20 women who complained of perimenopausal bleeding and diagnosed as complex endometrial hyperplasia with atypia. None of the women had received preoperative hormonal therapy. Patients with EH underwent total abdominal hysterectomy.

Five milliliters of venous blood samples were collected before any treatment and preoperatively for all women and one week postoperatively in hyperplasia groups. The endometrial specimens had been obtained by dilatation and curettage. Blood clots, debris and muscle tissue were rapidly dissected from the endometrial tissue. One part of the endometrial specimen was sent for pathologic examination and the other part was washed with ice-cold saline, immediately frozen and stored at −70 °C until analysis. After stripping away blood clots, debris and muscle tissue, the samples were thawed on ice, placed in 10 volume of ice-cold cell lysis buffer (pH 7.8, containing 100 mmol/l K_2 HPO_4–KH_2PO_4, 1 mmol/l DTT, 2 mmol/l EDTA, 1% Triton X100, and 0.75 μg/ml leupeptin) and homogenized. The cell lysate was centrifuged and the supernatant recovered and stored at −70° until analysis. The total protein in the prepared supernatant was measured by Bradford method using bovine serum albumin as a calibrator.

2.2. Determination of angiogenin by EIA

Quantitative determination of human angiogenin concentration in serum and tissues were done by a solid phase EIA (Quantikine, R&D System, USA). The assay employs the quantitative sandwich enzyme immunoassay technique (12).

2.3. Determination of ANG by Western Blotting (WB) technique

Twelve percentage of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to separate 20 μg sample protein of tissue supernatant. The gels were transferred to nitrocellulose (NC) filters in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 7.4) for 1 h at 60 V. NC sheets were washed and the unoccupied binding sites were saturated with blocking solution (Chromogenic Western Blotting kit, Biorad-Roche Diagnostics, GmbH, Germany) for 1 h at 37 °C. The sheets were then incubated with 0.1 mg/ml of either antihuman angiogenin monoclonal antibody (MOAB) (R&D Systems, USA) or normal mouse IgG serum (negative control) overnight at 4 °C. The membranes were washed with Tris buffer saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5). The antibodies that bound to the NC membrane were visualized by incubation with anti-mouse IgG-alkaline phosphatase conjugate for 90 min at room temperature (RT). Finally, the filters were incubated with alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.1 M Tris buffer) at RT until the developed bands were of desired intensity. By comparing the resulting developed NC with others in which normal mouse IgG serum was substituted for ANG MOAB, the ANG band was identified.

2.4. Statistical analysis

Anova test was used for statistical comparison of age and parity among the study groups. The non-parametric Kruskal–Wallis, and Mann–Whitney U rank sum tests were used for the statistical comparison of the ANG median value in the study groups. Spearman's rank correlations test was used to correlate serum and tissue ANG. Paired t test was used for the statistical comparison of the serum ANG values before

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