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Inhibin/activin-betaC Subunit in Human Endometrial Adenocarcinomas and HEC-1a Adenocarcinoma Cell Line

STEPHANIE D. KÄUFL[1], JOSEF MAKOVITZKY[2], CHRISTINA KUHN[1], SUSANNE KUNZE[1], UDO JESCHKE[1] and IOANNIS MYLONAS[1]

[1]*First Department of Obstetrics and Gynaecology, Ludwig Maximilians University Munich, Munich 80337, Germany;*
[2]*Department of Neuropathology, University of Heidelberg, Heidelberg, Germany*

Abstract. *Introduction: Inhibins and activins are important regulators of the female reproductive system. Recently, two novel inhibin subunits, named betaC (βC) and betaE (βE), have been identified. However, only limited data on the expression of the βC subunit in human endometrioid adenocarcinomas exist. Materials and Methods: Samples of uterine endometrioid adenocarcinomas were obtained and analysed by immunohistochemistry for the immunolabelling with an inhibin-βC antibody. Additionally, the endometrial cancer cell line HEC-1a was used to assess the inhibin-betaC expression with the use of immunofluorescence. Results: Expression of the inhibin-βC subunit was demonstrated at the protein level by means of immunohistochemical evaluation in human endometrioid adenocarcinomas and the HEC-1a cell line. Discussion: This study demonstrated, for the first time, that the novel inhibin/activin-βC subunit is expressed in human endometrioid adenocarcinomas and in the human endometrial carcinoma cell line HEC-1a. Whether this novel β-subunit has a substantial role in the pathogenesis and malignant transformation in human endometrium is still under investigation.*

Inhibin/activin proteins were primarily isolated from the gonads and identified as members of the transforming growth factor-beta (TGF-β) family (1-3). Recently, two additional β-subunits have been identified in humans to the already well-characterized inhibin-βA and -βB subunits (1-3). These novel β-subunits were named βC (4) and βE (5), sharing a 82% and 61% amino acid sequence similarity of the mature peptides from rat and mouse, respectively (6, 7).

The inhibin/activin βC protein was primarily found to be in human liver and prostate (8). Additionally, this subunit was identified in normal (9) and pathological placental tissue (10, 11). Moreover, a recent study has demonstrated an immunohistochemical staining reaction of the novel inhibin-βC subunit in normal human endometrium and in the human endometrial carcinoma Ishikawa cell line (12).

However, only limited data on the expression of the inhibin-βC subunit in human endometrial cancer tissue exist. The expression of inhibin-β subunits in endometrial cancer has become of importance, since activin signalling may be a promising target for therapeutic interventions (13). Therefore, the aim of this preliminary study was to evaluate the synthesis of the novel inhibin-βC subunit in human endometrial cancer tissues and in the human endometrial cancer cell line HEC-1a.

Materials and Methods

Tissue samples. Samples of human malignant endometrioid adenocarcinomas were obtained from the pathological archive of the First Department of Obstetrics and Gynecology of the Ludwig Maximilians University Munich. Endometrium samples were classified according to histological differentiation into well-differentiated (n=5), moderately differentiated (n=5) and poorly differentiated (n=5) endometrioid adenocarcinomas.

Immunohistochemistry. Immunohistochemistry was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex by using the goat-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) as previously described (9-11, 14, 15) with slight modifications. Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min and rehydrated in 100% of ethanol twice. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. After washing, slides were subjected to antigen retrieval for 5 min in a pressure cooker using sodium citrate buffer (pH 6.0), containing 0.1 M citric acid and 0.1 M sodium citrate in distilled

Correspondence to: Ioannis Mylonas, MD, Ph.D., Ludwig Maximilians University Munich, First Department of Obstetrics and Gynecology, Maistrasse 11, 80337 Munich, Germany. Tel: +49 8951604111, Fax: +49 8951604916, e-mail: ioannis.mylonas@med.uni-muenchen.de

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water. After cooling to room temperature, sections were washed twice in phosphate-buffered saline (PBS). Non-specific binding was blocked by incubating the sections with Ultra-V-Block (Lab Vision, Fremont, CA, USA) for 45 min at room temperature. Sections were then incubated at 4˚C overnight with the inhibin-βC polyclonal goat antibody (R&D Systems, Wiesbaden, Germany) at a dilution of 1:50 in Ultra-V-Block (Lab Vision). After washing with PBS, sections were incubated with biotinylated secondary anti-rabbit antibody (provided by Vector Laboratories) for 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (diluted in 10 ml PBS; Vector Laboratories) for 30 min and repeated washing steps with PBS, visualisation was performed with ABC substrate buffer (Vectastain Elite ABC kit; Vector Laboratories) and chromogen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) at 1 mg/ml concentration for 2 min. Sections were then counterstained with Mayer's acidic haematoxylin and dehydrated in an ascending series of alcohol (50-98%). After xylol treatment, sections were mounted. Negative controls were performed by replacing the primary antibody with normal goat IgG or rabbit IgG as isotype control in the same dilution compared to the primary antibody, respectively. Immunohistochemical staining was performed using human liver tissue as an appropriate positive control. Positive cells showed a brownish color and negative controls as well as unstained cells were stained blue. Sections were examined using a Leitz (Wetzlar, Germany) photomicroscope. Digital images were obtained with a digital camera system (JVC, Yokohama, Japan) and were saved on computer (Diskus software, Hilgers, Königswinter, Germany).

Cells and cell culture. The endometrial adenocarcinoma cell line HEC-1a (obtained from ATCC [LGC Promochem GmbH, Wesel, Germany]) has been established as a well-differentiated human endometrial adenocarcinoma cell line and is one of the most used endometrial cell lines to study the pathophysiology of endometrial cancer. Cells were grown in Quantum 263 medium (PAA, Pasching, Austria) supplemented with antibiotics at 37˚C in a humidified atmosphere with 5% CO_2 as previously described (9, 12, 16).

Immunofluorescence analysis. Cells grown on glass cover slips were fixed with acetone for 10min at room temperature and washed twice with PBS. Non-specific binding was blocked by incubating the sections with Ultra-V-Block (Lab Vision) for 15 min at room temperature as previously described (9, 12, 17). Thereafter, slides were incubated with inhibin-βC antibody (1:50 in dilution medium provided by DAKO) overnight at 4˚C, followed by a 1:500 diluted Cy3-conjugated donkey-anti-goat antibody (Dianova, Hamburg, Germany) for inhibin-βC as previously described (9, 12). The slides were finally embedded in mounting buffer containing 4,6-diamino-2-phenylindole (DAPI) resulting in blue staining of the nuclei. Slides were embedded with Vectashield mounting medium (Axxora, Lörrach, Germany) and examined with an Axiophot photomicroscope (Zeiss, Jena, Germany). Digital images were obtained with a digital camera system (Axiocam, Zeiss, Jena, Germany) and saved on a computer with the microscope software AxioVision (version 4.7., Zeiss, Jena, Germany).

Results

The specificity of the βC-subunit antibody was tested using appropriate positive controls including normal human liver specimens, confirming previous positive results (9, 10, 12, 18, 19). In endometrioid adenocarcinomas, inhibin-βC was primarily detected in malignant endometrial glandular epithelial cells, while immunostaining reaction in the stromal compartment was weaker (Figure 1).

The endometrial adenocarcinoma cell line HEC-1a is a malignant cell line derived from invasive endometrial adenocarcinoma. A positive staining reaction was observed in the analysed endometrial cancer cell line HEC-1a for inhibin-βC, being primarily localised in the cytoplasma (Figure 2).

Discussion

Inhibins and activins were initially characterised as endocrine and paracrine hormonal regulators of the hypothalamic-pituitary-gonadal axis. Meanwhile it is clear that they are expressed in a wide range of female reproductive tissues, including normal and pathological human endometrium (14, 15, 20-24). This preliminary study demonstrated an immunohistochemical staining reaction of the novel inhibin/activin-βC subunits in human endometrioid adenocarcinomas and in the human endometrial carcinoma cell line HEC-1a by immunohisto-chemical detection methods.

The inhibin/activin βC subunit was demonstrated in hepatocytes (4, 7), normal (9) and pathological placental tissue (10, 11), human endometrium (12), prostate, ovary, testes and pituary (8, 25). Additionally, inhibin-βC has a putative implication in endometrial pathogenesis and carcinogenesis as observed for human testicular, liver and prostate cancers (26). This preliminary study demonstrated, for the first time, the immunolabelling of this novel β-subunit in human endometrioid adenocarcinoma and in the HEC-1a endometrial cell line.

Although the precise role of this subunit is not yet elucidated, several possible functions have been suggested, including apoptosis (27, 28) and increasing the rate of DNA synthesis in primary rat hepatocytes (29). Additionally, this βC-subunit may function as an antagonist of activin function (8, 30), therefore regulating indirectly several well-known anti-proliferative functions of activin A (βA-βA) (31-36). Whether these functions are also applicable in normal or malignant endometrial tissue still remains to be clarified.

Interestingly, TGF-β has been recognised as a tumour suppressor in premalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in later stages of the disease (37). Furthermore, the inhibin-α subunit was an independent prognostic parameter in a large cohort analysis of human endometrial carcinomas (14), suggesting a putative tumour suppressive function in human endometrial cancer, as suggested in knock-out mouse models (38, 39). Whether this is also true for this novel subunit is still under investigation. However, the knowledge of the β-subunit expression patterns has become of tremendous importance, since activin signalling may be a promising molecular therapeutic for a variety of diseases, including malignant tumours (13).

In conclusion, this study demonstrated an immunohisto-chemical staining reaction of the novel inhibin-βC subunit in

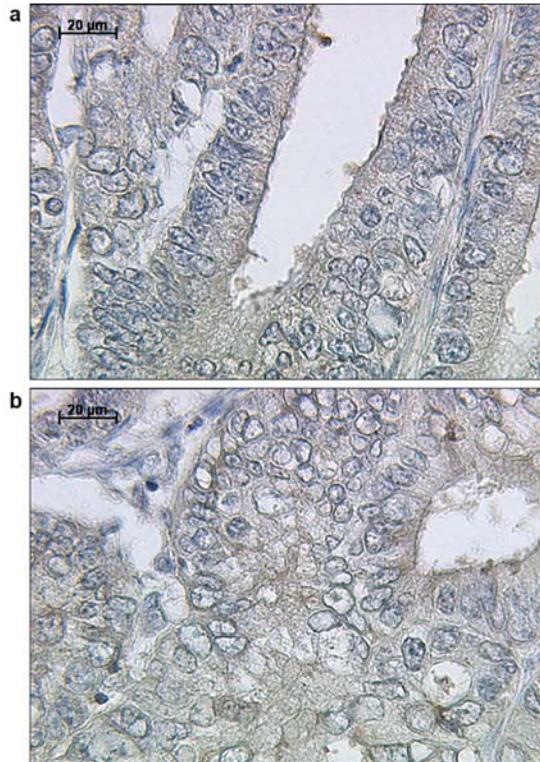

Figure 1. *Immunohistochemical staining reaction of inhibin-βC in human endometrial adenocarcinomas. Positive immunohistochemical staining reaction against inhibin-βC was demonstrated in well-differentiated (a) and poorly differentiated human endometrioid adenocarcinomas (b). Magnification factor: ×40.*

endometrioid adenocarcinomas and in the well-characterized endometrial carcinoma cell line HEC-1a by using immunohistochemical detection methods. Therefore, the inhibin-βC subunit may be substantially involved in the pathogenesis and malignant transformation in human endometrium. Moreover, since the precise function of this novel subunit in human endometrial carcinogenesis is not clear yet, the HEC-1a cell line can be used for further analysis regarding its pathophysiology in uterine cancers. Whether this novel subunit is a prognostic parameter in human endometrial cancer is still under investigation.

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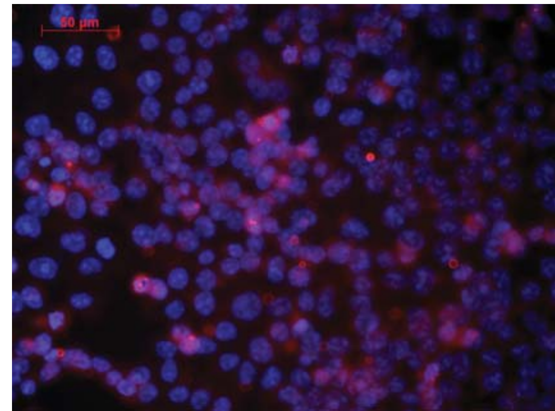

Figure 2. *Localisation of inhibin-βC expression in endometrial HEC-1a cancer cell line. The endometrial adenocarcinoma cell line Hec-1a was analysed by immunofluorescence for the expression of inhibin-βC, showing a positive cytoplasmic staining reaction for this subunit. Magnification factor: ×40.*

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