Expression of Inhibins in the Endometrial Carcinoma Cell Line RL-95-2 after Stimulation with Cortisol and Estradiol

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Abstract. Inhibins (INH) are dimeric glycoproteins composed of an alpha-subunit (INH-α) and one of two possible beta-subunits (INH-βA or -βB), with substantial roles in human reproduction and in endocrine-responsive tumours. The aim of the present study was the determination of the frequency and tissue distribution patterns of the inhibin/activin subunits in endometrial carcinoma cells of the cell line RL-95-2 after stimulation with estradiol and cortisol compared to unstimulated controls. Materials and Methods: Cells of the endometrial carcinoma cell line RL-95-2 were grown on quadrperm tissue slides and incubated with different concentrations (0.1 and 0.01 μmol/ml) of estradiol or cortisol. Expression of INH-α, βA and βB was analysed by immunocytochemistry with specific monoclonal antibodies directed against the inhibin subunits. Results: Expression of INH-α and -βB was higher in cortisol-stimulated RL-95-2 cells, whereas INH-βA expression was lower. In contrast to these, INH-βB expression was increased by estradiol while INH-α and -βA were unchanged under estradiol treatment. Conclusion: Expression of INH-subunits in RL-95-2 cells was described. Cortisol and estradiol showed an influence on INH expression. The RL-95-2 cell line could act as a useful model for the investigation of INH regulation, particularly for endometrial cancer.

Inhibins and activins are homologous to each other, sharing common β-subunits that have a nine-cysteine distribution pattern similar to the transforming growth factor-beta (TGF-β) family of proteins (1, 2). Inhibins, in contrast to activins, consist of an α-subunit and one of two possible β-subunits (βA or βB). The α-subunit can dimerize with either βA or βB to form inhibin A (α-βA) or B (α-βB), respectively. Activins are homodimers of β-subunits linked by a disulphide bond. Depending on the combination of the subunits, there are three isoforms of activin, namely activin A (βA-βA), activin B (βB-βB) and activin AB (βA-βB) (1, 2). Endometrial cancer is the most frequent gynaecological genital malignancy in the western world, with an increasing incidence in industrial nations, occurring in pre- and postmenopausal women (3). The traditional prognostic factors are histological type, tumour grade and metastatic infiltration. Although endogenous and exogenous sources of unopposed estrogen increase the risk of endometrial adenocarcinoma, and several molecular alterations have been recently identified, the molecular pathogenesis of endometrial carcinoma remains quite vague (3). However, the currently used diagnostic technology is insufficient to identify endometrial cancer patients with a poor prognosis. Inhibin has been demonstrated in normal human endometrium (4-6) as well as in endometrial hyperplasia and carcinoma, (6-9) although the precise role of inhibin/activin in the human endometrium remains unknown. Therefore, immunohistochemistry/cytochemistry of different specific markers like inhibins might be an interesting alternative to allow selection of high risk patients (10).

We recently demonstrated a higher immunohistochemical expression of the inhibin-α, -βA and -βB subunits in hyperplastic endometrial tissue compared to adenocarcinoma, suggesting an involvement of these subunits in endometrial pathogenesis (6, 8, 9). Furthermore, endometrial carcinoma cells of the cell line RL-95-2, derived from a Grade 2 moderately differentiated adenosquamous carcinoma of the endometrium (11), could act as a model to investigate the expression of inhibins. In a former study, we have found a significant correlation between inhibin with estradiol and cortisol in proliferative and early secretory glands of the endometrium and a significant correlation between inhibin, estradiol and cortisol in late secretory glands of the endometrium (4). Therefore, the aims of the present study were: a) the determination of the frequency and tissue distribution patterns of the inhibin-α, -βA and -βB subunits in RL-95-2 cells; and b) the assessment of these subunits under the influence of estradiol and cortisol in RL-95-2 cells.

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Materials and Methods

Cells. RL-95-2 cells were obtained from ATCC (ATCC® Number: CRL-1671™, LGC Promochem GmbH, Wesel, Germany) and grown in Dulbecco’s modified Eagle’s medium containing 10% foetal calf serum gold. This medium did not contain measurable amounts of cortisol or estradiol. Cells were grown on quadriperm cell culture slides as monolayer cell cultures.

Stimulation of RL-95-2 cells with estradiol and cortisol. RL-95-2 cells (1x10^5 cells/ml) were grown on quadriperm cell culture test slides for up to 72 h. Estradiol or cortisol were added to the cell cultures giving a final concentration of 0.01 or 0.1 μmol/ml and cultivated for an additional 48 h. Unstimulated cells were used as controls.

Immunocytochemistry. For the immunocytochemical characterization of INH subunits in endometrial tumour cells of the cell line RL-95-2, the Vectastain® Elite ABC Kit (Vector Laboratories, Burlingame, USA) was used for visualisation. Mouse monoclonal antibodies used for the experiments (Serotec, Oxford, UK) were: mouse anti-human INH-α, mouse anti-human INH-βA and mouse anti-human INH-βB.

For immunocytochemical evaluation the slides were air dried, washed in PBS, fixed in 4.0% formalin for 15 min, washed again in PBS for 5 min and incubated for 30 min in 0.3% H_2O_2. The slides were washed in distilled water and incubated with normal serum (ABC-Kit) for 30 min, followed by incubation with the described primary antibodies for one hour, and after washing, with diluted biotinylated secondary antibody for another 30 min. After incubation with the ABC Reagent for 30 min, H_2O_2/3,3’diaminobenzidine (Dako, Hamburg, Germany) was added for 2 min until brown staining of positive cells was observed. The slides were counterstained in hematoxylin. After xylol treatment, the slides were covered. Positively stained cells showed a brownish colour.

Evaluation and statistical analysis. The intensity and distribution patterns of specific inhibin-α, -βA and -β subunit immunocytochemical staining were evaluated using the semi-quantitative IRS score as described elsewhere (12, 13). The IRS score was calculated by multiplication of optical staining intensity (graded as 0= no, 1=weak, 2=moderate and 3=strong staining) and the percentage of positively-stained cells (0=no staining, 1=<10% of the cells, 2=11-50% of the cells, 3=51-80% of the cells and 4=>81% of the cells). The slides were examined by two independent observers, including a gynaecological pathologist (N.S.). Sections were examined using a Leitz (Wetzlar, Germany) photomicroscope. Digital images were obtained with a 3CCD colour camera (JVC, Victor Company of Japan, Japan) and were saved on computer. The results were evaluated using the non-parametric Mann-Whitney U rank-sum test (SPSS, Chigaco, IL, USA). Significance was assumed at p<0.05.

Results

Expression of INH subunits in RL-95-2 cells. In RL-95-2 endometrial carcinoma cells, a very faint expression of the INH-α subunit (Figure 1a) was found. In contrast, moderate expression of both the INH-βA (Figure 1b) and INH-βB (Figure 1c) subunits was apparent.

Stimulation of RL-95-2 cells with estradiol. After stimulation with different concentrations of estradiol (0.01-0.1 μmol/ml), no difference was found in the expression of the INH-α and INH-βA subunits, (Figure 2a) but strong up-regulation of the
INH-βB subunit (Figure 2b) in comparison to unstimulated controls was very apparent.

Stimulation of RL-95-2 cells with cortisol. After stimulation with different concentrations of cortisol (0.01-0.1 µmol/ml), up-regulation of both the INH-α (Figure 3a) and INH-βB (Figure 3b) subunits was found, compared to the unstimulated control. The expression of the INH-βA subunit (Figure 3c) was down-regulated by cortisol. Although differences in cortisol-stimulated cells and controls are not significant, we see a trend for the down-regulation of INH-βA (p=0.066). A summary of the stimulation experiments is shown in Figure 4.

Discussion

In this study, the expression of INH-subunits in RL-95-2 cells was described. Expression of the INH-α and -βB was increased in cortisol-stimulated RL-95-2 cells, whereas INH-βA expression was reduced in cortisol-stimulated RL-95-2 cells.
A positive correlation had already been shown between inhibin and cortisol, suggesting a link between these substances in human endometrial glandular cells which has not been demonstrated before in the human endometrium (4). Interestingly, a circadian rhythm of circulating inhibin correlating with that of cortisol has been shown in males (14). Munro et al. (15) suggested also a link between inhibin and cortisol production by demonstrating centripetal extension of immunoreactivity for inhibin-α in ACTH-stimulated hyperplastic renal cortex in Cushing’s disease. However, secretion of inhibin-like immunoreactive material and expression of inhibin-α mRNA can be regulated in vitro by ACTH in human adrenal glands (16). Because precise relationship and function between inhibin and cortisol in the human endometrium remain unknown, we used RL-95-2 cells as a model. In contrast to cortisol, INH-βB expression was increased by estradiol but INH-α and -βA expressions were unchanged. A positive correlation between inhibin and estradiol was shown in all menstrual phases, demonstrating an interdependence of these substances (4). Inhibin and estradiol seem to be the negative controls of FSH secretion that disappear at the time of luteal regression (17). However, little information about the correlation of inhibin and estradiol in the human endometrium exists. In the ovary, inhibin might have a paracrine action, although its effect and role in estradiol production is still controversial (18). Treatment with inhibin augments the LH-stimulated androgen production by human thecal cells in vitro (19). Interestingly, immunoneutralisation of endogenous inhibin in rat ovarian follicle culture results in a significant decrease in estradiol secretion (20). Whether a similar relationship exists between estradiol and inhibin in the human endometrium is still unclear.

Therefore we used RL-95-2 cells to investigate the relationship between estradiol and inhibins.

While inhibins/activins were initially characterised as endocrine and paracrine hormonal regulators of the hypothalamic-pituitary-gonadal axis it is now evident that they are expressed in a wide range of tissues including the human endometrium (21), cultured human endometrial cells (4, 5, 7), hyperplastic and malignant human endometrial tissue and cells (6, 8, 9, 22). In a former study, we demonstrated a significantly higher inhibin-α expression in the secretory than in proliferative endometrial tissue, confirming previous immunohistochemical (4, 21) and in vitro results (4, 5). Additionally, it was shown for the first time that inhibin-α is expressed in malignant endometrial tissues of different gradings. Although endometrioid adenocarcinomas showed positive immunohistochemical reaction with the inhibin-α subunit, their expression intensity was significant compared to normal human endometrial tissue, confirming previous results (8). Additionally, since inhibin-α has not been detected in poorly differentiated endometrioid adenocarcinomas (G3), it might be a tumour suppressor with crucial functions in endometrial cancer development.

Recently, we showed a lower inhibin-α expression in well-differentiated adenocarcinoma than in normal and hyperplastic endometrial tissue (8). We therefore speculate that the lower inhibin-α expression is used to bind with inhibin-βA subunit forming inhibin A, which probably has a substantial role in endometrial malignant transformation. However, it might be possible that an overproduction of tumoral inhibin-βA might lead to the formation of activin A, which is been secreted at a higher rate into circulation, as previously detected (7). This seems to be confirmed by the fact that in poorly-differentiated (G3) endometrioid cells.
adenoacarcinomas no inhibin-α subunit was detected, suggesting a production of activins rather than inhibins. Additionally the expressions of inhibin-β subunits were higher in G3 endometrial adenoacarcinomas compared to G1 tumours. Interestingly, the inhibin-βB subunit was higher in G3 adenocarcinomas compared to G2, suggesting a substantial function in malignant transformation (23).

In summary, an association between inhibin/activin-subunits and steroidogenesis has already been suggested (24). In normal human cultured endometrial glandular cells we showed a relationship between inhibin expression and estradiol or cortisol (4). However, the relationship of steroid hormones and inhibin expression in endometrial carcinoma cells has never been investigated. Within this study, we showed that the expression of INH-α was up-regulated under cortisol treatment. The expression of INH-βB was up-regulated under both estradiol and cortisol treatment, whereas INH-βA was down-regulated by cortisol.

References