

# Expression of the inhibin/activin subunits ( $\alpha$ , $\beta$ A and $\beta$ B) in normal and carcinogenic endometrial tissue: Possible immunohistochemical differentiation markers

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**Abstract.** Inhibins (INH) are dimeric glycoproteins, composed of an  $\alpha$ -subunit (INH- $\alpha$ ) and one of two possible  $\beta$ -subunits (INH- $\beta$ A or  $\beta$ B), with substantial roles in human reproduction and in endocrine-responsive tumors. The aims of this study were to determine the frequency and tissue distribution of INH- $\alpha$ ,  $\beta$ A and  $\beta$ B in normal and malignant endometria. Samples were obtained from normal (n=46), atrophic (n=8) and endometrioid carcinoma tissue (EC; G1=93; G2=32; G3=14). INH- $\alpha$  was significantly higher in normal compared to malignant endometrial tissue, showing a cyclical variation throughout the menstrual cycle. EC G3 did not express this subunit. INH- $\beta$ A and  $\beta$ B showed specific staining reactions within the tumor cells. The highest intensity of INH- $\beta$ A was observed in the normal secretory phase compared to adenocarcinomas ( $p<0.05$ ). For INH- $\beta$ B, the significantly highest expression was noted in EC G3 compared to EC G2 ( $p<0.05$ ) and atrophic endometrial tissue. In conclusion, INH- $\alpha$ ,  $\beta$ A and  $\beta$ B were immunolabeled in normal and malignant endometria. INH- $\alpha$  was expressed in a declining relationship in the transition from normal to tumor tissue, suggesting a tumor suppressive function in EC. A high expression of INH- $\beta$ B was observed in EC G3 compared to G2, suggesting an important role in the progression of endometrial carcinogenesis. However, the utilization of these subunits as specific tumor markers still remains unclear.

## Introduction

Endometrial cancer is the most frequent gynecological genital malignancy in the western world, with an increasing incidence in industrial nations, occurring in pre- and postmenopausal women (1). The traditional prognostic factors are the histological type, tumor grade and the depth of myometrial invasion. 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 unclear (1). However, the currently used diagnostic technology is insufficient in identifying endometrial cancer patients with a poor prognosis. Therefore, immunohistochemistry of different specific markers could be a better alternative for selecting high-risk patients (2).

Inhibins and activins are homologous with each other, sharing common  $\beta$ -subunits that have a nine-cysteine distribution pattern similar to the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of proteins (3,4). Inhibins, in contrast to activins, consist of an  $\alpha$ -subunit and one of two possible  $\beta$ -subunits ( $\beta$ A or  $\beta$ B). The  $\alpha$ -subunit can dimerize with either  $\beta$ A or  $\beta$ B to form inhibin A ( $\alpha$ - $\beta$ A) or B ( $\alpha$ - $\beta$ B), respectively. Activins are homodimers of  $\beta$ -subunits linked by a disulphide bond. Depending on the combination of the subunits, there are three isoforms of activin, namely activin A ( $\beta$ A- $\beta$ A), activin B ( $\beta$ B- $\beta$ B) and activin AB ( $\beta$ A- $\beta$ B) (3,4).

Inhibin has been demonstrated in normal human endometria (5-7) as well as in endometrial hyperplasia and carcinoma (7-13), although the precise role of inhibin/activin in human endometria still remains unknown. Meanwhile TGF- $\beta$  is recognized as a tumor suppressor in the pre-malignant stages of carcinogenesis with an additional dual role as a pro-oncogene in the later stages of disease leading to metastasis (14). The tumor suppressor activity of the inhibin- $\alpha$  subunit was first identified after the functional deletion of the inhibin- $\alpha$  gene in male and female mice that resulted in primary gonadal sex cord-stromal tumors (15). We recently demonstrated a higher immunohistochemical expression of the inhibin- $\alpha$ ,  $\beta$ A and  $\beta$ B subunits in hyper-

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plastic endometrial tissue compared to adenocarcinoma, suggesting an involvement of these subunits in endometrial pathogenesis (9-11). Furthermore, the knowledge of the expression pattern of the inhibin- $\alpha$  subunit in human endometrial tissue can be important, since the preferred secretion form of inhibin or activin is determined by this subunit.

Therefore, the aims of the present study were i) the determination of the frequency and tissue distribution patterns of the inhibin- $\alpha$ , - $\beta$ B and - $\beta$ B subunits in normal and malignant endometria, and ii) the assessment of these subunits as immunohistochemical markers for malignant endometrial adenocarcinoma.

## Materials and methods

**Tissue samples.** Samples of human endometrial tissue were acquired from 193 patients. Normal human endometria were obtained from premenopausal, non-pregnant patients undergoing gynecological surgery either by D&C or hysterectomy for benign diseases, mainly uterine leiomyomata. All women had a normal and regular menstrual cycle with no hormonal treatment for 3 months prior to surgery. All hyperplastic endometrial samples were excluded from this study. The endometrial samples were classified according to anamnestic and histological dating into the proliferative (day 1-14,  $n=27$ ), early secretory (day 15-22,  $n=10$ ) and late secretory phase (day 23-28,  $n=9$ ) as previously described (10,16,17). Additionally, 8 samples of atrophic human endometrial tissue from postmenopausal patients with no hormonal substitute who underwent hysterectomy or D&C for benign diseases, mainly urogenital prolaps, were analyzed. Samples of malignant endometrial tissues were obtained from the archival material from 139 women who had undergone hysterectomy or curettage at the 1st Department of Obstetrics and Gynecology, Ludwig-Maximilians-University Munich between 1990 and 1995. Patients with endometrioid adenocarcinoma received a modified radical hysterectomy, salpingo-oophorectomy or selective pelvic lymphadenectomy, with or without para-aortic lymphadenectomy. The endometrioid adenocarcinomas were graded according to the system of the World Health Organization as well-differentiated (G1;  $n=93$ ), moderately differentiated (G2;  $n=32$ ) and poorly differentiated (G3;  $n=14$ ).

**Immunohistochemistry.** Immunohistochemistry with inhibin/activin-subunits was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex with the use of the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) as previously described (16,18). The standardization, dilution and optimization of this protocol was primarily tested on normal premenopausal ovary tissue. Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min, rehydrated in an ascending series of alcohol (70%, 96% and 100%), and subjected to antigen retrieval on a high setting for 10 min in a pressure cooker in a sodium citrate buffer (pH 6.0), containing 0.1 M citrate acid and 0.1 M sodium citrate in distilled water. After

cooling, the slides were washed twice in PBS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with diluted normal serum (10 ml PBS containing 150  $\mu$ l horse serum; provided by Vector Laboratories) for 20 min at room temperature. The sections were then incubated at room temperature for 120 min with the primary antibodies (inhibin- $\alpha$ , clone R1, monoclonal mouse IgG2a, diluted in PBS 1:50; inhibin- $\beta$ A, E4, mouse IgG2b, diluted in PBS 1:50; inhibin- $\beta$ B, C5, mouse IgG2a, diluted in PBS 1:10; all three antibodies were obtained from Serotec, Oxford, UK). After washing with PBS, the slides were incubated in diluted biotinylated serum (10 ml PBS containing 50  $\mu$ l horse serum; provided by Vector Laboratories) for another 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex (reagent ABC, provided by Vector Laboratories) for another 30 min and repeated washing steps with PBS, visualization was performed with substrate and chromagen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8-10 min. The slides were counterstained with Mayer's acidic hematoxylin and washed in an ascending series of alcohol (50-98%). After xylol treatment the slides were covered. Negative controls were performed by replacing the primary antibody with normal mouse serum in the same dilution used for inhibin detection as previously described (16). The positive cells showed a brownish color and the negative controls as well as the unstained cells appeared blue.

**Immunohistochemical evaluation and statistical analysis.** The intensity and distribution patterns of the specific inhibin/activin-subunit immunohistochemical cytoplasmatic staining reactions were evaluated by two independent observers, including a gynecological pathologist (N.S.), using a semi-quantitative score as previously described (10). Briefly, the staining intensity score was graded as 0 = no staining; 1 = weak staining; 2 = moderate staining and 3 = strong staining. Digital images were obtained with a digital camera system (Olympus, Tokyo, Japan) and were saved on computer. The Mann-Whitney rank-sum test was used to compare the means of the staining intensity (SPSS; Chicago, IL, USA). A  $p$ -value of  $p \leq 0.05$  was considered significant.

## Results

The inhibin- $\alpha$ , - $\beta$ A and - $\beta$ B subunits were detected in normal human endometria, predominantly localized in the glandular and luminal epithelium, thus confirming previous results (10,11,16). Inhibin- $\alpha$  showed no or minimal expression during the proliferative phase (Fig. 1a), which increased with the progress of the menstrual cycle (Fig. 1b and c). Endometrioid adenocarcinomas were positive for inhibin- $\alpha$ , although with a lower immunohistochemical intensity than normal human endometria (Fig. 1d and e), while poorly differentiated carcinomas G3 did not demonstrate any inhibin- $\alpha$  immunoreaction (Fig. 1f). Normal human endometria also expressed the inhibin- $\beta$ A subunit (Fig. 2a-c), with the staining intensity being strongest in the late secretory phase (Fig. 2c).



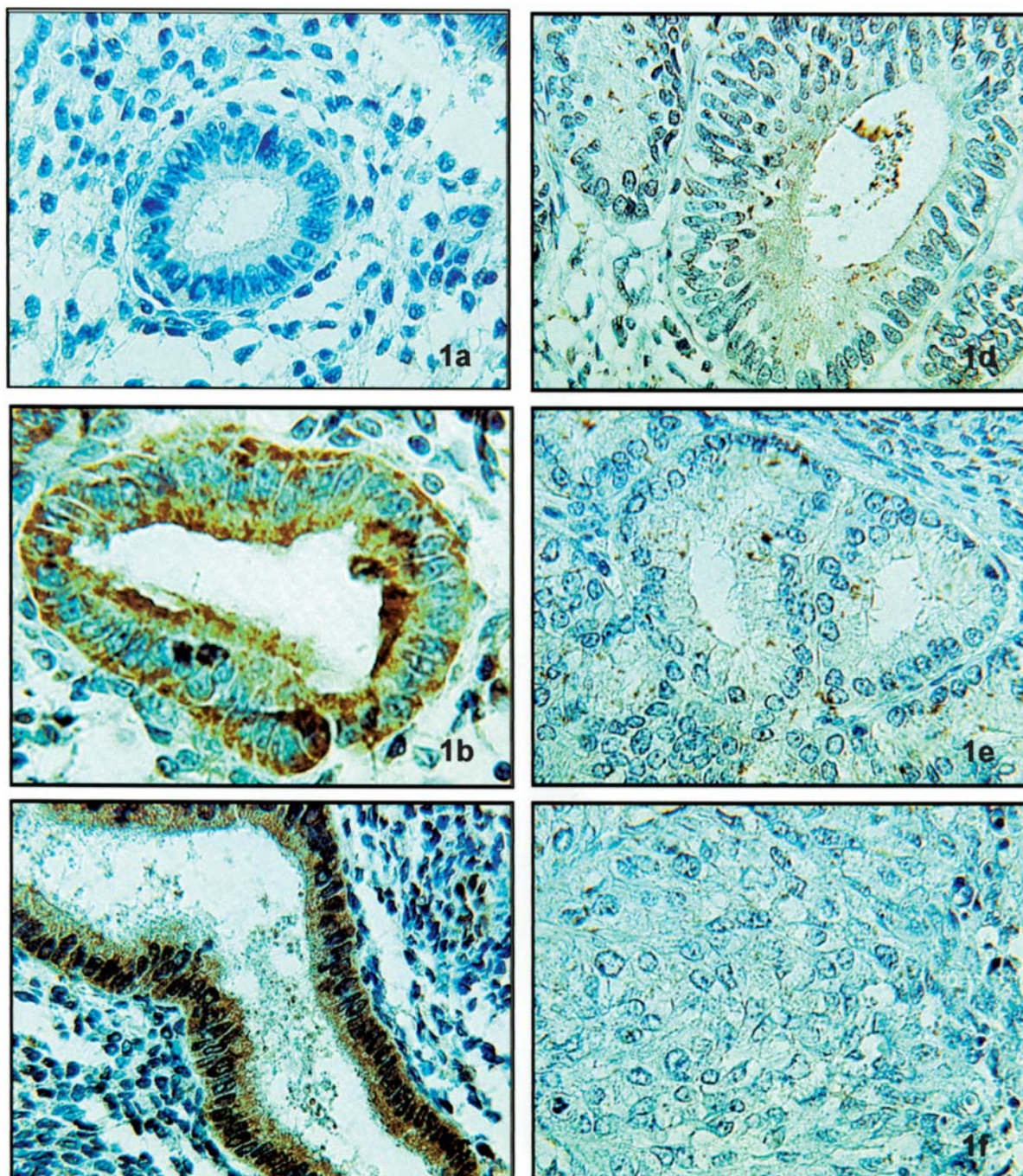


Figure 1. Expression of inhibin- $\alpha$  in normal and malignant human endometrial tissue. Inhibin- $\alpha$  showed no or minimal expression during the proliferative phase (a), which increased with the progress of the menstrual cycle (b-c). Endometrioid adenocarcinomas were positive for inhibin- $\alpha$ , although with a lower immunohistochemical intensity than normal human endometria (d-e), while G3 poorly differentiated carcinomas did not demonstrate any inhibin- $\alpha$  immunoreaction (f). Magnification x250.

Endometrioid adenocarcinomas were positive for inhibin- $\beta$ A (Fig. 2d-f). The inhibin- $\beta$ B subunit was also expressed in normal and malignant endometrial tissue (Fig. 3a-f), whereas a stronger intensity was observed between endometrioid adenocarcinomas G2 and G3 (Fig. 3e and f).

The immunohistochemical expression of inhibin- $\alpha$  was significantly higher during the early secretory phase ( $p < 0.05$ ) and the late secretory phase ( $p \leq 0.001$ ) compared to proliferative endometria (Fig. 4a). G3 endometrioid adenocarcinomas did not demonstrate any inhibin- $\alpha$  expression. A continuous decrease was noted from G1 and G2 endo-

metrioid adenocarcinomas to G3 adenocarcinomas ( $p < 0.05$ , respectively) (Fig. 4a). Atrophic endometrial tissue demonstrated a significant decline compared to the early secretory ( $p < 0.05$ ) and the late secretory phase ( $p < 0.01$ ) ( $p = 0.56$ ).

The immunohistochemical expression of inhibin- $\beta$ A was significantly higher during the late secretory phase compared to the proliferative and early secretory phase ( $p < 0.005$ , respectively). Inhibin- $\beta$ A expression was the highest in the late secretory endometrium compared to endometrioid adenocarcinomas (Fig. 4b). Inhibin- $\beta$ B was also expressed in human endometrial tissue (Fig. 4c). The immunohistochemical



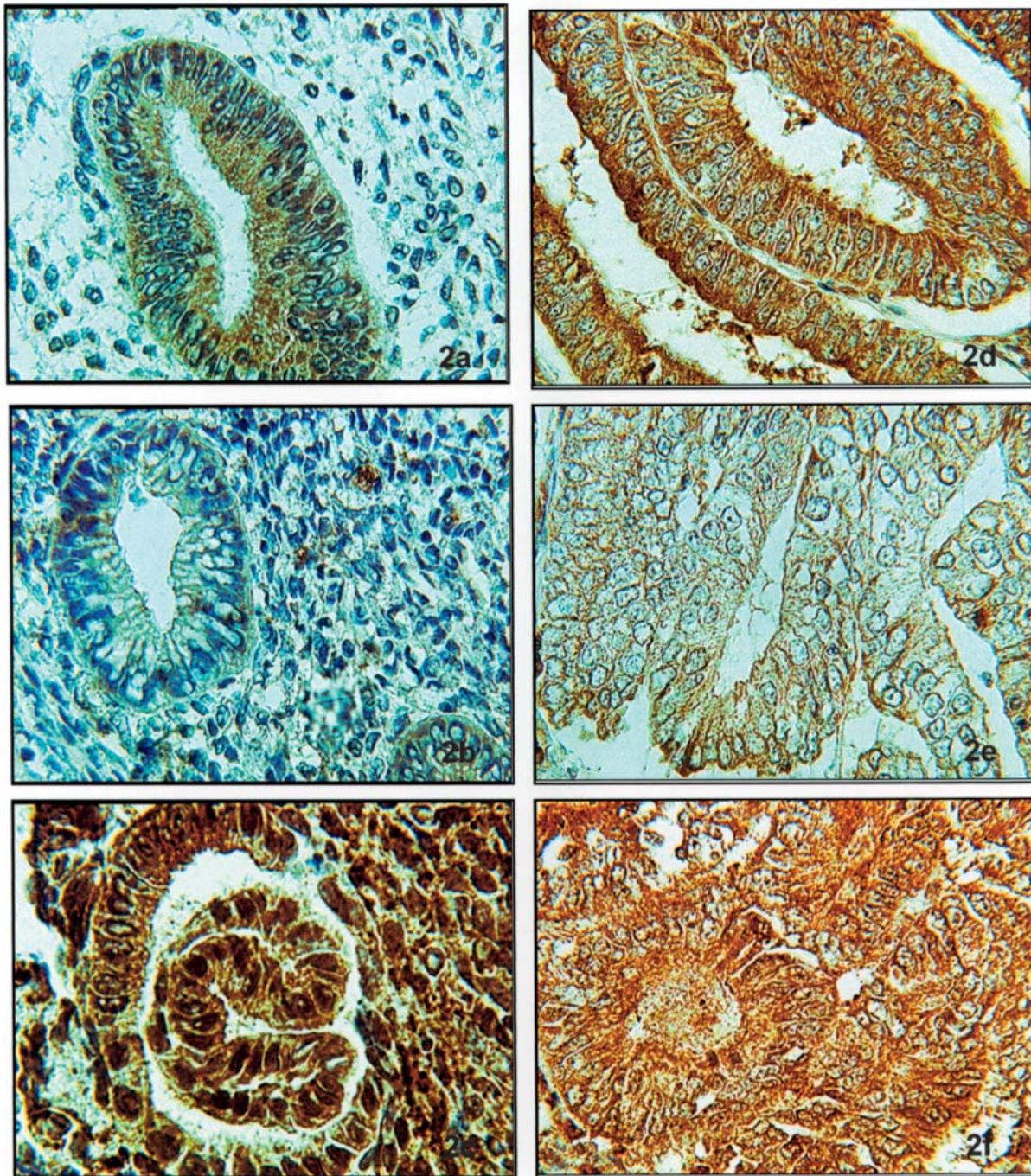


Figure 2. Expression of inhibin- $\beta$ A in normal and malignant human endometrial tissue. Normal human endometria also expressed the inhibin- $\beta$ A subunit (a-c), with the staining intensity being strongest in the late secretory phase (c). Endometrioid adenocarcinomas were positive for inhibin- $\beta$ A (d-f). Magnification x250.

expression of inhibin- $\beta$ B was higher during the late secretory phase compared to the proliferative and early secretory phase, however without statistical significance. An increase in the immunohistochemical staining reaction was observed from G2 to G3 adenocarcinomas ( $p < 0.05$ ). Additionally, atrophic endometrial tissue showed a significantly lower expression of inhibin- $\beta$ B than the late secretory phase ( $p < 0.05$ ) (Fig. 4c).

## Discussion

While inhibins/activins were initially characterized 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 breast cancer (18,19), the placenta (20-22), human endometria (5,7,13,16), cultured human endometrial cells (6,12,23), hyperplastic and malignant human endometrial tissue (7-11,13). We demonstrated a significantly higher inhibin- $\alpha$  expression in the secretory compared to the proliferative endometrial tissue, confirming previous immunohistochemical (6,16) and *in vitro* results (6,23). Additionally, it was shown for the first time that inhibin- $\alpha$  is expressed in malignant endometrial tissues of different grading. Although endometrioid adenocarcinomas showed a positive immunohistochemical reaction



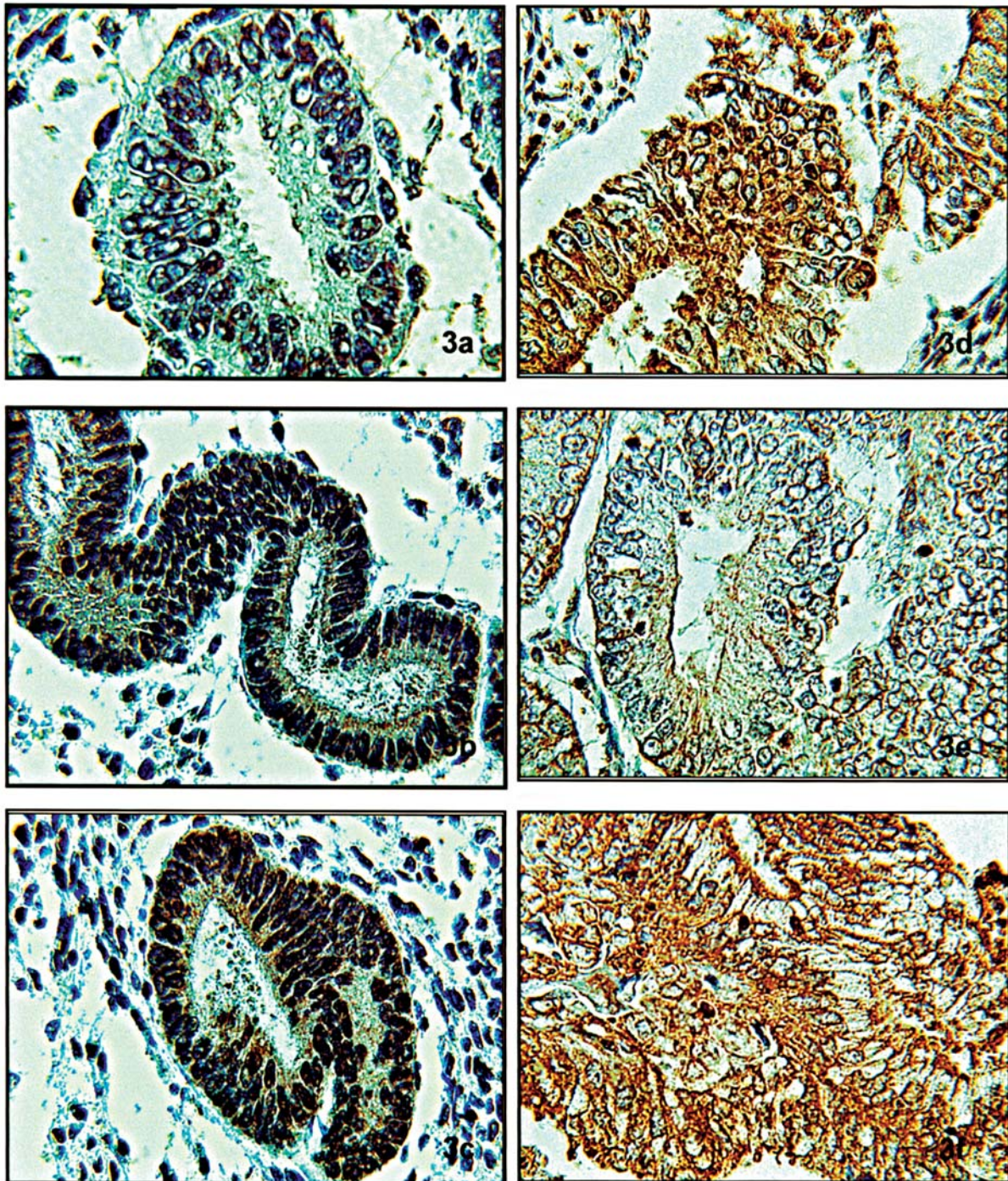


Figure 3. Expression of inhibin- $\beta$ B in normal and malignant human endometrial tissue. The inhibin- $\beta$ B subunit was also expressed in normal and malignant endometrial tissue (a-f), whereas a stronger intensity was observed between G2 and G3 endometrioid adenocarcinomas (e and f). Magnification  $\times 250$ .

with the inhibin- $\alpha$  subunit, their expression intensity was significantly lower compared to normal human endometrial tissue, thus confirming previous results (10,13). Additionally, since inhibin- $\alpha$  has not been detected in poorly differentiated endometrioid adenocarcinomas (G3), it might be a tumor suppressor with crucial functions in endometrioid adenocarcinoma development. Recently, we showed a lower inhibin- $\alpha$  expression in well-differentiated adenocarcinomas than in normal and hyperplastic endometrial tissue (10). We therefore speculate that the lower amount of inhibin- $\alpha$  expression is used to bind with the inhibin- $\beta$ A subunit

forming inhibin A, which could have a substantial role in endometrial malignant transformation. However, it might be possible that an overproduction of tumoral inhibin- $\beta$ A could lead to the formation of activin A, which is secreted in higher amounts into the circulation, as previously detected (12). This is confirmed by the fact that in poorly differentiated (G3) endometrioid adenocarcinomas no inhibin- $\alpha$  subunit was detected, suggesting a production of activins rather than inhibins. Additionally, the expression inhibin- $\beta$  subunits were higher in G3 endometrial adenocarcinomas compared to G1 tumors. The inhibin- $\beta$ B subunit was higher in G3



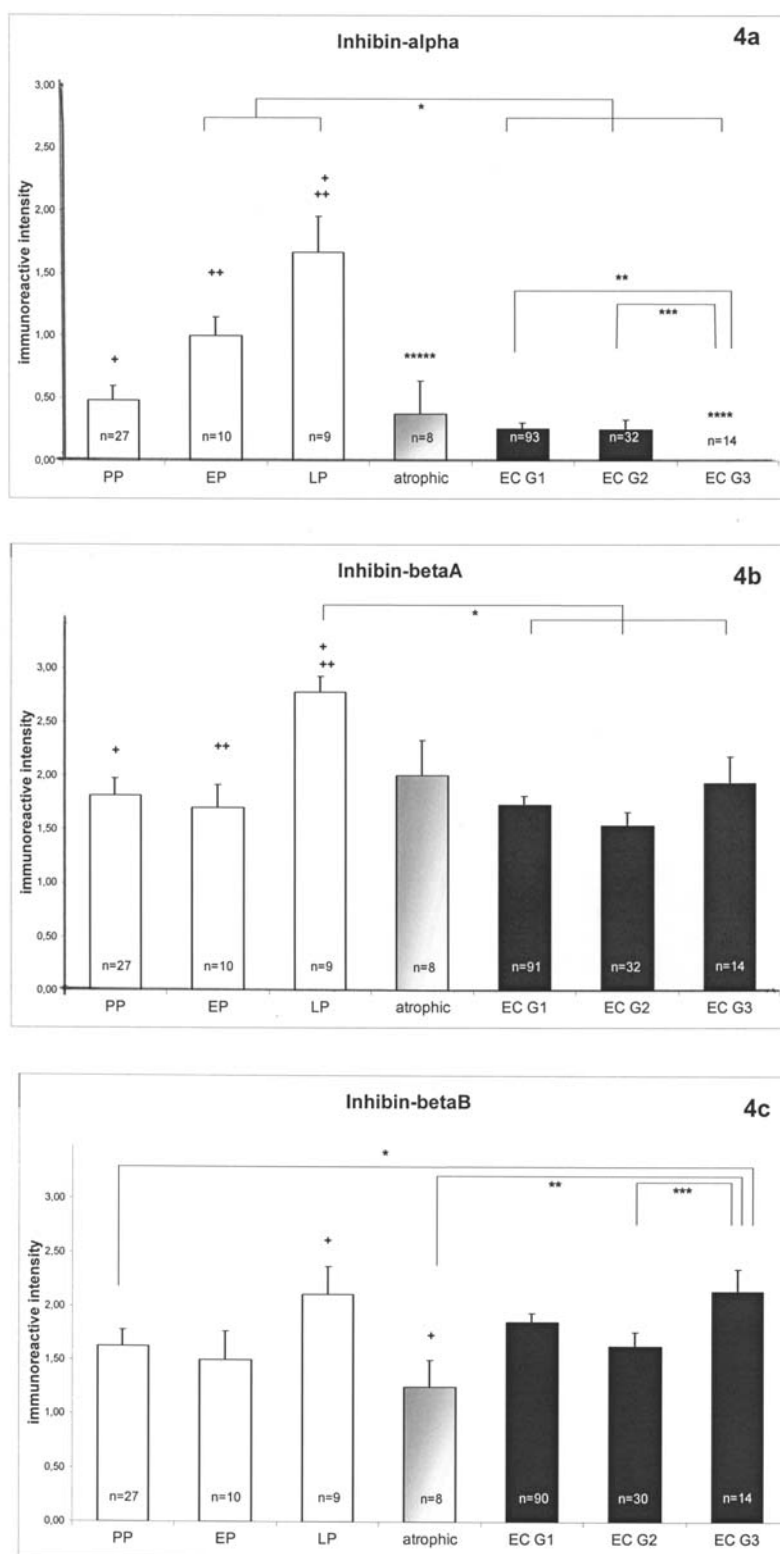


Figure 4. Inhibin- $\alpha$ , - $\beta$ A and - $\beta$ B in human endometrial tissue. (a) Inhibin- $\alpha$  in human endometrial tissue. The immunohistochemical expression of inhibin- $\alpha$  was significantly higher during EP ( $*p<0.05$ ) and LS ( $^{**}p\leq 0.001$ ) compared to PP. EC G3 did not demonstrate any inhibin- $\alpha$  expression, and was therefore significantly lower compared to all the other analyzed groups ( $^{****}$ ). A continuous decrease was noted from EC G1 to EC G3 ( $^{**}p<0.05$ ) and EC G2 to EC G3 ( $^{***}p<0.05$ ). Atrophic endometrial tissue ( $^{****}$ ) demonstrated a significant decline compared to ES ( $p<0.05$ ) and LS ( $p<0.01$ ), being higher than EC G3, although with no statistical significance ( $p=0.56$ ). (b) Inhibin- $\beta$ A in human endometrial tissue. The immunohistochemical expression of inhibin- $\beta$ A was significantly higher during LS compared to PP ( $*p<0.005$ ) and ES ( $^{**}p<0.005$ ). Inhibin- $\beta$ A expression was the highest in LS endometria, being significantly higher compared to endometrial carcinoma ( $p<0.001$  for EC G1 and EC G2;  $*p<0.05$  for EC G3). Although an increase of the immunohistochemical staining reaction was observed from EC G1 to EC G3, no statistical significance was noted. (c) Inhibin- $\beta$ B in human endometrial tissue. The immunohistochemical expression of inhibin- $\beta$ B was higher during LS compared to PP and ES, however without statistical significance. The inhibin- $\beta$ B staining reaction was significantly lower in PP ( $*p<0.05$ ) and atrophic endometrial tissue ( $^{**}p<0.05$ ) compared to EC G3. An increase in the immunohistochemical staining reaction was observed from EC G2 to EC G3 ( $^{***}p<0.05$ ). Additionally atrophic endometrial tissue showed a significantly lower expression of inhibin- $\beta$ B than LS ( $*p<0.05$ ). Values represent means  $\pm$  SEM. Significance is shown with a p-value of  $p<0.05$  (asterisks). PP, proliferative phase; ES, early secretory phase; LS, late secretory phase; EC G1, endometrioid adenocarcinoma grade 1; EC G2, endometrioid adenocarcinoma grade 2; EC G3, endometrioid adenocarcinoma grade III.

compared to G2 adenocarcinomas, suggesting a substantial function in malignant transformation.

Several autocrine and paracrine actions of inhibins/activins have been reported, although their precise role in human endometria still remains unclear. The expression of inhibin was also demonstrated in malignant endometria, suggesting a possible role for inhibin in endometrial proliferation and growth (5,13). However, the most important function of inhibin- $\alpha$  might be its action as a possible tumor suppressor gene, according to results from transgenic mouse models. The inhibin- $\alpha$  subunit gene is thought to be a tumor suppressor associated with ovarian cancer in a mouse model (15,24,25). However, with the loss of inhibin- $\alpha$  subunit production in mice, a marked increase of serum FSH was observed with elevated activin production in the ovary. The inhibin- $\alpha$ -deficient mice showed cachectic symptoms that were associated with the compensatory excessive secretion of activin (24). We observed no immunohistochemical expression of the inhibin- $\alpha$  subunit in G3 adenocarcinomas with a rise of the inhibin- $\beta$  subunits, suggesting a compensatory activin production in human endometrial cancer cells. Since inhibins and activins belong to the TGF- $\beta$  superfamily, it has been suggested that inhibin- $\alpha$  is a tumor-suppressive agent in benign and early-stage primary cancers, being silenced in tumor progression and reactivated as a pro-oncogenic factor in advanced and aggressive cancers (14). It still remains to be elucidated whether this is also true for endometrial adenocarcinomas. Additionally, it might be possible that the inhibin- $\alpha$  subunit is expressed and/or up-regulated in more malignant histological endometrial carcinomas (i.e. clear cell carcinomas). However, this also needs to be clarified.

Since activin A has the ability to enhance proliferation in certain cancer cell lines (26), a role for activin A in endometrial tumorigenesis has been suggested (8,27). It has also been indicated that the increased activin A production in human endometrial adenocarcinoma cells *in vitro* may not stimulate carcinoma cell proliferation or inhibit apoptotic signaling in carcinoma cells, suggesting that the insensitivity to the usual growth suppression signals induced by activin A could instead be a mechanism of the immortality of human endometrial adenocarcinoma cells (28). However, activin can inhibit angiogenesis, due to its function as a growth inhibitor of vascular endothelial cells (29). Therefore, the expression of the inhibin- $\beta$  subunits during carcinogenesis could play an important role in endometrial angiogenesis. Activin A has also been found at significantly higher concentrations in the culture media from cell lines, uterine washings and serum from patients with endometrial carcinoma, suggesting a secretion of activin A into the extracellular fluid and into the circulation (12). Since activin A can modulate cell growth, proliferation and apoptosis (30), a growth inhibitory role for activin A in pre-malignant transformation could be suggested. We demonstrated a differential expression of the  $\beta$ -subunits in malignant compared to normal endometrial tissue. The inhibin- $\beta$ B subunit was also upregulated in G3 compared to G2 carcinomas suggesting an important involvement of this subunit in endometrial carcinogenesis.

In conclusion, we showed the expression of the inhibin- $\alpha$ , - $\beta$ A and - $\beta$ B subunits in normal and malignant endometrial tissue. A continuous decrease in inhibin- $\alpha$  immunostaining

between G1 to G3 endometrial carcinomas was observed suggesting that this glycoprotein could be a possible parameter for assessing malignant transformation and could also act as a tumor suppressor gene in endometrioid adenocarcinomas. In uncertain and difficult pathological diagnoses, the inhibin/activin subunits could be a useful marker for further evaluation. Therefore, inhibin/activin immunostaining could be used to select subpopulations of patients with an increased risk of developing poorly differentiated endometrial cancer. Additionally, the intense expression of inhibin- $\beta$  subunits in poorly differentiated endometrial adenocarcinomas compared to G1 and G2 tumors suggests the utilization of these subunits as potential tumor markers. However, the prognostic significance of the inhibin/activin-subunits still remains unknown. It is still under investigation whether inhibin/activin can be used as specific markers in endometrial pathogenesis.

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