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Inhibin-betaA and -betaB subunits in normal and malignant glandular epithelium of uterine cervix and HeLa cervical cancer cell line

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Abstract
Introduction Inhibins, dimeric peptide hormones composed of an alpha-subunit and one of two possible beta-subunits (betaA or betaB), exhibit substantial roles in human reproduction and in endocrine-responsive tumors. However, it is still unclear if normal and cancerous cervical glandular epithelial cells as well as cervical cancer cell lines of glandular origin express the inhibin-betaA and -betaB subunits.
Materials and methods Normal cervical tissue samples and a total of 10 specimens of well-differentiated adenocarcinomas of the human cervix were analyzed for inhibin-betaA and -betaB subunit expression by immunohistochemical analysis. Additionally, the cervical carcinoma cell line HeLa was analyzed by immunofluorescence and RT-PCR analysis for the expression of inhibin subunits.
Results Immunolabeling of normal and malignant glandular epithelium of human cervical tissue revealed a positive staining reaction for the inhibin-betaA and -betaB subunits. Additionally, the cancer cell line HeLa synthesized both inhibin subunits. When compared to the normal cervical glandular epithelium, the expression of the inhibin beta subunits became significantly reduced in cervical adenocarcinoma tissues.
Discussion In conclusion, we demonstrated a strong, though differential expression pattern of inhibin-betaA and -betaB subunits in normal and malignant glandular epithelial cells of the human uterine cervix. Although the physiological role of inhibins is still quite unclear in cervical tissue, the expression of inhibin-beta-subunits might play an important role in cervical cancer carcinogenesis, since they are significantly down-regulated during pathogenesis in cervical adenocarcinomas.

Keywords Cervical cancer, adenocarcinoma · Cervical cancer cell line · Hela · Immunohistochemistry, immunofluorescence, RT-PCR, inhibin-betaA · Inhibin-betaB

Introduction

Cervical cancer is the second most common malignant disease among women worldwide, with the highest incidence occurring in developing countries [1, 2]. Approximately 80% of cervical cancers arise from squamous cells, while 15% are adenocarcinomas and 5% clear cell adenocarcinomas [2, 3]. Although cervical adenocarcinomas are thought to have a worse prognosis, there are no data showing they should be managed differently [2–5]. Several risk factors for the development of cervical cancer are recognized, including HPV infection [6–9]. However, cervical adenocarcinomas are believed to be different from the more common squamous cancer, with respect to the mechanism of carcinogenesis employed [3]. Additionally,

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no tumor markers are currently available for cervical adenocarcinoma [2, 10, 11].

Inhibins and activins are secreted polypeptides, representing a subgroup of the transforming growth factor-beta (TGF-β) superfamily of growth and differentiation factors [12, 13]. Inhibins are heterodimers that consist of an α-subunit and one of two possible β-subunits (βA or βB), resulting in the formation of either inhibin A (α-βA) or B (α-βB), respectively. Activins, on the other hand, are homodimers of β-subunits linked by a disulfide bond [12, 13]. Furthermore, two additional β-subunits have been identified in humans, βC and βE [13]. Although these novel subunits are synthesized in a wide range of normal and malignant tissues [14–18], their precise function still remains unclear.

Inhibin/activin-subunits have been detected in normal female tissue and endocrine tumors [19], including normal and pathological endometrial and placental tissue [20–30], suggesting that they have roles in cancer proliferation and growth [19, 31]. The βA-subunit was observed in adenosquamous tissue of endometrial carcinomas [32], while inhibin A, inhibin B and activin A were detected in normal and neoplastic human uterine tissues, including cervical cancer [27].

However, it is unclear whether, and if so to what extent, cervical epithelial cells also express these subunits. We recently demonstrated the expression of the novel βE-subunit in cervical cancer and cervical cancer cell lines, suggesting a substantial function in cervical pathogenesis [14]. Recently, both inhibin β-subunits demonstrated a differential expression in cervical intraepithelial neoplasia (CIN) and squamous cancer, suggesting important roles in cervical carcinogenesis [33]. Inhibin βA might be important during the progression of cervical intraepithelial neoplasia, while the inhibin βB-subunit could exert a substantial function during differentiation of cervical carcinomas [33].

The putative expression of inhibin βA- and βB-subunits in cervical cancer is of extreme importance, since activin signaling might be a promising target for therapeutic intervention [34]. Therefore, the aim of this study was to analyze the expression of the βA- and βB-subunits in normal and pathological glandular epithelial cells of the human uterine cervix, as well as the cervical carcinoma cell line HeLa, derived from a patient with cervical glandular carcinoma.

Materials and methods

Tissue samples

Four normal and 10 well-differentiated adenocarcinomas of the uterine cervix of a previous described group of patients were analyzed in this preliminary study [14, 35]. Samples of normal human uterine cervical tissue were obtained from 4 premenopausal, non-pregnant patients undergoing hysterectomy for uterine leiomyomata as previously described [14, 35]. Additionally, 10 specimens of well-differentiated (G1) adenocarcinoma of the cervix were obtained from the pathological archives of the 1st Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University Munich. This group is well-characterized group and has been previously used to assess inhibin-βC and -βE expression [14, 35].

Immunohistochemistry

Immunohistochemistry was performed using a combination of pressure cooker heating and the standard streptavidin–biotin–peroxidase complex using the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) as previously described [28, 29, 33] 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 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, USA) for 45 min at room temperature. Sections were then incubated at 4°C overnight with the inhibin-βA mouse antibody (mouse IgG2b, clone E4, Serotec, Oxford, UK), at a dilution of 1:50 in Ultra-V-Block (Lab Vision, Fremont, USA), or inhibin-βB mouse antibody (mouse IgG2a, clone C5, Serotec, Oxford, UK), at a dilution of 1:70 in Ultra-V-Block (Lab Vision, Fremont, USA). After washing with PBS, sections were incubated with biotinylated secondary anti-mouse 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, visualization was performed with ABC substrate buffer (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and chromagen 3,3′-diaminobenzidine (DAB; Dako, Glostrup, Denmark) at 1 mg/ml concentration for 4 min. Sections were then counterstained with Mayer's acidic hematoxylin 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 rabbit IgG as isotype control in the same dilution compared to the primary antibody, respectively. Immunohistochemical staining was performed using an appropriate positive control comprising ovaries containing follicular cysts [28]. Sections were examined using a Leica (Solms, Germany) photomicroscope and saved on computer. Positive cells showed a brownish color and negative controls as well as unstained cells were blue.

The intensity and distribution patterns of specific inhibin/activin-subunit immunohistochemical cytoplasmatic staining reaction was evaluated by two blinded, independent observers, including a gynaecological pathologist (N.S.), using a semi-quantitative score as previously described and used to asses the expression pattern of inhibin/activin-subunits [21, 22, 28, 29, 33]. 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 positive 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).

Cells and cell culture

The cervical cancer cell line HeLa (ATCC CCL2) is an ATCC-available cell line (ATCC-LGC Promochem GmbH, Wesel, Germany). HeLa cell line is an immortalized cell line derived from glandular cervical cancer. Cells were cultured in Quantum 263 medium (PAA, Pasching, Austria) supplemented with antibiotics at 37°C in a humidified atmosphere with 5% CO_2 as previously described [14, 33, 35, 36].

Immunofluorescence analysis

Cells grown on glass coverslips were fixed with acetone for 10 min at room temperature and washed twice with PBS. Non-specific binding was blocked by incubating the sections with Ultra-V-Block (Lab Vision, Fremont, USA) for 15 min at room temperature as previously described [14, 15, 33, 35]. Thereafter, slides were incubated with inhibin-βA antibody (1:100 in dilution medium provided by DAKO, Glostrup, Denmark) or inhibin-βB antibody (1:10 in dilution medium provided by DAKO, Glostrup, Denmark) overnight at 4°C, followed by a 1:500 diluted Cy3-conjugated goat-anti-mouse antibody (Dianova, Hamburg, Germany) for 30 min in room temperature. 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 a Zeiss (Jena, Germany) Axiophot photomicroscope. 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).

RT-PCR analysis

RNA was extracted from cells using the Nucleospin RNA II kit (Macherey–Nagel, Düren, Germany) as previously described [33]. Reverse transcription was performed with M-MLV reverse transcriptase and oligo-dT (Promega, Mannheim, Germany) as recommended by the supplier. PCR was performed in an Eppendorf Mastercycler with GoTaq (Promega, Mannheim, Germany). Primer sequences for inhibin-βA to amplify a 282 bp fragment were in 5′–3′ orientation: TGCCCTTGCTTTGGCTGAGA (forward primer) and ACTTTGCCCACATGAAGCTTT (backward primer) as previously described [33]. Additionally, primer sequences for inhibin-βB (333 bp) were GGCGAGCGG CGACTCAACCTAGA (forward primer) and CGTGTG GAAGGAGGAGGCAGAGC (backward primer) as previously described [33]. β-actin primers were from Stratagene (The Netherlands). PCR cycling was performed after a 5 min initiation at 94°C with 32 cycles of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C, followed by a 5 min extension at 72°C as previously described [33].

Statistical analysis

The IRS-scores of inhibin-βA and -βB immunohistochemical expression levels were compared using the non-parametric Mann-Whitney U test. Significance of differences was assumed at $p \leq 0.05$ at the two-sided test.

Results

Immunohistochemical analysis of inhibin βA and βB expression

The inhibin βA-subunit demonstrated a positive staining reaction in several cervical tissue samples (Fig. 1a–d). Normal cervical glandular epithelium demonstrated a positive inhibin βA (Fig. 1a) and βB (Fig. 1b) staining reaction. Cervical adenocarcinoma demonstrated a positive immunolabeling for inhibin βA (Fig. 1c) and βB (Fig. 1d). Interestingly, the staining intensity in adenocarcinomas appeared to be lower than in glandular epithelial cells. A statistical analysis of these observations revealed that the immunoreactive score (IRS) for inhibin βA and βB expression differed significantly between normal and malignant glandular epithelial cells ($p < 0.05$ each), whereas no statistical significance could be observed

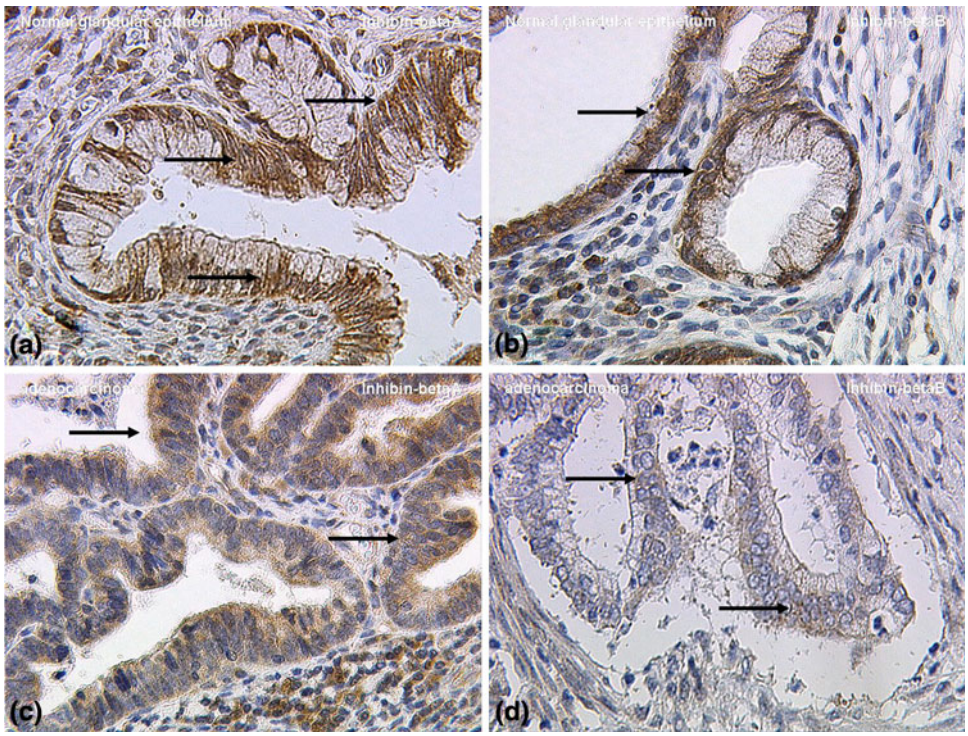

Fig. 1 Immunohistochemical staining reaction of inhibin βA and inhibin βB in normal and malignant glandular epithelium of human cervical tissue. Normal cervical glandular epithelium demonstrated a positive staining reaction for inhibin βA (**a**, ×250) and inhibin βB (**b**, ×250), while cervical adenocarcinomas also reacted positively but to a lesser extent, with the inhibin βA (**c**, ×250) and inhibin βB antibody (**d**, ×250)

between the normal squamous epithelium and squamous cervical carcinomas (Fig. 2).

Expression analysis of inhibin βA- and βB-subunits in the human cervical carcinoma cell line HeLa by immunofluorescence and RT-PCR analysis

Cervical carcinoma cells are malignant cells derived from invasive cervical carcinomas of different origins. The analysis of the expression of inhibin βA and βB in the human cervical cancer cell HeLa, which is derived from a cervical adenocarcinoma, revealed that both subunits had a low expression level (Fig. 3a–b). To verify inhibin βA and βB expression at the transcriptional level in HeLa cells, RT-PCR analysis of the expression of inhibin βA and βB mRNA was performed. Figure 4 shows that both subunits were expressed by HeLa cells, albeit at a lower level compared to the actin level.

Discussion

This preliminary report describes for the first time the immunohistochemical expression of inhibin βA- and βB-subunits in normal and pathological glandular epithelial tissue of the human cervix. In addition, inhibin βA and βB immunolabeling was significantly lower in malignant cervical glandular epithelium than in normal tissue. Moreover,

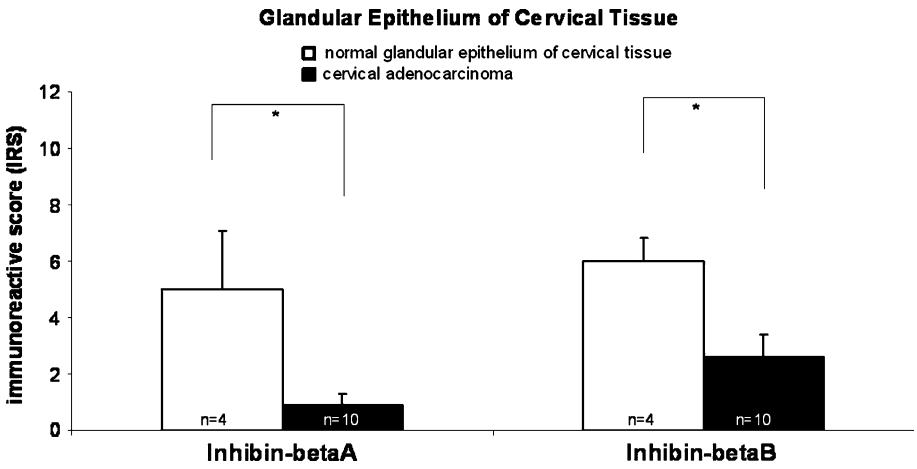

Fig. 2 Immunohistochemical analysis for inhibin βA- and βB-subunits. The immunoreactive score (IRS) for inhibin βA and βB decreased significantly in normal and malignant cervical glandular epithelium (*$p < 0.05$), Data represent mean ± SEM

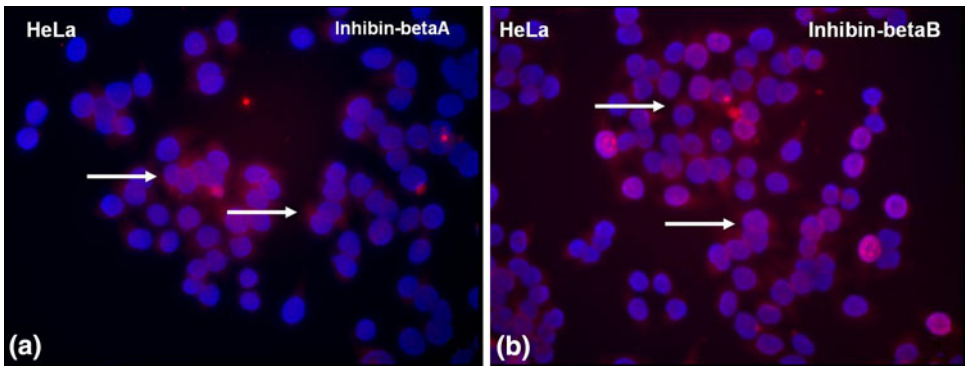

Fig. 3 Localization of inhibin βA and βB expression in HeLa cells. The cervical carcinoma cell line HeLa was analyzed by immunofluorescence, showing a cytoplasmatic positive staining reaction for inhibin βA (**a**; ×400) and inhibin βB antibody HeLa (**b**; ×400)

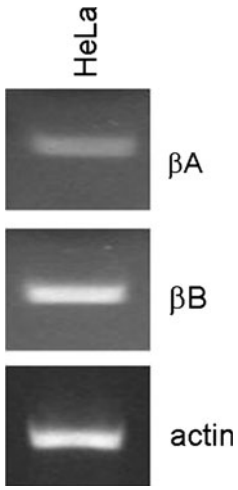

Fig. 4 Inhibin βA and βB expression in the HeLa cervical carcinoma cell line. HeLa cells were analyzed by RT-PCR analysis for the expression of inhibin βA- and βB-subunit. Expression of β-actin was used as a control

we observed the synthesis of these subunits in the HeLa cervical carcinoma cell line of epithelial origin using RNA amplification techniques.

Inhibins and activins have been primarily identified in human gonads and are also synthesized in endocrine tissues [19], including normal and pathological human placental [27, 28, 34, 35] and endometrial tissue [15, 23–29]. Their differential expression has suggested that they have an important role in malignant cell transformation [25, 27, 29, 31]. Recently, in a cohort group of 302 endometrial cancer patients, a differential immunohistochemical expression of the inhibin α-, βA- and βB-subunits has been demonstrated [29]. Although inhibin α immunoreactivity was an independent prognostic factor, expression of βA and βB did not correlate with patient survival [29]. However, by performing a sub-analysis of the inhibin βA-subunit in endometrioid adenocarcinomas, a significantly worse, cause-specific survival was demonstrated in patients with an intense inhibin βA expression [37]. Moreover, the inhibin βB-subunit constituted an independent prognostic parameter in uterine non-endometrioid cancer patients [26]. Therefore, both inhibin subunits might have important functions in human carcinogenesis.

Interestingly, TGF-β has been recognized as a tumor suppressor in premalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in later stages of the disease, leading to metastasis [38]. Regarding metastasis, inhibition of TGF-β suppresses experimental metastasis to multiple organs [39, 40]. Inhibin A, inhibin B and activin A were detected in normal and malignant human uterine tissues, including cervical cancer [27], and the neoplastic transformation of the human cervix might also be related to dysregulation of TGF-β, leading to loss of cell cycle control [41].

However, the most important function is the tumor suppressor activity of the α-subunit, which was first identified after the functional deletion of the inhibin α gene in mice [42, 43]. Interestingly, activin A inhibits cancer cell proliferation in various experimental models in vitro and in vivo [44–46]. Activin A inhibits telomerase activity in cancer cell lines, therefore contributing to the inhibition of cancer cell proliferation [47]. However, it was demonstrated that activin A is also capable of enhancing proliferation in certain cancer cell lines [48–50]. Additionally, inhibin resistance with a subsequent increased activin function could contribute to the aggressive behavior of ovarian cancer cells in vitro [51]. Therefore, the function of activins in different tissue and cell lines is still the subject of discussion [19]. Meanwhile, the putative functions of inhibin subunits in cervical pathogenesis and carcinogenesis remain unclear.

Expression analysis of these subunits in cervical tissue is scarce. Recently, we have observed novel βC- and βE-subunits in cervical cancer and cervical cancer cell lines [14, 35]. In addition, inhibin βA and βB are also expressed in cervical squamous epithelial cells [33]. Both inhibin β-subunits showed a differential expression in cervical intraepithelial neoplasia and squamous cancer, suggesting important roles in cervical carcinogenesis [33]. Therefore, inhibin βA might be important during the progression of cervical intraepithelial neoplasia, while the inhibin βB-subunit could exert a substantial function during differentiation of cervical carcinomas [33]. However, cervical

adenocarcinomas are believed to have a different identity to the more common squamous cancer, with respect to the mechanism of carcinogenesis [3]. The precise function of inhibin βA- and βB-subunits in cervical adenocarcinoma remains to be elucidated.

In conclusion, we demonstrated expression of inhibin βA- and βB-subunits in normal and malignant glandular epithelial cells of human cervical tissue, as well as in cervical cancer cell line HeLa. Although the physiological role of the subunits is still unclear in cervical tissue; however, they might play important roles in carcinogenesis, since they are significantly down-regulated during pathogenesis in cervical adenocarcinomas. Moreover, the synthesis of the inhibin βA and βB-subunits in the cervical carcinoma cell line of epithelial origin also facilitates the use of this cell line in elucidating their functions in cervical pathogenesis. Further studies on the prognostic value of the inhibin βA- and βB-subunits are warranted in this subtype of cervical cancer.

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Conflict of interest The authors declare that they have no competing interests.

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