Inhibin-α subunit in normal and malignant human cervical tissue and cervical cancer cell lines

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Abstract. Inhibins are dimeric glycoproteins composed of an α-subunit and one of two possible β-subunits (βA or βB), with substantial roles in human reproduction and in endocrine-responsive tumours. Four normal cervical tissue samples together with 10 specimens of well-differentiated squamous cervical cancer and adenocarcinoma of the cervix were immunohistochemically analysed for the expression of the inhibin-α subunit. Additionally, two cervical carcinoma cell lines (HeLa and CaSKi) were analysed with immunofluorescence and RT-PCR for the expression of the inhibin-α subunit. We demonstrated for the first time an immunolabelling of the inhibin-α subunit in normal and malignant cervical tissue, as well as cervical cancer cells. However, the immunoreactive reaction was albeit weak and mostly confined to mitotic cells in the analysed cervical tissues. Additionally, the expression pattern of this subunit was rather inconsistent. Therefore, the immunohistochemical evaluation of this subunit in cervical tissue cannot be used as a prognostic marker as suggested for endometrial cancer. However, since the expression of the inhibin-α subunit is minimal in HeLa cells as assessed by immunofluorescence and RT-PCR, the CaSKi cell line might be a better model for further functional experiments regarding cervical pathogenesis.

Introduction

Since the implementation of screening programs with the objective to prevent invasive cervical cancer by detecting its precursor cervical lesions, the incidence of this cancer has declined in the more developed countries. However, cervical cancer is still the second most common malignant disease among women worldwide with more than 500,000 new cancer cases every year, especially in less developed countries (1,2). Although several risk factors for the development of cervical cancer have been recognized, including HPV infection (3-6), the precise mechanism involved in carcinogenesis is still quite unclear and no effective tumour markers are available.

Inhibins and activins are secreted polypeptides, representing a subgroup of the TGFβ superfamily of growth and differentiation factors (7,8). 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. In contrast, activins are homodimers of β-subunits linked by a disulphide bond (7,8). Furthermore, two additional β-subunits have been identified in humans, βC and βE (7). Although these novel subunits are synthesised in a wide range of normal and malignant tissues (9-15), their precise function still remains unclear.

The inhibin subunits have been detected in female endocrine tumours, suggesting important roles in malignant cell transformation (16-20). However, one of the most intriguing functions of the inhibin-α subunit is its tumour-suppressor activity (19), which was first identified after functional deletion of the inhibin-α gene in mice (21). Inhibin-α knockout mice presented with gonadal stromal tumours, and even after these tumours were removed at an early age, these gonadectomised mice developed adrenal cortical sex steroidogenic tumours, demonstrating the tumour-suppressor function in the ovary and the adrenal gland (22).

We recently demonstrated the expression of the novel βC- (15) and βE-subunit (9) in cervical cancer and cervical cancer cell lines, suggesting a substantial function in cervical pathogenesis. Recently, both inhibin-βA and -βB subunits demonstrated a differential expression in cervical intraepithelial neoplasia (CIN) and squamous cancer, suggesting important roles in cervical carcinogenesis (23). Inhibin-βA appears to be important during the progression of cervical intraepithelial neoplasia, while the inhibin-βB subunit may exert a substantial function during differentiation of cervical carcinomas (23). Additionally, we demonstrated a differential expression pattern of inhibin-βA and -βB subunits in normal and malignant glandular epithelial cells, including cervical adenocarcinomas (24). Although endocrine-related cancers express the inhibin-α subunit (19,20,25), it is still unclear whether normal or cancerous cervical epithelial cells as well as cervical cancer cell lines can synthesise this subunit. Therefore, the aim of this study was to analyse the expression of the α-subunit in normal and pathological cervical tissue as well as cervical carcinoma cell lines.

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Materials and methods

Tissue samples. Samples of uterine cervical tissue were obtained from 4 premenopausal, non-pregnant patients undergoing hysterectomy for uterine leiomyomata. Additionally, 10 specimens of well-differentiated (G1) squamous cervical cancer and 10 tissue samples of well-differentiated (G1) adenocarcinoma of the cervix of a well-characterized group (9,15,23,24) were obtained from the pathological archives of the First Department of Obstetrics and Gynaecology, Ludwig Maximilian University of Munich.

Immunohistochemistry. Immunohistochemistry was performed using a combination of pressure cooker heating and the standard streptavidin-biotin-peroxidase complex by using the mouse-IgG Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) as previously described (16,25-27) with slightly 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, CA, USA) for 45 min at room temperature. Sections were then incubated at 4˚C overnight with the inhibin-α mouse antibody (mouse IgG2a, clone R1; Serotec, Oxford, UK) at a dilution of 1:50 in Ultra-V-Block. After washing with PBS, sections were incubated with biotinylated secondary anti-mouse antibody (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 chromagen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) at a 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 (16,26,27). Positive cells showed a brownish color and negative controls as well as unstained cells were blue.

Cells and cell culture. The cervical adenocarcinoma cell line HeLa (ATCC CCL2) and the cervical squamous carcinoma cell line CaSKi (ATCC CRL-1550) are ATCC-available cell lines (ATCC, LGC Promochem GmbH, Wesel, Germany). 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 (9,15,23,28).

Immunofluorescence analysis. Immunofluorescence analysis was performed as previously described (9,11-15,23,25). Briefly, 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) for 15 min at room temperature. Thereafter, slides were incubated with inhibin-α antibody (1:50 in dilution medium provided by Dako) overnight at 4˚C, followed by a 1:500 diluted Cy3-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany) for 30 min at 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) and saved on a computer system.

RT-PCR analysis. RNA was extracted from the cells using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). 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) as previously described (25,27). Primer sequences to amplify a 359-bp fragment of inhibin-α were in 5'-3' orientation: CCGGCCATCCCAGCATACACGC (forward primer) and GAGTTGAGCGTCGGGCTCTC (backward primer). 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.

Results

The inhibin-α subunit demonstrated no or minimal positive staining reaction in the normal and malignant cervical tissue (Fig. 1). Normal squamous epithelial cells demonstrated no or minimal reaction with the inhibin-α antibody, being primarily localised in mitotic cells (Fig. 1a). Squamous carcinomas also demonstrated no to minimal positive immunohistochemical staining reaction (Fig. 1b). Normal cervical glandular epithelium demonstrated also a minimal positive reaction primarily localised in the mitotic cells (Fig. 1c), while cervical adenocarcinomas showed no positive staining reaction (Fig. 1d). However, the immunoreactive reaction was albeit weak, inconsistent and mostly confined to mitotic cells in all analysed cervical tissues.

Cervical carcinoma cells are malignant cell lines derived from invasive cervical carcinomas of different origin. We therefore tested the expression of inhibin-α in the human cervical cancer cell lines HeLa and CaSKi. Immunofluorescence analysis of both cell lines expressed this α-subunit at the protein level. Expression of inhibin-α was found to be located primarily in the cytoplasm (Fig. 2). However, since the expression of the inhibin-α was minimal in the HeLa cells, the CaSKi cell line might be a better model for further functional experiments regarding cervical pathogenesis.

To verify inhibin-α expression at the transcriptional level in human cervical cancer cells of different histological origin,

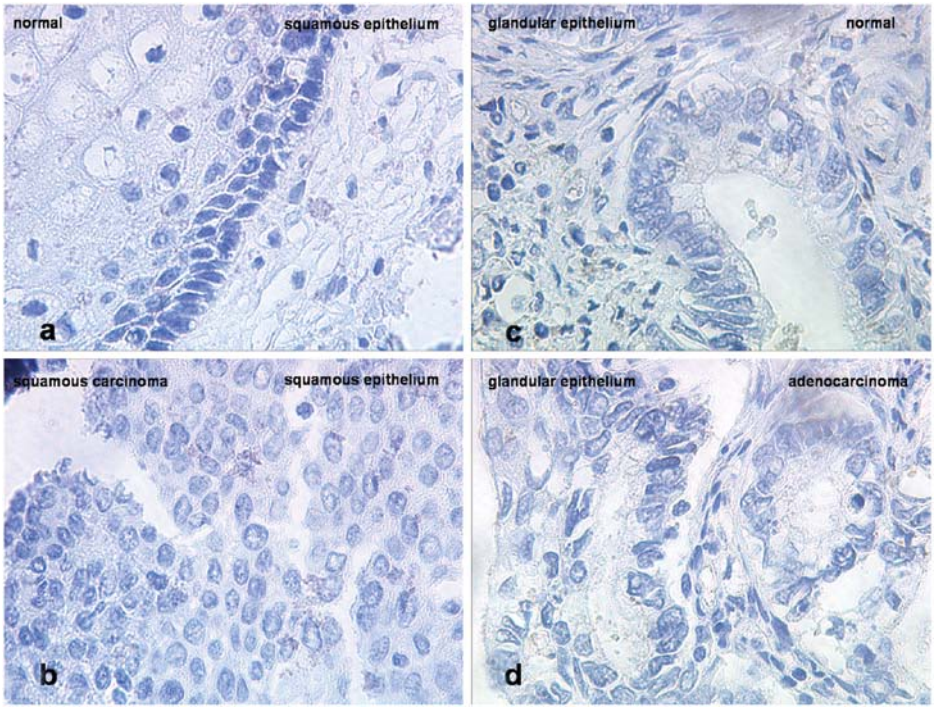

Figure 1. Immunohistochemical staining reaction of inhibin-α in normal and malignant cervical tissue. Normal squamous epithelial cells demonstrated no to minimal reaction with the inhibin-α antibody, being primarily positive in mitotic cells [(a), x400]. Squamous carcinomas also demonstrated no to minimal positive immunohistochemical staining reaction [(b), x400]. Normal cervical glandular epithelium demonstrated also a minimal positive reaction primarily localised in mitotic cells [(c), x400], while cervical adenocarcinomas showed no positive staining reaction [(d), x400]. However, the immunoreactive reaction was albeit weak and mostly confined to mitotic cells in all analysed cervical tissues.

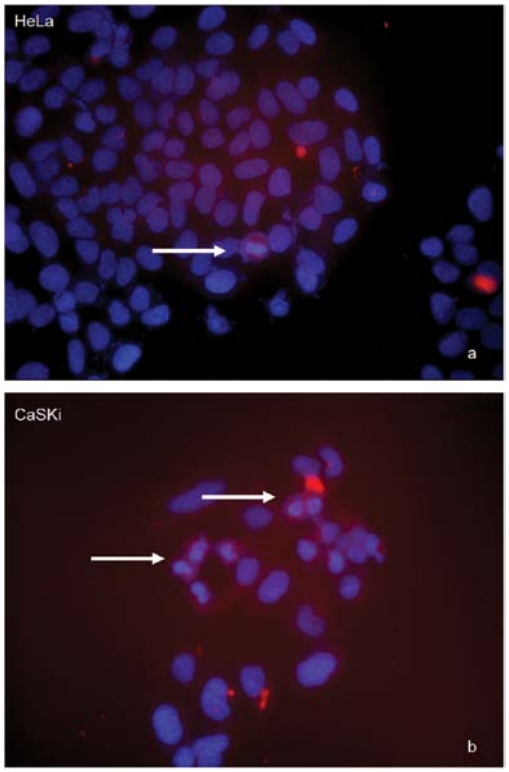

Figure 2. Localisation of inhibin-α expression in HeLa and CaSKi cells. The cervical carcinoma cell lines HeLa and CaSKi were analysed by immunofluorescence for the expression of inhibin-α, showing a cytoplasmatic positive staining reaction in HeLa [(a), x400] and CaSKi [(b), x400] cells. However, the expression of inhibin-α was less intense in the HeLa cells compared to the CaSKi cells. Notably, the labelling intensity was stronger in mitotic cells of both cell lines (arrows).

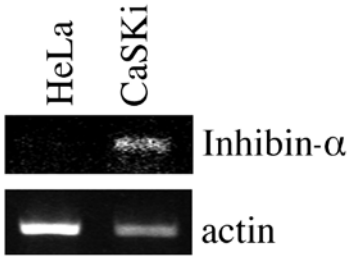

Figure 3. Inhibin-α expression in cervical carcinoma cell lines. HeLa and CaSKi cells were analysed by RT-PCR analysis for the expression of the inhibin-α subunit. Expression of β-actin was used as a control.

the squamous cervical cancer cell line CaSki and the cervical adenocarcinoma cell line HeLa were subjected to RT-PCR analysis for the expression of inhibin-α mRNA. Fig. 3 shows that this subunit is strongly expressed in CaSKi cells but poorly synthesised by HeLa cells. Therefore, the CaSKi cell line might be a better model for further functional experiments regarding cervical pathogenesis compared to the HeLa cell line.

Discussion

The inhibin/activin subunits have been detected in normal female reproductive tissue and endocrine tumours (16,19,20,26,29). We demonstrated in this preliminary study for the first time the immunohistochemical expression of the inhibin-α subunit in normal and pathological human cervical tissue. Moreover, we observed the synthesis of this subunit also in cervical carcinoma

cell lines of squamous and epithelial origins by using RNA amplification techniques. The putative expression of inhibin subunits in cervical cancer is of extreme importance, since activin signalling may be a promising target for therapeutic interventions (30,31).

Notably, TGF-β has been recognized as a tumour suppressor in premalignant stages of carcinogenesis with an additional dual role as a pro-oncogene in later stages of the disease, leading to metastasis (19,32). Regarding metastasis, inhibition of TGF-β suppresses experimental metastasis to multiple organs (33,34). Inhibin A, inhibin B and activin A were detected in normal and malignant human uterine tissues, including cervical cancer (35), and the neoplastic transformation of the human cervix might also be related to dysregulation of TGF-β, leading to loss of cell cycle control (36).

However, the most important function is the tumour-suppressor activity of the α-subunit (19), that was first identified after functional deletion of the inhibin-α gene in mice (21,22). Meanwhile, loss of inhibin-α expression resulted in a poorer survival of endometrial cancer patients, suggesting a tumour-suppressor function in human endometrium (16). Additionally, a pro-tumourigenic and pro-metastatic function for inhibin-α in prostate cancer has been described (37). However, the exact molecular mechanism by which the inhibin-α subunit is implicated in carcinogenesis is still not clear.

In prostate cancer, the inhibin-α promoter can be silenced by hypermethylation (38,39). Promoter hypermethylation, mostly described for cell cycle regulator proteins and E-cadherin expression, is a quite often observed gene silencing mechanism during human cancer progression (40). Furthermore, the inhibin-α subunit can be regulated by GATA and CCAAT/enhancer-binding protein-β transcription factors (41,42), but too little is known about the involvement and expression of these transcription factors in human cervical carcinomas. However, generally it is believed that inhibin-α is a tumour-suppressor gene silenced by mechanisms common to other tumour-suppressor genes (43).

The loss of the inhibin-α subunit production in mice resulted in a marked increase in activin production in the ovary. The inhibin-α sufficient mice presented with cachectic symptoms that were associated with the compensatory excessive secretion of activin (22). Additionally, inhibin resistance with a subsequent increased activin function may contribute to the aggressive behaviour of ovarian cancer cells *in vitro* (44). Inhibin-βA is overexpressed in lung adenocarcinomas and this overexpression is associated with a poorer survival, probably affecting promoter methylation and histone acetylation (45). Notably, an overexpression of activin A in esophageal squamous cell carcinoma resulted in a worse overall prognosis (46), probably leading to cell growth promotion, tumourigenicity, invasion and resistance to apoptosis in these squamous cancer cells (47). However, whether the inhibin subunits have similar functions in cervical pathogenesis and carcinogenesis remains unclear.

In conclusion, we demonstrated an expression of the inhibin-α subunit in normal and malignant cervical tissue, as well as cervical cancer cells. However, the immunoreactive reaction was albeit weak and mostly confined to mitotic cells in all analysed cervical tissues. Additionally, the expression pattern of this subunit was rather inconsistent. Therefore, the immunohistochemical evaluation of this subunit in cervical tissue cannot be used as a prognostic marker as suggested for endometrial cancer (16,18,25).

Although the physiological role is still quite unclear in cervical tissue, inhibin-α may play important roles in carcinogenesis. Moreover, the synthesis of this subunit in cervical carcinoma cell lines of squamous and epithelial origins allows the use of these cell lines in elucidating its functions in cervical pathogenesis and carcinogenesis. However, since the expression of the inhibin-α is minimal in HeLa cells, as assessed by immunofluorescence and RT-PCR, the CaSKi cell line may be a better model for further functional experiments regarding cervical pathogenesis.

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