Abstract. Background and aim: Inhibins are dimeric glycoproteins, belonging to the transforming growth factor beta (TGF-β) family, composed of an α-subunit (INH-α) and one of two possible β-subunits (βA or βB). Additionally two further β-subunits (βC and βE) have been cloned, although their function remains still quite unclear. The detection by immunohistochemistry of inhibin/activin subunits has been proposed as a useful marker of trophoblastic diseases. Interestingly, a complete mole cannot be easily differentiated from a partial mole. Therefore, the aim of this study was to determine expression changes of the five inhibin/activin subunits in partial and complete moles.

Materials and Methods: Histologically diagnosed complete (n=6) and partial (n=3) hydatidiform moles were immunohistochemically analyzed for INH-α, INH-βA, INH-βB, INH-βC and INH-βE subunits. The immunohistochemical reaction in intermediate trophoblast was analyzed with a semi-quantitative score (IRS) and statistical analysis was performed.

Results: Immunohistochemical reaction with INH-α, INH-βA, INH-βB, INH-βC and INH-βE subunits was demonstrated in hydatidiform moles. The immunohistochemical expression of INH-α, INH-βA, INH-βB, INH-βC and INH-βE subunits was significantly higher in complete compared to partial moles (p<0.05 each), while INH-α, INH-βB and INH-βE did not demonstrate any statistically significant differences. Conclusion: We demonstrated an immunohistochemical expression of all five inhibin/activin subunits in partial and complete hydatidiform moles. The expression of INH-βA and INH-βB determined immunohistochemically was significantly up-regulated in complete moles, suggesting the utilization of these antibodies as diagnostic differentiation markers between complete and partial moles.

Key Words: Inhibin/activin subunits, inhibin-α, inhibin-βA, inhibin-βB, inhibin-βC, inhibin-βE, complete hydatidiform mole, partial hydatidiform mole.
hydatidiform mole (18). Recently, it was suggested that serum inhibin A and activin A measurements might be of value in diagnosis and short-term follow-up of molar pregnancy (19). Inhibin-α and -β subunits are consistently co-expressed immunohistochemically in syncytiotrophoblast in complete and partial moles, suggesting that these glycoproteins might be useful tissue markers in the differential diagnosis of trophoblastic lesions (20).

Interestingly, complete moles can be reliably distinguished from non-molar pregnancy, but neither non-molar pregnancy nor complete moles are easily differentiated from partial mole (21, 22). However, limited data on histological expression of inhibin/activin subunits expression in these two forms of hydatidiform moles exists. Therefore, the aims of the present study were to determine expression changes of the five inhibin/activin subunits in partial and complete moles.

Materials and Methods

Placental tissues diagnosed by a gynecological pathologist as complete (n=6) and partial (n=3) hydatidiform moles were obtained from the First Department of Obstetrics and Gynaecology of the LMU Munich.

Generation of a polyclonal activin-βE peptide antibody. Anti-activin-βE polyclonal antibodies were generated in rabbits against a polypeptide of 16 amino acids of activin-βE (polypeptide-sequence: NH₂-CRWPGRPRQQSRTL-L-COOH; amino acid position 144 to 158; accession number: AAH05161). A primary dose of 200 μg activin-βE polypeptide was emulsified in Freund’s complete adjuvant (Sigma-Aldrich, Germany) and administered subcutaneously in rabbits. Three doses of the peptide emulsified in Freund’s incomplete adjuvant were administrated at intervals (6 weeks). After the third booster injection (14 days), blood was collected from the rabbit and the serum was separated. Antibodies were isolated using column chromatography with a protein A column (Amersham Pharmacia Biotech, Freiburg, Germany).

Immunohistochemistry. Immunohistochemistry on paraffin sections (7 μm) of the different placental tissue specimens was performed by incubating the slides in methanol/H₂O₂ (30 min) to inhibit endogenous peroxidase activity. Immunohistochemistry with inhibin-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) for inhibin-α, -βA and -βB as well as goat-IgG-Vectastain Elite ABC kit (Vector Laboratories) for inhibin-βC and -βE. The antibodies used are given in Table I.

Brieﬂy, parafﬁnin–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 sodium citrate buffer (pH 6.0), containing citrate acid 0.1M and sodium citrate 0.1M 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 μl horse serum; Vector Laboratories) for 20 min at room temperature. Sections were then incubated at room temperature for 120 min with the primary antibodies. Inhibin-α was diluted in PBS. After washing with PBS, the slides were incubated in diluted biotinylated serum (10 ml PBS containing 50 μl horse serum; Vector Laboratories) for another 30 min at room temperature. After incubation with the avidin-biotin peroxidase complex for another 30 min and repeated washing steps with PBS, visualisation was performed with substrate and the chromagen 3,3’-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 5-10 min. The slides were counterstained further with Mayer’s acidic hematoxylin and washed in a series of alcohol (50-95%). After xylol treatment the slides were covered.

Negative controls were performed by replacing the primary antibody with normal horse serum (Vector Laboratories). Positive cells showed a brownish color and the negative control, as well as unstained cells, appeared blue. The standardisation, dilution and optimisation of this protocol for inhibin-α, -βA and -βB was primarily tested on normal premenopausal ovary tissue, while negative controls included postmenopausal ovarian tissue. For inhibin-βC and inhibin-βE normal liver tissue was used as positive control.

Immunohistochemical evaluation and statistical analysis. The intensity and distribution patterns of specific inhibin/activin subunit immunohistochemical staining reaction was evaluated by two blinded, independent observers, including a gynecological pathologist (N. Ś.), using a semi-quantitative score (IRS score) as described elsewhere (23) and used in the evaluation of inhibin/activin subunit expression in normal human endometrial specimens (6). The IRS score was calculated as follows: IRS=SI x PP, where SI is the optical staining intensity (graded as: 0=no staining; 1=weak staining; 2=moderate staining and 3=strong

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>Inhibin-α</td>
<td>R1</td>
<td>mouse IgG2a</td>
<td>1:50</td>
<td>Serotec, Oxford, UK</td>
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<td>Goat</td>
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<td>R&amp;D Systems,</td>
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<tr>
<td>Inhibin-βE</td>
<td>Polyclonal</td>
<td>rabbit</td>
<td>1:4000</td>
<td>In house antibody</td>
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BioGenes, Berlin, Germany
staining) and PP the percentage of positively-stained cells. PP was estimated by counting ~200 cells (defined as 0=no staining; 1=<10% staining; 2=11-50% staining; 3=51-80% staining and 4=>81% 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 different IRS scores (SPSS; Chicago, IL, USA). Significance of differences of the means was assumed at \( p \leq 0.05 \).

**Results**

Immunohistochemical staining was performed using an appropriate positive control comprising ovaries containing follicular cysts or normal liver. Inhibin-\( \alpha \) stained positive with ovarian granulosa cells and theca interna cells, while inhibin-\( \beta A \) and -\( \beta B \) subunits also stained positively with human ovarian tissue as previously described (6). Inhibin-\( \beta C \) and -\( \beta E \) were immunohistochemically expressed in liver and ovarian tissue as described elsewhere (4, 5).

All five inhibin/activin subunits were detected in partial and complete hydatidiform moles. Inhibin-\( \alpha \) was primarily expressed in syncytiotrophoblast cells of partial (Figure 1A) and complete moles (Figure 1B). The \( \beta A \)-subunit was also detected in molar syncytiotrophoblast cells but with a weaker intensity in partial (Figure 1C) compared to complete moles (Figure 1D). The syncytiotrophoblast in partial and complete hydatidiform moles also demonstrated a positive immunohistochemical staining of the inhibin-\( \beta B \) subunit. Interestingly, the staining reaction was less in the partial (Figure 1E) than in the complete moles (Figure 1F). Inhibin-\( \beta C \) and -\( \beta E \) also demonstrated a positive staining reaction, albeit weaker than the other inhibin/activin subunits (Figures 1G-J).

The immunoreactive score for inhibin-\( \alpha \) demonstrated no significant differences between partial and complete moles (Figure 2), while the staining intensity of inhibin-\( \beta A \) and -\( \beta B \) subunit was significantly higher in complete hydatidiform moles than in partial ones (\( p < 0.05 \) each) (Figure 2). The immunoreactive score for inhibin-\( \beta C \) and -\( \beta E \) did not show any significant differences between either hydatidiform mole entities (Figure 2).

**Discussion**

While inhibins/activins were initially characterised as endocrine and paracrine hormonal regulators of the hypothalamic-pituitary-gonadal axis, it is now clear that they are expressed in a wide range of tissues, including normal and pathological placenta (10-13, 24). The human placenta expresses inhibin/activin mRNA (13) and inhibin/activin subunit protein throughout pregnancy, being the primary source of maternal circulating inhibin and activin levels (24).

Inhibin/activin subunits are also localized in trophoblast (10) and trophoblastic tumours (25, 26). The immunohistochemically detectable inhibin-\( \alpha \) subunit in placental tissue was mainly localized within the syncytiotrophoblast with positive staining of the decidua (10). Production of inhibin by these cells may account for raised serum levels during pregnancy. Inhibin can also be demonstrated in chorionicarcinoma and in nongestational trophoblastic tissue, including in hydatidiform mole with persistent polymorphic trophoblastic hyperplasia (15). The detection by immunohistochemistry of inhibin/activin subunits has been recently proposed, in association with beta-hCG, as a useful marker of trophoblastic neoplasia (25, 26). It has been suggested that measurements of serum inhibin A and activin A might be more useful in diagnosing and following up molar pregnancies than hCG (19). However, other research groups demonstrated that serum molecular forms of inhibins (inhibins A and B) might not be of relevance in the biological survey of patients with gestational trophoblastic diseases (27, 28). Additional immunohistochemical and serological studies are still needed to evaluate the measurement of inhibin/activin subunits in the clinical setting regarding trophoblastic lesions.

Using immunohistochemistry we demonstrated the expression of all five inhibin/activin-subunits in complete and partial hydatidiform moles. We observed a significant lower expression of the inhibin-\( \beta A \) and -\( \beta B \) subunit in partial compared to complete moles. Interestingly, expression of the inhibin-\( \alpha \), -\( \beta C \) and -\( \beta E \) subunits did not show any statistical significance. We therefore assume that the higher \( \beta A \) and \( \beta B \) subunit expression results in a higher activin A and activin B secretion. This assumption might explain the higher activin A concentration in serum in gestational trophoblastic diseases, while serological inhibin concentration (inhibins A and B) might not be of relevance in the survey of patients with hydatidiform moles. However, other possibilities like the formation of inhibin C or E as well as different activin molecules cannot be excluded and warrant further studies.

**Conclusion**

All five inhibin/activin subunits were observed in complete and partial hydatidiform moles using immunohistochemistry. Inhibin/activin subunits were distributed on the cell surface, playing a significant role in the pathogenesis of gestational trophoblastic diseases. The immunohistochemical reaction with inhibin-\( \alpha \) was similar in complete and partial moles. The inhibin-\( \beta A \) and -\( \beta B \) expression was significantly higher in complete compared to partial moles (\( p < 0.05 \) each), while inhibin-\( \beta C \) and -\( \beta E \) did not demonstrate any statistically significant differences. Therefore, the \( \beta A \)- and \( \beta B \)-antibodies can be used as diagnostic differentiation markers between complete and partial moles. Although the precise role of
these inhibin/activin subunits in human complete and partial hydatidiform moles is still unclear, they could be involved in autocrine/paracrine signalling, contributing to several aspects such as angiogenesis and tissue remodelling.

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Figure 1. Immunohistochemical staining reaction of inhibin/activin subunits in complete and partial hydatidiform moles. Magnification: ×100.

Figure 2. The immunoreactive score for inhibin-α demonstrated no significant differences between partial and complete moles, while the staining intensity of inhibin-βA and -βB subunits increased significantly between partial and complete hydatidiform moles *(p<0.05). The immunoreactive score for inhibin-βC and -βE did not show any significant differences between either hydatidiform mole entities. Data represent mean ±SEM. *Significance was assumed at p<0.05.
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References


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