The expression of inhibin/activin subunits in the human adrenal cortex and its tumours

L M A Munro, A Kennedy and A M McNicol

University Department of Pathology, Royal Infirmary, Glasgow G4 0SF, UK

(Requests for offprints should be addressed to A M McNicol, Department of Pathology, Glasgow Royal Infirmary University NHS Trust, Castle Street, Glasgow G4 0SF, UK)

Abstract

Inhibins and activins are dimeric proteins of the transforming growth factor-β superfamily which have been shown to be expressed in the adrenal cortex. Recent studies have suggested a role for these peptides in the pathogenesis and/or function of adrenal tumours. To investigate further their physiological and pathological roles, we have documented immunoreactivity for inhibin α, βA and βB subunits in normal adult and fetal human adrenals, in hyperplastic adrenals and in adrenal tumours.

In the normal and hyperplastic adult gland, diffuse immunopositivity was demonstrated for β subunits, suggesting that activins (βB dimers) can be expressed in all zones. Inhibin α was limited to the zona reticularis and the innermost zona fasciculata in the normal gland, extending centripetally into the zona fasciculata in hyperplasia, supporting a role for ACTH in the regulation of expression, and suggesting that expression of inhibins (αβ dimers) is restricted.

Immunopositivity for all three subunits was seen in both fetal and definitive zones of the fetal cortex, indicating that both inhibins and activins could be expressed in both.

Immunopositivity for all three subunits was seen in most adrenocortical tumours. Loss of immunopositivity for inhibin α in a subgroup of carcinomas might indicate a role in tumour progression. The greater intensity of staining for inhibin α in tumours associated with Cushing’s syndrome again suggests a link with cortisol production.


Introduction

Inhibins and activins are members of the transforming growth factor β family of proteins (Kingsley 1994). Inhibins are dimeric proteins which consist of an α and a β subunit (αβA, inhibin A; αβB, inhibin B), while activins are homodimers or heterodimers of the β subunits (βAβA, activin A; βBβB, activin B; βAβB activin AB). While characterised as endocrine and paracrine hormonal regulators of the hypothalamic–pituitary–gonadal axis (Ying 1988) it is now clear that they are expressed in a wide range of tissues including pituitary, bone marrow and central nervous system (Meunier et al. 1988) and placenta (Meunier et al. 1988, Petraglia 1997). They appear to have pleiotropic effects (Ying et al. 1997). For example, they may exert significant roles in embryogenesis including regulation of lateral asymmetry (Oh & Li 1997). They may also play a part in inflammatory processes (Hübner et al. 1997) and have been shown to inhibit hepatocyte growth in an autocrine manner by influencing apoptosis (Ying et al. 1997). The expression of the α subunit, and thus of inhibins, appears to be more restricted than that of the β subunit, and therefore of activins. However, where both are expressed, inhibins appear to oppose the actions of activins. Whether the more recently characterised β subunits βC (Hötten et al. 1995), βD (Oda et al. 1995) and βE (Fang et al. 1996) can dimerise to form mature inhibins and activins is at present unclear.

There is evidence that α, βA and βB subunits may be expressed in the adrenal cortex (Meunier et al. 1988, de Jong et al. 1990). Plasma immuno-reactive inhibin levels are higher in the adrenal veins than in vena cava or the peripheral circulation (Nishi et al. 1995) and normal adrenocortical cells in culture secrete inhibin-like immunoreactive material (Haji et al. 1991, Spencer et al. 1992). While this may reflect production of mature inhibin dimer it may also indicate the presence of free α subunit which may crossreact in the assay. Inhibin α mRNA has been demonstrated in the ovine (Crawford et al. 1987) and human (Voutilainen et al. 1991) adrenal cortex and its expression has been shown to be upregulated by adrenocorticotropic hormone (ACTH). Immunopositivity for inhibin α has been reported in the rat (Merchanthaler et al. 1991) and human (Spencer et al. 1992) adrenal cortex. There is less information on the expression of β subunits. Immunoreactivity for both βA and βB subunits has been demonstrated in human adult and fetal glands (Spencer et al. 1992), and studies using reverse transcriptase–polymerase chain reaction suggest that the mRNAs may be expressed at low levels (Voutilainen et al. 1991).
There has also been recent but conflicting information concerning the possible role of inhibins/activins in adrenal tumorigenesis. Studies from inhibin α deficient mice have indicated a putative tumour suppressor role (Matzuk et al. 1994) since these mice develop adrenocortical tumours when gonadectomized. In contrast, mice bearing an inhibin α promoter, Simian virus 40 T (SV40 T)-antigen fusion gene, show the same pattern of tumour development (Kananen et al. 1996). Secretion of inhibin immunoreactive material from human tumours has been reported as has immunopositivity for inhibin α (Chivite et al. 1998, McCluggage et al. 1998, Munro et al. 1998, Pelkey et al. 1998). Whether this reflects free α subunits or mature inhibin, as outlined above, is unclear, but immunopositivity for β subunits on tissue sections (Munro et al. 1998) indicates the potential for producing the mature dimers.

To investigate further the role of these peptides in the human adrenal cortex and its tumours, we have therefore documented immunoreactivity for α, βA and βB subunits in normal adult and fetal glands, and in a series of adrenocortical adenomas and carcinomas. In the normal gland, we have looked for zonal differences in expression. In tumours we have examined correlation with benign or malignant behaviour and with the hormonal profile.

Materials and Methods

Tissues

Normal adult human adrenal glands (n=6) were removed at surgery from patients with renal carcinoma or in the 1960s from patients with breast cancer who had had no endocrine therapy. Fetal adrenal glands (n=10) were obtained from spontaneous abortions occurring during the second and third trimester. Hyperplastic adrenals (n=3) were from patients with ACTH-dependent Cushing’s syndrome. Adrenal adenomas (n=17) and carcinomas (n=13) were characterised both by their behavioural patterns and on the morphological criteria of van Slooten et al. (1985). These were associated with Cushing’s syndrome (n=14), Conn’s syndrome (n=7) or were virilising (n=1) or non-functional (n=8). The latter two groups were assessed together. All material was fixed in formalin and embedded in paraffin wax.

Immunocytochemistry

Sections, 4 µm thick, were cut onto slides coated with aminopropyltriethoxysilane (Sigma, Poole, Dorset, UK), and were immunostained using a labelled streptavidin-biotin technique. Before staining, an antigen retrieval step was undertaken, with boiling for 8 min in citrate buffer pH 6·0 in a pressure cooker in a 650 W microwave oven. Endogenous biotin was blocked using a commercial kit (Biogenex Diagnostics, Finchhampstead, Berks, UK). The primary antibodies were a mouse monoclonal antibody to inhibin α subunit (Serotec, Kidlington, Oxford, UK) (1:200 overnight at 4 °C), and polyclonal antibodies raised in rabbit to inhibin βA (1:500) or βB (1:300) subunits (kindly gifted by Joan Vaughan and Dr Wylie Vale, La Jolla, CA, USA) (60 min at room temperature). The secondary antibodies were polyclonal antibodies raised in goat to mouse and rabbit immunoglobulins. These were labelled with biotin (Dako, Ely, Cambridge, UK). Sites of binding were visualised using streptavidin–peroxidase conjugate (Dako) with 3′-3′ diaminobenzidine (Sigma) as chromogen and 0·05% copper sulphate to intensify the staining. Sections were lightly counterstained with haematoxylin, dehydrated and mounted in piccolyte resin (Phase Separations Ltd, Deeside, Clywd, UK).

An ovarian granulosa cell tumour was used as a positive control for inhibin α (Stewart et al. 1997), placenta for βA (Minami et al. 1992) and pituitary for βB (Alexander et al. 1995). Negative controls included omission of primary antibodies and substitution of mouse immunoglobulin or non-immune rabbit serum as appropriate.

Analysis of staining patterns

In the normal and hyperplastic adult glands and in the fetal glands, the staining was assessed in a qualitative manner, with documentation of the zonal distribution and intensity of positive staining.

In the tumours, immunoreactivity was assessed in a semiquantitative manner as follows. Intensity of staining was classified as negative (0), weak (1), moderate (2) and intense (3). The extent of staining was classified as none (0), <10% of cells (1), 10–50% of cells (2) and >50% of cells (3). An histoscore was derived by multiplying the numerical values for extent and intensity. These were categorised as negative (0), low (1–3), medium (4–6) and high (7–9). Tumours were scored by one observer (L M A M) without knowledge of tumour behaviour or clinical syndrome. A random sample was reassessed (by A M McN), and no significant differences were identified between the two observers.

The distribution of histoscores was compared in adenomas and carcinomas, and among the three functional subgroups: Cushing’s, Conn’s and other.

Results

Normal gland

Adult cortex There were no obvious differences between the pattern seen in glands from patients with renal or breast carcinoma. Strong positive staining for α subunit was seen mainly in cells of the zona reticularis (ZR) with some extension into the inner fasciculata (ZF) (Fig. 1). In
some glands there was staining of occasional cells in the outer ZF, but the intensity was weaker. Positive staining was never seen in the zona glomerulosa (ZG). Diffuse positive staining of all three zones was seen with both \( \alpha \) and \( \beta \), the former being more intense than the latter.

**Hyperplastic adult cortex** In contrast to the normal gland, in addition to positivity of the inner cortex, immunopositivity for \( \alpha \) subunit extended in a centripetal fashion throughout most of the ZF. Although the intensity of staining varied it was in general as intense as in the normal gland (Fig. 3). The distribution of \( \beta \)A and \( \beta \)B subunits was similar to normal.

**Fetal adrenal** Over 50% of the cells of the fetal zone showed positive staining for \( \alpha \) subunit. There was variation in intensity of staining of individual cells, but in general the outer part showed the greatest intensity. Most of the cells of the definitive zone stained positively, but in places a rim of cells in the outermost part was less intensely stained and focally negative (Fig. 4). The majority of cells of both zones showed diffuse positivity for \( \beta \)A subunit. Immunostaining patterns were more variable with \( \beta \)B. Although in general there was widespread staining of both zones, the intensity varied between the zones but did not show any specific pattern with respect to gestation.

**Adrenocortical tumours**

**Analysis with respect to tumour behaviour** The distribution of histoscores is shown in Table 1. All adenomas expressed \( \alpha \) subunit, with two showing high levels of immunoreactivity, while the remainder split evenly between medium and low levels of immunoreactivity (Table 1, Fig. 5). In contrast to the normal gland, intensity of staining for \( \beta \)A was in general less than that for \( \beta \)B, none of the adenomas having a high histoscore for \( \beta \)A, while five did so for \( \beta \)B.

Immunopositivity for inhibin \( \alpha \) was more variable in carcinomas, with four having a high histoscore (Table 1, Fig. 6), and two being immunonegative. The patterns of \( \beta \)A staining were similar to adenomas. However, there...
was a relative loss of βB staining in carcinomas with five cases falling into the low category compared with one of the adenomas.

**Analysis with respect to clinical syndrome** The distribution of histoscores is shown in Table 2. Five out of fourteen of the tumours associated with Cushing’s syndrome had a high histoscore for inhibin α (Fig. 6) compared with none of the Conn’s tumours and one of the others. The two immunonegative cases were nonfunctional. A minority of cases of Cushing’s and Conn’s tumours showed a low histoscore for βA and βB while around half of the non-functional tumours did so in each case. In general, the staining was fairly diffuse, but inhibin α showed rather patchy staining in the Conn’s tumours (Fig. 5).

**Discussion**

Our data provide confirmation of the presence of immunoreactive inhibin/activin subunits in the human adrenal cortex. The distribution of inhibin α immunopositivity is similar to that reported by McCluggage et al. (1998) using the same antibody. However, other studies have reported different patterns in the human gland with immunopositivity present in scattered cells in the ZG and ZF (Spencer et al. 1992), or diffuse positivity, weak in the ZG, moderate in ZF and strong in ZR (Chivite et al. 1998). These differences may reflect the different antibodies used or the different techniques. We employed a heat-induced antigen retrieval technique which lowers the threshold of detection, and a monoclonal antibody which should provide specific and sensitive detection of the epitope. A single study in the rat documented scattered immunoreactive cells in ZG and ZR (Merchenthaler et al. 1991). This might reflect the technical differences outlined above or may indicate species differences in expression.

In the classical theory of zonation, the physiological secretion of cortisol is thought to be from the ZF and ZR. In the resting state, the compact cells of the ZR occupy only the inner rim of the cortex and our observations...
would suggest that expression of inhibin α is confined to that zone. We have, however, demonstrated centripetal extension of immunoreactivity for inhibin α in the ACTH-stimulated hyperplastic cortex in the cases with Cushing’s disease. These changes would fit with the observations that secretion of inhibin-like immunoreactive material and expression of inhibin α mRNA can be regulated in vitro by ACTH in human (Voutilainen et al. 1991, Spencer et al. 1992, Nishi et al. 1995) and ovine (Crawford et al. 1987) glands. Interestingly, a circadian rhythm of circulating inhibin has been reported which correlates closely with that of cortisol (Yamaguchi et al. 1991).

There is only one previous study documenting immunoreactivity for β subunits in the human adrenal cortex (Spencer et al. 1992) using the same antibodies as in this study. We demonstrated more widespread staining, the differences possibly explained again by our use of antigen retrieval. Diffuse immunoreactivity for activin A has been reported (Wada et al. 1996). Our data would also indicate the potential expression of activins throughout the cortex. Their physiological roles are unknown at present, as there are few published studies on their effects. It has been reported that activin A reduces secretion of cortisol and dehydroepiandrosterone (DHEA) from ACTH-stimulated bovine adrenocortical cells (Nishi et al. 1992) but, in contrast, it has no effect on adult human cells (Spencer et al. 1992). Clearly, further functional studies are required.

Our data on the distribution of α, βA and βB subunits in the fetal gland are similar to those of Spencer et al. (1992), although again we have demonstrated greater numbers of positive cells. It would appear, therefore, that both fetal and definitive zones are capable of producing both mature activins and inhibins. mRNAs for the three subunits have been demonstrated in fetal tissue (Voutilainen et al. 1991), although no spatial interpretation could be made from that study. Whether the negativity for α subunit seen in the outer definitive zone in some cases infers lack of inhibin expression would require further investigation. Activins may interact with other growth factors in the regulation of fetal adrenal growth with an inhibitory role (Spencer et al. 1990), specifically on the fetal zone (Spencer et al. 1992). Activin also appears to enhance the ACTH-induced shift from predominant DHEA sulphate production to increased cortisol production (Spencer et al. 1992). It may, therefore, have a role in the development of differentiated cortical function.

The data on the expression and possible role of inhibins/activins in adrenocortical tumours are conflicting. Matzuk et al. (1992) produced inhibin α deficient mice which developed sex cord stromal gonadal tumours with 100%
penetrance. When these were gonadectomised, they developed adrenocortical tumours (Matzuk et al. 1994), again with high penetrance, and the proposal was made that inhibin $\beta$ is a tumour suppressor gene for gonads and adrenal cortex. The observation that the same pattern of tumour development occurred in mice bearing a SV40 T-antigen transgene, driven by the inhibin $\beta$ promoter (Kananen et al. 1996), indicates physiological activation of the promoter for the inhibin $\beta$ subunit gene in the adrenal gland, which leads to the immortalisation of cells by expression of the SV-40 T antigen. In addition, there is evidence that, in the human, adrenocortical adenomas secrete inhibin-like immunoreactive material (Nishi et al. 1995) and that both adenomas and carcinomas immuno-stain positively for inhibin $\alpha$ (Chivite et al. 1998, McCluggage et al. 1998, Munro et al. 1998, Pelkey et al. 1998). This would suggest that inhibin $\alpha$ is not a tumour suppressor for the adrenal cortex in man. Our extended data support this, since positive staining was seen in all adenomas studied, indicating that loss of expression is not an early event in tumorigenesis. This pattern was seen in two of the other three published studies (Chivite et al. 1998, McCluggage et al. 1998, Pelkey et al. 1998) but in the Conn’s cases (Chivite et al. 1998) where 4 of 27 carcinomas were immunonegative, and they have found such a pattern in 2 of 4 further cases (our unpublished observations). This raises the possibility that loss of expression of inhibin $\alpha$ may be linked to tumour progression in some cases. Again, DNA sequencing would permit more detailed analysis. There are no previously published studies on expression of $\beta$ subunits in adrenocortical tumours. We have demonstrated immunopositivity for both, suggesting that the full range of inhibins and activins could be produced. However, lower intensity of $\beta B$ in carcinomas relative to adenomas might indicate a different balance of expression. This could lead to different interactions which might affect growth or function. Quantitative analysis of tumour extracts would be of interest to confirm the relative levels of expression.

Nishi et al. (1995) provided data to suggest that tumours associated with Cushing’s syndrome secreted higher levels of inhibin-like material than those causing other clinical syndromes again linking inhibin $\alpha$ to cortisol production. In our analysis, which was performed blind with respect to the clinical data, we demonstrated higher intensity of staining for inhibin $\alpha$ in the Cushing’s cases. This was also reported by Pelkey et al. (1998). This would be one of the patterns expected if Cushing’s tumours were secreting higher levels. The other pattern would be weak staining if they secreted, but did not store, the peptide. The more patchy distribution of inhibin $\alpha$ seen in the Conn’s cases is similar to that described by Chivite et al. (1998), although the reasons for this are unclear. McCluggage et al. (1998) did not report any differences between Cushing’s and Conn’s tumours in their studies.

### Table 2: Immunoreactivity index (histoscore) for inhibin subunits in adrenocortical tumours classified by clinical syndrome

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Number of cases</th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>Cushing's syndrome</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>$\beta A$</td>
<td></td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>$\beta B$</td>
<td></td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Conn's syndrome</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>$\beta A$</td>
<td></td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>$\beta B$</td>
<td></td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Virilisation/non-functional</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>$\beta A$</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>$\beta B$</td>
<td></td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

The histoscores, derived by multiplying the numerical value for the extent and intensity of staining, were categorised as negative (0), low (1–3), medium (4–6), high (7–9).
Our data suggest that inhibins and activins may have a physiological role as they are expressed in the normal adrenal cortex. The differential zonal distribution warrants further investigation. Their presence in adrenocortical tumours raises the possibility of a putative autocrine/paracrine role in growth and/or function. To gain further insight into their actions, it will be important to look also at the expression of the various activin receptors and of their binding proteins, follistatin and α₂-macroglobulin.

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