Inhibin/activin and ovarian cancer

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Abstract

Inhibin and activin are members of the transforming growth factor beta (TGFβ) family of cytokines produced by the gonads, with a recognised role in regulating pituitary FSH secretion. Inhibin consists of two homologous subunits, α and either βA or βB (inhibin A and B). Activins are hetero- or homodimers of the β-subunits. Inhibin and free α subunit are known products of two ovarian tumours (granulosa cell tumours and mucinous carcinomas). This observation has provided the basis for the development of a serum diagnostic test to monitor the occurrence and treatment of these cancers. Transgenic mice with an inhibin α subunit gene deletion develop stromal/granulosa cell tumours suggesting that the α subunit is a tumour suppressor gene. The role of inhibin and activin is reviewed in ovarian cancer both as a measure of proven clinical utility in diagnosis and management and also as a factor in the pathogenesis of these tumours. In order to place these findings into perspective the biology of inhibin/activin and of other members of the TGFβ superfamily is also discussed.

Introduction

In contrast to other gynaecological cancers, little progress has been made over the past decades in improving the life expectancy of women with ovarian cancer. Symptoms of ovarian cancer are vague and often confused with other abdominal ailments. As a consequence the majority of women with ovarian cancer (60–65%) are diagnosed at a late stage of the disease when the cancer has spread beyond the confines of the ovary. Their five-year life expectancy is 20–40%. If the cancer is detected while confined to the ovary, the corresponding life expectancy is >85% (Holschneider & Berek 2000). These statistics highlight the need to develop markers which detect ovarian cancers in the earliest stages of the disease.

Inhibin is a product of the normal ovary and of two ovarian tumours, granulosa cell tumours (GCT) and mucinous carcinomas. In addition, the inhibin α subunit, based on gene deletion studies in mice, has been postulated to be an ovarian tumour suppressor. This review examines the localisation and expression of the inhibin/activin subunits in the various ovarian cancers and explores their synthesis, regulation and signalling pathways. The development of inhibin diagnostic tests and their sensitivity/specificity characteristics are also discussed.

Inhibin and activin

Structure

Inhibin and activin are members of the transforming growth factor beta (TGFβ) family of cytokines of which over 40 members have now been identified (Kingsley 1994). Other members include Müllerian inhibitory substance, bone morphogenetic proteins (BMP) and growth differentiating factor (GDF) 9/9B. These cytokines are characterised structurally by similarities of primary sequence particularly with the location of the cysteine residues in the carboxy terminal region of the molecule, the presence of a ‘double cysteine knot’ architecture and the need for a dimeric structure (in most instances) to exhibit biological activity. Most members signal through a specific dimeric receptor complex linked to a serine/threonine kinase transduction system. Their biological activities are varied, with both stimulatory and inhibitory activities involved in proliferation and differentiation of many organ systems.

Inhibin consists of a dimer of two subunits (α and either βA or βB subunits) to form inhibin A and inhibin B (Fig. 1). The three subunits are products of separate genes located on chromosomes 2 (α and βB subunit) and 7 (βA subunit). There is 23–27% amino acid homology between the α subunit and either β subunit, with 64% homology between the β subunits (Kingsley 1994). There is also close
homology between species; in particular the $\beta$A and $\beta$B subunit sequences remain essentially unchanged (1 amino acid difference seen in sheep) in a large range of species.

Activins are dimers of the $\beta$ subunit, the most common being activin A, AB and B. Other activins (C, D, E) have been identified (Fang et al. 1997), although little is known about their biological significance. Activins C and E are highly expressed in liver; however their biological activity has not been established (Lau et al. 2000). The $\beta$C subunits are unable to dimerise with the $\alpha$ subunit to form $\alpha$-$\beta$C dimers (Mellor et al. 2000).

Inhibins A and B were originally isolated based on their ability to specifically suppress follicle-stimulating hormone (FSH) secretion from pituitary cells in vitro.

**Figure 1** Schematic diagram of precursor and mature forms of inhibin and its free $\alpha$ subunit. The mature form of activin is also presented. The 6 kDa region at the N-terminus of the $\alpha$-subunit is ‘Pro’, the 23 kDa region is $\alpha$N and the 20 kDa region is $\alpha$C. The 43 kDa N-terminal region of the $\beta$-subunit is Pro-$\beta$.
while activin was isolated based on its ability to stimulate FSH secretion from pituitary cells, an action now believed to be opposed by inhibin (Vale et al. 1990). It is still unclear if inhibin A and B exhibit qualitatively and quantitatively similar or dissimilar biological activities. Inhibin A and B show similar bioactivities in vitro with rat pituitary cells (Ling et al. 1986); however inhibin B exhibits 15–20% the activity of inhibin A in ovine pituitary cell cultures (Robertson et al. 1997). Furthermore, activin A inhibits the DNA synthesis stimulated by epidermal growth factor (EGF) in primary cultures of rat hepatocytes whereas activin B is without activity (Niimi et al. 2002). It is unclear if inhibin A and B or indeed the activin isoforms have comparable activities in the human or higher primates.

**Signalling pathways**

In contrast to that of inhibin, the activin signalling pathway is well characterised (see recent reviews by Massague & Chen 2000, Phillips 2003; Fig. 2). Activin initially binds to an activin type II receptor (of which there are 2 subtypes, ActRIIA and B) with high affinity and specificity. Following its binding, the complex recruits a second (type I) receptor (ActRIA or ActRIB, also known as ALK4 and ALK2 respectively) to form an activin-type I–type II receptor complex. This union promotes phosphorylation at a serine or threonine type I receptor which leads to the activation of a second messenger system involving protein mediators called Smads (sons of mothers against decapentaplegia) of which 8 types have been identified (Wrana & Attisano 2000). Activin specifically causes the association of Smad 2 and/or 3 with a companion Smad (co-Smad 4) which together are transferred to the nucleus. In conjunction with activating factors this leads to gene stimulation. Smads (2, 3 and 1, 5, 8) have also been identified as part of the TGFβ and BMP signalling pathways respectively, although TGFβ members can transduce a signal with different combinations of the various receptor and Smad subtypes. Inhibitory Smads (Smad 6 and 7) have also been identified although their roles are unclear.

The signalling pathway for inhibin is less clearly understood. To date no specific receptor has been isolated.
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nor specific threonine/serine kinases identified from genome searches (Pangas & Woodruff 2000). Based on the inhibition by inhibin of the stimulatory action of activin on pituitary FSH secretion and other cell systems where inhibin antagonises activin’s action (see Robertson et al. 2000), it has been suggested that inhibin may exert its biological effect by antagonising the binding or signalling of activin to the activin receptor rather than acting through a specific inhibin receptor. At high concentrations, inhibin has been shown to antagonise activin by binding to ActRII (Martens et al. 1997, Lebrun et al. 1999). This hypothesis was strongly supported by the observation that the association of inhibin with an ancillary binding protein (betaglycan, or TGF-β receptor type III) promoted inhibin binding to ActRII and thus was a very potent antagonist of activin binding and action (Lewis et al. 2000). The inhibitory action of inhibin on FSH secretion at the pituitary was therefore attributed to inhibin binding to betaglycan to inhibit stimulation of FSH secretion by activin.

However, while the involvement of betaglycan appears important, other observations suggest a more complex mechanism. Chong et al. (2000) identified a factor (p120 or inhibin binding protein (InhBP)) which, while it did not bind inhibin, facilitated the antagonism of activin signalling by inhibin (Bernard et al. 2002). Studies from our laboratory have shown that inhibin A but not inhibin B can bind to gonadal cell lines with very high affinity (Kd 50 pM) (Farnworth et al. 2001, Harrison et al. 2001) with limited crossreaction with activin (<0.1%). Based on crosslinking studies, four molecular weight species were identified, two of which were different molecular forms of betaglycan, while the other two species are unknown. These results suggest that there are additional (unknown) binding proteins involved in the binding of inhibin A to these cells. To date, we have not been able to identify high affinity binding proteins for inhibin B in a range of gonadal and other cell lines (unpublished observations). These studies again raise the question of whether the differences in biological activity between inhibin A and B are a function of separate binding systems.

Whether inhibin can exert its biological activity through its own receptor or through competition with other members of the TGFβ family is yet to be established. Studies have shown that in some systems both inhibin and activin have a stimulatory effect. Such observations are difficult to explain based on the activin inhibitory hypothesis. However, recently it has been shown that inhibin can also inhibit BMP signalling, suggesting a wider mode of action (Wiater & Vale 2003, Fig. 2). This observation may provide an explanation for the activin-independent stimulatory effects of inhibin noted above – inhibin may be exerting an effect through the BMP signalling system. Other binding proteins are also important. Follistatin (Phillips 2003) and follistatin-related peptide (FSRP) (Sidis et al. 2002) bind activin and BMP with high affinity although BMP has a lower affinity than activin with both proteins showing limited binding of inhibin. It is recognised that follistatin plays an important role in regulating activin activity in several biological systems (for review see Phillips 2003). A diverse range of other molecules can associate with the TGFβ/activin/BMP receptor subunits including cripto, a member of the EGF-CFC family of EGF-related proteins (Salomon et al. 2000). Cripto functions as a coreceptor for the activin type I receptor and is expressed in primary ovarian tumours (D’Antonio et al. 2002). Ninety percent of mucinous tumours of high malignant potential express cripto (Salomon et al. 2000). The potential role of these molecules in inhibin and/or BMP signalling has not been explored.

Endocrinology

The role of inhibin in vivo as a regulator of pituitary FSH secretion and the interplay of inhibin/FSH and possibly activin in regulating ovarian activity are now well understood (for reviews see Burger 1992, Baird & Smith 1993, Knight 1996). Inhibin exerts a specific inhibitory effect on FSH secretion, the effect of which is probably promoted by secreted ovarian steroids. In the late secretory phase of the human menstrual cycle, the fall in serum inhibin together with oestradiol and progesterone results in a rise in serum FSH which promotes ovarian granulosa cell proliferation. It is believed that FSH, in conjunction with endogenously produced activin (presumably activin B) and possibly oocyte-produced GDF (Jaatinen et al. 2002), would render the follicles FSH responsive by stimulating the production of FSH receptors on granulosa cells. During the early follicular phase, the elevated FSH levels promote follicular development with a parallel increase in serum activin (presumably activin B) and inhibin B (Groome et al. 1996a). By day 5–9 of the menstrual cycle, the circulating inhibin B levels are sufficiently high to generate a significant negative feedback effect on FSH production. As a consequence, FSH levels begin to fall. Meanwhile the dominant follicle which is now responsive to luteinising hormone (LH) produces more inhibin A than B such that by the time of ovulation little inhibin B is produced by the dominant follicle, although smaller developing follicles continue to do so. Following the LH surge, and with the luteinizing effect of LH, inhibin B and βB subunit production ceases. With the formation of the corpus luteum, inhibin A and activin A are produced and secreted together with
progesterone although this is not evident in other non-primate species.

The serum levels of inhibin B in the early follicular phase provide a good marker of those follicles which are likely to be responsive to FSH treatment, while serum inhibin A, like serum oestradiol, provides a good index of the stage of development of the dominant follicle (Eldar-Geva et al. 2000).

With the depletion of ovarian follicles at menopause, serum inhibin levels decrease to nondetectable levels (Burger et al. 1999). Serum FSH and LH (due to decreased ovarian steroid production) increase dramatically. Serum activin, since it is produced and secreted from a number of tissues other than the ovary, shows little change (Muttukrishna et al. 1996).

Localisation in the ovary

The inhibin subunits are differentially expressed in different cellular compartments of the normal ovary. Their location is outlined in Table 1. The α, βA and βB subunits as assessed by in situ hybridisation and immunocytochemistry are found in granulosa cells of preantral, small antral and large antral follicles, with the βB subunit identified more intensely in granulosa cells of preantral follicles compared with those of larger follicles (Roberts et al. 1993, Jaatinen et al. 1994, Pelkey et al. 1998). There is little evidence of subunit expression or protein in primordial granulosa cells.

The α subunit mRNA has been detected in theca cells surrounding preantral and small antral follicles although the α subunit protein is not readily detected (Roberts et al. 1993, Jaatinen et al. 1994, Pelkey et al. 1998). There is little evidence of subunit expression or protein in primordial granulosa cells.

In surface epithelia, α and βA subunit mRNA, as assessed by RT-PCR and in situ hybridisation, were detected in some but not all human ovaries investigated (Zheng et al. 1997). The α and β subunit mRNA and protein have been identified in luteinised stromal cells and ovarian cortex/hilar cells although the α subunit was more readily detectable. In oocytes (human and rat) neither βA nor βB mRNA were detectable while the α subunit is present at low levels.

Other components of the TGFβ receptor family signalling pathway, ActR (1A, IB, RIIA, RIIB), BMPR (1A, IB, II), MIS RII, Smads 1–8 and betaglycan, have been identified in the ovary (Shimasaki et al. 1999, Lewis et al. 2000, Findlay et al. 2002).

Action and regulation in the ovary

FSH, human chorionic gonadotrophin/LH and GDF9 stimulate inhibin A and B production by stimulating inhibin α, βA and βB gene expression in primary cultures of human granulosa cells in vitro (Roh et al. 2003). The βB subunit, and thus inhibin B, is also stimulated by TGFβ, activin A and BMP-2 (Eramaa et al. 1995, Jaatinen et al. 2002).

The role of inhibin in normal ovarian physiology is not well understood. Inhibin has no mitogenic effects in rat (Miro & Hillier 1996) or human granulosa cells (Rabinovici et al. 1990) in vitro nor effects on progesterone synthesis by monkey corpora lutea in vitro (Brannian et al. 1992). In theca cell lines, inhibin in the presence of forskolin stimulates androgen production (Rainey et al. 1996).

It has been shown that inhibin α subunit at high (μg/ml) concentrations similar to those found in follicular fluid is able specifically to compete with FSH for FSH receptors (Schneyer et al. 1991) and to promote oocyte maturation (Silva et al. 1999).

Activin, on the other hand, has diverse functions within the ovary. Activin stimulates proliferation and DNA synthesis of human granulosa cells in vitro (Rabinovici et al. 1990). Studies with rat granulosa cells (Miro & Hillier 1996) showed that activin stimulates DNA synthesis while FSH or the cAMP analogue alone did not. However, in combination with activin, FSH/cAMP analogue caused a dose-related increase in DNA synthesis. These studies suggest that FSH in the presence of activin is mitogenic. Activin has been shown to exert a number of effects related to granulosa cell function including steroid synthesis, FSH receptor and follistatin synthesis (see Findlay 1994 for review).

Serum inhibin and activin

Inhibin A and B are produced and in many cases secreted as precursor dimers of 105–120kDa (Fig. 1). The precursor α subunit is subject to cleavage at two serine protease sensitive sites, while the β subunits are primarily cleaved at one site. The 120 kDa inhibin precursor is thus cleaved to form a series of intermediate forms, with 30 kDa inhibin A/B forms being the most common mature products. It is the mature forms, in particular 30 kDa inhibin A, which have been extensively studied. The N region of the α subunit contains two glycosylation sites (human) while the mature β subunit contains none. The activin dimer precursor is cleaved primarily to the 25 kDa mature dimeric form.

The free α subunit is also secreted. It is found as the full precursor (Pro-αN-αC) or the more common cleavage product (Pro-αC) where the Pro- and αC regions are
Table 1 mRNA expression and cellular identification by *in situ* hybridization and immunohistochemistry of inhibin/activin members and follistatin in the normal human ovary.

<table>
<thead>
<tr>
<th>In situ hybridization</th>
<th>mRNA</th>
<th>Immunocytochemistry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primordial follicles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jaatinen et al. (1994)</td>
<td>+/-</td>
</tr>
<tr>
<td><strong>Granulosa cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preantral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Small antral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Large antral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td><strong>Thecal cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preantral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Small antral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Large antral</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
<tr>
<td>Atretic</td>
<td>++</td>
<td>Jaatinen et al. (1994)</td>
<td>++</td>
</tr>
<tr>
<td>Oocyte</td>
<td>++</td>
<td>Zheng et al. (1997)</td>
<td>++</td>
</tr>
<tr>
<td>Surface epithelium</td>
<td>4/7</td>
<td>Zheng et al. (1997)</td>
<td>++</td>
</tr>
<tr>
<td>Luteinized stromal cells</td>
<td>++</td>
<td>Roberts et al. (1993)</td>
<td>++</td>
</tr>
</tbody>
</table>

+++,, +, +/-, intensity of stain; -, no specific stain; 4/7, 4 of 7 specimens gave a positive reaction.
di-sulphide linked (Fig. 1). The presence of free αC or β fragments in biological samples is less commonly seen (Robertson et al. 1997).

In addition to the above forms, further processing of the mature forms has been reported, for example a truncated α-βA has been isolated from human follicular fluid (Hasegawa et al. 1994) and ovine testis extracts (Bardin et al. 1987) which may be a product of the purification procedure rather than occurring naturally. The 30 kDa inhibin A and Pro-αC present in serum of women at different stages of pregnancy undergo a reduction in molecular weight as pregnancy develops, which cannot be attributed to the effects of (de)glycosylation (Thirunavukarasu et al. 2002). This processing was attributed to increases in serum proteases in late pregnancy although their site of action is unknown. The 25 kDa activin A remained unchanged throughout pregnancy.

Assays are available for serum inhibin A, B, Pro-αC, and activin A (Groome et al. 1994, 1996a,b). Although these assays were developed for the mature forms they do detect the precursor forms, although in some cases it is unclear to what extent. These assays have been used to monitor serum inhibin levels in various physiological conditions and have been shown to be a useful diagnostic marker for Down’s Syndrome and for monitoring early conditions and have been shown to be a useful diagnostic marker for Down’s Syndrome and for monitoring early pregnancy although their site of action is unknown. The 25 kDa activin A remained unchanged throughout pregnancy.

Studies in plasma/serum show that a large proportion of either inhibin A or B is present as precursor forms (Robertson et al. 1997). Serum inhibin A is present primarily as αN-αC/βA and αC/βA, while inhibin B is found as αC/proβB-βB and αC/βB. The free α subunit is found primarily as Pro-αC with small amounts of Pro-αN-αC. αNαC/αA is cleaved in serum to αC/βA; however it is unclear if αC/proβB-βB is cleaved in serum.

Inhibin and activin in ovarian cancer

Two lines of research have implicated inhibin as an important factor in ovarian cancer: first, in a murine model (Matzuk et al. 1992) where the inhibin α subunit may be a tumour suppressor and secondly, in humans where ovarian tumours have been shown to synthesise inhibin.

Inhibin α subunit as a tumour suppressor

Matzuk and colleagues in a series of studies (Matzuk et al. 1992, 1994, 1996, Coever et al. 1996, Kumar et al. 1996, Cipriano et al. 2000) showed that the targeted deletion of the inhibin α subunit in mice resulted in ovarian tumours with very high penetrance and thus postulated that the inhibin α subunit was a tumour suppressor. This conclusion was complicated by the endocrine/paracrine changes that occurred with the loss of the α subunit. With suppression of the α subunit and hence suppressed dimeric inhibin synthesis, there was a marked increase in serum FSH which led to stimulation of activin production by the ovary. Activin levels were also elevated as formation of αβ dimers was not possible in the absence of the α subunit. The α inhibin-null mice have cachexia-like symptoms (Matzuk et al. 1994) that appear to result from excessive secretion of activin. Deletion of the ActRIIA gene ameliorates the cachexia, but not the tumour development (Coever et al. 1996).

However, the role for activin as a tumour promoter is open to question. Mice deficient in both the inhibin α subunit and FSH have substantially lower activin levels, yet ovarian tumours are still formed although at a lower rate (Kumar et al. 1996). Overexpression of FSH in mice does not result in ovarian tumours (Kumar et al. 1999). Additional studies (Cipriano et al. 2000) where α subunit-deficient mice were crossed with mice overexpressing follistatin, which would reduce the locally produced activins through its high affinity binding to activin, still showed tumour production, although at a lower rate compared with α subunit-deficient mice alone. The cachexia was also blocked. It appears that activin may

Table 2 Detection of inhibin α and β subunit mRNAs in ovarian tumours. Values in brackets refer to number of samples examined.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>α Subunit mRNA</th>
<th>α A Subunit mRNA</th>
<th>βB Subunit mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>49% (30)</td>
<td>89% (29)</td>
<td>100% (4)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>68% (25)</td>
<td>88% (26)</td>
<td>100% (5)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>40% (5)</td>
<td>80% (5)</td>
<td></td>
</tr>
<tr>
<td>GCT</td>
<td>100% (4)</td>
<td></td>
<td>100% (4)</td>
</tr>
</tbody>
</table>

Reviewed in Risbridger et al. (2001)

Table 3 Detection of inhibin α and β subunit by immunocytochemistry in ovarian tumours. Values in brackets refer to number of samples examined.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>α Subunit</th>
<th>βA Subunit</th>
<th>βB Subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>1% (124)</td>
<td>76% (96)</td>
<td>100% (15)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2% (85)*</td>
<td>93% (55)</td>
<td>100% (17)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>68% (22)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex cord: GCT</td>
<td>7% (69)</td>
<td>59% (29)</td>
<td></td>
</tr>
<tr>
<td>Sex cord: other</td>
<td>99% (227)</td>
<td>81% (21)</td>
<td>100% (4)</td>
</tr>
</tbody>
</table>

Reviewed in Risbridger et al. (2001) * Detected using R1 Mab; ** detected using other α subunit antisera.

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play a role as a tumour promoter; however other factors are clearly involved.

Gonadotrophins are essential for the development of these tumours in that when the inhibin-null mice are crossed with gonadotrophin-deficient mice (hpg mice), the tumours fail to develop in the double-null homozygotes (Kumar et al. 1996). Conversely, when FSHβ-deficient mice were crossed with inhibin α null mice, the tumours still developed but at a later stage (Kumar et al. 1999). The major difference between the two double knockouts is the retention of LH secretion in the latter line (Kumar et al. 1999). Transgenic mice over expressing LH develop granulosa cell tumours (Risma et al. 1999). Transgenic mice over expressing LH develop granulosa cell tumours (Risma et al. 1999) albeit with marked strain specificity. These tumours produce inhibin. Ovariectomy of inhibin α subunit-deficient mice (but not wild-type mice) results in the development of tumours in the adrenal cortex (Matzuk et al. 1992). The adrenal cortex is known to express inhibin α and β subunits and to be sensitive to adrenocorticotropic hormone (ACTH) and LH stimulation. Since ovariectomy causes serum FSH and LH levels to increase, a further possibility is that the elevated LH is responsible for the adrenal tumours; however the loss of the α subunit is also a critical factor. These studies suggest that in addition to the loss of the α subunit, LH and possibly FSH are important stimulators of these ovarian tumours. Whether activin is an intermediate in this process is yet to be resolved.

**Inhibin production by ovarian tumours**

Using a radioimmunoassay which detected all inhibin forms, Lappohn et al. (1989) reported elevated inhibin levels in stored sera from patients with GCT. Subsequently, Healy et al. (1993) observed that in postmenopausal women, where serum inhibin levels are normally very low or undetectable, levels were elevated in women with granulosa cell and mucinous tumours. The levels were sufficiently high and were detected sufficiently early in the progression of the disease to be used as a diagnostic test both in detection and in monitoring recurrence of the disease following treatment. These observations led to a series of studies exploring inhibin as a marker of other ovarian cancers.

**Localisation of inhibin and activin in ovarian tumours**

Based on their morphology, ovarian tumours can be divided into epithelial, stromal and germ cell tumours of which epithelial tumours represent 90% of the total. Epithelial tumours, in turn, have been further classified according to their morphology (serous, mucinous, endometrioid, clear cell or undifferentiated) with serous tumours the most prevalent. Among the sex cord tumours, GCT are the most common. Epithelial tumours (serous and mucinous) have also been classified as to whether they are benign, borderline or malignant.

The presence of α, βA, βB subunit mRNA (RT-PCR) and protein (immunocytochemistry) have been detected in GCTs and a range of other sex cord tumours e.g. Sertoli cell, Sertoli cell/Leydig cell tumours (Tables 1, 2). However, fibroma/thecomas, while showing evidence of α subunit immunoreactivity, showed no βA subunit reactivity (Choi et al. 2000). Inhibin α, βA and βB subunit mRNAs have been detected by RT-PCR (but not as yet by *in situ* hybridisation) in serous tumour tissue while βA and βB subunit immunoreactivity but not α subunit has been identified by immunocytochemistry (Zheng et al. 1997, 2000, Fuller et al. 1999, 2002a,b, Yamashita et al. 1999).

Inhibin α, βA and βB subunit mRNA (Zheng et al. 1997, Fuller et al. 1999) and βA and βB subunit protein have been readily identified in mucinous tumour tissue (Tables 1–3) (Gurusisinghe et al. 1995, Kommoss et al. 1998, Yamashita et al. 1999, Choi et al. 2000, Zheng et al. 2000). However, inhibin α subunit localisation by immunostaining in mucinous tumours is still unresolved, as marked differences have been observed between studies which are most likely attributable to differences in antibody selection and/or fixation procedures (Burger et al. 2001). Luteinised stromal tissue surrounding both mucinous and serous tumours showed α subunit immunocytochemical activity. Similar to mucinous tumours, endometrioid tumours show the presence of α and β subunit mRNA, and βA subunit by immunocytochemistry (Zheng et al. 1997, Kommoss et al. 1998, Choi et al. 2000) with the α subunit protein detected to a limited extent. The α subunit protein was not detected in germ cell tumours using the α subunit monoclonal antibody (R1 Mab) although positive responses have been obtained using an α subunit polyclonal antiserum (Cobellis et al. 2001).

It was suggested (Kommoss et al. 1998) that, based on the high number of tumours which showed a positive α subunit immunocytochemical response in the stroma, the observed elevated serum inhibin levels were a consequence of production and secretion by the stroma rather than the epithelial tumour cells. Serum inhibin levels correlate with the extent of the stroma in the tumour, with stroma in mucinous cancers being much more extensive than other tumour types e.g. serous. Zheng et al. (2000) examined the expression of α and βA subunits by RT-PCR following microdissection and immunocytochemistry in stromal and epithelial tissue of a range of ovarian tumours. In this study, βA subunit was expressed in the epithelium to varying degrees but not in the stroma, while α subunit immunoreactivity was localised to the stroma in a small
subset of tumours examined by microdissection. It was concluded that these epithelial cancer cells are not expressing the α subunit and that the α subunit is not a tumour marker per se. It follows, therefore, that for ovarian epithelial tumours, inhibin measurements may not be appropriate for monitoring the disease when the tumour has metastasised to other tissues. Stromal production of inhibin should not preclude the use of inhibin to monitor the presence of the tumour in the ovary. Conversely, in situ hybridisation studies have localised inhibin α-subunit mRNA to the epithelial cells in these cancers (Burger et al. 2001).

Of mice and men: is the inhibin α subunit a tumour suppressor?

Granulosa cell tumours develop in mice lacking the inhibin α gene yet human GCTs produce significant amounts of the dimeric inhibins. This paradox requires discussion. Watson et al. (1997) failed to find any evidence of loss of heterozygosity at the inhibin α subunit gene locus in a cohort of granulosa cell tumours. To reconcile this apparent contradiction, Matzuk et al. (1996) has suggested that human granulosa cell tumours may be resistant to inhibin. Such resistance could occur at the level of a receptor or in downstream signalling pathways. We (Fuller et al. 2002b) have examined the activin-inhibin receptor subunit genes in a cohort of ovarian tumours. We could not find clear evidence of loss of expression of any of the subunits examined in these tumours. The p120 gene (Chong et al. 2000) shows remarkable granulosa cell-specific expression with absence of expression in some tumours and high expression in other tumours. Although p120 is arguably an attractive candidate, Bernard et al. (2002) have recently cast some doubt on its role as an inhibin receptor.

The notion that loss of inhibin binding is pathogenic implies, given its apparent role as an antagonist, that a signal is constitutively active. It also follows that an inhibin-specific component of the receptor system must be lost. An alternate and arguably more plausible hypothesis is that the relevant pathway is constitutively activated, i.e. that some downstream component is mutated. As discussed above, there is a plethora of candidates (Findlay et al. 2002) but it is not clear which of these pathways is mitogenic and indeed which is relevant in these tumours. We have sought to clarify this question by defining granulosa cell tumours in terms of normal granulosa cell biology (Chu et al. 2000, 2002). Our data based on gene expression profiles suggest that granulosa cell tumours have a phenotype which is similar to that of the proliferating cells of the antral follicle, a stage which is FSH-dependent. One could argue that both the GDF9–BMP signalling pathway and the activin signalling pathway are potential candidate pathways in this context given that both have been shown to be inhibited by inhibin.

In a broader context, mutations in the TGFβ signalling pathway are a common feature of many cancers and there is recent evidence that this may extend to other members of the superfamily (Massague et al. 2000, Eng 2001). Loss of function mutations have been identified e.g. the TGFβ type II receptor in colorectal cancer, Smad4-DPC4 in chronic polyposis and pancreatic cancer, and the BMP type I receptor in colonic polyposis (Eng 2001). The mutations described are in ‘suppressor genes’ rather than activation of a pathway as is proposed for GCT. A recent study from Ala-Fossi et al. (2000) provides some support for the inhibin hypothesis; in a retrospective study of 30 patients who had previous surgery for GCT, they found that four patients whose tumours were negative for inhibin immunoreactivity had a far worse outcome than those who were inhibin positive. Unfortunately there was no information on inhibin levels. Inhibin-negative tumours were associated with the more advanced stage. Although loss of inhibin synthesis may, in this context, simply be an epiphenomenon of the malignant process, one could also argue, particularly in the context of the mouse models, that the absence of inhibin is of pathogenetic significance.

Wang et al. (2000) examined 32 ovarian cancers for mutations in the TGFβ signal transduction pathway. The tumours included a wide range of histologic types, but no stromal tumours. Thirty percent of the tumours did not express TGFβ receptor type I due to a range of genetic mutations. Recently, mutations of the activin type 1B receptor gene have been reported in pancreatic carcinoma.

Table 4 Detection of serum inhibins, activin A and CA125 by immunoassay in ovarian tumours. Values in brackets refer to number of samples examined.

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Total inhibin</th>
<th>Inhibin A</th>
<th>Inhibin B</th>
<th>Pro-αC</th>
<th>Activin A</th>
<th>CA125</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>18% (42)</td>
<td>5% (66)</td>
<td>2% (66)</td>
<td>15% (66)</td>
<td>50% (8)</td>
<td>94%</td>
</tr>
<tr>
<td>Mucinous</td>
<td>84% (27)</td>
<td>19% (42)</td>
<td>57% (42)</td>
<td>52% (42)</td>
<td>66% (3)</td>
<td>71%</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>54% (14)</td>
<td>18% (11)</td>
<td>9% (11)</td>
<td>30% (10)</td>
<td>0% (6)</td>
<td>91%</td>
</tr>
<tr>
<td>Sex cord: GCT</td>
<td>100% (16)</td>
<td>60% (15)</td>
<td>94% (18)</td>
<td>91% (11)</td>
<td>69% (13)</td>
<td>30%</td>
</tr>
</tbody>
</table>

1 Robertson et al. (2002b); 2 Robertson et al. (1997); 3 Lambert-Messerlian et al. (1999).
suggested that activin may be involved in tumour suppression in this epithelial tissue (Su et al. 2001).

**Role of other TGFβ family members in ovarian cancer**

Mullerian inhibiting substance (MIS) is a glycoprotein hormone member of the TGFβ superfamily which is produced postnatally in the granulosa cells of the ovary. It has been variously reported to both inhibit and stimulate granulosa cell proliferation in the developing follicle. Several studies have reported increased serum MIS levels in patients with both primary and recurrent GCT (Rey et al. 1996, Lane et al. 1999). Rey et al. (1996) examined serum MIS levels in a range of ovarian pathologies. In only GCT did the authors find elevations of MIS (in 8 of the 9 GCT examined). In these 8 patients, the MIS levels tracked with the α inhibin levels.

GDF9 and BMP15 (GDF9B) are two closely related members of the superfamily which are oocyte specific in rodent systems. They promote development of primordial follicles. There is evidence that they are also able to stimulate inhibin B synthesis in granulosa cells in vitro (Jaatinen et al. 2002). Both GDF9 and BMP15 have recently been shown to be expressed in human but not rodent granulosa cells where they act as inhibitors of luteinisation, i.e. differentiation (Yamamoto et al. 2002). Neither of these peptides has been characterised as yet in GCT. TGFβ itself is known to have effects on rodent granulosa cells in vitro but its precise role in vivo is unclear. Generally, the effects of TGFβ are inhibitory, and indeed Dunfield et al. (2002) demonstrated an inhibitory effect of TGFβ on primary cultures of ovarian epithelial cancer cells in culture.

Takakura et al. (1999) examined the status of three candidate tumour suppressor genes, including Smad2 and Smad4, on chromosome band 18q21. Allelic imbalance was identified in 41% of the cancers and in none of the borderline tumours examined, particularly at the Smad4 gene locus. They then examined the Smad4 gene in 42 primary ovarian epithelial tumours and 8 cell lines for mutations. Mutations were identified in 2 tumours and 3 cell lines. The investigations concluded that Smad4 may have a role in ovarian tumorigenesis.

**Clinical applications**

The diagnostic utility of serum inhibin assays is now well established. Immunocytochemical location of inhibin α subunit has been clinically useful in the identification of sex cord stromal tumours and differentiation from some epithelial cancers e.g. endometrioid cancers (Kommoss et al. 1998, Choi et al. 2000) and GCT metastases where diagnosis was difficult (McCluggage 2002).

**Serum patterns of inhibin in women with ovarian cancer**

The initial observations that serum inhibin was elevated in GCT and mucinous tumours were based on a serum radioimmunoassay which detected all α subunit-containing forms (McLachlan et al. 1986). To establish if the sensitivity and specificity characteristics of the assay in terms of detecting ovarian cancers could be improved, studies were undertaken to measure the different forms of inhibin in the various cancer types. Several approaches were used, based primarily on a differentiation between normal controls and women with ovarian tumours. All these studies were confined to women after the menopause (55+ years). Table 4 shows that the more specific assays for detecting serum inhibin A and B poorly discriminated between normal and cancer samples when compared with Pro-αC (free α subunit) assays, while the total inhibin assay which detects both dimeric and monomeric forms gave the best differentiation and was the preferred assay method. It is interesting to note that GCT produce large amounts of dimeric inhibin, inhibin B being the predominant form (Petraglia et al. 1998), while mucinous tumours primarily produce free α subunit.

Studies were undertaken to establish if the inhibin forms produced by ovarian tumours were structurally similar to those observed in serum from normal pre-menopausal subjects (Robertson et al. 2002b). Using a preparative PAGE method which separated inhibin forms according to molecular weight, the precursor and mature inhibin forms were detected using the various inhibin assays. Inhibin forms of similar size for known precursor and mature inhibin forms were detected. A similar pattern of inhibin α subunit immunoreactivity was detected in sera from women with mucinous and serous tumours. The dimeric inhibin forms could not be detected due to the low levels. These studies showed that the inhibins produced by ovarian cancers were similar to inhibins found in the serum of normal subjects and did not contain unusual processed forms as detected in sera from pregnant women (Thirunavukarasu et al. 2002). These results reinforce the finding that current total inhibin immunoassays are measuring all the inhibin forms produced by ovarian cancers.

**Serum inhibin as a marker of GCT and mucinous tumours**

After the menopause, inhibin levels are at or below the detection limits of inhibin assays. These low values provide a good baseline to compare with serum levels in postmenopausal women with ovarian cancer. Application of the total inhibin assay (Lappohn et al. 1989, Healy et al. 1993, Robertson et al. 1999a, 2001) and in particular
the more recently developed total inhibin ELISA (Robertson et al. 2002b) to serum from postmenopausal women with ovarian cancer showed that these assays were suitable for detecting GCT and a high proportion of mucinous carcinomas, but were less effective in detecting other epithelial carcinomas (Table 4). Additional studies determining serum inhibin levels in postmenopausal women with benign tumours (Robertson et al. 2002a) showed a range of responses – 10% for benign cysts, 54% for mucinous cystadenomas and 100% for thecomas.

To what extent the total inhibin assay detected cancers in the earlier stages cannot be established with any confidence at this stage due to the small number of samples available in these early stages. However, elevated inhibin levels were detected in many of these samples suggesting that the assay may be useful for detection during the earlier stages of the disease. More studies are clearly needed.

The inhibin test is also of value in monitoring the recurrence of GCT following surgery. Specific case studies (Jobling et al. 1994) have shown that the recurrence of the disease can be detected much earlier than by other procedures.

Application of the assay to women during their reproductive life has yet to be investigated as serum inhibin levels fluctuate markedly throughout the menstrual cycle. However, serum inhibin levels particularly in GCT can increase dramatically and may be sufficiently elevated to be adequately assessed once a baseline of normal values for younger women has been established.

It was observed that while serum inhibin was an effective marker of GCTs and mucinous tumours, another serum cancer marker, CA125, while less effective for these particular cancers was effective in detecting other epithelial cancers (serous, endometrioid, clear cell, undifferentiated). This complementarity of methods suggested that measurement of both markers should result in the increased detection of the majority of ovarian cancers. Studies have shown that the combination of both methods significantly increased the level of detection of ovarian cancers such that 95% of ovarian cancers were detected at 95% sensitivity (Robertson et al. 1999b, 2001, 2002b). Current studies are directed to the development of an ELISA test which is applicable to clinical diagnostic laboratories. A clinical diagnostic company is now developing the total inhibin kit and early results (Khosravi et al. 2003) are promising. It is hoped that serum inhibin and CA125 will be used as a pair in the detection of ovarian cancer and, where appropriate, the monitoring of these diseases following treatment.

In summary, the role of inhibin as a tumour suppressor in ovarian cancers is still unclear. Studies exploring the role of activin and gonadotrophins in this process suggest a permissive role for activin and FSH; however the data suggest that LH may be a key player. However, LH receptor mRNA levels in GCT are suppressed, questioning the critical role of LH (Chu et al. 2002). These data suggest that inhibin is involved as a tumour suppressor via some other mechanism.

Serum inhibin provides a good diagnostic marker of GCT and mucinous tumours and in combination with CA125 detects the majority of ovarian cancers. However, there is a question as to whether the elevated serum inhibin levels identified with various ovarian cancers, in particular epithelial tumours, are due to direct synthesis by the malignant cells or are produced as a result of secretion by the surrounding stromal tissue. While this does not negate the value of the inhibin test as a marker of ovarian cancers it does raise the question as to its value in monitoring ovarian tumours which have metastasised to other locations in the body.

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