

Inhibin/activin and ovarian cancer

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Abstract

Inhibin and activin are members of the transforming growth factor beta (TGF β) 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 β A or β B (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 TGF β superfamily is also discussed.

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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.

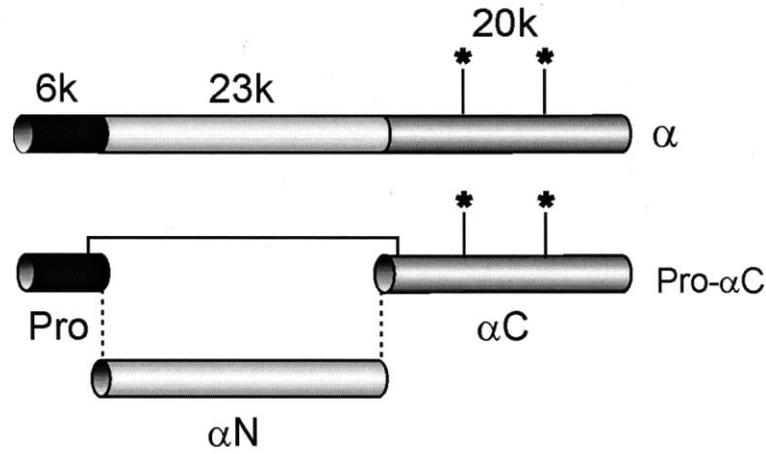
Inhibin and activin

Structure

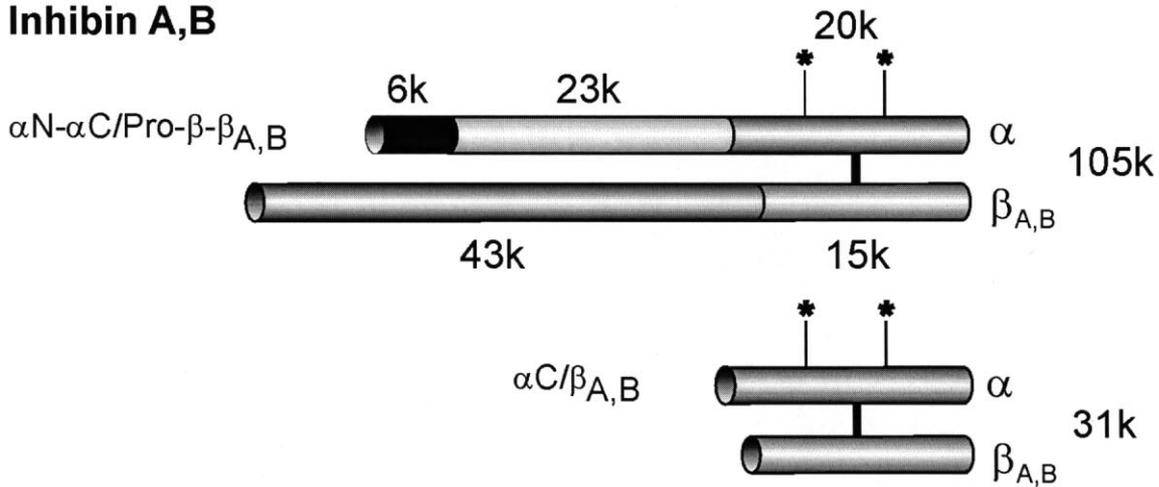
Inhibin and activin are members of the transforming growth factor beta (TGF β) family of cytokines of which over 40 members have now been identified (Kingsley 1994). Other members include Müllerian inhibitory substance, bone morphogenetic proteins (BMP) and growth differentiating factor (GDF) 9/9B. These 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 β A or β B subunits) to form inhibin A and inhibin B (Fig. 1). The three subunits are products of separate genes located on chromosomes 2 (α and β B subunit) and 7 (β A 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

Inhibin α Subunit



Inhibin A,B



Activin A, AB and B

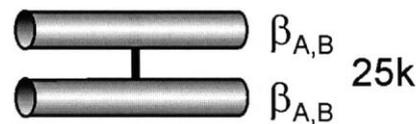


Figure 1 Schematic diagram of precursor and mature forms of inhibin and its free α subunit. The mature form of activin is also presented. The 6 kDa region at the N-terminus of the α -subunit is 'Pro', the 23 kDa region is α N and the 20 kDa region is α C. The 43 kDa N-terminal region of the β -subunit is Pro- β .

homology between species; in particular the β A and β B subunit sequences remain essentially unchanged (1 amino acid difference seen in sheep) in a large range of species.

Activins are dimers of the β 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 β C subunits are unable to dimerise with the α subunit to form α - β 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*

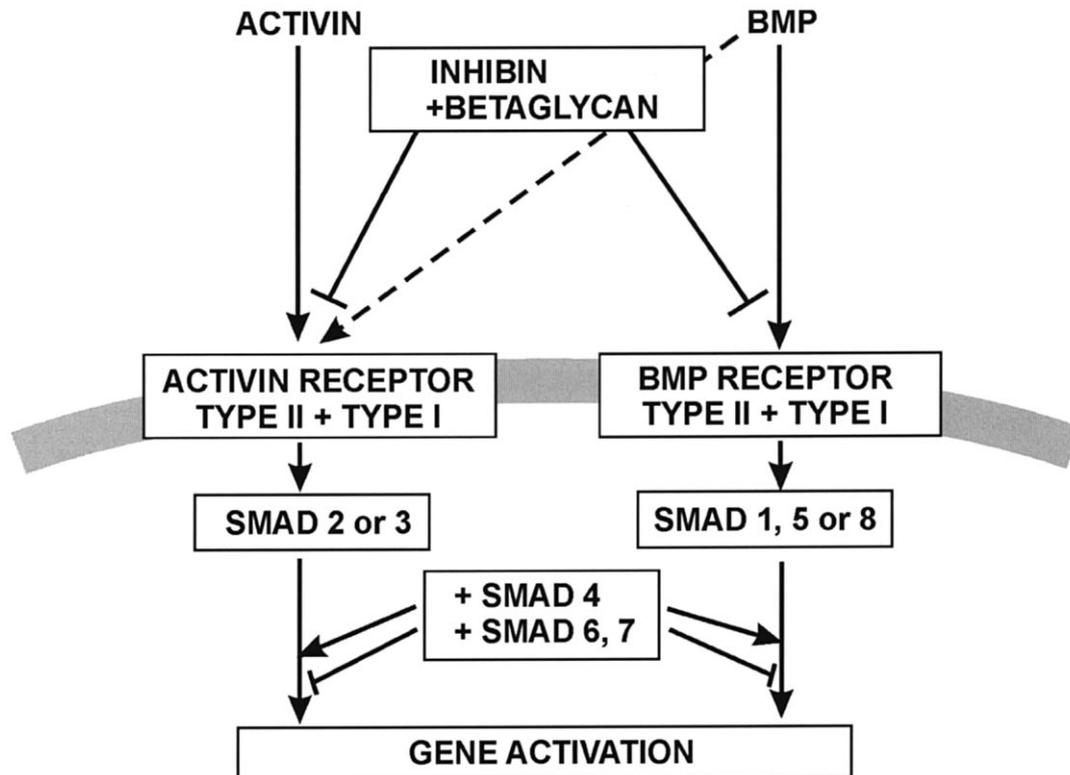


Figure 2 Signalling transduction system for activin and BMP and the inhibitory effects of betaglycan-bound inhibin on that process. Smads 6 and 7 exert an inhibitory action on the Smad pathway. Additional factors have been identified which can regulate this pathway and are not included above. Activin and BMP binding proteins (follistatin and FSRP) have neutralising activities by blocking access to their respective receptors. p120, as for betaglycan, has been identified as an ancillary protein involved in the binding of inhibin.

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 TGF β and BMP signalling pathways respectively, although TGF β 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

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 (K_d 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 TGF β 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 TGF β /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 β B 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 , β A and β B subunits as assessed by *in situ* hybridisation and immunocytochemistry are found in granulosa cells of preantral, small antral and large antral follicles, with the β B 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). Theca cells surrounding large follicles expressed α and β A subunit only. Inhibin subunits were not detected in atretic follicles (Jaatinen *et al.* 1994, Pelkey *et al.* 1998) although α subunit protein was identified in luteal cells surrounding atretic follicles. The α and β A subunit mRNA and protein and β B subunit protein have been detected in corpora lutea.

In surface epithelia, α and β A 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 β A nor β B mRNA were detectable while the α subunit is present at low levels.

Other components of the TGF β receptor family signalling pathway, ActR (IA, IB, RIIA, RIIB), BMPR (IA, 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 , β A and β B gene expression in primary cultures of human granulosa cells *in vitro* (Roh *et al.* 2003). The β B subunit, and thus inhibin B, is also stimulated by TGF β , 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 (μ g/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 105–120 kDa (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 α C 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- α N- α C) or the more common cleavage product (Pro- α C) where the Pro- and α C 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.

| | <i>In situ</i> hybridization | | | | mRNA | | | | Immunocytochemistry | | | | |
|--------------------------|------------------------------|-----------|-----------|---------------------------------|----------|-----------|-----------|----------------------------------|---------------------|-----------|-----------|----|-----------------------------------------------------------------|
| | α | β A | β B | Reference | α | β A | β B | Reference | α | β A | β B | FS | Reference |
| Primordial follicles | | | | | - | - | - | Jaatinen <i>et al.</i> (1994) | +/- | | | | Pelkey <i>et al.</i> (1998) |
| Granulosa cells | | | | | | | | | | | | | |
| Preantral | | | | | + | + | + | Jaatinen <i>et al.</i> (1994) | - | + | + | | Yamoto <i>et al.</i> (1992) |
| Small antral | ++ | + | ++ | Roberts <i>et al.</i> (1993) | + | + | ++ | Jaatinen <i>et al.</i> (1994) | + | + | + | + | Roberts <i>et al.</i> (1993), Pelkey <i>et al.</i> (1998) |
| Large antral | ++ | + | - | Roberts <i>et al.</i> (1993) | + | + | + | Jaatinen <i>et al.</i> (1994) | + | + | + | + | Roberts <i>et al.</i> (1993), Yamoto <i>et al.</i> (1992) |
| Thecal cells | | | | | | | | | | | | | |
| Preantral | | | | | | | | | | | | | |
| Small antral | ++ | - | - | Roberts <i>et al.</i> (1993) | | | | | - | + | - | + | Roberts <i>et al.</i> (1993) |
| Large antral | ++ | + | - | Roberts <i>et al.</i> (1993) | + | + | - | Jaatinen <i>et al.</i> (1994) | - | - | - | - | Roberts <i>et al.</i> (1993), Pelkey <i>et al.</i> (1998) |
| Corpus luteum | ++ | + | | Roberts <i>et al.</i> (1993) | | | | | + | + | + | + | Roberts <i>et al.</i> (1993), Pelkey <i>et al.</i> (1998) |
| Atretic | | | | | - | - | - | Jaatinen <i>et al.</i> (1994) | - | - | - | | Yamoto <i>et al.</i> (1992), Pelkey <i>et al.</i> (1998) |
| Oocyte | | | | | | | | | - | - | | | Zheng <i>et al.</i> (1997) |
| Surface epithelium | | | | | 4/7 | 4/7 | | Zheng <i>et al.</i> (1997) | - | - | | | Zheng <i>et al.</i> (1997) |
| Luteinized stromal cells | - | - | - | Roberts <i>et al.</i> (1993) | | | | | - | - | - | - | Roberts <i>et al.</i> (1993), Zheng <i>et al.</i> (1997) |

++, +, +/-, 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 α C 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 - β A 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- α C 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- α C, 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 follicular development in human assisted reproduction (e.g. Eldar-Geva *et al.* 2000).

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 α N- α C/ β A and α C/ β A, while inhibin B is found as α C/pro β B- β B and α C/ β B. The free α subunit is found primarily as Pro- α C with small amounts of Pro- α N- α C. α N α C/ α A is cleaved in serum to α C/ β A; however it is unclear if α C/pro β B- β B 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, Coerver *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

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

| Tumour | α Subunit mRNA | α A Subunit mRNA | β B Subunit mRNA |
|--------------|-----------------------|-------------------------|------------------------|
| Serous | 49% (30) | 89% (29) | 100% (4) |
| Mucinous | 68% (25) | 88% (26) | 100% (5) |
| Endometrioid | 40% (5) | 80% (5) | |
| GCT | 100% (4) | | 100% (4) |

Reviewed in Risbridger *et al.* (2001)

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 $\alpha\beta$ 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 (Coerver *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 3 Detection of inhibin α and β subunit by immunocytochemistry in ovarian tumours. Values in brackets refer to number of samples examined.

| Tumour | α Subunit | β A Subunit | β B Subunit |
|-----------------|------------------------|-------------------|-------------------|
| Serous | 1% (124) | 76% (96) | 100% (15) |
| Mucinous | 2% (85)* 68% (22)** | 93% (55) | 100% (17) |
| Endometrioid | 7% (69) | 59% (29) | |
| Sex cord: GCT | 99% (227) | 81% (21) | 100% (4) |
| Sex cord: other | 80% (255) | 50% (14) | |

Reviewed in Risbridger *et al.* (2001)

* Detected using R1 Mab; ** detected using other α subunit antisera.

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 FSH β -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.* 1995) 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 adrenocorticotrophin (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 , β A, β B 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 β A subunit reactivity (Choi *et al.* 2000). Inhibin α , β A and β B subunit mRNAs have been detected by RT-PCR (but not as yet by *in situ* hybridisation) in serous tumour tissue while β A and β B 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 , β A and β B subunit mRNA (Zheng *et al.* 1997, Fuller *et al.* 1999) and β A and β B subunit protein have been readily identified in mucinous tumour tissue (Tables 1–3) (Gurusinghe *et al.* 1995, Kommos *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 β A subunit by immunocytochemistry (Zheng *et al.* 1997, Kommos *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 (Kommos *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 β A subunits by RT-PCR following microdissection and immunocytochemistry in stromal and epithelial tissue of a range of ovarian tumours. In this study, β A 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 TGF β 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 TGF β 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 TGF β signal transduction pathway. The tumours included a wide range of histologic types, but no stromal tumours. Thirty percent of the tumours did not express TGF β 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.

| Tumour | Total inhibin ¹ | Inhibin A ² | Inhibin B ² | Pro- α C ² | Activin A ³ | CA125 ¹ |
|---------------|----------------------------|------------------------|------------------------|------------------------------|------------------------|--------------------|
| Serous | 18% (42) | 5% (66) | 2% (66) | 15% (66) | 50% (8) | 94% |
| Mucinous | 84% (27) | 19% (42) | 57% (42) | 52% (42) | 66% (3) | 71% |
| Endometrioid | 54% (14) | 18% (11) | 9% (11) | 30% (10) | 0% (6) | 91% |
| Sex cord: GCT | 100% (16) | 60% (15) | 94% (18) | 91% (11) | 69% (13) | 30% |

¹ Robertson *et al.* (2002b); ² Robertson *et al.* (1997); ³ Lambert-Messerlian *et al.* (1999).

suggesting that activin may be involved in tumour suppression in this epithelial tissue (Su *et al.* 2001).

Role of other TGF β family members in ovarian cancer

Mullerian inhibiting substance (MIS) is a glycoprotein hormone member of the TGF β 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 they 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. TGF β itself is known to have effects on rodent granulosa cells *in vitro* but its precise role *in vivo* is unclear. Generally, the effects of TGF β are inhibitory, and indeed Dunfield *et al.* (2002) demonstrated an inhibitory effect of TGF β 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 (Kommos *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- α C (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 premenopausal 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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