Paracrine factors relevant to the regulation of spermatogenesis – a review

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Introduction

In the last two decades, it has become increasingly evident that disturbances of the hypothalamic–pituitary–gonadal axis account for only a small percentage of the cases seen in male infertility clinics. In an attempt to clarify the pathophysiology of idiopathic male infertility and also for the development of new methods for male contraception, researchers have focused on local regulators of intratesticular events (Bartlett *et al.*, 1989; Hamilton and Waites, 1989). The number of factors implicated in paracrine regulation has been steadily increasing and it is essential that the data available are critically examined. The object of this review is to pinpoint the factors of potential or proven physiological significance and to concentrate on the more recent advances made in the field.

The two major areas of activity within the testis centre on steroidogenesis and spermatogenesis. The intratesticular control of steroidogenesis has already been extensively reviewed (Sharpe, 1990) and this paper will therefore concentrate on the regulation of Sertoli cell function and of the cycle of the seminiferous epithelium. A number of questions should be addressed. Does the process of initiation and maintenance of spermatogenesis require additional fine tuning by locally produced substances at the cellular level in the testis? Which of the large number of potential paracrine regulators of spermatogenesis described are of proven physiological significance? In assessing the large body of data available it is necessary to consider which techniques are best adapted to the study of paracrinology and what pitfalls are inherent in these techniques.

Does the process of initiation and maintenance of spermatogenesis require additional fine tuning by locally produced substances at the cellular level in the testis?

The complicated kinetics of the seminiferous epithelium have long attracted the attention of researchers. At a given time point, a cross-section of the testis reveals adjacent tubules at different stages of the cycle of the seminiferous epithelium. Once the complexity of intratesticular architecture is appreciated, it immediately becomes clear that FSH and testosterone, although generally accepted as the prime regulators of spermatogenesis, can simultaneously maintain the optimal intratesticular environment and also trigger the cascade of events essential for germ cell division and differentiated cell function. For this reason, efforts have been directed to studying intratesticular events. However, evidence supporting the hypothesis of paracrine regulation as an important entity in the testis is predominantly indirect and conclusive evidence is still lacking.

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Idiopathic infertility still represents one of the commonest diagnosis made. In this subgroup of patients with oligoazoospermia or azoospermia, there is no apparent hormonal deficiency: FSH concentrations are often normal or even high. Testicular biopsy often reveals maturation arrest or quantitatively inefficient spermatogenesis. A number of studies have been carried out on the pulsatile secretion of GnRH, and the subsequent release of FSH and LH as well as the gonadal feedback control of gonadotrophin secretion (Wagner *et al.*, 1984; Wagner and von zur Mühlen, 1987). If this represents the major pathophysiology underlying idiopathic male infertility, it would be reasonable to assume that pulsatile administration of physiological doses of LHRH would lead to normal semen parameters. However, in a double blind controlled study, serum FSH returned to normal concentrations but no improvement in semen parameters was observed after pulsatile LHRH administration (Bals-Pratsch *et al.*, 1989).

Other pathological phenomena have been proposed to underlie male idiopathic infertility. For example, isoforms of gonadotrophins, with different glycosylation patterns, altered bioactivity or both (Wang *et al.*, 1987; Jockenhövel *et al.*, 1990; Fauser *et al.*, 1990; Matzkin *et al.*, 1990), could account for the clinical findings outlined above. Alternatively, defects at the FSH or androgen receptor level can be implicated, for example, either at the level of ligand–receptor interaction (Namiki *et al.*, 1984; Buch *et al.*, 1991) or at the post-receptor level. In the latter case, defects may occur at the coupling site with second messenger systems or during protein phosphorylation. Errors in transcription, translation or post-translational modification of peptides can lead to alterations in the internal milieu that are detrimental to spermatogenesis.

Identification of defects occurring at the cellular level within the testis is difficult, however, when so little progress has been made in understanding the sequence of intracellular events and their regulation under normal physiological conditions. The number of factors or peptides isolated from testicular tissue or reported to affect Leydig or Sertoli cell function or spermatogenesis is constantly increasing but these substances have often not been characterized and sequenced. Furthermore, reports concerning the actions of intratesticular peptides are at times contradictory and much controversy has arisen as a result of methodological differences.

Which potential paracrine regulators of spermatogenesis described are of proven physiological significance?

The extent of the problem encountered in answering this question is immediately evident (Fig. 1). A long and ever growing list of factors implicated in the regulation of spermatogenesis

Fig. 1. Intratesticular paracrine and autocrine interactions of opioids. LC: Leydig cell; SC: Sertoli cell; GCs: germ cells; Enk: enkephalins; FSH: follicle-stimulating hormone; LH: luteinizing hormone; GnRH: gonadotrophin-releasing hormone; Gcd: germ cell development; β -Endo: β -endorphin; ACTH: adrenocorticotrophic hormone; Scd: Sertoli cell division; ABP: androgen binding protein; cAMP: cyclic adenosine monophosphate; PA: plasminogen activator; α -MSH: α -melanocytic stimulating hormone.

initially overwhelms the reader and immediately leads to the next question. Does the regulation of spermatogenesis require the interaction of all the reported factors or could the majority simply represent 'passive' components of the intratesticular milieu? Histologically, the testis has been divided into different cellular compartments (LeBlond and Clermont, 1952), but the establishment of different microenvironments with a regulated flow of biochemical information between the different compartments is obligatory if a large number of different regulators are to interact to coordinate steroidogenesis and spermatogenesis. This would then involve differential control of regional blood supply, cellular and endothelial permeability, and perhaps alteration in the biochemical half-life of peptides either via structural modification or via the presence of binding proteins. The possibility that such a multifactorial regulatory system operates within the testis has not been conclusively confirmed or refuted and the probability that this system exists will be assessed in the light of available evidence.

A paracrine regulator is by definition a factor produced by one cell type within an organ and having an effect on another cell type within the same organ. The effect at the target cell can

involve regulation of differentiated cell function or of cell growth and differentiation.

The roles of testosterone, LHRH-like peptides, interleukin 1 and other locally produced paracrine regulators of Leydig cell steroidogenesis have already been reviewed (Sharpe, 1990) and will not be discussed further here. A number of enzymes or factors for which no specific intratesticular function relating to either steroidogenesis or spermatogenesis has been identified have been implicated as paracrine regulators. These include renin and angiotensin (Parmentier *et al.*, 1983; Pandey *et al.*, 1984; Strittmatter and Snyder, 1984; Jaiswal *et al.*, 1985; Naruse *et al.*, 1985; Velletri *et al.*, 1985; Deschepper *et al.*, 1986; Singh *et al.*, 1987; Millan and Aguilera, 1988; Okuyama *et al.*, 1988; Sealey *et al.*, 1988), caeruloplasmin (Skinner and Griswold, 1983), calmodulin, actins and tubulins (Slaughter *et al.*, 1989), α_2 -macroglobulin (Stahler *et al.*, 1991; Kangasiemi *et al.*, 1992), cyclic protein-2 (Wright *et al.*, 1983; Wright and Luzarraga, 1986; Erickson-Lawrence *et al.*, 1990), gonadotrophin-binding inhibitors (Yang *et al.*, 1976; O'Shaughnessy, 1979; Dias *et al.*, 1981; Rojas *et al.*, 1981; Dias and Reichert, 1983), sulfated glycoproteins-1 and -2 (Kissinger *et al.*, 1982; Sylvester *et al.*,

1984; Sylvester *et al.*, 1991; Cheng and Bardin, 1987; Cheng *et al.*, 1987; Collard and Griswold, 1987; Cheng *et al.*, 1988; Collard *et al.*, 1988; Roberts *et al.*, 1991; Trasler *et al.*, 1992), plasminogen activator (Lacroix and Fritz, 1982; Hettle *et al.*, 1986; Hettle *et al.*, 1988; Vihko *et al.*, 1987, 1989; Nargolwalla *et al.*, 1990), transferrin (Holmes *et al.*, 1983; Djakiew *et al.*, 1986; Morales *et al.*, 1987; Toebosch *et al.*, 1987; Wauben-Penris *et al.*, 1988; Skinner *et al.*, 1989; Roberts *et al.*, 1991; Anthony *et al.*, 1991; Spaliviero and Handelsman, 1991), prodynorphins A and B (Douglass *et al.*, 1987; McMurray *et al.*, 1989) as well as thyrotrophin-releasing hormone (Pekary and Rosen, 1982; Pekary *et al.*, 1987), and corticotrophin-releasing hormone (Dave *et al.*, 1984; Yoon *et al.*, 1988). As no specific effect of these substances on the seminiferous epithelium or only effects as carriers have been described so far, their significance as paracrine regulators is yet to be established.

Notwithstanding, even after exclusion of the above-mentioned factors, there still remain a substantial number of peptides that have been detected in the testis and for which different functions have been postulated, based predominantly on the results of *in vitro* studies.

Pro-opiomelanocortin (POMC) and POMC-derived peptides

By means of *in situ* hybridization, POMC mRNA has been localized to Leydig cells, spermatocytes and spermatogonia (Chen *et al.*, 1984; Gizang-Ginsberg and Wolgemuth, 1985, 1987; Boitani *et al.*, 1988). Analysis of mRNA expression of POMC-like peptides in the testis revealed mRNAs of various sizes with expression of the authentic POMC mRNA as well as of a smaller form which could arise as a result of different transcription start sites or altered splicing of RNA (Chen *et al.*, 1984; Chen and Madigan, 1987; Jeanotte *et al.*, 1987; Kilpatrick *et al.*, 1987). The significance of these different forms of mRNA is not yet clear and the question naturally arises as to whether the short form of POMC is translated *in vivo*. Furthermore, direct evidence that the neuropeptide POMC is itself produced locally within the testis is still lacking.

However, the POMC-derived peptides β -endorphin, adrenal corticotrophic hormone (ACTH) and melanocyte-stimulating hormone (MSH) have been identified in the interstitial fluid and their secretion has been reported by Leydig cells both *in vivo* and *in vitro* (Valenca and Negro-Vilar, 1986; Fabbri *et al.*, 1988). These observations and the localization of opiate receptors on Sertoli cells (Boitani *et al.*, 1985; Fabbri *et al.*, 1989) strongly support the potential biological role of these peptides in mediating interactions between Leydig and Sertoli cells. This concept has been further extended following the demonstration that β -endorphin, MSH and ACTH have opposing effects on Sertoli cell function (Fig. 1). β -Endorphin inhibits FSH-stimulated Sertoli cell division and other FSH-regulated functions via modulation of adenylate cyclase activity (Boitani *et al.*, 1985; Gerendai *et al.*, 1986; Fabbri *et al.*, 1989), whereas ACTH and α -MSH stimulate cell division and Sertoli cell function, notably androgen-binding protein (ABP) and inhibin production (Bardin *et al.*, 1984; Boitani *et al.*, 1986). α -MSH also stimulates cAMP production and aromatase activity but inhibits plasminogen

activator production in cultures of juvenile rat Sertoli cells (Boitani *et al.*, 1988). The opposing effects of these related peptides on Sertoli cell function provide an interesting example of local regulation. However, the time frame in which these interactions occur also requires careful assessment, as cells may develop the capacity to react to different stimuli at different stages of development. *In vivo*, β -endorphin concentrations are low in the rat testis before 20 days of age, when division of Sertoli cells occurs, and rise to adult values between 20 and 60 days of age (Adams and Cicero, 1989), thus supporting *in vitro* evidence for β -endorphin-mediated inhibition of Sertoli cell division. However, it cannot be assumed that endorphins have a purely passive role in the adult testis inhibiting Sertoli cell division. Endorphins may stimulate GnRH production (Engelhardt, 1989), which in turn can regulate β -endorphin production in the adult testis (Fabbri *et al.*, 1989). Gonadotrophins and steroids can similarly regulate β -endorphin production within the testis, thus providing an excellent example of a closed loop system functionally regulating endorphin effects within the testis and simultaneously interacting with endocrine regulatory mechanisms.

Pro-enkephalins and enkephalins

Pro-enkephalins and enkephalins are another family of neuropeptides identified within the testis. Messenger RNA for the precursor, pro-enkephalin, is present in larger amounts than either POMC- or pro-dynorphin mRNAs in testicular tissue (Douglass *et al.*, 1987) and cDNA transcripts have been localized in both somatic and germ cell lines and also in Sertoli cells (Kilpatrick and Milette, 1986; Kilpatrick *et al.*, 1987). However, pro-enkephalin mRNA has not been found in mouse or rat tumour Leydig cells (McMurray *et al.*, 1989), although enkephalin immunoreactivity has been reported in Leydig cells throughout development (Engelhardt *et al.*, 1986). This discrepancy could be due to de-differentiation of tumour Leydig cells, inhibition of precursor expression or to both factors. A more likely explanation is age-dependent changes in peptide synthesis as suggested by the observation of changes in the expression of proenkephalin mRNA with age (Kilpatrick and Milette, 1986).

The detection of pro-enkephalin mRNA at puberty can be related to the appearance of round spermatids in the testis (Kilpatrick and Milette, 1986). As the signal is localized within round spermatids and pachytene spermatocytes, it is possible that proenkephalin regulates germ cell development. Alternatively, the germ cells produce proenkephalin which then feedback-regulates Sertoli cell function or further maintains germ cell differentiation and maturation. On the basis of pro-enkephalin mRNA analysis alone, however, it is not possible to rule out the possibility that the derivatives of pro-enkephalin and not the precursor itself represent the active elements regulating germ cell division or development on both processes. Enkephalin immunoreactivity has been reported in spermatogonia in juvenile and adult animals, and in primary spermatocytes and Sertoli cells in adult animals (Engelhardt *et al.*, 1986) which would imply that the derivatives may play an active role at least up to the level of primary spermatocytes. However, further analysis of transcription, translation and perhaps even post-translational

modification of the peptides, as well as localization of receptors to specific cell types is necessary before a clear picture emerges.

Enkephalins, like the POMC-derived peptides, may also play a dual role in the testis, affecting not only germ cell development but also somatic cell function. The production and secretion of testicular LHRH-like factor by Sertoli cells is increased by enkephalins (Saint Pol *et al.*, 1988); the LHRH-like factor in turn interacts with specific Leydig cell receptors modulating steroid production (Sharpe, 1986) and inhibiting β -endorphin production by Leydig cells (Fabbri *et al.*, 1989). Such a closed loop system of regulation within the testis could serve to maintain the right concentrations of β -endorphins and enkephalins at a given time. Germ cells or germ cell products, such as pro-enkephalin, could provide an additional level of control which is switched on at the time of appearance of pachytene spermatocytes and round spermatids, i.e. when the metabolic requirements of the seminiferous epithelium may be different. Recently conditioned media from pachytene spermatocytes and round spermatids have been reported to stimulate prepro-enkephalin gene expression in Sertoli cells *in vitro* (Fujisawa *et al.*, 1992). Although there is no conclusive evidence confirming the presence of such a complex regulatory system in the testis, the *in vitro* data discussed above together with evidence of hormonal regulation of enkephalin production in the testis (Saint Pol *et al.*, 1986, 1988; Yoshikawa and Aizawa, 1988; Kew and Kilpatrick, 1989) emphasises the potential biological importance of the neuropeptides in the testis.

Oxytocin and vasopressin

Although oxytocin and vasopressin have been localized immunohistochemically in the testes of different species (Wathes, 1984; Schmale and Richter, 1984; Guldenaar and Pickering, 1985; Kasson *et al.*, 1986; Ang *et al.*, 1991), evidence of intratesticular synthesis was lacking until recently due mainly to methodological problems.

Initial attempts using northern blot hybridization failed to provide evidence for oxytocin gene expression in the testis (Ivell *et al.*, 1986) and the evidence that vasopressin (AVP) is synthesized within the testis was limited until recently to one study where positive dot blotting and hybridization with an AVP probe was possible (Ivell and Morley, 1988). The polymerase chain reaction is a far more sensitive method for the detection of specific mRNA than northern blotting and recently this technique has been effectively applied to the testis (Foo *et al.*, 1991). An authentic RNA transcript identical to that found in the hypothalamus has been identified for oxytocin. However, two novel types of mRNA for testicular AVP have been identified. Exons II and III of the testicular RNA are identical to the corresponding exons of hypothalamic AVP mRNA. However, exon I, which usually encodes AVP and neurophysin-II in the hypothalamus, is not represented within the testis. These novel AVP RNAs are detectable by northern blotting and their expression seems to be developmentally regulated. Subsequent polysome analysis revealed that these messages are probably not translated, and hence not of physiological importance. Although the PCR provides a highly sensitive detection method for messages that are expressed at extremely low

levels, there is the danger of amplifying messages coding for sequences or peptides that under normal conditions *in vivo* are not translated.

However, a number of studies *in vitro* and *in vivo* have shown that AVP and oxytocin influence testicular function. Oxytocin has been reported to affect seminiferous tubule contractility in neonatal rats (Worley *et al.*, 1985), hypogonadal mice (Nicholson *et al.*, 1986) and in ethan-1,2-dimethane sulfonate (EDS)-treated rats (Nicholson *et al.*, 1987). Oxytocin and AVP regulate steroidogenesis (Adashi and Hsueh, 1981; Adashi *et al.*, 1984; Sharpe and Cooper, 1987; Tan and Kwan, 1987; Kwan and Grover, 1988; Nicholson *et al.*, 1991) and in addition AVP alters testicular LH and hCG receptor content (Pomerantz *et al.*, 1988). However, if AVP is not synthesized in the testis, the only source of intratesticular AVP is via the circulation and AVP then exerts, by definition, purely endocrine effects on the Leydig cell and is not a paracrine factor. Furthermore, if AVP is derived from the circulation, the question arises as to whether it can cross the blood–testis barrier and hence affect the cells of the seminiferous epithelium.

Inhibins and activins

In culture, both Leydig and Sertoli cells produce inhibin and activin (de Jong and Robertson, 1985; Tsonis and Sharpe, 1986; Bhasin *et al.*, 1989; Lee *et al.*, 1989; Risbridger *et al.*, 1989; Roberts *et al.*, 1989; Shaha *et al.*, 1989). These two peptides have been postulated to exert opposite effects on the pituitary–gonadal axis. However, since the first report on inhibin (McCullagh, 1932), the hormonal regulation and intratesticular actions of these peptides remain a matter of controversy.

Initially, testicular inhibin production as measured by bioassay and later by radioimmunoassay was reported to be regulated by FSH, androstenedione, insulin, epidermal growth factor (EGF) and adenosine (Le Gac and de Kretser, 1982; Conti *et al.*, 1988; Gonzales *et al.*, 1988; Morris *et al.*, 1988) (Fig. 2). However, analysis of mRNA expression revealed that FSH stimulates testicular inhibin α -subunit expression but has no effect on β B-subunit expression in immature rat Sertoli cells in culture (Toebosch *et al.*, 1988; Keinan *et al.*, 1989; Klaij *et al.*, 1990; Pineau *et al.*, 1990). However, FSH has been generally accepted as the prime regulator of inhibin production which subsequently feedback-regulates pituitary FSH production. The concept that other hormones or factors primarily regulate β -subunit expression was therefore new. An alternative explanation may be that immature cells cannot produce these peptides. Stage-specific expression of inhibin α - and β -subunits has been demonstrated in the adult testis using both immunohistochemical techniques and northern blot hybridization (Merchantaler *et al.*, 1987; Rivier *et al.*, 1988; Bhasin *et al.*, 1989), thus providing further evidence for age-dependent expression of both subunits and peptide production. Furthermore, inhibin secretion from adult rat seminiferous tubules in culture varies according to the stage of the seminiferous epithelium, changes that are detected by both radioimmunoassay and bioassay (Gonzales *et al.*, 1989). *In vitro* studies using Sertoli–germ cell cultures from 20-day-old rats have further demonstrated that germ cells, particularly early spermatids, stimulate inhibin mRNA expression

Fig. 2. Inhibin, activin and the transforming growth factors (TGF- α and TGF- β) and their role in the paracrine regulation of intratesticular events. LC: Leydig cell; SC: Sertoli cell; GCs: germ cells; LH: luteinizing hormone; FSH: follicle-stimulating hormone; SC and GC DNA-syn: Sertoli and germ cell DNA synthesis; GCP: germ cell products; EGF: epidermal growth factor; Vit A: vitamin A; Tr: transferrin; PA: plasminogen activator; PA-I: plasminogen activator inhibitor; Protein syn: protein synthesis.

and immuno- and bioactive inhibin secretion in Sertoli cells (Pineau *et al.*, 1990). However, *in vivo*, methoxyacetic acid treatment of adult rats with depletion of pachytene spermatocytes leads to an increase in inhibin secretion by Sertoli cells, whereas absence of late spermatids is clearly associated with a decrease in inhibin secretion by Sertoli cells and in circulating inhibin concentrations (Allenby *et al.*, 1991). One possible explanation for this discrepancy between *in vitro* and *in vivo* findings is that the cultured Sertoli cells were harvested from immature rat testes, whereas adult mature rats were used in the *in vivo* study. The demonstration of developmental changes in the expression of testicular inhibin mRNA and the secretion of the peptide (Keeping *et al.*, 1990) strongly supports this hypothesis and further emphasizes the pitfalls of extrapolating observations made in an *in vitro* system using immature cells to establish the physiological relevance of a given peptide in the adult *in vivo*.

There is sufficient evidence in the literature for both the production and secretion of inhibin by Sertoli cells, although the effect of different hormones and germ cells or germ cell products on synthesis and secretion do differ throughout development. The role of inhibin in the feedback regulation of pituitary FSH secretion has been extensively studied. Inhibin must be secreted from the testis, the major site of production of the

peptide, into the circulation to affect pituitary FSH secretion. Bidirectional secretion of inhibin by Sertoli cells in culture with preference for the luminal direction has been reported (Janecki *et al.*, 1988), whereas apically secreted inhibin is reabsorbed from the seminiferous tubule fluid into the blood in the region of the rete testis (Maddocks and Sharpe, 1989; Maddocks and Sharpe, 1990). However, despite FSH stimulation of inhibin secretion *in vitro*, the demonstration of a straightforward relationship between serum inhibin and FSH concentrations has been more difficult (Culler and Negro-Vilar, 1988; de Kretser *et al.*, 1989; Weinbauer *et al.*, 1989; Maddocks and Sharpe, 1990). Lack of specificity of the antisera available for the measurement of inhibin, interspecies variations and differential developmental regulation of inhibin secretion (Rivier *et al.*, 1988; Burger *et al.*, 1988; Abeyawardene *et al.*, 1989; Keeping *et al.*, 1990) could perhaps account for the conflicting reports. Significant suppression of serum FSH concentrations has, however, been achieved following the administration of recombinant inhibin to castrated rats (Robertson *et al.*, 1991), supporting the hypothesis that inhibin can act as a feedback regulator of pituitary FSH secretion.

The clinical observation of high concentrations of FSH in serum of patients with oligozoospermia and idiopathic

infertility has led to unsuccessful attempts to correlate concentrations of inhibin in serum to the degree of testicular damage (de Kretser *et al.*, 1989). As discussed above, germ cells modulate inhibin production by Sertoli cells and in patients with biopsy-proven Sertoli-cell-only syndrome or maturation arrest of spermatogenesis and high concentrations of FSH, changes in inhibin concentrations may be expected but have not been conclusively demonstrated. The development of more specific and sensitive assays for inhibin may eventually reveal disturbances in the serum inhibin profiles of selected patients. However, factors regulating the secretion of testicular inhibin into the circulation may modulate serum profiles such that any direct correlation to circulating FSH concentrations in a given sample taken at one time point is masked. A lag phase of as yet undefined duration may be present, as suggested by the observation that 4–8 h elapse after a single injection of recombinant inhibin to castrated rats before a fall in concentration of FSH in serum is observed (Robertson *et al.*, 1991).

Within the testis, the precise function of inhibin still needs to be established. The possibility that inhibin could interfere with FSH binding to its receptors in the testis has been raised, but not substantiated (Vijayalakshmi *et al.*, 1980). Inhibin has, however, been shown to inhibit the incorporation of labelled thymidine into testicular DNA both *in vitro* and *in vivo* (Franchimont *et al.*, 1981; van Dissel-Emiliani *et al.*, 1989). Inhibin, in addition to the previously postulated role in the feedback regulation of pituitary FSH secretion, may therefore also restrict FSH action at the testicular level. After the demonstration of a definite stimulatory effect of activin on pituitary cell FSH secretion and on circulating FSH concentrations in macaques and rats *in vivo* (Ling *et al.*, 1986; McLachlan *et al.*, 1989; Schwall *et al.*, 1989), the possibility that activin acts to oppose inhibin action within the testis has been investigated. Activin was shown to stimulate incorporation of [³H]thymidine into Sertoli and germ cell co-cultures from 21-day-old rats, and also to increase the 4C population of cells as shown by flow cytometric analysis, whereas inhibin does not affect either in this experimental model (Mather *et al.*, 1990; Mather and Krummen, 1992). In contrast, intratesticular injections of inhibin result in a significant reduction in spermatogonial numbers in the testes of adult mice and Chinese hamsters (van Dissel-Emiliani *et al.*, 1989). Although the presence of interspecies variation in the response to a peptide could account for these conflicting reports, the different observations are probably due to the experimental models used in these two studies.

The demonstration that mRNA for α -, β A- and β B-subunits is present in Sertoli cells but not in germ cells (Bhasin *et al.*, 1989; Roberts *et al.*, 1989; Shaha *et al.*, 1989; Kaipia *et al.*, 1991, 1992) and is expressed in a stage-specific manner supports a paracrine role for activin and inhibin in Sertoli–germ cell interaction. However, inhibin β A-subunits have been localized immunohistochemically to developing germ cells (Shaha *et al.*, 1989) with the result that autocrine effects of the peptides on germ cells cannot be excluded. As the level of mRNA expression may be too low to be detected by northern blot analysis, application of ribonuclease protection assays or of PCR could eventually help identify the presence or absence of inhibin or activin mRNA subunits in germ cells. An important limitation to the demonstration of both α and β mRNA subunits in a given tissue or cell population is that the presence of mRNA

for both subunits makes it impossible to distinguish between activin and inhibin production.

The cloning of the activin receptor (Mathews and Vale, 1991) was followed by the localization of a 4 kb mRNA for the activin receptor to isolated pachytene spermatocytes and round spermatids (de Winter *et al.*, 1992). Two activin receptor mRNAs (4 kb and 6 kb) have been identified in Sertoli cells from both immature and mature rats with low levels of expression evident in Leydig cells (de Winter *et al.*, 1992). The biological significance of the different messages for the activin receptor has yet to be investigated. One of the two messages could represent a transcript that is not translated as has been described in other instances (Ivell, 1992). However, alternative mRNA splicing has been shown to result in different activin receptor isoforms, belonging to the serine/threonine kinase receptor family, with species that differ in ligand binding affinity, cytoplasmic domain structure or both (Attisano *et al.*, 1992). Additional selectivity of activin and inhibin actions at a given time may be conferred through alteration of receptor gene expression and translation. Furthermore, identification of follistatin as an activin-binding protein in both the gonads and the pituitary (de Paolo *et al.*, 1991) suggests another possible mechanism for regulating activin actions.

Growth factors

Growth factors have been acknowledged to regulate somatic and germ cell division but recently it has become evident that growth factors also influence differentiated cell function. A number of extensive reviews discussing the role of different growth factors in the regulation of testicular function and the characteristics of these growth factors have been published by Bellvé and Zheng (1989) and Skinner (1991). The list of growth factors implicated in testicular paracrine regulation now includes Sertoli cell secreted growth factor (SCSGF), seminiferous growth factor (SGF), epidermal growth factor (EGF), transforming growth factors α and β (TGF- α and - β), insulin-like growth factors I and II (IGF-I and -II), acidic and basic fibroblast growth factors (aFGF and bFGF), β -nerve growth factor (β -NGF), platelet-derived growth factor (PDGF), and interleukin 1 (IL-1). This section of the review will therefore concentrate solely on those growth factors for which an effect on Sertoli cell function and Sertoli–germ cell interactions has been postulated or documented.

A prerequisite for the identification of growth factors important in the regulation of the seminiferous epithelial compartment should be the demonstration of the synthesis and secretion of the peptide in this compartment, demonstration of an effect or effects on differentiated cell function or somatic or germ cell division or on both, and the localization of receptors on the postulated target cells. Analysis of the literature reveals that of the above-mentioned growth factors, TGF- α and - β , EGF, IGF-I and IGF-II are the only factors that fulfil these criteria.

Transforming growth factor α (TGF- α)

TGF- α mRNA expression has been demonstrated in isolated peritubular and Sertoli cells (Skinner *et al.*, 1989) and secretion

of TGF- α by both cell types occurs *in vitro*. However, TGF- α acts predominantly in an autocrine fashion inducing proliferation and mixed cell aggregation solely in rat peritubular cells and not in Sertoli cells (Skinner *et al.*, 1989). The biological significance of these findings is not clear, but the presence of receptors for TGF- α or EGF on rat peritubular cells further supports the concept that TGF- α can regulate peritubular cell function. Although Skinner and co-workers could not demonstrate binding of radiolabelled EGF to TGF- α receptors on Sertoli cells, peritubular-Sertoli cell co-cultures produce transferrin on stimulation with TGF- α (Skinner *et al.*, 1989), an effect which they suggest is mediated by TGF- α -induced production of a paracrine factor, such as peritubular modifying substance (PmodS), which subsequently stimulates transferrin production by Sertoli cells (Norton and Skinner, 1989; Anthony *et al.*, 1991). However, direct effects of TGF- α on Sertoli cells may be possible as other groups have immunohistochemically localized the receptor to Sertoli cells in rats (Suarez-Quian *et al.*, 1989) and to the interstitium in humans (Stubbs *et al.*, 1990). Final confirmation of the presence or absence of EGF/TGF- α receptor synthesis by Sertoli cells has to await the application of sensitive hybridization protocols or of the polymerase chain reaction to the problem.

Transforming growth factor- β (TGF- β)

Five different isoforms of TGF- β , which are structurally related to inhibin and activin, have been isolated from different tissues (Deuvel, 1987; Sporn and Roberts, 1990). Within the testis, TGF- β 1 mRNA has been detected in peritubular cells and Sertoli cells, whereas peritubular cells also contain mRNA for TGF- β 2 in the immature rat testis (Skinner and Moses, 1989). Secretion of the isoforms by these cells in culture has not been demonstrated nor have receptors for TGF- β been localized within the testis. TGF- β has no effect on secretion of transferrin by Sertoli cells but promotes an increase in the production of radiolabelled secreted proteins by peritubular cells, and also stimulates peritubular cell migration and colony formation in culture (Skinner and Moses, 1989). This finding may be taken as indirect evidence for the presence of receptors for TGF- β on peritubular cells, but the possibility that other cell types contaminate the peritubular cell culture or of nonspecific interaction of TGF- β with other receptors cannot be excluded. Definite localization of the receptor is essential, as the lack of effects on Sertoli cell transferrin production does not exclude other TGF- β -mediated effects on the Sertoli cell and the presence of a receptor on Sertoli cells would stimulate further research in this area.

In other systems such as the ovary, TGF- α and TGF- β have been reported to have diametrically opposite effects on cell division and differentiated cell function and a similar role may be postulated for the TGFs in the testis. However, although TGF- α stimulates peritubular cell proliferation, TGF- β 1 had no effect either on peritubular or Sertoli cell proliferation in cells isolated from the mid-pubertal stage of development (Skinner and Moses, 1989). Re-examination of the expression of the growth factors at different stages throughout development revealed that TGF- β 2 expression occurs in both peritubular and Sertoli cells in the immature testis, whereas TGF- β 3 is expressed by both cell types throughout development. Stimulation of Sertoli

cells with FSH downregulates TGF- β 2 expression (Mullaney *et al.*, 1991) which would otherwise play a role as a growth inhibitor at the time of initiation of active germinal cell growth. In the adult testis, TGF- β 1 immunoreactivity has been observed in Sertoli cells, spermatocytes and spermatids at about the time when meiosis occurs, whereas TGF- β 2 immunoreactivity is present only in post-meiotic germ cells, suggesting a role for this isoform in spermatid differentiation (Teerds and Dorrington, 1992) in the adult rat. Although there is no firm evidence supporting interactions between TGF- α and TGF- β effects on somatic and germ cell populations in the testis *in vivo* at a specific stage of development, further elucidation of the interactions between the different isoforms of TGF- β may provide insight into the factors co-ordinating spermatogenesis and spermiogenesis in the seminiferous epithelium.

Epidermal growth factor (EGF)

Extirpation of mouse submaxillary glands results in a very low concentration of EGF in serum and impairment of spermatogenesis which is reversible in the presence of EGF (Tsutsumi *et al.*, 1986). Russell *et al.* (1990) were unable to quantitatively reproduce these effects, possibly owing to methodological differences in the morphological analysis of the testis. However, there is considerable evidence that EGF stimulates biochemical responses in Sertoli cells (Mallea *et al.*, 1986; Perez-Infante *et al.*, 1986; Spaliviero and Handelsman, 1991). Since EGF and TGF- α have a high degree of similarity in sequences (Savage *et al.*, 1972) and interact at one and the same receptor (Downward *et al.*, 1984), the reported effects could simply reflect an artefact due to EGF interaction at a receptor that normally sees only TGF- α . This hypothesis is supported by the inability to detect mRNA for EGF using a human cDNA probe in both Sertoli and peritubular cells (Skinner *et al.*, 1989). However, if the message for the growth factor is expressed only at low levels, the application of nuclease protection assays or PCR is essential before EGF is excluded as a paracrine regulator of testicular function. In addition, application of immunohistochemical techniques to the localization of EGF and TGF in the testis could help resolve the question, if antibodies of sufficient sensitivity and specificity are available. This line of investigation would be worth following, as in a study *in vivo* using an antiserum against EGF which has no crossreactivity with TGF- α , stage-dependent changes in intratesticular EGF or an EGF-like peptide have been reported in vitamin-A-deficient rats resubstituted with retinol and exhibiting stage-synchronization of spermatogenesis (Bartlett *et al.*, 1990).

The presence of EGF/TGF- α binding sites on Leydig, Sertoli, myoid and endothelial and different germ cell populations have been reported (Morris and Mather, 1984; Kris *et al.*, 1985; Suarez-Quian *et al.*, 1989; Stubbs *et al.*, 1990) in various animal species. Specificity of the antibodies for different epitopes on the EGF receptor, interspecies variations and age-dependent changes in the expression of EGF receptor could account for the different results obtained by the various groups.

Given the problems outlined above, it is not surprising that there is a lack of data on the effect of EGF in humans. It has been claimed that there is no correlation between seminal plasma EGF concentrations and age of the donor, number of spermatozoa, motility or period of sexual abstinence (Elson *et al.*, 1984). There

is also no difference in seminal plasma EGF concentrations in relation to sperm density, progressive motility of azoospermia in infertile compared with fertile men (Richards *et al.*, 1988). This could be due to the fact that the major proportion of the EGF in seminal plasma is from another source such as the prostate. Ethical reasons prevent studies on testicular EGF regulation in humans but the use of animal models to study the questions raised should elucidate the role of EGF in the regulation of spermatogenesis. Furthermore, studies *in vivo* of the stimulatory effect of EGF on Leydig cell steroidogenesis reported *in vitro* (Syed *et al.*, 1991) are obviously lacking.

Insulin-like growth factor-I (IGF-I)

There is evidence from several sources that IGF-I is present in the testis (Handelsman *et al.*, 1985; Benahmed *et al.*, 1987; Smith *et al.*, 1987) and mRNA transcripts for IGF-I have been identified among testis poly [A+] RNA by northern blot analyses (Casella *et al.*, 1987). The peptide has been identified immunohistochemically in and shown to be secreted by Leydig and Sertoli cells (Handelsman *et al.*, 1985; Smith *et al.*, 1987; Vanelli *et al.*, 1988), in immature rats and in adult humans (Hansson *et al.*, 1989) and to be present in primary spermatocytes in adult rats and humans (Vanelli *et al.*, 1988; Hansson *et al.*, 1989). A membrane receptor for IGF-I designated IGF type I receptor has been characterized and localized to Sertoli cells (Borland *et al.*, 1984; Vanelli *et al.*, 1988), Leydig cells (Handelsman *et al.*, 1985) and secondary spermatocytes and early spermatids (Tres *et al.*, 1986; Vanelli *et al.*, 1988). It could, therefore, be postulated that IGF-I plays an autocrine role in regulating Sertoli and Leydig cell function and may play a paracrine role in the cycle of the seminiferous epithelium.

Tissue IGF-I production is probably controlled by factors other than GH (D'Ercole *et al.*, 1984). FSH, LH or testosterone, alone or in combination, which appear to play a major role, as well as EGF and FGF, have been shown to regulate testicular IGF-I mRNA expression and peptide content in both immature and mature rats (Tres *et al.*, 1986; Chatelain *et al.*, 1987; Closset *et al.*, 1989; Spiteri-Grech *et al.*, 1991a, b).

Insulin-like growth factor-II (IGF-II)

Little is known about the production of IGF-II in the testis. The gene for IGF-II is not transcribed at detectable amounts in the rat testis when northern blot analysis is used (Murphy *et al.*, 1987). The physiological effects of IGF-II observed in the testis therefore probably depend on the interaction of the serum protein with endogenous cellular receptors, although the application of newer and more sensitive techniques should be used to address this question once more.

A type II membrane receptor (230 kDa) similar to the cation-independent mannose-6-phosphate receptor is present in testicular membrane preparations, specifically on Sertoli cell membranes (Borland *et al.*, 1984; Morgan *et al.*, 1987; Taylor *et al.*, 1987; Roth, 1988). Mouse pachytene spermatocytes and round spermatids have predominantly cation-dependent receptors (46 kDa) with low concentrations of the cation-independent form but more cell surface expression of the latter type than in Sertoli cells, which synthesize larger amounts of the cation-independent form but express very little on the cell surface (O'Brien *et al.*, 1988). Pachytene spermatocytes and spermatids

(steps 1–8) are capable of mannose-6-phosphate receptor mediated endocytosis and lysosomal enzyme processing, as shown by the accumulation of [¹²⁵I]-labelled lysosomal enzymes containing mannose-6-phosphate (O'Brien *et al.*, 1988). However, the question of whether the IGF-II/mannose-6-phosphate receptors on the germ cells are accessible to serum IGF-II or whether they transport mannose-6-phosphate containing lysosomal enzymes secreted by Sertoli cells into the central compartment of the seminiferous epithelium remains unanswered. Other functions reported include IGF-II-induced increases in Sertoli cell transferrin synthesis (Skinner and Griswold, 1982; Spaliviero and Handelsman, 1991) and increased lactate and protein production (Borland *et al.*, 1984). These effects may represent artefacts generated via interaction of IGF-II with type I receptors or may be specific biochemical effects mediated via interaction with the type II receptor. The latter possibility implies receptor linkage to different second messenger systems that mediate different cellular responses. Further studies concerning activation of second messenger systems after IGF binding to the different receptor types may clarify this.

Because of the methodological problems and the various affinities of the two types of receptor for the IGFs and insulin, the biological effects of the two factors on the testis should be discussed together (Fig. 3). Via interaction with type I receptors, IGF-I, IGF-II and insulin increase thymidine incorporation into Sertoli cell cultures prepared from 13-day-old rat testes, whereas IGF-II stimulates leucine incorporation into protein probably through interaction with type II receptors present on Sertoli cells (Borland *et al.*, 1984). FSH, IGF-I and insulin also stimulate Sertoli cell glucose uptake and lactate production (Mita *et al.*, 1985; Oonk *et al.*, 1985; Oonk, 1987; Oonk and Grootegoed, 1988). IGF-I may exert a priming effect on the capacity of the Sertoli cell to respond to FSH and furthermore stimulate mitogenesis in both immature Leydig and Sertoli cells. It can be argued that this is not a specific effect since insulin has similar effects but supraphysiological concentrations of insulin are necessary to achieve this and probably the growth-promoting effect of insulin is the result of interaction with tissue IGF-I receptors (Saez *et al.*, 1988). Similarly, pretreatment of porcine Leydig cells with nanomolar concentrations of IGF-I (or micromolar concentrations of insulin) increases the hCG receptor number and enhances steroidogenesis both directly (Lin *et al.*, 1986) and in response to gonadotrophins (Kasson and Hsueh, 1987). An additional level of control operates through hCG and LH regulation of Leydig cell IGF-I receptors (Lin *et al.*, 1988; Nagpal *et al.*, 1991). An LH-regulated IGF-mediated paracrine loop therefore exists in the testis. Whether a similar interaction between FSH, IGF-I and Sertoli cells is present and whether IGF-I produced by Sertoli cells is also secreted into the central compartment of the seminiferous tubule, thereby affecting germ cell differentiation, remains to be established. Recently, IGFs have been reported to stimulate apical transferrin secretion preferentially in a bicameral Sertoli cell culture system (Spaliviero and Handelsman, 1991), which provides evidence in support of IGF-mediated effects on germ cell differentiation. In rat Sertoli-spermatogenic cell co-cultures, immunoreactive IGF-I is associated with pachytene spermatocytes but not with spermatogonia or early meiotic prophase spermatocytes. *In vivo*, a strong and positive correlation between testicular IGF-I content and the presence of pachytene spermatocytes and spermatids has been

Fig. 3. The effects of insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) and the factors regulating their production at the cellular level in the testis. GH: growth hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; T: testosterone; Scd: Sertoli cell division; Pyr Lc: pyruvate lactate metabolism; Glu-up: glucose uptake; Thy-up: thymidine uptake; ABP: androgen-binding protein; PA: plasminogen activator; Tr: transferrin; Fe: iron; Tr-Fe: transferrin-iron complex; Gcd: germ cell development.

shown (Spiteri-Grech *et al.*, 1992), further stressing the potential biological importance of this peptide in regulating the seminiferous epithelium.

The presence of binding sites and the effect of various *in vitro* and *in vivo* treatments on IGF-I mRNA expression and peptide secretion strongly support a paracrine/autocrine role for IGF-I in the regulation of Sertoli-germ cell interactions. The role of the different IGF-binding proteins in modulating the actions of IGF-I and -II is also attracting increasing attention. Transcripts for IGFBP-3, -4 and -5 have been identified in the rat testis (Shimasaki *et al.*, 1989, 1990, 1991; Albiston and Herington, 1992). Peritubular cells in culture synthesize predominantly IGFBP-2, whereas Sertoli cells secrete mainly IGFBP-3 (Smith *et al.*, 1990). Differential regulation of IGFBP-3 and IGF-I secretion by FSH-stimulated Sertoli cells has been reported (Cailleau *et al.*, 1990; Smith *et al.*, 1990). The presence of a local feedback loop with FSH stimulating IGF-I production but inhibiting IGFBP-3 production which in turn may be stimulated by rising concentrations of bioactive IGF-I within the testis can therefore be postulated. Future research concerning the binding proteins may provide insights into how the biological availability of a given peptide is regulated at a given time under specific conditions within the testis.

Conclusion

In conclusion, paracrine regulation of testicular function is an interesting concept, but the nature of testicular architecture and

the multiple interactions occurring at a cellular level makes design of studies and interpretation of data generated difficult. Application of molecular biology techniques may help to answer some of the questions raised by this article, but caution is just as necessary in the interpretation of data generated by these methods as by the more traditional techniques previously used by morphologists and endocrinologists. The best approach to address the question of paracrine regulation of seminiferous epithelial function remains the combination of old and new methods in *in vitro* and *in vivo* studies. Observation of a given effect in an *in vitro* system using cells isolated from immature animals should certainly not lead to extrapolation to the adult animal *in vivo*. The question of differential regulation of gene expression throughout development and correlation to the development of specific functions or characteristics may provide important clues to the local regulation of testicular function. Immunohistochemistry, mRNA expression, and *in situ* hybridization suggest synthesis of a peptide but finally the demonstration of specific effects of the factors isolated from testicular tissue and subsequently characterized is essential. As a result of methodological and ethical problems, we are still a long way from identifying new pathophysiological mechanisms underlying male infertility. LH and FSH are generally acknowledged as prime regulators of male reproductive function, but increasing recognition of the importance of endocrine and paracrine interaction for normal testicular function is a step in the right direction. For the POMC-derived peptides, EGF/TGF- α , the inhibin-activin family and the IGFs, there is convincing evidence for such interactions. Time and effort are, however,

required before a clearly defined paracrine system integrating these and other factors into the complex regulation of testicular function is established.

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