REVIEW

Fine tuning of adrenocortical functions by locally produced growth factors

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Adult adrenal cortex is a differentiated endocrine tissue that undergoes permanent regeneration. Proliferation of the progenitor cells is restricted to the outer quarter of the cortex (Zajicek *et al.* 1986, Mc Nicol & Duffy 1987). After this initial proliferative step, adrenocytes are displaced centripetally, until they reach the junction to the medulla (the whole process takes about 100 days in the rat) (Zajicek *et al.* 1986). Half of the cells die on the way and the remainder are eliminated by apoptosis in the reticularis zone (Wyllie *et al.* 1973). During this displacement, adrenocytes acquire three distinct functional and morphological phenotypes characterized by different patterns of steroidogenic enzyme gene expression. Younger cells differentiate into glomerulosa cells, acquiring the capacity to synthesize aldosterone. Further along the way, adrenocytes become fasciculata cells, expressing P450c17 (steroid 17 α -hydroxylase) and synthesizing cortisol and/or corticosterone. In the innermost zone, the oldest adrenocytes acquire the reticularis phenotype, characterized in primates by the ability to synthesize adrenal androgens (dehydroepiandrosterone). Subcapsular remnants obtained after adrenal enucleation are able to regenerate differentiated cortical tissue with functional fasciculata and reticularis zones, even when grafted in ectopic locations (Skelton 1959, Belloni *et al.* 1990). The subcapsular glomerulosa zone thus probably contains what may be considered the adrenocyte stem cells. Two fairly recent reports suggest, however, that this may not be the only location of stem cells. Mitani *et al.* (1994) identified a layer of cells between the zona glomerulosa and the zona fasciculata that do not express aldosterone synthase and 11 β -hydroxylase and proposed that it represents the layer of adrenocyte stem cells. These cells were not isolated and therefore their potential for conversion into differentiated adrenocytes with a glomerulosa or fasciculata phenotype has not been proven. More recently, Thomas *et al.* (1997) succeeded in reconstituting a fully differentiated and vascularized adrenocortical organoid by transplanting clones of cells derived from a single bovine fasciculata cell, in the renal capsule of scid mice. Some 25% of the clones transplanted

were able to successfully form cortisol-secreting tissue, suggesting that some but not all of the fasciculata cells that possess the capacity to form a cell clone *in vitro* can be considered stem cells.

Several experimental approaches over the last few decades have clearly established that the pituitary hormone adrenocorticotrophin (ACTH) is the primary regulator of both fetal adrenal development and adult adrenal cortex homeostasis. Hypophysectomy of adult animals results in a dramatic regression of the adrenocortical tissue caused by a massive deletion of the fasciculata and reticularis zones through apoptosis while the glomerulosa zone remains intact and functional (Nussdorfer & Mazzochi 1972, Carsia *et al.* 1996). More delicate removal of the sole corticotrope and melanotrope cell populations from the pituitary gland, induced by ganciclovir treatment of transgenic mice expressing the herpes simplex virus thymidine kinase transgene under the control of the pro-opiomelanocortin promoter, results in a similar regression of the adrenal cortex tissue (Allen *et al.* 1995).

Besides its trophic action, ACTH is also a major regulator of adrenal cortex function, i.e. steroidogenesis. It stimulates the synthesis of cortisol by two distinct mechanisms. ACTH binding to its adenylate cyclase-coupled receptor MC2-R acutely increases steroid biosynthesis by stimulating cholesterol esterase, thereby releasing free cholesterol from stored cholesterol ester pools and making substrate available to the steroidogenic enzymes in the cell (Stocco & Clark 1996). ACTH is also able to chronically increase steroidogenesis by stimulating the transcription of several key cAMP-responsive genes that encode steroidogenic enzymes (Miller 1988, Simpson & Waterman 1988) and of the mitochondrial protein StAR (Steroidogenic acute regulatory protein), an essential component of the intramitochondrial cholesterol-transfer machinery (Clark *et al.* 1995).

In vitro studies of the proliferative action of ACTH using adrenocortical cell subpopulations from the glomerulosa or the fasciculata zones have generated controversial results. Some authors report a decrease in cell number and

cell proliferation (Ramachandran & Suyama 1975, Gill *et al.* 1980) whereas others suggest that it can stimulate adrenocortical mitogenesis (Menapace *et al.* 1987, Arola *et al.* 1993). In our hands, freshly prepared bovine fasciculata cells are mitogenically stimulated by ACTH but, after a few days of primary culture (in the absence of ACTH), become growth-inhibited by ACTH. The discrepancies in the literature may reflect differences in the age and differentiation status of the cultures used for the studies. They are also indicative that other regulatory factors, which are switched on or off during the primary culture, may regulate the mitogenic response to ACTH.

Besides ACTH, a number of other regulatory factors including angiotensin II, endothelin-1 and several growth factors have been shown to participate in the control of adrenal cortex homeostasis. The regulation of trophicity and steroidogenic function of the glomerulosa zone by angiotensin II has been particularly well documented. It has long been known that restriction of dietary sodium intake causes hypertrophy of the glomerulosa zone and increases aldosterone plasma levels (Quinn & Williams 1988). These changes appear to be mediated for a large part by the circulating renin-angiotensin system (Aguilera & Catt 1978). In addition, angiotensinogen, renin and angiotensin-converting enzyme are also expressed in the adrenal glomerulosa zone and constitute a local renin-angiotensin system able to generate angiotensin II within the tissue (Racz *et al.* 1992, Phillips *et al.* 1993). In contrast with the relatively high adrenal renin content, adrenal concentrations of angiotensinogen are low, not exceeding 10% of the amount of angiotensinogen present in plasma. Therefore the contribution of locally produced angiotensin II to the control of the glomerulosa phenotype is still unclear.

Given the variety of biological events triggered by ACTH in the adrenal cortex, it is reasonable to speculate that some (if not most) of these effects are induced by a variety of relay proteins synthesized and secreted by the different zones of the cortex, and/or that locally produced factors may synergize or antagonize the direct biological effects of ACTH in order to generate combinatorial responses in the different cell subpopulations. During the last decade, numerous investigations have contributed to the elucidation of the role of growth factors in the regulation of adrenocortical functions (Feige & Baird 1991, Mesiano & Jaffe 1997, Ho & Vinson 1997). These biological peptides are multifunctional in nature (Sporn & Roberts 1988) and, besides their original mitogenic function that originally determined their names, they often act as autocrine and/or paracrine regulators of differentiated functions.

In this paper, we will review the variety of adrenocortical functions regulated by growth factors and, in the second part, we will focus on the transforming growth factor β (TGF β)/TGF β receptor system to demonstrate

the important concept that extracellular activation of latent growth factors is a key regulatory step in the control of growth factor biological action.

Mitogenic effects of growth factors in the adrenal cortex

Studies of growth factor involvement in the regulation of fetal and adult adrenal development have addressed the questions of growth factor and growth factor receptor expression in these tissues, regulation of expression, and the effects of growth factors on proliferation of cultured cells. The roles of several growth factors, including fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and epidermal growth factor (EGF) have been examined. As shown below, fetal and adult adrenocortical cells appear to display quite different patterns of mitogenic responsiveness to these factors.

Fibroblast growth factors

FGF-2 (still often named basic FGF) is a potent mitogen for a variety of mesoderm- and neuroectoderm-derived cells, including adrenocortical cells. It is also a potent angiogenic and neurotrophic factor (Baird & Böhlen 1990, Bikfalvi *et al.* 1997). It is a member of a large family of structurally related peptides that comprises at least 14 members (Coulier *et al.* 1997). In contrast with other family members, FGF-1 and FGF-2 do not possess a signal peptide but are, however, secreted through a brefeldin A-resistant energy-dependent pathway distinct from the classical endoplasmic reticulum/Golgi secretory pathway (Mignatti *et al.* 1992, Florkiewicz *et al.* 1995).

Four different FGF receptors have been identified and named FGFR1 to FGFR4. Each one has an extracellular immunoglobulin loop-containing ligand-binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain. Differential mRNA splicing results in multiple forms of each receptor that differ in their binding specificities toward the different FGF family members (Goldfarb 1996). FGF-2 binds preferentially to FGFR1, FGFR3 and FGFR4 whereas FGF-1 binds to all four receptor types. Besides these high-affinity signal-transducing receptors, heparan sulphate proteoglycans (HSPGs) have been shown to bind to FGFs with a lower affinity. Binding to HSPGs is a prerequisite to binding to tyrosine kinase receptors in a number of cell types (Klagsbrun & Baird 1991, Yayon *et al.* 1991). However, the abundance of HSPGs at the cell surface also appears to be responsible for the trapping of large amounts of FGFs in a stable protease-resistant form (Saksela *et al.* 1988). The term crinopexy has been coined to describe this paradigm of factors that are secreted ('crino') and immediately adhere ('pexy') to the cell surface (Feige & Baird 1992, 1995).

The mitogenic effect of partially purified preparations of FGF-2 was first observed in the Y1 cell line (Gospodarowicz & Handley 1975). Further purification to homogeneity of both FGF-1 and FGF-2 allowed confirmation of the mitogenic action of both factors on primary cultures of adult bovine adrenocortical cells (Esch *et al.* 1985). FGF-2 and FGF-1 are active at concentrations ranging from 10 pg/ml to 1 ng/ml respectively. So far, FGF-2 has been found to be the most potent growth factor for adult adrenocortical cells.

FGF-2 has been purified from adult bovine adrenal cortex (Gospodarowicz *et al.* 1986), and cultures of bovine adrenocytes have been shown to synthesize and secrete FGF-2 but not FGF-1 (Schweigerer *et al.* 1987). It should be pointed out, however, that the cells used in this latter study had undergone several passages and were no longer stimulated to grow by fetal calf serum. We observed that freshly isolated bovine fasciculata adrenocytes (day 4 of primary culture) synthesize three forms of FGF-2 of molecular mass 18, 22 and 24 kDa (Fig. 1). All three forms were present in nuclei and to a lesser extent in microsomal and mitochondrial fractions whereas only the 18 kDa form was detectable in the cytosol. As characterized by many investigators, this shorter 18 kDa form represents the secreted mitogenically active form of FGF-2, and the biological function of the larger forms, which are targeted to the nucleus, is still elusive (Baird & Böhlen 1990, Bikfalvi *et al.* 1997). The distribution of FGF-2 in fetal and adult adrenals from various species has been investigated by several groups using immunohistochemistry. In the 18-day-old rat embryo, FGF-2 immunostaining is restricted to the fasciculata and reticularis layers of the cortex, whereas the glomerulosa and the adrenal capsule are devoid of FGF-2 (Gonzalez *et al.* 1990). Similarly, in human fetal adrenals, FGF-2 is present in the fetal zone but appears to be absent from the definitive cortical zone (Gonzalez *et al.* 1996). In contrast, FGF-2 protein is widespread over the three zones of the adult rat adrenal cortex and, in accordance with the concept of crinopexy, FGF-2 mRNAs are barely detectable in this tissue (Ho & Vinson 1995). The distribution of FGF-2 mRNA appears to be restricted to the most external layers of adrenocytes and to be related to the age and sex of the animals (Ho & Vinson 1997).

Bovine adult fasciculata adrenocytes express both a large number of HSPG FGF-2-binding sites (K_d about 400 pM, 230 000 sites/cell) and a limited number of high-affinity FGF-2 receptors (K_d about 2 pM, 4300 sites/cell) (Savona *et al.* 1991). The subtypes of FGF receptors expressed by adrenocortical cells have been characterized in human tissues by Northern blotting and immunohistochemistry. The highest concentrations of FGFR4 in 17–18-week-old human fetuses were found in the adrenal glands. No FGFR3 was detectable in the fetal adrenal, and FGFR1 and FGFR2 mRNAs were expressed at barely detectable levels (Partanen *et al.* 1991). In adult adrenals, steroido-

Figure 1 Immunodetection of FGF-2 isoforms in bovine adrenocortical subcellular fractions. Bovine fasciculata cells were grown for 4 days in primary culture in the presence of serum. After a PBS wash, cells (3×10^6 /10 cm plate) were lysed in 20 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.2 M sucrose buffer containing proteinase inhibitors (pepstatin, aprotinin and leupeptin) and homogenized using a Dounce homogenizer. Subcellular fractions were then prepared by differential centrifugation (800 g pellet: nuclei; 15 000 g pellet: mitochondria; 110 000 g pellet: microsomes; 110 000 g supernatant: cytosol). The pellets were frozen/thawed twice in RIPA buffer containing protease inhibitors and extracted for 30 min at 4 °C in this buffer. The suspension was centrifuged at 20 000 g and the supernatants were used for Western blot analysis. The cytosols were adjusted to the detergent concentrations present in RIPA buffer before Western blot analysis. Subcellular fractions derived from 3×10^6 cells were incubated with 0.1 ml heparin-agarose beads for 1 h at 20 °C. The beads were then washed successively with RIPA buffer, Tris-HCl buffer (pH 7.5)/600 mM NaCl, and RIPA buffer. The beads were then boiled in Laemmli sample buffer and the released proteins were separated by SDS-PAGE (15% gels). After transfer to nitrocellulose, the heparin-binding proteins from the different cell fractions were Western-blotted using a polyclonal anti-FGF-2 antibody (no 773, a gift from Dr A Baird, Salk Institute, La Jolla, CA, USA). Immunoreactive FGF-2 isoforms were revealed by chemiluminescence using the Renaissance kit (NEN Life Science, Les Ulis, France). Lane 1, nuclei; lane 2, mitochondria; lane 3, microsomes; lane 4, cytosol; lane 5, recombinant human FGF-2 (5 ng); lane 6, recombinant human FGF-1 (5 ng). Molecular masses (MW in kDa) of the immunodetectable FGF-2 isoforms are indicated on the left.

genic cells appeared to express FGFR2 and FGFR3, and all four receptors were detectable in the microvasculature (Hughes 1997).

Given the strong *in vitro* mitogenic activity of FGF-2 on adrenocortical cells, several authors have speculated that it may mediate the trophic action of ACTH. The expression of FGF-2 mRNA in human fetal adrenocortical cells was shown to be increased 2- to 3-fold by ACTH treatment (Mesiano *et al.* 1991). No such stimulation was observed in adult bovine fasciculata cells (J J Feige, I Vilgrain & S Souhelnskiy, unpublished observation) but the number of high-affinity FGF-2-binding sites was strongly stimulated by ACTH in a cAMP-dependent manner (Savona & Feige 1991). These observations argue in favour of a role for the FGF/FGFR system in the trophic action of ACTH. However, we await the results of targeted

disruption of the FGF-2 gene in mouse adrenals to confirm the role of FGF-2 *in vivo*. As FGF-2 is also a potent angiogenic and neurotrophic factor, the possibility exists that its trophic effects are due to effects on the growth and maintenance of the vascularization and innervation of the adrenal cortex.

Insulin-like growth factors

IGF-I and IGF-II are proinsulin-related peptides that affect growth and function of a wide variety of cell types in an autocrine, paracrine or endocrine manner (Daughaday & Rotwein 1989). IGF-I is present in plasma and mediates the somatotropic effects of growth hormone, thus promoting bone elongation during childhood. The biological function of IGF-II is less well defined, although its high serum concentration during fetal life and its decrease at birth argue for a role in fetal development. IGFs interact with two distinct IGF receptors. The type I IGF receptor is structurally related to the insulin receptor, has ligand-stimulated tyrosine kinase activity and binds both IGF-I and IGF-II with high affinity and insulin with lower affinity (Ullrich *et al.* 1986, Nissley & Lopaczynski 1991). The type II receptor, also known as the cation-independent mannose-6-phosphate receptor, lacks a tyrosine kinase domain, binds IGF-II and a number of lysosomal proteins with high affinity but does not bind IGF-I or insulin (Morgan *et al.* 1987, Nissley & Lopaczynski 1991). An additional degree of complexity is brought to the IGF system by the existence of six IGF-binding proteins (IGFBPs) which associate with IGFs with affinities equal to or higher than the high-affinity type I receptor (Shimasaki *et al.* 1991a,b) and modulate their bioavailability and their biological activity (Binoux 1996, Clemmons 1997, Spagnoli & Rosenfeld 1997).

Early studies using whole-body autoradiographic analysis of animals infused with radioiodinated insulin had indicated that significant amounts of this hormone bound to adrenal cortex (Bergeron *et al.* 1980). Further studies determined that type I IGF receptors are prominent in adult rat (Pillion *et al.* 1988), human (Pillion *et al.* 1989) and bovine adrenocortical cell membranes (Penhoat *et al.* 1988), whereas few, if any, insulin receptors are expressed. Type I IGF receptor mRNA is also abundantly expressed in the definitive, transitional and fetal zones of the adrenal cortex from rhesus monkey fetuses, from day 109 of gestation until term (occurring at day 165 ± 5) (Coulter *et al.* 1996). Its expression decreases to undetectable levels at term.

IGF-I and IGF-II exhibit opposite patterns of expression in fetal and adult adrenal tissues. In fetal adrenals, as in many other fetal tissues, the content of IGF-II mRNA is high and that of IGF-I mRNA is much lower (Han *et al.* 1988). *In situ* hybridization analysis revealed that IGF-II is expressed in all ACTH-responsive cortical cells, whereas IGF-I is only expressed in the capsule of midgestation

human fetal adrenals (Mesiano *et al.* 1993). In contrast, IGF-II mRNA is very low or undetectable in adult human adrenals (Mesiano & Jaffe 1997) whereas IGF-I mRNA is present (Mesiano *et al.* 1993). In the adult rat adrenal, IGF-I mRNA is mainly expressed in the zona fasciculata (Ho & Vinson 1995). Voutilainen & Miller (1987) first observed that the expression of IGF-II in cultured cortical cells from midgestation human fetal adrenals is strongly stimulated by ACTH and cAMP. In contrast, IGF-I mRNA is undetectable in these cells, even after ACTH treatment (Mesiano *et al.* 1993). In adult bovine adrenocortical cells, ACTH enhances IGF-I synthesis and secretion (Penhoat *et al.* 1989), whereas, in cultured adrenal cortical cells from a 6 week neonate, ACTH was shown to increase cortisol secretion but did not induce IGF-II (Mesiano & Jaffe 1992a). Except for IGFBP1, all IGFBPs are expressed to different levels in the fetal and adult adrenal cortex but do not appear to be regulated by ACTH (Ilvesmaki *et al.* 1993a, Coulter *et al.* 1996).

IGF-I and IGF-II are equipotent growth factors for human fetal adrenocortical cells (Mesiano *et al.* 1993). Their mitogenic effect appears to be co-operative with that of FGF-2 and EGF (Mesiano & Jaffe 1992a). In contrast, IGF-I is a poor mitogen for adult bovine adrenocortical cells but potentiates the strong mitogenic activity of FGF-2 (Penhoat *et al.* 1988). No mitogenic action of IGF-II on adult adrenocortical cells from any species has been reported so far.

Taken together, these data strongly implicate IGF-II as an important local regulator of fetal adrenal development, possibly relaying the specific trophic action of ACTH. The expression of IGF-II and its regulation by ACTH appear to be unique to fetal life since it is rapidly lost after birth. It is noteworthy, however, that IGF-II gene over-expression is detected in a large proportion of sporadic adrenocortical tumours, suggesting that the fetal IGF-II-dependent mitogenic response may be re-activated in the pathological cancer situation (Ilvesmaki *et al.* 1993b, Gicquel *et al.* 1994, 1997).

The pattern of IGF expression is shifted from IGF-II to IGF-I at birth but, in the neonate and adult adrenals, IGF-I does not appear to play an important role in the control of cell proliferation. IGF-I is clearly not upregulated during hypertrophy of the rat adrenal gland after either unilateral adrenalectomy or ACTH infusion (Townsend *et al.* 1990). As described below, IGF-I in adult adrenals probably deals with the regulation of steroidogenesis.

Other growth factors

A number of other growth factors have been found to be expressed in the adrenal cortex and to trigger an effect on cell proliferation.

Transforming growth factor α (TGF α) is a structural homologue of EGF which shares the same receptor. Both

EGF receptor and TGF α are expressed in human fetal and adult adrenals (Sasano *et al.* 1994, Smikle *et al.* 1996) whereas EGF is not. EGF and TGF α are potent mitogens for fetal and definitive zone cells from midgestation human fetal adrenals (Crickard *et al.* 1981, Mesiano *et al.* 1991) but do not stimulate the proliferation of adult bovine adrenocortical cells (Gospodarowicz *et al.* 1977, Hornsby *et al.* 1983). Like IGF-II, TGF α expression and function in the adrenal cortex appear to be restricted to the fetal period.

TGF β 1 and activin, two members of the TGF β superfamily, significantly inhibit the proliferation of human fetal adrenocortical cells from both the fetal and the definitive zone (Riopel *et al.* 1989, Spencer *et al.* 1990, Parker *et al.* 1992). No effect on the proliferation of adult bovine or ovine adrenocortical cells was observed (Feige *et al.* 1986, Hotta & Baird 1986, Rainey *et al.* 1988).

Endothelin-1 and atrial natriuretic factor have been shown to specifically inhibit the proliferation of adult adrenal glomerulosa cells (Cozza & Gomez-Sanchez 1990, Appel 1992).

General remarks

Overall analysis of the mitogenic responses of fetal and adult adrenocortical cells to the above factors leads to the conclusion that the pattern of growth stimulators and inhibitors is quite different in embryonic and adult states. Fetal adrenal cells (at least in the human species, which was mostly studied) respond like epithelial cells: they are stimulated to grow by EGF/TGF α , specific epidermal growth factors, and inhibited by TGF β , a factor that inhibits the proliferation of most epithelial cell types. In contrast, adult adrenocortical cells behave like mesenchymal cells in that they are insensitive to EGF/TGF α or TGF β and are optimally growth-stimulated by FGF-2, a potent mesenchymal cell mitogen. So, at birth, adrenocortical cells appear to switch from an epithelial-like to a mesenchymal-like phenotype. Several factors triggering such a switch have been identified using the bladder carcinoma cell line NBTII as a model (Valles *et al.* 1990). These include scatter factor/hepatocyte growth factor, FGF-1 (often named acidic fibroblast growth factor) and collagen used as a substratum for cell culture. It will be of great interest to define which epithelial/mesenchymal transition factor(s) is turned on soon after birth in the adrenal cortex and contributes to its reorganization.

Non-mitogenic effects of growth factors in the adrenal cortex

Insulin was long known as a hormone before its growth-promoting activity was characterized. Reciprocally, the first report of the non-mitogenic function of a well-established growth factor was made by Andrew Baird and

his collaborators when they reported that FGF-2, a potent mitogen for mesoderm-derived and neuroectoderm-derived cells, increases the secretion of thyrotrophin and prolactin by pituitary cells in response to thyrotrophin-releasing factor (Baird *et al.* 1985). It is now well established that growth factors are multifunctional and behave, in certain situations in certain tissues, as regulators of differentiated functions rather than as mitogens (Sporn & Roberts 1988). The adrenal cortex is no exception and several growth factors appear to be important regulators of steroidogenesis. Schematically, in this organ as in other endocrine tissues, systemic hormones may be considered the main regulators of steroidogenic functions, and growth factors appear to be in charge of the fine tuning of the hormonal response. Locally produced factors possess in common the property of being secreted and complexed in a latent form, and to require an activation step at the cell surface before acting as autocrine or paracrine regulators.

We will review the non-mitogenic actions of growth factors in the adrenal cortex and look at growth factor latency and activation using the TGF β system as an example.

Insulin-like growth factors

The expression of IGFs and IGF receptors in the adrenal cortex has been reviewed above. In adult bovine adrenocortical cells, besides its poor mitogenic effect, IGF-I enhances steroidogenic responsiveness to ACTH and angiotensin II (Penhoat *et al.* 1988). This effect is caused by an increase in ACTH and angiotensin II receptors as well as stimulation of the activity of several steroid hydroxylases (Penhoat *et al.* 1988, Dell *et al.* 1996). Similarly, long-term treatment of human adult adrenocortical cells with IGF-I or IGF-II results in enhanced ACTH receptor and steroidogenic enzyme mRNA expression and stimulates the acute steroidogenic response to ACTH 3- to 6-fold (Pham-Huu-Trung & Binoux 1990, L'Allemand *et al.* 1996). In contrast, exposure of human fetal adrenocortical cells from the fetal zone to IGF-II and oestradiol suppresses ACTH-induced cortisol synthesis but increases dehydroepiandrosterone sulphate (DHEAS) production (Mesiano & Jaffe 1992b). This differential effect results from specific upregulation of P450C17 expression. IGF-I thus appears to be an important factor for maintaining the differentiated phenotype of adult adrenocytes, and IGF-II appears to be an essential factor for maintaining the androgen-biosynthesis capacity of fetal adrenals.

TGF β

TGF β 1 is the prototype of a superfamily of structurally related homo- and hetero-dimeric peptides which includes functionally related members designated TGF β 1 to TGF β 5 and functionally distinct members such as activin, inhibin, müllerian inhibiting substance, bone

morphogenic proteins (BMP2 to BMP8), growth/differentiation factors (GDF1 to GDF9), glial cell line-derived neurotrophic factor and others (Kingsley 1994, Massagué *et al.* 1994). Most of these family members play an important role in embryonic organogenesis and have pleiotropic effects on adult differentiated cells. TGF β 1 is a strong inhibitor of epithelial cell proliferation, a very potent inducer of extracellular matrix accumulation (acting synergistically on the biosynthesis and degradation of matrix components), an immunosuppressor and a regulator of steroidogenic functions in various endocrine cell types (Roberts & Sporn 1991). Three types of specific TGF β 1 receptors have been identified on almost all mammalian cells (Lin & Lodish 1993). Type III receptor (also called betaglycan) is a proteoglycan that possesses a TGF β -binding site in its core protein similar to the one present in endoglin, an endothelial cell-specific membrane glycoprotein. Type III receptors are abundant and are considered to be storage/presentation receptors because they do not possess any signal-transduction module in their extremely short intracytoplasmic domain, and because binding of TGF β to betaglycan appears to increase its affinity for the signalling receptors TGF β RI and TGF β RII (Lopez-Casillas *et al.* 1993). Both TGF β RI and TGF β RII possess intrinsic serine/threonine protein kinase activity. After binding of TGF β 1 protein to TGF β RII, the two receptors TGF β RI and TGF β RII hetero-oligomerize, become phosphorylated and phosphorylate two cytoplasmic proteins named Smads (Smad 2 and Smad 3). Phosphorylated Smads form a hetero-oligomer with Smad 4 and are translocated to the nucleus where they associate with transcription factors to stimulate the transcription of specific TGF β -responsive genes (Attisano & Wrana 1996, Massagué *et al.* 1997). In addition, the existence of antagonistic Smads (Smad 6 and Smad 7), which bind to the TGF β -signalling receptors but do not become phosphorylated on ligand binding, has been reported very recently (Whitman 1997). A 400 kDa TGF β receptor has been described recently in mink lung epithelial cells and named TGF β RV. TGF β RV has a 40 times lower affinity for TGF β 1 than TGF β RI and TGF β RII and also binds IGFBP3 with high affinity (Leal *et al.* 1997). Cloning of this new receptor is expected to reveal whether it has intrinsic serine/threonine kinase activity.

We and the team of Baird first reported in 1986 that TGF β is a potent inhibitor of steroid production in primary cultures of adult bovine adrenocortical fasciculata cells (Feige *et al.* 1986, Hotta & Baird 1986). Several reports extended this observation to adult adrenocortical cells from the ovine and human species and to bovine glomerulosa cells (Rainey *et al.* 1988, 1990, Gupta *et al.* 1992, 1993, Lebrethon *et al.* 1994a, Naaman-Reperant *et al.* 1996). Although treatment of these different cell types with TGF β 1 in the ng/ml concentration range results, in every cell type, in inhibition of production of cortisol (for fasciculata cells) or aldosterone (for glomerulosa

cells), the intracellular targets of TGF β appear to differ from one species to another. In bovine fasciculata cells, low-density lipoprotein receptors (Hotta & Baird 1987) and angiotensin II receptors (Feige *et al.* 1987) appear to be downregulated by TGF β 1 whereas ACTH receptor number is not modified. In these cells, one main target of TGF β action was found to be cytochrome P450c17 (CYP_{17 α}), the mRNA and activity of which dramatically decline between 3 and 12 h after TGF β 1 addition (Perrin *et al.* 1991). In addition, we recently observed that cholesterol access to the inner mitochondrial membrane is also rapidly inhibited in response to TGF β . At day 2 of fasciculata cell primary culture, of the 80% inhibition of basal cortisol production, 40% appears to result from the inhibition of cholesterol import to the inner mitochondrial membrane and 40% from the inhibition of the 17 α -hydroxylation step (C Brand & S Bailly, unpublished observations). StAR, a recently cloned mitochondrial protein that is essential to the transfer of cholesterol from the cytoplasm to the inner mitochondrial membrane (Clark & Stocco 1996), is a candidate target of TGF β for its effect on mitochondrial cholesterol import (Brand *et al.* 1998). In sheep, distinct proteins are the targets of TGF β action. After 24 h of TGF β 1 treatment, ACTH-stimulated cAMP production, 11 β -hydroxylase and 21-hydroxylase activities were decreased (Rainey *et al.* 1988). Interestingly, 17 α -hydroxylase mRNA expression was reduced more than 10-fold after a 48 h TGF β treatment (Rainey *et al.* 1990), and a rapid effect of TGF β on the utilization of cholesterol pools was also observed (Rainey *et al.* 1988). Long-term TGF β treatment (5 days) of ovine cells also decreases the expression of P450_{sc} (Naaman-Reperant *et al.* 1996). In human adult fasciculata cells, TGF β 1 enhances angiotensin II AT1 receptors and 3 β -hydroxysteroid dehydrogenase but decreases P450_{17 α} expression (Lebrethon *et al.* 1994a). This results in a decrease in DHEAS production whereas basal or ACTH-stimulated cortisol production is not reduced (Lebrethon *et al.* 1994a). Similarly, TGF β preferentially inhibits DHEAS production in human fetal adrenal cells from both the fetal and the neocortical zones and has a more limited effect, if any, on cortisol production (Lebrethon *et al.* 1994b, Stankovic *et al.* 1994).

The TGF β 1 protein is present in abundant quantities in the most external zones of the adult bovine adrenal gland, with more prominent staining in the fasciculata than in the glomerulosa and much weaker staining in the reticularis (Keramidas *et al.* 1991). TGF β 1 protein has also been detected in the inner cortex of the adult mouse adrenal (Thompson *et al.* 1989), although very small amounts of TGF β 1 mRNA appear to be present in this tissue (Ho & Vinson 1995). Of the different TGF β isoforms, both TGF β 1 and TGF β 2 proteins are detectable in the fetal mouse adrenal whereas TGF β 3 is not (Pelton *et al.* 1991). In contrast, adult bovine adrenocortical cells express mainly TGF β 1, TGF β 3 slightly and TGF β 2 not at all, as determined by reverse transcription PCR (Chambaz *et al.*

Figure 2 Tentative model of the generation of latent and active forms of TGFβ in the supernatant of bovine adrenocortical fasciculata cell primary cultures. Unprocessed TGFβ is secreted in large quantities by bovine adrenocortical cells but it is not known whether this inactive pro-TGFβ can undergo maturation in the extracellular compartment. LTBP, LAP and mature TGFβ are found associated in a latent complex in bovine adrenocortical cell conditioned medium. This complex can be activated through interaction with thrombospondins and possibly through other mechanisms (e.g. plasmin degradation). Activation-dependent release of mature TGFβ results in its rapid binding to its high-affinity signalling receptors and the triggering of its biological effects (inhibition of cortisol production). Released TGFβ also binds rapidly to α₂-macroglobulin (α₂M) and forms a non-covalent complex that may be considered to be a circulating latent complex. This complex is slowly converted into a covalent association which is biologically inactive. This tentative model is based on observations reported in Souchelnitskiy *et al.* (1995).

1996). Given the redundancy of the effects triggered by these factors, these distinct patterns of expression may not reflect any difference in terms of biological effects. Bovine adrenocortical cells express all three types of TGFβ receptor, but TGFβRII is present in barely detectable amounts and probably represents the limiting protein in the TGFβ-binding system (Cochet *et al.* 1988, Feige *et al.* 1991). ACTH was observed to increase high-affinity TGFβ receptor number by a factor of 2 (Cochet *et al.* 1988). Similar observations were made on human fetal adrenal cells (Stankovic & Parker 1995). Bovine adrenocortical cells in primary culture secrete about 0.5 ng TGFβ-like activity/24 h per 10⁶ cells and all this TGFβ is in a latent form. TGFβ activity can be revealed by an acidification/neutralisation treatment (Keramidas *et al.* 1991, Souchelnitskiy *et al.* 1995). It is noteworthy that this amount of TGFβ would maximally inhibit steroidogenesis, if it was not complexed to latent proteins. The biochemical nature of these latent forms has been characterized recently. It appears that two distinct complexes coexist in the conditioned medium of adrenocortical cells: a 600 kDa complex containing α₂-macroglobulin and mature TGFβ1 and a 200–250 kDa complex containing latent TGFβ–

binding protein (LTBP), latency-associated protein (LAP; the precursor part of pro-TGFβ1) and mature TGFβ1 (Souchelnitskiy *et al.* 1995). Each complex appears to contain both covalent and non-covalent associations of the aforementioned proteins. Two physiological mechanisms of latent TGFβ activation have been reported in the literature: the proteolytic attack of LAP by plasmin and the interaction of latent TGFβ with thrombospondins (Flaumenhaft *et al.* 1993, Feige *et al.* 1996). Both latent TGFβ complexes present in the conditioned medium of bovine adrenocortical cells were incubated with either thrombospondin-1 or thrombospondin-2, and the release of active TGFβ was measured. It was observed that the α₂-macroglobulin–TGFβ complex remains latent whereas the LTBP–LAP–TGFβ complex becomes activated on incubation with either thrombospondin-1 or thrombospondin-2 (Souchelnitskiy *et al.* 1995). This activation probably requires additional cell surface components to take place, since no molecular interaction between purified thrombospondins and purified reconstituted small latent TGFβ was observed using surface plasmon resonance technology (Bailly *et al.* 1997). It should be noted that thrombospondins are synthesized and

Table 1 Latent forms of growth factors and their activation mechanisms

	Latent complex	Activation mechanism	References
Growth factor			
FGF-1, FGF-2	HSPG–FGF complex	Heparanases Urokinase Proteases	Bashkin <i>et al.</i> (1989); Whitelock <i>et al.</i> (1996) Odekon <i>et al.</i> (1992) Saksela & Rifkin (1990); Whitelock <i>et al.</i> (1996)
IGF-I, IGF-II	IGF–IGFBP complexes	Proteolysis	Collett–Solberg & Cohen (1996)
TGFβ1, TGFβ2	TGFβ–LAP–LTBP complex	Proteolysis of LAP by plasmin Indirect activation by thrombospondins	Flaumenhaft <i>et al.</i> (1993) Schultz–Cherry & Murphy–Ullrich (1993); Souhelnitskiy <i>et al.</i> (1995) Barcellos–Hoff & Dix (1996)
	TGFβ–α2-macroglobulin	Oxidation by reactive oxygen species Covalent complexes are not activatable Non-covalent complexes: interaction with heparin	McCaffrey <i>et al.</i> (1989)

differentially regulated by ACTH in bovine adrenocortical cells (Lafeuillade *et al.* 1996). Whereas thrombospondin-1 expression is inhibited by ACTH, thrombospondin-2 is one of the major secreted proteins induced by ACTH (Pellerin *et al.* 1993). Therefore these proteins probably represent physiological activators of latent TGFβ. In contrast, the α₂-macroglobulin–TGFβ complex may be restricted to the *in vitro* situation since we observed that α₂-macroglobulin synthesis is turned on when cells are placed in primary culture but is not active in freshly isolated cells (Negoescu *et al.* 1994). However, α₂-macroglobulin is present at high concentrations (2–4 mg/ml) in human plasma, and this circulating form of α₂-macroglobulin could play a similar role to that played by adrenocortical cell-secreted α₂-macroglobulin. From these data, we favour the hypothesis that TGFβ1 is synthesized and secreted by adrenocortical cells in a latent form comprising LTBP, LAP and mature TGFβ and is then activated at the cell surface by a thrombospondin-mediated mechanism. Released mature TGFβ can bind to its high-affinity receptors and transduce an inhibitory signal resulting in decreased steroidogenesis. Released mature TGFβ is also rapidly chelated by α₂-macroglobulin and slowly converted into a biologically inactive covalent complex (Fig. 2).

Le Roy *et al.* (1996) used an antisense oligonucleotide strategy to block the expression of TGFβ1 in bovine adrenocortical fasciculata cells. All of the TGFβ1 antisense oligonucleotide-treated cells exhibited a dramatically decreased TGFβ1 content. The expression of P450C17 and 3β-hydroxysteroid dehydrogenase was increased 2-fold and angiotensin II-induced cortisol production was also increased 2-fold as compared with sense oligonucleotide-treated cells (Le Roy *et al.* 1996). These experiments prove that an autocrine regulatory loop, by which TGFβ counteracts the steroidogenic action of ACTH and angiotensin II, exists in cultures of bovine adrenocortical cells. As underlined above, the *in vivo* situation may be somewhat different from the *in vitro*

situation and the physiological importance of TGFβ in the regulation of steroidogenesis remains to be established *in vivo*. TGFβ1 gene invalidation in the mouse results in multifocal inflammation and postnatal death, but does not induce gross developmental disorders (Shull *et al.* 1992, Kulkarni *et al.* 1993). We will have to wait for the results of conditional or tissue-specific gene knock-outs to understand more completely the specific role played by TGFβ *in vivo* in the adrenal cortex. The conservative site-specific recombination of bacteriophage P1, namely Cre-lox, can be used successfully in combination with homologous recombination to generate temporal- and cell type-restricted gene mutations or deletions *in vivo* (Orban *et al.* 1992, Kuhn *et al.* 1995, Wagner *et al.* 1997). The generation of transgenic mice expressing Cre recombinase under the control of an adrenocortical-specific gene promoter such as that of steroid 21-hydroxylase (Morley *et al.* 1996) should allow the engineering of mice with a TGFβ gene deletion restricted to the adrenal cortex. Also, the recently described fusion construct between Cre recombinase and a mutated ligand-binding domain of the oestrogen receptor resulting in a modified Cre recombinase, which is activated by tamoxifen but not by oestradiol, offers the possibility of generating conditional knock-out mice (Feil *et al.* 1996). Such a strategy should allow us to bypass the dramatic developmental effects of TGFβ gene invalidation and check the results of TGFβ gene invalidation at the adult stage.

The crinopexy paradigm

The implication of growth factors in the regulation of adrenal cortex proliferation and steroidogenic functions is now well documented. The existence of autocrine regulatory loops seems to be well demonstrated for at least three growth factors discussed in detail in this review: FGF-2, TGFβ1 and IGF-I. In every case, the expression of the growth factor and its receptors on the same adrenocortical

cell type and the characterization of the intracellular targets of its action have been determined. However, the observation that each of these factors is present in a latent form in the external milieu and requires an activation step to generate the biologically active factor has been vastly underestimated in the endocrinology field. As summarized in Table 1, growth factors form complexes with proteoglycans (HSPGs and FGFs) or with extracellular matrix-binding proteins (IGFBPs and IGFs; LTBP and pro-TGF β), which prevent them from binding to their signalling receptors.

We have proposed the term 'crinopectin' to define molecules that are secreted ('crino') by cells and adhere ('pectin') to specific pericellular structures where they become sequestered in a latent form, and the term 'crinopexy' to define the biological mechanisms by which cells regulate the bioavailability of crinopectins (Feige & Baird 1992, 1995). According to this paradigm, the biological signal induced by crinopectins is triggered by their release and/or activation from the storage sites rather than by their active synthesis. This would allow us to understand why the tissue distribution of a growth factor (e.g. abundant TGF β 1 protein in the fasciculata zone) may sometimes appear in contradiction with its expected biological activity (inhibition of P450_{17 α} expression induced by TGF β *in vitro*). The nature of the biological reactions that turn latent crinopectins into active receptor-binding growth factors is beginning to be deciphered (Table 1) but is still poorly characterized in adrenocortical cells. Understanding these mechanisms will, however, be necessary in order to obtain the complete picture of the regulation of adrenocortical function by growth factors.

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