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 17a-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 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 11b-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 adrenocorticotropic hormone (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 depletion 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 adenylyl 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 tropicity 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 (TGFβ)/TGFβ 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 secretary pathway (Mignatti et al. 1992, Forkiewicz 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).
FGFR3 was detectable in the fetal adrenal, and FGFR1 in human fetuses were found in the adrenal glands. No TGFβ was detectable in the fetal zone but appears to be absent from the adenohypophysis (Gonzalez et al. 1991). Similarly, in human fetal adrenals, FGFR-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, FGFR-2 mRNAs are barely detectable in this issue (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 (Kd about 400 nM, 230 000 sites/cell) and a limited number of high-affinity FGF-2 receptors (Kr about 2 pM, 4300 sites/cell) (Savona & Handley 1986). The subtypes of FGF receptors expressed by bovine 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 Souchelnitskiy, 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

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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 × 10⁶/10 cm plate) were lysed in 20 mM Tris–HCl (pH 7·5)/150 mM NaCl/0·2 M sucrose buffer containing proteinase inhibitors (pepsstatin, 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 × 10⁶ 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 (NEL 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.

MW (kDa)

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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 radiiodinated 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 adren 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 (TGFα) is a structural homologue of EGF which shares the same receptor. Both
EGF receptor and TGFβ are expressed in human fetal and adult adrenals (Sasano et al. 1994, Smikle et al. 1996) whereas EGF is not. EGF and TGFβ 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, TGFβ expression and function in the adrenal cortex appear to be restricted to the fetal period.

TGFβ1 and activin, two members of the TGFβ 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/TGFα, specific epidermal growth factors, and inhibited by TGFβ, 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/TGFα or TGFβ 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 TGFβ 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 adrenocortical cells, and IGF-II appears to be an essential factor for maintaining the androgen-biosynthesis capacity of fetal adrenals.

**TGFβ**

TGFβ1 is the prototype of a superfamily of structurally related homo- and hetero-dimeric peptides which includes functionally related members designated TGFβ1 to TGFβ5 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. TGFβ1 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 TGFβ receptors have been identified on almost all mammalian cells (Lin & Lodish 1993). Type III receptor (also called betaglycan) is a proteoglycan that possesses a TGFβ-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 TGFβ to betaglycan appears to increase its affinity for the signalling receptors TGFβRI and TGFβRII (Lopez-Casillas et al. 1993). Both TGFβRI and TGFβRII possess intrinsic serine/threonine protein kinase activity. After binding of TGFβ1 protein to TGFβRII, the two receptors TGFβRI and TGFβRII 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 TGFβ-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 TGFβ-signalling receptors but do not become phosphorylated on ligand binding, has been reported very recently (Whitman 1997). A 400 kDa TGFβ receptor has been described recently in mink lung epithelial cells and named TGFβRV. TGFβRV has a 40 times lower affinity for TGFβ1 than TGFβRI and TGFβRII 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 TGFβ 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 TGFβ1 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 TGFβ 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 TGFβ1 whereas ACTH receptor number is not modified. In these cells, one main target of TGFβ action was found to be cytochrome P450c17 (CYP17), the mRNA and activity of which dramatically decline between 3 and 12 h after TGFβ1 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 TGFβ. 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 17α-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 TGFβ for its effect on mitochondrial cholesterol import (Brand et al. 1998). In sheep, distinct proteins are the targets of TGFβ action. After 24 h of TGFβ1 treatment, ACTH-stimulated cAMP production, 11β-hydroxylase and 21-hydroxylase activities were decreased (Rainey et al. 1988). Interestingly, 17α-hydroxylase mRNA expression was reduced more than 10-fold after a 48 h TGFβ treatment (Rainey et al. 1990), and a rapid effect of TGFβ on the utilization of cholesterol pools was also observed (Rainey et al. 1988). Long-term TGFβ treatment (5 days) of ovine cells also decreases the expression of P450c17 (Naaman-Reperant et al. 1996). In human adult fasciculata cells, TGFβ1 enhances angiotensin II AT1 receptors and 3β-hydroxysteroid dehydrogenase but decreases P450c17 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, TGFβ 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 TGFβ1 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). TGFβ1 protein has also been detected in the inner cortex of the adult mouse adrenal (Thompson et al. 1989), although very small amounts of TGFβ1 mRNA appear to be present in this tissue (Ho & Vinson 1995). Of the different TGFβ isoforms, both TGFβ1 and TGFβ2 proteins are detectable in the fetal mouse adrenal whereas TGFβ3 is not (Pelton et al. 1991). In contrast, adult bovine adrenocortical cells express mainly TGFβ1, TGFβ3 slightly and TGFβ2 not at all, as determined by reverse transcription PCR (Chambaz et al. 1998).
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 10^6 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

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).
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 (HPSGs and FGs) or with extracellular matrix–binding proteins (IGFBPs and IGs; LTBP and pro–TGFβ), 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 TGFβ1 protein in the fasciculata zone) may sometimes appear in contradiction with its expected biological activity (inhibition of P450c17α expression induced by TGFβ 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.

Acknowledgements

We are grateful to Isabelle Dubard and Sonia Lidy for their editorial help and to colleagues from our laboratory for their contributions to this field. The research performed in our laboratory was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Commissariat à l’Energie Atomique (CEA/DSV/DBMS). C B is a recipient of a doctoral fellowship from CEA.

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Received 17 November 1997
Accepted 24 February 1998