

THE AUTO/PARACRINE REGULATION OF ENDOCRINE FUNCTIONS: A HISTORY OF TGF- β AND THE ADRENAL CORTEX

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RESUM

Aquesta breu revisió vol analitzar quinze anys de recerca que han portat a proposar el factor de creixement transformant beta (TGF- β) com un component endogen auto/paracrí de la regulació de les funcions diferenciades de teixits endocrins, com el còrtex adrenal. El TGF- β reuneix els criteris requerits per aquesta funció, és a dir: *a)* El pèptid TGF- β està produït per les cèl·lules adrenocorticals i expressat *in situ* en aquest teixit; *b)* el TGF- β reprimeix considerablement la capacitat esteroidogènica d'aquestes cèl·lules reprimint els marcadors clau de diferenciació; *c)* l'hormona sistèmica ACTH modula la resposta de les cèl·lules adrenocorticals al TGF- β ; *d)* la supressió de la síntesi de TGF- β suprimeix la inhibició de l'activitat esteroidogènica d'aquestes cèl·lules. El sistema del TGF- β adrenocortical és, al nostre entendre, el primer circuit regulador (*loop*) clarament establert en un teixit endocrí. Aquest viatge històric és un tribut al nostre amic José Sáez, que va participar activament en el coneixement d'aquesta història.

Paraules clau: còrtex adrenocortical, TGF- β , funcions diferenciades, regulació autocrina.

SUMMARY

This brief review is intended as a flash-back spanning over fifteen years of research and finally leading to the proposal that TGF- β could be an endogenous, auto/paracrine component in the regulation of the differentiated functions of an endocrine tissue, i.e., the adrenal cortex. TGF- β meets the criteria required for such a function: (i) the peptide is produced by adrenocortical cells and expressed in the tissue *in situ*; (ii) TGF- β strikingly down-regulates the steroidogenic capacity of these cells by repressing key differentiation markers; (iii) the systemic hormone ACTH modulates the adrenocortical cells' responsiveness to TGF- β , and (iv) the suppression of TGF- β synthesis releases the inhibition of the steroidogenic activity of these cells. The TGF- β adrenocortical cell system was, to our knowledge, the first autocrine regulatory loop clearly established in an endocrine tissue. This chronological account is a tribute to our friend, José Sáez, who actively contributed to this history.

Keywords: adrenocortical cortex, TGF- β , differentiated functions, autocrine regulation.

By the mid-eighties, the pituitary peptide adrenocorticotrophic hormone (ACTH) had been recognized for decades as the physiological agent controlling both the trophicity and the differentiated steroidogenic functions of the mammalian adrenal cortex in a positive way (Simpson and Waterman, 1988; Miller, 1988; Stocco and Clark, 1996). Corticosteroid secretion by adrenocortical cells is the product of a cascade of highly specific enzymatic reactions, starting with the side chain cleavage of cholesterol after its import into mitochondria and followed by a series of cytochrome P-450 supported hydroxylations (17α , 11β , 21 , ...). A limiting step of the pathway is considered to be the access of cholesterol to the inner mitochondrial membrane, which involves the Steroidogenic Acute Regulatory protein (StAR) and is acutely activated by ACTH through a cyclic AMP-protein kinase A signaling. At the same time, ACTH induces a long-term increase in the expression of the genes encoding the major steps of the steroidogenic cascade, such as steroid 17α hydroxylase (CYP $_{17\alpha}$) and the ACTH receptor itself (Le Roy *et al.*, 2000). In addition, at least in adrenocortical cells of bovine origin, angiotensin II (A-II) is an acute activator of steroidogenesis, although through a different intracellular signaling. By the early 90s, Fibroblast Growth Factor (FGF-2) had been shown to induce adrenocortical cell proliferation (Feige and Baird, 1991). In a search for new possible regulators of adrenal cortex trophicity, we presented the recently identified peptide TGF- β to bovine adrenocortical cells.

The name TGF (Transforming Growth Factor) came from the discovery that conditioned medium from virally transformed cells could induce normal cells to express a transformed phenotype, presenting the idea that a major player in the oncogenic process had been

identified. It was soon shown that this biological effect was due to the combination of two active peptides: TGF- α acting as a growth factor and TGF- β which appeared, in fact, as an inhibitor of epithelial cell proliferation (Sporn and Roberts, 1992).

Twenty years later, TGF- β is still a misleading name. While the peptide has been established as the archetype for a large family of multifunctional cytokines of great importance in the development and differentiation processes (Peralta-Zaragoza *et al.*, 2001), TGF- β has been found to act on many normal cellular processes. To our knowledge, TGF- β was the first peptide to meet the criteria of an auto/paracrine regulator of an endocrine tissue, i.e., a locally produced factor, regulating the actions of systemic hormones on their specific cellular targets.

This short review will cover 15 years of progress establishing TGF- β as a plausible autocrine regulator of adrenocortical functions. Following this line of investigation, our research Unit was accompanied by José Sáez's group in a constructive and stimulating competition, which was always respectful and which reinforced a warm and friendly relationship.

TGF- β IS A HIGHLY POTENT REPRESSOR OF ADRENOCORTICAL STEROIDOGENIC DIFFERENTIATED FUNCTIONS

Somewhat to our surprise, no effect at all was observed on serum or FGF supported DNA synthesis and proliferation, when TGF- β was added to bovine adrenocortical cells in cultures. In contrast, the addition of TGF- β rapidly resulted in a striking reduction in the steroidogenic capacity of the preparation (Feige *et al.*, 1986).

Basal, as well as ACTH and A-II activated cortisol production were inhibited in a time and dose-dependent fashion by TGF- β at picomolar concentrations. The effect was maximal after 12-15 hours and resulted in an average 50% cut in the response to ACTH and 90% in the response to A-II. Detailed study of the steroidogenic pathway pointed to 17 α hydroxylase as a major target of this negative effect (Feige *et al.*, 1987; Perrin *et al.*, 1990). Indeed, the cell contents in P-450_{17 α} mRNA and protein were strikingly reduced following 24 hours of TGF- β treatment, in agreement with a parallel loss of 17 α hydroxylase activity. In addition, the number of cell surface receptors to A-II was cut by half, explaining why TGF- β reduced the response to A-II more strongly than the steroidogenic response to ACTH (Feige *et al.*, 1987).

After spending one year in our group, a post-doctoral fellow (W. Rainey) joined José Sáez's laboratory and extended these seminal observations to adrenocortical cells of ovine origin, in which P-450_{17 α} was also shown to be a major target down-regulated by TGF- β (Rainey *et al.*, 1990). In these species, TGF- β reduced the expression of ACTH receptors and blocked their up-regulation by ACTH itself (Rainey *et al.*, 1989). It was also found that TGF- β -treated cells exhibited an impaired ability to use cholesterol for steroidogenesis. This point was later confirmed in bovine cells and could be explained by the fact that TGF- β repressed the expression of the StAR protein (Brand *et al.*, 1998a). The negative effect of TGF- β on steroidogenic functions was thereafter reported with normal, as well as tumoral adrenocortical cells of human origin, although it appeared that the specific down-regulated molecular targets varied somewhat in different species (Lebrethon *et al.*, 1994).

ADRENOCORTICAL CELLS EXHIBIT HIGH AFFINITY TGF- β RECEPTORS, WHICH ARE REGULATED BY ACTH

Since extra-cellularly added TGF- β was active, we searched for the presence of specific receptors at the surface of the adrenocortical cells. Two different TGF- β binding systems were characterized, of higher (Kd 10⁻¹⁰ M) and lower (Kd 10⁻⁸ M) affinities respectively. A most striking phenomenon was that a 24 hour ACTH treatment of these cells markedly increased (average two times) the number of high affinity receptors (Cochet *et al.*, 1988). More recently, the specific intracellular TGF- β signaling system, involving Smad proteins, was demonstrated in bovine adrenocortical cells, confirming their status as TGF- β target cells (Brand *et al.*, 1998).

These observations strongly suggested that ACTH exposure should result in the increased sensitivity of adrenocortical cells to the inhibitory action of TGF- β giving a basis for a negative regulatory feed-back control, limiting the positive actions of ACTH itself (Feige *et al.*, 1991; Langlois *et al.*, 1998).

TGF- β IS PRODUCED BY ADRENOCORTICAL CELLS, SUGGESTING ITS PARTICIPATION IN AN AUTOCRINE REGULATORY LOOP

Immunohistochemistry clearly established that TGF- β was expressed in bovine adrenocortical tissue, especially in the fasciculata-reticularis layers, corresponding to active steroidogenic zones (Keramidas *et al.*, 1991). Using a specific assay, biosynthesis and secretion of the peptide was unambiguously demonstrated. TGF- β mRNA appeared constitutively expressed in bovine adrenocortical cells, and a production of about 5 ng of peptide/24 hours/10⁶ cells could be assessed under basal culture conditions. This could result

in local concentrations well in the range of the functionally effective ones (see above).

When the molecular form of TGF- β secreted by the adrenocortical cells was examined in detail, it became clear that the peptide was not under a free moiety, as already reported from other biological sources (Savona *et al.*, 1994; Bailly *et al.*, 1997). Adrenocortical TGF- β appeared to be included in two major polypeptide complexes. One contained the Latent TGF- β Binding Protein (LTBP), known to be a major combination in blood platelets. The other was identified as a complex with α_2 -macroglobulin (α_2 -M), known to be a blood protein able to trap a number of cytokines (Feige *et al.*, 1996). However, TGF- β complexed with α_2 -M appeared to be active when added to adrenocortical cells (Keramidas *et al.*, 1992).

Together with α_2 -M, it was discovered that adrenocortical cells secrete another protein, whose production was greatly stimulated by ACTH. This corticotrophin-induced secreted protein was finally identified as thrombospondin 2 (TSP-2) (Pellerin *et al.*, 1993). Most interestingly, TSP-2 was shown to activate TGF- β when the peptide was complexed with LTBP (Souhelnitskiy *et al.*, 1995). In addition to ACTH, TGF- β itself appeared to stimulate adrenocortical TSP-2 secretion (Negoescu *et al.*, 1995), thus, contributing to an extra-cellular environment, which would favor the occurrence of biologically potent TGF- β .

TGF- β AND THE ADRENOCORTICAL CELLS: THE AUTO/PARACRINE MODEL

All the accumulated observations strongly suggested that endogenously produced TGF- β could be acting as a regulator of adrenocortical cell-differentiated functions, serving as a local relay in balance with the systemic hormone ACTH. TGF- β could represent an *in*

situ negative feed-back control loop, opposing most of the effects of ACTH. ACTH, in turn, could be controlling cell responsiveness to the peptide (Chambaz *et al.*, 1996).

The validity of this working hypothesis was definitively confirmed by José Saez's group, using an anti-sense oligonucleotide approach, which targeted the expression of TGF- β in bovine adrenocortical cells (Le Roy *et al.*, 1996). Conveniently chosen anti-sense was shown to enter the cells and to markedly inhibit TGF- β mRNA expression and peptide synthesis. Under these conditions, a large increase ($\times 2$) in the steroidogenic capacity of the cells, in response to an ACTH challenge, was observed, parallel to a similar increase in the expression of cyt P450_{17 α} hydroxylase. This clearly showed that the neutralization of the endogenous TGF- β signal resulted in the suppression (release) of a pathway, negatively regulating the steroidogenic differentiated functions in these cells. Thus, in this system TGF- β most likely represented an endogenous (autocrine) regulator, integrated as a negative local loop in the action of ACTH.

CONCLUSION

After more than fifteen years of experimental observation, TGF- β may be considered an autocrine regulator of adrenocortical cell-differentiated functions for solid reasons:

- Adrenocortical cells produce and secrete TGF- β which is present in the tissue *in situ*.

- These cells express TGF- β receptors and the corresponding specific intracellular signaling.

- TGF- β acts as a repressor of differentiated steroidogenic adrenocortical functions by negatively regulating the expression of specific cellular targets. TGF- β globally opposes the positive effects of ACTH on the same targets.

- ACTH controls the local TGF- β loop, e.g.,

by up-regulating the TGF- β receptors, thus, initiating the limitation of its own action.

— The suppression of TGF- β local production results in an amplification of the ACTH-effects, in agreement with a release of the local brake represented by the peptide. This last point convincingly establishes TGF- β as an *in situ* component in adrenocortical functions.

Using a similar approach, it was suggested that TGF- β contributed to an autocrine negative loop opposing the action of the trophic hormones LH/HCG in Leydig cells (Le Roy *et al.*, 1999). Knock-out of the TGF- β_1 gene in mice resulted in the early death of newborns with severe inflammatory syndrome. However, no targeted invalidation of the gene has yet been reported in endocrine tissue that could possibly support the autocrine model.

In addition to TGF- β a number of peptides have been shown to be produced by adrenocortical cells and may represent local relays in ACTH action, although they are not included in the local regulatory loop (Penhoat *et al.*, 1996; Feige *et al.*, 1998). This is the case with FGF-2, IGF-1 and IGF-2, possible relays for the trophic action of ACTH. One should point out that, as seen with TGF- β , these peptides are complexed in the extra-cellular milieu with binding proteins or proteoglycans. Thus, an additional mechanism of fine regulation may take place at that level, leading to the concept of crinopexy (Feige and Baird, 1995).

In addition to control of the differentiated steroidogenic functions of endocrine adrenocortical cells, locally produced factors may be crucial to the coordinated control of the very important vasculature of the gland. Such a paracrine role is clearly suggested for Vascular Endothelial Growth Factor (V-EGF), which is produced by endocrine adrenocortical cells and is the most powerful growth and differentiation factor for endothelial cells and angiogenesis (Thomas *et al.*, 2003, 2004). The role of locally produced TGF- β in combination with V-EGF in this process remains to be clarified.

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