Effects of prolonged infusion of basic fibroblast growth factor and IGF-I on adrenocortical differentiation in the autotransplanted adrenal: an immunohistochemical study

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Abstract

Adrenocortical regeneration after adrenal autotransplantation provides a model for the study of local autocrine/paracrine mechanisms involved in the growth and differentiation of the adrenal cortex. To study the possible involvement of some growth factors, namely basic fibroblast growth factor (bFGF, FGF-2) and insulin-like growth factor I (IGF-I), in cell differentiation, immunohistochemical and ultrastructural studies were carried out on adrenal autotransplants in adult male rats. To distinguish between fasciculata and glomerulosa-like cells with accuracy, tissue sections were immunostained with IZAb, which recognizes the inner zone antigen (IZAg) present in fasciculata and reticularis cells but absent from the glomerulosa, and by electron microscopy. IGF-I-treated animals exhibited a clear glomerulosa-like zone that was devoid of IZAb immunostaining. In this outer subcapsular area, ultrastructural examination showed cells containing mitochondria with irregular cristae resembling those of the fetal or immature glomerulosa cells. In contrast, no significant morphological differences were observed in bFGF-treated animals when compared with those from saline-treated controls, in both of which, IZAb immunostaining occurred in almost all adrenocortical cells, with no clear zonation or glomerulosa, as seen in the intact animal. Plasma aldosterone and corticosterone concentrations were lower in autotransplanted control animals than in intact controls, although plasma renin activities were similar. IGF-I treatment significantly increased aldosterone concentrations, whereas corticosterone and plasma renin activity were reduced. bFGF infusion further reduced plasma aldosterone, although plasma renin activity and corticosterone were unaffected. These results suggest that the two growth factors have different effects on zonal differentiation and function in the autotransplanted gland. In particular, bFGF, by reducing glomerulosa function, appears partly to replicate the actions of ACTH in normal animals. In contrast, IGF-I enhances the glomerulosa secreting phenotype and diminishes that of the fasciculata/reticularis, possibly replicating the actions of angiotensin II or a low sodium diet.

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Introduction

Adrenal gland autotransplantation provides a useful model to study adrenocortical regeneration in laboratory animals (Ingle & Higgins 1938, Belloni et al. 1990). This is particularly so as morphological zonation may also be reproduced (Saxe & Connors 1985, Vendeira et al. 1992).

Transplanted tissue may recover its functions, at least partially, but normally not reflecting morphological zonation, as neither glucocorticoid nor mineralocorticoid secretion is fully restored; this has been demonstrated in both humans (Prinz et al. 1989, Demeter et al. 1990, Lucon et al. 1993) and rodents (Belloni et al. 1991, Ganguly 1991, Sarria et al. 1995, Vendeira et al. 1996a).

As we have previously demonstrated (Vendeira et al. 1992), regeneration processes in the autotransplanted gland start at the periphery of the graft, proceeding from subcapsular glomerulosa-like cells. A partial adrenocortical zonation, with differentiated inner and outer areas, was observed at day 90 after autotransplantation, even in the absence of the medulla (Vendeira et al. 1996b). Conceivably, this may be enhanced by administration of substances that specifically stimulate the growth and steroidogenic capacity of the zona glomerulosa, perhaps including the wide range of neuropeptides that have been identified in
the capsule and zona glomerulosa of adrenal glands (Hinson et al. 1992, 1994, Malendowicz 1993, Nussdorfer 1996). Vascular products may also be involved, for example endothelin-1, which can both stimulate proliferation of the rat adrenal zona glomerulosa (Mazzocchi et al. 1992, 1997, Belloni et al. 1996) and potentiate aldosterone secretion (Cozza & Gomez-Sanchez 1990, Mazzocchi et al. 1990a, b, Hinson et al. 1991, Nussdorfer et al. 1997). Indeed, previous studies have emphasized a role for endothelin-1 in adrenals regenerating after autotransplantation (Vendeira et al. 1996a) or adrenal enucleation (Malendowicz et al. 1997).

Some growth factors are also known to be important. Much evidence supports the modulatory role of basic fibroblast growth factor (bFGF, FGF-2) and insulin-like growth factor-I (IGF-I) in the growth and regulation of a wide array of biological systems (Goustin et al. 1986, Han et al. 1987, Roith 1997, Bzikfaly et al. 1997, Ray & Melmed 1997). More specifically, in the context of steroidogenic organs, IGF-I is thought to induce and maintain differentiated functions of Leydig, ovarian granulosa and adrenocortical cells (Bergh et al. 1991, Penhoat et al. 1994). In the adrenal, this concept is strengthened by the fact that adrenocortical cells secrete IGF-I during proliferation induced by enucleation or unilateral adrenalectomy, or by adrenoventricular cells and medulla (Basile & Holzwarth 1993). In addition, after unilateral adrenalectomy, bFGF was localized immunohistochemically in cells of the rat adrenal zona glomerulosa and medulla (Basile & Holzwarth 1993, 1994). Taken together, these data support the view that growth and differentiation of the rat adrenal cortex may at least partly be mediated by these factors in an autocrine/paracrine manner (Mesiano et al. 1991, Ho & Vinson 1995). bFGF is also a potent mitogen of bovine adrenocortical cells and rat capsule glomerulosa in vitro (Gospodarowicz et al. 1977, Basile & Holzwarth 1993). In addition, after unilateral adrenalectomy, bFGF was localized immunohistochemically in cells of the rat adrenal zona glomerulosa and medulla (Basile & Holzwarth 1993, 1994). Taken together, these data support the role of bFGF in autocrine/paracrine stimulation and in the compensatory adrenal growth response.

As we have pointed out (Vendeira et al. 1996b), a more discriminatory morphological method of analysis is necessary for experimental regeneration studies. In this study, we describe the effects of chronic treatment with bFGF and IGF-I on the biochemistry and morphology of adrenal autotransplants. The specific antibody, inner zone antibody (IZAb), which labels the antigen IZAg, which is found only in fasciculata and reticularis cells of the rat adrenal cortex (Laird et al. 1988, Barker et al. 1992, Ho et al. 1994), was used as a tool for cellular analysis. Electron microscope studies were employed to give some insight into the ultrastructural alterations in the subcapsular glomerulosa-like cells.

Materials and Methods

Twenty-six male Wistar rats from the colony of the Gulbenkian Institute of Sciences (Oeiras, Portugal), with body weights of approximately 200g, were divided into four experimental groups. After intraperitoneal anaesthesia with sodium pentobarbital, the animals of three of these groups underwent bilateral adrenalectomy and adrenal autotransplantation as previously described (Vendeira et al. 1992). Briefly, bilateral adrenalectomy was performed by a subcostal extraperitoneal incision, and the adrenals were placed in a 0-9% sterile saline solution and cut into small pieces measuring 2 mm each including the capsule. All the fragments were immediately autotransplanted under the skin of the dorsal region. All animals were fed on a commercial diet and provided with 0-9% saline solution during the first 30 days after surgery, and subsequently with water until they were killed by decapitation. The rats were housed under normal laboratory conditions with regular diurnal light/dark alterations (12 h light/12 h darkness cycles). Seven days before being killed (at 90 days after the autotransplantation), the animals were chronically infused with either 0-9% saline solution (controls, five animals) or 0-9% saline containing bFGF (eight animals) or IGF-I (eight animals) (Bachem Feinchemikalien, Bubendorf, Switzerland). The delivery of the drugs was by intraperitoneal infusion after implantation of Alzet mini-osmotic pumps (model 2001) with a reservoir volume of 200 µl (Alza pharmaceuticals, Palo Alto, CA, USA). The rate of delivery was 0-2 µg/kg per h, with a pump rate of 1-0 µl/h. Animals were handled gently by the same operator to minimize stress responses. For the assessment of plasma aldosterone and corticosterone and plasma renin activity, trunk blood was collected. Plasma was separated by centrifugation and immediately stored at -25 °C until assayed. A group of five intact animals was also used to define normal IZAg expression as well as normal plasma steroid concentrations. Necropsy was carried out on all the animals to search for accessory adrenals. Adrenal grafts were removed and fixed in 4% formaldehyde in PBS (pH 7-4, 0·1 mol/l) for 18 h at 4 °C. Fixed adrenal tissue was dehydrated and then embedded in paraffin wax. Sections (5 µm) were cut and mounted on gelatine-coated glass slides. After being dewaxed, sections were washed in Tris-buffered saline (pH 7-5). They were then incubated for 30 min with IZAb (1:50), followed by biotinylated rabbit anti-mouse IgG and peroxidase-conjugated avidin (avidin–biotin complex; Dako Ltd, Copenhagen, Denmark). Visualization of the peroxidase activity was achieved by incubation for 20 min with 3,3’-diaminobenzidine (Sigma) and H2O2. Sections were counterstained with haematoxylin. Washed sections were then mounted with Entellan (Merck, Darmstadt, Germany) and viewed under a Leitz light microscope. To establish the specificity of the immunohistochemical staining, sections were also incubated with mouse IgG-I-
negative control serum (Dakopatts A/S Produktionsvej, Glostrup, Denmark) in place of the specific antibody described above. Twenty-four grafts from eight bFGF-treated and eight IGF-I-treated animals and nine from five saline-treated animals were used to determine the number of glomerulosa-like cell layers. Pieces of adrenal grafts were also fixed in 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·2) for 2 h at 4 °C, postfixed in 1% osmium tetroxide in veronal/acetate buffer (pH 7·2) for 2 h at 4 °C, and Epon embedded. Sections 1 µm thick were stained with methylene blue/azur II for light microscopy (Richardson et al. 1960) to identify the adrenal subcapsular and inner zones. Ultrathin sections from these areas were stained with alcoholic uranyl acetate (15 min) plus lead citrate (10 min) (Reynolds 1963) and examined in a Jeol 100 CX II electron microscope.

We used HPLC for corticosterone assays (Haughey & Jusko 1988, Swart et al. 1988) with absorbance detection at 254 nm. Aldosterone and plasma renin activity were determined by RIA (commercial kits purchased from Sorin Biomedica, Italy; intra-assay and interassay variations for aldosterone were 9·7% and 11·5% respectively and for plasma renin activity 7·6% and 9·1%). All experiments were carried out in duplicate. Mean values were compared by Student’s t-test. A P value of less than 0·05 was considered significant. In order to minimize circadian variations, all animals were killed and their trunk blood collected between 1400 and 1500 h.

Results

As previously observed (Ho & Vinson 1993, Pignatelli et al. 1995), immunohistochemical staining with IZAb in intact control animals was restricted to the inner adrenocortical zones (zona fasciculata and reticularis) (Fig. 1). The zona glomerulosa and the medulla were unstained. No specific immunohistostaining was seen in the non-specific mouse IgG-treated control sections. Ultrastructurally, glandular elements showed the usual features of steroid-secreting cells, containing mitochondria with tubular and vesicular cristae, abundant profiles of smooth endoplasmic reticulum, and lipid droplets.

No significant morphological differences could be found in autotransplanted glands after chronic 0·9% saline solution or bFGF infusion when compared with autotransplanted controls. IZAb immunohistostaining was observed in almost all of the regenerated adrenocortical tissue (Fig. 2a and b), with the exception of some small clusters of cells beneath the capsular tissue and adjacent well-vascularized connective tissue septal layers. There was no clear zonation as seen in the intact animal, although the immunonegative cells, which appeared to be glomerulosa-like, occasionally formed a layer one to two cells thick. Ultrastructurally most cells showed fasciculata characteristics, with mitochondria containing typical vesicular cristae. Identical findings were also obtained in the subcapsular area, although here a few isolated cells with glomerulosa-type features could be seen.

After chronic administration of IGF-I, a remarkable difference in glandular architecture was seen. In these glands, a glomerulosa-like zone was clearly evident as an extended subcapsular layer some three to seven cells thick. This zone was devoid of immunostaining, in contrast with the IZAb- positive zona fasciculata-like cells that constituted the inner cell population (Fig. 3). Electron microscope findings revealed that cells in the inner zone continued to exhibit mitochondria with the vesicular cristae, lipid droplets and smooth endoplasmic reticulum profiles typical of fasciculata cells (Fig. 4). However, in the outer subcapsular area, this experimental group showed cells with mitochondria resembling those of immature or fetal glomerulosa cells. Although exhibiting smooth endoplasmic reticulum profiles with evidence of hypertrophy and a lipid droplet depletion, these glomerulosa-like cells were mainly characterized by the presence of mitochondria with irregular tubular or tubulovesicular cristae but
lacking other features characteristic of reticularis cells, such as lipofuscin granules (Fig. 5). Chromaffin medullary tissue could not be found in any of the experimental procedures.

Prolonged infusion with IGF-I produced a significant rise in plasma aldosterone concentrations when compared with saline or bFGF infusion. Plasma renin activity was significantly decreased. In contrast, serum corticosterone was significantly lower in autotransplanted IGF-I-treated animals when compared with the other experimental groups (Table 1).

Discussion

The growth and differentiation of the adrenal cortex continues to present challenges for our understanding of the mechanisms involved. Most authors now believe that the three major zones are not immutable, and that cellular transformation, for example from glomerulosa to the fasciculata, or fasciculata to reticularis, occurs under normal conditions, and indeed is a feature of the cellular life history as the cells migrate centripetally from the peripheral part of the gland. Cellular transformation, however, occurs only at the specific sites where the zones adjoin, and consequently the zones retain their relative positions within the gland despite changes in relative abundance that may reflect physiological stimulation. The difficulty lies not only in identifying the factors that account for such cellular transformation, but also in explaining why they act only at such specific locations.

As the parenchymal cells are thought to migrate through the cortex, it might be supposed that the tissue-organizing factors originate in other structures that retain their position. These might, for example, include elements of the vascular system (Rosolowsky & Campbell 1994, Rubin & Levin 1994), or the innervation (De Léan et al. 1984, Gallo-Payet et al. 1987, Rebuffat et al. 1988, Hinson et al. 1992, Vizi et al. 1992, 1993, Malendowicz 1993, Bornstein et al. 1994, Hinson et al. 1994), and considerable evidence now exists that both can greatly affect adrenocortical function under experimental conditions. However, when glands are autotransplanted, these elements are lost (even if only temporarily), and still under appropriate conditions adrenocortical zonation can occur. Therefore the factors that regulate adrenocortical zonation must have another source.


After chronic stimulation with IGF-I, the adrenal neocortex exhibited an extended subcapsular area corresponding to the zona glomerulosa, and devoid of IZAb
immunoreactivity. The suggestion that IGF-I stimulates the zona glomerulosa is supported by a significant rise in plasma aldosterone concentrations. It is known from other studies that IGF-I exerts a potent proliferative effect on adrenocortical cells in vitro (Naaman et al. 1989), and furthermore that IGF-I is also synthesized within adrenal tissue, which contains a relative abundance of both IGF-I mRNA and the peptide (Hansson et al. 1988, Ho & Vinson 1995). IGF-I also stimulates steroidogenesis in isolated adrenocortical cells (Penhoat et al. 1994, Weber et al. 1997) and has been implicated in compensatory growth after unilateral adrenalectomy, as well as in adrenal regeneration and differentiation after bilateral adrenal enucleation (Jackson et al. 1991). Using non-radioactive in situ hybridization, IGF-I mRNA was located mainly in the zona fasciculata and adrenal medulla in untreated animals (Ho & Vinson 1995). After ACTH stimulation or sodium restriction, the translation of IGF-I mRNA is enhanced in zona glomerulosa, also supporting a local proliferative role for IGF-I. This seems plausible as IGF-I receptors have been identified in rat, bovine and human adrenal glands (Penhoat et al. 1988, Shigematsu et al. 1989, Arafah 1991, Weber et al. 1995, 1997). In our experiments, IGF-I administration increased the extent of IZAg non-expressing cells in the subcapsular area, suggesting its particular modulatory role in the peripheral areas of the regenerated adrenal cortex. This is in a sense similar to previous findings of IGF-I mRNA localization in the fetal adrenal (Han et al. 1987, Mesiano et al. 1993) in which a predominantly capsular localization was observed. The low plasma corticosterone

Figure 3 Autotransplanted adrenal gland (90 days). IZAb immunohistostaining (1:50) and avidin–biotin complex technique. IGF-I-treated animal. A glomerulosa-like zone devoid of immunohistostaining is clearly evident. C, Capsule; SC, subcapsular glomerulosa-like zone; IN, inner zone. Scale bar=10 µm.

Figure 4 Electron micrograph of adrenal cortex. Autotransplantation (90 days). IGF-I-treated animal. Glandular cells in the inner zone exhibited mitochondria (M) with vesicular cristae, lipid droplets (L) and smooth endoplasmic reticulum profiles (ER), typical of fasciculata cells. Scale bar=1 µm.
concentrations observed after chronic administration of IGF-I deserve a comment, because they do not appear to be consistent with the morphological evidence. This is, however, a common finding in adrenal-regenerated autotransplants, a situation in which a high plasma ACTH concentration is also observed (Wilkinson et al. 1981, Engeland 1984).

The morphological and biochemical analysis of adrenal grafts submitted to chronic bFGF administration showed some unexpected effects when compared with previous results. In fact, our observations revealed that bFGF produced no change in IZAg expression when compared with control autotransplanted animals. Immunohisto­staining was distributed in almost all of the regenerated adrenocortical tissue, with the exception of some small clusters of subcapsular cells. Interestingly, Basile & Holzwarth (1993, 1994) showed that, in the unilaterally adrenalectomized animal, in the remaining gland bFGF was preferentially located in the outer cortical area and adrenal medulla, while the expression of bFGF receptors was predominantly in the capsule and zona glomerulosa. Hence, bFGF may play a role in compensatory adrenal regeneration. In this study, we observed that plasma aldosterone concentrations were significantly lower than in control autotransplanted animals, and this finding suggests that bFGF partly replicates the actions of chronic ACTH in normal animals. The invariable plasma renin activities, when compared with controls, are intriguing.

### Table 1

Endocrine effects of prolonged infusion (7 days) with saline solution (SS), bFGF or IGF-I in autotransplanted (AT) rats. The data presented are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Intact rat</th>
<th>AT + SS</th>
<th>AT + bFGF</th>
<th>AT + IGF-I</th>
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<tr>
<td>Plasma aldosterone (pg/ml)</td>
<td>290 ± 59·1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81·15 ± 14·3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18·5 ± 8·8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>132·4 ± 10·8&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Serum corticosterone (µg/ml)</td>
<td>0·19 ± 0·02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0·09 ± 0·003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0·1 ± 0·01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0·05 ± 0·004&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma renin activity (ng/ml per h)</td>
<td>22·79 ± 3·54&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23·75 ± 3·81&lt;sup&gt;f&lt;/sup&gt;</td>
<td>18·37 ± 2·32&lt;sup&gt;i&lt;/sup&gt;</td>
<td>11·47 ± 3·46&lt;sup&gt;l&lt;/sup&gt;</td>
</tr>
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Statistical comparison of the data: a vs d, P = 0·001; b vs e, P = 0·005; c vs f, n.s.; d vs g, P = 0·01; e vs h, n.s.; f vs i, n.s.; g vs j, P = 0·01; e vs k, P = 0·01; f vs l, P = 0·01.
suggesting that even after a 7 day infusion of bFGF, the renin–angiotensin–aldosterone system still lacks adequate regulatory feedback.

As bFGF is also a potent angiogenic and neurotrophic factor (Flamme & Risau 1992, Bikitávi et al. 1997), it has been suggested that it may have a modulatory role in vascularization and innervation of the adrenal gland (Basile & Holzwarth 1994). We postulate that it may have a role in the neovascularization process in the autotransplanted adrenal cortex, which we have already described (Vendeira et al. 1992, 1996a), although as previously noted, neural pathways were not observed in the autotransplanted gland at least in the early steps of regeneration.

These findings strongly suggest that growth factors have an important role in regulating adrenocortical zonation. The factors that influence the extent and sites of their expression as well as those of their receptors will now require further study in order to firmly establish the mechanisms of proliferation and steroidogenic differentiation in the regenerating gland.

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Journal of Endocrinology (1999) 162, 21–29


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