EXPERIMENTAL STUDY

Differential production of adrenal steroids by purified cells of the human adrenal cortex is relative rather than absolute

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Abstract

Objectives: The adrenal cortex produces aldosterone, cortisol and androgens in response to ACTH and angiotensin II. To define the differential response of morphologically distinct cells of the adrenal cortex, we examined the phenotypical and functional characteristics of human adrenocortical cells.

Results: Tumour growth factor-β receptor-1 (TGFβ-R1) and CYP11 were found to be expressed predominantly in the zona fasciculata, whereas human leukocyte antigen (HLA-DR) and CYP17 were localised to the zona reticularis. The angiotensin II receptor, AT-1, was found to be predominantly expressed in the zona glomerulosa. Adrenocortical cells, separated by density, yielded two distinct fractions which displayed differential growth patterns. Lipid-rich cells of fraction I expressed TGFβ-R1 and produced significantly more cortisol relative to androstenedione than unseparated or fraction II cells, whereas lipid-poor cells of fraction II expressed HLA-DR and produced more androstenedione relative to cortisol in the presence of ACTH. Aldosterone production by fraction II was significantly greater than fraction I or unseparated cells. TGFβ-R1-positive fasciculata-type cells separated into fraction I and HLA-DR-positive cells consistent with reticularis cells separated into fraction II. Aldosterone-producing cells indicative of glomerulosa cells separated into fraction II.

Conclusions: Our findings are consistent with the concept that all adrenocortical cells are capable of producing a range of steroids, but the relative production of cortisol, androgen and aldosterone differs.

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Introduction

Anatomically, the adrenal cortex is divided into three zones. Zones of the adrenal cortex have functionally distinct roles in steroid hormone production. The traditional model has the outer zona glomerulosa synthesising mineralocorticoids, the zona fasciculata cortisol and the inner zona reticularis adrenal androgens. Aldosterone production is regulated by angiotensin II through activation of the phospholipid/calcium-dependent protein kinase C pathway and extracellular potassium-regulated calcium influx (1). Adrenocortico-trophic hormone (ACTH) modulates both cortisol and adrenal androgen production via a cAMP-dependent protein kinase A pathway (2). Although ACTH influences both cortisol and adrenal androgen production there are physiological and pathophysiological conditions where the production of these steroids is divergent (3, 4). It has therefore been suggested that some factor in addition to ACTH is involved in the modulation of adrenal androgen production. Many regulators have been implicated including the non-ACTH pro-opiomelanocortin fragments, β-endorphin and joining peptide, and also insulin-like growth factor (5–11). However, a specific adrenal androgen secretagogue has proven to be elusive.

It has been well established that aldosterone is produced by glomerulosa cells (12–14). However, classical comparisons of steroidogenesis between fasciculata and reticularis suggest that cells of both zones have the capacity to produce cortisol and androgens. It is possible, however, that within the fasciculata and reticularis zones there are varying proportions of cells types which produce exclusively either cortisol or androgens in response to the relevant secretagogues. Such cell-specific production of adrenal steroids may be pivotal to the differential regulation of cortisol and androgen in the adrenal cortex. Separation of the adrenal zonae, with characterisation of aldosterone-, cortisol-and androgen-producing cells has not been achieved to date. In order to define the zone-specific regulation of adrenal steroid production, the physical and functional characteristics of human adrenal cortical cells were examined. Specific cell surface markers for distinct cells of the adrenal cortex were identified, human leukocyte antigen (HLA-DR) and tumour growth factor-β
receptor-1 (TGFβ-R1), and utilised to distinguish separated adrenal cells. Examination of steroid production in the presence and absence of angiotensin II and ACTH indicates that functional differences between purified adrenocortical cells are relative rather than absolute.

Materials and methods

Preparation and stimulation of cell suspension
Twenty-three human adrenal glands surgically removed at the time of resection for renal transplantation were used for this study. Adrenal glands were prepared as described previously (5, 6). Excess fat and connective tissue were removed and the adrenal glands were diced. The tissue was incubated at 37°C for 20 min in Eagle’s Modified Essential Medium (EMEM) containing collagenase, type II (2 mg/ml). The tissue suspension was centrifuged at 400 g for 10 min and the supernatant discarded. The pellet was resuspended in 0.2% (w/v) BSA in EMEM and the cells passed through a nylon mesh (100 μm). The cell suspension was washed and centrifuged as before and the resultant pellet resuspended in 5 ml medium. The cell suspension was passed over a 30% Percoll gradient (Pharmacia) in parallel with density marker beads (Pharmacia) and centrifuged at 800 g for 30 min. The resultant fractions were removed, examined under the light microscope, counted and cell viability assessed at 98% using ethidium bromide acridine orange. The cell fractions and total unseparated cells from each adrenal were washed in EMEM, centrifuged and resuspended in EMEM containing BSA, 0.5% (w/v), calcium, 8 mmol/l, and ascorbic acid, 1 mmol/l, to a concentration of 10⁶ cells/ml in the presence and absence of α1-24 ACTH 10⁻⁷ mol/l or angiotensin II, 10⁻⁷ mol/l. Cells were incubated for 2 h at 37°C.

Steroid measurement
Cell suspensions were lysed by freeze thawing and centrifuged. Cortisol levels were measured by an ‘in house’ RIA, as previously described (4). Cell lysates were incubated with a specific anti-cortisol antibody (Guildhay, Guildford, Surrey, UK) and iodinated cortisol (Amer- sham) at 4°C overnight. Bound and free fractions were separated with 1% dextran-gelatin-coated charcoal in borate. Androstenedione and aldosterone were measured with commercially available RIA kits (Diagnostic Systems Laboratories, Heyford, Oxfordshire, UK). Androstenedione cross-reactivity with cortisol was <0.04%, and aldosterone cross-reactivity with cortisol was <0.01%.

Figure 1 Immunohistochemical stain for (a) TGFβ-R1, (b) HLA-DR, (c) AT-1, (d) CYP-17 and (e) CYP-11 in the human adrenal cortical cryosections counterstained with haematoxylin and matched controls. ZF, zona fasciculata; ZR, zona reticularis; ZG, zona glomerulosa; C, capsule. Brown staining demonstrates positive expression of antigen. TGFβ-R1, HLA-DR and AT-1 ×400; CYP-17 and CYP-11 ×200.
Acute cell culture

Fractionated and total unseparated human adrenal cells from five adrenals were grown at 37°C in 5% CO₂ for 24 h in supplemented RPMI (5 μg/ml insulin, 10 μg/ml transferrin, 30 nmol/l sodium selenite, 10 nmol/l hydrocortisone, 10 nmol/l β-oestradiol, 10 mM Heps, 2 mmol/l glutamine, 2% fetal calf serum (w/v)). Cells were seeded at 5 × 10⁵ cells/well in six-well culture plates (Costar, New York, NY, USA) on adherent coverslips (Nunc, Roskilde, Denmark). At the end of the incubation period cells were fixed on coverslips in absolute alcohol for 10 min.

Immunohistochemistry and cytochemistry

An immunohistochemical technique was employed for the identification and localisation of TGFβ-R1, HLA-DR and angiotensin receptor 1 (AT-1) in human adrenal gland cryosections and acute cell cultures of dispersed cells. Adrenal cryosections, 7 μm, or 24 h cultured adrenal cells, were immuno-blocked in serum for 90 min and incubated with a primary rabbit anti-human TGFβ-R1 (5 μg/ml), mouse anti-human HLA-DR (5 μg/ml), rabbit anti-human AT-1 (5 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-porcine CYP-17 (1:40) or rabbit anti-bovine CYP-11 (1:40) (from Professor Ian Mason, University of Edinburgh, Edinburgh, UK) for 1 h at room temperature and subsequently with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody, 1:1000, for 1 h at room temperature. Sections were developed in 3,3′-diaminobenzidine tetrahydrochloride for 7 min and counterstained with Mayer’s haematoxylin. Negative controls were performed either using blocking peptides to TGFβ-R1 or AT-1 (Santa Cruz) or by omitting the primary antibody, with no peroxidase staining observed.

Positive vs negative cells for TGFβ-R1 and HLA-DR immunoreactivity were counted per high-powered field (x400) on the light microscope for each of the fraction I and fraction II acute cell cultures. Positive cells were calculated as a percentage of total cells.

Chemicals

All chemicals were obtained from Sigma, unless otherwise specified.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test for non-parametric data, with significance accepted at the 5% level.
Results

Immunohistochemical identification of adrenocortical zonation

Steroid enzymes and candidate cell surface antigens were used to define distinct zones of the human adrenal cortex. TGFβ-R1 was expressed predominantly in the zona fasciculata cell layer (Fig. 1a) with some positive-staining cells projecting into the zona reticularis. HLA-DR-positive cells were found primarily within the zona reticularis (Fig. 1b). To confirm demonstrated zonal differences observed with TGFβ-R1 and HLA-DR, the key steroidogenic enzymes CYP-17 and CYP-11 were localised within the human adrenal. CYP-17, an enzyme concerned with cortisol and androgen production was localised to the zona fasciculata and to a greater extent the zona reticularis, but was found to be absent from the zona glomerulosa (Fig. 1d). CYP-11, an enzyme that facilitates the conversion of deoxycorticosterol to cortisol and 18-hydroxycorticosterone to aldosterone, was identified predominantly in the zona fasciculata and to a lesser extent the zona glomerulosa (Fig. 1e). AT-1 was found to be expressed in the zona glomerulosa cell layer. Focal nests of positive cells expressing the receptor were also observed in the outer cells of the zona fasciculata (Fig. 1c).

Phenotypical characterisation of dispersed human adrenocortical cells

Separation of human adrenal cell suspensions by density gradient centrifugation yielded two fractions, designated fraction I and fraction II with respective mean densities of 1.018 and 1.033 g/ml. Cells of fraction I and fraction II exhibited distinct morphological characteristics. Cells of fraction I were lipid-rich in appearance with a low nuclear to cytoplasmic ratio. Cells of fraction II were lipid-poor, had a large prominent nucleus and relatively sparse cytoplasm. Acute cell cultures of fraction I and fraction II displayed differential growth patterns. Cells of fraction I grew along the surface of the culture dish in a monolayer, while cells of fraction II grew in layered clumps away from the culture surface (Fig. 2). Cells from fraction I and II treated with anti-TGFβ-R1 or anti-HLA-DR exhibited differential staining: 96±0.69% (S.E.M.) of cells in fraction I displayed positive staining for TGFβ-R1 whereas 5.9±0.3% of cells in fraction II were TGFβ-R1-positive and 5.88±0.8% of cells of fraction I were HLA-DR-positive and 98.3±0.6% of cells of fraction II were HLA-DR-positive.

Functional characterisation of dispersed adrenocortical cells

Unseparated human adrenal cortical cells and cells of fraction I and fraction II produced aldosterone, cortisol and androstenedione, under basal conditions (Fig. 3). Cortisol and androstenedione production was significantly stimulated in unFractionation. Cells of fraction I produced significantly more cortisol relative to androstenedione than unseparated cells or fraction II cells in response to 2 h incubation with ACTH (Fig. 4a). Conversely, cells derived from fraction II produced more androstenedione relative to cortisol in comparison to fraction I in the presence of ACTH (Fig. 4b). Neither angiotensin II nor ACTH significantly stimulated aldosterone production in total unseparated adrenal cells or in fraction I and fraction II cells. Aldosterone production by fraction II was significantly greater than in fraction I or in total unseparated cells, basally and following incubation with ACTH or angiotensin II (Fig. 5a). Fraction II produced significantly more aldosterone relative to cortisol than did fraction I cells in the presence of angiotensin II (Fig. 5b).

Discussion

Separation of aldosterone-, cortisol- and adrenal androgen-producing cells is an important objective. The study of adrenocortical steroidogenesis has been hampered by the difficulty in obtaining purified human adrenal cells viable for functional studies. Cells of the adrenal cortex are morphologically diverse and have variable lipid content (15) providing an opportunity to separate these cells based on density. The relatively lipid-poor zonae glomerulosa and reticularis cells have a greater density than cells of the zona fasciculata. In this study, density gradient centrifugation of adrenocortical cells resulted in two discrete fractions with morphologically distinct characteristics under light microscopy.

In order to confirm successful separation, cell surface markers for the distinctive adrenocortical cells were defined and compared to the zonal expression of steroid enzymes. CYP-17, the steroid enzyme concerned with androgen and cortisol production, was expressed in the zona fasciculata and to a greater extent in the zona reticularis. CYP-11 was identified in the zona fasciculata and to a lesser extent in the zonae glomerulosa and reticularis. To date, little is known about the cell surface receptor profile of adrenocortical cells and in particular antigens that are expressed differentially on specific cells of each zone. TGFβ has been associated with glucocorticoid modulation and expression of the growth factor has been demonstrated in cells of the zona fasciculata (16, 17). Furthermore, treatment with TGFβ-R1 has been found to regulate the expression of adrenal cell-specific genes including ACTH receptors and 3β-hydrogenase (18, 19). Antigenic determinants recognised by HLA-DR monocolonal antibodies have been detected on cells of the zona.
We demonstrated by immunohistochemical examination of the human adrenal cortex that the TGFβ receptor, TGFβ-R1, was expressed on the zona fasciculata while HLA-DR was expressed predominantly in the reticularis. The angiotensin receptor, AT-1, was found to be expressed predominantly in the zona glomerulosa. Of interest, occasional focal nests of cells expressing AT-1 were observed in the zona fasciculata. AT-1 expression in human adrenal fasciculata cells has been previously described and angiotensin II has been found to stimulate cortisol expression and increase the expression of steroid enzyme mRNA.

The two cell fractions separated by density gradient centrifugation were cultured for 24 h to examine their phenotypical characteristics and growth patterns. Cells cultured from fraction I were found to express TGFβ-R1 and cells of fraction II predominantly HLA-DR. The two cell populations also displayed differential growth patterns. Cells of fraction I grew along the culture plate, appeared to have a high lipid content and had a low nuclear to cytoplasm ratio. In contrast, cells of fraction II grew away from the surface in clumps, were lipid-poor in appearance and had a high nuclear to cytoplasm ratio. Cells of fraction I and fraction II are consistent with the classical phenotypical characteristics and growth patterns.
The zone-specific expression of these enzymes would enable the regulation of steroid production by ACTH and angiotensin II (13, 27). In this study aldosterone production by fraction II cells was significantly greater than that by fraction I cells and the unseparated total cell population, basally and following stimulation with ACTH. Fraction II also produced relatively more aldosterone than cortisol in comparison to fraction I or unseparated cells in the presence of angiotensin II, suggesting that the compact, lipid-poor cells of the zona glomerulosa cells separated into fraction II by density centrifugation. There was, however, some aldosterone produced by fraction I cells. It is possible that an intermediate cell exists that retains the capacity to produce aldosterone, which because of morphological evolution separates to fraction I. Alternatively, cells of the human zona fasciculata may have a limited capacity to produce aldosterone, as has been previously described in bovine adrenocortical cells (28). The aldosterone response to ACTH was greater than that of angiotensin II following 2 h incubation: AT-1 may have been down-regulated during the cell purification procedure. ACTH has been shown previously to cause an acute increase in the production of aldosterone both in vivo and in isolated adrenal cells (27), although it acts chronically, however, to decrease plasma aldosterone levels (29). cAMP-dependent processes activated by ACTH have the capacity to down-regulate and interfere with the signal transduction mechanisms of AT-1 (30).

Total unseparated cells and cells of fraction I and fraction II produced cortisol and androstenedione, alone and in response to ACTH. However, fraction I produced comparatively more cortisol relative to fraction II and fraction II produced significantly more androstenedione in response to ACTH stimulation. Although both steroids are produced in response to ACTH in both cell fractions, the lipid-rich fasciculata cells of fraction I and lipid-poor reticularis cells of fraction II respond differently to the same secretagogue. Both cells must express the entire complement of steroidogenic enzymes necessary for the production of both cortisol and androstenedione. Therefore the ACTH regulation of these enzymes is likely to be at the transcriptional level. Moreover, although aldosterone is produced primarily by cells of fraction II, cells of fraction I also produced aldosterone in response to ACTH. To this point in time we have not achieved physical separation of the aldosterone- and androgen-producing cells of fraction II. This would permit insight into whether the aldosterone-producing cells also produce cortisol and androgens.

This is the first study to purify human adrenocortical cells based on density; the lipid-rich cells consistent with fasciculata origin separate into fraction I and the relatively lipid-poor cells consistent with glomerulosa and reticularis origin into fraction II. Although purified cells of the adrenocortex had a differential response to the classical secretagogues ACTH and angiotensin II, each cell type produced aldosterone, cortisol and adrenal androgens. Functional differences between adrenocortical cell types are therefore relative rather than absolute. This work supports the concept that adrenal cells in the various zones arise from a stem cell with evolution from glomerulosa to fasciculata and finally reticularis, rather than the independent development of each cell type.
zone. Zonae of the adrenal cortex are therefore composed of cells with the capacity to produce a variety of steroids in varying proportions rather than variable ratios of different cell types each dedicated to a single major product, e.g. cortisol, androgens or aldosterone.

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