Characterization of NCI-H295R Cells as an In Vitro Model of Hyperaldosteronism

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- Conn’s syndrome
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Abstract
In depth analysis of key molecular mechanisms involved in functional autonomy of aldosterone secretion is hampered by the lack of tumor cell lines that reflect functional characteristics of aldosterone producing adenomas. Herein, we describe the characteristics of the adrenocortical carcinoma cell line NCI-H295R and its suitability as a model of hyperaldosteronism in relation to different culture conditions. Steroid profiling revealed that NCI-H295R cells predominantly secrete cortisol, while aldosterone and other steroids are released at much lower concentrations. However, aldosterone output specifically increased in response to different stimuli such as ACTH and angiotensin II, and in particular to potassium in a dose dependent manner. NCI-H295R cells readily formed spheroids under specific culture conditions, a method widely used for the enrichment of progenitor cells. Unexpectedly, spheroid cells excelled with higher aldosterone concentration and higher expression levels of the steroidogenic enzymes StAR, 3βHSD, CYP17, SF-1, and the MC2-receptor. Further investigations revealed that this phenomenon is mainly attributed to epithelial growth factor (EGF) and particularly fibroblast growth factor (FGF), which are both essential ingredients in the spheroid culture medium. Aldosterone release under the combinatory influence of EGF and FGF was not higher than the effect of FGF alone. Spheroid growth per se, therefore, does not ensure an enrichment of less differentiated cell types in this cell line.

Introduction
Primary aldosteronism (PA) has been recognized as the most common cause of secondary hypertension, which is responsible for elevated blood pressure in 5–10% of all hypertensive patients [1]. While the classical hypokalemic form of PA is prevalent in less than 0.5% of patients with hypertension, more sensitive diagnostic screening approaches allow for the detection of the normokalemic variant of the disease which resulted in an increase of case detection and, thus, identification of PA as a relatively common disease [2].
On the contrary, the mechanisms accounting for autonomous aldosterone secretion are still only partly understood. All models currently being used in preclinical research have limitations. In vitro, the human NCI-H295R cell line is most commonly utilized [3,4], although it has been derived from an adrenocortical carcinoma and therefore inherently might not reflect the molecular and functional changes of true aldosterone producing adenomas [5]. Nevertheless, in contrast to other adrenocortical cell lines, such as the SW13 cells, these cells are known to be capable of aldosterone production to some extent. The aim of this study was to increase the knowledge about the characteristics of aldosterone production in NCI-H295R cells and their steroid profile under different culturing conditions.
In previous studies, the existence of subpopulations with distinct characteristics within a given tumor has been described. Some of these subpopulations seem to represent cells with progenitor or stem cell-like properties, and were claimed to be responsible for most of the tumor growth [6,7]. Interestingly, these subpopulations could also be found in clonally expanding cell populations, such as cell lines [8,9]. Therefore, besides analyzing the feasibility of NCI-H295R cells as an in vitro model for aldosterone excess, we investigated, whether a subpopulation of cells within the NCI-H295R adrenocortical carcinoma cell
line could be enriched by sphere formation, a process originally described to enrich for neural and other progenitor cells, including the adrenal medulla, and stem cell-like cancer cells [6, 8, 10-13].

Materials and Methods

Cell culture conditions
NCI-H295R monolayer cells and SW13 cells were grown at 37°C and 5 % CO2 in DMEM/F12 medium (Gibco Invitrogen, Carlsbad, CA, USA) containing 10 % FBS (HyClone, South Logan, UT, USA) and 2 mM l-glutamine (BioWhittaker Cambrex, East Rutherford, NJ, USA), and 10 μl/ml Pen/Strep (Gibco) as described previously [14]. Spheroid growth was induced by culturing the cells without serum in DMEM/F12 medium supplemented with B27, N2, 20 ng/ml hEGF, 20 ng/ml bFGF, and 10 μl/ml Pen/Strep (all Gibco). To measure growth factor effects on aldosterone release, either EGF or FGF was withheld from the medium in comparison to medium containing both factors, and in comparison to regular cell culture medium. For stimulation experiments, NCI-H295R monolayer cells were plated at a density of 50,000 cells in 24-well plates. After 72 h, the medium was replaced, and ACTH, angiotensin II, and potassium chloride (all Invitrogen) were added to the monolayer cells to achieve a representative standard for the expression analysis [6]. Spheroids were collected with a 100 μm mesh, fixed with PFA, dehydrated, and paraffin embedded. Tissue slides and coverslips were subsequently investigated by H&E staining.

For immunohistochemical analysis, cover slips and tissue sections were rehydrated, blocked with 0.3 % H2O2 in methanol for 10 min, and incubated with blocking buffer for 15 min. P450SCC primary rabbit anti-human antibody (kindly provided by Dr. Walter Miller, UCSF, CA, USA) was incubated overnight at 4°C after dilution in blocking buffer containing 3 % bovine serum albumin (Sigma, St. Louis, MO, USA), 5 % goat serum (Jackson Immuno Research Laboratories, West Grove, PA, USA), and 0.5 % Tween 20 (Calbiochem, San Diego, CA, USA). After rinsing for 15 min in PBS, secondary antibody (Goat anti-rabbit biotinylated IgG; Vector Laboratories, Burlingame, CA, USA) was applied for 30 min at room temperature. Bound primary antibody was detected using the VECTASTAIN Elite ABC Kit (Vector Laboratories) according to the manufacturer's protocol.

Real-time PCR
RNA from cell preparations was extracted using the Qiagen RNA mini kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. cDNA was transcribed using a reverse transcription kit (Promega, Mannheim, Germany) and 1.0 μg of total RNA.

Real-time PCR was performed using the FastStart DNA Master-Plus SYBR Green 1 reaction mix in the LightCycler 1.5 (Roche, Mannheim, Germany). The cycling conditions for real-time PCR included a preincubation step at 95°C for 10 min, followed by an amplification step, which consisted of 40-45 cycles at 95°C for 10 s, annealing (the temperature was primer dependent as given below) for 6 s and an extension step at 72°C, for which the time was calculated by the product length in bp divided by 25 (Roche, Indianapolis, IN, USA). Primer sequences as well as product lengths and annealing temperatures were as follows: P450SCC: 5'-GCACCGTGAGTCCGGTTTAT-3' and 5'-TCTCCAGACGTCTTCTT-3' (664 bp; 52°C); StAR: 5'-CAGGCAAGTGGGGACAAGT-3' and 5'-ATGACGCTGTGACTGTCG-3' (608 bp; 63°C); SF-1: 5'-TGACATCGACATGGACGAGT-3' and 5'-AGGCCCTCTTGATCCATAGAAGA-3' (390 bp; 58°C); 3βHSD 5'-CTAAGTTACCGCCCTTCTTG-3' and 5'-AAATCTCTCTCTGACATG-3' (285 bp; 50°C); Cyp 17 5'-CAA GCC AAG ATG AAT GCA GA-3' and 5'- CAT AAA CCG ATG TGG CTC GT-3' (438 bp; 50°C); MC2-receptor 5'-CATGGGCTATCTCAAGCCAC-3' and 5'-GAGATCTTCCGGTGAGAT-3' (360 bp; 55°C); CYP11B2 synthase 5'-GGCAGGCTGAGGAGCTGAC-3' and 5'-GGCATTGCGCAGCAG-3' (303 bp; 60°C). Melting curve analysis was performed between 65 and 98°C (0.1 °C/s) to determine the Tm of the amplified product and in order to exclude undesired primer dimers. Furthermore, the products were run on a 1 % agarose gel to verify the size of the amplified product. Each sample was run at least in triplicate. Quantification was normalized using HPRT (hypoxanthine phosphoribosyltransferase) as a housekeeping/reference gene. cDNA of 8 normal adrenals were pooled in equal volumes to achieve a representative standard for the expression analysis part of this study.

Statistical analysis
All results are expressed as mean ± SD. If not stated otherwise, all statistical comparisons were analyzed by one-way ANOVA and
independent samples T test. Statistical significance was defined as \( p < 0.05 \) and is indicated as a star (*) in the figures.

**Results**

**Steroid profile**

At baseline, ultra-pressure liquid chromatography tandem mass spectrometry revealed that cortisol was the predominantly secreted hormone in the supernatant of NCI-H295R cells (58.0 ± 5.1 ng/dl), whereas the other steroid hormones were released on a much lower level in similar range (Fig. 1a). Interestingly, potassium stimulation resulted in a distinct shift in the steroid profile due to a selective and significant 19.2-fold increase of aldosterone secretion (Fig. 1b) while most other steroid metabolites were not significantly changed in a potassium dependent manner.

**Aldosterone output of NCI-H295R cells at baseline and upon stimulation**

Baseline aldosterone levels measured in the supernatant of NCI-H295R cells grown as monolayers after 24 h of incubation ranged between 328–716 pg/ml, and were found to be responsive to stimulation by angiotensin II (131 ± 1.2 % vs. 100 ± 6.2 %; \( p < 0.01 \)), while incubation with ACTH increased aldosterone secretion only slightly (118 ± 6.4 %; \( p = 0.17 \); data not shown). However, the most pronounced aldosterone induction was observable upon potassium stimulation when aldosterone output increased from 352 ± 8.64 pg/ml at physiological potassium concentrations (5.2 mM) up to 8407 ± 1013 pg/ml at potassium concentrations of 20 mM in a dose dependent manner (Fig. 1c). Likewise, CYP11B2 expression gradually increased with increasing potassium concentrations up to a maximum of 411 ± 0.3 % at a potassium concentration of 15 mM (Fig. 1d). Further increase in potassium supplementation resulted in lower aldosterone concentrations and lower CYP11B2 expression levels, respectively, most likely due to potassium dependent cell death (Fig. 1c, d).

**Spheroid versus monolayer growth**

NCI-H295R cells were exposed to 2 different culture conditions: Regular serum containing medium and serum free medium supplemented with epithelial growth factor (EGF) and fibroblast growth factor (FGF). Adherent monolayer growth was observed (day 2 after plating), when cells were cultured in regular, serum containing medium, whereas they adopted spheroid-like structures after plating a minimum of 100 cells per well on a 24-well plate.
after 9 days

after 14 days

properties independent of culture conditions.

stained positive for P450SCC in immunohistochemical analyses.

Fixed and embedded spheroids and monolayer cells clearly

2 weeks (data not shown).

were signiﬁcantly higher in

plate in EGF and FGF containing serum free medium for a mini-

administration of growth factors (a + b, upper panels). After dissociation

and 9 more days in culture

and 23 more days in culture

b

▶ Fig. 2a, upper panels; ▶ Fig. 2b, upper panels).

NCI-H295R spheroids could be trypsinized and dissociated into

single cell suspension and continued to grow as monolayer cul-

ture, or re-formed spheroids at a much slower rate (23 more
days vs. 9 days), depending on the culture conditions they were

exposed to ( ▶ Fig. 2a, lower panel). Without dissociation, spher-

oid growth decreased over time and ceased after approximately

2 weeks (data not shown).

Fixed and embedded spheroids and monolayer cells clearly

stained positive for P450SCC (b, lower panel) indicating persistence of steroidogenic

properties independent of culture conditions.

Steroidogenic capacity of spheroids

Baseline aldosterone levels secreted by spheroids (S) and normal-

ized to total cellular protein were signifi-

cant higher in

dissociation and

spheroid growth decreased over time and ceased after approximately

2 weeks (data not shown).

Fig. 2  Spheroid vs. monolayer growth: NCI-H295R cells readily grew as

monolayer in their standard medium, or as spheroids in serum free me-

dium containing growth factors (a + b, upper panels). After dissociation

into single cells, spheroids can be regrown as spheroids or as adherent

cells, depending on cell culture conditions (a, lower panel). Both types of

cells stained positive for p450SCC (b, lower panel).

contrast, no expression of any steroidogenic enzyme analyzed

was detectable in SW13 cells, which is in agreement with their known lack of steroidogenic properties ( ▶ Fig. 3b).

To investigate the rationale behind the different steroidogenic

properties of NCI-H295R cells grown as monolayer vs. spheroids,

monolayer cells were incubated with the same concentrations of

growth factors as used for spheroid growth induction. While the

addition of EGF alone already led to a significant increase of

aldosterone release (2529±255.4 vs. 1206±44.4; p = 0.01), FGF treatment had an even greater effect, with aldosterone levels

similar to those observed for spheroid cells (4779±435.4; p < 0.01). The combination of EGF and FGF did not result in a

response greater than FGF treatment alone (4219±22.1; p = 0.35). Vice versa, when spheroid cells were switched to regu-

lar monolayer culture medium without growth factors, aldoste-

rone concentrations in the supernatant dropped to a level similar to that of monolayer cells (1013±31.4; p = 0.04; ▶ Fig. 4).

To exclude differences in aldosterone concentrations from differ-

ent amount of cells, results were related to total cellular pro-

tein levels for all conditions mentioned.

Discussion

Despite a number of potential drawbacks inherent to cell line

dependent model systems, adrenocortical NCI-H295R cells are

well established for their maintained steroid capabilities. Herein,

we speciﬁcally analyzed the suitability of these cells as an in vitro

model of autonomous aldosterone excess. Recently, endo-

crine properties of different strains, or subtypes of NCI-H295R

cells have been described [17]. These studies provided evidence that phenotypic variability and shifts through clonal selection have to be taken into account for the interpretation of experimental results. In addition, as shown herein, cell culture condi-
tions that might induce stem cell like properties have to be con-

sidered as modiﬁers of secretory abilities.

At baseline conditions, cortisol was the most abundantly

secreted hormone, which was expected and reﬂects the physio-

logical ratio of steroid hormones from the adrenal cortex. Inter-

estingly, potassium stimulation led to a relative decrease in
cortisol output. Overall, potassium induced effects on adrenal

glucocorticoid secretion are not well deﬁned and clearly influ-

enced by species-, cell- and context- (in vivo vs. in vitro) depend-

tent mechanisms with examples of potassium induced increased

[18], decreased [19], or unchanged [20] glucocorticoid levels.

While aldosterone output could be stimulated to some extent by

ACTH and angiotensin II, incubation with high levels of potas-

sium was followed by a pronounced, speciﬁc change in the rate

of steroid hormones from the adrenal cortex. Inter-

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[18], decreased [19], or unchanged [20] glucocorticoid levels.
from adrenocortical cells. In fact, in the presence of EGF and FGF in a serum-free medium NCI-H295R cells readily formed cell clusters, which progressively adopted the typical spheroid appearance. After dissociation and re-plating, the cells regrew as spheroids or as adherent cells, depending on the cell culture conditions they were exposed to. While we expected a less differentiated phenotype, surprisingly, spheroids seemed to retain the ability to produce steroids, as they expressed even higher levels of most of the steroidogenic enzymes tested, had greater aldosterone output, and grew slower than monolayer cells. Therefore, as confirmed by immunohistochemistry, RT-PCR and hormone measurements, spheroids from NCI-H295R cells seem to enrich for a rather differentiated phenotype.

In further experiments we analyzed potential direct effects of different culture conditions on spheroid and monolayer cell growth. Interestingly, when monolayer cells were exposed to the growth factors of spheroid medium, especially to FGF, their responsiveness to potassium significantly increased to a similar level as observed in spheroid cells. Vice versa, spheroid cells cultured in monolayer medium were characterized by aldosterone concentrations in the supernatant similar to that of monolayer cells. Taken together, most of the observed differences in aldosterone release between monolayer and spheroid cells seem to be attributable to FGF in the spheroid cell culture medium. This further confirmed the results of the expression analyses, that spheroid growth per se, does not necessarily ensure an enrichment of progenitor or stem cell like tumor cells in all cell types.
In conclusion, we have demonstrated the ability of NCI-H295R cells to specifically respond to different stimuli of the mineralocorticoid regulatory system, especially potassium, with a specific increase in aldosterone output. In addition, the cells easily grew spheroids, however, adopted surprisingly a rather differentiated phenotype, which is most likely related to FGF exposure. The highest aldosterone concentrations achieved were still approximately 50 times lower than those measured within the first 24 h of primary cells from aldosterone producing adenomas (data not shown). This finding underlines the need to continue the search for alternative, potentially more potent cell lines, preferentially from an aldosterone producing adenoma.

Conflict of Interest

The authors have nothing to disclose and have no conflict of interest.

References