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Development of a novel cell based androgen screening model

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ABSTRACT

The androgen receptor (AR) mediates the majority of androgen effects on target cells. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for glucocorticoid, mineralocorticoid and progesterone receptors (GR, MR and PR, respectively). As a result, many of the current AR screening models are complicated by inaccurate activation of reporters by one of these receptor pathways. Identification of more selective androgen testing systems would be beneficial for clinical, pharmacological and toxicologic screening of AR activators. The present study describes the development of a selective androgen-responsive reporter cell line that expresses AR but does not express GR, MR and PR. CV1 cells were stably transduced to express human AR and an androgen-responsive gaussia luciferase gene. Clonal populations of AR expressing cells were isolated. Quantitative RT-PCR (qPCR) and western analysis confirmed stable integration of AR in the most responsive clonal line which was named 'CV1-ARluc'. Stimulation of CV1AR-luc with androgenic ligands (testosterone and 5α-dihydrotestosterone) for 18 h caused an increase in luciferase activity in a dose-dependent manner. Other steroid hormones including aldosterone, cortisol, and progesterone did not stimulate luciferase response. The CV1-ARluc also increased luciferase activity when treated with human serum extracts. In conclusion, the CV1-ARluc cells provide a novel model system for screening of new AR agonists and antagonists and can determine the androgenic activity of human serum samples.

1. Introduction

Androgens are hormones that play an essential role in the differentiation and maintenance of primary and secondary male sexual characteristics [1]. The two main human androgens are testosterone (T), which is involved in the initial virilization phases of the human male embryo, and 5α-dihydrotestosterone (DHT), which is the active hormone in most androgen target tissues [2]. T is mainly synthesized by the testicular Leydig cells, in peripheral tissues, as well as to a lesser degree in ovaries and adrenals. T is converted to DHT by 5α-reductases and also can be converted to estradiol by aromatase. DHT is the most active physiologic androgen, inducing ten-fold higher androgen receptor (AR, *NR3C4*) bioactivity than T [3,4]. In addition, other endogenously produced steroids exhibit various degrees of androgenic activity [5,6]. Several synthetic androgen-related compounds (AR agonists and antagonists) have also been developed to modulate androgen signaling in therapeutic settings [7,8].

Androgens mediate their effects through binding and activation of the AR. AR is a member of the steroid nuclear receptor superfamily [9] and acts as a ligand-dependent transcription factor [10]. Among this family, five steroid receptors are known: estrogen (ESR, *NR3A1*), progesterone (PR, *NR2C3*), androgen, mineralocorticoid (MR, *NR3C2*) and glucocorticoid (GR, *NR3C1*) receptors. AR activates a wide range of target genes that encode proteins and noncoding RNAs, including regulatory microRNA species [11].

Similar to the other steroid receptors, unbound AR is located in the cytoplasm. Upon ligand binding, AR goes through a series of

Abbreviations: AR, androgen receptor protein; ARE, androgen response element; GR, glucocorticoid receptor protein; MR, mineralocorticoid receptor protein; PR, progesterone receptor protein; T, testosterone; DHT, 5α-dihydrotestosterone; Cort, cortisol; AD4, androstenedione; Prog, progesterone; 11OHT, 11-hydroxytestosterone; 11KT, 11-ketotestosterone; 11OHAD, 11-hydroxyandrostenedione; 11KAD, 11-ketoandrostenedione.

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conformational changes, dimerization and translocation to the nucleus, which is mediated by a nuclear localization signal. Translocated AR binds to androgen response elements (ARE). These ARE are characterized by a consensus (or near consensus) sequence 5′-TGTTCT-3′, which is located in the promoter or enhancer regions of AR gene targets. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for GR, MR and PR. The similarity of the response element for AR and the other steroid receptors, and particularly the wide-spread expression of the GR, has been problematic in the development of selective receptor screening assays.

The determination of androgen levels or the discoveries of new androgenic compounds are key elements for the diagnosis of a number of diseases in children and adults. Assays that detect bioactive serum androgens in a sensitive and selective manner benefit the diagnosis and treatment of several pediatric endocrine disorders, such as precocious puberty and ambiguous genitalia. In addition, androgen bioassays provide a screening tool for androgen abuse and endocrine disruptors [12]. Over the past 10 years, several bioassays were developed using different methods [13]. One of the first assays developed relied on a chloramphenicol acetyltransferase (CAT) reporter model [14]. This system was limited by experimental variation due to the transient nature of transgene expression. A luciferase reporter bioassay, using MDA-MB453 cells, was developed by Wilson et al. [15]. The major caveat of this assay was that it responds to AR as well as to GR agonists. Other androgen-reporter cell lines were developed but most of them were transiently transfected [16–18]. Transient transfection assays [19] can provide similar information with stable assays but may not reflect endogenous levels of receptor. A stable expression of AR in the cells can eliminate the need for repetitious transient transfections, reduce the variability associated with these transient assays and moreover be utilized for high-throughput studies. Until now, a selective androgen-responsive transcriptional activation assay has not been widely available.

The aim of this study was to develop a stable cell-based *in vitro* bioassay that expresses the human AR (hAR) gene with sensitive and selective reporter readout. For this purpose, a stable cell line was made with CV1 cells stably transduced with hAR and an MMTV promoter-driven luciferase reporter gene. The resulting model is selective for androgens and does not exhibit reporter activation by other steroid receptors. In addition the model appears useful to determine circulating androgenic bioactivity in human serum samples.

2. Materials and methods

2.1. Materials

T, DHT, cortisol (Cort), progesterone (Prog), aldosterone (Aldo), androstenedione (AD4), hydroxyflutamide (OHF) and the 11-keto and 11-hydroxy forms of androstenedione and T were purchased from Sigma (Missouri, USA). Coelenterazine used for the luciferase assay was purchased from Promega (Wisconsin, USA). Penicillin, streptomycin, hygromycin, geneticin (G418) and DMEM/F12 medium were purchased from Life technologies (New York, USA).

2.2. Cell line

The CV1 monkey kidney cell line was obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) from GE Healthcare Life Science (Utah, USA) and antibiotics including 1% penicillin/streptomycin. The cells were incubated under a humid atmosphere of 5% CO_2, at 37 °C, and the medium was changed every 3 days. CV1 cells were plated at a density of 20,000 cells/well (48 well dish) in growth medium and grown to 60% confluence after which they were treated for steroids activity.

CV1-ARluc cells were plated in a 48 wells culture plate in 500 μL of growth medium (10% FBS/DMEM-F12, G418 and Hygromycin). The cells were incubated under a humid atmosphere of 5% CO_2, at 37 °C. All treatments were performed with charcoal-stripped FBS serum to eliminate contaminating steroids.

2.3. Stable transduction

CV1 cells (20,000 cells/well) were plated about 18 h before transduction in a 48 well-dish. The lentivirus pBM14-MMTV with the *Gaussia Luciferase* gene was diluted 1:10 in DMEM/F12 medium and added to the flask with 8 μg/mL of polybrene. The flask was centrifuged at 1200 rpm for 80 min and after 4 h in a humidified 5% CO_2 incubator the cells were supplemented with 1 mL of DMEM/F12 containing 10% FBS without any antibiotics. After 48 h, the cells were selected in medium containing 1200 μg/mL of G418. The medium was changed three times a week. The obtained cells, named CV1-luc, were transduced with a lentivirus containing the hAR gene and the hygromycin selective gene. The stable transfection was performed as described above, using a multiplicity of infection (MOI) of 10 and 8 μg/mL of polybrene. 50 clones were obtained after 14 days of dual antibiotic (G418 and Hygromycin) selection. The clones were isolated using cloning rings (Sigma, Missouri, USA) and re-seeded and grown in a 48-well dish. After reaching 60% confluence, the cells were treated in DMEM/F12 containing 10% charcoal-stripped FBS and 10 nM of testosterone. After 24 h the treated cells were assayed for luciferase activity using the appropriate luminescence kit (Coelenterazine, Promega). The clone with the largest T induced luciferase activity was named CV1-ARluc and was used for further studies.

2.4. Isolation of RNA and qPCR analysis

The cells (25,000 cells/well) were grown for 24 h in 48 well culture. Total RNA was isolated from the cells previously plated using an RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and purity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For cDNA generation, 100 ng of total RNA was reverse transcribed using the High Capacity Kit (Applied Biosystems, Foster City, CA, USA). For qPCR, 12 ng of prepared cDNA was mixed with Fast Universal PCR Master Mix (Applied Biosystems). AR and peptidylprolyn isomerase A (PPIA) primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). PPIA was used as the housekeeping control gene.

2.5. Protein extraction and protein assay

Cells were lysed in 200 μL Mammalian Protein Extraction Reagent (Pierce Chemical Co., Illinois, USA), and the protein content was estimated by the bicinchoninic acid (BCA) protein assay using the BCA protocol (Thermo Scientific, Illinois, USA).

2.6. Western analysis

CV1 and CV1-ARluc cell lines were plated at a density of 75,000 cells/well (24 well-dish), in growth medium. Samples were lysed with lysis buffer (2% sodium dodecyl phosphate, 62.5 μM Tris, 0.04% bromophenol blue, 0.5% dithiothreitol) and heated at 95 °C for 5 min. Proteins were then loaded (20 μg) on 10% bis-Tris gel and electrophoresed for 1 h before transferring to polyvinylidene difluoride membranes. The membranes were then blocked