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Construction and characterization of a reporter gene cell line for assessment of human glucocorticoid receptor activation

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ABSTRACT

Glucocorticoids are widely used drugs in human pharmacotherapy. There is an increasing demand for tools allowing detection of the ligands for glucocorticoid receptor (GR), with regard to pre-clinical drug testing and environmental applications. We constructed human luciferase reporter gene cell line AZ-GR derived from HeLa human cervix carcinoma cells, which were stably transfected with reporter plasmid containing three copies of glucorticoid response element (GRE) upstream of luciferase reporter gene. We isolated five dexamethasone-responsive clones, and we further characterized two most responsive ones (AZ-GR). Dose–response analyses were performed with 22 different natural and synthetic steroids and the values of EC_{50} were calculated. AZ-GR cells displayed high specificity and sensitivity to glucocorticoids, very low responsiveness to mineralocorticoids, but no responsiveness to estrogens, gestagens or androgens. Time-course analyses revealed that AZ-GR cells allow detection of GR activators soon after 14 h of the treatment (6–10-fold induction by 100 nM dexamethasone). Functionality of AZ-GR cells was not affected with cryopreservation. Generated reporter gene cell lines fully maintained responsiveness to glucocorticoids for 32 days in the culture and over 16 passages without significant alterations. The sensitivity of the assay allows high throughput format using 96-well plates.

Collectively, we present here glucocorticoid-responsive stable reporter gene cell line that allows high throughput, rapid, sensitive and selective detection of GR activators, with possible use in pre-clinical research and environmental applications.

1. Introduction

Glucocorticoid receptor (GR) is a ligand-inducible transcriptional factor that belongs to the subfamily of nuclear hormone receptors. It exists in two isoforms, GRα and GRβ that are generated by different splicing of exon 9 (Hollenberg et al., 1985). Transcriptionally active is isoform GRα that binds glucocorticoids, whereas GRβ does not bind the ligands and inhibits GRα-mediated gene transcription (Kino et al., 2009). In the resting state, GRα is localized in the cytosol in the complex with chaperone proteins. Upon the ligand binding, it translocates to the nucleus where it binds to glucocorticoid response elements in DNA as a homodimer GRα/GRα (Dvorak and Pavek, 2010). Glucocorticoids are massively used drugs for their anti-inflammatory and immunosuppressive effects. Synthetic (exogenous) glucocorticoids include dexamethasone, beclomethasone, betamethasone, triamcinolone etc. The most important physiological (endogenous) ligand of human GR is cortisol (hydrocortisone).

There is a need for the reliable, selective, sensitive and high throughput tools for monitoring glucocorticoid activity, at least for two purposes: (i) Pre-clinical applications, i.e. in search of new glucocorticoids. (ii) Environmental applications, i.e. monitoring of steroid hormones in soils and waters. Gene reporter assays are routinely used tools for assessment of activation of transcription factors or receptors. In the current paper, we used HeLa cells that have fully functional endogenous GR, and we generated AZ-GR cells stably transfected with the plasmid containing multiple GREs fused to luciferase reporter gene.

Several luciferase reporter gene assays for assessment of steroid receptor transcriptional activity have been developed. Israel and Kaufman transfected reporter gene plasmid containing multiple GREs to Chinese hamster ovarian cells. The resulting cell line showed high induction by dexamethasone (Israel and Kaufman 1989). Stably transfected cell line called MDA-kb2 was derived from MDA-MD-453 cells that were transfected with MMTV.luciferase.neo reporter gene construct. The MDA-kb2 cell line was used to screen chemicals for androgen receptor and GR-mediated activity (Wilson et al., 2002). Mori et al. constructed reporter cell line

Abbreviations: DEX, dexamethasone; DMSO, dimethylsulfoxide; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HeLa, human negroid cervix epitheloid carcinoma cells.

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hGR-Luc for the purpose of lipophilic chemical screening. This cell line was constructed by genomic recombination in stable genetic modified HeLa55 cells using a uniquely designed donor vector harboring an exchange cassette comprised of the human GR gene, its response element, and a luciferase reporter gene (Mori et al., 2008).

In the current paper, we have developed stably transfected human luciferase reporter gene cell lines AZ-GR allowing measurement of GR transcriptional activity. The cell lines are selective for glucocorticoids and the luciferase activity is possible to measure after 14 h of incubation. The assay is very sensitive and allows high throughput format (96-well plates). AZ-GR cell line remained fully functional over 16 passages and 32 days in culture. The responsiveness to glucocorticoids was retained in cryopreserved cells after thawing as compared to fresh cells.

2. Materials and methods

2.1. Compounds and reagents

Fugene HD transfection reagent was from Roche (Basel, Switzerland). DMSO, hygromycin B, dexamethasone, beclometasone, betamethasone, cortisol, corticosterone, prednisolone, methylprednisolone, testosterone, estradiol, diethylstilbestrol, 4-hydroxyta- moxifen, spironolactone, aldosterone, 17α-hydroxyprogesterone and progesterone were purchased from Sigma–Aldrich (Prague, Czech Republic). Raloxifene hydrochloride, tamoxifen citrate salt, genistein, cyproterone acetate, danazol, triamcinolone and mifepristone were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Luciferase lysis buffer was from Promega (Hercules, CA). All other chemicals were of the highest quality commercially available.

2.2. Plasmid

Reporter plasmid pGL-4.27-GRE was constructed as follows: Three copies of tandem GREs (**GGTACA**TTT**TGTTCT GGTACA** GTA **CGTCCT** GTTCT **GGTACA**AAC**TGTTCT**) were synthesized and inserted using KpnI-XhoI enzymes into pGL4.27 [luc2P/minP/Hygro] vector (Cat. Nuber E8451) from Promega (Hercules, CA).

2.3. HeLa Cells

Human Negroid cervix epitheloid carcinoma cells HeLa (ECACC No. 93021013) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal calf serum, 100 U/ml streptomycin, 100 μg/ml penicillin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.4. Transfection of HeLa cells and selection process

HeLa cells were transfected with pGL-4.27-GRE (2 μg) reporter plasmid using Fugene HD reagent and seeded at the density of 8×10^5 cells in 60 mm culture dishes in 5 ml of the DMEM culture medium. Following 36 h of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin B (0.2 mg/mL). The medium was changed every 3–4 days for the period of 3 weeks, until a polyclonal population was selected. Thereafter, the cells were transferred to 10 cm culture dishes at the density of 500–1000 cells per dish and cultured for additional 2 weeks in the presence of hygromycin B until small colonies were visible. 17 colonies were subcloned into a 48-well tissue culture plate to obtain monoclonal populations (two best clones 2 and 15 are termed AZ-GR cells). Hygromycin B resistant clones were treated with glucocorticoids, mineralocorticoids, estrogenes, androgens and gestagens. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (Ref. 91997/ENV/10).

2.5. Gene reporter assay

AZ-GR cells were seeded on 96-well plates in density 20.000 cells per well. Following 16 h of incubation, cells were treated with tested compounds as described in detail in figure legends. After the treatments, cells were lysed and luciferase activity was measured in 96-well plate format, using Tecan Infinite M2000 plate luminometer.

2.6. Statistical Analyses

Student's pair *t*-test was applied. The values of EC_{50} were determined using free software ED50plus v1.0 (http://www.free-downloads-center.com/download/ed50plus-v1-0-2434.html). Fitting of dose–response curves was performed by GraphPad software.

3. Results

3.1. Selection process and generation of AZ-GR clones

HeLa cells were transfected with pGL-4.27-GRE reporter plasmid and stably transfected clones were selected under the pressure of hygromycin B, as described in Methods section. We found slight morphological differences between parent HeLa cells and stably transfected cells. HeLa cells have an epithelial like morphology and elongated shape, while transfected cells were rather oval (Fig. 1).

We isolated seventeen hygromycin B - resistant clones and we tested the responsiveness of the clones to synthetic glucocorticoid dexamethasone. For this purpose, stably transfected clones cells

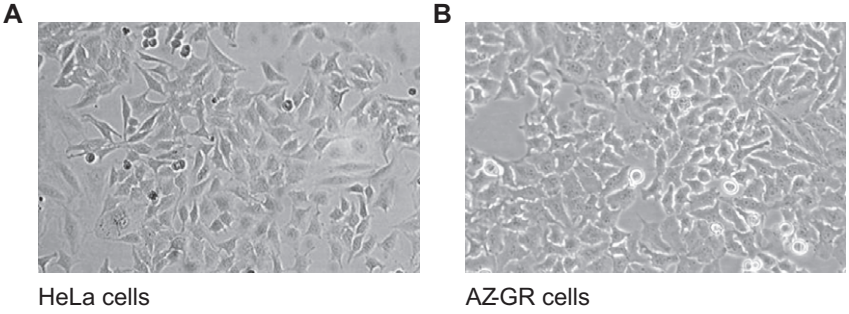

Fig. 1. Morphology of AZ-GR cells and HeLa cells Phase contrast micrographs of parent HeLa cells (at 7th passage) and AZ-GR cells (5th passage, clone 2).