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A DRUG-BASED CELLULAR ASSAY (DBCA) FOR STUDYING CYTOTOXIC AND CYTOPROTECTIVE ACTIVITIES OF THE PRION PROTEIN: A PRACTICAL GUIDE

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Abstract

Although a great deal of progress has been made in elucidating the molecular identity of the infectious agent in prion diseases, the mechanisms by which prions kill neurons, and the role of the cellular prion protein (PrP^C) in this process, remain enigmatic. A window into the normal function of PrP^C, and how it can be corrupted to produce neurotoxic effects, is provided by a PrP deletion mutant called Δ CR, which produces a lethal phenotype when expressed in transgenic mice. In a previous study, we described the unusual observation that cells expressing Δ CR PrP are hypersensitive to the toxic effects of two cationic antibiotics (G418 and Zeocin) that are typically used for selection of transfected cell lines (1). We have used this drug sensitizing effect to develop a simple Drug-Based Cell Assay (DBCA) that reproduces several features of mutant PrP toxicity observed *in vivo*, including the rescuing activity of wild-type PrP. In this manuscript, we present a detailed guide for executing the DBCA in several, different experimental settings, including a new slot blot-based format. This assay provides a unique tool for studying PrP cytotoxic and cytoprotective activities in cell culture.

Keywords

prion protein; mutant PrP; cellular assay

1. INTRODUCTION

Prion diseases are infectious, neurodegenerative disorders that affect humans and animals (2). They are associated with the accumulation of PrP^{Sc}, a misfolded form of the cellular prion protein (PrP^C) (3). PrP^C and PrP^{Sc} share the same amino acid sequence but differ in their conformations and biochemical properties, with PrP^{Sc} showing higher β -sheet content and a greater tendency to form insoluble, protease-resistant aggregates. PrP^C is a normal cellular glycoprotein of 33–35 kDa that is attached to the outer surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The physiological function of PrP^C is still unclear (4). There is evidence that an alteration in the normal function of PrP^C may play some role in prion pathogenesis, but the underlying mechanisms remain uncertain (5). The development of cell culture models for genetic and infectious prion diseases has been fundamental for understanding many biochemical and cell biological properties of

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PrP^C, and the mechanism by which it is converted into PrP^{Sc}. However, it has proven difficult to reproduce the toxic effects of PrP^{Sc} using cell culture systems, a problem that has limited our ability to study the molecular mechanisms of prion induced neurodegeneration.

Important insights into the physiological activity of PrP^C and how it might be altered in the disease state come from studies of transgenic (Tg) mice expressing forms of PrP deleted in the flexible, N-terminal tail (Δ 32-121; Δ 32-134; Δ 94-134 and Δ 105-125, the latter also referred to as Δ CR PrP) (6-8). All of these mice spontaneously develop ataxia, accompanied by cerebellar neurodegeneration and/or white matter pathology, a phenotype that is dose-dependently suppressed by co-expression of wild-type (WT) PrP, either from the endogenous *Prn-p* locus or from a second transgene. Interestingly, Tg(Δ CR) mice, which express the smallest deletion, display the most severe phenotype, characterized by massive degeneration of cerebellar granule cells, vacuolization of the white matter in the brain and spinal cord, and death within 1 week on the PrP-null background (8). These data suggest that removal of the central region of PrP, encompassing residues 105-125, endows the protein with a powerful neurotoxic activity that is antagonized by the presence of WT PrP (4).

To gain mechanistic insights into the toxicity of deleted forms of PrP, it was necessary to study this activity in a cell culture system. We have now developed such a system, based on the ability of mutant PrP molecules to hypersensitize a variety of transformed cell lines, as well as differentiated neural stem cells, to the toxic effects of two classes of antibiotics: aminoglycosides (such as G418) and bleomycin analogues (such as Zeocin) (1). We refer to this assay as the Drug-Based Cell Assay (DBCA).

The DBCA mimics several features of mutant PrP toxicity observed in Tg mice. Most importantly, the drug-sensitizing effect of Δ CR PrP can be suppressed by co-expression of WT PrP, paralleling the ability of WT PrP to rescue the neurodegenerative phenotype of Tg(Δ CR) mice. In addition, there is a correlation between the pathogenicity of the mutant molecules *in vivo* and the strength of their drug-sensitizing effects *in vitro*, with Δ CR PrP displaying the greatest toxic potential in both settings. Such a strong correlation between the effect of mutant PrP molecules in the DBCA and in Tg mice suggests that similar cellular mechanisms are operative in both contexts. The DBCA provides a powerful assay for studying PrP cytotoxic and cytoprotective activities in an *in vitro* format. Importantly, this assay can be performed rapidly using standard blotting and imaging devices available in most laboratories. We have recently improved the DBCA by optimizing the time of drug treatment, sample processing and data acquisition, with the objective of using the DBCA for structure-function analyses and for high-throughput screening to identify small molecules inhibitors of Δ CR PrP toxicity. Here we provide a practical guide for executing the DBCA in multiple experimental formats, including new and rapid one that utilizes slot blotting.

2. CELL CULTURE

In all the protocols presented here, the DBCA was performed using HEK293 cells (ATCC CRL-1573) stably expressing WT or Δ CR PrP. Δ CR PrP also induces drug hypersensitivity in a variety of other cell types, including mouse neuroblastoma cells (N2a), Chinese hamster ovary cells (CHO), and differentiated mouse neural stem cells (NSC) (1); the assay could, in principle, be optimized for these cell types as well. In all cases, stable rather than transient expression of PrP molecules showed the best results. The DBCA can also be adapted to detect the effect of other PrP mutants (e.g. Δ 32-134) that show lower toxicity *in vivo* (unpublished data).

2.1. Materials and reagents

- 25 cm² flasks

- Plastic 24-well plates
- Maintaining medium (M-medium): α -minimum Eagle's medium/Dulbecco's modified Eagle's medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, non-essential amino acids, penicillin/streptomycin, 50 μ g/ml hygromycin.
- Treatment medium (T-medium): α -minimum Eagle's medium/Dulbecco's modified Eagle's medium (1:1) containing 10% fetal bovine serum, 2 mM glutamine, non-essential amino acids, penicillin/streptomycin, and 500 μ g/ml Zeocin or G418 (Invitrogen)

2.2 Procedure

Stably transfected HEK293 cells are grown in 25 cm² flasks and are maintained under hygromycin selection (50 μ g/ml in M-medium). Cell density is a crucial determinant of the efficiency of the DBCA. Therefore, all cell clones used in the assay should be at similar density before the treatment with Zeocin or G418. In order to obtain the maximum drug-sensitizing effect, cells are split one day before drug treatment in plastic 24-well plates at 30–60% confluency, depending on the toxicity readout (see below). For most experiments, 24-well plates are preferred over 48- or 96-well plates, because cell density can be more easily controlled in these plates (it normally takes 2-3 days for cells at 30% confluency to reach full confluency in a 24-well plate). However, the DBCA was recently optimized for 384-well plates, in order to perform high-throughput drug screening (unpublished data). Twenty-four hours after plating, cells are treated with 500 μ g/ml of Zeocin or G418 (depending on the final readout) in T-medium. The length of treatment varies from 2-96 hrs, depending on the toxicity readout to be used (Fig. 1). The DBCA can be performed using three different readouts based on cell viability (MTT), cell death/DNA fragmentation (TUNEL), or DNA damage response (H2AX phosphorylation). Detailed information for executing each one of these assays will be presented in the next three sections.

3. MTT

The MTT assay is a colorimetric test which has been widely used for quantifying metabolically active cells (9). This assay is based on the activity of mitochondrial dehydrogenases, which convert the tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), a water-soluble, yellow dye, into water-insoluble formazan crystals, which appear purple and can be solubilized with dimethyl sulfoxide (DMSO). The amount of formazan produced can be easily quantitated by measuring the absorbance at 570 nm in a standard microplate reader.

3.1. Materials and reagents

- Phosphate-buffered saline (PBS), pH 7.4
- MTT buffer (154 mM NaCl, 5.6 mM KCl, 1 mM MgCl₂, 2.3 mM CaCl₂, 8.6 mM HEPES, 5.6 mM glucose)
- MTT solution: 1 mg/ml MTT (Sigma) suspended in MTT buffer
- Dimethyl Sulfoxide (DMSO) (Sigma)
- Plastic 24-well plates
- Plate reader (e.g., Bio-Rad model 3550)

3.2. Procedure

1. Plate HEK293 cells expressing WT or Δ CR PrP in 24-well plates (50–60% confluency) and let them grow for 24 hours in M-medium.
2. Replace M-medium with T-medium, which should then be changed every 24 hours. The MTT assay (see next step) can be performed anytime between 2 and 4 days after treatment, although the largest difference between WT and Δ CR cells is usually detected by performing the assay after 2 days.
3. Remove the T-medium.
4. Wash the cells once with PBS
5. Add 0.5 ml MTT solution, and then place the cells in an incubator at 37°C for 30 minutes.
6. Remove the MTT solution and incubate the cells in 0.5 ml DMSO for 10 minutes in the incubator.
7. Transfer 100 μ l of each sample into a 96-well plate and read the absorbance at 570 nm. If the DMSO solution is too dark, dilute all the samples 1:1 with fresh DMSO to bring the samples into the linear range of the plate reader.
8. Quantify the data as the A_{570} values for Zeocin-treated cells expressed as a percentage of the A_{570} values for untreated cells.

4. TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a method commonly used to detect dying cells. This assay is based on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labeled dUTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA (10). Such DNA fragmentation occurs during certain forms of cell death like apoptosis. Therefore, Δ CR-dependent cell hypersensitivity to Zeocin and G418 can be conveniently detected by TUNEL staining using a standard fluorescent microscope.

4.1. Materials and reagents

- Phosphate-buffered saline (PBS), pH 7.4
- Fixing solution: 4% Paraformaldehyde
- Permeabilization solution: 0.5% Triton X-100
- TMR Red *in situ* cell death detection kit, TUNEL (Roche, Cat. N. 12156792910)
- 4',6-diamidino-2-phenylindole (DAPI, Invitrogen); 300 nM in PBS.
- Plastic 24-well plates
- Inverted fluorescence microscope (e.g. Nikon TE2000-E2)
- Glass coverslips
- Gel Mount (Biomed)

4.2. Procedure

1. Seed HEK293 cells expressing WT or Δ CR PrP on 13 mm glass coverslips inserted in 24-well plates. Cells should be at 30–40% confluency in M-medium.

2. After 24 hours, replace the M-medium with 1 ml of T-medium (containing Zeocin or G418). Since pH changes can interfere with drug activity, medium needs to be replaced every 24 hours. The TUNEL assay should be performed 24 to 48 hours after Zeocin treatment.
3. Remove medium.
4. Wash each well twice with PBS.
5. Add 0.5 ml fixing solution and incubate in the dark at room temperature for 30 minutes.
6. Remove the fixing solution and wash twice with PBS.
7. Add 0.5 ml permeabilization solution and incubate 5 minutes.
8. Wash twice with PBS.
9. Deposit 50 μ l of the TUNEL reaction mixture onto a flat, parafilm-covered tray.
10. Remove each glass coverslip from the well and flip it up-side-down onto the drop of TUNEL mixture.
11. Incubate in the dark at 37 °C for 1 hour in a humidified incubator.
12. Wash each coverslip twice with PBS.
13. For counterstaining nuclei, repeat steps 9–12, replacing the TUNEL mixture with DAPI.
14. Mount each coverslip on a microscope slide with Gel Mount.
15. Data can be acquired with a standard fluorescent microscope capable of detecting the wavelength of the Texas-Red fluorophore and DAPI (excitation/emission wavelengths of 350/470 and 596/615 respectively) and software for cell counting. Multiple fields per coverslip should be analyzed, scoring a total of least 500–1,000 cells per sample. We acquired images with an Image Express Micro High Content Imager (Molecular Devices). Images (9 fields/well; ~1,000 cells total) were taken at 20 magnification. Data were analyzed using multi-wavelength cell scoring software (MetaExpress, Molecular Devices) to count both DAPI-positive cells and TUNEL-positive cells. The threshold was set to 50 gray levels above background, and minimum and maximum cell widths were 10 and 20 nm. The number of TUNEL-positive cells as a percentage of the number of DAPI-positive cells was calculated for each well.

5. γ -H2AX

This readout is suitable for detecting hypersensitivity to Zeocin, but not to G418. H2AX is a variant of histone H2A that is rapidly phosphorylated at Ser 139 (to produce γ -H2AX) following treatment of cells with agents (such as Zeocin) that induce double-strand DNA breaks. Since multiple molecules of H2AX surrounding each break are phosphorylated, detection of γ -H2AX represents an extremely rapid and sensitive readout of DNA damage (11). We previously took advantage of this phenomenon to measure the effect of Zeocin on Δ CR PrP-expressing cells, using either Western blotting or immunofluorescence staining to measure γ -H2AX (1). Here, we describe a new protocol for detecting γ -H2AX by slot-blotting, which further increases sensitivity and decreases the time of execution.

5.1. Materials and reagents

- Phosphate-buffered saline (PBS), pH 7.4

- Plastic 24-well plates
- Plate reader (e.g., Bio-Rad model 3550)
- T-medium (containing 500 µg/ml of Zeocin)
- Lysis buffer: Igepal (NP40) 0.5%, sodium deoxycholate 0.5%, PBS pH 7.4, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (Roche)
- Tris Buffered Saline (TBS) pH 7.4: 20 mM Tris, 500 mM NaCl
- TTBS: 0.1% Tween-20 in TBS
- Blocking solution: 5% nonfat dry milk in TTBS
- Bio-Dot SF microfiltration apparatus (Biorad)
- Chemidoc XRS (Biorad)
- Primary antibody: Anti γ -H2AX (BioLegend, San Diego, CA), 1:2,000 in blocking solution
- Secondary antibody: Goat anti-mouse IgG (Sigma), 1:5,000 in blocking solution
- QuantityOne software (Biorad)

5.2. Procedure (Fig. 2)

1. Plate HEK293 cells expressing WT or Δ CR PrP in 24-well plate (50% confluence in M-medium).
2. After 24 hours, replace the medium with 1 ml of T-medium.
3. Incubate the cells at 37°C for 2 hours.
4. Wash each well twice with PBS.
5. Add 100 µl of lysis buffer to each well, wait 2 minutes, then transfer each sample to a 1.5 ml tube.
6. Quantify total protein in each sample by using the BCA protein assay kit (Pierce).
7. For each sample, dilute
8. µg of total protein in 200 µl TBS.
9. Place the Bio-Dot SF apparatus under negative pressure by attaching it to a vacuum source.
10. Assemble the apparatus using three sheets of Bio-Dot filter paper (Biorad) and a nitrocellulose membrane (Biorad) previously wetted in TBS.
11. Apply 100 µl of TBS to all the sample wells.
12. As soon as the buffer solution drains from all the wells, disconnect the vacuum.
13. Load 200 µl of each sample.
14. Apply a gentle vacuum until the wells are drained.
15. Wash each well once by adding 200 µl of TBS before disassembling the apparatus.
16. Incubate the membrane 10 minutes with Ponceau S solution (Fluka).
17. Wash the membrane three times with distilled water and acquire images of the Ponceau-stained bands (using e.g., the Chemidoc XRS, Biorad)

18. De-stain the membrane with TTBS.
19. Incubate the membrane 30 min with blocking solution.
20. Incubate the membrane with the primary antibody in blocking solution for 1 hour.
21. Wash the membrane three times for 5 minutes with TTBS.
22. Incubate the membrane with the secondary antibody in blocking solution for 1 hour.
23. Wash the membrane three times for 10 minutes with TTBS.
24. Wash an additional time with TBS.
25. Develop the membrane with Immobilon Western (Millipore): Mix equal volumes of Luminol Reagent and Peroxide Solution. Place the blot protein side up in a clean container, and add the HRP substrate onto the blot. Cover the blot with a clean plastic wrap. Incubate the blot for 5 minutes at room temperature.
26. Acquire images with Chemidoc (Biorad).
27. Quantify the intensity of each band using image analysis software (e.g., QuantityOne, Biorad) normalizing the γ -H2AX signal for the corresponding ponceau signal. Data are then expressed as the normalized γ -H2AX signal in Zeocin-treated cells as a percentage of the value in untreated cells.

6. RESULTS

6.1. MTT

HEK293 cells stably expressing empty vector, Δ CR PrP or WT PrP were treated with 500 μ g/ml of Zeocin for 96 hours, and cell viability was measured using the MTT assay. We found that Zeocin caused 60% loss of viability in Δ CR PrP-expressing cells compared to untreated controls, while no substantial effect (<10%) was observed in cells expressing WT PrP or empty vector (Fig. 3). The simplicity of the readout, which requires only a plate reader to collect data, makes this assay easy to perform. However, MTT is just one of several possible readouts for detecting cell viability. Since cell expressing Δ CR PrP die within few days of Zeocin treatment, one alternative to MTT is would be to count the total number of cells in the well, for example using an automated, high-content microscopic imaging system. We anticipate that such technological improvement of the DBCA, coupled with the optimization of the assay for 384-well plates, will allow rapid screening of compound libraries to identify small molecules that inhibit Δ CR PrP-dependent toxicity.

6.2. TUNEL

Using the same experimental set-up described above, we also measured cell death based on TUNEL. We found that after 24 hr of treatment with 500 μ g/ml of Zeocin more than 25% of cells expressing Δ CR PrP were TUNEL positive, compared to <2% for cells expressing WT PrP or vector (Fig. 4). The fold-difference between Δ CR and control cells was significantly greater using TUNEL than MTT, suggesting that the former assay has a higher sensitivity for scoring cell death, most likely because of the lower background levels in the absence of drug treatment. Therefore, the TUNEL readout could potentially be used to detect the drug-hypersensitizing effect of other PrP mutants that are less toxic *in vivo*.

6.3. γ -H2AX

The effect of Zeocin can be conveniently detected by measuring the levels of phosphorylated H2AX. Once again, we treated HEK293 cells stably expressing empty

vector, Δ CR PrP or WT PrP with 500 μ g/ml of Zeocin, but this time only for 2 hrs. Samples were then analyzed by slot-blotting, detecting the γ -H2AX signal with a specific antibody. We found a robust increase γ -H2AX only in Δ CR cells treated with Zeocin, and not in any of the control cells (Fig. 5). The Zeocin-dependent increase in γ -H2AX was prevented by inclusion of 30 mM of KCl in the incubation medium, which was previously shown to suppress the hypersensitizing effect of Δ CR PrP (1).

Detection of γ -H2AX by slot blotting is simpler and faster than detection by Western blotting or immunofluorescence staining, and would be compatible with low to medium throughput screening approaches to identify inhibitors of Δ CR PrP toxicity.

7. CONCLUDING REMARKS

Although Δ CR is an artificial molecule, it is likely to act by subverting a normal physiological function of PrPC, similar to what has been postulated for PrPSc. Thus, insights derived from the study of Δ CR and related molecules will most likely have applicability to understanding the pathogenesis of prion diseases, and to working out effective therapies for these disorders. The fact that the expression of Δ CR PrP renders a variety of transformed cell lines hypersensitive to Zeocin and G418, allowed us to define a novel, robust cellular assay to investigate the biological activity of Δ CR and related neurotoxic molecules.

We previously reported that the drug-hypersensitizing effect of Δ CR PrP can be suppressed by membrane depolarization, suggesting that Δ CR PrP facilitates accumulation of cationic drugs down an electrochemical gradient. This conclusion is supported by the recent observation that Δ CR PrP, as well as PrP molecules carrying disease-associated point mutations in the central region, induce spontaneous ionic currents in a variety of cell types (12). These results suggest that mutations in the central region of PrP alter the activity of membrane channels or transporters responsible for drug uptake. Alternatively, the mutant PrPs may alter the permeability of the plasma membrane in such a way as to allow entry of inorganic and organic cations.

In conclusion, the DBCA represents a useful tool for dissecting the function of PrPC and how mutant molecules can subvert it to produce toxicity. It is significantly more sensitive and robust than other published assays for detecting the toxicity of deleted forms of PrP and Doppel (13,14). In this article we have provided a comprehensive description of the DBCA that will assist other laboratories in performing this assay. We expect that the DBCA will facilitate investigation of the mechanisms underlying mutant PrP toxicity, and will form the basis high throughput screening projects to identify compounds that inhibit that toxicity.

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FIGURE 1. Possible DBCA read-outs

HEK293 cells are grown in a 25 cm² flask, split into 24-well plates and 24 hours later treated with 500 μ g/ml Zeocin. (A) Cells are treated with Zeocin or G418 for 48 hours, and cell viability is measured by MTT reduction using a standard microplate absorbance reader. This assay requires approximately 96 hours. (B) Cells are treated with drugs for 24 hours, and cell death is measured by TUNEL using a standard fluorescence microscope, or an automated cell imaging system. This assay requires approximately 4 days. (C) Cells are treated with Zeocin for 2 hours, and the amount of phosphorylated H2AX (γ -H2AX) is quantitated by slot blotting. This assay can be done in 1 day.

FIGURE 2. Slot blot-based assay for γ -H2AX
(see text for details)

FIGURE 3. MTT assay

Cells expressing ΔCR PrP, WT PrP or vector were treated with 500 µg/ml Zeocin for 96 hours, and viability was assayed by MTT. (A) Representative wells containing HEK 293 cells that express Vector (Vec), WT PrP or ΔCR PrP, with and without Zeocin treatment, after application of the MTT reduction assay. (B) Quantification of the MTT assay. Data are expressed as the viability of Zeocin-treated cells as a percentage of the viability of untreated cells. ΔCR PrP-expressing cells treated with Zeocin exhibit a 60% decrease in cell viability, while cells expressing WT PrP or empty vector show no loss of viability. Bars show means ± S.D. (n = 3 independent experiments). *Significantly different from control cells (p<0.001).

FIGURE 4. TUNEL assay

Cells expressing Δ CR PrP, WT PrP or vector were treated with 500 μ g/ml Zeocin for 24 hours, and then stained by TUNEL (red) (E-H) to reveal fragmented DNA and with DAPI (blue) (A-D) to reveal nuclei. (I) The number of TUNEL-positive cells, expressed as a percentage of the number of DAPI-stained cells, was determined in nine wells (total of 1000 cells) for each sample group. The bars show means $\pm$ S.E. ($n = 3$ independent experiments). The number of TUNEL-positive cells was 13 times higher in Δ CR cells than in WT cells.

*Significantly different from control cells ($p < 0.001$).

FIGURE 5.

γ -H2AX assay. Cells expressing WT or Δ CR PrP were treated with 500 μ g/ml Zeocin for 2 hours, and DNA damage was detected by slot blot using an antibody against γ -H2AX. The γ -H2AX signals (right-hand panels) were normalized to the Ponceau signals (left-hand panels). Zeocin treatment of cells expressing Δ CR caused a large increase in γ -H2AX levels, which was suppressed by inclusion of 30 mM KCl, which causes membrane depolarization.