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process of expression cloning. After sequential rounds of transfection, selection and harvests, episomes are repeatedly recovered, but with an expected increased representation of fewer cDNA species per clone, demonstrating the dependability of this method in the context of expression cloning experiments (Table 2 and Figure 2).

This alternative method of harvesting episomes offers three advantages. First, since this method is based upon a commercial kit, the standardized reagents and columns can contribute to the consistent yields, similar to other methods (4–6). Second, unlike Hirt's method, in which inhibitors to bacterial transformation have occasionally been detected, inhibitors to electroporation are not found in harvests collected from MCF-10A cells with this method. Third, use of the DNA purification

resin and mini columns makes this method more efficient. This modified Promega protocol can be accomplished in 1 h and 15 min (including the added extraction and precipitation steps), which compares favorably with Hirt's protocol, in which at least 3 h, and up to 2–3 days are required if the recommended overnight incubations are included (3–5).

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Optimization of Single-Cell Gel Electrophoresis (SCGE) for Quantitative Analysis of Neuronal DNA Damage

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ABSTRACT

Neuronal death can be induced by DNA-damaging agents and occurs by apoptosis involving a specific signal-transduction pathway. However, to our knowledge, methods for the quantitative determination of DNA damage in individual neurons have not yet been described. Here we optimize the single-cell gel electrophoresis (SCGE) or "comet"-assay to measure DNA damage within individual neurons growing in dissociated cell culture. In addition, we have written a macro for the NIH Image program to determine the tail moment of individual comets. We have calibrated this method using γ -irradiated (0–16 Gy) cerebral cortical neurons from the rat central nervous system. Neuronal DNA damage (in the form of DNA strand breaks) occurs in a linear, dose-dependent manner, which can be quantitatively determined in vitro using the SCGE assay. These data demonstrate that the SCGE assay is an effective method to measure DNA damage in individual neurons and may be highly useful for the study of neuronal DNA damage formation, repair and apoptosis.

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INTRODUCTION

Neuronal apoptosis can be induced both in vitro and in vivo by exposure to DNA-damaging agents such as radiation, anticancer agents and oxidative stress (1,2,5,6,14). However, methods for the quantitative determination of DNA damage in individual neurons have not been described. Determination of nuclear DNA strand-break formation following treatment with DNA-damaging agents has classically been determined using the alkaline elution assay (9) or pulsed-field gel electrophoresis (PFGE) (5). However, both alkaline elution and PFGE are quantitative assays for whole cell populations and cannot be used for determination of DNA damage within single cells. In addition, alkaline elution requires the pre-labeling of DNA with radioisotopes and, since neurons are nondividing, this technique is virtually impossible to per-

form unless neuronal precursors are pre-labeled during fetal development (a technically difficult and variable procedure). Therefore, we used single-cell gel electrophoresis (SCGE) or the "comet-assay" (16) to directly determine the level of DNA strand-break formation within individual neurons in culture immediately following treatment with γ -radiation, a well-characterized DNA-damaging agent that induces both DNA single-strand breaks (SSB; the predominant form of damage) and double-strand breaks (DSB).

SCGE can be used to monitor both DNA damage and DNA repair. It is based on the principles of DNA migration following electrophoresis and alkaline filter elution to quantitatively determine the degree of DNA strand-break formation. The assay entails the gel electrophoresis of lysed cells entrapped in a layer of agarose to visualize the mobility of damaged DNA. As

reviewed by Fairbairn et al. (3), there are seven major steps in the SCGE assay: (i) slide and tissue preparation, (ii) cell lysis, (iii) DNA unwinding, (iv) electrophoresis, (v) staining and (vi) DNA visualization and recording and (vii) analysis/quantification of DNA damage. While the general principles and procedures for this assay are established, many different variations have been published based on the need to adjust each of the individual steps to optimize the results for a particular experimental condition. To our knowledge, conditions that optimize the SCGE assay for DNA damage to single neurons have not yet been described. Although the SCGE assay has been previously utilized for assessing DNA damage in brains of adult animals (12,13,20), the procedures measure damage to each of the cell types in the adult brain (e.g., neurons, glia, endothelial cells and macrophages), thus preventing assess-

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ment of damage in neurons. Therefore, we have optimized each step of the SCGE assay to examine the formation of DNA strand breaks caused by γ -irradiation (γ -IR) in pure primary cultures of rat cerebral cortical neurons.

MATERIALS AND METHODS

Figure 1 shows a simple schematic diagram of the SCGE assay procedure. To prepare slides, 150 μ L of 0.7% agarose (SeaKem® Gold; FMC Bio-Products, Rockland, ME, USA) were applied to two locations on fully frosted slides (Fisher Scientific, Pittsburgh, PA, USA) in a 9°C cold room. A very thin agarose layer was quickly formed by the rapid application of the liquified agarose to the cold slide, followed by the addition of a 24 $\times$ 40-mm, No. 1 thickness microscope glass coverslips (Fisher Scientific) quickly pressed upon the agarose drop. The agarose was allowed to solidify for 20 min in the cold room, and then the glass coverslips were removed. This first layer of agarose facilitated adequate adhesion, and omission of this layer resulted in agarose layers that floated away after subsequent addition of buffers during the assay.

Primary cerebral cortical neurons were cultured as previously described (14), plated at 2×10^6 cells/dish in poly-L-lysine (100 μ g/mL; Sigma Chemical, St. Louis, MO, USA) coated 35-mm petri dishes and incubated at 37°C in humidified 5% CO₂/95% O₂ for at least 18–24 h before treatment. Neurons (1–3 days in vitro) were γ -irradiated (1.2 Gy/min; Cs¹³⁷ γ -source), washed twice with isotonic phosphate-buffered saline (PBS) containing 0.5 mM EDTA and harvested by trypsinization. Cells were then pelleted by centrifugation at 1000 $\times$ *g* and resuspended in cold PBS at a density of approximately 5×10^5 cells/mL. With time, DNA repair processes would result in a reduction in measured strand breaks. Therefore, to minimize DNA repair, the cells were irradiated on ice, kept cold during the wash and harvest steps, and the time between γ -IR and SCGE analysis was kept to a minimum (approximately 10 min). For the SCGE assay, 25 000 cells per condition (ca. 50 μ L) were quickly added to 450 μ L of

0.7% low-melting point agarose diluted in 0.1 M PBS and maintained at 37°C. Dilution of the agarose in an isotonic solution is essential to maintain membrane integrity during agarose embedding. From this suspension, 2500 cells per condition (approximately 50 μ L) were then applied atop the first solidified agarose layer and flattened with a 22 $\times$ 22-mm, No. 1 thickness microscope glass coverslips (VWR Scientific, W. Chester, PA, USA) and allowed to solidify for 20 min in the cold room.

After the coverslip was removed from the cell/agarose layer, the cells

were lysed by the addition of a freshly made alkaline lysis solution (1% *N*-lauryl sarcosine, 1% Triton® X-100, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base and 1 *N* NaOH, pH ca. 10.5), which was added directly upon the top agarose layer (covering the entire layer) and allowed to incubate for 1 h. We found that a lysis solution with a high pH (≥ 13.0) resulted in comet formation under control conditions and enhanced comet formation under experimental conditions. These results are consistent with the alkali-mediated conversion of alkali-labile sites into DNA strand

Figure 1. Schematic diagram of the SCGE assay. Cultured neurons are treated with a DNA damaging agent and harvested, and live cells are embedded within agarose on a glass slide. Cells are then lysed, and DNA is “unwound” under alkaline conditions followed by electrophoresis and ethidium bromide staining. Damaged DNA migrates towards the anode, resulting in an appearance of a comet, which can be analyzed quantitatively for DNA strand breaks.

Figure 2. Induction of comet formation in cortical neurons. (A and B) Treatment of neurons with γ -IR results in comet formation in cortical neurons in vitro. The SCGE assay was utilized to determine whether strand break formation occurred in γ -IR neurons. Purified neuronal cultures were prepared and subjected to SCGE analysis following treatment with γ -IR at 1 day in vitro (DIV). Neurons grown in 35-mm petri dishes were irradiated using a Gammacel-40 Irradiator (Nordion International, Kanada, ONT, Canada) containing a Cs^{137} γ -source (1.2 Gy/min). Following ethidium bromide staining, nuclei were visualized under fluorescein epifluorescence, and images were captured for comet moment analysis. (A) A nucleus from a control cortical neuron after the SCGE assay, demonstrating little comet tail formation and an intact, round nucleus with a thin penumbra of DNA staining. (B) A nucleus from a cortical neuron following treatment with 4 Gy γ -IR and subjected to SCGE. γ -IR resulted in significant comet tail formation, with lengthening of the tail containing more intense DNA staining and reduction of nuclear size. Bar = 20 μm . (C) γ -IR induces dose-dependent comet formation in cortical neurons. SCGE was performed immediately after exposing 1-DIV cultures of purified cortical neurons with various doses of γ -IR (0–16 Gy). Tail moment was determined for approximately 40–49 neurons per condition, and data are expressed as mean $\pm$ standard error of the mean (SEM) normalized to control within each experiment. Results from two representative experiments are shown, and the effects of γ -IR were significant ($P < 0.0001$ by one-way analysis of variance [ANOVA]).

breaks, suggesting that neurons undergo significant DNA base modification under basal conditions (4,9). After lysis, the cells were then subjected to a DNA-unwinding solution (0.3 M NaOH, 1 mM EDTA, pH ca. 11.5) for 20 min. This high-salt and high-pH alkaline “unwinding” solution allows DNA denaturation and unwinding/relaxation of supercoiled chromatin. We found that an unwinding time of 20 min was ideal, with longer times (40–60 min) resulting in enhanced comet formation in control nuclei. Following unwinding, the slides were subjected to electrophoresis at 1.7 V/cm for 10 min (Sub-Cell® DNA Gel Electrophoresis Apparatus; Bio-Rad, Hercules, CA, USA) using the above unwinding solution as the gel-running buffer. The slides were just barely immersed in the buffer to prevent the agarose from floating away. After electrophoresis, the slides were then stained by application of 20 $\mu\text{g/mL}$ of ethidium bromide for 1 min. Following a rinse with sterile water, the nuclei were visualized using a Zeiss 20 $\times$ Plan-Neofluar objective using epifluorescent illumination (fluorescein filter sets; excitation 450–490 nm, dichroic cutoff 510 nm, emission 515–565 nm) on a Zeiss Axioplan® microscope (both from Carl Zeiss, Thornwood, NY, USA). Images of nuclei in random, nonoverlapping fields were captured using an MTI CCD-300 camera (Dage-MTI, Michigan City, IN, USA) and a Macintosh® Quadra® 700 (Apple Computer, Cupertino, CA, USA) with a Model LG-3 frame-grabber board (Scion, Frederick, MD, USA). Images were then analyzed with Version 6.1 of NIH Image (available by anonymous file transfer protocol [FTP] from: zippy.nimh.nih.gov) and further processed with Adobe® Photoshop® Version 5.0 software (Adobe Systems, San Jose, CA, USA).

The amount of migrating, damaged DNA is a function of both the number of DNA strand breaks and the size of the strands. After ethidium bromide detection, damaged DNA can be visualized as a bright migrating “tail” observed emanating from the nucleus forming a structure very reminiscent of a “comet”; hence, the descriptive “comet-assay”. DNA damage was quantified by determining the “tail-mo-

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ment", a function of both the tail length and intensity of DNA in the tail (7,15). Images were captured using on-chip integration, with the gain and black level set to maximize contrast. We consistently found that with a dynamic range of 8 bits (at the gain setting necessary to capture the comet tail), the intensity of the DNA in the center of the nucleus was such that some pixels were saturated (although the tail was always unsaturated). Thus, our calculations of comet moment (8) were based on the DNA in the tail and do not take into account the unmigrated DNA in the nucleus (7). Once a gain setting was established, it was not changed during the session.

RESULTS

We utilized alkaline SCGE (which denatures DNA) to determine the level of DNA strand breaks in cultured neurons treated with γ -radiation. Since the alkaline conditions used here for the SCGE denature DNA, our results should reflect total DNA strand-break formation. Depending on cell type and culture conditions, it has been determined that γ -IR causes approximately 3–8 DNA SSB per cell per rad of γ -rays (11,17,19) and approximately one SSB per 1.3×10^7 nucleotides (10) [a value 23 times that of DNA DSB formation (9)]. Therefore, the predominant form of DNA damage produced by γ -radiation is the SSB.

Figure 2 demonstrates the results of the SCGE assay performed with primary cerebral cortical neurons immediately following γ -IR. After ethidium bromide staining and pseudo-color image enhancement, neuronal nuclei under control conditions appeared very bright and round with intact nuclei surrounded by a thinly stained penumbra of DNA (Figure 2A). Also, very little migration towards the anode (left of image) is evident, suggesting that little DNA damage (in the form of DNA strand breaks) occurs in neurons under basal conditions. However, following treatment with 4 Gy of γ -IR, there is significant elution of DNA from the nucleus, forming a "comet" tail emanating from the nuclear core and stretching in the direction of the anode (Figure 2B). In addition, γ -IR caused a simulta-

neous reduction in the size of the ethidium bromide-stained nucleus.

According to Hellman et al. (7), the most reliable indicator of DNA damage was computed as the integrated density in the comet tail multiplied by the distance from the center of the nucleus to the center of mass of the tail. We therefore calculated comet moment using a macro written for the NIH Image program (available from: http://www2.umdj.edu/~geller/lab/Comet_Scoring_Macro.txt). This macro is based on an equation modified from Hellman's et al. (7). The macro is comprised of two steps: (i) location of the center of the nucleus and (ii) outlining the furthest extent of the comet tail (see instructions available at the above Web site). As demonstrated in Figure 2C, treatment of cortical neurons with γ -IR resulted in a significant, linear, dose-dependent induction of comet formation [correlation coefficient (r) = 0.998].

DNA damage induced by γ -IR is a highly reproducible, currently accepted calibration method for the SCGE assay (8). Treatment of neurons with 8 Gy (800 rads) of γ -IR resulted in a tail-moment of approximately 9 times that of control (Figure 2C). We found that, although the absolute comet moment was somewhat variable from day to day, the percentage increases with γ -IR were consistent. In addition, we found a dose-related increase in comet moment after treatment of neurons with increasing concentrations of hydrogen peroxide (H_2O_2) (data not shown). Thus, we find it advantageous to include a standard DNA damage event, such as a fixed dose of γ -IR or H_2O_2 , in each experiment to allow calibration of damage by novel agents. These data are consistent with previous results demonstrating the effects of γ -IR and H_2O_2 on DNA strand-break formation in human fibroblasts and Chinese hamster ovary cells analyzed by the SCGE assay (8,18).

DISCUSSION

These results demonstrate that the SCGE assay can be used to reliably and consistently quantify DNA damage in neurons of the central nervous system in vitro. Treatment of neurons with γ -IR, a well-characterized DNA-damag-

ing agent, produced significant comet formation in a linear, dose-dependent manner. The SCGE assay is advantageous for analysis of DNA damage in neurons, since only a small number of cells are needed, which is an important advantage considering that many primary neuronal culture protocols yield small numbers of neurons. In addition, cells are immediately embedded in agarose following treatment, thereby reducing physical manipulation of DNA and reducing the occurrence of mechanical shearing, which may produce false-positive results. Moreover, this method allows direct investigation of the extent of DNA damage in individual cells and is useful for a variety of different neuronal types, including cultured rat cerebellar granule neurons and sympathetic neurons (data not shown). Taken together, these data demonstrate that the SCGE assay can be successfully used to quantify the level of DNA strand-break induction in single neurons by a particular condition or chemical agent and may be highly useful during investigations of neuronal apoptosis, DNA damage formation and mechanisms of DNA repair in neurons.

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