Analytical Biochemistry 414 (2011) 287–293

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A modified fluorimetric neutral filter elution method for analyzing radiation-induced double strand break and repair

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ARTICLE INFO

Article history:
Received 18 December 2010
Received in revised form 18 March 2011
Accepted 24 March 2011
Available online 29 March 2011

Keywords:
Neutral filter elution
DNA damage and repair
PicoGreen
Lymphocyte

ABSTRACT

Neutral filter elution assay is one of the methods used for detection of DNA double strand breaks (DSBs). However, it is laborious, expensive, and hazardous (radiolabeled precursors for DSB detection and scintillation counter for quantification), making it a less preferred method for DSB detection. In the present study, an attempt was made to improve the existing neutral filter elution assay by making use of fluorescent dye (PicoGreen) and microfiltration assembly for eluting the fragmented DNA, thereby reducing the cost and time required for the assay. We studied the effect of dye dilution, pH conditions, and cell number as a part of method standardization. X-ray dose–response and repair kinetics in lymphocytes as well as cell lines were studied for validating the sensitivity of the assay. A linear dose–response relationship for DSBs was observed at a cell number of 4×10^5 cells, a dye dilution of 500-fold, and at pH 10. Repair kinetics revealed a time-dependent repair of DSBs up to 360 min of posttreatment, indicating its usefulness in DSB repair studies. In conclusion, the present modified method is more efficient (in terms of cell number), cost effective, less time-consuming, and less hazardous compared to the existing method.

Radiation-induced double strand breaks (DSBs)[1] in DNA are considered to be the most critical damage that can induce genomic instability. Repair of DNA DSBs is considered to be biologically significant when compared against other types of DNA damages. Erroneous rejoining of DSBs is a causative factor for mutagenesis, chromosomal aberrations, and tumorigenesis [1]. Therefore it is essential to quantify the induction of DSBs and to estimate the repair capacity to predict the radioresponse. Currently the most efficient methods being used for DSB analysis include pulsed field gel electrophoresis (PFGE) [2], neutral comet assay [2], γ-H2AX foci analysis [3], and to a certain extent neutral filter elution [4]. But all these methods noted here have certain advantages as well as disadvantages. PFGE is used for detection of DSBs in genomic DNA of higher molecular weight. Further, the instrumentation for PFGE is expensive and the procedure is laborious. The comet assay is a sensitive method for detecting strand breaks at the single cell level, but experimental variability and the length of time to obtain assay results are important issues to be considered for human biomonitoring studies [2]. γ-H2AX foci analysis by microscopy is by far the most sensitive technique available [5] for detecting the accumulation of phosphorylated H2AX formed due to DSB, but the disadvantages include the inability to analyze the DSBs induced at the higher end of the survival curve dose range, the need for a high-end microscope with fluorescence filters and a software for foci analysis, the time required for processing as well as image analysis make it a difficult method for damage analysis. Recently, several other investigators demonstrated the usefulness of flow cytometric analysis of H2AX phosphorylation as a measure of DSB, which is very rapid and also sensitive [6,7]. However, the instrumentation and antibodies used in this procedure are expensive. Neutral filter elution is an easy and facile procedure but requires less manipulation and has been used by many earlier investigators [8,9]. Unfortunately the method used [10] for detection of DNA DSBs using neutral filter elution includes several pitfalls such as the requirement of a large number of cells and radioactive precursors for DNA labeling, and it is more time-consuming for fragmented DNA elution using a vacuum pump. In the present study, an attempt was made to modify the existing method by adapting centrifugation according to Leanderson et al. [11] instead of the vacuum-based elution method [4]. Another important modification was the introduction of fluorescent (PG)

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[1] *Abbreviations used:* DMEM, Dulbecco-modified Eagle medium; DMSO, dimethyl sulfoxide; DSB, double strand break; PBS, phosphate-buffered saline; PG, PicoGreen; PFGE, pulse field gel electrophoresis; dsDNA, double stranded DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OTM, olive tail moment.

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doi:10.1016/j.ab.2011.03.033

dye to stain the eluted double stranded DNA instead of the conventional radiolabeled DNA precursors.

Materials and methods

Chemicals

Tris–hydrochloride, ethylenediaminetetraacetate disodium salt dihydrate (Na_2EDTA), *N*-lauroylsarcosine, Triton X-100, L-glutamine, proteinase-K, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute-1640 (RPMI), and Dulbecco-modified Eagle medium (DMEM) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was purchased from Pan-Biotech (Aidenbach, Germany). Ficoll-Paque was purchased from GE Healthcare Biosciences AB (Uppsala, Sweden). Quant-iT PicoGreen (PG) dsDNA reagent was purchased from Molecular probes, Invitrogen (Eugene, OR, USA). All other chemicals were purchased from Qualigens Fine Chemicals (Mumbai, India).

Lymphocyte isolation

In the present study, human peripheral blood lymphocytes (HPBLs) were obtained by collecting the blood from consenting healthy donors. The study was approved by the Institutional ethical committee (Kasturba Hospital Ethics Committee, Manipal) and informed consent was obtained from all the participants. The selection of donor was done according to the current guidelines of International Programme on Chemical Safety (IPCS) in humans. Peripheral blood (4–5 ml) was collected aseptically by vein puncture from fasting healthy individuals in heparinized sterile tubes (mean age 42.3 ± 8.5). Lymphocytes were isolated using Ficoll-Paque method according to manufacturer's instructions (GE Healthcare Biosciences AB).

Cell line and cultures

Human osteosarcoma cells (SaOS2) and human cervical cancer cells (SiHa) were procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown in 25 cm^2 flasks (Falcon, Becton Dickinson, USA) with loosened caps, containing DMEM supplemented with 10% FCS, 1% L-glutamine and 50 μg/ml gentamycin sulfate at 37 °C in a humidified 5% CO_2 incubator (NuAire incubator, Plymouth, MN, USA).

Irradiation

Cells were irradiated in PBS with different doses of radiation by using a 160 kV X-ray machine (Faxitron CP160, USA) at a dose rate of 1 Gy/min.

Filter assembly

The design of the microfiltration unit was originally marketed by Bioanalytical Systems (West Lafayette, IN, USA); however, as this unit was not commercially available, we constructed a similar device. The unit consists of 4 components, viz. sample compartment, rubber washer, filter support, and a sample collection tube (Fig. 1a) and are assembled as shown in the Fig. 1b. All the components of the filter assembly except the washer were made from polypropylene. The 0.8 μm polycarbonate membrane filter (Millipore, Ireland), cut into a circle of diameter 0.91 cm, was placed in the filter support and a rubber washer was placed on the membrane to avoid leakage of fluid. The lysis of cells is done in the sample compartment and the eluted fraction containing fragmented DNA is collected in the sample collection tube.

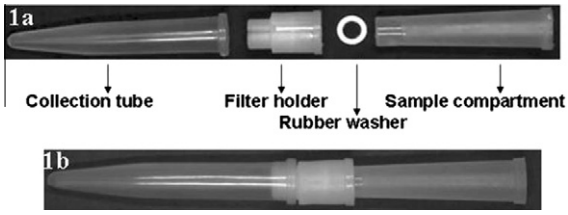

Fig.1. (a) Components of microfiltration unit; (b) an assembled microfiltration unit.

Neutral filter elution for radiation-induced DSB analysis

The filtration unit was assembled as shown (Fig. 1b). Neutral filter elution was performed using the same general principles of the standard method [4], but with several technical modifications [11]. Briefly, 4×10^5 cells treated with X-ray irradiation were taken and transferred on to polycarbonate membrane filter mounted in the filtration assembly. The filtration apparatus was placed in 15 ml conical centrifuge tubes and centrifuged at 1000*g* for 2 min. After the centrifugation, the filtrate was discarded and the cells were lysed with 300 μl of lysis reagent (0.5% Sarkosyl, 0.5% Triton X-100, 10 mM Tris–HCl, 1 mM Na_2EDTA, and 0.2 mg/ml proteinase-K, pH 10). Cells are lysed for 1 h at 55 °C and centrifuged at 30*g* for 4 min to remove the lysate. To elute the damaged double stranded DNA fractions from the membrane, 300 μl of lysis reagent was added and centrifuged after 5 min at 50*g* for 6 min. Simultaneously 4×10^5 cells were taken in a 1.5 ml centrifuge tube and lysed with 300 μl of lysing solution for 1 h at 55 °C. The initial DNA damage was analyzed 30 min after irradiation for 5 and 10 Gy and the rejoining of the DSBs was evaluated after 180 min for peripheral blood mononuclear cells and 180 and 360 min for the cell lines. The fraction of DSB containing DNA eluted was assessed in Nunc 96-well black microplates (Roskilde, Denmark) by fluorescence measurements. A 100 μl of the fraction eluted was added with 100 μl of PG (500-fold diluted) and kept for 10 min at room temperature. Fluorescence readings were taken at excitation of 480 nm and emission of 520 nm using Tecan Infinite M200 series Microplate reader (Salzburg, Austria).

Data analysis

Total fragmented DNA (in percentages) was calculated by comparing fraction of DNA eluted in relation to total DNA values. Mean values for eluted DNA (filtrate) and total DNA were taken and analyzed for DNA damage. DNA double strand breaks as neutral eluted fraction were calculated using the mean values of 2 trials,

$$\%\ \text{damaged DNA} = \frac{\text{fluorescence of the filtrate}}{\text{fluorescence of total DNA}} \times 100.$$

The statistical significance between the treatments was evaluated by one-way analysis of variance and with Bonferroni's post hoc test using GraphPAD InStat Software (USA).

Clonogenic assay

This assay was performed according to the method of Puck and Marcus [12]. A fixed number (5×10^5) of exponentially growing SaOS2 and SiHa cells were inoculated into several individual 25 cm^2 culture flasks and allowed to grow. Twenty-four hours later, these cultures were exposed to different doses (0–8 Gy) of radiation. Briefly, cells from the above groups were trypsinized and the single cell suspensions were counted using a hemocytometer and