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Broad spectrum detection of DNA damage by Repair Assisted Damage Detection (RADD)

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ABSTRACT

Environmental exposures, reactive by-products of cellular metabolism, and spontaneous deamination events result in a spectrum of DNA adducts that if un-repaired threaten genomic integrity by inducing mutations, increasing instability, and contributing to the initiation and progression of cancer. Assessment of DNA adducts in cells and tissues is critical for genotoxic and carcinogenic evaluation of chemical exposure and may provide insight into the etiology of cancer. Numerous methods to characterize the formation of DNA adducts and their retention for risk assessment have been developed. However, there are still significant drawbacks to the implementation and wide-spread use of these methods, because they often require a substantial amount of biological sample, highly specialized expertise and equipment, and depending on technique, may be limited to the detection and quantification of only a handful of DNA adducts at a time. There is a pressing need for high throughput, easy to implement assays that can assess a broad spectrum of DNA lesions, allowing for faster evaluation of chemical exposures and assessment of the retention of adducts in biological samples. Here, we describe a new methodology, Repair Assisted Damage Detection (RADD), which utilizes a DNA damage processing repair enzyme cocktail to detect and modify sites of DNA damage for a subsequent gap filling reaction that labels the DNA damage sites. This ability to detect and label a broad spectrum of DNA lesions within cells, offers a novel and easy to use tool for assessing levels of DNA damage in cells that have been exposed to environmental agents or have natural variations in DNA repair capacity.

1. Introduction

Nucleic acids are continuously subjected to modification by endogenous and exogenous sources. The formation and retention of these nucleic acid modifications or adducts can threaten the fidelity of the genome by altering the nucleic acid structure, changing base pairing and promoting the likelihood of insertions, deletions and translocations. Detection and removal of DNA damage is essential for maintaining genomic integrity and a tailored and lesion specific DNA damage response (DDR) has evolved for signaling the enzymatic recognition of DNA adducts and corrdinating repair by a suite of DNA repair pathways. Mutations in genes involved in DNA repair are linked to aging and genetic diseases, as well as cancer predisposition, and these mutations can also alter treatment outcomes [1–4]. Therefore, assessment of DNA damage formation and persistence in cells aids in the determination of the genotoxic or carcinogenic potential of chemical or environmental exposures and may identify subpopulations vulnerable to exposure effects. This potential has led to the development of assays that monitor and measure the formation and retention of DNA adducts within a genome, in order to assess the functional DNA repair capacity (reviewed in [5–7]).

Liquid chromatography and mass spectrometry have been used extensively to identify and quantify DNA adducts. These methods have allowed precise quantitation of adduct levels in purified DNA samples and have significantly advanced our understanding of the structure and lifetime of DNA adducts. However, these techniques require expert users, expensive equipment, often employ isotopic labeling for precise quantitation, and require microgram quantities of isolated DNA [6,8,9]. While there are distinct advantages to utilizing these techniques to measure specific adducts, there are issues with DNA isolation procedures introducing further DNA damage and in the standardization of measurements [10].

Abbreviations: RADD, repair assisted DNA damage detection; TUNEL, Terminal deoxynucleotidyl transferase nick end labeling; ISEL, In situ end label; DDR, DNA damage response; TdT, terminal deoxynucleotidyl transferase; dUTP, deoxyuracil triphosphate; CSK, cytoskeletal buffer; CPD, cyclyobutane pyrimidine dimer; BrdU, bromodeoxyuridine

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More accessible forms of DNA damage and adduct detection are antibody based strategies, comet assays, and enzymatic detection by terminal deoxynucleotidyl transferase (TdT). Antibody strategies can be applied to isolated DNA, in cells, or in fixed tissues. While antibodies exist for strand break signals (γH2AX or 53BP-1) and some DNA lesions (6-4 photoproducts, cyclobutane pyrimidine dimers (CPD), etc.), these techniques are limited by the variation and specificity of available antibodies and may be difficult to multiplex due to incompatibilities in fixation or staining procedures. Comet assay or Single Cell Gel Electrophoresis allows more specific strand break detection in cells, eliminating the requirements for specific antibodies, and with modifications can detect alkali labile sites, oxidative base damage, and DNA cross-linking [11,12]. However, comet assay has been difficult to standardize and reproduce from lab to lab, though comet chip technologies and automated image processes are improving these shortcomings [13–15].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and in situ (DNA) end labeling (ISEL) [16–18] have also been employed extensively over the past 20 years to detect DNA strand breaks during apoptosis and in some cases DNA damage across a variety of biological samples [19–21]. However, just like the other methods, there are drawbacks to using TUNEL or ISEL because they are highly specific for 3′-OH ends. Several TUNEL modifications have emerged extending its ability to detect other types DNA ends (i.e., 3′-PO_4) or improve DNA damage detection by incorporating FPG to excise oxidative DNA adducts [22].

While all of these techniques are used extensively in the literature to assess DNA damage and adduct formation, each has significant limitations for broad spectrum detection of DNA damage. This gap in methodologies has led us to develop the Repair Assisted Damage Detection (RADD) assay, which harness the action of specific DNA repair enzymes to recognize and excise DNA adducts throughout the genome. Once the DNA adduct has been removed, the adduct position is tagged by insertion of a biotinylated deoxyuridine triphosphate (dUTP). This method has proven viable for detecting DNA lesions on isolated DNA [23], and here we demonstrate for the first time that this detection scheme can be extended to fixed cells to measure DNA damage *in situ*.

The assay provides a novel platform for the characterization of nuclear DNA damage within and across different cell lines by scoring the DNA lesion load. The experiments outlined herein demonstrate that RADD is a robust and novel assay for the measure of nuclear DNA damage and has the potential to be used to investigate specific DNA repair mechanisms, to address risk assessment for both environmental toxicology and cancer etiology, and to evaluate DNA targeted cancer therapies.

2. Material and methods

2.1. Cell culture

A375P cells were purchased from the American Type Culture Collection (ATCC CRL-3224) and maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose (Hyclone #SH30022.01) and supplemented with 10% fetal bovine serum (Atlanta Biologicals # S11550) and 1% sodium pyruvate (Gibco # 11360-070). Chinese hamster ovary (CHO-K1) cells were received from Dr. Samuel H. Wilson at the National Institute of Environmental Health Sciences and grown in minimal essential medium (MEM, Hyclone #SH30265FS) supplemented with 10% FBS. All cells were maintained in a 5% CO_2 incubator at 37 °C and fewer than 10 cell passages were utilized experimentally. Mycoplasma testing was regularly performed using Lonza MycoAlert® and no contamination was detected.

2.2. Cytotoxicity

Cytotoxicity was determined by growth inhibition assay. A375P cells were plated at a density of 4×10^4 cells per well in a 6-well plate (Greiner Bio-One #657165) or 35 mm dish (Falcon #353001) and treated the following day. Prior to DNA damage the cells are washed with Dulbecco's phosphate buffered saline (PBS, Cellgro #21-031-CV) or Hanks' balanced salt solution (HBSS, Hyclone #SH30031.02) for UV and $KBrO_3$ damage, respectively. Cells were either exposed to a specified dose of UVC (254 nm) using the Spectroline® Spectrolinker XL-1000, or exposed to potassium bromate ($KBrO_3$, Sigma Aldrich #309087) diluted in media for 1 h. Following the damage induction cells were washed one time in HBSS, and growth media was replaced. Cells were maintained in a 5% CO_2 incubator at 37 °C until untreated control cells reached approximately 90% confluency, typically 4-5 days. The cells were then briefly treated with 0.25% Trypsin (Life technologies #25200-056), resuspended in 1 mL of PBS, and counted with Bio-Rad TC20 automated cell counter. Results are presented as the ratio of the number of cells in treated well to cells in control well (% control survival) with error bars representing the standard error of the mean (SEM).

2.3. Repair Assisted Damage Detection

A375P cells were plated at 2.5×10^6 in a 6-well plate with coverslips (VWR #48366-227) or 35 mm glass fluorodishes (World Precision Instruments #FD35-100). The following day the DNA damage was induced as described in the cytotoxicity methods with the UV and $KBrO_3$ doses indicated, and cells were either immediately processed for damage detection or allowed to repair for the indicated times. At the indicated time, cells were washed twice with PBS and incubated with cytoskeletal buffer (CSK, 100 mM NaCl (Fisher Scientific #7647-14-5), 300 mM sucrose (VWR #0335-1KG), 10 mM PIPES pH 6.8 (Amresco #108321-27-3), 3 mM $MgCl_2$ (Amresco #7786-30-3), 0.5% Triton X-100 (Sigma-Aldrich #T8787)) on ice for 5 min, followed by three washes with PBS. The samples were then incubated with 2% formaldehyde (Amresco #M134) in PBS for 10 min at room temperature (~23° C) and washed three times with PBS. They were next treated with 0.25% Triton X-100 (Sigma-Aldrich #T8787) in PBS for 10 min again at room temperature and washed twice with PBS, followed by one wash with sterile deionized H_2O. The DNA damage processing mix contains enzymes purchased from NEB (UDG #M0280S, Fapy-DNA glycosylase # M0240S, T4PDG #M0308S, Endo IV # M0304S, Endo VIII # M0299S) and prepared in 1 x Thermpol buffer (NEB # B9004S) and incubated at 37 °C in a humidified incubator for one hour. Next, the gap filling mix, again prepared in 1 x Thermpol buffer, is added to the DNA damage processing mix, and incubated for an additional hour at 37° C. The RADD enzymes and their functions are outlined in Table 1 and the sequential DNA damage processing and gap filling reactions are outlined in Table 2. The enzyme cocktails were then washed with 1% bovine serum albumin (BSA, Jackson ImmunoResearch # 001-000-162) in PBS three times and blocked with 5% goat serum (Invitrogen #31873) in PBS for 30 min at room temperature. The blocking serum was then aspirated, and the goat anti-biotin FITC conjugated antibody (Sigma-Aldrich #F6762) is diluted 1:400 in 5% goat serum in PBS and incubated at room temperature for 1 h, protected from light. The cells were washed three times with 1% BSA (Jackson ImmunoReseach #001-000-162) in PBS, dried briefly, and mounted in Prolong® Gold with DAPI (Life Technologies #P36931) following the manufacturer's instructions.

2.4. Laser micro-irradiation

Laser micro-irradiation was performed as previously described [24]. Briefly, CHO-K1 cells were plated at 3×10^4 cells per chamber in an 8 chamber slide (Nunc LabTek II, ThermoFisher # 12-565-338) and sensitized with 10 μM bromodeoxyuridine (BrdU, Sigma Aldrich #B5002) for 24 h prior to micro-irradiation. During micro-irradiation, cells were placed in a microscope stage incubator and maintained at 37°