Approach for assessing total cellular DNA damage

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The capability to relate phenotypic effects to damage associated with either the mitochondrial or nuclear genome is especially useful under a number of circumstances. Potential hazardous exposures can be evaluated for genotoxicity and related to diseases, particularly cancer. The correlation of DNA damage with adverse health effects is also important in evaluating the safety of various chemical agents and prospective therapeutics. Many techniques exist that afford the ability to identify and measure cellular DNA damage upon exposure to a suspected genotoxic agent; however, quite often these techniques are limited either by the advanced instrumentation and skill needed to perform the analyses or the amount of time needed and limited information obtained regarding the types of DNA damage generated. Recent advances in cellular-based methods have resulted in the timely and straightforward collection of reliable and specific data regarding levels of damage and the identity of the damage products. Antibodies developed for DNA damage lesions allow for the direct measurement of those lesions within a population of exposed cells, while the automation of the single-cell gel electrophoresis (comet) assay and the use of scoring software have led to rapid and standardized data collection. This essay describes the usefulness of these approaches, while providing a brief experimental overview of the techniques.

Introduction

Many endogenous and environmental factors lead to the damage and degradation of cellular components. The presence of damaged proteins, lipids, and DNA is correlated with a variety of disease states and the aging process, demonstrating that the disruption of cellular function through the accumulation of damage products interferes with physiological function. Damage to DNA is especially harmful due to the mutations that can result if left unrepaired. Consequently, cells have developed systemic defense mechanisms that link the process for repairing DNA damage to both cell cycle regulation and controlled cell death (1–6). Cells experiencing oxidative stress conditions demonstrate an increase in the expression of the genes involved in DNA repair (7–9). However, once the capacity of the repair pathways is exceeded, significant problems arise. Generally, low to moderate levels of damage are countered by enzymes of the base excision repair (BAR) pathways, while high levels of damage to cellular components, including DNA, elicit an apoptotic response. High levels of DNA damage have been correlated with aging (6), type II diabetes (7,8), carcinogenesis (10,11), and autoimmune diseases (12). In each case, results have been reported that associate low levels of DNA repair enzymes, cellular anti-oxidants, and altered gene expression as consequences of oxidative stress and DNA damage. For these reasons, the ability to associate levels of DNA damage with deleterious effects is of particular interest in the medical community. Information from these measurements could potentially be developed for quantifying the level of genotoxicity induced by environmental toxins, and carcinogens, or perhaps to diagnose the early stages of disease.

Somatic human cells possess two separate genomes that are packaged and housed separately. Nuclear DNA is present as a single copy and is packaged with proteins that both condense and protect the genome. Damage to the nuclear genome is perceived to be more deleterious as there is only one copy. The circular 16.6-kb mitochondrial genome (mtDNA) is found free within the mitochondria. Due to the oxidative environment of the inner mitochondria, damage occurs frequently. This effect is partially countered by the presence of hundreds to thousands of copies of the genome in each mitochondrion. Current work has begun to use damage and mutations within mitochondrial DNA as cancer biomarkers (13). The capacity to assess damage within the two genomes and link that damage to an array of phenotypic effects would be very useful.

Potential hazardous exposures [e.g., smoking (14), pesticides (15), ultraviolet (UV)-light (16)] can be evaluated for genotoxicity and related to carcinogenesis. This association is also particularly important in determining the safety of various chemical agents and prospective therapeutic pharmaceuticals. Many techniques exist that afford the ability to identify and measure cellular DNA damage upon exposure to a suspected genotoxic agent (17). These methods include micronuclei (18), chromosome aberration test (19), unscheduled DNA synthesis (20), and the bacterial mutation test (21), each of which may be used in regulatory evaluations of potential genotoxic agents. These methods, although reliable, are often limited in their tissue

application, require large sample volume, and in some cases, offer only partial data regarding primary DNA lesions. Often, the existing genotoxicity tests are limited to evaluating damage to nuclear DNA or the cell as a whole. However, in order to thoroughly understand genotoxic effects, damage to each genome should be evaluated separately.

Recent advances in cellular-based methods have resulted in the timely collection of reliable and specific data regarding levels of damage and the identity of the damage products. Antibodies developed for specific DNA damage lesions now allow for the direct measurement of those lesions within a population of exposed cells. The automation of the single-cell gel electrophoresis (comet) assay and the use of scoring software have also led to rapid data collection. Developments within the comet assay methodology have led to its acceptance and utilization by health authorities, who regard it as at least equivalent to existing techniques (i.e., micronuclei, chromosome aberration test) with regulatory acceptance (22). Under similar experimental conditions, it has also been demonstrated that the levels of DNA damage measured using the comet assay are comparable with those observed using high-performance liquid chromatography (HPLC) analyses (23).

Recent work in the Kelley laboratory has involved cell penetrating peptidoconjugates featuring a peptide portion and a DNA-intercalating fluorescent dye, thiazole orange (TO) (Reference 24 and unpublished data). Alterations in the peptide sequence attached to TO affords the localization of these compounds to the nucleus and/or the mitochondria (unpublished data). Upon photoexcitation, the TO portion of the compounds is capable of generating singlet oxygen ($^1O_2$), a reactive oxygen species known to damage DNA (25,26). Photoexcitation of these compounds led to considerable cytotoxicity; however this effect can be attributed to a range of biological factors, including, but not limited to, DNA damage.

Previous work involving our TO-peptidoconjugates has demonstrated their capability to damage synthetic oligonucleotides and plasmid DNA (27,28). Given the toxicity of the compounds in cellular studies, we enlisted the techniques described here to determine if similar damage occurs within cells. The rapid data collection, simple instrumentation, inexpensive cost, straightforward methods, and breadth of information concerning damage to both the nuclear and mitochondrial genome provided reason for their use in our studies. The work described here was performed to demonstrate a correlation between cytotoxicity and genome-specific DNA damage.

Measuring Nuclear DNA Damage Using the Comet Assay

The comet assay is a relatively versatile, sensitive, and simple test applied to genotoxicity studies. The advantages of this particular technique include its application to various tissues or cell types and the low cost and short time needed to perform the measurement. One particular drawback of the method is the experimental variability. In recent years, automated data collection and analysis, along with efforts to generate a common comet assay protocol (29), have led to refined guidelines for data collection and analysis.

Recently, a comet assay protocol has been published that thoroughly describes the experimental guidelines mentioned above (30). Several alternate methods with slight variations in experimental conditions or additional steps involving DNA repair enzymes or nucleases may also be applied in the assay to monitor particular types of damage (31). Through varying a parameter such as the pH of either buffer used in the assay, one may measure a range of damage products including double-strand breaks, single-strand breaks, oxidized bases, and abasic sites. The differential measurement of these lesions is particularly important, because the cell is not equally efficient in repairing these products. Therefore, the capacity to identify different types of DNA damage when measuring genotoxicity is advantageous, since it provides insight into the ability of a cell to repair the damage generated. More explicit types of damage are also measurable through the implementation of repair enzymes. Specifically, nucleases such as formamidopyrimidine-DNA glycosylase will recognize formamidopyrimidine and 8-oxo-guanine (8oxoG) moieties and generate strand scission at that site. Use of such enzymes allows for further visualization of specific damage products. Optional variations of this assay and repair enzymes that have been successfully applied are highlighted in Table 1.

In general, the most widely applied version of this assay takes advantage of the relative instability of DNA damage products under alkaline conditions. By performing electrophoresis at pH > 13.0, base oxidation and direct strand breaks are visualized through the denaturation of DNA followed by the separation of fragmented (i.e., damaged) portions from those which are intact. Separation is based upon the electrophoretic mobility of DNA within the solid matrix causing the smaller, more mobile, damaged DNA to appear as a trailing tail from the condensed undamaged DNA (hence the term comet assay). Levels of DNA within the tail are directly proportional to the degree of nuclear DNA damage.

The comet assay was utilized to evaluate the presence of DNA damage induced by intracellular localization of oxidative stress generated by our cytotoxic conjugate molecules. Cell-penetrating peptidoconjugates bearing TO—a DNA binding intercalative fluorophore capable of generating the reactive oxygen specie $^1O_2$ upon photoexcitation—were evaluated for their capacity to damage nuclear DNA. Cells were incubated in the dark with 5 μM

Table 1. Variations of the Comet Assay

	pH 7.0	pH 12.3	pH > 13.0	DNA Repair Assay[a]
DNA Damage Monitored	Double strand breaks	Double strand breaks Single strand breaks Delayed repair sites	Double strand breaks Single strand breaks Delayed repair sites Alkali labile sites	Strand breaks Oxidized bases Abasic sites Modified bases Mismatches
Lysing Buffer	EDTA (30 M) SDS (0.5%–2.5%) Proteinase K 10 mg/mL >1 h	NaCL (1.0 mM) NaOH (30 mM) 0.5% N-laurylsarcosine 2 mM EDTA >1 h	EDTA (100 mM) NaCl (2.5 M) Tris (10 M), pH 10.0 N-laurylsarcosine 1% Triton® X-100 Proteinase K 10 mg/mL >1 h	EDTA (100 mM) NaCl (2.5 M) Tris (10 mM), pH 10.0 N-laurylsarcosine 1% Triton X-100 >1 h
Unwinding Buffer	Acetic acid (90 mM) EDTA (2–5) mM Tris (40–120) mM pH 7.0 Duration: (2–17) h	EDTA (1–2) mM NaOH (30 mM) pH 12.3 Duration: 1 h	EDTA (1 mM) NaOH (300 mM) pH > 13.0 Duration: 20–60 min	No unwinding necessary. Alternatively, place in trough containing buffer at 4°C for 5 min. Treat with enzyme two times at 37°C for 5 min. Stop reaction with DMSO at 4°C.
Electrophoresis	0.5–0.57 V/cm 25 min	0.5–0.67 V/cm 25 min	0.8–1.5 V/cm 25 V/300 mA (10–60) min	20 V for 24 min 300 mM NaOH 1 mM EDTA

Variations of the comet assay used to measure specific types of DNA damage or repair. Conditions for each of the steps involved in the comet assay are listed. SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.
[a]Enzymes used in this assay include, but are not limited to, endonucleases III, IV, V, VIII, exonuclease III, Fgp protein, uracil-N-glycosylase, MutY DNA glycosylase, TDG enzyme, 3-mA-DNA glycosylase I & II, and T4 endonuclease 4.

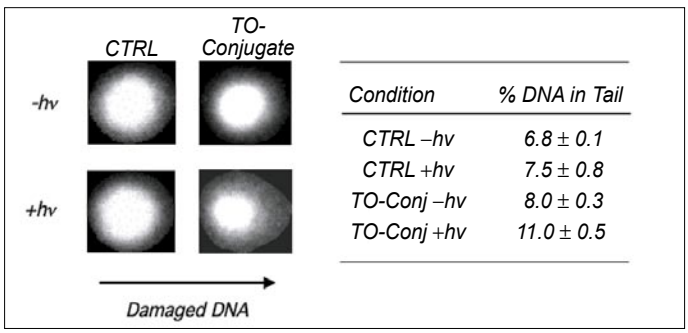
conjugate for 90 min in culture media to allow for uptake. Cells were then irradiated in a darkroom at the λ_{max} of TO (501 nm) for 20 min using an Oriel Spectral Illuminator (150 W Xe, power = 1.8 mW/cm^2; Newport Corporation, Irvine, CA, USA) and immediately processed for analysis via the comet assay as described above. Several controls were prepared under identical conditions, with either the lack of light or conjugate.

Data collection and analysis was performed using a fully integrated computer-controlled optical microscopy system (AutoComet™ III; TriTek, Sumerduck, VA, USA) and software specific for comet scoring (AutoComet freeware v1.5; TriTek). The automation of this process permitted rapid data collection, yielding a range of 300–1000 comets scored per sample, per experiment. This method also removed any scorer-related bias from the experimental results.

Results of the comet assay confirmed the generation of significant oxidative DNA damage within the nucleus via photoexcitation of the cell-penetrating compound (Figure 1). While there are several values in the literature used to denote damage levels, our values are reported as %DNA in tail ($\%DNA_T$) due to its standard acceptance within the community. This value is obtained using the following formula,

$$\%DNA_T = (I_T \div I_C) \times 100$$

where I_T is the intensity of the total number of pixels in the tail of the comet, and I_C represents the total pixel intensity of the comet as a whole. Our results denote a significant increase in the level of DNA in the tail for the irradiated sample that was incubated with the cytotoxic compound. All other conditions (i.e., compound and no light, no compound and light) exhibit no significant increase in DNA damage relative to the baseline represented by the damage within untreated cells. The level of damage observed is



Condition	% DNA in Tail
CTRL –hv	6.8 ± 0.1
CTRL +hv	7.5 ± 0.8
TO-Conj –hv	8.0 ± 0.3
TO-Conj +hv	11.0 ± 0.5

Figure 1. Nuclear DNA damage as assessed by the comet assay. (A) Images depicting the common morphology of cells measured for levels of DNA damage under the specific conditions listed (CTRL, control). The greatest level of fragmented DNA is observed in samples treated with the localized, singlet oxygen generating, thiazole orange (TO)-conjugate and light at 501 nm. (B) Average values of %DNA in tail under each condition listed. Numeric value was determined by calculating the amount of fluorescence in the tail of a given cell divided by the total fluorescence of that cell. Measurements were calculated using AutoComet software and represent three trials, with at least 300 cells scored per trial. Only comets within one standard deviation of the mean were scored per experiment. Error represents standard deviation between trials.

modest in comparison to reported values for other genotoxic agents (23). However, relative to the short exposure time for the assay, the values are significant and correlate well with the cytotoxic effects observed when irradiated at the λ_{max} of TO (Reference 24 and unpublished data). The significance of our results refers to the standard deviation of the mean, which is the same as the combined standard uncertainty of the mean for the purposes of this work. Thus, results of the comet assay suggest that the cytotoxicity of our compounds may be attributed, in part, to a genotoxic effect.

Our results demonstrate the advantageous aspects of the comet assay. By means of this assay, we processed a large number of cells in each experiment, increasing the level of certainty in our results. The approach also provides information on damage among the total population of cells, while imparting the capacity to monitor the distribution of damage from cell to cell. Alternatively, other methods, including HPLC and gas chromatography/mass spectrometry (GC/MS), were not successful in measuring cellular DNA damage. These techniques rely upon isolation and processing of cellular DNA, which may have led to the loss of the unstable damage products to be measured. In using the comet assay, this experimental step is removed, reducing the level of sample processing. For our system, this resulted in the quantitative measurement of nuclear DNA damage.

Mitochondrial DNA Damage Visualized via Anti-8oxoG Staining

The comet assay is a very useful method in evaluating the extent of nuclear DNA damage within a cell. However, this approach is not optimal for providing information concerning the damage produced within the mitochondrial genome. Therefore, one must exploit a separate assay in the measurement of mtDNA damage. Several alternate methods can be used in accessing damage through first isolating the mtDNA and then measuring damage using assays such as quantitative PCR (32) or base composition analysis using liquid chromatography coupled to a mass spectrometer (33). These techniques, although informative, are laborious and time-consuming due to the initial isolation of either the mitochondria or the mtDNA. We chose to implement an immunochemical approach in our studies, which allows for the measurement of mtDNA damage in situ and without initial separation from the cellular components. This approach greatly reduces sample processing, which decreases the potential for DNA damage to be generated or products lost through the analytical process itself.

In recent years, the use of selection techniques has led to the identification of unique antibodies chosen for binding against specific targets of interest (34). One particular set of targets includes DNA damage lesions. Several commercially available antibodies selected or developed for binding against a number of common DNA damage products [e.g., 8oxoG, (6-4)-dipyrimidine photoproducts, benzopyrene-diol epoxide-DNA] have been used for the measurement of these lesions within biological samples (35–37). The high binding affinity and specificity of these antibodies provide the capacity to quantitate individual types of damage products within a sample using microscopy or enzyme-linked immunosorbent assay (ELISA). Tissue or cellular localization of these damage products may be determined in a qualitative manner using microscopy, while more quantitative data can be generated with ELISA or automated image analysis software.

The presence of elevated levels of oxidative damage to the mitochondrial genome was investigated through utilizing the specificity of a designed monoclonal antibody for the recognition of 8oxoG along with a fluorophore-labeled immunoglobulin to visualize localization of damage (34–36). Anti-8oxoG was used to stain fixed cells that had been treated with a mitochondrial localizing TO-peptide conjugate. Under the oxidative conditions of our assay, the low oxidation potential of guanine—in relation to the remainder of the DNA structure—would presumably lead to elevated levels of 8oxoG. It is for this reason that anti-8oxoG was chosen for our studies.

Briefly, cells were incubated with the conjugate molecules as they had been when performing the comet assay. Samples were either incubated in the dark or subjected to confined oxidative stress through irradiation at 501 nm. After irradiation, mitochondria were fluorescently stained with 300 nM MitoTracker 588 (Invitrogen, Carlsbad, CA, USA) for 20 min. All cells were then immediately fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton® X-100 for 10 min at room temperature and treated with 100 µg/mL RNase A for 1 h at 37°C to remove cellular RNA. Proteinase K (10 µg/mL for 10 min at room temperature) was used to digest cellular proteins and liberate bound DNA. A brief treatment with 2 M HCl for 5 min, followed by neutralization with 1 M Tris-base allowed for the relaxation of cellular DNA. These preceding steps ensure the availability of DNA for antibody binding. Staining for 8oxoG was completed by incubation with 4 µg/mL anti-8-oxo-dG monoclonal antibody (Trevigen, Gaithersburg, MD, USA) overnight in 0.1% bovine serum albumin (BSA), after initially blocking with 2% BSA. Cellular localization of 8oxoG was visualized by staining with 10 µg/mL Alexa Fluor® 633 goat anti-mouse immunoglobulin G (IgG) (Molecular Probes; Invitrogen) in 2% BSA for 2 h at room temperature.

Confocal microscopy confirmed the presence of elevated levels of 8oxoG within the mitochondria for those cells irradiated in the presence of the cell penetrating TO-peptide conjugate, while minimal levels of 8oxoG are visible in the mitochondria of cells treated with conjugate but without

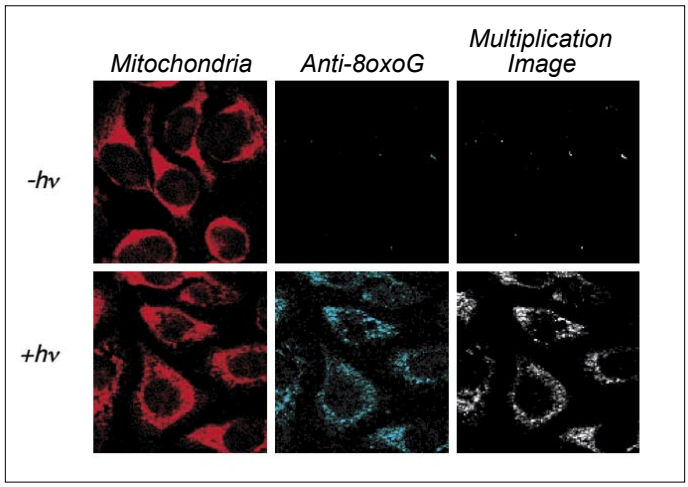


Figure 2. Confocal microscopy images of cells incubated with thiazole orange (TO)-peptidoconjugate for 90 min prior to treatment. Cells were either incubated in the dark or irradiated with 501 nm light for 10 min (1.8 mW/cm^2). Mitochondria of cells were stained with MitoTracker 588 prior to fixation (left), followed by staining with an anti-8-oxo-guanine (8oxoG) monoclonal antibody and secondary Alexa Fluor 633 goat anti-mouse immunoglobulin G (IgG) (middle). Co-localization of mitochondrial and 8oxoG staining is represented as a multiplication image at right.

irradiation (Figure 2). Confirmation of 8oxoG presence in mitochondria was obtained by staining the mitochondria with MitoTracker 588. Using ImageJ software, the pixel intensity and location of the anti-8oxoG and Mitotracker were multiplied in each image to provide verification and information regarding the certainty with which these dyes co-localized. These results support the hypothesis that the cytotoxic effects of our mitochondria-penetrating conjugates are associated with an increased occurrence of DNA damage within that genome.

Common cellular DNA damage methods monitor the effects of suspected genotoxic agents on nuclear DNA only. The immunochemical approach detailed here provides a general means by which to measure mtDNA damage in parallel to that generated in the nucleus. Although damage to the nuclear genome is perceived to be more deleterious than those within the mitochondrial genome, many mutations within the mitochondrial genome have been associated with disease (38). For this reason, it is imperative that mtDNA damage be considered when measuring genotoxicity. The immunochemical method does not require the separation of mitochondria from cellular components, thereby reducing the sample preparation and potential for the loss of damage products or introduction of additional DNA damage after exposure to the suspected genotoxin.

Summary

Many techniques are currently implemented in the measurement and assessment of cellular genotoxicity. Often times these tests suffer from a number of shortcomings or drawbacks including a lack of versatility with respects to damage products or tissue types and time-consuming experimental procedures. Efforts of late have lead to the enhancement of the well-documented comet assay and have also generated a number of very specific antibodies for DNA damage markers. These advances have provided simple means by which to visualize and measure DNA damage by a number of straightforward methods, which have become more popular within the medical and regulatory communities. As a result, publications involving these techniques have risen, while commercially available products incorporating the technology have become more readily available. Although the use of these methods has become more commonplace, the implementation of several procedures in measuring DNA damage will provide conclusive evidence regarding levels of genotoxicity. The purpose of this essay is not to suggest that the techniques highlighted are superior to others, but rather to generate insight into the strengths of the methods, how they are executed, and the information obtained. However, the methods described provide an example of a versatile approach to evaluate genotoxicity in a fast and inexpensive manner.

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