The Bromodeoxyuridine Comet Assay: Detection of Maturation of Recently Replicated DNA in Individual Cells


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Abstract

The single-cell gel electrophoresis (Comet) assay is a relatively simple method of measuring DNA single strand breaks and alkali-labile sites in individual cells. We have combined this with bromodeoxyuridine (BrdUrd) labeling of DNA and immunolocalization of the BrdUrd to assess DNA replicative integrity on a single-cell basis. We show that the existence of strand discontinuities in recently replicated domains of DNA, caused during semiconservative replication or exacerbated by the arrest of replicative polymerases at UV irradiation- or chemical-induced lesions, can be detected in individual cells. Data obtained from BrdUrd-Comet assays are consistent with biochemical data derived with a range of techniques showing that DNA replication involves the creation of strand breaks or gaps adjacent to recently replicated material, and that DNA damage prolongs the duration of such discontinuities where DNA polymerases are stalled opposite lesions (R. T. Johnson et al., The Legacy of Cell Fusion, pp. 50–67, Oxford: Science Publications, 1994; R. B. Painter, J. Mol. Biol., 143: 289–301, 1980.). Compared with standard biochemical techniques, the BrdUrd-Comet assay is simple and suitable for the accurate and automatable assessment of replicative integrity in very small numbers of mammalian cells, such as may be obtained by biopsy.

Introduction

In DNA replication, strand discontinuities inevitably occur at the leading and lagging strand growth points. In normal replication, DNA polymerases rapidly move on. Label incorporated into DNA during a brief pulse is initially close to strand discontinuities, but during pulse-chase experiments, such label is soon incorporated into high molecular weight, continuous DNA, far distant from any discontinuities. The process by which this occurs is known as DNA maturation. After DNA damage, replication is affected. DNA polymerases initially stall at noncoding damage sites (1–3). Through the process of PRR, polymerases bypass lesions or avoid them through retrospective gap filling (4). One striking case of deficiency in DNA maturation is the Indian muntjac line SVM84, which (compared with normal DM87 Indian muntjac cells) are hypersensitive to UV irradiation and are deficient, although not entirely defective, in UV PRR capacity (5). In DM87 cells, the PRR capacity is largely caffeine sensitive, whereas in SVM 84 cells, the residual PRR is caffeine insensitive (6).

PRR in these cases, and in other mammalian cells, has normally been studied by biochemical methods; [1H]thyidine pulse-labeling, followed by alkaline sucrose gradient centrifugation (7), or by chromatography on BND-cellulose or hydroxyapatite (8). These techniques, however, are time consuming and require both radioactive labeling of DNA and the use of large cell populations (~10^7 per data point) for which only an average figure can be obtained.

An alternative method for measuring DNA strand breaks and alkali-labile sites in single cells, the Comet assay, has been in use for some years (9, 10). In this assay, single cells are embedded in agarose on frosted slides, lysed in denaturing buffer, subjected to brief electrophoresis, stained with DNA intercalating dye, and observed with fluorescence microscopy. Undamaged DNA is unable to enter the agarose gel and is retained in the cavity formed by the lysed cell; damaged DNA streams down the electrophoretic field and forms the “tail” of a Comet. Software packages have been devised to quantitate the distribution of DNA between damaged Comet tails and undamaged Comet “heads.” Modifications of the Comet assay have been developed to detect more complex DNA lesions, provided they are endonuclease sensitive (11). More recently, we have combined the Comet assay with fluorescence in situ hybridization to allow detection of damage within defined gene sequences (12).

We have combined this method with pulse-labeling of replicating DNA with the thymidine analogue BrdUrd and immunological localization of BrdUrd within the heads and tails of Comets to assess replicative integrity on a single-cell basis. The BrdUrd-Comet assay is described using UV-irradiated or caffeine-treated muntjac fibroblasts, DM87 and SVM84, and human T lymphocytes.

Materials and Methods

Isolation and Growth of Human T Lymphocytes. Lymphocytes were extracted from whole blood according to the method described previously (13). For all experiments using human T lymphocytes, the Raji (TK^−) Burkitt’s lymphoma cell line (14) was included as a control and maintained in exponential growth in RPMI 1640 supplemented with 15% fetal bovine serum and antibiotics.

Fibroblast Growth and Irradiation. The Indian muntjac fibroblast cell lines, DM87 (American Type Culture Collection, MD) and SVM84 (a gift from Professor K. Sperling, Berlin, Germany) were plated at a density of 1 × 10^4 cells/60-mm Petri dish in complete medium as described (5) and grown for 48 h to ensure exponential growth prior to irradiation. Cells were exposed to UV irradiation (254 nm) using 2 J/m^2 and incubated in complete medium at 37°C for 1 h to recover prior to BrdUrd pulsing. Caffeine (Sigma, Dorset, United Kingdom) was used at a concentration of 2 mM and was added 30 min before UV irradiation, where indicated.

BrdUrd Labeling and Processing of Cells. Medium was removed from exponentially growing cells (with or without irradiation) and replaced with fresh medium containing 100 μM BrdUrd (Sigma) at 37°C for 15 min. Medium containing BrdUrd was washed off with PBS (pH 7.4); cells were either processed for the Comet assay or, in chase experiments, given medium containing each of 200 μM thymidine, 2′-deoxyctydine, 2′-deoxyguanosine, and 2′-deoxycadenosine (Sigma). Fibroblasts were harvested by brief trypsinization, and lymphocytes were harvested by centrifugation, both were resuspended in ice-cold PBS at 5 × 10^7 to 1 × 10^8 cells/mL, respectively. Cells were washed in cold PBS, and the cell pellet was resuspended in 100 μl of 0.25% low melting point agarose at 37°C. The cell suspension was spread onto a standard Comet assay slide (15), and the slides were immersed in a neutral lysis buffer, adapted from that described by Blin and Stafford (16): 2.5 M LiCl,
0.03 M EDTA, 10 mM Tris, and 0.1% LiDS (pH 8.0), prewarmed to 37°C before the addition of 0.03 mg/ml proteinase K. Slides were incubated at 37°C overnight for protein digestion and cell lysis. This lysis solution was used because of the superior solubility of LiDS compared with SDS. Slides were then transferred to alkaline lysis solution (2.5 mM NaCl, 100 mM Na2EDTA, and 10 mM Tris, pH 10) for 1 h at 4°C.

**Comet Electrophoresis and Staining.** Slides were removed from lysis solution, drained, and placed in a horizontal electrophoresis tank, side by side with gaps filled with blank slides. The tank was filled with fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) to a level just covering the slides, and the DNA was left to unwind for 40 min before electrophoresis. Electrophoresis was conducted for 20 min at 25 V and 300 mA. After electrophoresis, the slides were drained and flooded with three changes of neutralization buffer (0.4 M Tris), followed by one wash in PBS to allow removal of alkali and detergents that would interfere with immunostaining. The incorporated BrdUrd was immunolocalized by adding 25 μl/gel of mouse monoclonal anti-BrdUrd (10 μg/ml; Boehringer Mannheim) to the gels, with coverslips (18 × 18-mm) applied on top of the gels, allowing the antibody to spread evenly. Slides were incubated in the dark at room temperature for 1 h. After removal of the coverslips, the primary antibody was gently washed off with three changes of PBS and one wash with PBS/0.1% BSA before addition of 25 μl/gel of secondary antibody (5 μg/ml sheep anti-mouse IgG, fluorescein conjugated; Boehringer Mannheim), which was incubated and washed off as before. The gels were then counterstained with 25 μl of propidium iodide (0.75 μg/ml; Sigma) and covered with a coverslip (22 × 50-mm) for image analysis.

**Comet Analysis.** Observations were made at a final magnification of ×400 (Nikon ×40 Fluor lens) using an epifluorescence microscope (Olympus BH2) equipped with an Hitachi KP571 CCD camera interfaced through a Matrox IP8 board, using Hewlett Packard Super VGA and Komet 3.0 software (Kinetic Imaging Ltd., Liverpool, United Kingdom). Propidium iodide was visualized using a Chroma filter at excitation 540 nm and emission 635 nm. Fluorescein was visualized using a Chroma filter at excitation 480 nm and emission 535 nm. Densitometric and geometric parameters of each Comet were calculated and recorded for statistical analysis using Microsoft Excel, version 5.0, software.

The percentage of DNA in the Comet tail for each cell, which is indicative of the number of replicons in the replicating DNA, was measured. Results were expressed as the distribution of the percentage of Comet tail DNA in 25 cells on each Comet slide. The incorporation of the BrdUrd label into the Comet head after the chase period is indicative of DNA maturation and is represented by a reduction in percentage of tail DNA for each of 25 Comets.

**Results.**

**BrdUrd-Comet Assay Development.** Several improvements of the initially tested assay were made by altering lysis and staining conditions. The standard Comet assay for the detection of DNA strand breaks is carried out under alkaline conditions (15); however, use of neutral lysis facilitated nuclear matrix protein digestion using proteinase K at 37°C. This improvement to the technique, which is not normally used in the alkaline Comet assay, greatly improved cell lysis and BrdUrd-Comet formation (Fig. 1). In addition, the use of LiCl and LiDS in place of NaCl and SDS, commonly used for cell lysis, also provided greater cell lysis because of the greater solubility of LiDS compared with SDS; in neutral lysis, SDS was found to precipitate at 37°C. Comet detection was improved with the application of a second-stage antibody to detect BrdUrd-labeled DNA. The resulting Comets, using the improved protocol, displayed greater movement of DNA strands through the agarose gel with greater differentiation between Comet head and tail.

**Calibration of Assay Against Fibroblasts of Known PRR Propensity.** Treated and control SVM84 and DM87 fibroblasts were pulsed with BrdUrd for a short time and either processed directly for the BrdUrd comet assay, or the incorporated BrdUrd was chased with normal nucleotides for a period of 1 h.

After UV irradiation (2 J/m²) of SVM84 cells, the amount of BrdUrd-labeled DNA moving into the Comet head was reduced compared with the nonirradiated cells. No further effect was seen in the presence of caffeine (Fig. 2). These results confirm the maturation deficiency of UV-irradiated SVM84 fibroblasts (5).

In the nonirradiated DM87 cells, the level of Comet tail BrdUrd-labeled DNA was reduced in the DM87 cells after 1-h chase. This competency in DNA maturation is similar, regardless of the presence of caffeine in the nonirradiated DM87 cell line (Fig. 3, nonirradiated cells).

In the absence of caffeine, UV irradiation alone (2 J/m²) induced an increase in the percentage of DNA in the BrdUrd-Comet tail of DM87 cells (Fig. 3, irradiated cells). However, after a 1-h chase period with normal nucleotides, there is a retardation of maturation in the presence of caffeine compared with cells exposed to UV irradiation alone in the absence of caffeine (Fig. 3, irradiated cells). These results confirm the reduced efficiency of DNA maturation in UV-irradiated, but not nonirradiated, DM87 cells exposed to caffeine (6).

**PRR after Low-Dose Hydrogen Peroxide Detected by the BrdUrd Comet Assay in Human T Lymphocytes.** UV irradiation produces large helix-distorting lesions (17); we have also exposed isolated T lymphocytes to hydrogen peroxide, an agent that induces small base lesions. After 72 h of culture, H₂O₂ induced a measurable increase in BrdUrd-Comet tail moment in these cells. This is indicative of a delay in maturation occurring in these cells after exposure to a low dose of the DNA-damaging agent, H₂O₂ and occurs prior to the onset of measurable or lethal DNA damage, as detected by Comet analysis of bulk DNA (Fig. 4).

**Discussion.**

In this study, we have developed a novel assay for the detection of DNA synthesis and maturation in single cells. We have demonstrated that the novel BrdUrd-Comet assay can be further used to detect delays in replication on a damaged template after UV irradiation or after exposure to caffeine. Caffeine has been shown to potentiate cytotoxic, mutagenic, and chromosome-damaging effects of DNA-damaging agents by alternative mechanisms in different cell types;
inhibition of postreplication recovery has been reported in caffeine-sensitive DM fibroblasts (6). In the present study, caffeine-treated DM87 cells demonstrated a marked increase in the amount of BrdUrd-labeled DNA in the Comet tail (Fig. 3), which is indicative of an increase in the number of replicons, a known mechanism for the action of caffeine in DNA maturation delay (6). Caffeine retarded maturation on a damaged, but not on an undamaged, template in DM87 cells exposed to UV irradiation (Fig. 3). This is consistent with the alkaline sucrose sedimentation data described in muntjac fibroblasts (6), where caffeine was shown to block any bypass of UV-induced damage during replication. SVM cells, on the other hand, have been shown to be replication incompetent after exposure to low fluences of UV irradiation and were unaffected by the presence of caffeine (7, 8). Our data confirm these findings (Fig. 2).

Data shown in this study, using the BrdUrd-Comet assay, is therefore parallel to the biochemical data derived earlier and offer a simple

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**Fig. 2.** BrdUrd-Comet analysis of SVM84 fibroblasts after exposure to UV irradiation. Nonirradiated and UV-irradiated (2 J/m² with 1-h recovery period) cells were pulsed with BrdUrd in culture for 15 min and either processed immediately for the BrdUrd-Comet assay or chased with 200 μM of all four deoxynucleotides for 1 h prior to Comet processing. Results are expressed as: A, frequency distribution plots of BrdUrd-Comet percentage of tail DNA; a, BrdUrd pulse alone; b, BrdUrd pulse with chase; and c, BrdUrd pulse with chase in the presence of caffeine; B, representative Comet images, where identical fields are shown with green fluorescein staining (BrdUrd labeling) and red propidium iodide staining (DNA labeling) using appropriate fluorescent filters; a, BrdUrd pulse alone; b, BrdUrd pulse after UV irradiation; c, BrdUrd pulse with chase; and d, BrdUrd pulse with chase after UV irradiation.
alternative to other laborious and time-consuming biochemical methods that involve either tedious centrifugation or meticulous preparation of human DNA. In addition, small numbers of cells are sufficient for analysis, and the technique is therefore potentially suitable for the analysis of single cells derived from small patient biopsies.

We have also exposed isolated T lymphocytes to hydrogen peroxide, an agent that induces small base lesions (18) and plays a role in a variety of physiological and pathological processes, in particular aging and carcinogenesis (19). Cells exposed to a low dose of the DNA-damaging agent had more DNA breaks adjacent to fluorescently labeled repair sites visible in the Comet tail than control cells, indicating the sublethality of this low dose and the ability of the cells to repair any strand breaks formed. Indeed, this effect is visible prior to the onset of measurable DNA damage, evident by the lack of DNA

Fig. 3. Frequency distribution curves of BrdUrd-Comets of DM fibroblasts exposed to caffeine. Nonirradiated and UV-irradiated (2 J/m² with 1-h recovery period) cells were pulsed with BrdUrd in culture for 15 min and either processed immediately for the BrdUrd-Comet assay or chased with 200 µM of all four deoxynucleotides for 1 h prior to Comet processing. Results are expressed as frequency distribution plots of BrdUrd-Comet percentage of tail DNA. Where indicated, caffeine (2 mM) was present for 30 min prior to irradiation and during BrdUrd pulse and chase periods. a, BrdUrd pulse only; b, BrdUrd pulse with chase; c, BrdUrd pulse with chase in the presence of caffeine.

Fig. 4. BrdUrd-Comet images of isolated human T lymphocytes. Cells were cultured for 72 h and processed for BrdUrd-Comet analysis. Control cells were stained with PI (a) and BrdUrd (b) and double exposed for propidium iodide and BrdUrd staining (c) cells treated with 10 µM H₂O₂ at 4°C prior to processing and stained with PI (d) and for BrdUrd (e) are shown.
damage (Comet formation) in propidium iodide-stained whole DNA in these cells.

The present data on PRR in the two muntjac cell lines represent a semiquantitative analysis of the rates of DNA maturation. Further development of the assay are ongoing to determine the lengths and activity of labeled DNA molecules. Furthermore, the BrdUrd-Comet assay is applicable to the detection of DNA repair after early DNA damage induced by hydrogen peroxide in isolated T lymphocytes.

References
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