DNA breakage detection-fluorescence *in situ* hybridization in buccal cells

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Abstract

DNA breakage detection-fluorescence *in situ* hybridization (DBD-FISH) is a recently developed technique that allows cell-by-cell detection and quantification of DNA breakage in the whole genome or within specific DNA sequences. The present investigation was conducted to adapt the methodology of DBD-FISH to the visualization and evaluation of DNA damage in buccal epithelial cells. DBD-FISH revealed that DNA damage increased significantly according to H_2O_2 concentration (r^2=0.91). In conclusion, the DBD-FISH technique is easy to apply in buccal cells and provides prompt results that are easy to interpret. Future studies are needed to investigate the potential applicability of a buccal cell DBD-FISH model to human biomonitoring and nutritional work.

Introduction

The DNA breakage detection/fluorescence *in situ* hybridization (DBD-FISH) is a recently developed technique that allows cell-by-cell detection and quantification of DNA breakage in the whole genome or within specific DNA sequences. In this technique, cells are embedded within an inert agarose matrix on a slide and lysed, to remove membranes and proteins; the resulting nucleoids are exposed to a controlled denaturation step of alkaline incubation. The alkali transforms DNA breaks into restricted single-stranded DNA (ssDNA) motifs, initiated from the ends of the DNA breaks, which can be detected using hybridization with specific or whole-genome fluorescent DNA probes. As the number of DNA breaks increases in a target region, more ssDNA is produced and more probe hybridizes, resulting in a more intense FISH signal that can be quantified using image analysis systems.[1-3]

These lesions are traditionally known as alkaline-labile sites (ALS). The presence of ALS in the genome of various cell types may vary between cells with conventionally conformed genomes,[4] and may change if the cell is under stress conditions, such as radiation[2] or human papilloma virus.[5,6] Recently, the higher resolution of DBD-FISH respect to comet assay has been demonstrated.[7] The cells used most commonly in the DBD-FISH technique in human studies are the peripheral blood lymphocytes. However, buccal cells are an excellent sample invasive not to monitor human exposure to occupational and environmental genotoxicants, because are in direct contact with ingested or inhaled pollutants.[8] Buccal cells are reportedly more sensitive than peripheral blood lymphocytes to the cytogenetic damage induced by cigarette smoke,[8] other environmental mutagens,[9] and oral cancer.[10]

The present investigation was conducted to adapt the methodology of DBD-FISH to the visualization and evaluation of DNA damage in buccal epithelial cells. This model is an attractive and potentially useful tool for investigating the *in vitro* and *in vivo* damaging effects of dietary agents, lifestyle choices, and chemical agents on DNA.

Materials and Methods

Buccal cell sampling

Buccal cells were collected from five individuals via gentle brushing of the inside part of the lower lip using a cytological brush after washing the mouth several times with tepid distilled water, to remove exfoliated dead cells.

The brushes were stirred in 15 mL plastic tubes containing 1 mL of phosphate-buffered saline (PBS). Cells to be used for DBD-FISH were washed twice, centrifuged at 1500 rpm for 10 min at room temperature, and resuspended in PBS.

Written informed consent was obtained from each subject; the Ethical and Research Committee of Centro de Investigación Biomédica del Noreste (CIBIN), IMSS has approved all experimental procedures.

Slide preparation

The material was submerged in 5 mL of cold PBS. The samples were brought to the laboratory under cold conditions and were processed within 1 h of sampling. A trypan blue test was performed in 20 mL aliquots of buccal cells, to determine the percentage of viable cells for each individual. All samples showed viability ≥85%. The cell suspension was mixed with low-melting-point agarose at 37°C, to give a final concentration of 0.7%. The mixture (15 mL) was pipetted onto slides pretreated with agarose, covered with glass cover slips (24 mm × 24 mm), and left at 4°C for 5 min. Cells were treated with proteinase K (1 mg/mL) for 20 min at 37°C, followed by washing with PBS.

Treatment with H_2O_2

Slides were exposed to three concentrations of H_2O_2 (0.03, 0.3, and 3%). Immediately after, the cover slips were removed and the slides were incubated in lysing solution. The protein-depletion, alkaline-unwinding, and alkaline treatments were performed as described previously. This was used to investigate the dose response of DBD-FISH score in the produce.

DBD-FISH

Slides were ready for processing after gentle removal of the cover slips. In DBD-FISH, it is important not to allow the slides to dry, as the protocol is a continuous process. Basically, the method consists of a protein-depletion procedure followed by treatment with an alkaline solution to produce ssDNA. In this case, for depletion of proteins from buccal cells, slides were treated with a basic solution containing 2 M NaCl, 0.05 M Ethylenediaminetetra acid (EDTA), 0.4 M Tris-base, and 1% SDS at 43°C for 25 min. Slides were incubated horizontally, to avoid chromatin loss. After protein removal, the resultant nucleoids were washed in 0.9% NaCl for 10 min. For ssDNA production, protein-depleted slides were incubated in an alkaline unwinding solution (0.03 M NaOH and 1 M NaCl) for 2.5 min at room temperature. After neutralizing with 0.4 M Tris-HCl (pH 7.5) for 5 min, nucleoids were washed in TBE buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA;

pH 8.3) for 2 min. For ssDNA stabilization, slides were dehydrated in sequential 70, 90, and 100% ethanol baths for 2 min each, and then air dried.

FISH

Whole-genome DNA probes were obtained from lymphocyte pellets using a DNA isolation kit for mammalian blood (Roche Diagnostics Corporation, Indianapolis, IN, USA). One microgram from each DNA sample was labeled with digoxigenin-11-dUTP using a commercial nick translation kit (Roche Diagnostics Corporation). The digoxigenin-labeled whole-genome probes were denatured and incubated with the slides. After overnight incubation at room temperature, slides were washed twice at room temperature in 50% formamide/2× SSC (pH 7) for 5 min and in 2×SSC (pH 7) for 3 min. The hybridized DNA probe was detected via 30 min incubation with fluorescein isothiocyanate-labeled avidin (1:400) (Roche Diagnostics Corporation). The slides were counterstained with propidium iodide (1 mg/mL) in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Image and statistical analyses

DBD-FISH results were analyzed on all slides using a Digital Image Analysis platform based on a Leica DMLB fluorescence microscope equipped with three low-pass band filters for visualization of green, red, and blue fluorescence. Images were acquired using a Leica DF-35 16-bit black-and-white CCD camera in a 16-bit TIFF format. Image analysis was performed to compare the fluorescence intensities obtained after DBD-FISH. For this purpose, integrated density (ID) (area × gray scale) after background subtraction was calculated using the Leica Q-Win image analysis software. After background subtraction, 50 different cells were measured for each treatment.

The Kruskal-Wallis test was used to investigate any possible differences between ID and the different treatments. Pearson correlation coefficient was using to determine the grade of relation between the concentrations different of H_2O_2 and DNA damage. A value of $P<0.05$ was considered significant.

Results and Discussion

DBD-FISH experiments

DBD-FISH performed under mild alkaline denaturation conditions for protein removal and ssDNA production led to the detection of regions of buccal cell nuclear damage in cells without treatment with H_2O_2. These regions represented the background detected by DBD-FISH for this cell type and were considered as normal levels of DNA damage or the constitutive DNA damage for this cell type (Figure 1A).

The buccal cell did not present cellular disintegration nor resistance to controlled lysis.[11]

Dose-response studies

The results of our analyses suggest that damage is an inherent structural characteristic of DNA in buccal cells and is present at low, but detectable, levels after DBD-FISH. However, increased levels of DNA damage correlated with high concentrations of H_2O_2 ($r^2=0.91$) (Figure 1; Table 1). Although the molecular biology and significance of constitutive DNA damage are not well understood, some observations support the hypothesis that these genomic regions escape the normal DNA configurations and may be transient structural features of cells. This constitutive DNA damage may change if the cell is exposed to mutagenic agent.[2,5,6]

Our results demonstrated than DBD-FISH using controlled lyses could be applied to the analysis of the global DNA damage in buccal cells induced by mutagenic agents (for example H_2O_2). In fact, the present study was performed, analyzing the overall genome, using a whole genome probe. However, many different specific probes could be hybridized

In conclusion, the DBD-FISH technique is easy to apply in buccal cells and provides prompt results that are easy to interpret. This protocol has great potential for studying DNA damage and repair linked to specific sequences in human biomonitoring and nutritional work. Once the technique has been established, further investigations are needed to achieve information on the sensitivity of the technique regarding, *e.g.,* i) the recording of susceptibility to DNA damage in different chromatin sequences using specific DNA probes and/or ii) the establishment of chromosomal dose-response curves for classical damaging agents, such as ionizing radiation.

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Table 1. Comparison of the area, grey level, and integrated density (ID) after fluorescence densitometry in buccal cells treated with varying concentrations of H_2O_2.

Group (H_2O_2)]	Fluorescence analysis		
	Area (X±SD)	Grey level (X±SD)	ID (X±SD)
Control	$124^{E2} \pm 54^{E2}$[b,c,d]	$403^{E3} \pm 287^{E3}$[b,c,d]	$924^{E7} \pm 731^{E7}$[a,c,d]
0.03%	$358^{E2} \pm 97^{E2}$[b,c,d]	$674^{E3} \pm 376^{E3}$[a,c,d]	$2339^{E7} \pm 1161^{E7}$[a,c,b]
0.3%	$489^{E2} \pm 167^{E2}$[a,b,d]	$1709^{E3} \pm 781^{E3}$[a,b,d]	$4633^{E7} \pm 1246^{E7}$[a,b,d]
3.0%	$863^{E2} \pm 213^{E2}$[a,b,c]	$2750^{E3} \pm 941^{E3}$[a,b,c]	$7849^{E7} \pm 1161^{E7}$[a,b,c]

ID, area x grey level. [a]Different to control (P=0.0001); [b]different to 0.03% (P=0001); [c]different to 0.3% (P=0.0001); [d]different to 3% (P=0.0001).
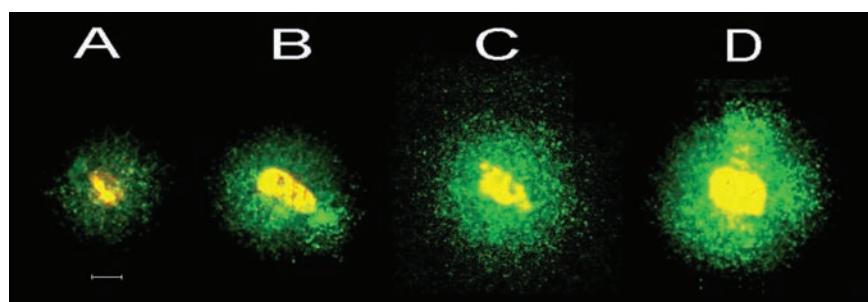

Figure 1. Buccal cells after DBD-FISH without treatment (A) and after treatment with varying concentrations of H_2O_2 (0.03% (B), 0.3% (C), and 3% (D). Scale bar: 10 μm.

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