

Standardization of the comet assay technique on FRTL5 Cells

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Abstract

The comet assay is a sensitive and rapid method for DNA strand break detection in individual cells. The principle of break detection, using either the alkaline or neutral version of the assay, makes it a good technique for studying both double and single strand DNA breaks. Furthermore, the possibility of following DNA damage at different time moments also makes it possible to investigate the cell repair mechanisms. This explains why in the last few years there has been a tremendous increase in the number of laboratories which started to use this technique.

The technique was first created for lymphocyte cells and later on has been used on many other cell types, growing both in suspension and adherent. To date, no one has applied this technique on normal differentiated endocrine cells, such as FRTL5 cells (Fisher Rat Thyroid Cells).

The aim of this study has been to standardize the alkaline version of the Comet Assay technique on FRTL5 cells by studying the kinetics of DNA-damage and DNA-repair after different doses of UV-C (254 nm). FRTL-5 cells not only resulted very sensitive to UV-C ($p < 0.05$ at 5 J/m^2), but were also able to repair most of their DNA damage very rapidly (within one hour) as shown by a significant exponential regression in comet length.

Finally, the successful measurement of biomarkers of UV-C on thyroid cells established the comet assay as a valuable tool in measurement of DNA damage and repair. Any radiation, or other damaging agents, interacting with living organisms could cause DNA damages which, depending upon dosages and kinetics of exposure, may or may not be completely repaired.

KEYWORDS: Comet assay, FRTL-5, UV-C radiation.

1. Theoretical Background

Rydberg and Johanson first introduced the comet assay technique in the 1978 [1]. In 1986 Singh first made microgels slides and electrophoresing under alkaline conditions [2]. This removed the DNA supercoiling and denatured the DS DNA to SS DNA. With this modification he obtained a dose-response curve with respect to length of DNA migration. The resulting images were subsequently named 'comets' because of their appearance and their total length was considered directly related to the DNA damage.

From that moment a range of applications of the comet assay have been covered, from investigations of the physiochemical behavior of DNA, through studies of cellular responses to DNA damage, to biomonitoring of human population [3]. The possibility of following DNA damage at different times also made it possible to investigate the cell repair mechanisms. This method is now widely used.

We have decided to study the effect of UV-C radiation (far ultraviolet light: 254 nm) on thyroid cells because they are supposed to be very sensitive to radiation [4]. FRTL5 cells (Fisher Rat Thyroid Cells) are defined as a differentiated, non-transformed, cloned thyroid cells in long-term culture [5]. We have applied the alkaline version of the comet assay technique on resuspended FRTL-5 cells in order to study SS breaks. UV-C induced strand breaks are not formed directly, instead the incision step of excision repair leads to strand-break formation and yield of breaks representing an equilibrium between the incision and strand rejoining steps is

found [6]. We have decided to consider both DNA damage, by measuring total comet length, and the kinetics of cellular repair after different doses of UV-C (254 nm).

2. Material and Methods

2.1. Cell culture

FRTL5 cells for routine maintenance were grown in Ham's modified F-12 medium supplemented with 5% calf serum, gentamicyne (50 $\mu\text{g/ml}$), and a hormone mixture consisting of insulin (10 $\mu\text{g/ml}$), Hydrocortisone (0.36 ng/ml), transferrine (5 $\mu\text{g/ml}$), somatostatine (10 ng/ml), glycil-L-histidyl-L-lysine acetate (2 ng/ml), TSH (10 mU/ml). The cells were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C .

2.2. Irradiation

Radiation at 254 nm was obtained from a low-pressure mercury lamp (Spectroline Model ENF-240C). The fluence rates (J/m^2) were measured by a radiometer (Int. Light IL1700). Cells were irradiated on an uncovered plate (30 mm diam.) in 0.5 ml PBS medium.

2.3. Comet assay

After irradiation approximately 20000 cells were suspended in 10 μl PBS and mixed with 50 μl of

0.5 % low-melting agarose solution at 45°C (Amresco). For repair studies, approximately 300000 FRTL5 cells, after irradiation, were resuspended in usual medium and incubated in an atmosphere of 95% air and 5% CO₂ at 37°C. At different time points microgels were prepared by placing different layers of cells and agarose mixture on frosted slides. Slides were then placed in cold lysing solution (2.5M NaCl, 1% sodium N-laurylsarcosinate, 100 mM disodium EDTA, 10mM Tris base, at pH 10) with 1% Triton X-100. After 12 hours of lysis (overnight) the slides were treated with DNAase-free Proteinase-K (Amresco) in lysing solution for 2 hours at 37°C. Slides were put horizontally in an electrophoretic assembly (Hoefer Model No HE 100). One liter of electrophoretic alkaline buffer was added (1% 8-Quinolol, 10mM tetrasodium-EDTA, 2% DMSO, 300mM NaOH, pH 10) and left for 20 min. (unwinding time). Electrophoresis (0.4 V/cm) and recirculation (100ml/min) were started and left for 60 min.

After electrophoresis slides were washed with a neutral solution (400nm Trizma hydrochloride, 1M ammonium acetate, pH 7.4) and immersed in absolute ethanol for 30 min and in 70% ethanol for 60 min. Slides were dried and stained with 35 µl of 1M YOYO-1 in distilled water. After staining slides were observed with a fluorescent microscope (Carl Zeiss Large Fluorescent Microscope) and length was determined using an eyepiece micrometer.

3. Results

Irradiation of cells with different UV-C dosages showed an increase in DNA migration.

Figure 1 summarizes results of UV-C induced single-strand breaks. Each histogram shows the frequency distribution of length of DNA migration from 50 representative cells per UV-C dose. A significant increase in mean length of DNA migration pattern is observed even at very small doses (5J/m²).

For the study of DNA repair kinetics, cells were studied at time zero and one hour after irradiation with 5 J/m² (data not shown). Interestingly, most of the cells decreased significantly their migration pattern after one hour. This would suggest that most of the damage by UV-C is repaired within one hour.

4. Discussion

The comet assay technique has turned out to be a very sensitive method for the detection of SS breaks. The high sensitivity could be attributed both to the type of cell used (FRTL-5) or to the type of technique (comet assay). In favour of the second hypothesis, also other kind of cells such as lymphocytes, are significantly damaged by doses in the order of 5 J/m² [7]. In order to evaluate the thyroid cells sensitivity, in future experiments we are planning to compare the effect of UV-C in different kinds of cells.

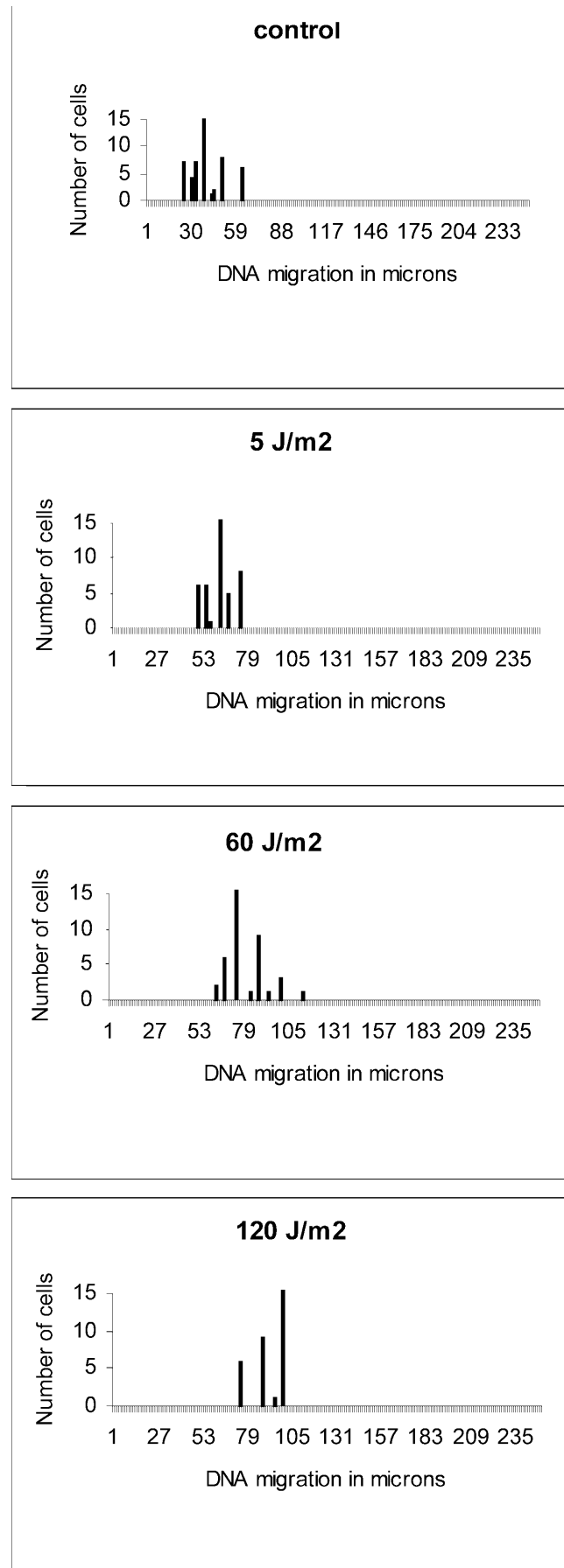


Fig. 1 – Frequency distribution histograms of length of DNA migration from 50 representative cells. Each histogram represents cells after irradiation with different UV-C doses (0, 5, 60 and 120 J/m²). Cells were analyzed immediately after exposure (time 0).

Regarding the repair capacity, FRTL 5 cells irradiated with 5 J/m² showed a significant regression in DNA migration length within one hour. These data confirm previous studies performed on lymphocytes [8]. The possibility of studying DNA repair in FRTL5 cells represents a great challenge for future applications of this technique.

The data obtained confirm also the relevance of different technical parameters chosen for preparing microgel slides using FRTL-5 cells. Different trials have been done (data not shown) to study the effect of varying different parameters. For example, a lower proteinase-K concentration decreased DNA migration pattern especially in irradiated cells. This caused a decrease in the technique sensitivity. At 5 J/m² cells appeared similar to the control cells and there was not a significant increase in DNA migration pattern.

5. Conclusions

For measuring DNA strand breaks, the comet assay has turned out to be a very sensitive method and thyroid cells, being very similar to human cells, could easily be used as a good model for studying the effect of any radiation or other damaging agents

which, interacting with living organisms, could cause reversible as well as irreversible DNA damage.

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