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Study of DNA integrity in alpha-particle radiobiological experiments using thin CR-39 detectors

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Abstract

Thin CR-39 detectors (with thickness of $\sim 20\,\mu m$) were prepared from commercially available CR-39 SSNTDs through chemical etching in 1 N NaOH/ethanol at 40 °C. Custom-made petri dishes, with a hole drilled at the bottom and covered with a piece of thin CR-39 detector, were used for culturing HeLa cells. Alpha particles (from an ^{241}Am source) were irradiated from the bottom of the CR-39 detector. The hit frequency of cell nuclei by alpha particles was obtained from the positions of the etched alpha-particle tracks. The effect of alpha-particle irradiation was quantified using the results of terminal dUTP nick end-labeling (TUNEL) assay, which characterizes strand breaks in DNA.
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1. Introduction

Ionizing radiation produces many kinds of DNA damages including double-strand breaks (DSBs) and single-strand breaks (SSBs). Among these, DSB is the most lethal and mutagenic. As shown in Fig. 1, unrepaired DNA damages will likely lead to cell deaths (Wang et al., 2000), while incorrectly repaired DNA damages may induce carcinogenesis (Bernstein et al., 2002).

Apoptosis is a physiological suicide mechanism that preserves homeostasis. Cleavage of genomic DNA during apoptosis may yield double-stranded, low-molecular weight DNA fragments (mono- and oligo-nucleosomes) as well as (SSBs) ("nicks") in the DNA. In the terminal dUTP nick-end labeling (TUNEL) reaction, the enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of fluorescein-labeled deoxyuridine triphosphate (dUTPs) to the free 3′-OH DNA ends in a template-independent manner as shown in Fig. 2. The fluorescein labels are then detected and quantified by fluorescence microscopy or flow cytometry. In the present study, TUNEL assay together with flow cytometry were used to investigate the effects of alpha particles on cells in terms of creation of strand breaks. Flow cytometry characterizes single cells as they pass through a laser beam at high speed. The laser beam provides a light source for scattering and at the same time excites the fluorescent molecules used to label the cells.

In the present paper, a special method was developed to use homemade thin CR-39 detectors as cell-culture substrates to record the positions where the alpha-particles hit the nuclei of cultured cells and to use TUNEL assay to quantify strand breaks. As a demonstration of the method, HeLa cervix cancer cells were employed.

2. Methodology

2.1. Thin CR-39 detectors

CR-39 detectors are one of the most commonly used solid-state nuclear track detectors (SSNTDs). A recent review on SSNTDs can be found in Nikezic and Yu (2004). In the present work, the alpha particles pass through the substrate to strike the cells so the substrate should be thin enough to allow passage of alpha particles. According to the SRIM program (Ziegler, 2003), the range of 5 MeV alpha particles in CR-39 is 28.77 μm. However, the thinnest commercially available CR-39 SSNTDs
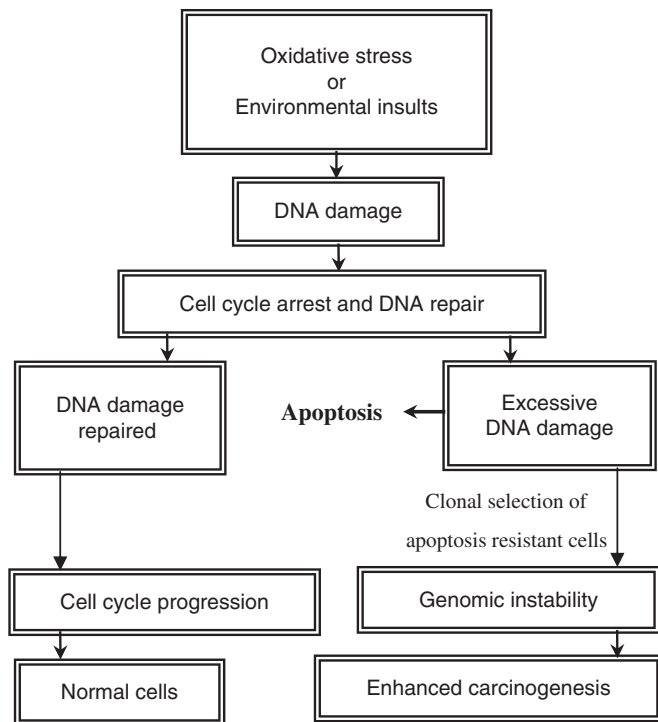

Fig. 1. Roles of DNA damage, DNA repair, cell cycle arrest and apoptosis in carcinogenesis.
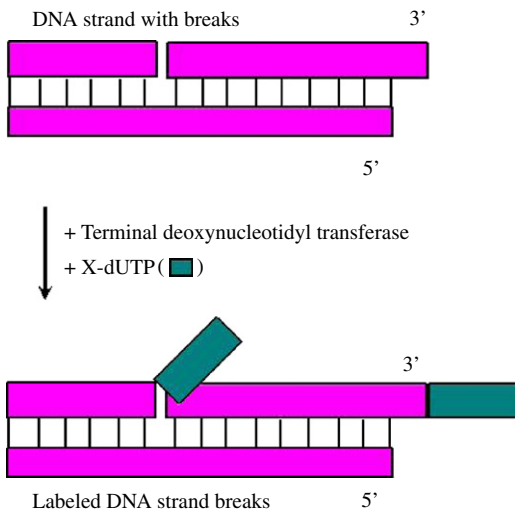

Fig. 2. DNA of fixed cells labeled by the addition of fluorescein-labeled dUTP (deoxyuridine triphosphate) catalyzed by TdT (terminal deoxynucleotidyl transferase).

are $\sim 100 \, \mu$m thick. We prepared thin CR-39 detectors from CR-39 SSNTDs with a thickness of $100 \, \mu$m (from Page Mouldings (Pershore) Limited, Worcestershire) by chemical etching in 1 N NaOH/ethanol at $40 \, ^\circ$C (Chan et al., 2007). These thin CR-39 detectors had a root-mean-square roughness of less than $0.006 \, \mu$m. They were glued by epoxy (Araldite$^\circledR$ Rapid, England) to the bottom of petri dishes (Orange Scientific) with a diameter of 5 cm, with a hole of 1 cm diameter drilled at the

bottom, to form the cell dishes (see also Søyland et al., 2000; Gaillard et al., 2005).

2.2. Markers for alignment of images

As will be described in the following, two sets of images were superimposed to obtain accurate positions of alpha-particle hits on the cells. The first set contained images of cells while the second set contained images of the tracks corresponding to the 5 MeV alpha particles used to irradiate the cells. In the present investigations, a special method was developed to use markers for alignment of these two sets of images. Before culturing cells onto the custom-made cell dishes, the bottom of the CR-39 detectors were irradiated by 1 MeV alpha particles for 10 min. The alpha source employed in the present study was a planar ^{241}Am source (main alpha energy = 5.49 MeV under vacuum) with an activity of 4.26 kBq. After irradiation, the bottom side of the cell dishes was etched by 14 N KOH solution at $37 \, ^\circ$C (corresponding to a bulk etch rate of $0.64 \pm 0.01 \, \mu$m/h determined by the masking method, Ho et al., 2003; Yasuda et al., 1998) for 3 h 40 min to reveal the tracks. These tracks were used as markers for alignment only, and are referred to as "base tracks".

The tracks formed on CR-39 by 1 and 5 MeV alpha particles after chemical etching showed distinct characteristics and were thus conveniently distinguished from one another. For example, 1 MeV alpha-particle tracks had larger track openings and were shallower than 5 MeV alpha-particle tracks.

2.3. Cell cultivation and irradiation

After introducing the markers, the thin CR-39 cell dishes were sterilized by submerging them into 75% (v/v) ethyl alcohol for 2 h and then in absolute alcohol. These cell dishes were then used for culturing National Institutes of Health HeLa cervix cancer cells which were obtained from American type culture collection. The cell line was maintained as exponentially growing monolayer at low passage numbers in Dulbecco's modified eagle medium (D-MEM) supplemented with 10% fetal bovine serum, 1% (v/v) penicillin/streptomycin (Gibco, Karlsruhe, Germany). The cells were cultured at $37 \, ^\circ$C in a humidified atmosphere containing 5% CO_2. The cells were trypsinized for 4 min with 0.5/0.2% (v/v) trypsin/EDTA (ethylenediamine-tetra-acetic acid; Gibco), adjusted to a concentration of about 3×10^5 cells ml^{-1}, and plated out on the CR-39 cell dishes. Images of these cells together with the markers were captured, and are referred to as the CM (cells and markers) images.

The CR-39 cell dishes, with the cells inside, were then irradiated from the bottom with 5 MeV alpha particles under normal incidence through a collimator for 1 h (to give a fluence of about 12,700 alpha particles per cm^2). The residual energy of the alpha particles after passing through the CR-39 detector could be calculated using the SRIM program (Ziegler, 2003). CR-39 detectors with different thickness could be used if the response of cells to different alpha-particle energies were needed.
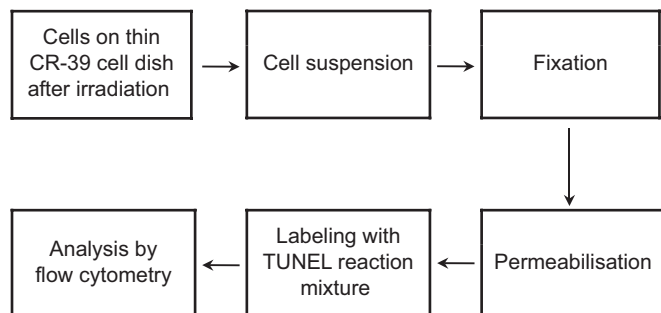
Fig. 3. The flow chart of the TUNEL assay procedures.

2.4. TUNEL assay

A TUNEL assay flow chart is shown in Fig. 3.

After 5 MeV alpha-particle irradiation, adherent cells on the custom-made thin CR-39 cell dishes were trypsinized for 4 min with 0.5/0.2% (v/v) trypsin/EDTA and these cells were collected from the cell dishes immediately. The collected cell suspensions would be transferred into a 1.5 ml micro-centrifuge tube and washed by phosphate buffered saline (PBS) once. The washed cell suspension was then fixed by adding freshly prepared fixation solution (2% paraformaldehyde in PBS) and resuspended well with the centrifuged cells. The incubation conditions were 1 h at 15–25 °C. The cells were then washed once with PBS and resuspended in 1 ml permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 10 min on ice (2–8 °C). After permeabilisation, all the cells were washed twice with PBS.

A positive control sample was then prepared by adding DNase I (grade I) to digest the DNA for 15 min at 15–25 °C. After that, the cells in all samples were resuspended in 50 μl/tube TUNEL reaction mixture made of 90% (v/v) label solution (Roche Diagnostics GmbH, Germany) and 10% (v/v) TUNEL enzyme (Roche Diagnostics). The cell suspension was then incubated for 1 h at 37 °C in a humidified atmosphere in dark for reaction to take place.

The two negative controls were unirradiated samples in a TUNEL buffer without terminal transferase and those in TUNEL reaction mixture. To study the effect of light from the microscope on the cells during taking images before irradiation, the samples of cells that had been exposed to light for the same time interval of taking images were also stained with the TUNEL reaction mixture. The samples were then washed twice in PBS.

The cells were then transferred in a specialized tube to a final volume of 500 μl in PBS. The samples were analyzed by flow cytometer (Becton DICKINSON, FACSCalibur cytometer) by counting 10,000 cells for each sample to detect TUNEL signals.

2.5. Hit positions of 5 MeV alpha particles

After the cells were trypsinized from the CR-39 detector, the bottom sides of the cell dishes were etched by a 14 N KOH solution at 37 °C for 6 h to reveal tracks to record the positions

of alpha-particle hits. After this etching, there were two types of alpha-particle tracks on the CR-39 detector: (1) 1 MeV alpha particle tracks (markers) which had been etched for a total of 9 h 40 min (3 h 40 min + 6 h) and (2) 5 MeV alpha-particle tracks which had been etched for 6 h. Images of these tracks (hereafter referred to as the FT, final tracks, images) were captured. The FT images would be superimposed with the CM images previously captured through the alignment of the "base tracks" as markers.

3. Results and discussions

3.1. Alpha-particle hit positions

The alpha-particle hit positions on the cells or otherwise can be determined from the superposition of CM images and FT images. An example is shown in Fig. 4. The number of alpha-particle hit cell nuclei can be obtained by counting the number of cell nuclei overlapping with the 5 MeV alpha-particle tracks. Moreover, the residual energies of the alpha particles that hit the cell nuclei would be dependent on the thickness of the pre-etched thin CR-39 detector. For example, the original thickness of the thin CR-39 detector corresponding to the residual energy of 0.39 MeV is 29 ± 1 μm. As a result, the final thickness of the thin CR-39 detector is 26.6 μm after 3 h 40 min etching. Finally, the residual energies were calculated by interpolation from the output of the SRIM program (Ziegler, 2003). The number of alpha particles hitting the cell nuclei and the energies of these alpha particles could be used as references to study the DNA strands broken by alpha-particle radiation.

3.2. Results of TUNEL assay

Fig. 5 shows sample results from the flow cytometer, which give the strengths of fluorescence signals from the samples. M1 represents the region without TUNEL signals while region M2 represents the region with TUNEL signals. The negative control involved unirradiated cells treated with the TUNEL reaction mixture, and had 1.84% of signals in the M2 region.

The sample irradiated for 1 h by alpha particles with a residual energy of 0.68 MeV on cells had 42.66% of signals in the M2 region and was thus, after adjustment for the 1.84% of signals from the negative control in the M2 region, (42.66 − 1.84)% = 40.82% relative TUNEL positive. TUNEL positive corresponded to DNA strand breaks in the sample, which could be used to study the effects of alpha particles on the cells. Similarly, the positive control was 85.51% TUNEL relative positive. It is remarked that the TUNEL positive signals were determined relative to the negative signals measured by flow cytometer each time. Therefore, all the steps for the TUNEL assay should be performed and all the reagents should be prepared on the same day. In this way, the number of samples used for determining TUNEL signals was inevitably restricted.

Fig. 6 shows the flow cytometer results from unirradiated cells (0.50% TUNEL positive) and unirradiated cells exposed to light from the microscope employed for capturing the cell images (0.66% TUNEL positive), with both samples stained
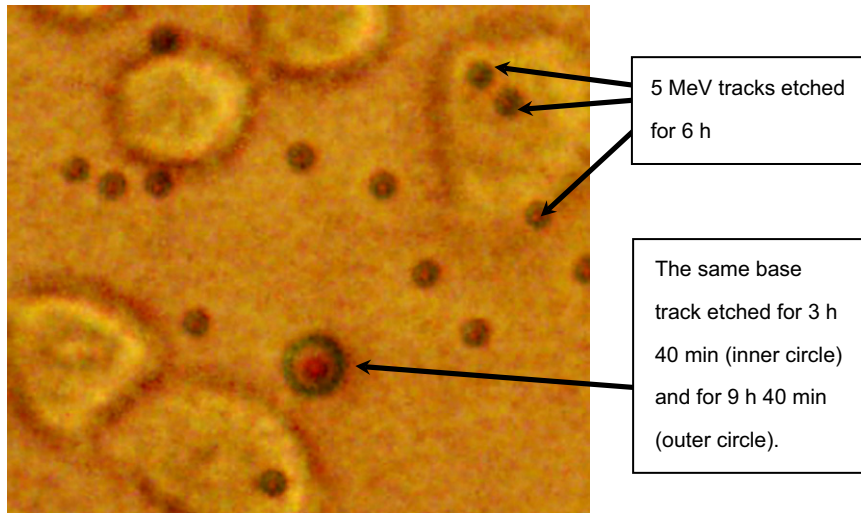
5 MeV tracks etched
for 6 h

The same base
track etched for 3 h
40 min (inner circle)
and for 9 h 40 min
(outer circle).

Fig. 4. Superposition of the "cells and markers" image with the "final tracks" image through the alignment of the "base tracks" as markers.
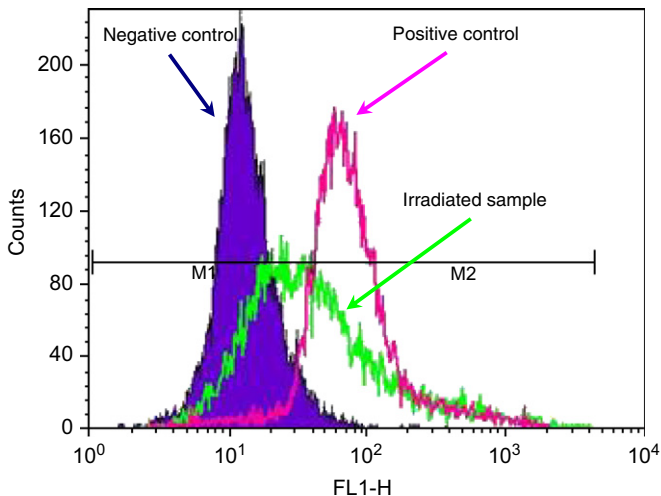

Fig. 5. Sample results from the flow cytometer that give the strengths of fluorescence signals from the samples. The x-axis gives the strength of fluorescence signals while the y-axis gives the number of cells counted. Region M1 represents the region without TUNEL signals while region M2 represents the region with TUNEL signals. The three peaks correspond to the negative control (1.84% TUNEL positive), the sample irradiated for 1 h by alpha particles with a residual energy of 0.68 MeV on cells (40.82% TUNEL positive) and the positive control (85.51% TUNEL positive) stained by TUNEL reactive mixture, respectively.
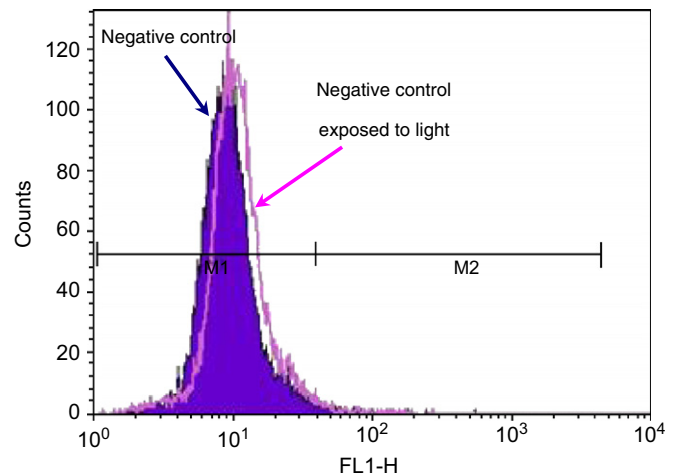

Fig. 6. The TUNEL results of the negative control of unirradiated cells (0.50% TUNEL positive) and the negative control of unirradiated exposed to light from the microscope (0.66% TUNEL positive).

with the TUNEL reaction mixture. The similarity of the results demonstrated that exposure to light from the microscope would not induce DNA damages in cells. The average value for negative controls exposed to light from the microscope was $0.80 \pm 0.40\%$ TUNEL positive.

4. Conclusions

In the present paper, we described a method based on thin CR-39 detectors and TUNEL assay to study DNA integrity in alpha-particle radiobiological experiments. The thin CR-39

detectors were prepared as cell-culture substrates to record the positions where the alpha-particles hit the nuclei of cultured cells. TUNEL assay together with flow cytometry were used to investigate the effects of alpha particles on cells in terms of creation of strand breaks.

With this proposed methodology, we can study in future, for example, the dependence of the amount of strand breaks on the number or percentage of hit cell nuclei as well as the alpha-particle energies incident on the cell nuclei (the latter being related to the linear energy transfer values), and the bystander effect through the TUNEL signals from non-targeted cells. These results will be reported in a separate paper.

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