Detection of Apoptotic Frequency in Chinese Hamster Ovary (CHO-K1) Cells after Gamma-Irradiation Using Both Neutral Comet Assay and *terminal desoxynucleotidyl transferase (TdT)* Assay

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Abstract

Objectives: Two assay methods, namely the neutral Comet assay and *terminal desoxynucleotidyl transferase (TdT)* assay, were carried out for comparison to investigate the capability of using the neutral Comet assay as an alternative for detection of apoptosis.

Materials and methods: Chinese hamster ovary-K1 (CHO-K1) cells were exposed to gamma-rays with different doses and then the frequencies of apoptotic cells were determined at given points of time using the neutral Comet assay and *TdT* assay.

Results: Apoptotic frequency of CHO-K1 cells after gamma-irradiation is dependent on both time after irradiation and radiation dose using either the neutral Comet assay or *TdT* assay. There are differences between the data obtained using the neutral Comet assay and *TdT* assay ($p < 0.01$, Student's t-test).

Conclusions: The neutral Comet assay appears to be an appropriate tool for detection of radiation-induced apoptosis at the early stage of the process. Compared to the other methods such as the *TdT* assay, the neutral Comet assay is a rapid, simple and economical method for detection of apoptosis.

Key words: detection; apoptotic; Comet assay; Chinese hamster ovary-K1 cells; gamma-irradiation

1. Introduction

There has been an increasing interest in the role of apoptosis in physiology and pathology. Over the years, there have been several methods of detection of apoptosis. They include methods based on morphological changes, DNA (deoxyribonucleic acid) fragmentation, membrane alterations and cytoplasmic changes. Each method has its advantages and disadvantages. The "Comet assay", or single cell gel electrophoresis, was originally described by Ösling and Johanson (1) as a simple and rapid technique to detect DNA strand-breaks in individual cells. From this technique, the alkaline Comet assay which measures alkali-labile sites and single strand-DNA breaks and the neutral Comet assay which measures double strand-DNA breaks, were developed. The former was developed by Singh et al. (2) and the latter by Olive et al. (3). Radiation induced-apoptosis is accompanied by degradation of cellular genomic DNA (4–8). This process was detected by methods for identifying double strand-DNA breaks such as *terminal desoxynucleotidyl transferase (TdT)* assay and Comet assay. Thus, the

Comet assay seems to be a suitable method for detection of apoptosis (9–13), but few reports have been published, so we conducted a study on detection of apoptosis induced by gamma rays in Chinese hamster ovary (CHO-K1) cells using the neutral Comet assay and *TdT* assay with multiple step comparison and expense comparison.

2. Materials and Methods

2.1. Cell culture and radiation

CHO-K1 cells were cultured in Ham's-F12 medium supplemented with glutamin in a 90×20 mm dish. Before irradiation, the cells were trypsinized, resuspended in fresh medium and then transferred into tubes with a concentration of 10^5 cells per 1 ml. The samples were exposed to Cobalt 60-gamma rays with doses of 8 or 16 Gy at dose rate of 2 Gy/min at room temperature. After irradiation, the cells were cultured in 50×5 mm Petri dishes with 2 ml medium and incubated at 37°C up to three days for detection of apoptosis at 24 hour intervals.

2.2. Single cell gel electrophoresis (Comet assay)

The procedure was carried out as Olive et al. (10, 11) described with some modifications. Briefly, after centrifuge of the cell suspension, the cells were mixed with phosphate buffered saline (PBS: NaCl 8 g, KCl 0.2 g, Na₂PO₄ 1.15 g, KH₂PO₄ 0.2 g/1000 ml distilled water, pH 7.4). An equal volume of cell

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suspension was mixed with 2% low melting point agarose, then dropped on slides pre-covered with 1% normal melting point agarose. Cover slides were used. When the gel set, the cover slide was removed. The embedded cells were then lysed in lysis buffer with 2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 10% DMSO, 1% sodium lauryl sarcosinate, 1% Triton X-100) for 30 min for protein removal, then washed in buffer with 90 mM Tris, 90 mM boric acid, 2 mM EDTA-2Na (TBE (Tris, boric acid, EDTA) buffer)), (pH 8) prior to electrophoresis. The slides were subjected to electrophoresis in TBE buffer at a current of 0.01 A and voltage of 25 V for 30 min. After electrophoresis, the slides were treated with 0.03 M NaOH for RNA digestion for 5 min, then neutralized in 0.4 M Tris-HCl (pH 7.4). Just before scoring, slides were stained with ethidium bromide (20 mg/ml), then scored on a fluorescent microscope at a magnification of 400X. The comets with completely separated and nearly separated tails were classified as complete apoptotic (C (complete)) and undergoing apoptosis (U (undergoing)) cells, respectively. At least 500 cells were analyzed for each sample to obtain apoptotic frequencies.

2.3. Terminal deoxynucleotidyl transferase (TdT) assay

The assay was performed according to the manufacturer's procedure using S7100 Apo Tag[®] peroxidase Kit (Intergen). Briefly, cell suspension was dropped on slides pre-coated with bovine serum albumin (BSA), then air dried until TdT staining. Digoxigenin-labelled nucleotides were added to apoptotic generated 3'-OH site by enzyme *TdT*. Cells undergoing apoptotic DNA fragmentation were then labelled using anti-digoxigenin antibody and then counter-stained with 0.05% (w:v) methyl green. The slides were mounted under glass coverslips in mounting medium, then observed under light microscope at a magnification of 400X. Brown cells were classified as apoptotic cells. The blue cells were unapoptotic cells. At least 1000 cells were scored to obtain apoptotic frequency for each sample.

3. Results and Discussion

3.1. Comet image of apoptotic cells

By single cell gel electrophoresis of gamma ray-irradiated CHO-K1 cells, we noticed that apoptotic cells could be clearly distinguished from unapoptotic cells by the pattern of the comet image (3, 10). The comet image of apoptotic cells has the tail separated from the comet head as previously indicated (3, 8, 10). The apoptotic cells could be classified into two types based on the DNA migration patterns: completely apoptotic cells and cells undergoing apoptosis. The former with apoptotic DNA fragmentation were clearly identified by the complete separation of the tail from the small comet head. The latter had tails nearly separated from the comet head. These patterns of comet image of apoptotic cells depend on the stage of the apoptotic process. The results are shown in Tables 1 and 2.

3.2. Comparison between TdT and the Comet assay

In comparison between the two methods, Tables 2 and 3 show that there are differences in apoptotic frequencies obtained by the Comet assay and *TdT* assay at all three given points of time ($p < 0.01$, Student's *t*-test). Data in Table 3 express only frequencies of completely apoptotic cells (C) using the *TdT* assay, because the *TdT* assay is incapable of detecting cells undergoing

Table 1 Percentages of cells undergoing apoptosis (mean $\pm$ SD (Standard of deviation)) among CHO-K1 cells exposed to gamma rays using Comet assay. Data are mean of results from three independent experiments.

Time (h)	Dose (Gy)	
	8 Gy	16 Gy
24	0.17 $\pm$ 0.01	0.33 $\pm$ 0.22
48	1.11 $\pm$ 0.37	1.61 $\pm$ 0.28
72	1.06 $\pm$ 0.14	1.80 $\pm$ 0.10

Table 2 Percentages of completely apoptotic cells (mean $\pm$ SD) among CHO-K1 cells exposed to gamma rays using Comet assay. Data are mean of results from three independent experiments.

Time (h)	Dose (Gy)	
	8 Gy	16 Gy
24	0.51 $\pm$ 0.01	0.67 $\pm$ 0.02
48	2.23 $\pm$ 0.12	3.06 $\pm$ 0.18
72	0.87 $\pm$ 0.03	4.80 $\pm$ 0.04

Table 3 Percentages of apoptotic cells (mean $\pm$ SD) among CHO-K1 cells exposed to gamma rays using TdT assay. Data are mean of results from three independent experiments.

Time (h)	Dose (Gy)	
	8	16
24	0.63 $\pm$ 0.24	1.44 $\pm$ 0.43
48	9.47 $\pm$ 1.77	21.07 $\pm$ 2.00
72	24.18 $\pm$ 6.85	44.11 $\pm$ 4.52

apoptosis. The time required for apoptosis depended on the kinds of cells and agents. The purpose of classifications 'C' and 'U' here was to explain why there were differences in data, especially at 48 h and 72 h between the two methods. The differences were greater at later times because at the late stage of the apoptotic process, especially at high doses of irradiation, most of the small DNA strand-breaks had migrated far away from the comet head even before electrophoresis. In such a case, the fluorescent density of the comet became too dim to be recognized (8, 14). As a result of that, apoptotic frequencies detected at 72 h by Comet assay are lower than those at 48 h, whereas apoptotic frequencies detected at 72 h by *TdT* assay are much higher than the frequencies at 48 h. Our results were in accordance with some others (13, 15). The results indicated that the Comet assay is suitable for apoptotic detection at an early stage. Meanwhile, the *TdT* assay has an advantage in apoptotic detection at a late stage (complete apoptosis), compared with the Comet assay. Although the *TdT* assay is more sensitive than the Comet assay, the importance of detecting the early stage of apoptosis should be emphasized because apoptotic cells become permeable (like necrotic cells) or break up into many apoptotic bodies, making their detection more complicated (16).

4. Conclusions

In summary, the neutral Comet assay is a suitable method for apoptosis detection in CHO-K1 cells induced by gamma rays at early stages of the process. The Comet assay can be applied to investigate apoptosis in various cells at 24 h as a useful tool. The method can also visualize the degree of apoptotic DNA fragmen-

tation in individual cells. Moreover, compared with other methods such as *TdT* assay, the Comet assay has the advantages of simplicity and economy.

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