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Limitations of the single-cell gel electrophoresis assay to monitor apoptosis in U937 and HepG2 cells exposed to 7β-hydroxycholesterol

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Abstract

The single-cell gel electrophoresis (comet) assay is a method which allows the detection of DNA strand breaks in individual cells. It has been suggested that the single cell gel electrophoresis assay, as an index of DNA fragmentation during cell death, may be applied to monitor apoptosis. The aim of the present study was to determine if the pattern of DNA fragmentation determined by the single cell gel electrophoresis assay can be used to discriminate between the mode of cell death in two cell lines (U937, a human monocytic blood cell line and HepG2, a human hepatocarcinoma cell line) which were treated with 30 μM 7β-hydroxycholesterol (7βOHC) over a 48 hr period. The single cell gel electrophoresis assay was compared with more established methods for the determination of apoptosis such as morphological examination, flow cytometry and DNA laddering. The percentage of maximally damaged nuclei as measured by the single cell gel electrophoresis assay was found to be similar at 48 hr in both U937 and HepG2 cells when treated with 7βOHC. However, morphological examination, flow cytometry and DNA laddering techniques showed that 7βOHC induced apoptosis in U937 cells but not in HepG2 cells. Thus, although the alkaline single cell gel electrophoresis assay detected DNA strand breaks occurring during cell death, these breaks were observed only when the process was fairly well advanced and a major part of the cells had lost membrane permeability. Therefore the present report demonstrates that the single cell gel electrophoresis assay, used in isolation, cannot accurately be used to distinguish between the mode of cell death induced by 7βOHC in U937 cells (apoptosis), or HepG2 cells (cell lysis).

Keywords: Oxysterol; Apoptosis; Cell death; Comet assay; U937 cells; HepG2 cells

1. Introduction

Apoptosis is an active, physiological process of cell death, which occurs in an orderly, controlled manner [1]. During the process of apoptotic cell death the nuclear chromatin becomes condensed, the cytoplasm shrinks and blebs appear on the surface of the cell membrane. The DNA is cleaved to nucleosome-sized fragments of approximately 180 base pairs, which results in the formation of a ladder-like pattern when the DNA is subjected to electrophoresis in an agarose gel [2]. This ladder-like pattern is regarded as the biochemical hallmark of apoptosis [3]. A number of the other assays used to measure apoptosis also do so by detecting DNA fragmentation. Such assays include flow cytometry, which measures hypodiploid cells and the TUNEL assay which labels free DNA ends *in situ* using terminal deoxynucleotidyl transferase [4].

The single cell gel electrophoresis, or comet, assay is a rapid technique for the direct visualisation of DNA fragmentation in individual cells and has been suggested as a method for the detection of apoptosis [5–7]. The single cell gel electrophoresis assay may be carried out under alkaline conditions (pH < 10) which detects strand breaks and alkali labile sites, or using a neutral pH, which is believed to detect mostly double strand breaks. However, it has been noted that agents which cause extensive single strand breaks can also produce comet tails in the neutral assay, due to relaxation of DNA supercoiling [8]. The extensive fragmentation of DNA which occurs during apoptosis allows most of the DNA to be drawn away from the nucleus during electrophoresis while the nucleus itself becomes smaller. Nuclei which are considered to be apoptotic are therefore distinguished from undamaged nuclei (which are intact and

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Abbreviations: TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling of fragmented nuclear DNA *in situ*; 7βOHC, 7β-hydroxycholesterol; EtBr, Ethidium bromide.

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spherical) by their distinctive appearance, as there is almost total migration of DNA from the nucleus (comet head) into the comet tail [7,9,10]. However, not all comets that have this distinctive appearance result from cells that have undergone apoptosis [11].

The single cell gel electrophoresis assay is a powerful tool for the measurement of both DNA strand breaks and oxidative base damage and has recently become available commercially for these purposes (Trevigen). The single cell gel electrophoresis assay is also being used increasingly to measure the occurrence of apoptosis [6,12,13]. Indeed, the application notes for the commercially available kit suggest that the assay can be used to measure the occurrence of apoptosis. The aim of this study was to compare the effectiveness of the single cell gel electrophoresis assay as a measure of apoptosis, with the more established methods such as DNA ladder detection on agarose gels, flow cytometry and the morphological examination of nuclei after Hoechst 33342 staining. The oxysterol, 7β-hydroxycholesterol (7βOHC) was employed to induce cell death. 7βOHC is not genotoxic *per se* [14] and has previously been shown to induce apoptosis and cell lysis in a number of cell lines [15–17]. The oxysterol is commonly found in foods of animal origin and is especially concentrated in highly processed foods, such as powdered egg yolks [18]. This study was undertaken in two human cell lines, U937, a human monocytic cell line which has previously been shown to undergo apoptosis when treated with oxysterols including 7βOHC [15,17] and HepG2, a human hepatoma cell line. The alkaline version of the single cell gel electrophoresis assay was used for this study.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. unless otherwise stated. Tissue culture plastics were supplied by Costar. Information on the purity of the 7βOHC (purity >95%) was obtained from the Sigma Chemical Co. Cell lines were obtained from the European Collection of Animal Cell Cultures.

2.2. Maintenance of cell lines

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Human hepatoma HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 1% non-essential amino acids. The cells were grown at 37°/5% CO_2 in a humidified incubator. Both cell lines were screened for mycoplasma contamination by the Hoechst staining method [19] and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

2.3. Treatment of cells with oxysterols

HepG2 cells and U937 cells were adjusted to a density of 1×10^5 cells/mL and 7βOHC was added to the tissue culture medium to give a final concentration of 30 μM. 7βOHC was dissolved in ethanol for delivery to cells. Control cultures contained ethanol only, the final concentration in the cultures did not exceed 0.3% (v/v). Cells were seeded into either 6-well culture dishes for morphological analysis of nuclei or 10-cm dishes (HepG2 cells) and 25 cm^2 flasks (U937 cells) for DNA fragmentation analysis by gel electrophoresis. Samples were incubated for up to 48 hr at 37°/5% CO_2. In the case of HepG2 cells, both floating and attached cells were collected for analysis in each assay.

2.4. Cell viability

The viability of the cells was assessed over 48 hr by the fluorochrome-mediated viability assay as previously described [20]. Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate (FDA) and ethidium bromide (EtBr), then incubated at 37° for 5 min before being layered onto a microscope slide. Under these conditions, live cells fluoresce green, whereas dead cells fluoresce red. Samples were examined at 200X magnification on a Nikon fluorescence microscope using blue light (450–490 nm). 200 Cells were scored from each slide and cell viability was expressed as the percentage of viable (green) cells.

2.5. Determination of cellular glutathione levels

The cellular level of glutathione was measured according to the method of Hissin & Hilf [21]. Briefly 4×10^6 cells were centrifuged at 100,000 *g* for 25 min. The supernatant was diluted in phosphate-EDTA buffer (pH 8) and mixed with 1 μg/μL solution of *o*-phthalaldehyde. After incubation at room temperature for 15 min, the fluorescence at 420 nm was detected after activation at 350 nm. Protein was determined by the bicinchoninic acid (BCA) method [22].

2.6. Single cell gel electrophoresis assay (comet assay)

The single cell gel electrophoresis assay was performed as previously described [14,23]. Briefly 30 μL of a single cell suspension was embedded in low melting point agarose on a microscope slide. Samples were then placed in lysis solution [containing 2.5 M NaCl, 100 mM EDTA, 10 mM *tris*(hydroxymethyl)aminomethane, 1% (v/v) Triton X-100, and 10% (v/v) dimethyl sulphoxide, pH 10] for 1 hr at 4°, followed by 40 min in alkaline solution (1 mM EDTA/300 mM NaOH) at 4°, to allow DNA unwinding to occur. Electrophoresis was performed, without changing the alkaline solution, at 20 V for 25 min. The slides were neutralised

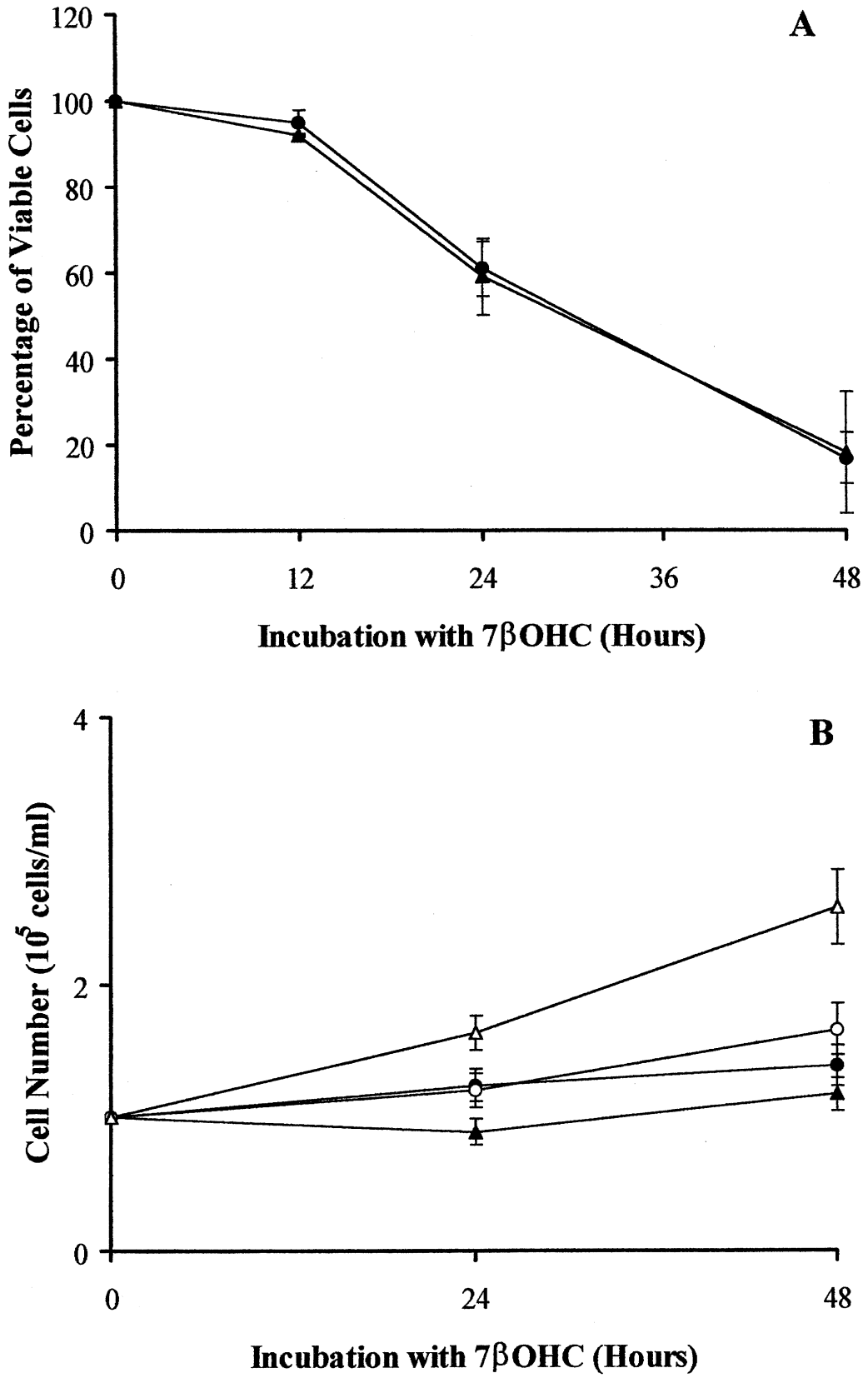

Fig. 1. Cytotoxic effects of 7β-hydroxycholesterol (7βOHC) on U937 cells (triangles) and HepG2 cells (circles). Viable cells (A) were determined using the FDA (fluorescein diacetate)/EtBr method as described in materials and methods and expressed as a percentage of the untreated control cell samples. Cell number (B) was determined by haemocytometer counting. Samples were incubated for either 12, 24, or 48 hr (A) or 24 and 48 hr (B) in the presence (black symbols) or absence (open symbols) of 30 μM 7βOHC. Each point represents the mean $\pm$ SE of data from at least three independent experiments.

Table 1
Effect of a 12-hr incubation with 7β-hydroxycholesterol (7βOHC) on cellular glutathione levels

	Glutathione (nmol/mg protein)	
Cell Line	Control	30 μM 7βOHC
U937	52.9 ± 3.7	13.4 ± 2.4*
HepG2	50.3 ± 2.7	52.9 ± 5.2

U937 or HepG2 cells were sonicated and centrifuged as described in the materials and methods. σ-Phthalaldehyde was added to the supernatant and fluorescence was detected at 420 nm following activation at 350 nm. Protein was determined by the BCA method. Results represent the mean ± SE of data from at least three independent experiments.
* $P < 0.01$.

(400 mM *tris*(hydroxymethyl)aminomethane, pH 7.4) and stained with ethidium bromide. Slides were coded and nuclei were scored visually under fluorescent light (Nikon, 100X magnification). The level of DNA strand breaks was expressed as arbitrary units. A total of 100 nuclei were scored per slide, there were 2–3 slides per sample and each experiment was repeated 2–3 times. Nuclei were analysed and given a score from 0 (undamaged nucleus) to 4 (severely damaged nucleus). Thus a negative control sample would have a score of 0, ranging to 400 for a maximally damaged sample. This visual method of analysing comet assay slides has been employed by several research groups for a number of years, and has been validated against image analysis systems in both our own and other laboratories [24,25].

2.7. Morphological analysis of cell nuclei

Nuclear morphology of control and oxysterol-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. After oxysterol treatment, approximately 4×10^5 cells were centrifuged at 200 *g* for 10 min to form a pellet. Hoechst 33342 stain (200 μL, 5 μg/mL PBS) was added and the samples incubated at 37°/5% CO_2 for 1 hr. Stained samples were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope 400X magnification). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation [26].

2.8. DNA fragmentation assay

Detection of small DNA fragments was done according to the method of Swat *et al*. [27] with some modifications. Briefly, 2×10^6 cells were harvested and the pellets were lysed with 20 μL of a solution containing 50 mM tris(hydroxymethyl)aminomethane, 10 mM EDTA and 0.5% (w/v) sodium lauryl sarcosinate. RNAse A (0.25 mg/mL) was added and the samples incubated at 50° for 1 hr. The condensate was spun down and proteinase K (5 mg/mL) added. The samples were incubated at 50° for a further hour before being loaded into the wells of a 1.5% agarose gel. A 100–1500bp DNA standard (Promega) was used to assess DNA fragmentation. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M *tris*(hydroxymethyl)aminomethane, 0.45 M boric acid and 2 mM EDTA, pH 8), at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining, and photographed using a digital camera (Kodak).

2.9. DNA labelling and flow cytometric analysis

Cells (2×10^6) were harvested (200 *g*) and the pellet was fixed in 70% (v/v) ethanol overnight at −20°. The cells were then centrifuged and re-suspended in 200 μL of PBS containing 50 μg/mL propidium iodide and 5 Kunitz RNAse at 4° for 60 min. The sheath fluid was ISOTON II balanced electrolyte solution (Coulter). Data acquisition and analysis (10,000 cells) were performed with an Epics Elite Cell Sorter (Coulter) [28].

2.10. Statistics

All data points are the mean values (±SE) of at least 3 independent experiments. Where appropriate, data were analysed by the Student's *t*-test.

3. Results

3.1. Effect of 7βOHC on U937 and HepG2 cell number and membrane permeability

Incubation with 7βOHC (30 μM) was toxic to both U937 and HepG2 cells. Increasing the time of incubation with the oxysterol from 12–48 hr resulted in a decrease in cell viability as assessed by a decrease in the number of cells able to exclude EtBr (Fig. 1A). The cell number in the 7βOHC-treated samples was also decreased relative to the untreated controls (Fig. 1B). Although the oxysterol had similar effects on membrane integrity of the two cell lines, cellular levels of glutathione were influenced differently (Table 1). 7βOHC treatment significantly reduced cellular glutathione levels of U937 cells after 12 hr, but had no effect on HepG2 cells.

3.2. Effect of 7βOHC on U937 cell death

In U937 cells, the percentage of condensed and fragmented nuclei increased with increasing time of oxysterol incubation (Fig. 2A), and was significantly different from control after 12 hr ($P < 0.01$), before cell lysis was evident (Fig. 1A). Extensive DNA laddering, considered to be a hallmark of apoptotic cell death, was demonstrated after 24-hr treatment using agarose gel electrophoresis (Fig. 3). Flow cytometry was also used to determine U937 DNA

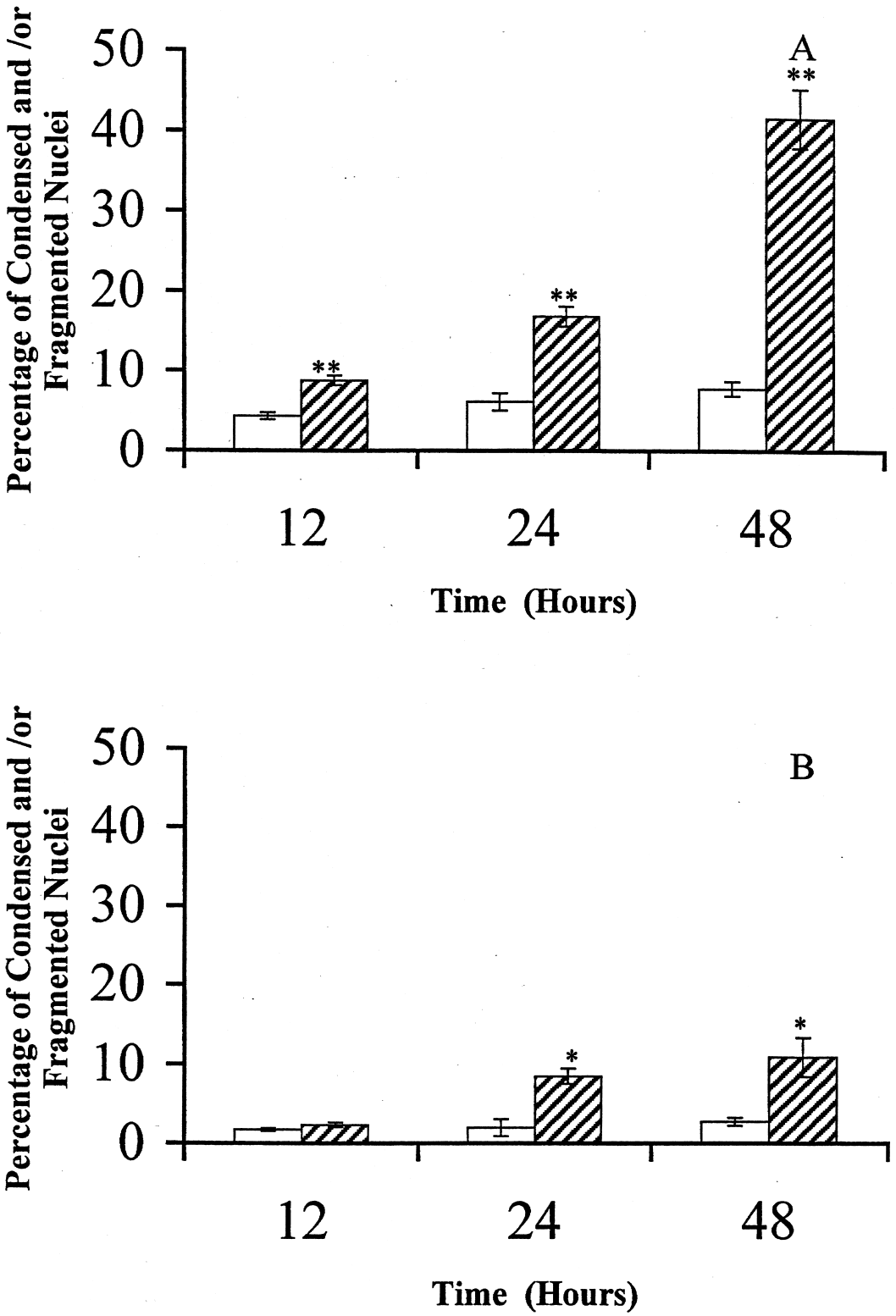

Fig. 2. Generation of condensed and fragmented nuclei by 7β-hydroxycholesterol (7βOHC) in either U937 cells (A) or HepG2 cells (B). Nuclei were stained with Hoechst 33342 as described in materials and methods, and the number of apoptotic nuclei expressed as a percentage of the total number. Samples were incubated for either 12, 24, or 48 hr in the presence (hatched bars) or absence (open bars) of 30 μM 7βOHC. Each point represents the mean ± SE of data from at least three independent experiments. ($*P < 0.05$, $**P < 0.01$).

content. Following incubation with 7βOHC, a sub-G1 peak (found below the peak representing G0/G1 cells) was detected and was significantly different from the control samples ($P < 0.01$). This indicated cells with a hypodiploid

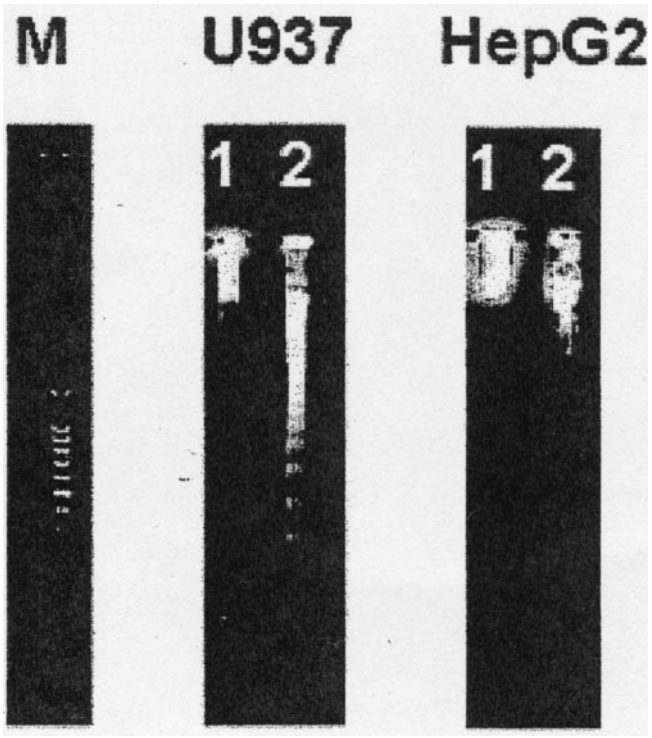

Fig. 3. Induction of DNA fragmentation by 7β-hydroxycholesterol (7βOHC) in either U937 cells or HepG2 cells. DNA was isolated and electrophoresed in 1.5% gels (3 V/cm) as described in materials and methods. After staining with ethidium bromide, gels were visualised under UV light on a transilluminator (312 nm) and photographed using a digital camera (Kodak). Lane 1: control, Lane 2: 30 μM 7βOHC.

DNA content, characteristic of a population undergoing apoptosis. The percentage of cells found in the sub-G1 peak is shown in Table 2.

The single cell gel electrophoresis assay detected a shift over time from mostly undamaged nuclei (class 0 & 1) to mostly extensively or maximally damaged nuclei (class 4; Fig. 4) in U937 cells treated with 7βOHC. The increase in the percentage of maximally damaged comets (class 4) over the 48 hr treatment period agreed well with the results obtained by staining the cells with Hoechst 33342 as shown in Fig. 2A ($r^2 = 0.986$). Previous studies have suggested that these class 4 or maximally damaged nuclei can be produced by apoptotic or necrotic cells [29]. In addition to the distribution of damage throughout the sample, the overall level of DNA damage was also assessed. Although the alkaline single cell gel electrophoresis assay detected DNA strand breaks occurring during cell death (there was a small but significant increase in DNA migration after U937 cells had been incubated with 7βOHC for 12 hr, ie, before cell lysis had occurred) these breaks were extensive only when the process was fairly well advanced and a major part of the cells had lost membrane permeability (Fig. 6).

Table 2
Effect of a 48-hr incubation with 7β-hydroxycholesterol (7βOHC) on the percentage of hypodiploid cells generated in U937 and HepG2 cells.

U937		HepG2	
Control	30 μM 7βOHC	Control	30 μM 7βOHC
2.7 ± 0.3	39.0 ± 4.3*	11.2 ± 3.8	4.7 ± 2.3

U937 and HepG2 cells were harvested, fixed and stained as in the materials and methods. Hypodiploid cells were determined by flow cytometry. Results represent the mean ± SE of data from at least three individual experiments.

* $P < 0.01$.

3.3. Effect of 7βOHC on HepG2 cell death

Although 7βOHC was as cytotoxic to HepG2 cells as it was to U937 cells (Fig. 1), no DNA laddering was evident in the liver cells (Fig. 3) after a 24 hr incubation period with the oxysterol. DNA ladders were still not visible even after 48 hr incubation with the oxysterol (data not shown). For the determination of DNA content by flow cytometry, both floating and attached HepG2 cells were collected for analysis and the proportion with a sub-G1 DNA content determined. There was no difference between the percentage of cells in the sub-G1 peak either in the presence or absence of 7βOHC treatment (Table 2).

There was a significant increase in the percentage of condensed and fragmented nuclei in the 7βOHC-treated HepG2 cells after 24 hr and 48 hr treatment, but not after 12 hr (Fig. 2B). However these values (percentage increase) were considerably lower compared to those obtained in U937 cells (Fig. 2A). In 7βOHC-treated HepG2 cells, the single cell gel electrophoresis assay detected an increase in the extent of DNA strand breaks and the percentage of maximally damaged nuclei (Fig. 5) over time and the correlation with Hoechst staining as shown in Fig. 2B was good ($r^2 = 0.998$). However the single cell gel electrophoresis assay detected almost twice the number of maximally damaged (class 4; approx 20%) comets compared to the Hoechst method of detecting condensed and fragmented nuclei (approx 10%). Also, in contrast to the U937 cells, there were more undamaged (class 0) HepG2 nuclei present after a 48-hr incubation with 7βOHC.

When the overall level of DNA damage was considered (arbitrary units, Fig. 6) there was little difference between the values obtained for HepG2 cells and those obtained from U937 cells, despite the fact that the U937 cells had undergone apoptosis and the HepG2 cells had apparently lysed. From these data alone it was not possible to determine which of the two cell types had undergone apoptosis.

4. Discussion

7βOHC is an oxysterol commonly found in highly processed foods and human plasma. Although not genotoxic *per se*, oxysterols can be extremely toxic to different cell types, causing cell lysis and apoptosis to occur [14,30,31]. In the present study, similar results were obtained for cell

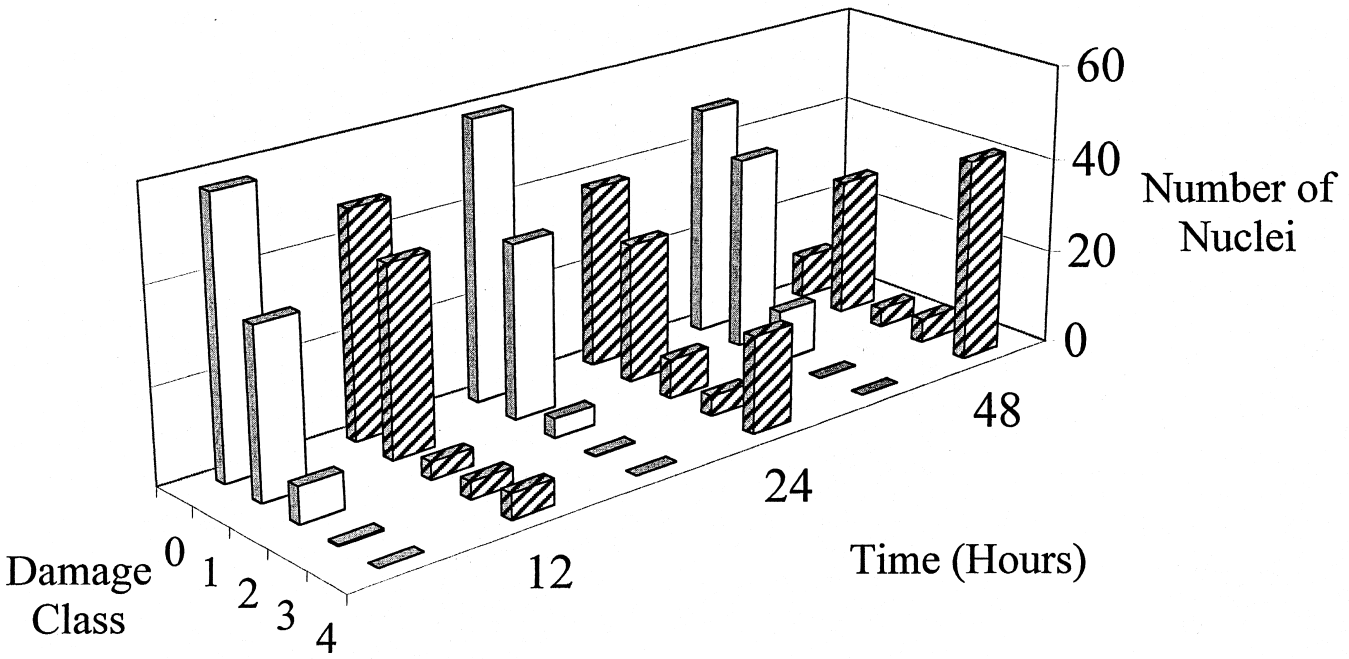

Fig. 4. Distribution of DNA strand breaks induced by treatment with 7β-hydroxycholesterol (7βOHC) in U937 cells. Samples were incubated for either 12, 24, or 48 hr in the presence (hatched bars) or absence (open bars) of 30 μM 7βOHC. Samples were processed for the single cell gel electrophoresis assay as described in materials and methods, stained with EtBr, and classified into five different categories of damage. Damage classification ranged from undamaged (0) to maximally damaged (4). Each point represents the mean $\pm$ SE of data from 4 independent experiments.

membrane permeability and DNA strand breakage when both U937 and HepG2 cells were treated with 30 μM 7βOHC. However the mode of cell death in the two cell lines was clearly different. The results obtained suggest that 7βOHC induced apoptosis only in the U937 cell line. This was concluded from the presence of DNA laddering, the depletion of glutathione, believed to be an early event in oxysterol-induced apoptosis [32], the increase in the percentage of hypodiploid cells and the increase of condensed and fragmented nuclei in U937 cells exposed to 7βOHC. In

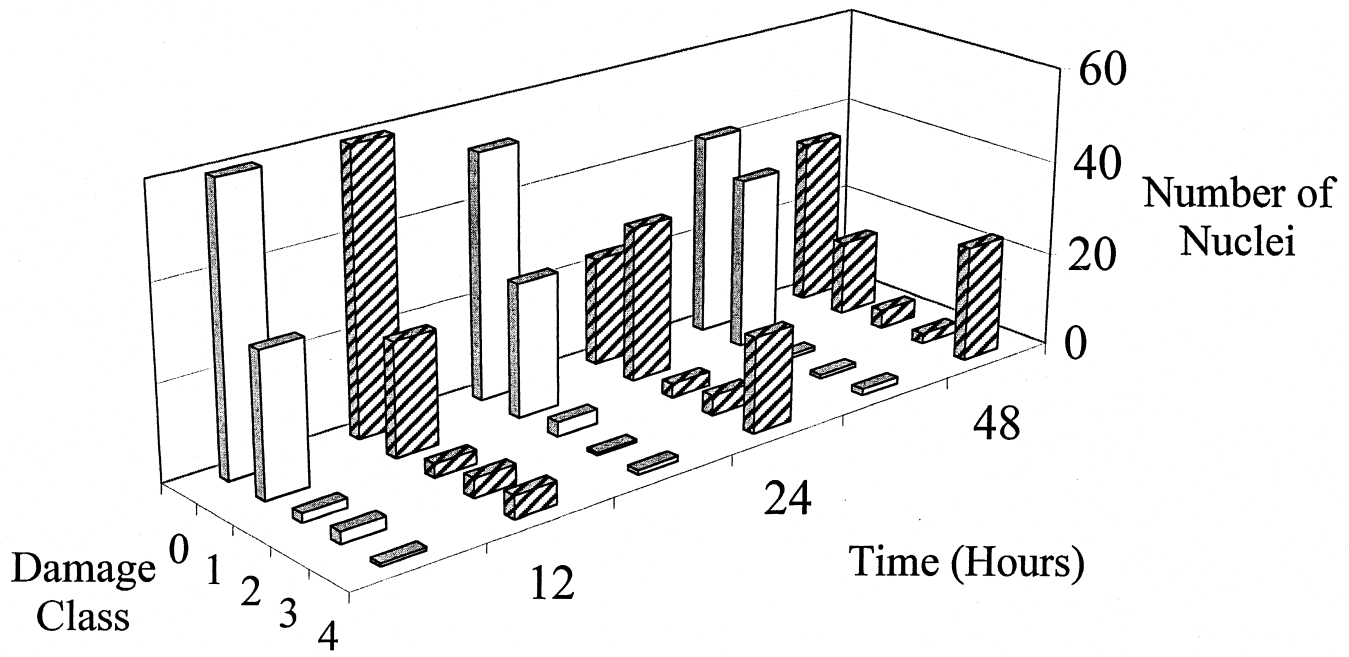

Fig. 5. Distribution of DNA strand breaks induced by treatment with 7β-hydroxycholesterol in HepG2 cells. Legend as for Fig. 4.

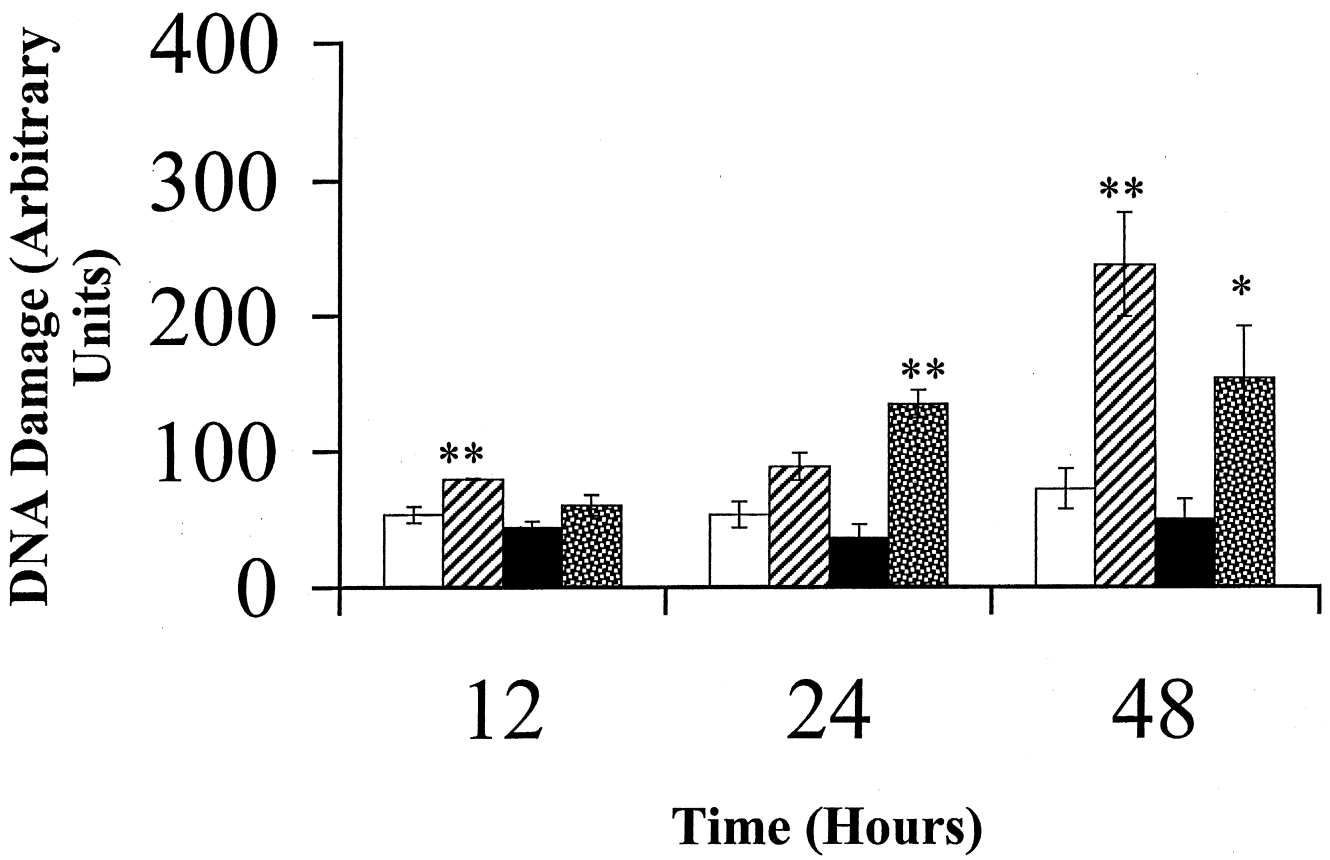

Fig. 6. Level of DNA strand breaks (arbitrary units) as determined by the single cell gel electrophoresis assay following treatment with 30 μM 7β-hydroxycholesterol (7βOHC). DNA damage was assessed as described in materials and methods. Arbitrary units were calculated according to the equation ($1n_1 + 2n_2 + 3n_3 + 4n_4$), where N = the number of nuclei in the respective DNA damage category. U937 control cells (open bars); U937 cells incubated with 7βOHC (hatched bars); HepG2 control cells (solid bars); HepG2 cells incubated with 7βOHC (stippled bars). Each point represents the mean ± SE of data from 4 independent experiments ($*P < 0.05$, $**P < 0.01$).

contrast, there was no evidence that 7βOHC had induced apoptosis in HepG2 cells. There was no increase in either hypodiploid cells, condensed and fragmented nuclei, nor was there DNA laddering evident. 7βOHC appears to be one of the most cytotoxic oxysterols and has been shown to induce either apoptosis or necrosis depending on the cell type [16,17,33]. Liver cells appear to be especially sensitive to killing by 7βOHC. Derivatives of this oxysterol have been shown to delay the development of hepatocarcinoma in mice [34] while in culture, proliferating liver epithelial cells and fibroblasts, as well as hepatoma cells were found to be lysed by treatment with 7βOHC (50–80 μM) [35,36].

In the present report, we used the single cell gel electrophoresis assay in an attempt to distinguish between different types of cell death, as cell death is accompanied by DNA fragmentation, either randomly or non-randomly. As the single cell gel electrophoresis assay has been reported to be a good method of detecting apoptosis, not only as a result of its early detection of strand breaks, but also because of the distinctive shape of the comets produced [29] we had hoped to see an increase in DNA strand breaks prior to cell lysis in U937 cells undergoing apoptosis. A small increase in DNA strand breaks was detected in U937 cells (Fig. 6). However, we were unable to distinguish the mode of cell death between the two cell types, based solely on the data obtained from either total DNA damage (arbitrary units) or class 4 nuclei.

The single cell gel electrophoresis assay has been suggested as an economical and simple alternative to the more established methods for the early detection of apoptosis *in vitro* with the advantage that it allows the detection of apoptosis in the individual cell [6,13]. Olive *et al*. [7] compared both the neutral and the alkaline versions of the single cell gel electrophoresis assay to conventional agarose gel electrophoresis and flow cytometry in TK6 human B lymphoblast cells which had been induced to undergo apoptosis by exposure to radiation. These workers showed that both the neutral and alkaline single cell gel electrophoresis assay produced similar results which correlated well with the results obtained by flow cytometry. Since then, the single-cell gel electrophoresis assay has been adopted as a method to measure apoptosis in a number of cell types. However there have been some reports suggesting a poor correlation between this assay and other methods of detecting apoptosis. Siles *et al*. [37] compared the single cell gel electrophoresis assay with the TUNEL assay and agarose

gel electrophoresis as a measure of radiation induced apoptosis in eight human cancer cell lines and also found no correlation between the single cell gel electrophoresis assay and the other assays used in identifying apoptosis. A second study by Mars *et al*. [38] using human peripheral blood lymphocytes also showed large differences between strand breaks identified by the comet assay (10%) and by the TUNEL method (63%). The results reported in the present study also showed discrepancies between the comet assay and other methods of detecting apoptosis, particularly for the HepG2 cells. A recent review by Tice *et al*. [39] has also pointed out that determination of the mode of cell death using the comet assay, based on comet shape alone, may be an oversimplification. Vasquez and Tice [40] have demonstrated that in terminally apoptotic or necrotic cells, the DNA is so extensively fragmented that under the electrophoretic conditions of the comet assay it is simply being electrophoresed out of the gel, thus underestimating the population of these cells. These workers have developed a neutral diffusion assay [41] which does not rely on an electrophoresis stage to detect dying cells, but have been unable to distinguish between apoptotic and necrotic nuclei.

In conclusion, the single cell gel electrophoresis assay is a powerful tool for the measurement of DNA strand breaks and oxidatively damaged sites. Moreover, its flexibility as an assay looks to be extended with application of techniques such as staining with bromodeoxyuridine, or fluorescence in-situ hybridisation. However the present report supports the work of others in suggesting that use of this assay alone, at a time when the DNA cleavage processes during cell death are still not fully delineated, may not be accurate enough to determine whether a cell population is undergoing apoptosis or necrosis, particularly in non lymphocyte or lymphocyte-derived cell types. The present study demonstrates that the single cell gel electrophoresis assay alone was unable to distinguish between 7βOHC-induced apoptosis in U937 cells and necrosis in HepG2 cells.

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