

Analysis of DNA Fragmentation in Epidermal Keratinocytes using the Apoptosis Detection System, Fluorescein



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Apoptosis - programmed cell death - encompasses the controlled death of a variety of cells, both in development and in adult-stage tissues, and it has evolved into a popular and fast-growing field of study. In this report, we used the Apoptosis Detection System, Fluorescein, available from Promega, for the specific detection and quantitation of apoptotic keratinocytes in populations of apoptotic and nonapoptotic cells.

Introduction

The epidermis is a multilayered epithelial cell layer that is composed primarily of keratinocytes. Differentiated keratinocytes are continuously lost from the surface of the epidermis and are replaced by proliferating basal keratinocytes. Stratified squamous epithelia must maintain a balance between proliferation and death (reviewed in reference 1). *In vivo*, terminal differentiation of stratified squamous epithelia is characterized by numerous morphological changes. Ultimately, keratinocytes in the upper layers of the epidermis become enucleated and lose their intracellular organelles. The specific molecular mechanisms that govern the terminal differentiation and enucleation of keratinocytes are poorly understood. Recent studies (2-4) suggest that terminal differentiation in the epidermis may constitute a specialized form of apoptosis. However, the role of apoptosis in the maintenance of human epidermal tissue remains unknown.

Apoptosis refers to specific morphological changes that occur during the orderly self-destruction of cells. The morphological changes include condensation and compartmentalization of nuclear and cytoplasmic material into structurally-preserved, membrane-bound fragments or blebs (reviewed in reference 5). Apoptosis is distinct from necrosis, another form of cell death, where mitochondrial swelling and leakage of lysosomal enzymes occurs. In addition to the morphological hallmarks of apoptosis, a biochemical marker of apoptosis is the formation of a distinct pattern of the genomic DNA, which can be visualized in gel electrophoresis with ethidium-bromide staining, and resembles a "ladder" (6). Cleavage between the nucleosomal bundles of genomic DNA results in multiples of approximately 200bp in length that comprise the "rungs" of the DNA ladder.

Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick End-Labeling, or TUNEL, is frequently used to detect breaks in DNA which are characteristic of apoptosis (2). TdT is used to add dUTP that is conjugated to a detection molecule, such as biotin or digoxigenin, to the 3'-end of single-stranded DNA. Fragmented DNA then can be detected by adding streptavidin or a secondary antibody that recognizes the labeled nucleotide. Although simple to perform, this two-step detection system can be problematic due to reduced positive signal detection and increased nonspecific staining from the secondary detection molecule. The Apoptosis Detection System, Fluorescein, available from Promega (Cat.# G3250), eliminates these problems by using fluorescein-12-dUTP^{**}. The Apoptosis Detection System, Fluorescein combines all the necessary reagents in a one-step TUNEL assay for detecting fragmented DNA. Here we report the detection of apoptotic cells in human neonatal foreskin tissue sections and monolayers of cultured human keratinocytes, and FACSTM analysis of human keratinocytes following suspension. In this study, we modified the system protocol (7) in order to optimize the detection of apoptosis in cultured keratinocytes and epidermal tissues.

***Manufactured for Promega by NEN[®] Life Science Products under U.S. Pat. Nos. 5,047,519 and 5,151,507.*

Detection of apoptosis in epidermal tissue sections

Human neonatal foreskin was treated with a halogenated aromatic hydrocarbon known to induce apoptosis in thymocytes (8) for 24 hours prior to fixation. Following overnight fixation in 4% paraformaldehyde and dehydration in a series of solutions of increasing alcohol concentration (30%, 50%, 70%, 85%, 95% and 100%, two times), the pre-treated tissue was embedded in ParaplastTM X-tra Tissue Embedding Media. Sections of the embedded tissue (6 μ m) were placed onto gelatin-coated slides. To prepare for incubation with the labeling reagents, the paraffin was removed from the tissue sections by submersion in xylene two times, for 10 minutes each. Tissue sections were rehydrated by sequential placement in a series of solutions of decreasing alcohol concentration (100%, two times, 95%, 85%, 70%, 50% and 30%) for 3 minutes each. Following a 5-minute rinse with 0.85% NaCl, the sections were re-fixed for exactly 20 minutes in 4% paraformaldehyde. The tissue was permeabilized with 100 μ l of 20 μ g/ml Proteinase K diluted in 50mM Tris, pH 7.5, and 5mM EDTA. Tissue sections were treated with Proteinase K solution for exactly 7.5 minutes and then were submersed in PBS for 5

minutes to remove residual enzyme. The timing of Proteinase K digestion is critical to ensure adequate permeabilization for labeling while preserving tissue architecture.

After a post-fixation in 4% paraformaldehyde and a rinse in PBS, 5 minutes each, the tissue sections were incubated in 80 μ l of Equilibration Buffer for 10 minutes at room temperature. During the equilibration, the reaction buffer was prepared by combining 90 μ l of Equilibration Buffer and 10 μ l of Nucleotide Mix being careful to protect both from exposure to direct light. Two microliters of TdT were added to the reaction buffer just prior to use. The Equilibration Buffer was removed by gentle aspiration and replaced with 50 μ l of the TdT reaction buffer. For the TdT reaction, the slides were covered with aluminum foil and incubated at 37°C for 1 hour in a humidified chamber. A second set of tissue sections was incubated with 50 μ l of the reaction buffer without TdT as a negative control. As a positive control, a third set of slides was treated with 1 μ g/ml of RQ1 RNase-Free DNase (Cat.# M6101) for 10 minutes at room temperature to induce nonspecific breaks in the DNA as substrate for the Apoptosis Detection System, Fluorescein. While protecting the sections from direct light, the reaction was stopped by immersing the slides in 20mM EDTA, instead of 2X SSC as stated in the system protocol (7), at room temperature for 10 minutes. The sections were counterstained for 10 minutes with the DNA-specific dye Hoechst 33258 (1 μ g/ml in PBS), which intercalates DNA in all nuclei. The slides were washed with PBS, and a small amount of VECTASHIELD[®] mounting medium was placed on each section to preserve fluorescence and prevent rapid photobleaching. The tissue sections were covered with glass coverslips and sealed with clear nail enamel.

Tissue sections were examined using a Nikon[®] fluorescent microscope equipped with an epi-illumination single band emitter filter cassette for the separate illumination of green (FITC) and blue (ultraviolet) fluorescence. Fluorescein-12-dUTP, once conjugated to the 3'-OH ends of fragmented DNA, stains the nuclei of apoptotic cells green. Microscopic images were captured with an Image Point[™] cooled CCD Video camera and Image-Pro Plus[™] software on a Gateway 2000[®] computer. Figures 1 and 2 were created from the digitized images using Adobe Photoshop[™] software.

Figure 1 presents representative slide sections of human neonatal foreskin treated with the halogenated aromatic hydrocarbon (xenobiotic) to induce apoptosis, and processed for DNA strand breaks using the Apoptosis Detection System, Fluorescein. **Figure 1B** shows the green fluorescent staining of apoptotic nuclei following pre-treatment with the xenobiotic. The inset shows a 3X enlargement, of the panel image, of apoptotic cells in the tissue (marked by the white arrow). **Figure 1C** shows the same area of **Figure 1B** except an ultraviolet filter was used to illuminate the blue fluorescence from all nuclei stained with Hoechst 33258. As evidenced by this panel, there are a number of cells in the tissue that are not fluorescein-labeled as in **Figure 1B**. There is specific detection of apoptotic nuclei as evidenced by the minimal background staining in tissue sections incubated without TdT enzyme (**Figure 1E**) even though there are many cells in the microscopic field (**Figure 1F**). All of the cells in sections treated with RQ1 RNase-Free DNase are fluorescently tagged (compare Panels H and I).

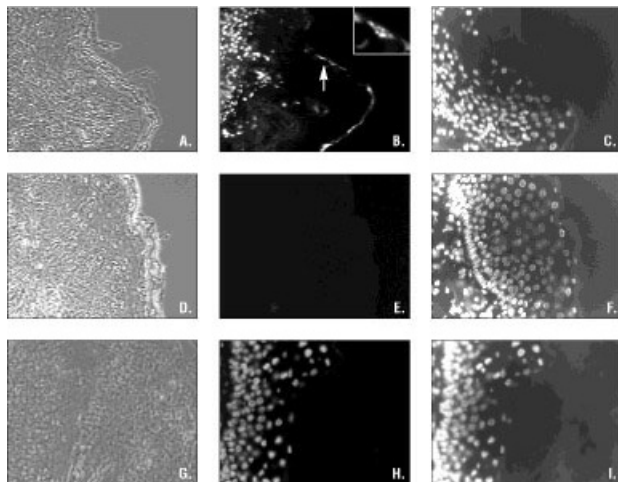


Figure 1. *In vivo* analysis of DNA fragmentation in human neonatal foreskin. Xenobiotic-treated human epidermal sections (6 μ m) were processed according to the protocol as outlined in the text. DNA strand breaks were treated with fluorescein-12-dUTP at the 3'-OH ends in TdT reaction buffer at 37°C for one hour with TdT (Panels A-C and G-I) or without TdT (Panels D-F). Sections in Panels G-I were treated with RQ1 RNase-Free DNase as a positive control. Tissue sections are positioned with the dermis to the left and the epidermis to the right. Fluorescent images were viewed using an epi-illumination single band emitter filter cassette to illuminate phase contrast (Panels A, D and G), fluorescein (Panels B, E and H) and Hoechst 33258 (Panels C, F and I).

Detection of apoptosis in cultured keratinocytes

Propidium iodide (PI), which is selectively taken up by cells that have lost membrane permeability, is used routinely to distinguish dead cells from apoptotic cells. However, whether apoptotic cells actually lose membrane permeability is in dispute. It is advantageous to fix cells after a specific treatment, but addition of nucleotide-specific PI makes it cumbersome to identify a small population of apoptotic

cells in a high background of viable cells. The Apoptosis Detection System, Fluorescein alleviates such problems by fixing the cells immediately after treatment and specifically labeling those cells with DNA strand breaks on the 3'-ends.

While DNA fragmentation is a very rare event in nonconfluent, adherent cultures, the loss of adhesion associated with cultures in suspension (in semi-solid medium) is a potent inducer of apoptosis in normal human keratinocytes (4,9). However, we have found that confluent adherent cultures exhibit an increase in DNA fragmentation compared to nonconfluent control cultures using a 3'-end-labeling technique (9). Although quite sensitive, this technique requires radioactive materials which can be costly and inconvenient. The Apoptosis Detection System, Fluorescein is a more cost-effective reagent that is able to detect the increase in DNA fragmentation associated with confluent human keratinocytes in culture.

Human keratinocytes were cultured on glass coverslips for 5 days after reaching confluency. The cell-coated coverslips were rinsed three times with PBS and fixed with 4% paraformaldehyde at 4°C for 20 minutes. Coverslips were rinsed with PBS and the cells were made permeable by incubating in 0.2% Triton[®] X-100 in PBS at 4°C for 15 minutes. A one-step fixation and permeabilization procedure of a 3:1 solution of acetic acid: methanol (v/v) was found to increase background fluorescence, and, therefore, was abandoned. Following permeabilization, we included a 10-minute incubation at room temperature with DNase-free RNase ($\geq 50\mu\text{g/ml}$; Qiagen). This optional step is important if samples are to be counterstained. PI intercalates RNA as well as DNA making it difficult to visualize the green fluorescence in a high background of orange (Figure 2B). To avoid this situation, the samples were counterstained with Hoechst 33258.

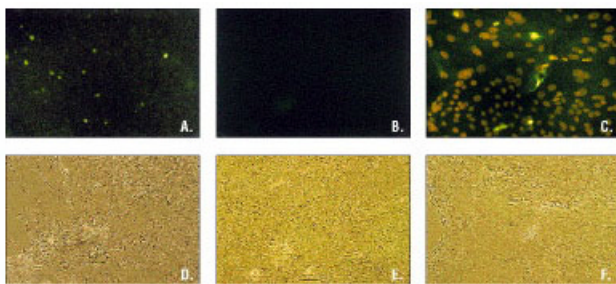


Figure 2. *In vitro* analysis of DNA fragmentation in five-day post-confluent human keratinocytes. Normal human keratinocytes were grown on glass coverslips until five days after reaching confluency. The cells were incubated as described in the text either with TdT (Panels A and C) or without TdT (Panel B). Following terminal transfer of fluorescein-12-dUTP to fragmented DNA, some cells were counterstained with propidium iodide (Panel C). Fluorescent images were viewed as described in Figure 1 for phase contrast (Panels D-F) and fluorescein (Panels A-C).

Cells affixed to coverslips were incubated with 80 μl of Equilibration Buffer at room temperature for 10 minutes. During the equilibration, the reaction buffer was prepared by combining 90 μl of Equilibration Buffer and 10 μl of Nucleotide Mix. Care was taken to protect the reaction buffer containing fluorescein-12-dUTP from exposure to direct light. Two microliters of TdT were added to the mixture just prior to use. The Equilibration Buffer was replaced with 50 μl of the TdT reaction buffer and the samples were incubated at 37°C for 1 hour in a humidified chamber. A set of coverslips was incubated with 50 μl of reaction buffer without enzyme as a negative control (for background fluorescence).

The labeling reaction was terminated by addition of approximately 200 μl of 20mM EDTA. The coverslips were rinsed twice with PBS and once with Milli-Q[®]-pure water to eliminate excess salts on the glass prior to sealing. The coverslips were placed, cell side down, in a drop of VECTASHIELD[®] and sealed to glass slides with clear nail enamel. A small population of cells was labeled specifically by the Apoptosis Detection System, Fluorescein, while cells incubated without the TdT were not labeled (compare Panels A and C to Panel B). A second set of coverslips was counterstained with PI (0.01 $\mu\text{g/ml}$ in PBS) to verify that all cells were being labeled by the fluorescein. PI, in decreasing concentrations from 1 $\mu\text{g/ml}$ to 0.001 $\mu\text{g/ml}$, was tested to optimize the degree of staining (orange) without hindering the fluorescein (green). Figure 2F shows a representative slide of cells counterstained with PI (orange) demonstrating that fluorescein-12-dUTP (green) tags cells that morphologically appear to be apoptotic. All of the cells in Figure 2F stained orange due to the PI. However, only a few cells stained green. Furthermore, cells incubated without TdT were stained by PI and not by fluorescein-12-dUTP.

Detection of apoptosis in keratinocytes in suspension using FACSTM

Flow cytometry using FACSTM is the most rapid and precise method to sort apoptotic and healthy cells in culture, and can be used to separate cells from replicative and differentiating compartments. Because cells remain intact after sorting by FACSTM, markers of differentiation and apoptosis can be readily determined in sorted cells. We used FACSTM analysis to examine whether the Apoptosis Detection System, Fluorescein, would be useful in separating subpopulations of cultured keratinocytes that contain fragmented DNA. Our laboratory has previously demonstrated that suspension cultures of keratinocytes placed in semi-solid medium for 10-24 hours undergo nucleosomal fragmentation characteristic of apoptosis (4). Using these culture conditions for keratinocytes as a method for inducing apoptosis, cells were recovered from serum-free, semi-solid medium by repeated dilution with serum-free medium, and were assayed for fragmented DNA using the Apoptosis Detection System, Fluorescein. Adherent keratinocytes were removed from surface

cultures and treated in an identical fashion as a negative control experiment.

Recovered keratinocytes from adherent and suspension cultures were fixed in 1% formaldehyde for 20 minutes on ice followed by permeabilization with 0.2% Triton[®] X-100 in PBS for 15 minutes on ice. It is critical to use cold reagents for FACS[™] analysis to prevent the keratinocytes from clumping which can be a severe problem in flow cytometric analyses. The cells were rinsed three times with cold PBS and the cell numbers were counted using a hemocytometer. Two million cells were placed in 80 μ l of Equilibration Buffer at room temperature and the reaction buffer was prepared as above. Although cell numbers need not be exact, it is important to control the number of cells in the reaction to ensure that the reagents are not limiting. Following removal of the Equilibration Buffer, each sample was combined with 50 μ l of TdT reaction buffer and incubated in a 37°C water bath for 1 hour. Samples must be protected from direct light, and gentle agitation every 15 minutes is critical to prevent cells from settling out of solution. The reaction was terminated by the addition of 1ml of 20mM EDTA. Samples were washed three times with cold PBS containing 0.1% Triton[®] X-100 and 5 μ g/ml BSA. These washes helped remove excess fluorescein molecules and prevented cell adhesion. After the final wash, cells were resuspended in approximately 500 μ l of wash buffer on ice and analyzed immediately by flow cytometry.

In FACS[™], forward scatter (light intensity) correlates directly with cell size in many cell types, and this parameter has been used successfully to categorize cultured keratinocytes according to their status of differentiation (10). The Apoptosis Detection System, Fluorescein, was tested by FACS[™] to distinguish terminally differentiating cells, with fragmented DNA based on their light scattering characteristics, from all other cells. If a particular population of cells has incorporated the fluorescein-12-dUTP into their DNA, this population should display distinct characteristics of scatter that produce a visible shift in the number of fluorescently-labeled keratinocytes following suspension.

We analyzed keratinocyte samples using an EPICS[®] II flow cytometer in the Flow Cytometry Laboratory, Wisconsin State Laboratory of Hygiene. For FITC measurement, a 525nm band-pass filter and an argon laser were used. The total population of adherent (Panel A) and suspended (Panel B) keratinocytes analyzed by flow cytometry is depicted in [Figure 3](#). Both forward and side-scatter (granularity measure) were measured in arbitrary units on a linear scale. Forward and side-scatter were "gated" to exclude dirt and clumped cells; gating was used identically on all flow cytometry analyses. (Gating is used routinely in FACS[™] analyses to isolate cells of interest.) At least 1×10^6 gated cells were analyzed per treatment. A histogram depicting the number of cells staining positive for fluorescence was established for each treatment and an overlay of each histogram, for adherent and suspension cultures, was created ([Figure 3C](#)). The overlay demonstrates a shift in the number of apoptotic cells following a loss of adhesion.

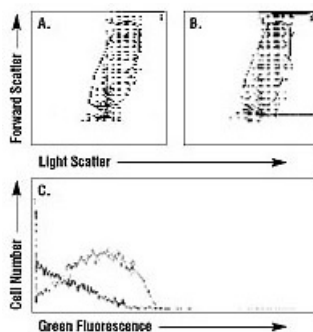


Figure 3. Flow cytometric analysis of DNA fragmentation using human keratinocyte cells from adherent and suspension cultures. Nonconfluent, adherent keratinocytes were treated with serum-free medium, or were removed from surface culture with trypsin and suspended in serum-free medium that was rendered semi-solid with 1.7% methylcellulose, for 18 hours. Panels A and B are representative dot plots of the total population of keratinocytes in adherent (Panel A) or suspension (Panel B) cultures. At least one million cells were gated and analyzed per treatment. Arbitrary values are plotted on a linear scale. Panel C is an overlay of histograms for both adherent (solid line) and suspension (dotted line) cultures. The diagram was corrected for cell number between treatments. Arbitrary fluorescence values are plotted on a logarithmic scale.

Summary

Our *in vivo* and *in vitro* analyses of normal human keratinocytes demonstrate that the Apoptosis Detection System, Fluorescein, is a useful and convenient system for analyzing DNA fragmentation, a biochemical marker of apoptosis. By using TdT, dUTP nucleotides conjugated to fluorescein are transferred to the 3'-OH end of fragmented DNA. This allows for the specific detection of DNA strand breaks characteristic of apoptosis in mixed populations of cells. The system provides all the components, except common laboratory buffers such as PBS, for the detection of apoptosis exploiting the TUNEL assay. We believe that the Apoptosis Detection System, Fluorescein, will become a useful tool in the continuing analysis of apoptosis in epidermal tissues.

Editor's Note: See also the article on Apoptosis that appeared in Promega Notes 57 for additional experiments using the Apoptosis Detection System, Fluorescein.

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Ordering Information

Product	Cat.#
Apoptosis Detection System, Fluorescein	G3250

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