Using Terminal Deoxynucleotidyl Transferase (TdT) Enzyme to Detect TUNEL-Positive, GFP-Expressing Apoptotic Cells

Jennifer Jewell, B.S., and Andrea M. Mastro, Ph.D.
The Pennsylvania State University

Abstract

This article describes a method for adapting the TUNEL assay to a co-culture system where some of the cells express GFP. In addition, all cells can be visualized with a third compatible dye in order to determine the percentage of each cell type undergoing apoptosis.

Introduction

Apoptosis is a naturally occurring cellular event that helps maintain organism homeostasis. A cell can trigger apoptosis in a neighboring cell through signaling processes such as Fas and Fas-Ligand interaction. Human osteoblasts express Fas, and human breast cancer cells may express Fas-Ligand. When breast cancer cells metastasize to the bone, they may induce osteoblast apoptosis through the Fas signaling pathway. We investigated the effects of breast cancer cell-osteoblast interaction in a co-culture system using TUNEL analysis.

During apoptosis, the chromatin condenses against the nuclear envelope, and the DNA inside the nucleus becomes fragmented. Terminal Deoxynucleotidyl Transferase (Cat.# M1871), when used with a fluorescent marker, allows detection of cells with apoptotic DNA fragmentation. TdT is an enzyme that catalyzes the repetitive addition of dNTPs to the 3′-OH end of a DNA fragment. In TUNEL analysis, fluorescently conjugated dUTPs are added to the 3′-OH groups of the DNA fragments, making the apoptotic cells visible by fluorescent microscopy.

Promega's DeadEnd™ Fluorometric TUNEL System (Cat.# G3250, formerly called Apoptosis Detection System, Fluorescein) includes most of the reagents necessary to visualize apoptotic nuclei. However, this system was of limited utility for marking apoptotic cells in a cell line that contains the green fluorescent protein (GFP), because GFP and FITC emissions are nearly impossible to distinguish from each other. By using TdT enzyme separately and creating a nucleotide mix with FluoroLink™ Cy®5 dUTP (Molecular Probes, Cat.# PA 55022), we were able to visualize both GFP labels and apoptotic nuclei.

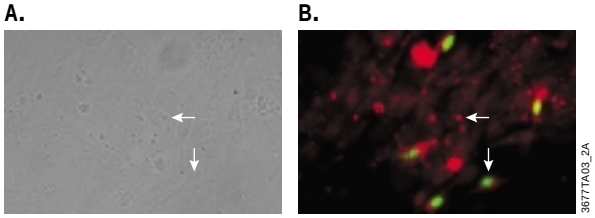

Figure 1. TUNEL assay using Cy®5 dUTP. hFOB cells were differentiated for 3 days and then co-cultured with GFP-MDA-MB-435 breast cancer cells for 48 hours. Cells were treated with TdT incubation buffer and visualized with Cy®5. Staining was specific to apoptotic cells with fragmented nuclei. **Panel A.** Phase contrast image. **Panel B.** Superimposed image of the same field photographed for GFP and Cy®5 TUNEL. White arrows indicate GFP-MDA-MB-435 cells. Yellow arrows indicate hFOB 1.19 cells.

Methods

We developed controls recommended in the *DeadEnd™ Fluorometric TUNEL System Technical Bulletin*, #TB235. Negative slides were prepared by omitting the TdT enzyme from the nucleotide mix, and positive slides were prepared by treating cells with DNase I. DNase I treatment results in positive staining for all cells on a slide by introducing 3′ OH nicks in the cellular DNA. Partial positives can be obtained by inducing apoptosis in cell cultures by treatment with cyclohexamide and staurosporine.

For adherent cells, we adapted the method described in the *DeadEnd™ Fluorometric TUNEL System Technical Bulletin*, #TB235, to produce far-red-labeled fragmented nuclei (Table 1). Figure 1 shows typical Cy®5 staining of apoptotic nuclei using this protocol. To determine the percent of cells undergoing apoptosis, we stained cell nuclei with SYTOX® Orange using the protocol described in Table 2 (Figure 2).

Osteoblast cells, specifically hFOB cells, were seeded on round glass coverslips, coated with 0.5mg/ml gelatin in a 24-well dish. When the cells began to differentiate, GFP-MDA-MB-435 breast cancer cells were added. Some wells received other nonbreast cancer cells or no cells. After 48 hours of co-culture, we performed the TUNEL assay.

Results

The level of apoptosis in the hFOB cells cultured alone ranged from 1 to 10 percent, depending on the assay. Co-culture with MDA-MB-435 cells for 48 hours increased apoptosis levels several fold. The level of apoptosis in the breast cancer cells, distinguished by the presence of GFP fluorescence, remained low (1–2%), regardless of the presence or absence of the hFOB cells.

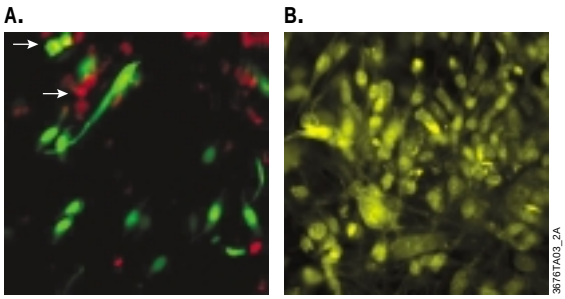

Figure 2. SYTOX® Orange staining of co-cultured hFOB and breast cancer cells. Panel A. TUNEL stained slide of GFP-MDA-MB-435 breast cancer cells on a lawn of hFOB 1.19 cells. The slide was photographed for the two fluorochromes separately and the images superimposed. **Panel B.** The slide was next stained with SYTOX® Orange to visualize all the nuclei in the field. Arrows indicate a TUNEL-positive hFOB (red) and a TUNEL-positive GFP-MDA-MB-435 cell (yellow).

Table 1. Method for Staining Co-Cultured Cells for Apoptotic Nuclei.

1. Remove cell culture medium and wash in PBS.
2. Fix cells with 4% paraformaldehyde in PBS for 20 minutes at 4°C.
3. Wash 3 times for 10 minutes each in PBS.
4. Permeabilize with 0.2% Triton® X-100 in PBS for 5 minutes at room temperature.
5. Wash 3 times for 5 minutes each in PBS.
6. Prepare DNase-positive sample according to the method described in Technical Bulletin #TB235.
7. Remove PBS from cells and add 100µl of Equilibration Buffer for 10 minutes at room temperature.
8. While cells are equilibrating, prepare TdT incubation buffer.
9. Remove Equilibration Buffer and add 50µl TdT incubation buffer. Incubate at 37°C in a dark, humidified chamber for 60 minutes. A plastic coverslip can be placed on top of the round coverslip to evenly distribute the buffer.
10. Add 2X SSC to cells to terminate the reaction. Incubate for 15 minutes at room temperature.
11. Wash 3 times for 5 minutes in PBS.
12. Wash in ultra-pure water and mount on glass slide with VECTASHIELD® Mounting Medium (Vector Labs, Cat.# H-1000).

Summary

This adaptation of the TUNEL protocol provides a useful method for detecting apoptosis with a fluorescent marker in a system where a cell line is expressing GFP. The use of a compatible third stain, such as SYTOX® Orange allows the researcher to determine the percent of apoptotic nuclei in the population of cells.

Table 2. Method for Staining Cell Nuclei with SYTOX® Orange.

1. Remove coverslip from slide by immersing in warm PBS for several minutes.
2. Wash 2 times for 5 minutes each in buffered saline. Do not use a buffer that contains phosphate, as phosphate interferes with SYTOX® Orange staining.
3. Stain cells with SYTOX® Orange at a final concentration of 0.5–1.0µM for 10 minutes at room temperature.
4. Wash 2 times for 5 minutes each in buffered saline.
5. Wash in ultra-pure water and mount on glass slides with VECTASHIELD® Mounting Medium (Vector Labs, Cat.#H-1000).

Table 3. Composition of Buffers and Solutions.

Equilibration Buffer

200mM	potassium cacodylate (pH 6.6 at 25°C)
25mM	Tris-HCl (pH 6.6 at 25°C)
0.2mM	DTT
0.25mg/ml	BSA
2.5mM	cobalt chloride

Nucleotide Mix

100µM	ATP
10mM	Tris-HCl (pH 7.6)
1mM	EDTA
120µM	FluoroLink™ Cy®5-dUTP

Prepare solution in the dark, on ice.
The 120µM concentration of the Cy®5-dUTP fades quickly.

TdT Incubation Buffer (for each 50µl reaction)

45µl	Equilibration Buffer
5µl	Nucleotide Mix
25 units	TdT Enzyme

Protocols

DeadEnd™ Fluorometric TUNEL System Technical Bulletin #TB235, Promega Corporation
www.promega.com/tbs/tb235/tb235.html

Ordering Information

Product	Size	Cat.#
DeadEnd™ Fluorometric TUNEL System[a]	60 reactions	G3250
Terminal Deoxynucleotidyl Transferase*	300 units	M1871
	1,500 units	M1875

*For Laboratory Use.

[a] For Research Use. Products may be covered by pending or issued patents. Please visit our Web site for more information.