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mm; Sigma Chemical, St. Louis, MO, USA) is placed inside the tube and held just above the suspension of cells by two larger magnets (external magnets can be between 1.5 and 2.5 in; Sigma Chemical) held outside of the tube (Figure 1). This enables the small magnet inside to be maintained along the edge of the tube and prevents it from touching the sample port during acquisition. A calibrated drop of agonist, of a volume between 5 and 10 μL , is placed on the magnet inside the tube immedi-

Figure 1. Method of agonist addition using magnetic stir bars. Small magnetic stir bar held above cell suspension inside tube by larger exterior magnetic stir bar. Calibrated drop of agonist is placed on small magnet by pipet once magnet is in place.

Figure 2. Comparison of flow cytometric analysis of Ca^{2+} mobilization using magnet technique to addition of agonist by pipet. Flow cytometric analysis of THP-1 cells loaded with fluo-3 and stimulated with ionomycin. Arrow indicates addition of agonist. Thick line represents addition of ionomycin by pipet. Thin line represents addition of ionomycin by magnet.

ately prior to acquisition. The tube is then quickly placed on the sample port with the side arm of the cytometer in place to acquire data. In Figure 2, the agonist used was ionomycin (4 μg ; Sigma Chemical). After approximately 15–30 s of acquisition of baseline fluorescence, the external magnets are used to lower the magnet inside, allowing the addition of substrate. These magnets may also be used to mix the sample with the cells by moving the small magnet inside the tube. After addition of the agonist, a beaker containing approximately 75 mL of water at 37°C is placed on the side arm to keep the sample warm and stabilize the sample tube on the sample port.

As illustrated in Figure 2, this method of analysis of cellular calcium mobilization allows for the continuous acquisition of data. The ability to observe the entire course of calcium mobilization allows the recording of events that are transient and occur within seconds of addition. This simple method is used routinely in our laboratory to measure calcium mobilization (7) in a wide variety of cells, including both cell lines and primary lymphocytes as well as a wide variety of stimuli, such as chemokines, anti-CD3 and ionomycin, without extensive and expensive modifications of the flow cytometer (3,5).

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Use of Green Fluorescent Protein in Studies of Apoptosis of Transfected Cells

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Apoptosis is a genetically controlled response associated with numerous biological processes, such as organ development, oncogenesis and immune responses (1). Extensive studies have

been undertaken in recent years to understand the molecular basis of apoptosis. Through genetic, biochemical and other approaches, many molecules associated with apoptosis pathways have been identified. Such molecules include CED3, CED4, CED9, Bcl2 family members, RIP, TRADD, MORT1/FADD and ICE protease family members, MACH/FLICE (6). One of the most commonly used approaches contributing significantly to the identification and characterization of the apoptosis-inducing proteins is transient expression of the protein of interest and assessment of whether the protein can induce apoptotic cellular morphological changes and cell death in transfected cells. An essential component of this method is the identification of transfected cells, because most transfection procedures do not deliver functional DNA to a majority of the cells in a monolayer. One commonly used method is to co-transfect the plasmids expressing the protein of interest together with a plasmid expressing LacZ protein (4,7,8). The two plasmids are mixed in various ratios so that most, if not all, cells transfected with LacZ plasmid will also contain plasmid expressing the putative apoptotic or anti-apoptotic gene of interest. At various times after transfection, the cells are fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). The numbers and morphology of blue cells after various experimental permutations are compared to determine what effects the expression of the exogenous proteins has had on the cells. Although the X-gal-staining procedure has become a standard and has served well many important studies of apoptosis, there are a few drawbacks. First, the cells need to be processed using fixatives; consequently, data from only one time point can be collected from any population of cells. Second, the washing steps during fixation and staining often result in the loss of cells, which may affect quantitation of cell killing. Third, cell scoring after fixation is based on fixed (i.e., dead) cells rather than living cells. Thus, it is possible that the fixation or staining process may produce some artifacts. Finally, the fixation and staining take several hours to overnight for the color devel-

opment and visualization of results.

To circumvent these problems, we tested whether we could use the green fluorescent protein (GFP) instead of LacZ as a marker to study apoptosis of transfected cells. GFP is a fluorescent protein originally isolated from jellyfish (11). The protein in the living cells emits green fluorescent light upon excitation by UV light without need for any further processing. GFP does not appear to significantly bleach upon prolonged UV exposure and does not increase cell damage by UV light. The original gene has been mutated to optimize its expression and emission of fluorescent light in mammalian cells (10). The gene has been widely used in many cell biology studies, such as protein distribution, protein trafficking and gene expression (5,11,12). We show that GFP is useful in studying apoptosis in transiently transfected cells. As shown in Figure 1D, 24 h after transfection, the cells transfected with control plasmid (empty vector) together with the GFP-expressing plasmid show normal morphology. The cells are flat and well-attached to the plate. In contrast, the cells expressing the death-inducing proteins, tumor necrosis factor receptor (TNF-R) and Fas (2,3), are all rounded, and some are fragmenting with the small cytoplasmic blebs that are characteristic of apoptosis (Figure 1, E and F). In addition, by 48 h post-transfection, most of the GFP-stained cells in Panels B and C had died and floated away from the cell monolayer. In contrast, Panel A looked essentially the same at 24 and 48 h except that some of the GFP-expressing cells had divided (data not shown).

The use of GFP as a marker to study apoptosis in a transient transfection system offers several advantages over the commonly used LacZ marker. The morphology of the transfected cells can be easily scored on living cells without fixation and staining. Based on morphology, the numbers of normal and apoptotic cells can be readily obtained. Because the cells can be scored under normal conditions of growth, the system can be used to collect data kinetically on the same cells at different time points after transfection, to study the progression of apoptosis and to compare the kinetics of cell death induced

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by different proteins. In fact, we found that the cells that underwent apoptosis and detached from the monolayer still maintained their GFP positivity. This transition of GFP-positive cells from monolayer to medium can also be used as an index for measuring apoptosis. In addition, the system can show the progression of the morphological changes of apoptotic cells, such as the blebbing of cell membranes (Figure 1, E and F). Such kinetic studies of the morphology cannot be achieved in systems using LacZ as a marker. Furthermore, whenever necessary, the transfected cells can be counterstained with propidium io-

dide or other fluorescent cell-staining reagents to study more detailed cell structural changes associated with apoptosis. Transfected cells can also be sorted by flow cytometry on the cell sorter using GFP as an indicator. This procedure is especially useful when more detailed studies such as electron microscopic examinations of the apoptotic cells are required.

In conclusion, we have shown and discussed how the GFP marker system is faster, easier and less expensive than the currently widely used LacZ system in apoptosis studies. GFP is easy to use, and the only equipment required is a

fluorescence microscope, which is widely available in most research institutes.

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Figure 1. Morphological changes of Fas- or TNF-R-transfected cells as revealed by co-transfected GFP. Human embryonic kidney (HEK) 293 cells were transfected with 0.3 μ g of the pAd-Bgl II-GFP plasmid and in: (A and D) 4 μ g pcDNA-T7 of control plasmid not expressing any foreign protein (9); (B and E) 0.5 μ g pcDNA-FLAG-TNF-R (4) and 3.5 μ g pcDNA-T7 plasmid DNAs; (C and F) 0.5 μ g pcDNA-FLAG-Fas (3) and 3.5 μ g pcDNA-T7 plasmid DNAs. Transfections were done using Trans-LT2 reagents (PanVera, Madison, WI, USA) following the manufacturer's protocol. Twenty-four hours after transfection, the cells were observed and photographed with a fluorescein isothiocyanate (FITC) filter using a DiaphotTM 200 Fluorescent Microscope (Nikon, Melville, NY, USA). Panels D, E and F represent the fluorescent images, and the phase images of the same fields are shown in Panels A, B and C, respectively. The arrows in Panels A and D indicate GFP-positive cells. In Panels B and C, the phase and fluorescent images were photographed simultaneously, and the GFP-positive cells are readily visible. pAd-Bgl II-GFP is a plasmid used either for inserting foreign genes for cloning into adenovirus-based vectors or for transient transfections. The coding sequence for GFP was obtained from a humanized GFP vector, pRSGFP-N1 (kindly provided by Dr. Greg Ryan, Albert Einstein College of Medicine) and inserted between the cytomegalovirus (CMV) promoter and the 3' poly(A) site, which have been cloned into pAd-Bgl II (from Dr. M.J. Imperiale, University of Michigan).

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Nonradioactive, Agarose Minigel Procedure for Telomeric Repeat Amplification Protocol

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Telomeres, the ends of chromosomes, are composed of many kilobases of TTAGGG repeats in vertebrates. These repeats are added by the enzyme telomerase, a ribonucleoprotein DNA polymerase that synthesizes the repeats based on its RNA template. A sensitive assay combining telomerase activity and the polymerase chain reaction (PCR) known as the telomeric repeat amplification protocol (TRAP) has been developed (6,10). In this assay, telomerase adds TTAGGG repeats to a telomerase substrate (TS) oligonucleotide that has no telomeric sequence; the enzyme pauses after adding each repeat, presumably to permit repositioning of its RNA template prior to synthesizing the next repeat. This generates a DNA ladder of 6-bp addition products that is then sensitively detected by polyacrylamide gel electrophoresis (PAGE) after PCR amplification (using a second CX oligonucleotide as reverse primer).

Telomerase activity has been detected in tumor, germ and stem cells but not in adult somatic cells. Thus, the enzyme may play a significant role in aging and in the pathogenesis of cancer

(2,6). Because of this widespread interest, improvements to TRAP are continually being developed, e.g., internal standard, fluorescent TS primer and improved CX-ext primer (7,9,12). We report fluorometric detection of TRAP DNA ladders by high-resolution agarose gel electrophoresis.

The human embryonic kidney (HEK) 293T cell line and the human monocytic macrophage Mono Mac 6 cell line were cultured as described (3, 5). Telomerase extracts were obtained from cells by the procedure of Kim et al. (6) with minor modifications (1,10, 11). Cells were counted, harvested and treated with ice-cold TRAP lysis buffer at 10^6 cells/100 μ L buffer. The TRAP lysis buffer contained 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, 25 mM 2-mercaptoethanol, 1 ng/mL leupeptin and 50% glycerol in diethyl pyrocarbonate (DEPC)-treated water. The lysate was kept on ice for 30 min and then centrifuged at $16000\times g$ for 20 min at 4°C in a Model 5415 Eppendorf® Microcentrifuge (Brinkmann Instruments, Westbury, NY, USA). Afterwards, 80 μ L of supernatant were collected, flash-frozen in liquid nitrogen and stored at -75°C until used.

Detection of telomerase activity from cell extracts was performed in a two-step process: (i) telomerase-mediated extension of the TS nucleotide and (ii) PCR amplification of the resultant product with both TS and the reverse CX primers (1,10,12). The oligonucleotides TS (5'-AATCCGTCGAGCAGAGTT-3') and CX (5'-CCCTTACCC-TTACCCTTACCCTAA-3') (both from BioSynthesis, Lewisville, TX, USA) were each dissolved in DEPC-treated water at 0.1 μ g/ μ L. Each thin-wall PCR tube contained 20 μ L of a reaction mixture that included 16.5 μ L of TRAP reaction buffer (20 mM Tris-HCl, pH 8.2, 63 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mg/mL 1 $\times$ bovine serum albumin [BSA] and 0.05% Tween® 20), 1 μ L of TS (0.1 μ g), 0.5 μ L of dNTPs (10 mM stock) and 2 μ L cell extract (from 1000 cells). The telomerase extension reaction was completed in a Model DB 66925 Temp.Tronic® Thermal Cycler (Barnstead/Thermolyne,