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SHORT TECHNICAL REPORTS

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Simultaneous In Situ Detection of Apoptosis and Necrosis in Monolayer Cultures by TUNEL and Trypan Blue Staining

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ABSTRACT

A method for simultaneously detecting membrane permeability (characteristic of necrosis) and DNA fragmentation (characteristic of apoptosis) is described. By combining a common dye-exclusion method (Trypan Blue) with a commercially available terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) labeling kit, we have succeeded in developing a novel methodology for obtaining permanently mounted slides of monolayer cell cultures double-labeled for DNA fragmentation and cell lysis. This method should facilitate in situ studies of cell death by allowing for a more accurate quantification of total toxicity in monolayer cell cultures and perhaps further enhance our understanding of the different mechanisms of cell death as well.

INTRODUCTION

In another report, we describe an in situ method for the permanent fixation and mounting of Trypan Blue-labeled human cortical neurons grown in monolayer culture and exposed to the neurotoxin c-PAF (14). Subsequently, we have been able to expand the versatility of this method by staining for apoptotic cell death in situ by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method immediately after Trypan Blue staining, thus giving us double-labeled slides that have been analyzed for both cell membrane integrity and nonrandom DNA fragmentation.

The idea of using an additional label in conjunction with Trypan Blue is certainly not novel, but typically the alternate label is used to indicate the viable

cell population to better contrast viable vs. nonviable cells, rather than being used to indicate another class of nonviable cells. Examples include the use of Trypan Blue in sequence with a neutral red (viability) stain (4) or the more common Trypan Blue/fluorescein diacetate spectrophotometric methodology (13). Furthermore, methods of determining apoptotic vs. necrotic cell death have been explored only fairly recently as investigators have begun to recognize the importance of identifying different mechanisms of cell death. Although there are numerous assays designed to independently identify either nonrandom DNA fragmentation (apoptosis) or cell lysis (necrosis), there are few, if any, available methods that can simultaneously identify and distinguish between the two modes of cell death, despite the widespread use that such assays would have.

Not only would such an assay be useful, but if an estimation of total cell toxicity is desired, such an assay is even crucial. Most cytotoxicity assays designed for in situ cell culture use, such as Trypan Blue, function on the principle that dying cells have a permeable plasma membrane and thus will not be able to exclude certain dyes (hence the name "dye-exclusion" assays). Many cells that are in the early stages of apoptosis, however, will not be labeled by these standard cytotoxicity assays. This is because during the apoptosis of cells in vitro, the membranes of apoptotic bodies do not lyse until very late in (or even after) the apoptotic pathway (9,11). Therefore, to make a determination of *total* cell toxicity, cytotoxicity assays that test for a lack of membrane integrity, such as Trypan Blue, must be used in combination with assays for apoptosis. Furthermore, although DNA fragmentation by specific endonucleases into 180-bp oligonucleosomal units is a hallmark for many forms of apoptosis, it is by no means a prerequisite for apoptosis. Some cell types show no further degradation beyond 300 and/or 50 kbp (12) (fragment sizes that are typically a prerequisite to further digestion into 180-bp nucleosomal units), while others show digestion to 20-kbp fragments with modifications at internucleosomal regions rather than the formation of

Table 1. Method for Simultaneous Detection of Apoptosis and Necrosis in Monolayer Cultures

1. Culture cells on glass (or plastic) coverslips suitable for ICC and microscopy as per the standard culture protocol. Treat with the desired conditions in parallel wells for varying periods of time to evaluate the potentially different time course for apoptotic vs. necrotic cell death.
2. Stain with Trypan Blue, fix and rinse the cells in accordance with the protocol described by Perry et al. (14).
3. Perform TUNEL staining using the ApopTag® kit (Oncor, Gaithersburg, MD, USA) exactly as described in the protocol with the following modifications:
 - A. First, modify the ApopTag method to permit immunostaining directly in 24-well cell culture plates with the cells growing on coverslips at the bottom of the wells. Cut the plastic coverslips supplied by Oncor to fit the wells.
 - B. In step 2 of the ApopTag protocol, quench in Levamisole (1 drop of 100×/5 mL phosphate-buffered saline [PBS]; Vector Laboratories, Burlingame, CA, USA) for 5 min instead of H₂O₂.
 - C. At step 6, use anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim, Mannheim, Germany), diluted 1:500 in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5, 1% (wt/vol) blocking reagent (Boehringer Mannheim), in place of the anti-digoxigenin-peroxidase reagent. [Although this method was independently developed, the same concept of substituting anti-digoxigenin-AP for anti-digoxigenin-peroxidase in the ApopTag procedure has also been used by others (1).]
 - D. At step 8, use a New Fuchsin Chromagen-Substrate System (Dako, Carpinteria, CA, USA).
 - E. Omit the methyl green counterstain in step 10, and dehydrate the coverslips in sequential ethanol washes (1 min each in 70% and 95%, 3× 1 min in 100%) and then clear in PRO-PAR Clearant (Anatech, Battle Creek, MI, USA), a xylene substitute, for 3× 1 min.
4. Finally, mount the coverslips on glass slides with a compatible permanent mounting solution such as REFRAx Mounting Medium (Anatech).

double-stranded fragments (15). Still other cell lines show morphological features of apoptosis without any DNA fragmentation (10,16). These pieces of evidence suggest that apoptosis without DNA degradation may occur in other cell types as well. In such instances, where apoptotic cells would not be detected by assays for apoptotic DNA fragmentation, a conventional assay for cell lysis used in conjunction with an assay for apoptosis might prove useful in detecting apoptotic cells after their membranes have been compromised. Therefore, it is clear that to obtain a more accurate quantitative assessment of total cell toxicity, both necrotic and apoptotic, combined methodologies must be used, with the assumption that some dying cells missed by one method

of detection will be accounted for by the other. Here we present a method allowing for a simultaneous assessment of membrane integrity and DNA fragmentation in monolayer cell cultures.

MATERIALS AND METHODS

Table 1 describes the materials and methods used in our method for simultaneous *in situ* detection in monolayer cell cultures.

RESULTS

We developed this method because we became interested in comparing the occurrence of apoptotic vs. necrotic

cell death in response to various neurotoxic insults. To illustrate our technique, we treated cultures of primary human cerebral cortical neurons transplanted from second-trimester fetal tissue with 1 mM H₂O₂ for 10 min, followed by incubation at 37°C for 18 h in normal growth media, then assaying by Trypan Blue and TUNEL methodologies. In the past several years, much attention has been focused on oxidative stress as being a key initiator of neuronal death in numerous neurodegenerative diseases (8). It has also been shown that the same oxidative stress insult can produce either apoptosis or necrosis in the same cell population, dependent upon the intensity of the insult, with higher doses causing greater necrotic cell death, and lower doses resulting in more apoptotic death (3,6). Low doses of H₂O₂, in particular, have been shown to induce apoptosis in cultured rat cortical neurons (17). Therefore, we hypothesized that a 1 mM dose of H₂O₂ would be high enough to induce necrosis, yet low enough to cause apoptotic death as well. Additionally, the random DNA degradation that characterizes necrotic neuronal cell death is not sufficiently prevalent to be detectable by the TUNEL method until 24–48 h after necrotic insult (7). Therefore, using an 18-h time point in this model system, any TUNEL staining will be specific for apoptotic cell death.

Figure 1 shows primary human fetal neuronal cultures treated with either vehicle control or 1 mM H₂O₂ for 10 min, followed by incubation in their normal growth media for 18 h, and then double-stained by Trypan Blue and TUNEL. Eighteen hours after treatment with vehicle control, an abundant, healthy, viable cell population with only rare TUNEL or Trypan Blue staining is observed (Figure 1A). In contrast, 18 h post-treatment with 1 mM hydrogen peroxide, significant damage to the cultures is apparent, with visible cell debris and morphologic damage, a vast decrease in cell numbers, an obvious increase in Trypan Blue and TUNEL staining (Figures 1, B and C).

DISCUSSION

We know of only one other currently

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published method that attempts to simultaneously visualize those cells dying by apoptosis and those dying by necrosis. This method, originally published by Dive et al. (5), utilizes multiparameter flow cytometry and several fluorescent dyes to distinguish between apoptotic and necrotic cells. Although Gold et al. (7) described the combined use of in situ tailing and nick translation techniques to differentiate between cellular apoptosis and necrosis, they did not incorporate a two-color, double-labeling technique into their methodol-

ogy to simultaneously identify apoptosis and necrosis occurring within the same sample. The technique we describe here is novel in that it describes the simultaneous in situ detection of nonrandom DNA fragmentation (apoptosis) and loss of cell membrane integrity (necrosis) in monolayer cell cultures, as well as fixation and mounting for a permanent record of the staining. Because of these features, this method is both unique and important in that it can immunohistochemically identify apoptotic and necrotic cell bodies, provide a permanent record of the staining for cytological and morphometric analysis by conventional light microscopy and even allow for ultrastructural analysis by electron microscopy before the staining procedure.

Ankacrona et al. (2) observed that knowing the inciting stimulus or even the final pathological outcome does not guarantee the proper mechanism of cell death can be identified. For example, one inherent and unavoidable source of error in any method designed to distinguish necrotic death from apoptotic death is the observation that in many cases cells that died by apoptotic means will later necrose, and thus will be identified by methodological indicators for necrosis. If this cell is therefore interpreted to have died by necrosis, without consideration of the possibility that it may have undergone a post-apoptotic necrosis, then a false conclusion would have been drawn as to this cell's mode of death. In light of the data shown in Figure 1, this method may serve to resolve some of these sources of confusion and error. Here, the overwhelming majority of labeled cell bodies stained for either Trypan Blue or TUNEL reagent, and only rarely did both stains localize within a single cell. This specificity of labeling again suggests a high degree of correlation between TUNEL staining and apoptosis in this model system and is consonant with the findings of Gold et al. (7) that TUNEL staining does not label necrotic neuronal cell death until 24–48 h after a necrotic insult. Therefore, this method may make it possible to more accurately predict the mode of cell death based upon the final observed pathological outcome in vitro. Taken together, these findings further suggest

Figure 1. Cultures of primary human fetal cortical neurons double-stained by TUNEL and Trypan Blue. Apoptotic bodies show a positive red TUNEL stain (New Fuchsin substrate), necrotic bodies stain with Trypan Blue. Cell cultures were treated with either vehicle (A) or 1 mM H₂O₂ (B and C) for 10 min, then incubated in normal growth media for 18 h. Panels B and C represent two different fields receiving the same treatment conditions, both illustrating the kind of staining obtained with the method described here. All tissue was taken in strict accordance with National Institutes of Health (NIH) and University of Rochester guidelines for the use of human tissues. Photographs were taken under 132× magnification, using an Olympus BX50 light microscope with ND25 and LBD filters and Nomarski optics (Olympus, Melville, NY, USA) to enhance contrast and add depth.

that if double-labeling of individual cells is observed, with red TUNEL staining overlaying Trypan Blue, it is likely because of secondary necrosis of apoptotic bodies, rather than TUNEL staining of nonrandom DNA degradation in necrotic cells. Thus, this method might also prove useful for identifying secondary necrosis in cell populations.

The method we have provided here can serve as a useful supplement to all methods currently in use. Although it is still clear that a time-lapsed analysis of cell death in vitro, using video electron microscopy, would provide the most unquestionable diagnosis of a cell's mode of death, such methods are prohibitively labor-intensive. However, because techniques for identifying the mode of cell death must be combined with a rigorous analysis of the morphological features that accompany cell death, we have developed a technique that will both accommodate time-lapsed observation and allow for later morphological analysis based upon the cell's final pathological outcome. In these ways, the method we have presented here will complement the other methods to distinguish between apoptosis and necrosis, and will also facilitate the necessary morphological analyses.

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