Methods for DNA Strand Breaks Detection

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Abstract: The study of Deoxyribonucleic Acid (DNA) damage holds a wide interest within both basic and applied fields of research. Elucidating the mechanisms involved in the generation of DNA damage and the consequences of this damage, will have an enormous impact on multiple fields of scientific research and will ultimately lead to a better understanding of human disease. In this review article, a variety of experimental molecular biology techniques will be described.

Key words: DNA strand breaks, flow and laser scanning cytometry, limitations, nick translation and electron microscopy, T4 DNA ligase, TUNEL assay, ultrasound

INTRODUCTION

Advances in medicine and medical technology have resulted in a tremendous improvement in health and welfare. However, we are still faced with various diseases that are difficult to treat using contemporary medicine (Namiri et al., 2010). A number of exogenous and endogenous toxic agents may damage DNA, leading to genomic instability and transcriptional infidelity. Genetic or acquired defects in DNA repair mechanisms also contribute to exacerbate DNA damage (Rolig and McKinnon, 2000). The assessment of cellular DNA damage is crucial in many areas of biology including immunology, developmental biology, aging, cancer and environmental science (Freeman et al., 1986). At least two types of cell death are the consequence of extensive and irreversible DNA damage, i.e., apoptosis and necrosis. Apoptosis plays a central part in normal tissue homeostasis and has a role in a variety of clinical diseases characterized by either increased or decreased cell survival (Fauziah et al., 2010) (Fig. 1). Apoptosis is a widespread phenomenon, which plays an important role in many physiological events as well as pathological processes (Ansari et al., 1993). In order to establish that apoptosis is occurring, other criteria must also be used (Walker et al., 1995). The cell shrinkage is a ubiquitous characteristic of programmed cell death that is observed in all examples of apoptosis, independent of the death stimulus (Parichehr et al., 2008). Morphologic criteria for apoptosis include cell shrinkage, blebbing of the cell surface, chromatin condensation and margination, nuclear pyknosis and late fragmentation into apoptotic bodies, with remarkable preservation of the integrity of cell membranes and organelles. All of these changes are due to cleavage of various cytoplasmic and nuclear substrates (Budihardjo et al., 1999). Although the nuclear breakdown is widely considered to be a hallmark of apoptosis, its mechanism remains poorly understood (Montague and Cidlowski, 1996). DNA damage also occurs in necrosis. At variance with apoptosis, necrosis is not a programmed event, is characterized morphologically by early swelling, disintegration of membranes and organelles and absence of chromatin condensation (Majno and Joris, 1995). Indeed, the DNA damage that occurs in necrosis is not easily distinguishable from that of apoptosis. Thus, there has been tremendous progress in molecular biology techniques to understand the mechanism of DNA damage. In this article, different methods for DNA damage detection will be described.

LABELING DNA BREAKS USING TERMINAL TRANSFERASE (TUNEL ASSAY)

Apoptotic and programmed cell death are characterized by and indeed were first discovered from observations of, remarkable morphological changes that occur in the nucleus (Lockshin et al., 1998). Thus, light and electron microscopy were the first tools for the detection of apoptosis. This characteristic collapse of chromatin and ultimately the structural organization of the nucleus are triggered by the degradation of DNA, which is an active process and occurs prior to death of the cell. The degradation of DNA was subsequently found to be mediated by endonucleolytic activity that generated a specific pattern of fragments (Wyllie, 1980). DNA fragmentation is quite variable within cells and some cell types produce only High Molecular Weight (HMW) fragments. The latter observations formed the basis of a
A convenient *in vitro* biochemical technique for the routine detection of apoptosis by resolving the fragmented DNA by conventional or pulsed field agarose gel electrophoresis. However, this technique requires relatively large amounts of material and DNA extraction. Subsequently, a variety of techniques have emerged to detect apoptotic DNA fragmentation *in situ* by exploiting the fact that the hydroxyl group at the 5' or 3' ends of the small DNA fragments becomes exposed. Nucleotide analogues can be attached to the ends by several enzymes, with Terminal deoxynucleotidyl Transferase (TdT) being the most popular (Gavrieli *et al*., 1992; Mundle *et al*., 1994). The assays are typically fluorescence-based, either by the direct incorporation of a nucleotide to which a fluorochrome has been conjugated, or indirectly using fluorescent dye conjugated antibodies that recognize biotin or digoxigenin tagged nucleotides. The assays have been formatted for light and confocal microscopy as well as flow cytometry, thereby greatly facilitating the detection and quantitation of apoptosis *in situ*. The first end-labeling protocol developed for the detection of DNA fragmentation in apoptosis was the Terminal Uridine Nucleotide End Labeling (TUNEL) technique of Gavrieli *et al*. (1992). This method exploited the ability of the enzyme, terminal transferase, to add biotin-conjugated nucleotides onto the 3' OH of a DNA strand. By using either a fluorescently tagged or radioactively labeled nucleotide analog, the DNA fragments become detectable.
Limitations: DNA fragments with 3'-OH ends can be produced in a number of situations where apoptosis is not occurring. For example, some forms of DNA damage produce DNA breaks or nicks with 3'-OHs. Moreover, the DNA degradation that occurs during necrosis also produces fragments with 3'-OH that would be labelled by TUNEL or ISEL (In situ End Labeling). Over-reliance on these techniques has led to considerable controversy in studies in brain where, following some insults, both apoptosis and necrosis occur simultaneously making it very difficult to establish and quantitate true apoptotic cell death (Roy and Sapolsky, 1999; Gilmore et al., 2000; Chun, 2000; Darzynkiewicz, 1993). It is evident, therefore, that TdT-based labelling techniques should not be used as the sole criterion for establishing the nature of the cell death mechanism. In order to establish that apoptosis is occurring; other criteria must also be used. Since it is possible to use multiple fluorochromes in the same experiments, another marker such as the appearance of annexin on the cell surface can be used simultaneously. Once it has been established that the cell death is indeed apoptotic, then the TdT-based assays can be used for routine quantitation by microscopy or flow cytometry (Walker et al., 1995; Darzynkiewicz, 1993).

ANALYSIS OF APOPTOSIS BY FLOW AND LASER SCANNING CYTOMETRY

One advantage of strand break labeling with fluorochromes is that such cells can rapidly be analyzed by flow cytometry. When cellular DNA content also is measured in these cells, the bivariate analysis of such data provides information about DNA ploidy or the cell cycle phase specificity of apoptosis (Gorczyca et al., 1992; Gorczyca et al., 1993). Laser Scanning Cytometer (LSC) is the microscope-based cytofluorimeter which allows one to measure rapidly, with high sensitivity and accuracy, fluorescence of individual cells (Kamentsky, 2001; Darzynkiewicz et al., 1999). The instrument combines advantages of both flow and image cytometry. Cells staining on slides eliminates their loss that otherwise occurs during repeated centrifugations in sample preparation for flow cytometry. Another advantage of LSC stems from the possibility of localization of cells on slide for their visual inspection or morphometry after the initial measurement of large cell population and electronic selection (gating) of cells of interest. Visual examination is of particular importance because the characteristic changes in cell morphology (Kerr and Wyllie, 1972) are still considered the gold standard for positive identification of apoptotic cells. Furthermore, the measured cells can be bleached and re-stained with another set of dyes (Bedner et al., 2001). The cell attributes measured after re-staining can be correlated with the attributes measured before, on a cell by cell basis (Bedner et al., 2001; Li and Darzynkiewicz, 1999) (Fig. 3). Fixation and permeabilization of the cells are the initial essential steps to success-fully label DNA strand breaks. Cells are briefly fixed with a cross linking fixative such as formaldehyde and then permeabilized by suspending them in ethanol or using detergents in the subsequent rinses. By cross linking low MW DNA fragments to other cell constituents, formaldehyde prevents extraction of the fragmented DNA, which otherwise occurs during repeated centrifugations and rinses required by this procedure (Gorczyca et al., 1992; Darzynkiewicz et al., 1992; Darzynkiewicz et al., 1997). The 3'OH-termini of the DNA breaks serve as primers and become labelled in this procedure with Bromodeoxyuridine (BrdU) when incubated with 5-Bromo-2'-deoxyuridine-5'-Triphosphate (BrdUTP) in a reaction catalyzed by exogenous Terminal deoxynucleotidyl Transferase (TdT) (Li and Darzynkiewicz, 1995; Li et al., 1996). The incorporated BrdU is immunocytochemically detected by BrdU antibody conjugated to FITC (Li and Darzynkiewicz, 1995). The latter is a reagent widely used in studies of cell proliferation to detect BrdU incorporated during DNA replication (Dolbeare and Selden, 1994). The overall cost of reagents is significantly lower and sensitivity of DNA

Fig. 3: Detection of apoptotic cells after labeling DNA strand breaks with BrdUTP. U-937 cells were untreated (A) or treated with tumor necrosis factor-α in the presence of cycloheximide (B) (Li and Darzynkiewicz, 2000; Bedner et al., 2000)
strands breaks detection is higher when BrdU TP is used as a marker, compared to the alternative labeling with biotin (or digoxigenin) or directly fluorochrome-tagged deoxynucleotides (Li and Darzynkiewicz, 1995). This method is useful for clinical material, such as obtained from in leukemias, lymphomas and solid tumors (Halicka et al., 1997) and can be combined with surface immunophenotyping.

The procedure of DNA strand break labelling is rather complex and involves many reagents. Negative results, therefore, may not necessarily mean the absence of DNA strand breaks but may be due to methodological problems, such as loss of TdT activity, degradation of BrdU TP, etc.

**Nick translation and electron microscopy:** (Rigby et al., 1997) Introduced nick translation procedure in 1977. This technique is used to label purified DNA. This method relies on combined 5'-3' polymerase and 5'-3' exonuclease activities of Escherichia coli DNA polymerase I. In addition to DNA damage detection (Hashimoto et al., 1995) the method was adapted to study the location of DNase I-sensitive regions within cellular DNA. (Levitt et al., 1979) Selectively labeled nuclease sensitive sites in DNA in isolated nuclei by nick-translation using treatment with low concentrations of DNase I. It was recently adapted to the electron microscopic level in order to discriminate between active and inactive regions of inter-phase chromatin (Thiry, 1991). The technique is applied to the ultrathin sections of biological material and includes two successive steps, the enzymatic labeling reaction followed by an immunocytochemical detection step. The newly synthesized DNA strands are visualized by an indirect immunogold labelling technique. Nick translation using mild digestion with DNase I allows preferential labelling of actively transcribing DNA regions. When DNase I treatment is omitted the technique detects the pre-existing single-stranded DNA breaks and can be used for visualization of DNA damage in situ at the electron microscopic level (Sugimoto et al., 1999).

**Using T4 DNA ligase to detect DNA breaks and apoptosis:** In situ ligation technique was developed to label a particular type of DNA damage, namely, full double strand breaks, directly in fixed tissue sections (Didenko and Hornsby, 1996; Didenko et al., 1998; Didenko et al., 1999). The technique uses double-stranded oligoprobes with specific ends, which are attached by T4 DNA ligase to the ends of DNA breaks (Fig. 4).

In situ ligation can be combined with TUNEL, allowing co detection of free 3'OH groups in situ and with immunohistochemical staining of cellular proteins. The types of double-strand breaks which can be detected by in situ ligation occur in various cellular processes, the majority of which are related to cell damage and death.

The basics of in situ ligation labeling of apoptotic cells rely on the fact that double-strand breaks produced by apoptotic endonucleases are either blunt-ended or have short 3' or 5' extensions. Applications of in situ ligation to the investigation of the other sources of double strand breakage in DNA are currently lacking. These other sources of double strand breaks include free radicals, ionizing radiation and various radiomimetic and chemothapeutic agents, capable of inducing the breaks directly, or indirectly as a result of the damaged DNA repair, recombination and replication. In addition, two physiological forms of intentional double strand breakage, generating a small number of breaks, occur in lymphoid cell differentiation during V(D)J recombination and class switch recombination (Lieber et al., 1998). However detection of these breaks is problematic unless specialized methods of signal enhancement are used.

**Combination of comet and TUNEL assays:** There are many biological circumstances that require the use of small cell samples. To extract DNA damage information from these samples, techniques that rely upon the evaluation of DNA damage at the level of single cells is required. The Single Cell Gel Electrophoresis (SCGE) or “comet” assay is the most widely applied method for the detection of DNA damage in single cells. In this approach damaged DNA in individual cells is electrophoresed away from a nucleus into an agarose gel followed by staining.

The analysis of DNA damage in single cells was first reported in the studies of (Ryberg and Johanson, 1997). In this technique DNA damage was detected by:

- Lysing cells embedded in agarose on slides
- Promoting partial DNA unwinding
- Staining DNA with a dye

This method was improved by the subsequent addition of an in situ agarose gel electrophoresis step (Ostling and Johanson, 1984) to allow easier discrimination between damaged and undamaged DNA. This approach was further refined by (Singh et al., 1988)
Ultrasound: The high frequency ultrasonic detection of programmed cell death has been demonstrated in vitro, in situ and in vivo using a variety of different apoptosis inducing methods (Czarnota et al., 2000) (Fig. 5). This method provides a useful image based adjunct for the detection of programmed cell death in a laboratory setting as well as being a powerful potential clinical tool which can be used to monitor tumour responses to treatment. Data collected to date indicates that this capability of high-frequency ultrasound is based on interactions of high-frequency ultrasound waves with the chromosomal nuclear material in cells, which undergoes structural changes of condensation and subsequent fragmentation with the process of programmed cell death (Czarnota et al., 2000) (Fig. 5). The ultrasound-based approach to detecting apoptosis has a number of potential applications which range from embryological studies of development where apoptosis plays an important role, to assessing organ viability for the purposes of transplantation, a situation where the presence of programmed cell death is correlated to clinical outcome (Wood, 1998).

REFERENCES


