Diphenylamine assay - Summary

Drug Discovery and Activity Testing

The method is suitable for the determination of the fragmented DNA from apoptotic cells induced by putative antineoplastic drugs.

Objective & Application

TYPE OF TESTING	:	Screening
LEVEL OF ASSESSMENT	:	Efficacy, Toxic potential
PURPOSE OF TESTING	:	Drug evaluation

Apoptosis is the major mode of death induced by antineoplastic agents. DNA fragmentation is a reliable index of apoptosis as this parameter is a hallmark of the apoptogenic process. The use of diphenylamine is suitable to quantify colorimetrically the amount of DNA fragmented from apoptotic cells. Thus, this method can be used to screen apoptogenic compounds.

Basis of the Method

Primary or stabilized cell cultures growing in suspension are incubated with the apoptogenic compound for different times. Fragmented (low-molecular-weight) and intact (high-molecular-weight) DNA are separated by centrifugation. The low-molecular-weight DNA in the supernatant is precipitated. The DNA contained in the pellet and in the supernatant fractions is hydrolized and incubated with diphenylamine, a dye interacting specifically with the sugar moiety of deoxyribonucleic acids. The amount of DNA present in each of the two fractions is determined at 600 nm with the use of a spectrophotometer. Fragmentation is calculated as the percentage of total DNA (supernatant and pellet) recovered as low-molecular-weight DNA in the supernatant.

Experimental Description

Biological and Endpoint Measurement:

To assess the amount of DNA fragmentation induced by an external agent:

APOPTOTIC DEATH: DNA fragmentation

Endpoint Value:

Optical density at 600 nm

Experimental System:

Human and higher eukaryotes' cells

The method has been applied in our laboratory to the following cell lines:
- Human acute promyeloblastic HL-60 cell line
- Human promyelocytic NB4 cell line
- Human ovarian cancer cell lines, SKOV-3, OVCAR-3, A2780
- Human chronic myelogenous leukemia K562 cell line

The culture conditions for each of these cell lines are standard as they grow either in suspension in RPMI 1640 (HL-60, NB4, K562) supplemented with 10% fetal calf serum (FCS) or in adherence in DMEM (SKOV-3, OVCAR-3, A2780) containing 10% FCS. The culture conditions are not critical for the assay. In addition, in principle, the assay can be performed on any kind of cultured cell line.

PROCEDURE:

-Cells (approximately 0.5-1 x 106 in 2-10 ml of serum-containing medium) growing in suspension in T25 flat bottom flasks are harvested.
-The cell suspension is centrifuged in Eppendorf tubes at 13,000 x g for 2 min and washed with cold PBS (phosphate buffered saline) two times, by resuspension and centrifugation.
- Then cells are lysed with 100 :l of 10 mM Tris, pH 7.5, 1 mM EDTA and 0.2% Triton X-100 at 4°C for 15 min.

- Cells are centrifuged at 13,000 x g for 20 min at 4°C.
- Centrifugation resistant low-molecular-weight DNA in the supernatant is carefully removed by aspiration with a fine needle. and precipitated with 12.5% (v/v) TCA (trichloroacetic acid) for 18 hr.
- High-molecular-weight DNA in the pellet is mixed with 300 :l of cold 12.5% TCA for 18 hr.
-Samples are then centrifuged at 13,000 x g at 4°C for 5 min and DNA in the precipitates is extracted with 20 :l of 1 M perchloric acid at 70% for 20 min.
- Diphenylamine reagent is prepared mixing 1 ml of15 mg/ml diphenylamine in glacial acetic acid: sulphoric acid (67:1 v/v) with 5 :l of acetaldeide (16 mg/ml in water).
- Then,120 :l of diphenylamine reagent are added to each Eppendorf and samples are vortexed and incubated at 37°C for 18 hours.
- From each sample, 120 :l are transferred to flat-bottomerd 96-well plates and the absorbance at 600 nm is measured on an automated plate reader.

- DNA fragmentation is calculated as percentage of total DNA (pellet and supernatant) recovered as low-molecular-weight DNA in the supernatant.

Critical parameters

The DPA colorimetric assay requires a relatively high number of cells to obtain reliable results, as at least 1 m g DNA must be measured. One of the most critical drawbacks of the method is the fact that the centrifugal separation technique works only for the extensive typical internucleosomal fragmentation of DNA, but not for rare events of random double-stranded cleavage or single-stranded nicking. Moreover, this method cannot apply to those apoptotic events where no DNA fragmentation occurs.

MATERIALS

DPA solution: Add 10 ml glacial acetic acid to 150 mg diphenylamine in a 50 ml polypropylene tube and mix thoroughly by repeated inversion until complete dissolution. Add 150 ml concentrated sulfuric acid and mix thoroughly. Add 50 ml acetaldehyde solution and mix thoroughly.

CAUTION: prepare fresh and use within 60 min. DPA is an irritant: wear appropriate protection.

Acetaldehyde solution: Add 16 mg acetaldehyde to 10 ml deionized water to have a 16 mg/ml stock solution. Store at 2-8°C for < 1 year.

Diphenylamine D3409 can be purchased from Sigma (St. Louis, MO, USA)

MODIFICATIONS INTRODUCED

In the case that an automated plate reader is not available in the laboratory, it is possible to adjust the volume reaction in order to read samples manually in a spectrophotometer.

Test Compounds

The test compounds are organic chemicals, peptides or proteins

Discussion

Advantages:

The method is quantitative and simple. The use of an automated plate reader allows the analysis in parallel of large number of samples. After centrifugation, samples may be frozen and this allows accumulation, storage and transport of samples. The assay is non-radioactive.

Limitations:

Diphenylamine is a very dangerous reagent since it is a potent carcinogen. Thus, this compound have to be manipulated very carefully. The method is less sensitive, in respect to other DNA-fragmention methods, such as TUNEL assay. The whole procedure is lengthy requiring two days to be completed.

Status

The assay is used to establish the apoptotic potential of antineoplastic drugs. At the experimental level, the method is used to asess whether a test compound kills cells through a necrotic or an apoptotic process. Among the tests used for the determination of apoptosis this is one of the least popular, although it gives quantitative results. The test is widely accepted by the scientific community as any other test whose

endpoint is the determination of DNA fragmentation. Measurement of DNA fragmentation with the DPA colorimetric assay is preferentially used to evaluate apoptosis in resting cells or in other cell populations where DNA labeling is impossible or difficult.

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