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Evaluation of Apoptosis in Immunotoxicity Testing

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Abstract

Immunotoxicity testing is important in determining the toxic effects of chemical substances, medicinal products, airborne pollutants, cosmetics, medical devices, and food additives. The immune system of the host is a direct target of these toxicants, and the adverse effects include serious health complications such as susceptibility to infections, cancer, allergic reactions, and autoimmune diseases. One way to investigate the harmful effects of different chemicals is to study apoptosis in immune cell populations. Apoptosis is defined as the programmed cell death, and in general, this process helps in development and maintains homeostasis. However, in the case of an insult by a toxicant, apoptosis of the immune cells can lead to immunosuppression resulting in the development of cancer and the inability to fight infections. Apoptosis is characterized by cell shrinkage, nuclear condensation, changes in cell membrane and mitochondria, DNA fragmentation into 200 base oligomers, and protein degradation by caspases. Various methods are employed in order to investigate apoptosis. These methods include direct measurement of apoptotic cells with flow cytometry and in situ labeling, as well as RNA, DNA, and protein assays that are indicative of apoptotic molecules.

Keywords

Apoptosis; Immunotoxicity; Caspase; Bcl-2

1. Introduction

Immunotoxicity is defined as the adverse effects to the immune response of the host caused by exposure to a toxicant (1). It is caused by chemical substances, medicinal products, airborne pollutants, cosmetics, medical devices, and food additives (2). Exposure to these substances results in many health complications such as susceptibility to cancer, autoimmune diseases, allergic reactions, and inability to fight infections (2). It is important to assess the toxic effects of environmental chemicals, medical device materials, and newly discovered drugs before they induce significant adverse effects on living organisms. Some of the adverse effects on the immune system include: (1) reduction in antibody production, (2) reduction in cytokine secretion, (3) inability to distinguish between self and nonself, (4) hypersensitivity, and (5) induction of apoptosis in immune cell populations (3). Therefore, precise testing of immunotoxicity is required for the identification of hazardous materials and substances.

One reliable way to measure immunotoxicity is to measure apoptosis in immune cell populations. Apoptosis is the process of programmed cell death, during which many changes occur such as membrane blebbing, cell shrinkage, mitochondria leakage, and DNA fragmentation into oligonucleosomal size of ~200 base pairs (4, 5). Apoptosis differs from another form of cell death, which is known as necrosis, in that the latter is characterized by random DNA fragmentation and cell swelling, resulting in lysis and release of cellular contents that induce an inflammatory response. The molecular changes of apoptosis are classified under two different pathways of apoptosis (4, 5). The intrinsic pathway, which occurs via the mitochondria, is initiated by an imbalance in antiapoptotic and proapoptotic members of the Bcl-2 family of proteins. Some of the proapoptotic molecules include Bad, Bid, Bax, Bim, and Bcl-xS while the antiapoptotic molecules are Bcl-2 and Bcl-xL. A shift toward proapoptotic factors results in the permeability of the mitochondrial membrane and cytochrome c leakage from the intermembrane space of the mitochondria into the cytosol. Cytochrome c then combines with pro-caspase 9 and apoptotic protease activating factor-1 (APAF-1) in the presence of adenosine triphosphate (ATP) to form the apoptosome. Active caspase 3 is produced in the apoptosome by cleavage of the pro-caspase 3, and this effector caspase activates endonucleases that cleave the DNA as well as the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP) (6). Caspase 9 activity can be antagonized by the inhibitor of apoptosis proteins (IAPs). The antiapoptotic effect of IAPs is neutralized upon release of the mitochondrial protein known as the second mitochondrial-derived activator of caspase or direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Smac/DIABLO) which acts by sequestering the IAPs (7).

The extrinsic pathway is triggered by the ligation of death receptors belonging to the tumor necrosis factor (TNF) receptor family (e.g., TNF-R1 or CD95/Apo-1/Fas) with their respective ligands, TNF or FasL. This receptor–ligand interaction initiates the recruitment of a cascade of signaling molecules including the adaptor proteins TNF-R-associated death domain (TRAD) or Fas-associated death domain (FADD), which along with the recruitment of the procaspase 8 and 10 results in the formation of Death Inducing Signaling Complex (DISC). In the DISC, active caspase 8 and 10 are produced, which are the initiator caspases that activate caspase 3, resulting in apoptosis (4). The two apoptotic pathways are linked through the proapoptotic molecule, Bid, a member of the Bcl-2 family of proteins found in the cytosol. Bid is cleaved by caspase-8 to form a truncated protein tBid, which translocates to the mitochondria, where it induces the release of cytochrome *c* and causes mitochondrial dysfunction leading to activation of caspase 9. Signaling through the death receptors is inhibited by recruitment of an inhibitory protein, FLICE-like inhibitor protein (FLIP), to the DISC.

2. Materials

2.1. Annexin V/Propidium Iodide Staining

1. 12 × 75 mm round bottom culture tubes (VWR International, Westchester, PA).
2. Annexin-V-FLUOS Staining Kit (Roche Applied Science, Indianapolis, IN).
3. Phosphate Buffered Saline (PBS) (VWR International, Westchester, PA).

4. Annexin-V-FLUOS labeling solution: 20 µL Annexin-V-Fluos labeling reagent, 20 µL PI solution, 1 ml incubation buffer. All of these solutions are included in the kit.

2.2. TUNEL Assay

1. 12 × 75 mm round bottom culture tubes (VWR International, Westchester, PA)
2. In situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN).
3. Staining Buffer: Phosphate Buffered Saline (PBS) with 1% FBS (Atlanta Biologicals, Lawrenceville, GA).
4. Fixation Solution: freshly prepared 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) in PBS.
5. Permeabilization Solution: freshly prepared 0.1% Triton-×-100, 0.1% sodium citrate in PBS (Sigma Aldrich, St. Louis, MO).
6. Labeling solution: 22.5 µl of labeling solution (nucleotide mix), 2.5 µl of enzyme (TdT). Both the labeling solution and the enzyme are the components of the kit.

2.3. In situ TUNEL Assay

1. Xylene (Sigma Aldrich, St. Louis, MO).
2. Ethanol (100%, 95%, 85%, 70%, and 50% diluted in deionized water).
3. PBS (VWR International, Westchester, PA).
4. 4% methanol-free formaldehyde (Polysciences, Warrington, PA) in PBS.
5. 0.85% NaCl solution.
6. Proteinase K buffer (BD Biosciences, San Josa, CA).
7. DNase I (RNase-free) (Promega, Madison, WI).
8. DNase I buffer (Promega, Madison, WI).
9. DeadEnd Fluorometric TUNEL System including equilibration buffer, incubation buffer and 20× SSC (Promega, Madison, WI).
10. Propidium iodide (Sigma Aldrich, St. Louis, MO).
11. Anti-Fade Kit (Invitrogen, Carlsbad, CA).

2.4. Gel Electrophoresis for Detection of DNA Fragmentation

1. TE buffer pH 7.4 (Sigma Aldrich, St. Louis, MO).
2. TTE solution: TE buffer pH 7.4 with 0.2% Triton-×-100 (store at 4°C).
3. NaCl 5 M, ice cold.
4. Isopropanol, ice cold.
5. Ethanol at 70%, ice cold.
6. Bromophenol Blue (Sigma Aldrich, St. Louis, MO).

7. TBE buffer for electrophoresis (Sigma Aldrich, St. Louis, MO).
8. Ethidium bromide solution (Sigma Aldrich, St. Louis, MO).
9. Electrophoresis-grade agarose (Sigma Aldrich, St. Louis, MO).
10. DNA molecular weight markers (Invitrogen, Carlsbad, CA).

2.5. Mitochondrial Membrane Potential

1. DiOC6: 3,3-dihexyloxacarbocyanine iodide (Invitrogen, Carlsbad, CA).

2.6. Detection of Caspase 3/7 Activity

1. Apo-ONE homogenous caspase-3/7 assay kit including 100× substrate buffer (Promega, Madison, WI).
2. Blank: Apo-ONE caspase 3/7 reagent, cell culture medium only.
3. Negative control: Apo-ONE caspase 3/7 reagent, vehicle-treated cells.

2.7. Protein Assay

1. Ammonium persulfate (APS) (Sigma Aldrich, St. Louis, MO).
2. Tetramethylethylenediamine (TEMED) (Sigma Aldrich, St. Louis, MO).
3. Sodium dodecyl sulfate (SDS) (Sigma Aldrich, St. Louis, MO).
4. Acrylamide (Sigma Aldrich, St. Louis, MO).
5. Tris-HCl: 0.5 M and 1.5 M (Sigma Aldrich, St. Louis, MO).
6. N′N′-bis-methylene-acrylamide (Sigma Aldrich, St. Louis, MO)
7. Glycerol (Sigma Aldrich, St. Louis, MO)
8. Glycine (Sigma Aldrich, St. Louis, MO)
9. Bromophenol blue (Sigma Aldrich, St. Louis, MO)
10. Methanol
11. NaCl
12. Tween-20 (Sigma Aldrich, St. Louis, MO)
13. Nonfat dry milk
14. 2 beta-mercaptoethanol (Sigma Aldrich, St. Louis, MO)
15. BCA protein assay kit (Pierce, Rockford, IL)
16. Enhanced chemilluminescence (ECL) Reagent (GE HealthCare, Piscataway, NJ)

2.7.1.Western Blot Solutions

1. Transfer buffer: 3.03 g Tris base, 14.4 g glycine, 200 ml ethanol, adjust the volume to 1000 ml.

2. 10× TBS: 24.2 g Tris base, 80 g NaCl, adjust the volume to 1000 ml and pH to 7.6.
3. TBS-T 0.1%: 1 ml of 50% Tween-20, 500 ml 1× TBS
4. 5% Blocking solution: 15 ml 10× TBS, 135 ml deionized (DI) water, 7.5 g nonfat dry milk, mix well, and while stirring add 300 μl of 50% Tween-20.
5. APS: 100 mg ammonium persulfate, 1 ml DI water.
6. Stripping Solution: 3.125 ml 0.5 M Tris-HCl pH 6.8, 5 ml 10% SDS, 180 μl 2-betamercaptoethanol**,** 16.7 ml DI water. Incubate in 50°C water bath for 15 min.
7. 30% acrylamide (300 ml): 87.6 g acrylamide, 2.4 g N′N′-bis-methylene-acrylamide, then complete it to 300 ml with DI water.
8. 10% SDS: Gently dissolve 10 g SDS in 90 ml of DI water and bring it up to 100 ml.
9. 1.5 M Tris-HCl (pH 8.8): 27.23 Tris base, 80 ml DI water, adjust the pH to 8.8 and bring the volume up to 150 ml.
10. 0.5 M-HCl (pH 6.8): 6 g Tris-base, 60 ml DI water, adjust pH to 6.8 and bring the volume up to 100 ml.
11. Sample Buffer: 0.6 ml DI water, 2 ml 0.5 M Tris-HCl pH 6.8, 5 ml 32% glycerol, 1.6 ml 20% SDS, 0.0002 g bromophenol blue. Prior to use, add 8% 2-mercaptoethanol.
12. 5× SDS-PAGE: 15 g Tris base, 72 g Glycine, 5 g SDS, adjust the volume to 1000 ml.

2.8. RT-PCR Analysis of Gene Expression

2.8.1. Extraction of Total RNA Using Trizol Reagent

1. Trizol Reagent (Invitrogen Corporation Carlsbad, California, Life Technologies, Frederick, MD) can be used to extract total RNA from cells or tissues as per the manufacturer’s instruction.
2. Chloroform (Sigma, St. Louis, MO).
3. Isopropanol (Sigma, St. Louis, MO).
4. Ethanol.
5. TE buffer (Sigma Aldrich, St. Louis, MO)
6. Polypropylene microcentrifuge tubes.

2.8.2. Extraction of Total RNA Using RNeasy Mini Kit

1. RNeasy Mini kit (Qiagen, Valencia, CA).
2. RNase-free DNase(Qiagen Inc., Valencia, CA).
3. Ethanol (Sigma Aldrich, St. Louis, MO).
4. Polypropylene microcentrifuge tubes.

5. Sterile, RNase-free pipette tips.
6. Disposable gloves.

2.8.3. RT-PCR for Gene Expression

1. PCR tubes (Bioplastics, eEnzyme LLC, Gatthersburg, MD).
2. Mineral Oil (Sigma Aldrich, St. Louis, MO).
3. Bio-Rad iCycler PCR unit (BioRad Laboratories, Hercules, CA).
4. iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA).
5. PCR reagent kit, Epicenter Master mix (Epicenter, Madison, WI).
6. Moloney murine leukemia virus reverse transcriptase (Amersham, Arlington Heights, IL).
7. Oligonucleotide primers (Integrated DNA Technology, Coralville, IA).
8. AlphaImager™ (Alpha Innotech Corporation, San Leandro, CA).

3. Methods

A variety of approaches in combinations is used to confirm apoptosis in immune cell populations. Using several methods will also help to determine which pathways are involved and which proteins play a role. For in vitro experiments, a time and a dose response should be performed in all of the experiments in order to determine the optimal time and dose for the induction of apoptosis. If primary immune cells are used directly from in vivo experiments following injection of an apoptosis-inducing chemical, it may be difficult to measure apoptotic cells because the phagocytes in vivo rapidly clear them. To overcome this, we have shown that in vitro culture of such cells for an additional 18–24 h followed by analysis of apoptosis can help to detect apoptotic cells because in such culture conditions, the cells undergoing apoptosis are less likely to come in contact with phagocytic cells (8, 9).

3.1. Annexin V/Propidium Iodide Staining

As described previously, during apoptosis a cell undergoes many changes. One of the early characteristics of apoptosis is the translocation of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer one. Annexin V binds to PS with high affinity in the presence of Ca^{2+}. Thus, fluorochrome-conjugated Annexin V can be used as a sensitive probe for the detection of PS on the cell surface. Annexin-V-Fluos stain identifies the apoptotic cells, while Propidium Iodide (PI) stains the DNA of necrotic cells.

3.1.1. Procedure

1. Collect the cells exposed to the agent either in vivo or in vitro (see Note 1) and place 10^6 cells per 12 × 75 mm round bottom culture tube.

[1]The immune cells may be cultured in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of vehicle or different concentrations of the toxicant for 24–48 h for the detection of apoptosis.

2. Add 2 ml of phosphate buffered saline (PBS) per tube and wash the cells at 200×*g* for 10 min.
3. Discard supernatant and resuspend the cell pellet in 100 μl of Annexin-V-Fluos labeling solution.
4. Incubate for 10–15 min at room temperature.
5. In order to remove any excess fluorochrome, add 2 ml of phosphate buffered saline (PBS) per tube and wash the cells at 200×*g* for 10 min.
6. Determine fluorescence, using a flow cytometer. Cells that are Annexin V+ PI− are the early apoptotic cells, Annexin V+ PI+ are late apoptotic cells and Annexin V− PI+ are necrotic cells.

3.2. TUNEL to Detect DNA Fragmentation

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) kit is used to detect single- and double-strand breaks in DNA in individual cells, which is a marker for early apoptotic cells. The kit employs the enzyme TdT, which catalyzes the addition of modified deoxyribonucleotides (dUTP) to the 3′OH ends of single- and double-stranded nicks. The dUTP is fluorescently labeled and can therefore be detected with a flow cytometer.

3.2.1. Procedure

1. Collect treated cells and place 10^6 cells per 12 × 75 mm round bottom culture tube.
2. Add 2 ml of staining buffer per tube and wash the cells at 200×*g* for 10 min.
3. Discard supernatant and resuspend the cell pellet in 100 μl of fixation solution (4% paraformaldehyde in PBS) per tube. The fixation cross-links the DNA to the cellular constituents so it is not lost during the permeabilization step.
4. Incubate the cells at 4°C for 30 min.
5. Wash the cells twice by adding 2 ml of staining buffer per tube and by centrifugation at 200×*g* for 10 min.
6. Add 100 μl of permeabilization solution per tube and incubate at room temperature for 5 min. This permits the enzymes to enter the cell through the permeable membrane.
7. Wash the cells twice by adding 2 ml of staining buffer per tube and by centrifugation at 200×*g* for 10 min.
8. Freshly prepare labeling solution, add 25 μl per tube, and incubate at 37°C for 1 h.
9. Wash the cells twice by adding 2 ml of staining buffer per tube and by centrifugation at 200×*g* for 10 min (see Note 2)

3.3. In Situ TUNEL Staining

The principle of this assay is identical to that described in TUNEL method, in which apoptotic cells can be visualized in frozen or paraffin-embedded tissue sections, smears, cytospin, or adherent cells. The assay can be fluorometric or colorimetric, depending on the incubation buffer mix. The following assay procedure is used for the fluorometric staining of paraffin-embedded sections.

3.3.1. Procedure

1. Wash paraffin-embedded sections twice by immersing in xylene in a Coplin jar for 5 min. This step deparaffinizes the tissue sections.
2. Immerse the slides in 100% ethanol containing Coplin jars for 5 min at room temperature.
3. Perform graded ethanol washes by immersing slides in 100%, 95%, 85%, 70%, and 50% ethanol respectively, for 3 min per wash. This step rehydrates the tissue sections.
4. Wash the slides first by immersing in PBS for 5 min at room temperature.
5. Place the slides in 4% methanol-free formaldehyde in Coplin jars for 15 min at room temperature. This step fixes the tissue sections.
6. Wash slides with PBS for 5 min. Repeat twice.
7. At this step, dry the slides and place them on a flat surface.
8. Prepare 20 μg/ml Proteinase K solution and add 100 μl to each slide in order to cover the tissue section. Incubate slides for 8–10 min at room temperature.
9. Wash slides with PBS for 5 min at room temperature.
10. Place the slides in 4% methanol-free formaldehyde in Coplin jars for 15 min at room temperature.
11. Once again, wash the slides with PBS for 5 min at room temperature in order to remove 4% methanol-free formaldehyde.
12. Tap the slides on a stack of paper towels in order to remove excess liquid.
13. Add 100 μl of equilibration buffer in order to cover the tissue sections.
14. Incubate at room temperature for 5–10 min.
15. Prepare rTdT incubation buffer by mixing 45 μl of equilibration buffer, 5 μl of nucleotide mix, and 1 μl of rTdT enzyme per reaction. 50 μl is required per reaction.

[2]Positive control slides should be prepared for in situ TUNEL assay. After step 11 in subheading 18.2.3, add 100 μl of DNase I buffer to the slide and incubate at room temperature for 5 min. Then, remove the excess liquid, add 5–10 units/ ml of DNase I in DNase buffer to the slides, and incubate at room temperature for 10 min. Wash the slide at least three times with deionized water in a Coplin jar and continue with the staining steps. It is important to use separate Coplin jars for the positive control, since DNase contamination may affect the experimental slides.

16. Thaw the nucleotide mix on ice, always keep the incubation buffer on ice and protect it from light (see Note 3).
17. Using a paper towel, blot around the tissue in order to remove some of the equilibration buffer.
18. Add 50 μl of incubation buffer on each section and cover the tissue section with a plastic cover slip. The cover slip ensures even staining of the sections.
19. Place the slides in a humidified chamber, cover with foil and incubate at 37°C for 60 min.
20. Make up 2× SSC from stock by mixing 5 ml of 20× SSC with 45 ml of deionized water and fill a Coplin jar with the solution.
21. Remove the coverslips and immerse slides in 2× SSC for 15 min at room temperature.
22. Wash three times with fresh PBS for 5 min at room temperature. This step ensures the removal of unincorporated fluorescein-dUTP.
23. Prepare 40 ml of 1 μg/ml propidium iodide in PBS and fill a Coplin jar.
24. Place slides in PI solution for 15 min at room temperature in the dark.
25. Wash with deionized water for 5 min at room temperature. Repeat this step two more times.
26. Remove excess water from the slides.
27. Add one drop of antifade solution to the area.
28. Mount the slides using glass coverslips.
29. Seal the edges with clear nail polish and let them dry for 5–10 min.
30. Immediately, analyze slides under a fluorescent microscope. The green fluorescence of fluorescein can be viewed at 520 ± 20 nm and the red fluorescence of PI can be viewed at >620 nm.

3.4. Gel Electrophoresis for Detection of DNA Fragmentation

1. Dispense 1–5 × 10^6 cells in tubes.
2. Centrifuge the cells at 200×*g* at 4°C for 10 min.
3. Add 0.5 ml of TTE solution to the pellet and vortex vigorously. This procedure allows the release of fragmented chromatin from nuclei after cell lysis with Triton-×-100 and disruption of the nucleus following Mg^{++} chelation by EDTA in the TTE solution.

[3]The colorimetric DeadEnd TUNEL system kit may be obtained from a commercial source such as Promega (Cat. #G7130). The assay is very similar to the fluorometric assay, except that the rTdT reaction mix includes biotinylated nucleotide mix. Additional steps are necessary such as blocking the endogenous peroxidase, addition of peroxidase-labeled streptavidin followed by the substrate, hydrogen peroxide, and the chromogen, 3,3′-diaminobenzidine (DAB), provided in the kit.

4. Centrifuge tubes at 20,000×*g* for 10 min at 4°C to separate fragmented DNA from intact chromatin.
5. Transfer supernatants to new tubes.
6. Add 0.5 ml of TTE solution to the pellet.
7. Add 0.1 ml of ice-cold 5 M NaCl to the 0.5 ml volume present in tubes having the supernatants or pellet and vortex vigorously. The addition of the salt removes histones from DNA.
8. Add 0.7 ml of ice-cold isopropanol to each tube and vortex vigorously.
9. Allow precipitation to proceed overnight at −20°C.
10. Pellet DNA by centrifugation for 10 min at 20,000×*g* at 4°C.
11. Discard supernatants by inverting the tubes.
12. Rinse the pellets by adding to each tube 0.5–0.7 ml of ice-cold 70% ethanol.
13. Centrifuge the tubes at 20,000×*g* for 10 min at 4°C.
14. Discard supernatants by rapidly inverting tubes.
15. Air dry the tubes for about 3 h.
16. Dissolve DNA by adding to each tube 20–50 μl of TE solution at 37°C for 1–3 days.
17. Mix DNA with 10× loading buffer to a final concentration of 1×.
18. Place the samples in a heating block at 65°C for 10 min.
19. Immediately load 10–20 μl of to each well of a standard 1% agarose gel containing ethidium bromide 0.5 mg/ml.
20. Appropriate DNA molecular weight markers should be included. Ethidium bromide is a potential carcinogen and should only be handled while wearing gloves.
21. Run the gel electrophoresis in TBE buffer at the desired voltage. The migration of samples is followed by the migration of bromophenol blue dye contained in the loading buffer.
22. Stop the electrophoresis when the dye reaches about 3 cm from the end of the gel.
23. To visualize DNA, place the gel on a UV transilluminator and take photos of the gel. Wear eye and skin protection when UV is on. DNA laddering is characteristic of apoptosis.

3.5. Mitochondrial Membrane Potential

During the earliest stages of apoptosis, even before the exposure of phosphatidylserine, there is loss in mitochondrial membrane potential and the mitochondria become leaky. This leads to translocation of cytochrome c into the cytosol and further advancement of the intrinsic apoptotic pathway. One easy way to measure mitochondrial membrane potential is with a potential-sensitive, membrane-permeable, lipophilic cation dye called 3,3-dihexylox-

acarbocyanine iodide ($DiOC_6$), which exhibits fluorescence after accumulation into energized systems.

3.5.1. Procedure

1. Culture immune cells at a density of 5×10^6 cells/ml with the toxicant.
2. Add 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) at a final concentration of 40 nM 15 min prior to the end of incubation time.
3. After the incubation time, harvest the cells and place in 12 × 75 mm round bottom culture tubes.
4. Assess mitochondrial membrane potential ($\Delta\psi_m$) by using a flow cytometer with excitation and emission settings of 488 and 525 nM. A decrease in the fluorescence intensity is indicative of a loss of mitochondrial membrane potential (see Note 4).

3.6. Detection of Caspase Activity

The extrinsic apoptotic pathway is characterized by the activation of caspase 8, while the intrinsic pathway is characterized by the activation of caspase 9, and both of these pathways intersect at the activation of caspase 3. The existence of these proteins is directly proportional to the amount of apoptosis, and it can be measured with available reagent kits or with immunoblotting. Please refer to the next subheading (18.2.7 Protein assays) for immunoblotting protocols. The Caspase-Glo Assays from Promega use the luminogenic caspase-8 tetrapeptide substrate (Z-LETD-aminoluciferin), the caspase-9 tetrapeptide substrate (Z-LEHD-aminoluciferin), or the caspase-3/7 substrate (Z-DEVD-aminoluciferin), and a stable luciferase in buffer, which induces cell lysis and can detect luciferase activity. Upon cleavage of the substrate by the caspase, aminoluciferin is liberated and can contribute to the generation of light in a luminescence reaction. This section provides the assay protocol for the Apo-ONE homogenous caspase 3/7 assay kit.

3.6.1. Procedure

1. Incubate the blank, the negative control and the experimental samples in a 96 well-plates.
2. Thaw 100× substrate buffer to room temperature.
3. Prepare the Apo-ONE caspase 3/7 reagent by making a 1:100 dilution.
4. Add 100 μl to each well of the 96-well plate.
5. Mix the contents of the plate with a shaker at 300–500 rpm for 30 s.
6. Incubate the plate at room temperature between 30 min to 18 h.
7. Perform a time response in order to determine the optimal time for caspase activity.

[4]In the mitochondrial membrane potential detection assay (Subheading 18.2.5), expose the cells to 5 μmol/L carbamoyl cyanide *m*-chlorophenylhydrazone for 15 min at 37°C for a positive control.

8. Measure the fluorescence of the wells with excitation wavelength at 499 nm and emission wavelength at 512 nm by using a plate reader.

3.7. Western Blotting for Protein Estimation

An increase in some important proapoptotic proteins and/or a decrease in the antiapoptotic molecules within a cell is an indicator of apoptosis. In the intrinsic pathway, the Bcl family proteins play a significant role as well as the leakage of cytochrome c from the mitochondria into the cytosol. Both in the intrinsic and extrinsic pathway of apoptosis, the effector caspase 3 plays a significant role, and thus, the cleavage of procaspase 3 to caspase 3 is assayed. PARP, an enzyme that is responsible for DNA repair, is also cleaved by caspase 3. Thus, caspase 3 activity is studied by estimating PARP cleavage. Furthermore, the cleavage of procaspase 8 and 10 is indicative of the involvement of the death receptor pathway, while cleavage of procaspase 9 denotes the induction of the mitochondrial pathway. Detection of the proapoptotic truncated Bid suggests cross-talk between the death receptor and mitochondrial pathways. All of these proteins can be quantitated by preparing lysates from cells as well as the cytosolic and mitochondrial fractions and immunoblotting for the protein of interest.

3.7.1. Procedure

1. Place the small plate onto a 1 mm spacer plate and slide the plates into the frame.
2. Tighten the clamps and put on the stand with the short plate facing you.
3. Insert a comb and make sure to leave 1 cm between the bottom of the loading lanes and the top of the resolving gel.
4. Prepare a resolving gel: 3.4 ml DI water, 2.5 ml 1.5 M Gel Buffer, 4 ml 30% acrylamide, 100 μl 10% SDS
5. In the last minute of gel preparation, add 50 μl 10% APS and 5 μl TEMED for the resolving gel.
6. Pour the resolving gel and add a few drops of 0.1% SDS in order to form a uniform-shaped gel. Cover the gel with water.
7. Once the gel polymerizes, drain the water and remove excess water with filter paper.
8. Prepare the stacking gel.
9. Stacking gel: 6.1 ml DI water, 2.5 ml 1.5 M Gel Buffer, 1.3 ml 30% acrylamide, 100 μl 10% SDS
10. In the last minute of gel preparation, add 50 μl 10% APS and 10 μl TEMED for the stacking gel.
11. Pour the gel on top of the resolving gel and push the comb so that the top of the wells corresponds to the top of the glass.
12. Place the gel into the electrode assembly with the short plate on the inside.
13. Fill the center of chamber with buffer.

14. Denature samples as well as the ladder by boiling or using the PCR machine at 95°C for 3 min.
15. Load the ladder and the samples at the concentration of 15 μg of protein/lane.
16. Fill the empty wells as well as the outside part of the chamber with loading buffer and try to avoid air bubbles.
17. Run the samples on high at 200 V until the samples reach the buffer.
18. Saturate Whatman papers, the transfer membrane, and the brillo pads with transfer buffer.
19. In order to perform the gel transfer, disassemble the apparatus, and wash the chamber.
20. Place a Whatman paper on a brillo pad then place both on the black side of the sandwich device.
21. Separate the plate carefully, remove the gel and cut the bottom of the first lane.
22. Put the stacking gel on the Whatman paper with the cut on the bottom right corner.
23. Cut the right corner of the transfer membrane and place it onto the gel.
24. Place another wet Whatman paper on the gel, followed by the brillo pad, and close the chamber.
25. Put the black chamber into the transfer chamber containing an ice block and add enough transfer buffer until it reaches the top of the first row of holes. Run at 100 V for 1–2 h.
26. Separate the membrane from the gel, place it in the block solution protein side up and incubate on a shaker for 1 h.
27. Wash the membrane three times with 0.1% TBS-T by shaking at room temperature for 10 min each.
28. Prepare the primary antibody solution in 5% blocking solution, add it to the membrane, and shake at 4°C overnight.
29. Wash the membrane three times with 0.1% TBS-T by shaking at room temperature for 10 min.
30. Prepare the secondary antibody solution in 5% blocking solution, add it to the membrane, and shake at room temperature for 1 h.
31. Wash the membrane three times with 0.1% TBS-T by shaking at room temperature for 10 min.
32. Freshly prepare enhanced chemilluminescence (ECL) solution according to the manufacturer’s directions.
33. Cover the blot with the solution, keeping the protein side up.v
34. Incubate for 1 min.

35. Remove air bubbles.

36. Develop the blot by using a fast film, usually XOMAT for 2–20 min.

3.8. RT-PCR Analysis of Gene Expression

Yet another way to detect the presence of the apoptotic molecules such as Fas and FasL is to use reverse transcriptase-polymerase chain reaction (RT-PCR). However, this is a semiquantitative assay and does not depict the protein expression. Earlier studies have used an extraction procedure that involved the partitioning of RNA into aqueous and organic phases for separation. In more recent studies, commercially available cartridges that use adsorption technology to purify RNA are used. Unlike the older techniques, the recent methods are time-saving and yield good quality total RNA without major concerns for its degradation. We describe both of the methods here.

3.8.1. RNA Extraction Using Trizol Reagent

1. Isolate total RNA from immune cells using Trizol Reagent.
2. Homogenize 100 mg of tissue in a glass homoginizer in 1 ml of Trizol reagent. Mix the homogenate with repetitive pipetting.
3. Transfer to a 2 ml polypropylene microfuge tube.
4. Pellet 5 million cells in a 2 ml microfuge tube and lyse in 1 ml of Trizol reagent with repetitive pipetting.
5. After five minutes at room temperature to allow complete dissociation of nucleoprotein complexes, add 0.2 ml of chloroform.
6. After the closing the lid, vigorously shake the tubes for 15 s.
7. After standing at room temperature for 15 min, spin tubes at 12,000×*g* for 15 min at 4°C to separate RNA containing aqueous phase (top) from DNA (interface) and protein containing organic phase (bottom).
8. Transfer the aqueous phase to a fresh tube and precipitate RNA by mixing with 0.5 ml of isopropanol.
9. After standing at room temperature for 10 min, centrifuge at 12,000×*g* for10 min at 4°C to pellet RNA.
10. Wash RNA precipitate (not often visible) by suspending in 1 ml of 75% ethanol.
11. Vortex the tube and centrifuge at 7,500×*g* for 5 min at 4°C.
12. After removing ethanol, partially air dry the RNA pellet for 3–5 min and dissolve in 20–30 μl of double distilled water or TE buffer by warming at 55°C for 20 min.
13. The optical density at 260 nm of 1.0 corresponds to 0.04 μg/μl of RNA. The A260/280 ratio should be ≥1.8 (see Note 5).

3.8.2. Extraction of Total RNA Using RNeasy Mini Kit—The commercially available RNAeasy Mini kit can isolate RNA longer than 200 bp within 30 min of cell lysis, without

using alcohol precipitation or chloroform extraction steps. It uses a silica-based membrane and the speed of microspin technology to isolate highly pure RNA that is suitable for a wide range of applications.

Procedure

1. First lyse biological samples and homogenize in the presence of the denaturing reagent guanidine isothiocyanate containing buffer, which immediately inactivates RNases to ensure purification of intact RNA.
2. Add ethanol to provide appropriate binding conditions.
3. Apply the sample to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away.
4. Elute high-quality RNA in 30–50 μl water.
5. Remove any residual DNA contamination that might affect PCR reaction by using a RNase-free DNase.

3.8.3. RT-PCR—The gene expression of proapoptotic and antiapoptotic genes can be measured by RT-PCR.

Procedure

1. Use 10 μg of RNA from each sample for reverse transcription to synthesize cDNA, using Moloney murine leukemia virus reverse transcriptase
2. Subject the cDNA samples to PCR amplification using synthetic oligonucleotide primers for proapoptotic genes such as Fas, FasL, or β-actin. Use β-actin as an internal control along with other genes of interest in PCR tubes together with PCR reagents supplied in Epicenter Master mix kit in amounts recommended by the manufacturer. The conditions of the PCR vary and may be as follows: denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 2 min. Totally, 30 cycles are performed. The following primers will be used: Fas sense primer, 5′-GCACAGAAGGGAAGGAGTAC-3′; Fas antisense primer, 5′ GTCTTCAGCAATTC-TCGG GA-3′ (amplified fragment, 455 bp); FasL sense primer, 5′-GAGA-AGGAAA-CCCTTTCCTG-3′; FasL antisense primer, 5′-ATATTCCTGGTGCCCATGAT-3′ (amplified fragment, 940 bp); mouse β-actin sense primer, 5′-ATCCTGACCCT-GAACTACCCCATT-3′; and β-actin antisense primer, 5′-GCA-CTGTAGTTTCTCTTCGACACGA-3′ (amplified fragment, 464 bp).
3. Electrophorese the PCR products through a 1.5% agarose gel containing ethidium bromide (Subheading 18.2.4).

[5]RNA is very sensitive to degradation by RNases and, therefore, precautions should to taken to prevent their introduction by wearing gloves, using autoclaved distilled water and buffers and dry baked plastic or glassware (Subheading 18.2.8). If necessary, prepare all the solutions with DEPC (diethyl-pyrocarbonate)-treated water, using 0.1% (v/v) DEPC in distilled water (dH_2O). Allow the DEPC-treated water to incubate overnight at room temperature then autoclave the DEPC-treated water prior to use. Do not use DEPC-treated water to dissolve the final RNA pellet, as it interferes with optical density readings.

4. Quantitatively measure the PCR products by taking a digital photograph of the gel exposed to UV light, using the imager that contains the UV source, a digital camera, and the software for computing the intensity of each band.

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