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Lipid Peroxidation Models

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Introduction

Lipids are important targets of active oxygen species and free radicals and the oxidation products of lipids have a variety of biological effects. Accordingly, lipid peroxidation has often been studied *in vitro* to elucidate its mechanism and products and to assess antioxidant activity against it. Subjects which must be considered are how lipid peroxidation is induced in a particular type of reaction medium, and how the extent of lipid peroxidation is measured, because antioxidant efficacy is highly dependent on these factors.

Preparation of Liposomes

Liposomes comprising phospholipids with and without free cholesterol are often used as a model of biological membranes [1].

Protocol

1. Prepare chloroform or methanol solution of phosphatidylcholine (3 mM), free cholesterol (1 mM) and lipid-soluble additive, if any in a 50-mL pear-shaped flask.
2. Remove solvent under reduced pressure by rotary evaporation to obtain a thin film on the flask wall.
3. Add an equal volume of phosphate-buffered saline (pH 7.4) and slowly peel off the lipid film by shaking to obtain multilamellar vesicles.
4. If required, small unilamellar vesicles may be obtained by sonicating the above suspension in ice water.
5. Oxidize liposomes with a suitable oxidant.

Preparation of Liposomes for Uptake by Macrophages

Protocol

1. Prepare chloroform solution of dioleoyl phosphatidylcholine (DOPC; 25 mM), phosphatidylserine (PS, bovine brain; 2.5 mM), dicetyl phosphate (DCP; 1 mM) and lipid-soluble additive, if any (ex. fluorescent compound).
2. Place DOPC (40 µL), PS (40 µL), and lipid-soluble additive in a 50-mL pear-shaped flask.
3. Remove solvent under reduced pressure by rotary evaporation at 37°C to obtain a thin film on the flask wall.
4. Add sterilized glucose solution (0.3 M, 1 mL; 37°C) and peel off the lipid film by shaking to obtain multilamellar vesicles.
5. Sonicate the suspensions (20 W, 5 min) in an ice-cold water-bath.
6. Filter with a 0.22 µm Mille-GV filter (Millipore).

Preparation of Oxidized LDL

Protocol

1. Isolate LDL (1 .019 < d < 1.063) from human or animal plasma by centrifugation.
2. Dialyse with PBS containing EDTA (100 µM) at 4°C for more than 12 h.
3. Determine concentration of LDL by measuring protein concentration of LDL with a BCA kit (Pierce).
4. Place LDL (0.25-4.0 mg protein mL^{-1}), PBS (10 mM, pH 7.4), and EDTA (100µM) in a brown bottle with a cap.
5. Add oxidizing species such as copper salt ($CuCI_2$, $CuSO_4$ 1-50 µM), AAPH (0.5-5 mM), SIN-1 (20µM-1 mM) or lipoxygenase (-5 nktal mL^{-1}) as PBS solution or MeO-AMVN (50-500µM) as acetonitrile solution (less than 1% v/v).
6. Incubate at 37°C with gentle mixing.
7. Remove an aliquot at appropriate time.
8. Stop oxidation by cooling on ice.
9. Remove oxidizing species by use of a PD-10 column (Pharmacia).
10. Sterilize by filtration with through a 0.22-µm Mille-GV filter (Millipore).

Preparation of Macrophages

Protocol

1. Administer thioglycollate (40.5g L^{-1}, NIH Thioglycollate Broth, DIFCO; 1 mL) intraperitoneally (26-gauge needle) to mouse (ICR mouse, 6-8 weeks).
2. After 3 days kill the mouse by cervical dislocation.
3. Sterilize abdomen with ethanol (70% v/v) and incise the abdominal skin.
4. Inject ice-cold RPMI 1640 (5 mL) into the peritoneum (22-gauge needle).
5. Massage abdomen to suspend peritoneal cells in the medium.
6. Collect the medium, as much as possible, by means of a syringe (22-gauge needle) and transfer the contents into a 50-mL Falcon tube (pretreated with FCS and cooled on ice to prevent adhesion of macrophages).
7. Centrifuge at 250 g for 5 min at 4°C.
8. Wash cells 2 or 3 times with ice-cold PBS.
9. Incubate cells on non-fluorescent cover glass (1×10^5 cells cm^{-2}, 35 mmφdish) with RPMI 1640 (5% FCS, 50 units mL^{-1} penicillin, 50 µg mL^{-1}, streptomycin; 1 mL) for 1 h (37°C, 5% v/v CO_2 incubator).
10. Wash twice with PBS (37°C) to remove non-adherent cells.
11. Incubate (37°C, 5% v/v CO_2) with the same medium.

Addition of Liposomes

Protocol

1. Add one-twentieth of the volume of the liposome solution to the macrophages.

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2. Incubate (37°C, 5% v/v CO_2 incubator) for 6 h.
3. Wash twice with cold PBS.
4. Observe cells by fluorescence microscopy.
5. Dry the cells at room temperature for 10 min.
6. Add Triton X-100 (2%, 50 μL cm^{-2}) and dissolve the cells at room temperature for 20 min.
7. Measure the fluorescent intensity by spectrofluorimetry at an appropriate wavelength [2].

Addition of Fluorescent Probe (DiI)-labelled LDL

Protocol

1. Add LDL (2 mg protein mL^{-1}, 3 mL) to lipoprotein-deficient serum (Sigma; 5 mL).
2. Add Dil (3, 3'-dioctadecylindocarbocyanine, Molecular Probes; 3 mg mL^{-1}, 100 μL)
3. Incubate at 37°C overnight.
4. Isolate Dil-labelled LDL by ultracentrifugation.
5. Dialyse against PBS (100 μM EDTA pH 7.4) for 48 h.
6. Adjust LDL concentration to approx. 2 mg protein mL^{-1} PBS.
7. Oxidize LDL according to the Section Preparation of oxidized LDL, above.
8. Sterilize LDL by filtration through a Mille-GV 0.45 μm filter (Millipore).
9. Add LDL solution (1/200-1/500, v/v) to macrophage culture medium.
10. Incubate for 24 h (37°C, 5% v/vCO_2).
11. Wash five times with PBS containing BSA (2 mg mL^{-1}) and once with PBS (pH 7.4).
12. Fix cells with 3% formalin in PBS (pH 7.4) at room temperature for 30 min.
13. Observe cells by microscopy with epifluorescent illumination and a rhodamine filter package.

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