

Mast Cell Activation by Lipoproteins

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Summary

Mast cells are activated by a number of agents that act independently from immunoglobulin E (IgE)-mediated hypersensitivity. One of these agents is oxidized low-density lipoprotein (oxLDL). OxLDL has been implicated in the pathogenesis of atherosclerosis and has been shown to induce microvascular dysfunction by the activation of mast cells. In this chapter, we describe the method for isolation of human LDL, oxidation of LDL, and demonstrate that oxLDL activates mast cells by measuring messenger ribonucleic acid (mRNA) levels and protein levels of interleukin (IL)-8 an inflammatory cytokine. IL-8 is a potent chemoattractant for neutrophils and monocytes, which would result in a chronic inflammatory response. IL-8 mRNA levels were measured by reverse-transcription polymerase chain reaction and protein levels by enzyme-linked immunoassay.

Key Words: HMC-1; mast cells; lipoproteins; cell culture; ELISA; interleukin-8; ultracentrifugation; mRNA.

1. Introduction

The ability of human mast cells to produce a variety of cytokines induced by multiple stimuli suggests that mast cells can participate in immunological processes other than immunoglobulin E (IgE)-mediated hypersensitivity reactions. In support of this view, several studies suggest a role for mast cells in vascular inflammation and arteriosclerosis (1–4), wound healing (5), and rheumatoid arthritis (6). Mast cells are activated to release histamine and cytokines by a number of agents, including anti-IgE, compound 48/80, calcium ionophore A23187, stem cell factor, and phorbol myristate acetate (PMA [7,8]). In this chapter, we use a unique activating agent, namely oxidized low-density lipoprotein (oxLDL). In this method, we describe the isolation and oxidation of human plasma LDL, culture of the human mast cell line HMC-1, the experimental design for activating mast cells using LDL and oxLDL, and verification

From: *Methods in Molecular Biology*, vol. 315: *Mast Cells: Methods and Protocols*
Edited by: G. Krishnaswamy and D. S. Chi © Humana Press Inc., Totowa, NJ

of activation by measuring interleukin (IL)-8 protein secretion by mast cells using enzyme-linked immunoassay (ELISA) and by changes in mRNA for IL-8 using reverse transcription-polymerase chain reaction (RT-PCR).

2. Materials

1. Fresh human plasma from normolipidemic subjects.
2. Density solution 1.006 g/mL: add 12.46 g of KBr to 5 mL of a 10% solution of ethylene diamine tetraacetic acid (EDTA) and bring volume to 1 L.
3. Density solution 1.063 g/mL: add 91.98 g of potassium bromide (KBr) to 5 mL of a 10% solution of EDTA and adjust the volume to 1 L.
4. LDL dialysis buffer (phosphate-buffered saline [PBS]): 0.01 M sodium phosphate, 0.15 M NaCl, and 0.01% EDTA, pH 7.4.
5. Dialysis tubing: spectrapor membrane tubing, 10,000 MW cutoff (Spectrum, Los Angeles, CA).
6. BCA Protein Reagents: Pierce BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).
7. Mast cell culture media: RPMI 1640 media, 5% heat inactivated fetal bovine serum, 0.01 M HEPES, 50 μ g/mL gentamycin, 0.05 mM 2-mercaptoethanol, 5 μ g/mL insulin transferrin solution and 2 mM L-glutamine.
8. Mast cell activator, PMA-ionomycin: 50 ng/mL PMA plus 5 μ M Ionomycin.
9. Beckman Ultracentrifuge and Type 80 Ti rotor: Beckman Instruments, Palo Alto, CA.
10. Agarose gel electrophoresis equipment.
11. Thermal Cycler.
12. 96-well plate reader.

3. Methods

The methods described in **Subheadings 3.1–3.3.** outline: (1) culture of the human mast cell line HMC-1; (2) isolation and oxidative modification of human plasma LDLs; and (3) activation of mast cells by oxLDLs.

3.1. Culture of the Human Mast Cell Line HMC-1

The human mast cell line HMC-1 ([9]; kindly provided by Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN) was grown in mast cell culture media using standard sterile technique. Cells are counted and then seeded in culture flasks at a density of 20,000 cells/mL of media and recultured every 4 d. HMC-1 grow in suspension and their numbers double approximately every 24 h. Cell viability is measured using trypan blue exclusion and is usually greater than 95%. (See Chapter 16 for more information on the culture of HMC-1.)

3.2. Isolation and Oxidative Modification of Human Plasma LDL

Blood is collected from healthy fasting volunteers by venipuncture into tubes containing 1.5 mg of EDTA per milliliter of blood. Blood is centrifuged at

2000g for 10 min to separate the plasma from the cellular components of the blood. Lipoproteins are separated from plasma by sequential ultracentrifugal flotation as described in detail (10).

3.2.1. Isolation of LDL

1. Fill Beckman OptiSeal tubes with fresh human plasma and place the tubes into a Beckman Type 80 Ti rotor (see **Note 1**).
2. Centrifuge the plasma, which has a density of 1.006 g/mL, for 24 h at 14°C in a Beckman L5 75B ultracentrifuge, type 80 Ti rotor, at 112,000g to float the very low-density lipoproteins (VLDL) to the top of the tube (see **Note 2 [10]**).
3. Remove the VLDL from the top of the tube after the 24-h centrifugation using a Beckman tube slicing device (see **Note 3**). Discard the VLDL fraction.
4. Place the infranatant solution in a sterile plastic culture tube and adjust the density to 1.063 g/mL by adding KBr to float the LDL. Add 0.818 g of KBr for each milliliter of infranatant solution (see **Note 4**). Dissolve the KBr by gentle inversion of the tube. Fill the OptiSeal tubes with the solution at a density of 1.063 g/mL and centrifuge for 24 h at 14°C in a Beckman L5 75B ultracentrifuge at 112,000g to float the LDL to the top of the tube. Remove the LDL fraction ($d = 1.006\text{--}1.063$ g/mL) from the top of the tube using a Beckman tube slicing device and place into a sterile plastic culture tube. Dialyze the LDL at 4°C against 4 L of LDL dialysis buffer to remove high levels of salt. After dialysis, determine the protein content of LDL using the Pierce BCA protein assay (Pierce, Rockford, IL) with human serum albumin as the standard (see **Note 4**).

3.2.2. Oxidation of LDL

1. In preparation for oxidation, dialyze a portion of the LDL sample against 4 L of PBS at 4°C without EDTA, because the oxidation process will not work in the presence of EDTA which acts as an antioxidant.
2. Adjust the LDL solution (free of EDTA) to a protein concentration of 200 μ g/mL, and then incubate overnight, with gentle stirring, at 37°C in the presence of 5 μ M CuSO₄ as the oxidizing agent (see **Note 5**).
3. Concentrate the LDL solution to less than 2 mL using a Centriprep Concentrator (Centricon 30, Amicon, Beverly, MA). Verify the protein content of the oxLDL using the BCA protein assay. Confirm the oxidation of LDL by electrophoresis in an agarose gel using the Ciba-Corning Lipoprotein electrophoresis system following the manufacturer's instructions. Visualize the lipoproteins using fat Red 7B provided in the Ciba-Corning kit. OxLDL migrates three to four times faster in the agarose gel than native LDL (see **Note 6; Fig. 1**).

3.3. Activation of Mast Cells by oxLDL

3.3.1. Experimental Setup to Measure Activation of Mast Cells by oxLDL

1. Dispense the cultured HMC-1 into 60 $\times$ 15-mm culture dishes with a final concentration of 2 million cells in a total volume of 2 mL.

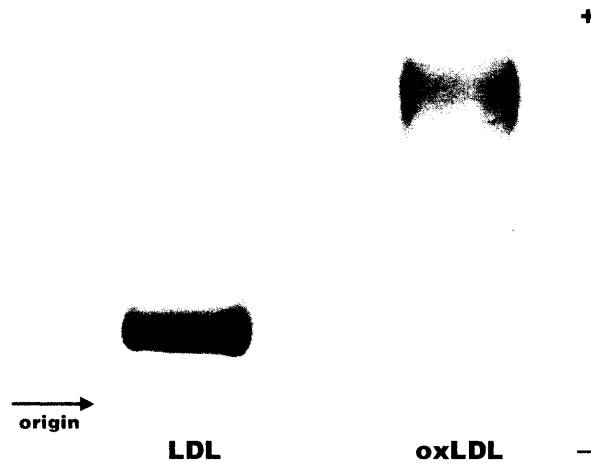


Fig. 1. Agarose electrophoresis of LDL and oxLDL. Lipoproteins were stained with Oil Red O. OxLDL migrates four times faster than LDL.

- Incubate the cells for 24 h at 37°C in a humidified atmosphere of 5 % CO₂, with varying concentrations of native LDL (25, 50, and 100 µg /mL) and oxLDL (25, 50, 100, and 200 µg/mL) in triplicate. Activate HMC-1 using PMA-ionomycin as a positive control. Keep three dishes of HMC-1 in unsupplemented media as a negative control.
- Harvest the cells after 24 h the cells by low speed centrifugation. Freeze the cell pellets in liquid nitrogen for safe storage. Freeze the culture media at -80°C. Assay the cell culture media for IL-8 protein levels by ELISA. Purify the cellular RNA from the frozen cell pellets and assay for IL-8 mRNA by RT-PCR.

3.3.2. Mast Cells Activated With oxLDL Express-Elevated Levels of IL-8 mRNA

Mast cell mRNA for IL-8 is measured, after treatment of cells with native and oxLDL, by RT-PCR (*see* Chapter 16). RNA was extracted and assessed for expression of IL-8 (Sense 5'ATG ACT TCC AAG CTG GCC GTG GCT 3' antisense 5' TCT CAG CCC TCT TCA AAA ACT TCT 3'; DNA synthesis Core Facility, John Hopkins University, Baltimore, MD) The RT-PCR is performed as previously described (*11*). Briefly RT-PCR is performed with a GeneAmp kit from PerkinElmer (Branchburg, NJ) using a PerkinElmer thermal cycler. Cycles for RT consists of one cycle each of 42°C for 20 min, 99°C for 10 min and 5°C for 5 min. PCR consists of initial denaturation at 95°C for 2 min, denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension

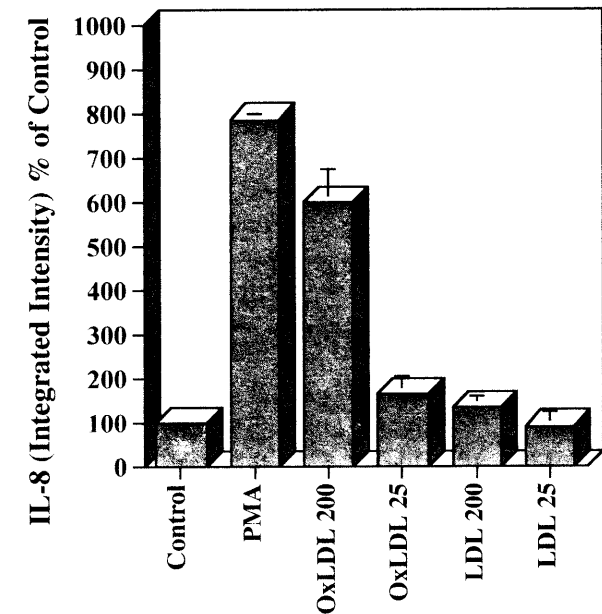


Fig. 2. Measurement of IL-8 mRNA by RT-PCR as a marker for activation of mast cells by lipoproteins. Mast cells were stimulated to express IL-8 mRNA after activation with PMA + ionomycin as a positive control and by oxLDL but not by native LDL. A representative agarose gel from RT-PCR for IL-8 is shown as an insert at the top of the figure. Integrated intensity was determined by scanning the gel. HMC-1 mast cells were incubated with 25 and 200 µg/mL of native LDL and 25 and 200 µg/mL of oxLDL.

at 72°C for 90 s. Samples are run on agarose gels and photographs are scanned to measure the intensity of the bands. OxLDL treatment of mast cells results in a concentration-dependent increase in IL-8 mRNA (**Fig. 2**).

3.3.3 Mast Cells Activated With oxLDL Secrete IL-8 Protein Into the Media

Human IL-8 protein secreted into the media by mast cells was assayed using an ELISA kit developed by R & D Systems (Quantikine®, R&D Systems, Minneapolis, MN) according to the manufacturer's protocol (*see* Chapter 16). OxLDL treatment of mast cells results in a significant concentration-dependent increase in IL-8 protein as measured by ELISA (**Fig. 3**).

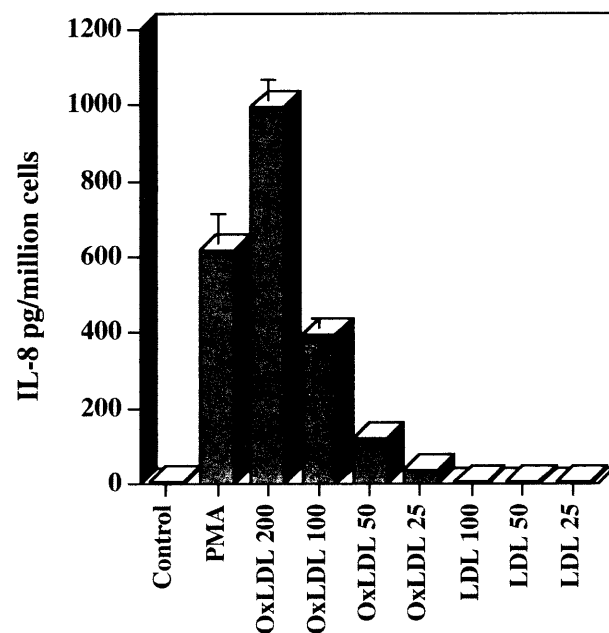


Fig. 3. Measurement of IL-8 protein by ELISA as a marker for activation of mast cells by lipoproteins. Mast cells were stimulated to secrete IL-8 into the media after exposure to PMA + ionomycin as a positive control and by increasing concentrations of oxLDL for 24 h. Native LDL did not stimulate secretion of IL-8. HMC-1 mast cells were incubated with 25, 50, and 100 $\mu\text{g/mL}$ of native LDL and 25, 50, 100, and 200 $\mu\text{g/mL}$ of oxLDL.

4. Notes

1. Other types of centrifuge tubes can be used, such as the Beckman QuickSeal tubes, but these tubes require a heat-sealing device, which is more difficult to use than the OptiSeal tubes. It is important that reagents and tubes that come in contact with the LDL are free of endotoxin because small amounts of endotoxin can activate mast cells.
2. Any ultracentrifuge or rotor combination may be used for this lipoprotein isolation method as long as they can achieve an average g -force of 112,000 g . It is critically important to minimize vibration of the rotor containing the plasma during and after the centrifugation. All density solutions are prepared in pyrogen free water. For accuracy of the density solutions, the KBr is dried by heating at 100°C for 2 h then stored in a dessicator. Densities of all of the solutions is verified using a Mettler PAAR Density Meter.
3. If a tube-slicing device is not available, the lipoprotein fractions can be removed from the top of the tube using a syringe and a small bore needle. Aspirate slowly with the needle bevel in the down position.

4. At this step, it is important that the dialysis buffer contains EDTA to protect LDL from oxidation. LDL also should be protected from light and be used within 1 wk of preparation.
5. It is important not to be too vigorous in the stirring action during the oxidation step. Stirring too vigorously will cause the LDL to precipitate out of solution; the solution becomes very cloudy if this happens.
6. If the oxLDL does not migrate faster than the native LDL, the EDTA was not completely removed during dialysis. Repeat the dialysis and the oxidation steps.

Acknowledgments

The authors thank Dr. Butterfield for providing the HMC-1 cell line used in these studies. We also thank Karen Cantor her technical support. This work was supported by grants from East Tennessee State University RDC, Cardiovascular Research Institute and the Department of Internal Medicine.

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