Evaluation of foam cell formation in cultured macrophages: an improved method with Oil Red O staining and DiI-oxLDL uptake

Suowen Xu · Yan Huang · Yu Xie · Tian Lan · Kang Le ·
Jianwen Chen · Shaorui Chen · Si Gao · Xiangzhen Xu ·
Xiaoyan Shen · Heqing Huang · Peiqing Liu

Received: 18 February 2010 / Accepted: 8 July 2010 / Published online: 16 November 2010
© Springer Science+Business Media B.V. 2010

Abstract Macrophage-derived foam cell formation elicited by oxidized low-density lipoprotein (oxLDL) is the hallmark of early atherogenesis. Detection of foam cell formation is conventionally practiced by Oil Red O (ORO) staining of lipid-laden macrophages. Other methods include 1,1'-dioctadecyl-3,3,3'-tetramethylindocyanide percholorate (DiI)-labeled oxLDL (DiI-oxLDL) uptake and Nile Red staining. The purpose of the present study is to report an optimized method for assessing foam cell formation in cultured macrophages by ORO staining and DiI-oxLDL uptake. After incubation with oxLDL (50 µg/ml) for 24 h, the macrophages were fixed, stained with ORO for just 1 min, pronounced lipid droplets were clearly observed in more than 90% of the macrophages. To test the in vivo applicability of this method, lesions (or foam cells) of cryosections of aortic sinus or primary

mouse peritoneal macrophages from ApoE deficient mice fed a high cholesterol diet were successfully stained. In another set of experiments, treatment of macrophages with DiI-oxLDL (10 µg/ml) for 4 h resulted in significant increase in oxLDL uptake in macrophages as demonstrated by confocal microscopy and flow cytometry. We conclude that the optimized ORO staining and fluorescent labeled oxLDL uptake techniques are very useful for assessing intracellular lipid accumulation in macrophages that are simpler and more rapid than currently used methods.

Keywords Atherosclerosis · Macrophages · Foam cell · Oil Red O · DiI-oxLDL

Introduction

Atherosclerosis, the most common cause of myocardial infarction, stroke and cardiovascular mortality, is an inflammatory-immunomodulatory disease process (Ross 1999; Lusis 2000; Wuttge et al. 2001; Libby 2002; Hansson 2009). Scavenger receptors-mediated uptake of oxidized LDL (oxLDL) by macrophages resulting in foam cell formation represents the hallmark of early atherosclerosis (Moore and Freeman 2006). Atherogenic modifications of LDL include, in addition to oxidation, acetylation, retention and aggregation. Among these modified LDL, oxLDL is the most typical and physiological stimulus of macrophage foam cell formation (Parthasarathy et al. 2010).

Suowen Xu and Yan Huang contributed equally to this work.

S. Xu · Y. Huang · T. Lan · K. Le · J. Chen ·
S. Chen · S. Gao · X. Xu · X. Shen · H. Huang ·
P. Liu (✉)

Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-Sen University (Higher Education Mega Center), 132 East Wai-huan Road, 510006 Guangzhou, People's Republic of China
e-mail: liupq@mail.sysu.edu.cn

Y. Xie

Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Zhongshan 2 Rd., 510080 Guangzhou, People's Republic of China

The assessment of foam cell formation is of primary interest as an important end-point analysis for evaluating atherogenesis both in vitro and in vivo (Scholz et al. 2004; Beckers et al. 2007; Li et al. 2008; Ma et al. 2008; Lee et al. 2009). Morphometric methods involving the staining of *en face* arterial tree (Kobayashi et al. 2004) or aortic sections from aortic sinus (Kobayashi et al. 2004) or innominate artery (Teupser et al. 2004) with Oil Red O (ORO) followed by computer-assisted image analysis are commonly used for in vivo studies. Based on the published literature, the standard protocol for ORO staining is summarized in Table 1. Briefly, the standard staining protocol mainly consists of fixation (10% formalin or 3.7–4% paraformaldehyde, 10 min to 1 h (Li et al. 2008)), ORO staining (10 min to 4 h (Scholz et al. 2004; Li et al. 2008)), and counterstaining. Additionally, for quantification of the capacity of macrophages to uptake oxLDL, a fluorescent method was developed, i.e., DiI-labeled oxLDL (DiI-oxLDL) uptake by fluorescent microscopy or flow cytometry. For a detailed description of this method and its application, we refer the reader to the excellent article by Teupser et al. (1996). As far as this method is concerned, different time of DiI-oxLDL stimulation was used in various studies, ranging from 3 h (Ide et al. 2006), 6 h (Sawamura et al. 1997) to 24 h (Lian et al. 2008). In addition, the concentration of DiI-oxLDL varied from 5 µg/ml (Moriwaka et al. 2010), 10 µg/ml (Wuttge et al. 2001), 20 µg/ml (Nagy et al. 1998), to 80 µg/ml (Li et al. 2004). Despite DiI-oxLDL can be obtained from commercial suppliers (such as Intracell Inc.), home-made DiI-oxLDL is preferred and widely used in atherosclerotic research for the relatively low cost.

Our objective was to evaluate an optimized method for assessing oxLDL-elicited (50 µg/ml, 24 h of incubation) foam cell formation by staining with the neutral lipid-targeting lysochrome Oil Red O (ORO; Sigma) for 1 min, as well as by DiI-oxLDL

uptake (10 µg/ml, 4 h of incubation) using confocal microscopy and flow cytometry.

Materials and methods

Chemicals and reagents

The DMEM medium and other cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate (DiI) and DAPI were from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). All other chemicals were of analytical reagent grade or better and were obtained from Sigma-Aldrich or other commercial suppliers.

Cell culture

RAW 264.7 cells, a murine macrophage cell line (ATCC, Rockville, MD), were cultured in 60-mm petri dishes at a density of 1×10^6 cells/ml in DMEM medium supplemented with 100 IU/ml of penicillin G, 100 µg/ml streptomycin, 2 mmol/l L-glutamine, and 10% (vol/vol) FBS, and incubated in a humidified atmosphere of 5% CO₂ in an incubator at 37 °C. Sub-confluent cells were serum starved for 24 h then incubated with oxLDL for the indicated time periods.

Preparation of aortic sinus from apolipoprotein E knockout (ApoE KO) mice

Homozygous male ApoE KO mice on C57BL/6J background and age-matched wild-type C57BL/6J mice were purchased from Peking University Experimental Animal Center (Beijing, China) after being originally obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed under a 12-h light/dark cycle in specific-pathogen free facility.

Table 1 Summarized optimized staining protocol for Oil Red O (ORO) in comparison to the conventional ORO staining method

Treatment	Conventional ORO staining	Optimized ORO staining
Fixation	10% formalin (30 min)	10% formalin (10 min)
Rinsing	3 × 30 s deionized water	PBS rinsing 1 min; Isopropanol rinsing 15 s
Staining	15 min ORO working solution	1 min in ORO working solution
Rinsing	3 × 30 s deionized water	15 s isopropanol
Rinsing	10 min in running tap water	3 × 3 min in PBS

Starting from 6 weeks, the mice were fed a high cholesterol diet (HCD, 10% fat, 1.25% cholesterol, 0% cholic acid) for 16 weeks. Aortic sinus (aortic root) cryostat sectioning, was performed according to the modified method of Paigen et al. (1987). Briefly, the hearts from each treatment group were harvested with about 1 mm of proximal aorta attached. The top half of the heart with aorta root was embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and snap-frozen in liquid nitrogen. Serial sections were cut from the proximal 1 mm of the aortic root. Sequential 25- μ m sections were cut until the appearance of the 3-aortic valve cusps at the junction of the aorta and the heart. From this point on, serial 8- μ m sections were collected until tricuspid valves were no longer visible.

Isolation and cultivation of mouse peritoneal macrophages

Resident mouse peritoneal macrophages were obtained from male ApoE KO mice and age-matched C57BL/6J mice fed a HCD for 16 weeks. The mice were sacrificed by cervical dislocation, in accordance with Institutional Animal Care and Use Committee. Sterile ice-cold phosphate-buffered saline (PBS) was injected into the cavity of each mouse by peritoneal lavage. This fluid was carefully collected and centrifuged at 1,000 rpm for 6 min. After centrifugation, the supernatant was then withdrawn, and the cell pellet was resuspended in RPMI 1640 medium (containing 100 IU/ml of penicillin, 100 μ g/ml of streptomycin and 100 μ g/ml of L-glutamine) and plated in 6-well tissue culture plates (Costar) at 1.5×10^6 cells per well. Cells were incubated in a humidified CO₂ (5%) incubator at 37 °C for 2–3 h to allow adherence, and non-adherent cells were rinsed away with pre-warmed RPMI 1640 and 2 ml of complete RPMI 1640 medium (supplemented with 10% fetal bovine serum) was added. Medium with all additions were replaced daily and macrophages were used within 5 days from harvesting.

LDL isolation

Human native-LDL (nLDL, $d = 1.019$ – 1.063 g/ml) was prepared from the plasma of fasted normolipidemic volunteers by sequential density gradient ultracentrifugation as described in our previous report (Xu et al. 2009). Briefly, venous blood from healthy

adult donors was obtained from Guangzhou Blood Center (Blood type: O-RhD positive). Blood was collected in EDTA coated tubes and immediately centrifuged at $1,750 \times g$ at 4 °C. Plasma was condensed with polyethylene glycol (PEG) 20,000 and adjusted to a density of 1,200 g/ml by adding solid NaBr, with gentle stirring (IKA Workshop, Germany), after the previous addition of EDTA (1 mM final concentration). The plasma solution was then distributed into 9-ml polycarbonate centrifuge tubes and a discontinuous density gradient was made by overlaying the plasma solution (3.25 ml) with 3.25 ml phosphate-buffered saline ($d = 1.063$ g/ml, NaBr adjusted) and 2.50 ml phosphate-buffered saline ($d = 1.019$ g/ml, NaBr adjusted), saturated with nitrogen. The plasma was ultracentrifuged at 50,000 rpm for 5 h at 4 °C in a Ti-90 fixed-angle rotor (OPTIMA LE-80K; Beckman Instruments, Fullerton, CA, USA). After centrifugation, the tubes were carefully removed from the rotor and placed in the vertical position. The yellow-orange LDL fraction stayed in the infranatant. The LDL fraction was collected by suction using a 1 ml syringe. The collected LDL was dialyzed in the dark for 24 h at 4 °C against 3 changes of 1 l of 0.01 M PBS or 0.15 M NaCl (PH7.4).

Oxidative modification of LDL

To generate copper-oxidized LDL (oxLDL) (Xu et al. 2009), native LDL diluted in PBS to a concentration of 2 mg/ml was incubated with copper sulphate (5 μ M, final concentration) for 24 h at 37 °C, protected from light. The oxidation was terminated by adding Na₂EDTA to a final concentration of 0.24 mM and then dialyzing against phosphate buffered saline for 48 h. The extent of oxidation was determined by measuring thiobarbituric acid-reactive substances (TBARS) value according to the manufacturer's instructions (Jianchen Biotech, Nanjing, China). In our experimental setting, the TBARS value of ox-LDL was 12.50 ± 0.68 versus 0.65 ± 0.26 nM per 100 μ g protein in the native LDL preparation ($P < 0.001$). The purity and charge of both LDL and oxLDL were evaluated by examining the relative electrophoretic mobility (REM) in 0.5% agarose gel containing 0.05 M barbital buffer (PH 8.6). The distance of oxLDL migration was compared to that of native LDL and expressed as REM (2.0 ± 0.5 , Arbitrary Units).

Fluorescent labeling of LDL

LDL was labeled with the fluorescent probe DiI and then oxidized to DiI-oxLDL as described previously (Lian et al. 2008), with minor modifications. Briefly, LDL was incubated overnight at 37 °C under nitrogen and light protection with 50 µL of DiI (3 mg/ml in DMSO) for each milligram of LDL protein. The LDL must be labeled before oxidation. For preparation of DiI-oxLDL, DiI-LDL (0.1 mg/ml) was incubated with 5 µM Cu²⁺ (final concentration) at 37 °C in dark for 24 h. Unbound dye and copper ions from the oxidation step described above which would otherwise be cytotoxic were removed by ultracentrifugation at 50,000 rpm for 5 h at 4 °C. The DiI-oxLDL distributed in the middle layer was re-isolated and subjected to dialysis against phosphate buffered saline containing 0.24 mM EDTA. The protein content of native LDL, oxLDL and DiI-oxLDL were determined using BCATM Protein Assay Kit (Pierce) and expressed as micrograms per milliliter of solution. All lipoproteins were filter sterilized, stored at 4 °C in dark and used within 3 weeks.

Optimized in vitro foam cell assay by ORO staining

Reagents and equipments

Oil Red O stock solution and working solution 0.5 g Oil Red O powder (Sigma) was dissolved in 80 ml isopropanol (100%) in water bath with a temperature of 56 °C overnight. The container was sealed to prevent the evaporation of isopropanol. The final volume of the stock was adjusted to 100 ml and mixed under gentle stirring (IKA Working Group, Germany). Before staining, the stock solution was pre-warmed to 60 °C and filtered with number 1 filter paper (Whatman, UK). A working solution was prepared by diluting the stock solution 6:4 with deionized water, allowing to stand for 10 min at room temperature and then filtered (0.22 µm, Millipore). The working solution should be made freshly and used within 2 h of preparation.

60% isopropanol 60 ml isopropanol was mixed with 40 ml of ddH₂O

Microscope equipped with imaging system The photomicrographs were taken with Image Pro-Plus

6.0 software (Cybernetics) equipped on a microscope (Olympus IX71, Tokyo, Japan).

Staining procedures (less than 0.5 h)

Foam cell induction Cells were plated in 24-well tissue culture plates (Costar) at a density of 1×10^5 cells/ml, when reaching sub-confluence, the macrophages were incubated with oxLDL (50 µg/ml) for 24 h (Beckers et al. 2007), after that, the medium was aspirated and cells were rinsed twice with 0.01 M PBS (Boster, Wuhan, China).

Cell fixation The cells were fixed in 10% phosphate buffered formalin for 10 min.

Rinsing The cells were rinsed in PBS once (1 min) then rinsed in 60% isopropanol for 15 s to facilitate the staining of neutral lipids.

Oil Red O staining The cells were stained with filtered Oil Red O working solution at 37 °C for 1 min in darkness.

Destaining The cells were destained with 60% isopropanol for 15 s.

Rinsing Then washed with PBS for 3 times, 3 min each.

Mounting on microscope Positive-staining (red) cells were macrophage-derived foam cells, which were observed via light microscope (Olympus) and then photographed using Image Pro-Plus 6.0 software (Media Cybernetics).

Optimized cellular uptake of DiI-oxLDL

Uptake of DiI-oxLDL was studied either with confocal microscopy or fluorescence-activated cell sorting (FACS). For confocal microscopy, the cells were grown overnight on culture slides (Warner Instruments) and then incubated with different concentrations of DiI-oxLDL for 4 h. Thereafter, the cells were washed 3 times with PBS-2 mg/ml BSA and twice with PBS. For nucleus staining, the cells were incubated for 10 min with 10 µg/ml DAPI (Sigma) and washed 3 times with PBS. The cells were then placed for confocal microscopy (LSM710, ZEISS, Germany).

For FACS, the cells were incubated with DiI-oxLDL (10 µg/ml medium) for 4, 6, and 24 h. To test the specificity of uptake, the cells were pre-treated

Fig. 1 Representative photomicrographs of lipid-laden macrophages or atherosclerotic lesions by optimized ORO staining. **A** RAW 264.7 macrophages were incubated with oxLDL (50 µg/ml) for 24 h then stained with ORO for different time points as indicated; **B** The cells were incubated in the absence (Ctrl group) or presence of indicated concentrations of oxLDL for 24 h. Cells were then fixed with 10% formalin and stained with ORO for 1 min, original magnification: $\times 640$; **C** ORO

staining of aortic sinus from C57BL/6J mice (**a**) or apoE KO mice (**b**) after 16 weeks of high-cholesterol diet feeding. Aortic lesions were indicated with *blue arrows* and aortic valve leaflets were indicated with *red arrows*. Original magnification $\times 50$. ORO staining of mouse peritoneal macrophages isolated from C57BL/6J mice (**c**) or apoE deficient mice (**d**) after 16 weeks of high-cholesterol diet feeding, original magnification: $\times 640$

with 50-fold excess of unlabeled oxLDL for 30 min then incubated with DiI-oxLDL for 4 h. Thereafter, the cells were washed 3 times with PBS and twice with PBS-2 mg/ml BSA and then were detached with trypsin (0.25% trypsin, 0.02% EDTA). The cells were harvested with RPMI 1640/10% FBS, centrifuged at 1,000 rpm $\times$ 5 min, washed twice with PBS. FACS was performed with a FACScan (Becton–Dickinson). Data were calculated by subtracting the cell autofluorescence from the fluorescence of the treated samples and expressed as mean fluorescence intensity (MFI).

Statistical analysis

Results were presented as means $\pm$ standard deviation (SD). For analysis of differences between various cell treatments, one-way linear analysis of variance

(ANOVA) was used. Differences were considered statistically relevant at $p < 0.05$.

Results and discussion

The time of ORO staining is crucial in examining foam cell formation. We first examined the effect of different time of staining on revealing foam cell formation. As shown in Fig. 1A, after staining with ORO working solution for 1, 15, 30 min, 1 h, only 1 min staining exhibits significant lipid accumulation (indicated by black arrows). Secondly, we examined the dose-dependency of oxLDL on foam cell formation. After incubation with oxLDL for 24 h, RAW264.7 macrophages assumed the morphological appearance of foam cells with ORO-stained lipid droplets present

not only in the perinuclear area, but also distributed throughout the cytosol of most cells (Fig. 1B); in contrast, no lipid droplets were found in the untreated cells. The visual abundance of lipid droplets grossly correlated with the concentration of oxLDL. To determine whether this staining protocol applied to in vivo foam cell formation, frozen sections of aortic sinus or primary mouse peritoneal macrophages from ApoE KO mice or age-matched control C57BL/6J mice were stained by ORO as described above. As shown in Fig. 1C, obvious lipid accumulation was observed in sections or macrophages from ApoE KO mice after 16 weeks of high cholesterol diet feeding. While, negative staining was observed in sections or macrophages from control mice.

In cellular DiI-oxLDL uptake studies by confocal microscopy, it was observed that DiI-oxLDL can be taken up by macrophages in a dose-dependent manner, with 10 $\mu\text{g}/\text{ml}$ achieving the maximal fluorescence (Fig. 2). Similar results were obtained using flow cytometric analysis of DiI-oxLDL uptake in macrophages (data not shown). As shown in Fig. 3, macrophages ingested DiI-oxLDL in a time-dependent manner. After incubation with DiI-oxLDL from

Fig. 2 Representative images of DiI-oxLDL uptake in RAW 264.7 macrophages by confocal microscopy. RAW264.7 macrophages were incubated in the absence (Ctrl group) or presence of indicated concentrations of DiI-oxLDL for 4 h. Cells were then washed, fixed, and examined with a $\times 63$ oil immersion objective using a 546 nm filter set. DiI-oxLDL uptake was shown in red and counterstained with DAPI (blue). Bar = 20 μm

Fig. 3 Representative flow cytometric analysis of DiI-oxLDL uptake by macrophages in different time periods. Cells were stimulated in the absence (Ctrl group, autofluorescence group) or presence of DiI-oxLDL (10 $\mu\text{g}/\text{ml}$) for 4, 6, 24 h, then detached with trypsin, collected, washed and subject to flow cytometry as described in “Materials and methods” section. To test the specificity of uptake, the cells were pre-treated with 50-fold excess of unlabeled oxLDL for 30 min then incubated with DiI-oxLDL for 4 h

4, 6 to 24 h, oxLDL uptake into macrophages was significantly increased (MFI 29.5 ± 1.1 , 39.8 ± 1.6 , 69.9 ± 1.9 vs. 4.7 ± 1.1 , all $p < 0.001$, compared with control group), while the addition of 50-fold excess of unlabeled oxLDL competed the uptake of DiI-oxLDL (MFI 12.6 ± 2.1 vs. 29.5 ± 1.1 in DiI-oxLDL 4 h group, $p < 0.001$), further confirming the specificity of this binding.

Cardiovascular disease, currently the leading cause of morbidity and mortality in developed countries, will soon become the preeminent health problem worldwide (Registered and Login 1997). Atherosclerosis—a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries—constitutes the single most important contributor to this growing burden of cardiovascular disease (Lusis 2000; Libby 2002). It is widely accepted that atherosclerosis is not only a lipid deposition disease, but also involved in inflammatory responses (Ross 1999; Libby et al. 2002). The first stage of atherosclerosis is initiated by the monocytes adhering to endothelium, then migrating to the intima, subsequently differentiating to macrophages and the macrophages avidly taking up lipids thus becoming foam cells (Libby et al. 2002). Additionally, fatty streaks, which represents the earliest grossly visible atherosclerotic lesions, consist mainly of macrophage foam cells that have taken up massive

amounts of cholesterol (Glass 2002). Thus, accumulation of cholesterol-laden foam cells in the arterial wall represents a hallmark and key event of early atherogenesis.

Currently, non-fluorescent and fluorescent methods are developed to investigate foam cell formation. The former primarily includes ORO staining and Sudan III/IV staining. The later involves several fluorescent staining including Nile Red dye, DiI-oxLDL. ORO staining is a well-established and classical method to examine foam cell formation in macrophages from different origins (murine macrophage cell line RAW 264.7, J774 A.1, human THP-1, human monocytes-derived macrophages, bone marrow-derived macrophages) and smooth muscle cells. It specifically stains triglycerides and cholesteryl oleate. Additionally, it can be used for quantifying the extent of foam cell formation through extracting the dye with isopropanol and measuring the absorbance spectrophotometrically at 510 nm (Ramirez-Zacarias et al. 1992; Scholz et al. 2004). Meanwhile ORO staining can combine with immunofluorescence for automatic quantification of lipid droplets (Koopman et al. 2001). In the present study, we introduce a simple and rapid staining method of lipids. Compared with the conventional ORO staining method (Kohn et al. 1996; Koopman et al. 2001; Jian-ling et al. 2009), the optimized method shortens the time of cell fixation from 30 to 10 min and most importantly, shortens the staining time from 10 min or even longer to 1 min, meanwhile produces better photos. It was observed that a longer staining period (15, 30 min or 1 h) produced less stained lipids, and this could be caused by the fact that the lipids were dissolved in isopropanol rather than stained by ORO (Fowler and Greenspan 1985). In dose-dependency experiments, oxLDL stimulated foam cell formation in a dose-dependent manner (Fig. 1B). Compared with conventional ORO staining, we used 60% isopropanol to facilitate lipids infiltrating into macrophages and also destained with 60% isopropanol to eliminate non-specific staining to furnish a clear background. ORO staining is also widely used for assessing lesion size of aortic sinus or arterial tree. As shown in Fig. 1C, 1 min is long enough to stain lesions of aortic sinus or mouse peritoneal macrophages from experimental atherosclerotic animals (ApoE KO mice), further extending the present optimized method to in vivo atherosclerosis-related study. As for another non-

fluorescent staining method—Sudan staining, which was primarily used in earlier studies (Leary 1935; Schwartz and Mitchell 1962; Adams and Bayliss 1975), was gradually replaced by ORO staining, because ORO staining has simpler manipulation and produces better staining results.

Among the fluorescent staining methods, the fluorescent dye Nile Red and DiI are routinely used. Nile Red primarily stains hydrophobic structures, when used in lipid staining, it can detect neutral lipid deposits, presumably unesterified cholesterol, not usually seen with ORO or other traditional lipid stains (Fowler and Greenspan 1985). But the lipids accumulated in foam cells are primarily triglyceride and cholesterol ester. Thus, the staining spectrum does not correspond to the current research aim, but the dye can be applied in aqueous medium, which avoids the dilemma imposed by most fat stains that must be dissolved in organic solvents which may also dissolve the lipids that the dye is intended to stain (Fowler and Greenspan 1985). However, the interaction of Nile Red with cellular membranes network and the other hydrophobic structures probably gives rise to background fluorescence. DiI-oxLDL uptake by macrophages as determined by fluorescent microscopy or flow cytometry was a useful tool to detect oxLDL uptake. Different stimulation time of DiI-oxLDL and varying concentrations of DiI-oxLDL were used in published articles. In the present study, we determined the optimal incubation time (4 h) of DiI-oxLDL and the optimal concentration (10 $\mu\text{g/ml}$) of DiI-oxLDL. In order to readily observe the uptake of DiI-oxLDL, DAPI was used to counterstain the cell nuclei.

In conclusion, we demonstrate a simple and rapid technique for assessing foam cell formation by optimizing ORO staining and DiI-oxLDL uptake studies. Our optimized technique simplifies and facilitates a key end-point analysis in atherogenesis.

Acknowledgments This study was supported by research grants from National Natural Science Foundation of China (No: 30472022), National Major Project “Key New Drug Creation and Manufacturing Program” (No: 2009ZX09102-152, 2009ZX09303-007). The authors would like to gratefully acknowledge the skillful technical assistance of Dr. Jing-Hua Ou, Dr. Xiao-Lu Duan for help with confocal and fluorescent microscopy. FACS was performed at the Flow Cytometry Core Facility of School of Life Sciences, Sun Yat-sen University (Guangzhou, China). We would like to extend our gratitude to all members of Department of Pharmacology and Toxicology

for technical assistance and helpful discussions. Suowen Xu received “New Investigator Awards” from Ministry of Education of the People’s Republic of China.

References

- Adams C, Bayliss O (1975) Lipid histochemistry. In: Glick D, Rosenbaum RM (eds) *Techniques of biochemical and biophysical morphology*. Wiley, New York, vol 2, pp 100–156
- Beckers L, Heeneman S, Wang L, Burkly LC, Rousch MM, Davidson NO, Gijbels MJ, de Winther MP, Daemen MJ, Lutgens E (2007) Disruption of hedgehog signalling in ApoE $-/-$ mice reduces plasma lipid levels, but increases atherosclerosis due to enhanced lipid uptake by macrophages. *J Pathol* 212:420–428
- Fowler S, Greenspan P (1985) Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: comparison with oil red O. *J Histochem Cytochem* 33:833–836
- Glass C (2002) The macrophage foam cell as a target for therapeutic intervention. *Nat Med* 8:1235–1242
- Hansson GK (2009) Atherosclerosis—an immune disease: the Anitschkov lecture 2007. *Atherosclerosis* 202:2–10
- Ide N, Keller C, Weiss N (2006) Aged garlic extract inhibits homocysteine-induced CD36 expression and foam cell formation in human macrophages. *J Nutr* 136:755S–758S
- Ii H, Oka M, Yamashita A, Waku K, Uozumi N, Shimizu T, Sato T, Akiba S (2008) Inhibition of cytosolic phospholipase A(2) suppresses production of cholesterol ester through the reesterification of free cholesterol but not formation of foam cells in oxidized LDL-stimulated macrophages. *Biol Pharm Bull* 31:6–12
- Jian-ling T, Xiong-zhong R, Hang L, Xue-mei L, Moorhead J, Varghese Z, Xue-wang L (2009) Endoplasmic reticulum stress is involved in acetylated low-density lipoprotein induced apoptosis in THP-1 differentiated macrophages. *Chin Med J* 122:1794–1799
- Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, Katagiri H, Majima M, Yokode M, Kita T, Narumiya S (2004) Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. *J Clin Invest* 114:784–794
- Kohn A, Summers S, Birnbaum M, Roth R (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271:31372
- Koopman R, Schaart G, Hesselink M (2001) Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* 116:63–68
- Leary T (1935) Pathology of coronary sclerosis. *Am Heart J* 10:328–337
- Lee TS, Lin CY, Tsai JY, Wu YL, Su KH, Lu KY, Hsiao SH, Pan CC, Kou YR, Hsu YP, Ho LT (2009) Resistin increases lipid accumulation by affecting class A scavenger receptor, CD36 and ATP-binding cassette transporter-A1 in macrophages. *Life Sci* 84:97–104
- Li L, Sawamura T, Renier G (2004) Glucose enhances human macrophage LOX-1 expression: role for LOX-1 in glucose-induced macrophage foam cell formation. *Circ Res* 94:892–901
- Lian TW, Wang L, Lo YH, Huang IJ, Wu MJ (2008) Fisetin, morin and myricetin attenuate CD36 expression and oxLDL uptake in U937-derived macrophages. *Biochim Biophys Acta* 1781:601–609
- Libby P (2002) Inflammation in atherosclerosis. *Nature* 420:868–874
- Libby P, Ridker P, Maseri A (2002) Inflammation and atherosclerosis. *Circulation* 105:1135
- Lusis AJ (2000) Atherosclerosis. *Nature* 407:233–241
- Ma KL, Ruan XZ, Powis SH, Chen Y, Moorhead JF, Varghese Z (2008) Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. *Hepatology* 48:770–781
- Moore KJ, Freeman MW (2006) Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol* 26:1702–1711
- Moriyama N, Ide N, Weiss N (2010) Aged garlic extract inhibits CD36 expression in human macrophages via modulation of the PPARgamma pathway. *Phytother Res* 24:602–608
- Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* 93:229–240
- Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA (1987) Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 68:231–240
- Parthasarathy S, Raghavamenon A, Garelnabi MO, Santanam N (2010) Oxidized low-density lipoprotein. *Methods Mol Biol (Clifton)* N J 610, 403–417
- Ramirez-Zacarias J, Castro-Munozledo F, Kuri-Harcuch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochem Cell Biol* 97:493–497
- Registered A, Login P (1997) Global mortality, disability, and the contribution of risk factors: global burden of disease study. *Lancet* 349:1436–1442
- Ross R (1999) Atherosclerosis—an inflammatory disease. *N Engl J Med* 340:115–126
- Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T (1997) An endothelial receptor for oxidized low-density lipoprotein. *Nature* 386:73–77
- Scholz H, Aukrust P, Damas JK, Tonstad S, Sagen EL, Kolset SO, Hall C, Yndestad A, Halvorsen B (2004) 8-isoprostane increases scavenger receptor A and matrix metalloproteinase activity in THP-1 macrophages, resulting in long-lived foam cells. *Eur J Clin Invest* 34:451–458
- Schwartz C, Mitchell J (1962) The morphology, terminology and pathogenesis of arterial plaques. *Postgrad Med J* 38:25
- Teupser D, Thierry J, Walli AK, Seidel D (1996) Determination of LDL- and scavenger-receptor activity in adherent and non-adherent cultured cells with a new single-step fluorometric assay. *Biochim Biophys Acta* 1303:193–198
- Teupser D, Pavlides S, Tan M, Gutierrez-Ramos JC, Kolbeck R, Breslow JL (2004) Major reduction of atherosclerosis in fractalkine (CX3CL1)-deficient mice is at the

- brachiocephalic artery, not the aortic root. *Proc Natl Acad Sci USA* 101:17795–17800
- Wuttge DM, Romert A, Eriksson U, Torma H, Hansson GK, Sirsjo A (2001) Induction of CD36 by all-trans retinoic acid: retinoic acid receptor signaling in the pathogenesis of atherosclerosis. *FASEB J* 15:1221–1223
- Xu S, Fu J, Chen J, Xiao P, Lan T, Le K, Cheng F, He L, Shen X, Huang H, Liu P (2009) Development of an optimized protocol for primary culture of smooth muscle cells from rat thoracic aortas. *Cytotechnology* 61:65–72