(Dau)、长春新碱和秋水仙碱均有交叉抗性. 抗性株倍增时间延长，G_1 期细胞增加，细胞内积聚的 Dau 明显减少，粉防已碱和维拉帕米增加 Dau 在抗性细胞内的积聚. 抗性细胞端着丝粒染色体增多，染色体畸变增加，过度表达多药抗性的 *mdr*-1 基因和 P-糖蛋白 150 kDa. **结论**：抗 Har 的 HL60 细胞为多药抗药性株，过度表达 *mdr*-1 基因和 P-糖蛋白.

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Oxidized low-density lipoproteins induce apoptosis in macrophages[1]

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KEY WORDS DNA; apoptosis; LDL lipoproteins; peritoneal macrophages

AIM: To examine whether oxidized low density lipoproteins (ox-LDL) might induce apoptosis in mouse peritoneal macrophages (MPM). **METHODS**: Low density lipoproteins (LDL) were isolated from healthy human plasma by ultracentrifugation and oxidized by $CuSO_4$ 10 μmol·L^{-1}. MPM were incubated in a medium containing ox-LDL, LDL, or phosphate-buffer solution (PBS) as control. DNA fragmentation was visualized by agarose gel electrophoresis and determined quantitatively using Hoechst 33258 fluorochrome. **RESULTS**: Ox-LDL, not LDL, elicited typical apoptotic morphological changes (shrinkage of cytoplasm and condensation of chromatin) and DNA fragmentation in a time- and dose-dependent manner. Incubation for 24 h was necessary for ox-LDL 200 mg protein ·L^{-1} to induce DNA fragmentation, and the maximal effect reached at 72 h. The DNA fragmentation after 24-h incubation with ox-LDL at concentrations of 100, 200, and 400 mg protein·L^{-1} amounted to 6.0 % ($P > 0.05$), 9.3 % ($P < 0.05$), and 30.9 % ($P < 0.01$), respectively *vs* PBS control. Dextran sulfate, a scavenger receptor blocker and cycloheximide, a protein synthesis inhibitor, exhibited no effect on DNA fragmentation. However, antioxidant butylated hydroxytoluene (BHT) 20 μmol·L^{-1} completely inhibited Cu^{2+}-mediated oxidation of LDL as well as the apoptosis-inducing effect of Cu^{2+}-exposed LDL. Lysophosphatidylcholine (LPC), an active component in ox-LDL, at concentration up to 60 μmol·L^{-1}, did not elicit DNA fragmentation in MPM. **CONCLUSION**: Ox-LDL induces apoptosis in MPM without involving LPC.

Macrophages can take up modified low density lipoproteins (LDL) to form foam cells[1]. As the atherosclerotic plaque develops, increasing number of macrophage derived foam cells appear in the artery wall. Some of these macrophages die and form the bulk of necrotic gruel at its base[2].

Apart from necrosis, there is an another type of cell death, *ie*, programmed cell death (PCD), and many pathological conditions and chemical agents, such as anticancer drugs, hyperthermia and viral infection can induce PCD[3,4].

Oxidized low density lipoproteins (ox-LDL), potent atherogenic lipoproteins, exist in atherosclerotic lesions, and can be regarded as endogenously biosynthesized cytotoxins. Its potential toxicity to endothelial cells and smooth muscle cells was demonstrated[5]. Ox-LDL triggered PCD in lymphoblastoid cells[6]. This study was aimed to investigate whether ox-LDL might induce apoptosis in macrophages.

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MATERIALS AND METHODS

Cell culture Mouse peritoneal macrophages (MPM) were obtained from ♂ Balb/c mice aged 6 - 12 wk and cultured in M199 medium with 10 % heat-inactivated fetal bovine serum (FBS).

Preparation of LDL[7] LDL was isolated from fresh normal human plasma by ultracentrifugation at 119 000 × *g* in the presence of edetic acid 0.2 $mmol \cdot L^{-1}$. Then LDL was filtered aseptically (0.2 μm) into dialysis tube and dialyzed in phosphate-buffer solution (PBS) at 4 ℃ for 36 h. Dialyzed LDL was concentrated and stored at 4 ℃ in dark. LDL prepared under these conditions is referred to as native LDL (n-LDL). Before oxidation, n-LDL was dialyzed against PBS to remove edetic acid, then it was oxidized by exposure to $CuSO_4$ 10 $\mu mol \cdot L^{-1}$ at 37 ℃ for 24 h. The entire procedure was performed under sterile conditions. The oxidation extent of ox-LDL was estimated by measuring increases in thiobarbituric acid reacting substance (TBARS) content, absorption at 234 nm, and electrophoretic mobility on agarose gel[8].

Morphological determination of apoptosis The cells were examined microscopically by hematoxylin eosin stain.

DNA fragmentation[9] MPM were harvested by centrifugation at 200 × *g* for 10 min, and lysed in lysis buffer 1.5 mL (0.5 % vol/vol Triton X-100 and edetic acid 20 $mmol \cdot L^{-1}$, Tris 5 $mmol \cdot L^{-1}$, pH 8.0) for 15 min. The lysed cells were centrifuged at 15 000 × *g* for 20 min to separate intact DNA from fragmented DNA. The pellet was sonicated for 10 s in another 1.5 mL lysis buffer. DNA in supernatant and pellet was determined using Hoechst 33258 fluorochrome, which bound DNA quantitatively to form a fluorescent complex. The data were expressed as the % of DNA appeared in supernatant to the total cellular DNA.

DNA electrophoresis[10] At end of incubation, cellular DNA was extracted by salting-out. DNA was electrophoretically fractionated on 1.5 % agarose gel and visualized by ethidium bromide.

Statistics The results were expressed as $\bar{x} \pm s$, assessed by ANOVA and *t*-test.

RESULTS

Morphological changes After incubation with ox-LDL 200 $mg \cdot L^{-1}$ for 48 h, MPM was fixed and observed microscopically. Ox-LDL-treated MPM showed a typical apoptosis. The cell volume was reduced, indicating shrinkage of cytoplasm, but the plasma membrane remained well defined, in agreement with trypan blue exclusion. The chromatin became condensed and nucleus marginated to the periphery of the cell membrane (Fig 1).

Fig 1. Morphological changes of MPM induced by ox-LDL. HE stain, × 600.

DNA electrophoresis Preincubating MPM with ox-LDL 200 mg protein$\cdot L^{-1}$ elicited a "ladder" of DNA bands representing integer multiples of the internucleosomal DNA length (about 180 base pair, bp) in a time-dependent manner, whereas n-LDL had no such effect up to 48 h (Fig 2).

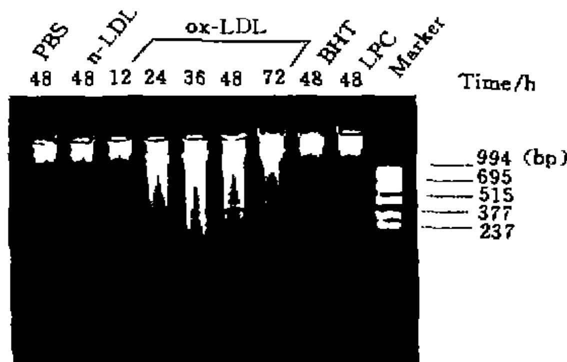

Fig 2. Agarose gel electrophoresis of ox-LDL-induced DNA fragmentation in MPM.

Significant DNA fragmentation (9.3 %) was first seen after 24 h of incubation in the presence of ox-LDL 200 $mg \cdot L^{-1}$, and reached near 30 % with ox-LDL 400 $mg \cdot L^{-1}$ (Tab 1).

Protein synthesis and scavenger receptor Pretreatment of the MPM with a protein synthesis inhibitor, cycloheximide (CH) 1 $mg \cdot L^{-1}$, for 1 h

Tab 1. Dose-dependent effect of ox-LDL on DNA fragmentation. $n=5$, $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* PBS.

Pretreatment	DNA fragmentation/%
PBS	5.4 ± 0.5
ox-LDL 100 mg·L^{-1}	6.0 ± 1.0
ox-LDL 200 mg·L^{-1}	9.3 ± 1.3^b
ox-LDL 400 mg·L^{-1}	30.9 ± 3.1^c

before adding ox-LDL did not affect the effect of ox-LDL (200 mg·L^{-1}) on DNA fragmentation. Coincubation with a scavenger receptor antagonist, dextran sulfate (DS) 20 mg·L^{-1} during pretreatment of the MPM with ox-LDL (200 mg·L^{-1}) exerted no effect on the DNA fragmentation (Tab 2).

Tab 2. Effect of protein synthesis inhibitor (cycloheximide, CH) and scavenger receptor blocker (dextran sulphate, DS) on DNA fragmentation. $n=5$, $\bar{x}\pm s$. $^cP<0.01$ *vs* PBS.

Pretreatment	DNA fragmentation/%
PBS	5.4 ± 0.5
ox LDL 200 mg·L^{-1}	30.4 ± 2.3^c
ox-LDL 200 mg·L^{-1} + DS 20 mg·L^{-1}	28.5 ± 1.6^c
ox-LDL 200 mg·L^{-1} + CH 1 mg·L^{-1}	31.1 ± 3.3^c

Lysophosphatidylcholine (LPC) Pretreatment of MPM with LPC at concentration even up to 60 μmol·L^{-1} for 48 h did not elicit DNA fragmentation (Fig 2).

Effect of butylated hydroxytoluene (BHT) BHT 20 μmol·L^{-1} completely inhibited Cu^{2+}-mediated increase in LDL TBAR material (Tab 3), as well as the DNA fragmentation-inducing effect of Cu^{2+}-exposed LDL (Fig 2), and abolished morphological change elicited by Cu^{2+}-exposed LDL.

Tab 3. Effect of antioxidant (BHT) on the oxidation of ox-LDL induced by Cu^{2+}. $n=5$, $\bar{x}\pm s$. $^cP<0.01$ *vs* n-LDL.

Pretreatment	MDA equivalent, μmol/g protein
n-LDL	2.15 ± 0.3
$CuSO_4$ 10 μmol·L^{-1}	6.33 ± 0.29^c
$CuSO_4$ 10 μmol·L^{-1} + BHT 20 μmol·L^{-1}	2.23 ± 0.28

DISCUSSION

Our observations showed that ox-LDL caused the death of MPM by a process that involved the morphological changes and DNA fragmentation characteristic of apoptosis. This effect of ox-LDL was not mediated by scavenger receptor, and not dependent upon newly synthesized protein. LPC was not involved in this event.

The cytotoxicity of ox-LDL to various kinds of cells including MPM was well documented, but until now, few experimental studies have been done of the mechanism of the cytotoxic process induced by ox-LDL[5]. Chemical-induced cytotoxicity is currently believed to occur by one of 2 mechanisms, *ie*, apoptosis or necrosis[3]. Apoptosis is considered to be a controlled mechanism of cell death and is associated with defined morphological changes and classical nucleus alterations[12]. Results in this study demonstrated that pretreating MPM with ox-LDL elicited typical apoptotic morphological changes including shrinkage of cytoplasm, condensation of chromatin and margination of nucleus in a time-dependent manner[6]. These results are in accordance with Heid's ultrastructural observation that exposure to ox-LDL induced marked apoptotic morphological change in MPM[13], however his study did not show whether ox-LDL could trigger DNA fragmentation which is an important indicator of apoptosis. The present study constitutes the first report that ox-LDL induced a "ladder" pattern of DNA bands representing integer multiples of the inter-nucleosomal DNA length (about 180 bp) in MPM.

Several lines of evidences have suggested that during the oxidative modification of LDL, phosphatidylcholine (PC) was hydrolyzed by an intrinsic LDL-associated phospholipase A_2 to become LPC[11], LPC in ox-LDL could be transferred and incorporated into cell surface membrane in an apoprotein-independent manner. The transferred LPC can modulate various cell function[14]. However the present study showed that LPC at concentration even up to 60 μmol·L^{-1} did not elicit DNA fragmentation indicating that LPC was not responsible for the effects of ox-LDL.

The fact that scavenger receptor blocker and protein synthesis inhibitor did not affect the effect of ox-LDL on DNA fragmentation rseasoned that apoptosis-inducing effect of ox-LDL was not dependent upon scavenger receptor, and that *de novo* protein synthesis in the MPM was not required. These findings, plus our further results that antioxidant butylated hydroxytoluene (BHT) completely inhibited Cu^{2+}-mediated oxidation of LDL as well as the apoptosis-inducing effect of Cu^{2+}-exposed LDL suggest that lipid peroxidation products of LDL might be an important factor for the action of ox-LDL.

In conclusion, MPM exposed to ox-LDL died primarily by apoptosis, and LPC was not involved in this event. This fact may have implications in the understanding of macrophage cell death in the atherosclerotic plaque.

REFERENCES

1 Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 1979; **76**: 333-7

2 Aqel NM, Ball RY, Waldmann H, Mitchinson MJ. Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies. J Pathol 1985; **146**: 197-204.

3 Ucker DS. Death by suicide: one way to go in mammalian cellular development? New Biol 1991; **3**: 103-9.

4 Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF, Sikorska M. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. Cancer Res 1991; **51**: 1078-85.

5 Hessler JR, Morel DW, Lewis LJ, Chisholm GM. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. Arteriosclerosis 1983; **3**: 215-22.

6 Isabelle EB, Robert S, Anne NS. Necrosis and apoptosis induced by oxidized low density lipoproteins occur through two calcium-dependent pathways in lymphoblastoid cells. FASEB J 1994; **8**: 1075-80.

7 Li YJ, Li YJ, Wu JX, Yu XJ, Yan YF. Protective effect of tetramethylpyrazine against damages of aortic endothelial cells elicited by low-density lipoproteins. Acta Pharmacol Sin 1994; **15**: 407-10.

8 Galle J, Bassenge E, Busse R. Oxidized low density lipoproteins potentiate vasoconstrictions to various agonists by direct interactions with vascular smooth muscle. Circ Res 1990; **66**: 1287-93.

9 Fuchs BA, Pruett SB. Morphine induces apoptosis in murine thymocytes *in vivo* but not *in vitro*: involvement of both opiate and glucocorticoid receptors. J Pharmacol Exp Ther 1993; **266**: 417-23.

10 Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, *et al*. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res 1994; **75**: 426-33.

11 Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL, Steinberg D. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. Proc Natl Acad Sci USA 1985; **82**: 3000-4.

12 Walker NI, Harmon BV, Gobe GC, Kerr JFR. Patterns of cell death. Methods Achiev Exp Pathol 1988; **13**: 18-54.

13 Reid VC, Mitchinson MJ, Skepper JN. Cytotoxicity of oxidized low density lipoprotein to mouse peritoneal macrophages: An ultrastructural study. J Pathol 1993; **171**: 321-8.

14 Kugiyama K, Kerns SA, Morrisett JD, Roberts, Henry PD Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low density lipoproteins. Nature 1990; **344**: 160-2.

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氧化型低密度脂蛋白诱导巨噬细胞凋亡

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关键词 DNA 片断；细胞凋亡；低密度脂蛋白；腹腔巨噬细胞

A**目的**：研究氧化型低密度脂蛋白（ox-LDL）诱导巨噬细胞凋亡． **方法**：超速离心法分离人血浆 LDL，以 $CuSO_4$ 10 μmol·L⁻¹氧化．观察 ox-LDL 对培养小鼠腹腔巨噬细胞的损伤作用． 琼脂糖凝胶电泳和 Hoechst 33258 荧光密度法定性与定量分析 DNA 降解． **结果**：Ox-LDL 诱导巨噬细胞典型凋亡形态学变化，DNA 降解呈时间和剂量依赖性． 放线菌酮 D 1 mg·L⁻¹和硫酸葡聚糖 20 mg·L⁻¹对此作用无影响． BHT 20 μmol·L⁻¹可取消 $CuSO_4$ 对 LDL 的氧化作用和 ox-LDL 引起的 DNA 降解． 溶血性磷酯酰胆碱 60 μmol·L⁻¹无诱导凋亡作用． **结论**：Ox-LDL 能诱导巨噬细胞凋亡．

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