*Free Rad. Res.*, Vol. 29, pp. 389–398
Reprints available directly from the publisher
Photocopying permitted by license only





# OxLDL-induced Macrophage Cytotoxicity is Mediated by Lysosomal Rupture and Modified by Intralysosomal Redox-active Iron

WEI LI[a,b,*], XI MING YUAN[a,b] and ULF T. BRUNK[a]

[a]*Division of Pathology II, Department of Neurobiology and Locomotion;* [b]*Clinical Research Centre, Faculty of Health Sciences, Linköping University, Sweden*

Accepted by Prof. B. Halliwell

*(Received 15 June 1998; In revised form 13 July 1998)*

Oxidized low density lipoprotein (oxLDL) is believed to play a central role in atherogenesis. LDL is oxidized in the arterial intima by mechanisms that are still only partially understood. OxLDL is then taken up by macrophages through scavenger receptor-mediated endocytosis, which then leads to cellular damage, including apoptosis. The complex mechanisms by which oxLDL induces cell injury are mostly unknown. This study has demonstrated that oxLDL-induced damage of macrophages is associated with iron-mediated intralysosomal oxidative reactions, which cause partial lysosomal rupture and ensuing apoptosis. This series of events can be prevented by pre-exposing cells to the iron-chelator, desferrioxamine (DFO), whereas it is augmented by pretreating the cells with a low molecular weight iron complex. Since both DFO and the iron complex would be taken up by endocytosis, and thus directed to the lysosomal compartment, the results suggest that the normal contents of lysosomal low molecular weight iron may play an important role in oxLDL-induced cell damage, presumably by catalyzing intralysosomal fragmentation of lipid peroxides and the formation of toxic aldehydes and oxygen-centered radicals.

*Keywords:* Atherosclerosis, low molecular weight iron, lysosomes, macrophages, oxidized LDL, oxidative stress

## INTRODUCTION

Oxidative modification of LDL leads to its scavenger receptor-mediated endocytic uptake by macrophages, with the ensuing transport to their acidic vacuolar compartment (lysosomes). OxLDL, in contrast to LDL, is partly resistant to degradation by lysosomal hydrolases, which causes it to accumulate within lysosomes.[1–4] Although the exact mechanisms behind the cytotoxicity of oxLDL are unknown, it has been found that oxLDL is toxic to different arterial wall cells and induces both apoptotic and necrotic cell death.[5–9]

We have recently shown that the cytotoxic effects of oxLDL are due, at least partly, to its ability to destabilize lysosomal membranes, resulting in the release of lysosomal contents into

*Corresponding author. Division of Pathology II, University Hospital, Linköping, S-581 85 Sweden. Tel.: +46-13-221517. Fax: +46-13-221529. E-mail: weili@pat.liu.se.

the cytosol with induction of apoptosis or necrosis.[10,11] These findings are consistent with other recent reports about the role of lysosomal proteolytic enzymes as initiators of apoptosis.[12–18]

OxLDL fragments spontaneously to form aldehydes and oxygen centred radicals ($LO^{\bullet}$, $LOO^{\bullet}$, $HO^{\bullet}$), but it is even more rapidly and efficiently degraded through the catalytic activity of redox-active low molecular weight iron.[19] Low molecular weight iron is known to be generally present in the acidic vacuolar compartment of most cells, due to the degradation of iron-containing metalloproteins by normal auto- and heterophagocytotic activity.[20,21] Theoretically, increased amounts of lysosomal, low molecular weight iron, caused, for example, by enhanced degradation of metalloproteins, would magnify the oxLDL-toxicity by further increasing the radical-induced destabilization of the lysosomal membranes.

Pronounced iron sequestration, mainly in the form of ferritin, has been observed in the cytosol and lysosomes of macrophages and foam cells of human atheroma. Such iron-loaded macrophage/foam cells have been found to undergo pronounced apoptosis.[22] The role of such increased cellular iron in oxLDL-induced cytotoxicity *in vivo* remains unknown.

The aims of the present study were to estimate whether lysosomal redox-active iron is involved in oxLDL-induced toxicity and lysosomal damage in macrophages, and to examine the relationships between oxLDL-induced lysosomal rupture and macrophage apoptosis.

## MATERIALS AND METHODS

### Chemicals

F-10 culture medium and fetal bovine serum (FBS) were from GIBCO (Paisley, UK). Glutamine, penicillin-G, and streptomycin were from Flow (Rickmansworth, UK), and acridine orange (AO) was from Gurr (Poole, UK). Desferrioxamine mesylate (DFO) was from Ciba-Geigy (Basel, Switzerland). All other reagents used were obtained from standard sources and of the highest purity available.

### Cell Cultures

Murine macrophage-like J-774 cells were cultured in F-10 with 10% (v/v) fetal bovine serum (FBS), glutamine (2 mmol/L), penicillin G (100 units/ml), and streptomycin (100 μg/ml) in 75 ml plastic culture flasks (Costar, Cambridge, MA, USA). They were grown at 37°C in a humidified atmosphere (5% $CO_2$ in air). The cells were subcultivated once a week and used for experiments 24 h after transfer into 35 mm plastic petri dishes (Costar) with or without coverslips.

### Preparation of LDL and its Oxidative Modification

Human LDL ($1.025 < d < 1.050$ g/ml) was freshly isolated by sequential ultracentrifugation from plasma of normolipidaemic donors as described before.[23] It was prepared in the presence of ethylenediamine tetraacetic acid (EDTA, 1.4 mg/ml) to inhibit lipid peroxidation. Finally, it was dialyzed for 24 h at 4°C under nitrogen against 0.01 mol/L phosphate buffer with 0.16 mol/L NaCl, pH 7.4. Aliquots of LDL solutions were photo-oxidized in oxLDL by ultraviolet light (254 nm) for 3 h at room temperature, as described before.[10]

### Experimental Procedures

Cells were divided into four groups and grown for 24 h after subcultivation in F-10 culture medium containing 10% FBS. Two groups were treated, in complete culture medium, either with different concentrations of DFO (100–500 μM) for 1 h, or with an insoluble iron phosphate complex (obtained by adding a concentrated $FeCl_3$ solution to the culture medium. Final iron-concentration was 50 μM) for 2 h, and then exposed to oxLDL for another 24 or 48 h. Another group of cells was exposed for 24 or 48 h only to oxLDL.

Cells without any of these treatments were used as controls. During the oxLDL-exposure, the cells were kept in F-10 medium containing 5% FBS. Plasma membrane permeability was determined by the trypan blue dye exclusion test at the end of the oxLDL-exposure.

### Detection of Apoptosis

To examine nuclear morphology, cells were stained according to the Wright-Giemsa method and viewed by light microscopy. The percentage of apoptotic nuclei (condensed or fragmented) was determined by randomly counting at least 500 cells from each determination. Apoptotic cells were also detected by the TUNEL-technique using the ApopTag *in situ* apoptosis detection kit according to the manufacturer's instruction (Oncor Inc., Gaithersburg, MD). In brief, cells were fixed with neutral buffered formalin containing 0.5% Triton X-100 for 20 min. Incubation (1 h) with the terminal deoxynucleotidyl transferase enzyme was followed by incubation with anti-digoxigenin-peroxidase for 30 min. The cells were examined by light microscopy after staining with 0.05% diaminobenzidine and 300 cells were randomly counted in each determination ($n = 2$).

Cellular DNA fragmentation was evaluated by agarose gel electrophoresis. After 48 h of treatment with oxLDL, DNA was prepared as described.[24] The resulting DNA preparations were electrophoresed for 2 h at 70 V on a 1.5% agarose gel and stained with 2 μg/ml ethidium bromide. Bands were visualized and photographed with UV transillumination.

### Estimation of Lysosomal Integrity Using Acridine Orange Vital Staining

Living J-774 cells, growing on coverslips, were incubated for 15 min in a 2 ml acridine orange (AO) solution (5 μg/ml in complete F-10 medium) at 37°C. After incubation, the cells were rinsed with complete F-10 medium at room temperature and then kept in fresh, complete F-10 medium at 22°C for another 10 min. The intensity of red lysosomal fluorescence from 50 to 100 individual cells/coverslip (indicating the number of intact lysosomes) were then assayed by static cytofluorometry, as described previously.[10] The AO-stained cells were also viewed by confocal microscopy.[11]

### Statistics

Statistical comparisons were made by using the two-tailed Student's *t*-test. Results were considered significant if $p < 0.05$.

## RESULTS

### Influence of DFO on Cell Growth and Cell Viability

DFO is a fungal product, isolated from *Streptomyces pilosus*, which is a potent iron-chelator and able to prevent iron-catalyzed radical reactions in different cell models. The used concentration of DFO in different experiments have been quite varying. During the current experiments we realized that an optimal concentration of DFO is critical when using proliferating cells. As shown in Table I there is a significantly decreased

TABLE I Effect of DFO on growth and viability of J-774 cell

| DFO concentration | 100 μM | 200 μM | 400 μM | 500 μM |
|---|---|---|---|---|
| Dye exclusion (% of control) | 100.9 ± 1.2 | 99.2 ± 1.5 | 96.2 ± 1.6 | 95.9 ± 1.6 |
| Total number of cells (% of control) | 86.0 ± 5.8 | 82.2 ± 7.8 | 80.1 ± 6.6 | 79.1 ± 5.1* |

Cells were treated with different concentrations of DFO for 1 h under otherwise normal culture conditions. Plasma membrane permeability was evaluated by the trypan blue dye exclusion test after another 24 h under normal culture conditions, while the total number of cells was also counted in each culture. Values are means ± SE ($n = 4$).
*Significantly different from the control, $p < 0.05$.

proliferation following exposure of J-774 cells to DFO for 1 h in concentrations >400 μM. In this study we used 200 μM DFO as an optimal concentration for J-774 cells under the conditions we applied.

### OxLDL-Cytotoxicity and Protective Effect of DFO

The toxicity of oxLDL to J-774 cells was assayed using the trypan-blue dye exclusion test. As shown in Figure 1, exposure of the cells to 100 μg/ml oxLDL resulted in a significant loss of cell viability, when measured after a 24 h. This test would underestimate the loss of viability because apoptotic cells do not take up trypan blue until they are in a post-apoptotic necrotic stage.

The effects of pretreatment with iron or DFO on the toxicity of oxLDL were examined as well. Cells were initially exposed to either DFO or an iron-complex, and then to oxLDL as described in the **Experimental Procedure** section. Pre-treatment of the cells with DFO afforded significant protection against the toxic effects of oxLDL, whereas the oxLDL induced cytotoxicity was enhanced in cells pre-loaded with iron (Figure 1).

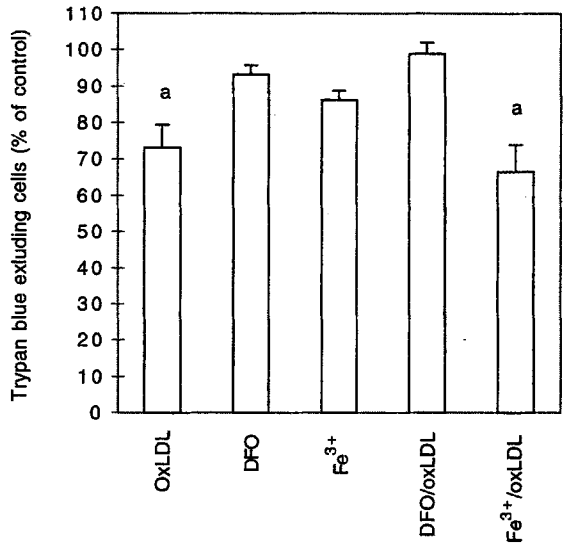



FIGURE 1 Effects of DFO and an iron–phosphate complex on the plasma membrane permeability of oxLDL-exposed cells. Cultures were initially treated (or not) with 200 μM DFO for 1 h, or with 50 μM ferric iron for 2 h, and then exposed (or not) to oxLDL (100 μg/ml) for another 24 h. Plasma membrane permeability was assayed by the trypan-blue dye exclusion test. Values are means ± SE ($n = 4$–5). (a) Significantly different from both control and DFO pre-treated cells, $p < 0.05$.

### Macrophage Apoptosis

Oxidized-LDL has been documented to be a DNA-damaging agent in various types of cells. In the present study, macrophage apoptosis was induced by exposure to oxLDL, and the ability of DFO to modulate apoptosis was examined. The principal morphological features associated with apoptosis were condensed or fragmented nuclei and cell shrinkage. Pre-treatment with DFO significantly suppressed these apoptotic morphological changes (Figure 2 and Table II). The TUNEL-procedure showed enhanced apoptosis following exposure to oxLDL, both after 24 and 48 h, compared to control or DFO pre-treated cells. After 24 h oxLDL-exposed cells showed 16.3% positivity, while controls showed 3.5% and DFO/oxLDL treated cells 10.3%. After 48 h the number of TUNEL positive cells were lower in both oxLDL treated (10.8%) and DFO/oxLDL treated (2.3%) cells. We presume that this decline is due to phagocytosis by surviving neighbour cells and detachment of dead cells.

DNA fragmentation-pattern was observed by electrophoresis in the cells treated with oxLDL, whereas DNA fragmentation was partially prevented by DFO pretreatment (Figure 3). The cleavage of DNA into nucleosomal fragments of 180–200 base pairs has been considered to be one of the most important characteristics of apoptosis. However, it has recently been proposed that DNA is initially cleaved into 50–300 kilobase pairs and that the larger DNA fragments are subsequently degraded into small fragments in the apoptotic process. Our results with DNA electrophoresis are in agreement with the latter proposal, since the oxLDL-treated cells showed both large and small DNA fragments.

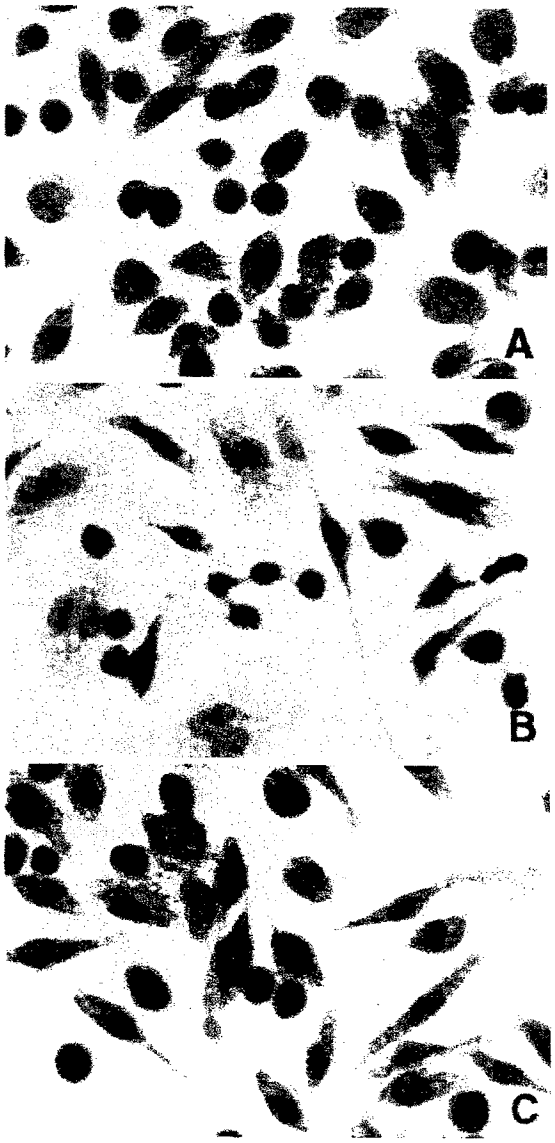



FIGURE 2 Light microscopy of J-774 cells stained according to Wright-Giemsa. A: control cells cultured for 48 h without treatment; B: cells exposed to oxLDL (100 μg/ml) for 48 h; C: cells pre-treated with 200 μM DFO for 1 h and then exposed to oxLDL for another 48 h. Note the large number of cells with apoptotic appearance in the oxLDL group. There are some apoptotic cells in the DFO/oxLDL group as well. (See Color plate I at the end of this issue.)

## Lysosomal Stability

To study the influence of oxLDL alone, or in combination with DFO or ferric iron, on lysosomal damage, cells were pre-treated either with DFO for 1 h or with an iron complex for 2 h, and

TABLE II Percentage of morphologically apoptotic cells after 24 and 48 h of exposure to oxLDL

| Treatment | 24 h | 48 h |
|---|---|---|
| None (control) | 5.06 ± 0.8 | 9.98 ± 1.2 |
| OxLDL | 18.49 ± 0.9 (a) (b) | 30.51 ± 6.1 (d) |
| DFO/oxLDL | 11.97 ± 1.3 (c) | 14.04 ± 1.1 |

Cells were pre-treated (or not) with 200 μM DFO for 1 h and then exposed (or not) to 100 μg/ml of oxLDL for 24 or 48 h. At the time decided, the cells were stained according to the Wright-Giemsa method, and analyzed by light microscopy. Shrunken cells with condensed/fragmented nuclei were considered to be apoptotic. Data are means ± SE of at least three experiments.
(a) Significantly different from control cells, $p < 0.001$.
(b) Significantly different from DFO pre-treated cells, $p < 0.05$.
(c) Significantly different from control cells, $p < 0.01$.
(d) Significantly different from both control and DFO pre-treated cells, $p < 0.05$.

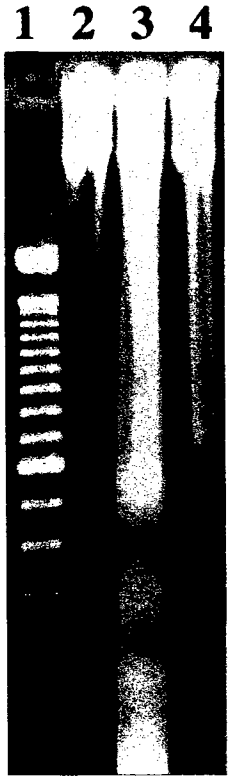
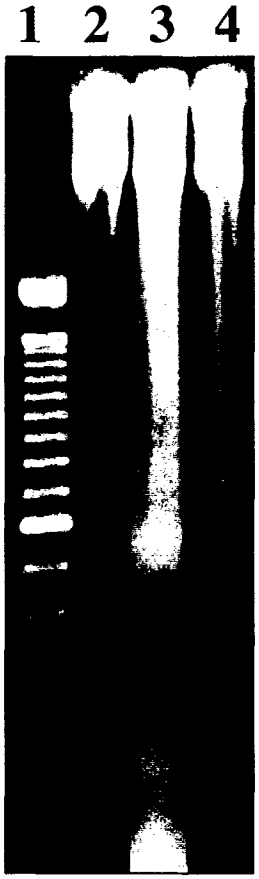



FIGURE 3 DNA fragmentation induced by oxLDL and its partial suppression by DFO pre-treatment. Cells were lysed, treated with proteinase K, RNase A, and assayed by 1.5% agarose gel electrophoresis. Lane 1: 100 bp DNA ladder marker; lane 2: control cells; lane 3: cells treated with 100 μg/ml oxLDL for 48 h; lane 4: cells pre-treated with 200 μM DFO and then with oxLDL. (See Color plate II at the end of this issue.)


**Colour Plate I (see page 393, figure 2)** Light microscopy of J-774 cells stained according to Wright-Giemsa. A: control cells cultured for 48 h without treatment; B: cells exposed to oxLDL (100 μg/ml) for 48 h; C: cells pre-treated with 200 μM DFO for 1 h and then exposed to oxLDL for another 48 h. Note the large number of cells with apoptotic appearance in the oxLDL group. There are some apoptotic cells in the DFO/oxLDL group as well.




**Colour Plate II (see page 393, figure 3)** DNA fragmentation induced by oxLDL and its partial suppression by DFO pre-treatment. Cells were lysed, treated with proteinase K, RNase A, and assayed by 1.5% agarose gel electrophoresis. Lane 1: 100 bp DNA ladder marker; lane 2: control cells; lane 3: cells treated with 100 μg/ml oxLDL for 48 h; lane 4: cells pre-treated with 200 μM DFO and then with oxLDL.

then exposed to oxLDL for another 24 h. Lysosomal stability was determined using the acridine orange uptake test. A significant decrease in AO-induced red fluorescence was seen in oxLDL exposed cells, indicating that lysosomal membranes were damaged with resultant destroyed proton gradient over the lysosomal membranes. We have previously shown that the uptake of the lysosomotropic weak base-acridine orange is decreased when the lysosomes have ruptured. Such decreased uptake of acridine orange is paralleled by redistribution of their contents to the cytosol.[11,14,16–18] Pre-treatment of the cells with DFO significantly reduced the lysosomal damage, whereas pre-treatment with the iron complex increased lysosomal damage (Figure 4). The protective role of DFO against oxLDL-induced lysosomal damage was clearly illustrated by confocal scanning laser microscopy (Figure 5). OxLDL treated cells showed decreased

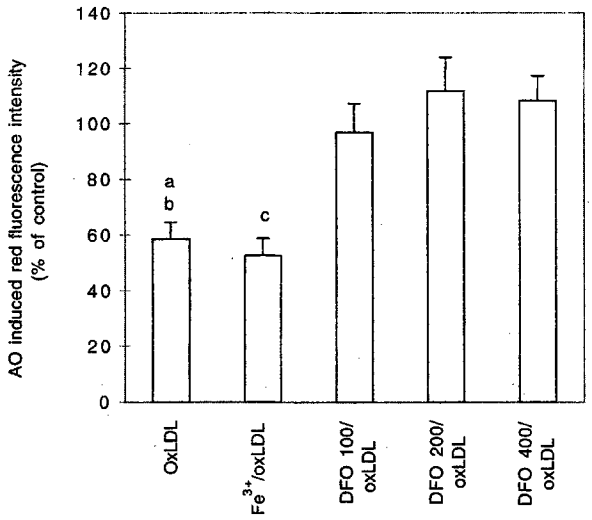



FIGURE 4 Determination of lysosomal integrity using the AO-uptake test. The intensity of AO-induced red lysosomal fluorescence was assayed by static cytofluorometry. Values are means ± SE ($n = 3$–4). Cells were cultured at normal conditions (control), exposed to 100 μg/ml oxLDL for 24 h (OxLDL), or pre-treated with either 50 μM iron–phosphate complex for 2 h ($Fe^{3+}$/oxLDL) or with different concentrations of DFO for 1 h before the exposure to oxLDL (DFO/oxLDL). (a) Significantly different from control cells, $p < 0.01$. (b) Significantly different from DFO pre-treated cells, $p < 0.05$. (c) Significantly different from both control and DFO pretreated cells, $p < 0.05$.

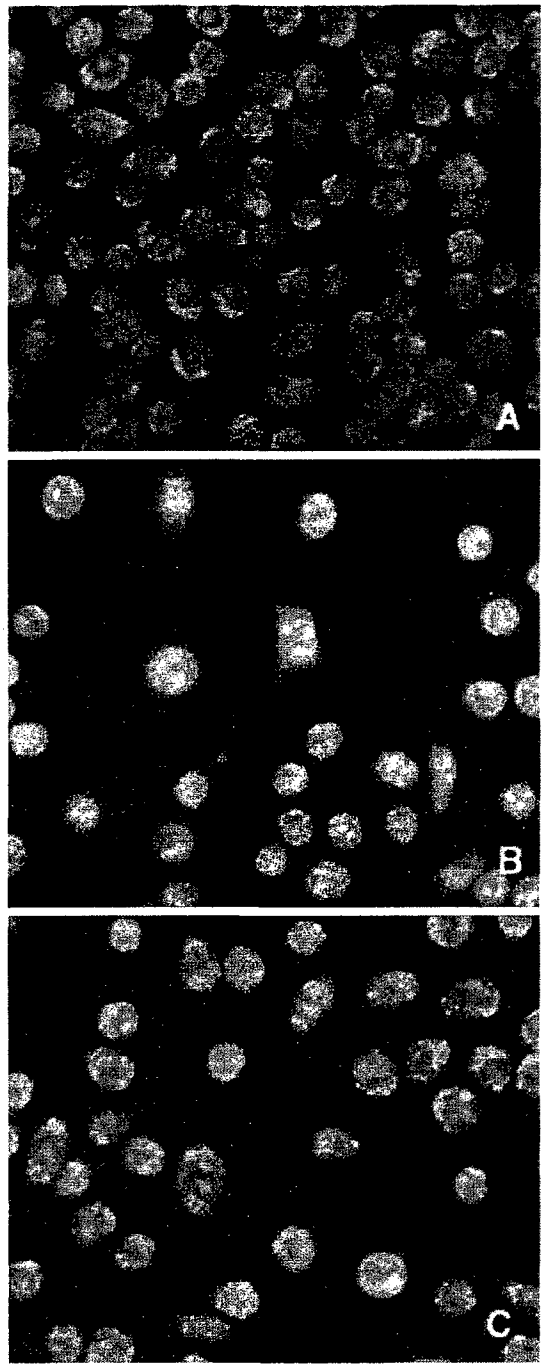
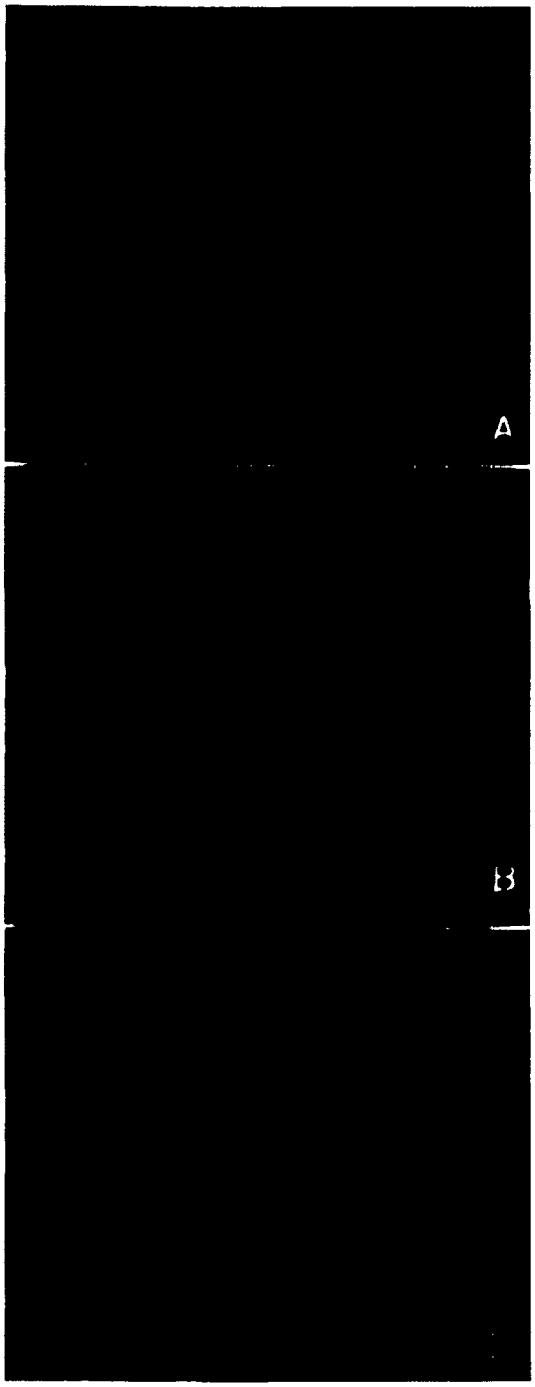



FIGURE 5 Confocal scanning micrographs of AO-exposed cells. The treatments of the cells were the same as described for Figure 4 (A: control cells cultured for 24 h without treatment; B: cells exposed to oxLDL (100 μg/ml) for 24 h; C: cells pre-treated with 200 μM DFO for 1 h and then exposed to oxLDL for another 24 h). (See Color plate III at the end of this issue.)




**Colour Plate III (see page 394, figure 5)** Confocal scanning micrographs of AO-exposed cells. The treatments of the cells were the same as described for Figure 4 (A: control cells cultured for 24 h without treatment; B: cells exposed to oxLDL (100 μg/ml) for 24 h; C: cells pre-treated with 200 μM DFO for 1 h and then exposed to oxLDL for another 24 h).

cytoplasmic granular red fluorescence and increased cytosolic and nuclear green fluorescence, indicating that the oxLDL-induced partial lysosomal rupture also had resulted in lowered cytosolic pH. The better preserved red granular fluorescence of DFO pre-treated cells reflects the prevention of lysosomal destabilization by DFO.

## DISCUSSION

The results of this study provide evidence for the involvement of intralysosomal low molecular weight iron in the induction of oxLDL-induced macrophage injury through lysosomal destabilization. It was found that the iron-chelator DFO inhibited oxLDL-induced cytotoxicity, whereas it was potentiated by an endocytosed insoluble iron phosphate complex.

OxLDL-cytotoxicity has been extensively studied. It was recently shown that oxLDL and several oxidation products of cholesterol are able to induce DNA fragmentation and apoptosis in arterial wall cells, and that apoptosis is associated with the activation of the interleukin-1 $\beta$-converting enzyme (ICE)-like protease caspase 3 (CPP32).[25,26] A catalytic interaction between normal, lysosomal low molecular weight iron with hydroperoxycholesterols, e.g. 7$\beta$-hydroperoxycholesterol (7$\beta$-OOH-chol) from oxLDL, leading to chain peroxidation production, may be a critical step in this process. Our results suggest that oxLDL is further cleaved intralysosomally into toxic aldehydes and oxygen-centred radicals. The former would presumably be responsible for the inactivation of lysosomal enzymes that we and others previously have described,[1–4,11] whereas the radicals may be responsible for the labilization of lysosomal membranes that results in leakage of hydrolytic enzymes into the cytosol.[14,16–18]

Leakage of lysosomal hydrolytic enzymes into the cytosol is reported to result in apoptosis.[13,14–18] In a previous study on oxLDL cytotoxicity, the rupture of lysosomes was found to result in relocation of hydrolytic enzymes to the cytosol, including cathepsin-D.[11] This is supposedly the initiating event for the apoptotic process that seems to be a consequence of oxLDL uptake, and modulated by the intralysosomal concentration of low molecular weight iron.

Control cells, and not only the iron-exposed ones, were sensitive to oxLDL. This may be explained by the fact that most secondary lysosomes contain some amount of redox-active iron, since both phagocytosed and autophagocytosed iron-containing metalloproteins are continuously degraded inside the lysosomal compartment of all cells and especially in macrophage.[14,16,20]

Desferrioxamine (DFO) is a potent iron chelator that is taken up only by endocytosis, and it is, thus, selectively transported to the acidic vacuolar compartment.[27,28] Intralysosomally, DFO binds low molecular weight iron, thereby preventing iron-mediated redox-reactions and protecting the stability of the lysosomal membranes. It has been also reported that DFO may scavenge some radical species directly.[29] In addition, DFO is a potent inhibitor of copper and photo-oxidized LDL cytotoxicity on endothelial, smooth muscle cells, and fibroblasts.[7–9] It has also been evidenced that an intracellular iron-dependent lipid peroxidation pathway is the mechanism of oxLDL induced cell damage.[7,8] Our data are consistent with these findings, and we also show that the iron chelator DFO protects lysosomal damage by oxLDL, whereas pre-treatment with an iron complex enhances the cytotoxic effects of oxLDL. Taken together, these findings suggest that transition metals play a role both in oxLDL-induced cell injury and lysosomal damage. DFO has been found to inhibit cell proliferation in several type of cells, and even to induce apoptosis.[30,31] This is not surprising since lysosomal DFO would be expected to bind all iron passing through the lysosomal compartment, resulting in severe iron-starvation. However, at a suitable concentration, and for a limited period of time, DFO does have cytoprotective activity during oxidative stress by chelating intralysosomal iron

and preventing intralysosomal Fenton chemistry and the resulting lysosomal destabilization.[16]

The stability of lysosomes in relation to cellular degeneration has been a matter of dispute ever since the discovery of these organelles by Christian de Duve, who, somewhat provocatively, nicknamed them "suicide bags" in order to emphasize their potential harmfullness to the living cell.[32,33] Today, however, the prevailing general opinion is that lysosomes are rather stable organelles that break down only late during the process of cellular degeneration and do not initiate damage to still living cells by releasing their numerous hydrolytic enzymes into the cytosol. One of the main reasons for this opinion seems to be that lysosomes look ultrastructurally normal, even in cells with otherwise advanced degenerative alterations. However, it has been demonstrated that lysosomes showing undamaged membranes by transmission electron microscopy nevertheless may have leaked a substantial amount of the marker enzyme, acid phosphatase, into the cytosol.[34] It has also been shown that minor lysosomal destabilization produces degenerative alterations that are rapidly and efficiently repaired by autophagocytotic processes in a short period of time, suggesting that minor lysosomal leakage might be a rather common but reversible phenomenon.[35] Not until the damage to the lysosomal compartment has become substantial, are the cells no longer able to survive.[21] These observations are in agreement with findings that the same type of injury may induce proliferation, apoptosis, or necrosis, depending upon the magnitude of the impact.[36]

Because intact lysosomes are found in apoptotic cells, it has been claimed that the rupture of lysosomes does not induce apoptosis. However, all lysosomes do not burst simultaneously[37] and our results indicate that apoptosis is the result of an initial rupture of a limited number of lysosomes. A nearly total loss of intact lysosomes is only observed during late, post-apoptotic necrosis.[14,16–18]

The exact mechanism by which lysosomal leak/rupture might induce apoptosis is not clear. Perhaps the most plausible explanation would be that lysosomal proteolytic enzymes, such as cathepsin-D, once released into the cytosol, act in concert with the caspases, which are known to require proteolytic activation. Such a scenario would be in agreement with recent reports on the role of cathepsin-D in apoptosis, which show an enhanced expression of this well-known endoprotease in programmed cell death, thereby adding it to the list of proteases that may be mediators of apoptosis.[13,17] Another attractive candidate for the mechanism by which lysosomes become involved in the apoptotic process is the ceramide-activated pathway. Monney *et al.*[12] recently proposed that an acidic vesicular compartment, reminiscent of lysosomes, generates at least two pathways that account for the caspase-3 activation and apoptosis induced by TNF-$\alpha$. It was found that exposure of human monocyte-derived macrophages to oxLDL raised the levels of the sphingolipid ceramide.[38] The ceramide content of atheroma lesional LDL was also found to be enriched 10–50-fold compared with plasma LDL ceramide.[39] These findings all point to the existence of a lysosome-related ceramide pathway that is involved in atherogenesis. Further studies are needed to verify this hypothesis.

*In conclusion*: We have shown that the induction of apoptosis in macrophages by oxLDL is associated with iron-mediated, oxidative damage to lysosomes, which leads to lysosomal rupture. DFO can, at least partially, prevent this process, whereas it is aggravated by endocytosed low molecular weight iron. Our findings also indicate that the partial rupture of the acidic vacuolar compartments may be a relevant pathway in programmed cell death, and that such rupture may be induced by a variety of factors. Furthermore, our results raise the possibility that dysregulated iron metabolism and the pronounced apoptosis of macrophages in atheromas, reported by us and others, may be causally linked to the concentration of low molecular weight redox-active iron in lysosomes.

## Acknowledgement

We would like to thank Ms. Karin Roberg and Ms. Irene Svensson for their skilled technical help, and Ms. Britt Sigfridsson and Ms. Ylva Svensson for the preparation of low density lipoproteins. This work was supported by the Swedish Medical Research Council (No. 4481 and 6962).

## References

[1] G. Hoppe, J. O'Neil and H.F. Hoff (1994) Inactivation of lysosomal proteases by oxidized low density lipoprotein is partially responsible for its poor degradation by mouse peritoneal macrophages. *Journal of Clinical Investigation*, **94**, 1506–1512.

[2] J. O'Neil, G. Hoppe, L.M. Sayre and H.F. Hoff (1997) Inactivation of cathepsin B by oxidized LDL involves complex formation induced by binding of putative reactive sites exposed at low pH to thiols on the enzyme. *Free Radical Biology and Medicine*, **23**, 215–225.

[3] M. Lougheed, H. Zhang and U.P. Steinbrecher (1991) Oxidized low density lipoprotein is resistant to cathepsins and accumulates within macrophages. *Journal of Biological Chemistry*, **266**, 14519–14525.

[4] W. Jessup, E.L. Mander and R.T. Dean (1992) The intracellular storage and turnover of apolipoprotein B of oxidized LDL in macrophages. *Biochimica et Biophysica Acta*, **1126**, 167–177.

[5] S.J. Hardwick, L. Hegyi, K. Clare, N.S. Law, K.L.H. Carpenter, M.J. Mitchinson and J.N. Skepper (1996) Apoptosis in human monocyte-macrophages exposed to oxidized low density lipoprotein. *Journal of Pathology*, **179**, 294–302.

[6] I. Escargueil-Blanc, O. Meilhac, M.T. Pieraggi, J.F. Arnal, R. Salvayre and A. Negre-Salvayre (1997) Oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway. Prevention by aurintricarboxylic acid. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **17**, 331–339.

[7] J.P. Thomas, P.G. Geiger and A.W. Girotti (1993) Lethal damage to endothelial cells by oxidized low density lipoprotein: role of selenoperoxidases in cytoprotection against lipid hydroperoxide- and iron-mediated reactions. *Journal of Lipid Research*, **34**, 479–490.

[8] M.D. Coffey, R.A. Cole, S.M. Colles and G.M. Chisolm (1995) *In vitro* cell injury by oxidized low density lipoprotein involves lipid hydroperoxide-induced formation of alkoxyl, lipid, and peroxyl radicals. *Journal of Clinical Investigation*, **96**, 1866–1873.

[9] J.R. Guyton, M.L. Lenz, B. Mathews, H. Hughes, D. Karsan, E. Selinger and C.V. Smith (1995) Toxicity of oxidized low density lipoproteins for vascular smooth muscle cells and partial protection by antioxidants. *Atherosclerosis*, **118**, 237–249.

[10] X.M. Yuan, W. Li, A.G. Olsson and U.T. Brunk (1997) The toxicity to macrophages of oxidized low-density lipoprotein is mediated through lysosomal damage. *Atherosclerosis*, **133**, 153–161.

[11] W. Li, X.M. Yuan, A.G. Olsson and U.T. Brunk (1998) Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **18**, 177–184.

[12] L. Monney, R. Olivier, I. Otter, B. Jansen, G.G. Poirier and C. Borner (1998) Role of an acidic compartment in tumor-necrosis-factor-alpha-induced production of ceramide, activation of caspase-3 and apoptosis. *European Journal of Biochemistry*, **251**, 295–303.

[13] L.P. Deiss, H. Galinka, H. Barissi, O. Cohen and A. Kimichi (1996) Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO Journal*, **15**, 3861–3870.

[14] U.T. Brunk, H. Dalen, K. Roberg and H.B. Hellquist (1997) Photo-oxidative disruption of lysosomal membranes causes apoptosis of cultured human fibroblasts. *Free Radical Biology and Medicine*, **23**, 616–626.

[15] V.L. Sheen and J.D. Macklis (1994) Apoptotic mechanisms in targeted neuronal cell death by chromophore-activated photolysis. *Experimental Neurology*, **130**, 67–81.

[16] H.B. Hellquist, I. Svensson and U.T. Brunk (1997) Oxidant-induced apoptosis: a consequence of lethal lysosomal leak? *Redox Report*, **3**, 65–70.

[17] K. Roberg and K. Öllinger (1998) Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. *American Journal of Pathology*, **152**, 1151–1156.

[18] U.T. Brunk and I. Svensson (1998) Oxidative stress, growth-factor starvation, and Fas-activation may all cause apoptosis through lysosomal leak. *Redox Report*, (in press).

[19] B. Halliwell and J.M.C. Gutteridge (1989) Lipid peroxidation: a radical chain reaction. In *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford, pp. 188–218.

[20] U.T. Brunk, C.B. Jones and R.S. Sohal (1992) A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. *Mutation Research*, **275**, 395–403.

[21] U.T. Brunk, H. Zhang, K. Roberg and K. Öllinger (1995) Lethal hydrogen peroxide toxicity involves lysosomal iron-catalyzed reactions with membrane damage. *Redox Report*, **1**, 267–277.

[22] X.M. Yuan, W. Li, A.G. Olsson and U.T. Brunk (1997) Ferritin accumulation in human atheromas and its relation to apoptosis. XIth International Symposium on Atherosclerosis 1997, Paris. *Atherosclerosis*, 134–258.

[23] R.J. Havel, H.A. Eder and J.H. Bragdon (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *Journal of Clinical Investigation*, **34**, 1345–1353.

[24] M.J. Rogers, K.M. Chilton, F.P. Coxon, J. Lawry, M.O. Smith, S. Suri, R.G. Russell (1996) Bisphosphonates induce apoptosis in mouse macrophage-like cells *in vitro* by a nitric oxide-independent mechanism. *Journal of Bone & Mineral Research*, **11**, 1482–1491.

[25] S. Dimmeler, J. Haendeler, J. Galle and A.M. Zeiher (1997) Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. *Circulation*, **95**, 1760–1763.

[26] M.P. Ares, M.I. Porn-Ares, J. Thyberg, L. Juntti-Berggren, P.O. Berggren, U. Diczfalusy, B. Kallin, I. Bjorkhem, S. Orrenius and J. Nilsson (1997) $Ca^{2+}$ channel blockers verapamil and nifedipine inhibit apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells. *Journal of Lipid Research*, **38**, 2049–2061.

[27] J.B. Lloyd, H. Cable and C. Rice-Evans (1991) Evidence that desferrioxamine cannot enter cells by

passive diffusion. *Biochemical Pharmacology*, **41**, 1361–1363.
[28] B.T. Olejnicka, K. Öllinger and U.T. Brunk (1997) A short exposure to a high-glucose milieu stabilizes the acidic vacuolar apparatus of insulinoma cells in culture to ensuing oxidative stress. *APMIS*, **105**, 689–698.
[29] I. Morel, J. Cillard, G. Lescoat, O. Sergent, N. Pasdeloup, A.Z. Ocaktan, M.A. Abdallah, P. Brissot and P. Cillard (1992) Antioxidant and free radical scavenging activities of the iron chelators pyoverdin and hydroxypyrid-4-ones in iron-loaded hepatocyte cultures: comparison of their mechanism of protection with that of desferrioxamine. *Free Radical Biology and Medicine*, **13**, 499–508.
[30] E. Porreca, S. Ucchino, C. Di Febbo, N. Di Bartolomeo, D. Angelucci, A.M. Napolitano, A. Mezzetti and F. Cuccurullo (1994) Antiproliferative effect of desferrioxamine on vascular smooth muscle cells *in vitro* and *in vivo*. *Arteriosclerosis and Thrombosis*, **14**, 299–304.
[31] D. Hileti, P. Panayiotidis and A.V. Hoffbrand (1995) Iron chelators induce apoptosis in proliferating cells. *British Journal of Haematology*, **89**, 181–187.
[32] C. deDuve and R. Wattiaux (1966) Functions of lysosomes. *Annual Review of Physiology*, **28**, 435–492.
[33] C. deDuve (1969) The lysosome in retrospect. In: Dingle, J.T. Fell, H.B. *Lysosomes in Biology and Pathology*. Vol 1. North Holland: Amsterdam, 3.
[34] U.T. Brunk and J.L. Ericsson (1972) The demonstration of acid phosphatase *in vitro* cultured tissue cells. Studies on the significance of fixation, tonicity and permeability. *Histochemical Journal*, **4**, 349–363.
[35] U.T. Brunk, H. Zhang, H. Dalen and K. Öllinger (1995) Exposure of cells to non-lethal concentrations of hydrogen peroxide induces degeneration-repair mechanisms involving lysosomal destabilization. *Free Radical Biology and Medicine*, **19**, 813–822.
[36] J.M. Dypbukt, M. Ankarcrona, M. Burkitt, A. Sjöholm, K. Ström, S. Orrenius and P. Nicotera (1994) Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *Journal of Biological Chemistry*, **269**, 30553–30560.
[37] E. Nilsson, R. Ghassemifar and U.T. Brunk (1997) Lysosomal heterogeneity between and within cells with respect to resistance against oxidative stress. *Histochemical Journal*, **29**, 857–865.
[38] R. Kinscherf, R. Claus, H.P. Deigner, O. Nauen, C. Gehrke, A. Hermetter, S. Russwurm, V. Daniel, V. Hack and J. Metz (1997) Modified low density lipoprotein delivers substrate for ceramide formation and stimulates the sphingomyelin-ceramide pathway in human macrophages. *FEBS Letters*, **405**, 55–59.
[39] S.L. Schissel, J. Tweedie-Hardman, J.H. Rapp, G. Graham, K.J. Williams and I. Tabas (1996) Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein. Proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *Journal of Clinical Investigation*, **98**, 1455–1464.