New pathogenetic hypothesis for Wolman disease: possible role of oxidized low-density lipoproteins in adrenal necrosis and calcification

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Wolman disease is an inherited metabolic disease, characterized by a severe deficiency of the acid lipase and a massive lysosomal storage of triacylglycerols and cholesteryl esters, associated with hepatosplenomegaly, adrenal calcification and nearly always fatal in the first year of life. Cultured human lymphoblastoid cells and human adrenal cells are able to promote the formation of mildly oxidized low-density lipoproteins (LDL), which in turn exhibit a non-negligible cytotoxic effect on these cells. In contrast, fibroblasts induce only very low levels of LDL oxidation. Comparative experiments have shown that the cytotoxic effect of oxidized LDL was higher to Wolman-disease cells than to controls. The oxidative ability of Wolman cells was similar to that of normal ones. The over-cytotoxicity of mildly oxidized LDL to Wolman cells resulted from the higher uptake of mildly oxidized LDL through the LDL-receptor pathway, which is only poorly down-regulated in Wolman cells subsequently to the block of the lysosomal degradation of LDL-cholesteryl esters. In cultured adrenal cells, oxidized LDL induced a sustained rise in intracellular $[Ca^{2+}]$ which is directly involved in the cellular

damage and cell death induced by oxidized LDL [Nègre-Salvayre and Salvayre (1992) *Biochim. Biophys. Acta* 1123, 207–215]. This Ca^{2+} peak is followed by a dramatic deposition of calcium in damaged or/and dead cultured adrenal cells, quite similar to that observed in Wolman-disease adrenal cortex. The cell-induced LDL oxidation and the subsequent cytotoxic effect can be prevented, at least in part, by antioxidants such as α -tocopherol and nordihydroguaiaretic acid. These findings support the hypothesis that the Wolman-disease adrenal damage (necrosis and calcification) could result from the association of the following events: mild oxidation of LDL by adrenal cells, over-uptake of mildly oxidized LDL by Wolman cells (resulting from the block of the lysosomal degradation of cholesteryl esters in Wolman cells), and cytotoxicity related to the amount of mildly oxidized LDL internalized by cells. The reported data also suggest that LDL oxidation induced by adrenal cells and their subsequent cytotoxicity can be prevented (in part) by antioxidants, and the potential therapeutic use of antioxidants in Wolman disease is discussed.

INTRODUCTION

Wolman disease is a rare autosomal recessive lysosomal-storage disorder characterized by a severe deficiency of the acid lipase and a massive lysosomal storage of triacylglycerols and cholesteryl esters [1–4]. In normal cells, low-density lipoproteins (LDL) taken up via the receptor-mediated pathway are directed to lysosomes and are degraded by the acid lysosomal enzymes [5,6]. In intact living cultured fibroblasts from Wolman disease, the lysosomal degradation of LDL-neutral lipids is almost completely deficient [7,8] and the neutral lipids accumulated in the lysosomes of Wolman fibroblasts are mainly from exogenous (lipoproteins) origin, whereas endogenously biosynthesized neutral lipids do not participate in the lysosomal storage [8,9]. One of the most striking features of Wolman disease is the extensive calcification of adrenal cortex, in which the lipid storage is associated to necrotic areas [4,10]. This prominent alteration of the adrenal cortex could be related to the large influx of LDL in adrenal-cortex cells as suggested by the large number of receptors and the high rate of cholesterol metabolism in this tissue [11]. The mechanism of lipid accumulation in the lysosomal compartment is well understood [4], but the pathophysiology of

Wolman disease remains unclear. In Wolman disease, the presence of ceroid [12] (as in other lipidoses [13]) and of oxygenated sterols [14] strongly suggests the occurrence of a lipid-peroxidation process. The presence of calcification in necrotic areas of Wolman-disease adrenal cortex suggests the occurrence of a cytotoxic process. As neutral lipids are probably not cytotoxic in themselves, and as ceroid accumulates in Wolman cells, we hypothesized that lipid-peroxidation derivatives might be the cytotoxic compounds potentially involved in pathogenesis of Wolman disease. This hypothesis is supported by the fact that lipid-peroxidation products are cytotoxic [15], and that oxidized LDL promote a rise in cellular thiobarbituric acid-reacting substances (TBARS) [16] and are cytotoxic to cultured cells [17–19].

We report here that: (i) LDL exhibit a higher cytotoxic effect on Wolman lymphoblastoid cells than on normal cells; (ii) human lymphoblastoid cells and adrenal cells promote LDL oxidation (leading to formation of minimally oxidized LDL); (iii) oxidized LDL are cytotoxic to cultured cells in a dose-dependent manner; (iv) the higher cytotoxicity of oxidized LDL to Wolman cells results from their higher LDL uptake; (v) the LDL oxidation and the subsequent cytotoxicity are blocked by

Abbreviations used: LDL, low-density lipoproteins; $[Ca^{2+}]$, free cytosolic Ca^{2+} concn.; NDGA, nordihydroguaiaretic acid; CFDA, carboxyfluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Fluo-3/AM, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxy]-2-[2-amino-5-methylphenoxy]ethane-*NNN'*-tetra-acetic acid, penta-acetoxymethyl ester; Quin-2/AM, 2-[[2-bis-(carboxymethyl)amino-5-methylphenoxy]-methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline tetrakis-acetoxymethyl ester; TBARS, thiobarbituric acid-reacting substances; apo, apolipoprotein.

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antioxidants; (iv) the cytotoxic effect of oxidized LDL is associated to a sustained rise in free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) and to later calcium deposition in dead adrenal cells.

MATERIALS AND METHODS

Chemicals

[^3H]Cholesteryl oleoyl ether (38 Ci/mmol) was purchased from Amersham (Paris, France). α -Tocopherol, nordihydroguaiaretic acid (NDGA), tetramethoxypropane, BSA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO, U.S.A.). Carboxyfluorescein diacetate (CFDA), Fluo-3/AM and Quin-2/AM (systematic names of these two given in title-page footnote) were from Molecular Probes (Eugene, OR, U.S.A.). RPMI 1640, Phenol Red-free RPMI 1640, fetal-calf serum, glutamine, penicillin and streptomycin were from Seromed (Paris, France). Ultrosor HY and G were from IBF (Villeneuve-la-Garenne, France), and Hydragel was from Sebia (Issy-les-Moulineaux, France). Other reagents were obtained from Merck (Darmstadt, Germany) or Prolabo (Paris, France).

Cell culture

Lymphoblastoid cell lines [established by Epstein-Barr-virus (B95/8) transformation of blood B lymphocytes] and fibroblasts were obtained from healthy subjects and from a patient affected with Wolman disease, as previously reported [20]. The adrenal-cortex adenocarcinoma cell line SW-13 (A.T.C.C. CCL 105) was from A.T.C.C. (Rockville, MD, U.S.A.).

Lymphoblastoid cells, fibroblasts and adrenal cells were grown in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) and lipoprotein-free serum substitute 2% Ultrosor (Ultrosor HY for lymphoblastoid cells or Ultrosor G for fibroblasts) or 10% heat-inactivated fetal-calf serum, when indicated.

LDL isolation, labelling of LDL with [^3H]cholesteryl oleoyl ether and LDL uptake

LDL from pooled sera were isolated by sequential ultracentrifugation [21], dialysed against 0.15 M NaCl containing 0.3 mM EDTA, sterilized by filtration (0.2 μm -pore Millipore membrane) and stored at 4 °C under nitrogen (up to 3 weeks), all these procedures as previously indicated [19]. LDL were labelled with [^3H]cholesteryl oleoyl ether [approx. 2×10^5 d.p.m./mg of apolipoprotein B (apoB)] by a procedure derived from that of Roberts et al. [22], isolated again by ultracentrifugation, dialysed and incorporated into the cell culture medium.

The cellular uptake was evaluated as previously indicated [19]: briefly, cells were incubated for 12 h with radiolabelled LDL (with [^3H]cholesteryl oleoyl ether) and washed twice with PBS. A portion of the cell culture was used for determining the cell-associated radioactivity by liquid-scintillation counting (Packard model Tricarb 2100).

LDL oxidation

Two alternative procedures were used to promote LDL oxidation: by incubation with cultured cells as indicated in the text, or by u.v. irradiation (254 nm, 0.5 mW/cm², for 2 h) under the standard conditions previously indicated [23,24]. Oxidation conditions were defined to obtain 'mildly oxidized LDL' containing 3–6 nmol of TBARS/mg of apoB. TBARS were determined fluorimetrically as described by Yagi [25] under the previously

used conditions [19,23] with a standard of tetramethoxypropane. Oxidized LDL were immediately incorporated into the culture medium.

Cytotoxicity determination

The cytotoxicity was determined by using the CFDA test, as described by McGinnes et al. [26] or the MTT test, as described by Denizot and Lang [27], under the conditions previously used [16,28]. Briefly, cells distributed in 24-microwell-tissue culture plates (approx. 3×10^5 cells in 1 ml of culture medium per microwell) were incubated with variable concentrations of LDL or mildly oxidized LDL. After incubation for 48 h, the cell viability was determined by using CFDA and MTT tests (the two tests were always performed simultaneously, but, as the results were similar, we reported only the results of one test in the Figures).

Determination of calcium [Ca^{2+}]

$[\text{Ca}^{2+}]_i$ was determined by using Quin-2/AM. Adrenal cells were incubated in 25 mM Hepes-buffered (pH 7.35) RPMI 1640 medium (containing 0.4 mM Ca^{2+}) with 0.5% BSA and 5 μM Quin-2/AM for 1 h before $[\text{Ca}^{2+}]_i$ determination. After washing once with RPMI 1640 and twice with PBS buffered with Hepes (25 mM), cells were immediately used to determine the fluorescence (F) and the calibration of the dye response (by measuring F_{min} and F_{max}) exactly as described by Arslan et al. [29] and as previously used [16,28].

For fluorescence-microscopy studies, Fluo-3/AM was used as indicated by the manufacturer (2 μM in 0.5% dimethyl sulphoxide and 0.02% Pluronic acid F-127) [30].

Lipid analysis and protein determination

Lipids were extracted by the procedure of Folch et al. [31], and lipid extracts were used for determining free and esterified cholesterol mass by g.l.c. (Packard, model 439) equipped with methylsilicone capillary column SP2100 (20 m $\times$ 0.3 mm internal diam., used isothermally at 250 °C) by the procedure of Ambert et al. [32], under the previously used conditions [33] and using 5 α -cholestane as internal standard.

Proteins were determined as described by Lowry et al. [34].

Statistical analysis of the data was done by Student's t test.

Cytochemistry and electron microscopy

Calcium deposition in cells was detected by AgNO_3 by the procedure of Von Kossa as described by Ganter and Jolles [35].

Ultrastructural scanning-microscopy studies of cultured cells were performed under the previously described conditions: cells were fixed in glutaraldehyde in 0.4 M cacodylate/HCl buffer (pH 7.4) at room temperature, dehydrated by ethanol and isoamyl acetate, then gold-coated and examined by electron microscopy using a JEOL 25 microscope [19,29].

RESULTS

Cytotoxicity of native LDL to cultured Wolman-disease lymphoblastoid cells (Figures 1 and 2)

When lymphoblastoid cells were incubated in the presence of native LDL, we observed a cytotoxic effect progressively increasing with LDL concentration. This cytotoxicity of LDL to Wolman-disease lymphoblastoid cells was significantly higher than that to the normal ones (Figure 1a). As shown in Table 1,

Figure 1 Cytotoxicity of native LDL to lymphoblastoid cells (a, black symbols) and to fibroblasts (b, white symbols) from the normal subjects (N; squares) or from a patient affected with Wolman disease (W; circles)

Cells grown in lipoprotein-free medium (containing 2% Ultrosor G), were incubated in the presence of increasing concentration of native LDL for 48 h. The viability was evaluated by the CFDA test and expressed as percentage of controls (cells grown in the absence of LDL). Results are means $\pm$ S.E.M. of five separate experiments. Differences between Wolman-disease cells and controls evaluated by Student's test: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Table 1 Lysosomal storage of neutral lipids in, and cytotoxicity of native and oxidized LDL to cultured cells grown in a serum-free medium or serum-supplemented medium

Cells were grown for 3 weeks before the experiments under standard conditions either in the presence of fetal-calf serum (complete medium) or in a lipid-free culture medium in the presence of 2% Ultrosor G or HY. Cholesterol and cholesteryl esters were determined by g.l.c. and are expressed as nmol/mg of cell protein. The cytotoxic effect was evaluated by the MTT test on cells grown for 48 h in the presence of LDL or oxidized LDL (200 μ g of apoB/ml) and are expressed as percentage of control (cells grown in the standard medium, i.e. RPMI containing 2% Ultrosor, but no LDL). Values are means $\pm$ S.E.M. of two separate experiments.

Culture conditions	Cellular cholesterol (nmol/mg of cell protein)		MTT test (%)	
	Cholesterol	Cholesteryl esters	LDL	Oxidized LDL
Serum-free Ultrosor medium				
Lymphoblastoid cells				
Normal	86 $\pm$ 20	15 $\pm$ 4	75 $\pm$ 5	32 $\pm$ 7
Wolman	70 $\pm$ 15	18 $\pm$ 2	50 $\pm$ 6	12 $\pm$ 8
Fibroblasts				
Normal	56 $\pm$ 7	4 $\pm$ 1	100 $\pm$ 7	22 $\pm$ 6
Wolman	35 $\pm$ 5	11 $\pm$ 5	98 $\pm$ 6	10 $\pm$ 5
Serum-supplemented medium				
Lymphoblastoid cells				
Normal	84 $\pm$ 20	24 $\pm$ 9	80 $\pm$ 5	37 $\pm$ 8
Wolman	68 $\pm$ 15	64 $\pm$ 9	58 $\pm$ 8	19 $\pm$ 6
Fibroblasts				
Normal	50 $\pm$ 10	10 $\pm$ 4	103 $\pm$ 9	27 $\pm$ 7
Wolman	35 $\pm$ 7	60 $\pm$ 9	99 $\pm$ 7	15 $\pm$ 8

Wolman cells grown in a serum-supplemented medium accumulated cholesteryl esters, in lysosomes as previously shown [33], but the over-cytotoxicity of LDL to Wolman lymphoblastoid

Figure 2 Oxidation of LDL (a) and subsequent cytotoxicity evaluated in the test system consisting of Wolman fibroblasts (b)

(a) TBARS formation in culture medium containing increasing concentrations of LDL incubated for 24 h without (0; ---) or with cells (—) (lymphoblastoid cells, LCL, black symbols; fibroblasts, Fbl, white symbols) from normal subjects (squares) or a patient with Wolman disease (circles). Inset: time course (up to 48 h) of LDL oxidation by lymphoblastoid cells (■) and fibroblasts (□), at a fixed concentration of LDL (200 μ g of apoB/ml). (b) MTT test of cytotoxicity of LDL-containing conditioned culture medium of lymphoblastoid cells from normal subjects (□) or a Wolman-disease patient (○), or LDL-free medium (····) on Wolman fibroblasts (test system). The viability was evaluated by the MTT test and expressed as percent of the control (without any lipoprotein). Results are means $\pm$ S.E.M. of three separate experiments.

cells was not influenced by the lysosomal storage of cholesteryl esters. In contrast with lymphoblastoid cells, we detected no appreciable cytotoxicity of native LDL (up to 200 μ g of apoB/ml) to fibroblasts (Figure 1b).

Human lymphoblastoid cells were able to oxidize LDL, as shown by TBARS formation (Figure 2a) and by the depletion of endogenous tocopherols (results not shown). Under the culture conditions used, human lymphoblastoid cells induced a time- and dose-dependent increase in TBARS in LDL-containing media, whereas human fibroblasts promote only negligible levels of TBARS (about similar to LDL oxidation in the cell-free Ultrosor culture medium) (Figure 2a). It is noteworthy that LDL-free culture medium of lymphoblastoid cells contained no significant level of TBARS (result not shown). Lymphoblastoid cells from Wolman disease and from normal subjects exhibited the same oxidative ability, thus suggesting that the over-cytotoxicity observed in Figure 1 did not result from an over-oxidation of LDL by Wolman cells. When the LDL-containing medium conditioned (for 24 h) by lymphoblastoid cells from normal subjects or from Wolman disease was transferred to cultured fibroblasts, it exhibited a dose-dependent cytotoxicity, whereas LDL-free medium conditioned by lymphoblastoid cells was not cytotoxic (Figure 2b). This suggests that the cytotoxic effect was due to LDL oxidized by lymphoblastoid cells and that the lack of LDL 'auto-cytotoxicity' in fibroblast culture observed in Figure 1(b) resulted probably from a lack of LDL oxidation by fibroblasts, rather than from a specific cellular resistance.

Cytotoxicity of oxidized LDL to cultured Wolman and normal cells (Figure 3)

LDL mildly oxidized under standardized conditions exhibited a dose-dependent cytotoxicity to fibroblasts (Figure 3a), in contrast with native LDL (Figure 1b): this clearly demonstrates that fibroblasts are susceptible to the toxic effect of oxidized LDL and supports the idea that the lack of cytotoxicity of LDL to

Figure 3 Comparative susceptibility of Wolman and normal fibroblasts to the cytotoxicity of oxidized LDL

(a) Susceptibility of fibroblasts from normal subjects (squares) or Wolman-disease patients (circles) to the cytotoxic effect of increasing concentrations of mildly oxidized LDL (standard u.v.-C oxidation system). Differences between Wolman-disease cells and controls were evaluated by Student's *t* test: ***P* < 0.01 and ****P* < 0.001. In (b), intrinsic susceptibility of normal (N, squares) or Wolman (W, circles) fibroblasts to oxidized LDL or to native LDL (triangles), expressed as % cytotoxicity (100 - MTT) versus amount of internalized LDL (determined by using increasing concentrations of radiolabelled LDL under the conditions indicated in the Materials and methods section). Results are means ± S.E.M. of four separate experiments.

fibroblasts (Figure 1b) resulted from the lack of LDL oxidation. Oxidized LDL exhibited a significant over-cytotoxicity to Wolman fibroblasts (Figure 3a), which did not result from an artefactual effect, since the same batch of oxidized LDL was used in these experiments. The uptake of native or mildly oxidized LDL was significantly higher in Wolman cells than in normal cells: for instance, at 100 and 150 μg of apoB/ml, the cellular uptake in Wolman cells was $150 \pm 10\%$ of that in normal cells (results not shown), in agreement with the data of Goldstein et

al. [7]. However, the intrinsic susceptibility to the cytotoxic effect of oxidized LDL was similar for Wolman and normal fibroblasts, as shown by the same threshold dose (1.2 ± 0.2 μg of apoB/mg of cell protein, evaluated by the intercept with the abscissa) (Figure 3b). Quite similar results (not shown) were obtained with lymphoblastoid cells. Moreover, a hypothetical role of lysosomal storage in the susceptibility of Wolman cells to the cytotoxic effect of the mildly oxidized LDL was not supported by the data reported in Table 1, which suggest that the over-cytotoxicity observed in Wolman cells is relatively independent of the accumulation of neutral lipids in these cells. In conclusion, all these data suggest that the over-cytotoxicity of mildly oxidized LDL to Wolman cells resulted probably from the higher uptake of mildly oxidized LDL by these cells.

LDL oxidation by adrenal cells, subsequent cytotoxicity and Ca^{2+} deposition (Figures 4–6)

As adrenal degeneration is one of the earliest and most prominent lesions occurring in Wolman disease, it was decided to investigate (i) the ability of adrenal cells to induce LDL oxidation, and (ii) the susceptibility of adrenal cells to the cytotoxic effect of oxidized LDL.

The human adrenal cell line SW-13 was able to induce the formation of mildly oxidized LDL, as shown by TBARS formation (Figure 4a), without significant alteration of the electrophoretic mobility until after 18 h of incubation, under the experimental conditions used here (inset of Figure 4a) (these features are characteristic of mildly oxidized LDL, which have been shown to be taken up by cells through the apoB/E receptor pathway [19]). When transferred to cultured fibroblasts, this adrenal-conditioned medium (containing adrenal-oxidized LDL) was more cytotoxic to Wolman fibroblasts than to controls (Figure 4b). Similarly, adrenal-oxidized LDL were cytotoxic to adrenal cells themselves (Figure 4c) and induced severe morphological alterations, such as cell retraction and holes and tears in the plasma membrane (Figure 4e), by comparison with adrenal cells grown under standard conditions (Figure 4d). These data

Figure 4 LDL oxidation by cultured adrenal cells (SW-13) (a) and subsequent cytotoxicity of adrenal-oxidized LDL to cultured fibroblasts (b) and to cultured adrenal cells (c)

(a) Time course of TBARS formation in the standard culture medium (2% Ultrosor G) containing a fixed concentration of LDL (200 μg of apoB/ml) incubated in the presence of SW-13 cells for increasing periods of time. Upper inset: TBARS formation with increasing LDL concentration in the culture medium incubated for 24 h in the presence (squares) or absence (crosses) of cells. Lower inset: electrophoresis on agarose (Hydragel) of native LDL (a) and mildly adrenal-oxidized LDL (18 h incubation in the presence of cells) (b). (b) and (c) cytotoxicity of increasing concentrations of adrenal-oxidized LDL (mildly oxidized LDL obtained by 18 h incubation with adrenal cells) to cultured fibroblasts (b) from normal subjects (squares) or from a Wolman-disease patient (circles) and to cultured adrenal cells (c). Results are means ± S.E.M. of three separate experiments: ****P* < 0.001. (d) and (e) scanning electron micrographs showing the morphological changes of adrenal cells induced by adrenal-oxidized LDL (200 μg of apoB/ml for 48 h) (e) in comparison with control adrenal cells grown in lipoprotein-free standard medium (d) (magnification × 400).

Figure 5 Rise in $[Ca^{2+}]_i$ induced in adrenal cells by mildly oxidized LDL

(a) Time course of $[Ca^{2+}]_i$ in adrenal cells grown in the standard culture medium containing mildly oxidized LDL (circles) or native (non-oxidized) LDL (triangles) ($200 \mu\text{g}$ of apoB/ml). In (b) and (c), $[Ca^{2+}]_i$ imaging of adrenal cells stained by Fluo-3/AM at the $[Ca^{2+}]_i$ peak (b) and at the resting state (c).

allow us to conclude that: (i) adrenal cells are able to produce mildly oxidized LDL; (ii) adrenal cells are susceptible to the cytotoxic effect of oxidized LDL; and (iii) adrenal-oxidized LDL are more cytotoxic to Wolman cells than to controls.

The cytotoxicity of adrenal-oxidized LDL to adrenal cells was mediated by a broad, delayed and sustained rise in $[Ca^{2+}]_i$ (Figure 5). The number of dead cells increased progressively after the $[Ca^{2+}]_i$ peak, in agreement with our previous data, which showed that the $[Ca^{2+}]_i$ peak is directly involved in the genesis of cytotoxic events leading to cell death [20,33]. When adrenal cells were incubated under the conditions of Figure 4(c), i.e. in the presence of (non-pre-oxidized) LDL, a $[Ca^{2+}]_i$ rise was also observed, but at a later time, 24–30 h (results not shown), because of the delay for cell-induced LDL oxidation (Figure 4a).

After incubation for 72 h under these latter conditions, cytochemical techniques showed a dramatic increase in intracellular calcium deposition in dying (Figure 6b) and dead adrenal cells (Figure 6c). It is noteworthy that these calcium deposits were quite similar to the 'finely granular deposits' and 'dense lumps' observed in Wolman-disease adrenal glands [4].

Protection by antioxidants (Table 2)

As LDL oxidation was a primary cause of the cytotoxicity of LDL to cultured cells, the cytotoxicity (and the over-cytotoxicity to Wolman cells) should be inhibited by antioxidants. As expected, lipophilic antioxidants such as α -tocopherol and NDGA were able to inhibit significantly the adrenal-cell-induced LDL oxidation and subsequently to decrease their cytotoxicity to Wolman fibroblasts (Table 2).

DISCUSSION

It is well established that the cause of Wolman disease is acid lipase deficiency, but the pathogenesis of Wolman disease is largely unknown. As necrosis and calcification of adrenal cortex are early and prominent lesions in Wolman disease, we hypothesized that the mechanism of adrenal cell damage might stand at

Figure 6 Cytochemistry of calcium deposits (appearing as black granular deposits) induced in adrenal cells by adrenal-oxidized LDL

(a) Lack of calcium deposits in control adrenal cells grown for 48 h in lipoprotein-free medium. (b) and (c) presence of calcium deposits in adrenal cells grown for 48 h in presence of adrenal-oxidized LDL ($200 \mu\text{g}$ of apoB/ml): (b) finely granular calcium deposits in a damaged cell still attached to the culture dish; (c) dense lumps of calcium deposits in a detached (floating) dead cell (magnification $\times 1000$).

Table 2 Inhibition of the cell-induced LDL oxidation (TBARS) and of the subsequent cytotoxicity (MTT test) by antioxidants

The culture medium with or without LDL was incubated with adrenal cells for 12 h in the absence or presence of antioxidants, α -tocopherol or NDGA. Then a sample was used for determining the TBARS content, and the medium was transferred on to cultured Wolman fibroblasts for a further 48 h. The cytotoxicity was evaluated by the MTT test at the end of this 48 h period. Results are means $\pm$ S.E.M. of three separate experiments. *** $P < 0.001$.

LDL (μg of apoB/ml)	Antioxidant (μM)	TBARS (nmol/ml)	MTT test (%)
None	None	0.05 ± 0.01	100 ± 9
LDL ($200 \mu\text{g}/\text{ml}$)	None	4.0 ± 0.4	58 ± 5
LDL ($200 \mu\text{g}/\text{ml}$)	α -Tocopherol ($100 \mu\text{M}$)	1.3 ± 0.2	$87 \pm 6^{***}$
LDL ($200 \mu\text{g}/\text{ml}$)	NDGA ($10 \mu\text{M}$)	1.3 ± 0.2	$79 \pm 6^{***}$

the cross-road between the specificity of the physiology of adrenal-cortex cells and the specific defect of Wolman disease. In adrenal cells, plasma LDL are very effectively taken up, owing to the large number of LDL receptors in these cells, are degraded into the lysosomes, and the liberated cholesterol takes part in the down-regulation of the LDL receptor [11]. In Wolman cells, the acid lipase deficiency [7,8] induces a sequestration of LDL-cholesteryl esters in lysosomes, and thus a defect of the down-regulation of the LDL receptor [7], and finally a sustained high rate of LDL uptake which increases in turn the lysosomal storage. Thus, in the affected tissues (e.g. adrenal cells) of Wolman disease, the cytotoxic events may result from the over-uptake of LDL-associated toxic molecules. One possibility was the transformation of LDL into a cytotoxin by oxidation [16–19].

The data reported in this paper clearly demonstrate that immortalized lymphoblastoid cells from Wolman disease were more susceptible to the cytotoxic effect of native LDL and oxidized LDL than were those from normal subjects. The cytotoxic effect of native LDL resulted probably from their oxidation by cells, since: (i) the LDL-containing culture medium conditioned by fibroblasts (which induce very little LDL oxidation) was not cytotoxic to fibroblasts; (ii) those conditioned by lymphoblastoid or adrenal cells (two cell types which induce LDL oxidation) were cytotoxic to fibroblasts; (iii) LDL oxidized by other methods [19] are cytotoxic to fibroblasts; (iv) antioxidants inhibit LDL oxidation and the subsequent cytotoxic effect.

The over-cytotoxicity of native LDL to Wolman-disease lymphoblastoid cells could be explained by three concomitant facts. (i) Lymphoblastoid cells are able to induce the formation of mildly oxidized LDL. (ii) Wolman lymphoblastoid cells exhibited a significant over-uptake of native LDL or mildly oxidized LDL (in contrast with extensively oxidized LDL [36]), subsequent to the lack of down-regulation of the LDL receptor in Wolman cells due to the cholesterol sequestration in lysosomes. (iii) The cytotoxic effect of oxidized LDL is directly related to the amount of oxidized LDL internalized by the cell [23]. As the cytotoxic effect of oxidized LDL on lymphoblastoid cells is directly related to the amount of oxidized LDL internalized by the cell (above a threshold dose) [19], the over-uptake of mildly oxidized LDL may easily explain the over-cytotoxicity of oxidized LDL to Wolman cells.

Adrenal cells possess the highest density of LDL receptors, compared with the other organs [11], and are able to induce the formation of mildly oxidized LDL (the present paper), which are taken up through the LDL-receptor pathway and are cytotoxic in a dose-dependent manner [19]. In normal adrenal cells, the number of LDL receptors is strongly regulated (maximal variation by about 800–1000%) by LDL (down-regulation) and by corticotropin (up-regulation) [11]. In contrast, in Wolman adrenal cells the two regulatory mechanisms are very probably impaired subsequently to the lysosomal defect: (1) the cholesterol-induced down-regulation of the LDL receptor is probably impaired in Wolman adrenal cells, as demonstrated in Wolman fibroblasts [7]; (2) the early adrenal failure occurring in Wolman disease [4,37] should stimulate the corticotropin secretion, which in turn should up-regulate the LDL receptor of adrenal cells [11]. The lack of down-regulation in Wolman cells should result in a dramatic and permanent over-uptake of (non-oxidized) LDL and of LDL mildly oxidized *in situ* by adrenal cells. Subsequently the over-uptake of mildly oxidized LDL leads to the cytotoxic threshold dose being exceeded [19], the antioxidant cellular defences being overwhelmed and finally to the Wolman-disease adrenal cell being killed.

Oxidized LDL elicited in lymphoblastoid and endothelial cells

a delayed and sustained $[Ca^{2+}]_i$ rise which was dependent on extracellular Ca^{2+} and was directly involved in the cytotoxicity [16,29], in agreement with other model systems of chemically induced cell death (for reviews, see [38,39]). The mechanism of the $[Ca^{2+}]_i$ rise induced by oxidized LDL is unknown, and seems to be different (A. Nègre-Salvayre and R. Salvayre, unpublished work) from that elicited by redox-cycling agents [40]. In adrenal cells, toxic doses of oxidized LDL induced a similar $[Ca^{2+}]_i$ rise, which is followed by a dramatic intracellular deposition of calcium (possibly bound to cellular calcium-binding proteins) in the damaged cells. This cellular calcium accumulation is quite similar to the characteristic calcium deposits observed in the adrenal cortex in Wolman disease [4,10].

Finally, another interesting feature is the ability of lipophilic antioxidants to decrease the cytotoxic effect of oxidized LDL. In agreement with previously reported data with other cellular or cell-free systems [41,42], lipophilic antioxidants such as α -tocopherol and NDGA are able to inhibit the adrenal-cell-induced LDL oxidation and the subsequent cytotoxicity.

In conclusion, the reported data support the hypothesis that the mechanism of the cytotoxicity involving LDL oxidation and over-uptake of mildly oxidized LDL (resulting from the block of the lysosomal degradation of cholesteryl esters in Wolman cells) could be relevant to the genesis of adrenal lesions. However, to date, oxidized LDL have not been detected *in vivo* in the adrenal gland, and it cannot be excluded that lipid peroxidation could occur inside the cell, in the stored lipids, leading finally to the intracellular formation of toxic lipid peroxidation products. In both cases (oxidation of LDL or of intracellular stored lipids), this novel pathogenetic hypothesis of Wolman disease suggests the idea to test drugs that are able to decrease the oxidation of LDL and/or the cytotoxicity of oxidized LDL [42–44] as a new therapeutic approach for Wolman disease.

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