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Oxidized Lipoproteins May Play a Role in Neuronal Cell Death in Alzheimer Disease

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ABSTRACT

Oxidative stress in the central nervous system (CNS) may cause oxidation of lipoprotein particles. The oxidized lipoproteins may damage cellular and subcellular membranes, leading to tissue injury and cell death. Human low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) are oxidized by transition metal ions, such as Cu^{2+}. Using PC 12 cells, we tested the cytotoxicity of oxidized LDL and VLDL. Cell death was increased in a dose-dependent manner. Antioxidants added to the incubation medium, such as vitamins E or C, or resveratrol showed some protection. Results indicated that oxidized lipoproteins may serve as an oxidative stressor, which may initiate the neuronal cell death leading to the manifestation of Alzheimer disease (AD).

Index Entries: Oxidized low-density lipoprotein; very low-density lipoprotein; Apo E; lipid peroxides; PC12 cells; Alzheimer disease.

INTRODUCTION

Most of the lipids in the central nervous system (CNS) are actively synthesized in the CNS itself and deposited in large amounts during the early phase of development. The observation that lipids are taken up by brain cells throughout life suggests the presence of a mechanism for lipid transport and cholesterol homeostasis in the brain similar to the vascular system (Pitas et al., 1987). The presence of lipoprotein particles in the

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cerebral spinal fluid (CSF) has been demonstrated (Pitas et al., 1987; Osman et al., 1995). On the other hand, Apo E, a major cholesterol and lipid-carrying protein secreted by astrocytes, plays a very important role in distribution of cholesterol and phospholipids during membrane remodeling associated with synaptic plasticity and compensatory synaptogenesis (Poirier et al., 1991, 1993).

Oxidative stress in the CNS may cause oxidation of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) fractions. Human LDL can be very easily oxidized in the presence of metal ions, such as Cu^{2+} or Fe^{3+} (Esterbauer et al., 1992). One effect of oxidized LDL (or VLDL) is cytotoxicity, which has been demonstrated in various cell types in vitro, including fibroblasts (Hessler et al., 1979; Chisolm et al., 1994; Escargueil-Blanc et al., 1994).

Several studies have suggested that oxidative stress may be involved in the pathogenesis of Alzheimer disease (AD) (Lyras et al., 1997; Sayre et al., 1997). Oxidized lipoproteins may interact with the Apo E receptor and be internalized and degraded via a lysosomal pathway (Goldstein et al., 1983). However, during the course of internalization and degradation, the oxidized lipoproteins may induce a cytotoxic effect on the target cells, causing cell death and plaque formation in a manner similar to foam cell death during atherogenesis (Goldstein et al., 1979). In view of the involvement of Apo E in neuritic plaque and neurofibrillary tangle formation (Rebeck et al., 1993; Strittmatter et al., 1993a,b), it is possible that oxidized lipoproteins directly participate in the pathogenesis of AD.

Rat adrenal pheochromocytoma cells (PC12 cells) possess properties similar to those of neurons and are found to have Apo E receptors resembling the LDL and LDL-related protein (LRP) receptors (Kim et al. 1996). The purpose of the present study was to examine whether oxidized LDL and VLDL are toxic to PC12 cells and, if so, to assess the protection by antioxidants against oxLDL or oxVLDL cytotoxicity. The results may have significant implications by showing that oxidized lipoproteins may be a major risk factor for the onset of AD.

MATERIALS AND METHODS

Cell Culture

Rat pheochromocytoma cells (PC12 cells) were grown routinely at 37°C under an atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% fetal calf serum, and 50 U/mL penicillin/streptomycin (Cheng et al., 1996). The cells were used in various experiments 4–5 d after subculturing, prior to becoming confluent.

Preparation of Native LDL

LDL was isolated from human serum by sequential density ultracentrifugation according to the method of Hatch and Lees (1968). Serum

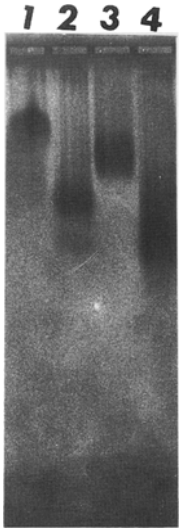

Fig. 1. Electrophoretic pattern of native and oxidized LDL/VLDL. LDL or VLDL was isolated and incubated with $CuSO_4$ as described in Materials and Methods. After agarose gel electrophoresis, the bands of VLDL (lane 1), oxVLDL (lane 2), LDL (lane 3), and oxLDL (lane 4) are shown after Sudan Black staining.

was adjusted to the desired density by the addition of a saturated solution of sodium bromide. Two lipoprotein fractions were prepared: VLDL, $d < 1.019$ LDL, $d = 1.019$–1.063 g/mL. Protein concentration was determined using Peterson's modification of the micro-Lowry method.

Preparation of Oxidized LDL

LDL was dialyzed against PBS and then oxidized using copper sulfate (20 μ*M*) for 24 h at 37°C. The incubation mixture was dialyzed against PBS to remove $CuSO_4$ and other oxidative products, such as H_2O_2, malondialdehyde, and so forth. Oxidation of LDL was confirmed using gel electrophoresis on 0.6% agarose gel in 0.05 *M* barbital buffer at pH 8.0. Typical relative electrophoretic mobilities for the oxidized LDL were within the range 3.0–4.0, compared with native LDL (1.0) (Fig. 1). Similar results were obtained when copper sulfate was replaced with the same concentration of ferric chloride.

MTT Calorimetric Assay

This method is based on the reduction of the yellow dye, salt 3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into blue formazan product, mainly by the mitochondrial enzyme dehydrogenase. The amount of formazan produced is proportional to the number of living cells. Cells were maintained in 96-well plates. After the appropriate treatment, MTT dye was added (0.5 mg/mL), and the plates were incu-

bated at 37°C for 3 h. Then the solution was removed, and the blue formazan produced was solubilized in dimethyl sulfoxide. The optical density of the blue formazan formed was determined at 540 nm using an Anthos Labtec plate reader and expressed as percent of control.

Lactate Dehydrogenase (LDH) Leakage

Cell lysis was determined by assessing leakage of LDH into the medium using the spectrophotometric method (Cheng et al., 1996), which monitors the rate of oxidation of β-nicotinamide adenine dinucleotide, reduced form (NADH) coupled to the reduction of pyruvate to lactate, at 340 nm. LDH activity was measured in the medium, and activity was expressed in U/L.

RESULTS

Effect of Oxidized LDL and VLDL on Cell Survivability

PC12 cells were treated with oxidized LDL and oxidized VLDL for 48 h, and cell survivability was assessed by LDH release. Oxidized LDL and VLDL induced a significant degree of cell death after cells were incubated with the oxidized lipoproteins for 48 h (Fig. 2). Neither native LDL nor VLDL showed any effect on PC12 cells. We have also checked whether trace amounts of Cu^{2+} remained with oxLDL and oxVLDL, which may have contributed to the cell death. Therefore, we added a trace amount of Cu^{2+} (10 μ*M*) to the medium together with LDL or VLDL to examine whether additional amounts of copper would cause cell death. As shown in Fig. 2, Cu^{2+} at 10 μ*M* did not significantly induce cell death after 48 h of incubation. The effect of cell death by oxLDL or oxVLDL appeared to be dose-dependent as shown in Fig. 3. OxVLDL was more effective in inducing cell death at much lower concentrations than oxLDL. At 50 μg/mL, oxVLDL increased cell death by 50%. However, it took double that concentration of oxLDL to induce the same level of cell death.

The Protective Action of Three Antioxidants on oxLDL-Induced Cell Death

If the oxidized LDL-induced cell death is owing to oxidative stress on PC12 cells, addition of antioxidants may protect the cells from oxidative damage and prevent cell death. Resveratrol has been demonstrated recently to be a very effective antioxidant in protecting living cells from lipid peroxidation and cell death (Chanvitayapongs et al., 1997). Figure 3 shows that resveratrol greatly protected the cells from cell death.

We have also employed another assay system, the MTT method, to monitor the survivability of cells. As shown in Fig. 4, oxidized VLDL treat-

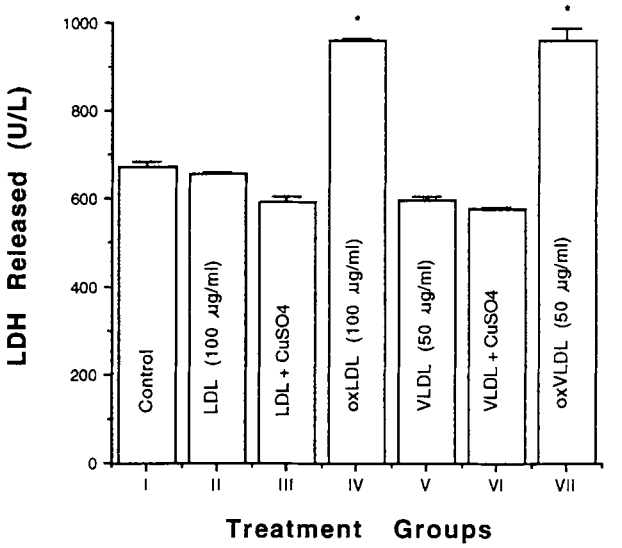

Fig. 2. Oxidized LDL and oxidized VLDL-induced cell death in PC12 cells. PC12 cells were incubated for 48 h in the presence of native LDL (100 µg/mL, Group II) or VLDL (50 µg/mL, Group V) alone or together with 10 µ*M* $CuSO_4$ (Group III or Group VI, respectively), and LDH released to the medium serves as an index for cell death. Groups IV and VII represent the cells incubated with oxidized LDL (100 µg/mL) or oxidized VLDL (50 µg/mL) for 48 h, respectively. Data were expressed as mean ± SD. *$p < 0.001$ Groups IV or VII as compared with control (Group I).

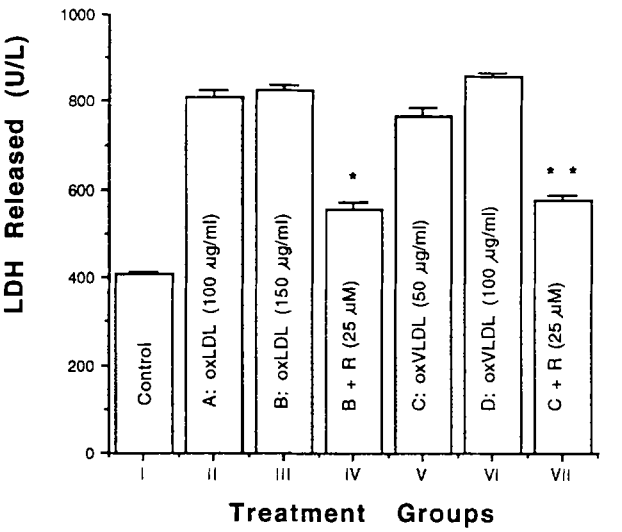

Fig. 3. Protective effect of resveratrol on oxLDL/oxVLDL-induced cell death in PC12 cells. PC12 cells were incubated with oxidized LDL (100 µg/mL, Group II or 150 µg/mL, Group III), or oxidized VLDL (50 µg/mL, Group V; 100 µg/mL, group VI) for 48 h and LDH release assayed as an index of cell death. Group IV represents the cell death after the addition of 25 µ*M* of resveratrol to the medium containing 150 µg/mL of oxLDL, and Group VII represents the protective effect of resveratrol (25 µ*M*) added to the culture medium containing 50 µg/mL of oxVLDL. Data were expressed as mean ± SD. *$p < 0.01$ for Group III vs IV; **$p < 0.01$ for Group VI vs VII.

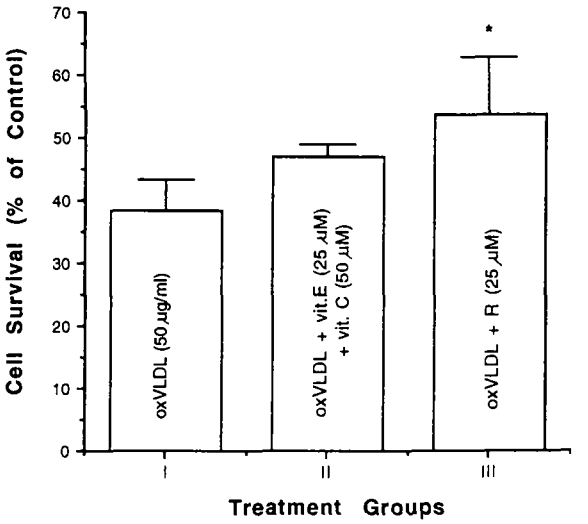

Fig. 4. Effect of antioxidants on survivability of PC12 cells after oxVLDL insult as measured by MTT assay. The cell survivability was assayed after 48 h of incubation of PC12 cells with 50 µg/mL of oxVLDL in the absence (Group I) or the presence of various antioxidants. Group II was the same as Group I with the addition of vitamin E (25 µ*M*) and vitamin C (50 µ*M*). Group III was the same as Group I with the addition of resveratrol (25 µ*M*). Data are expressed as percent of control. $^*p < 0.05$ Group III as compared with Group I.

ment at 50 µg/mL induced almost 70% cell death in 48 h. Resveratrol again was very effective in protecting cells from death. The combination of vitamin E and vitamin C appeared to be less effective in preventing cell death.

DISCUSSION

There is evidence for the existence of lipoprotein particles in the CSF (Osman et al., 1995; Pitas et al., 1987). In the CNS, phospholipid and cholesterol, together with Apo E, may form HDL-like particles and be taken up by cells through endocytosis for the purpose of cell membrane remodeling and synaptogenesis during development or cellular injury. Since brain phospholipids are enriched with polyunsaturated fatty acids, they may be vulnerable to free radical attack. Lipoproteins can be oxidatively modified by endothelial cells to generate lipoproteins containing oxidized lipid, which can be broken down to lysophospholipids and induce propagation of peroxidation (Steinberg et al., 1989). Large amounts of oxidized lipoprotein particles were detected in patents with traumatic brain injury (Borovic et al., 1995). The cell-mediated oxidative modification of lipoproteins can be mimicked by incubating LDL in a medium containing a sufficient amount of copper or iron (Steinbrecher et al., 1987). In this study, not only have we demonstrated that copper induced lipoprotein oxida-

tion as shown in the shifting of lipoprotein bands in Fig. 1, but we have also demonstrated that Fe^{3+} or other oxidative stressors, such as H_2O_2, may also induce a similar pattern of lipoprotein oxidation. Since the brain of AD patients may encounter high levels of iron and other oxidative stressors, it is possible that oxidized lipoprotein levels may be higher in these patients than in controls.

Oxidized LDL has been demonstrated to exert cytotoxic effects on endothelial cells, lymphoblasts, smooth muscle cells, and skin fibroblasts (Hessler et al., 1979; Chisolm et al., 1994; Escargueil-Blanc et al., 1994). In this study, we have demonstrated that oxLDL and oxVLDL are also very potent in inducing PC12 cell death as shown in Figs. 2 and 3, as measured by intracellular LDH release. The mechanism of cell death induced by oxLDL and oxVLDL is not yet fully understood. Many different components of oxLDL or oxVLDL might be responsible for this toxicity, including lipid peroxides, aldehyde, and oxidized cholesterol. It appears that lipid peroxide may play an important role in inducing cell death, since it was demonstrated earlier that lipid peroxide induces lipid peroxidation and cell death in PC12 cells (Chanvitayapongs et al., 1997). In that earlier study, we used *t*-butyl hydroperoxide (*t*-BuOOH) as a model to study the effect of lipid peroxide on cell viability. However, *t*-BuOOH is an amphipathic molecule, which may not completely resemble the fatty acyl hydroperoxides formed in the lipoprotein particles. Whether acyl hydroperoxide, malondialdehyde, 4-hydroxynonenal, oxidized cholesterol, or other oxidized lipoprotein particles are causing lipid peroxidation and cell death in PC12 cells remains to be seen. This work is now in progress in our laboratory. In the present study, we have shown that oxidative damage may play an important role in oxLDL- or oxVLDL-induced cell death, since antioxidants partially protect the cell from these insults. We have also shown that oxVLDL is more potent in inducing cell death than oxLDL. It is possible that VLDL contains a higher proportion of polyunsaturated fatty acid and thus possesses more lipid peroxide after Cu^{2+} treatment. However, both oxLDL and oxVLDL induced cell death, which points to the fact that it is oxidized lipids in the lipoproteins that caused the cell death. It does not matter whether the oxidized lipids resided in LDL or VLDL or other lipoprotein particles.

The protection of antioxidants against oxLDL/oxVLDL-induced cell death was confirmed by another assay system—the MTT assay (Fig. 4). The MTT assay depends on the activity of mitochondrial dehydrogenase to reduce MTT, so that only the "live" mitochondria in living cells exhibit high activity. Therefore, the MTT assay has been popularly used as an index of cell death owing to its simplicity and the advantage that multiple samples can be treated at the same time in a 96-well plate. In this study, similar trends were shown in the results from both LDH release and MTT assay systems. Recently, the occurrence of an LDL receptor in the blood–brain barrier (BBB) has been demonstrated (Dehouck et al., 1994), indicating that the LDL can be transcytosed through the BBB by a

receptor-mediated mechanism, especially during abnormal circumstances, such as cholesterol depletion (Dehouck et al., 1997). Therefore, the work reported here not only suggests that CNS-generated lipoproteins may participate in oxidative damage to the neural cells, but also that blood LDL may be involved in brain damage through this transport mechanism from blood to the brain.

AD is characterized by the deposition of extracellular β-amyloid (A_β) and the accumulation of intracellular neurofibrillary tangles (NFT). Apolipoproteins were found in neuritic plaques and neurofibrillary tangles (Strittmatter et al., 1993a,b). It is possible that the oxidative stress in the CNS causes the oxidative modification of lipoprotein particles, which damage the cellular and subcellular membrane, leading to the binding of apoprotein in decomposed membraneous complexes.

There are three isoforms of Apo E (E2, E3, and E4). The Apo E4 allele is markedly increased in sporadic and late-onset familial AD (Saunders et al., 1993). It is possible that Apo E4 may interact with τ protein and A_β more readily than the other two isoforms because of extra-positive charge on its arginine groups at positions 112 and 158 (Roses, 1996). The differential binding of Apo E to A_β and/or τ in the development of A_β deposition and/or neurofibrillary tangle formation may explain the pathogenetic role of Apo E in AD.

PC12 cells possess properties similar to those of neurons and are found to have Apo E receptors resembling the LDL and VLDL receptors (Kim et al., 1996). Although PC12 cells are catecholaminergic and may not be the major cell type involved in the pathogenesis of AD, the present results may be applicable to neuronal cells in general, especially in view of our recent results showing that oxLDL/oxVLDL also induce cell death in primary neuronal cells (unpublished data).

The fact that antioxidants are effective in protecting PC12 cells from oxLDL/oxVLDL-induced cell death indicates that reactive oxygen species (ROS) are involved in the process. Resveratrol is most potent in protecting PC12 cells from oxidative injury by *t*-BuOOH as compared with vitamin E or vitamin C alone (Chanvitayapongs et al., 1997). However, the combination of vitamin E and vitamin C was more effective in protecting the cells from lipid peroxidation and cell death than vitamin C or E alone (unpublished results). Here we have demonstrated that antioxidants are effective protective agents and that resveratrol is the more effective antioxidant as compared with vitamins E/C. Resveratrol has been shown to inhibit LDL oxidation and to prevent the cytotoxicity of modified LDL (Frankel et al., 1993). We have now demonstrated that resveratrol can effectively prevent oxLDL/oxVLDL-induced cell death. Resveratrol has been shown also to exhibit anti-inflammatory action (Arichi et al., 1982; Chung et al., 1992) and inhibit tumorigenesis activities (Jang et al., 1997). It is an amphipathic molecule, and thus is able to provide more effective oxidative protection for cellular and subcellular components than most commonly employed antioxidants, such as vita-

mins E and C. Resveratrol is an important ingredient in grapes and red wine, and is present in high concentration in some oriental herbal medicines, such as Kojo-kon, used in treating fever, hyperlipidemia, atherosclerosis, and inflammation (Arichi et al., 1982; Chung et al., 1992). Based on its ability to reduce cell death caused by oxidized lipoprotein particles, it may have beneficial value in preventing neurodegenerative diseases in general and AD in particular.

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