Expression of the Very Low-Density Lipoprotein Receptor (VLDL-r), an Apolipoprotein-E Receptor, in the Central Nervous System and in Alzheimer's Disease

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Abstract. The very low density lipoprotein receptor (VLDL-r) is a cell-surface molecule specialized for the internalization of multiple diverse ligands, including apolipoprotein E (apoE)-containing lipoprotein particles, via clathrin-coated pits. Its structure is similar to the low-density lipoprotein receptor (LDL-r), although the two have substantially different systemic distributions and regulatory pathways. The present work examines the distribution of VLDL-r in the central nervous system (CNS) and in relation to senile plaques in Alzheimer disease (AD). VLDL-r is present on resting and activated microglia, particularly those associated with senile plaques (SPs). VLDL-r immunoreactivity is also found in cortical neurons. Two exons of VLDL-r mRNA are differentially spliced in the mature receptor mRNA. One set of splice forms gives rise to receptors containing (or lacking) an extracellular O-linked glycosylation domain near the transmembrane portion of the molecule. The other set of splice forms appears to be brain-specific, and is responsible for the presence or absence of one of the cysteine-rich repeat regions in the binding region of the molecule. Ratios of the receptor variants generated from these splice forms do not differ substantially across different cortical areas or in AD. We hypothesize that VLDL-r might contribute to metabolism of apoE and apoE/Aβ complexes in the brain. Further characterization of apoE receptors in Alzheimer brain may help lay the groundwork for understanding the role of apoE in the CNS and in the pathophysiology of AD.

Key Words: Alzheimer’s disease; apoE; Apolipoprotein receptors; VLDL receptor.

INTRODUCTION

Recent genetic evidence suggests that inheritance of the e4 allele of the apolipoprotein E (apoE) gene is associated with Alzheimer’s disease (AD) (1–6). Individuals with apoE e4 have higher amounts of Aβ deposits and an earlier age of onset of AD (4, 7). Senile plaques contain apoE (8, 9). Although these data are well established, the biological mechanism underlying the apoE e4-mediated increased risk for AD is unknown. One hypothesis suggests that apoE binds Aβ, and the complex is cleared by apoE receptors (4). Alternatively, apoE could act as a pathological chaperone enhancing Aβ deposition (10). ApoE has also been suggested to interact with tau in an isoform-dependent fashion (11). Because apoE is not synthesized by neurons (12), this hypothesis invokes the presence of apoE receptors on neurons to provide intraneuronal access for apoE.

In light of these hypotheses, and to further explore the pathophysiologically basin of apoE effects in the central nervous system (CNS) we have examined the expression of apoE receptors in the AD brain. Four receptors have been described in peripheral organs which mediate internalization of apoE-containing complexes: the low density lipoprotein receptor (LDL-r), the LDL receptor-related protein (LRP) (13, 14), megalin (gp330) (15), and the very low density lipoprotein receptor (VLDL-r) (16, 17).

A recent report suggests a genetic link between VLDL-r and AD (18). There is a polymorphic trinucleotide repeat in the 5′ untranslated region of the VLDL-r gene. Okiuzumi et al reported that in a Japanese population there is an over-representation of the allele containing 5 repeats. Although this report has not been confirmed in other populations (19), it raises the intriguing possibility that, at least in some contexts, alterations in VLDL-r expression might influence AD pathophysiology.

The VLDL receptor’s structure is similar to that of LDL-r, although its function and regulation are distinct (16). As predicted by amino acid sequence, both molecules contain the following five domains: (a) a cysteine repeat-rich ligand binding region, (b) an epidermal growth factor (EGF) precursor homology region, (c) an O-linked glycosylation region, (d) a transmembrane region, and (e) an intracellular NPXY sequence needed for internalization via clathrin-coated vesicles. The two receptors exhibit an approximately 50% homology at the primary sequence level. VLDL-r is distinguished from LDL-r primarily by the presence of one additional cysteine-rich repeat in the putative ligand binding domain. This difference alters the binding specificity considerably: LDL-r binds and internalizes apoE and apoB-containing lipoproteins such as LDL, whereas VLDL-r is similar to LRP and megalin in interacting with proteinases, proteinase inhibitors, and lipases, as well as apoE-rich particles such as VLDL, β-VLDL, and LDL, but not LDL particles (16, 17, 20, 21). As for all identified members of the LDL receptor
gene family, binding of these ligands is effectively blocked by the 39kDa Receptor Associated Protein (RAP)(22, 23).

Two separate exons of VLDL-r have been identified as subject to alternative splicing in mammals (16, 24). One, exon 16, encodes the O-linked glycosylation region in the extracellular domain just distal to the transmembrane domain. The functional difference between receptors containing and lacking this region remains unknown, though it has been suggested that such O-linked glycosylation regions may serve as molecular "extenders", allowing an extracellular binding site to protrude beyond a clustered cell-surface microenvironment (25). Deletions in the homologous region of the LDL receptor have also been implicated in some forms of familial hypercholesterolemia (26). The other set of variants arises from alternative splicing of exon 4, which encodes one of the cysteine-rich repeats in the putative ligand-binding region of the molecule. The form of VLDL-r lacking exon 4 has been described in rat as being present in brain but in no other tissue examined, including heart, skeletal muscle, white and brown fat, ovary, testis, and salivary gland (24). While the absence of this portion of the molecule is presumed to influence ligand binding, its functional significance is similarly uncertain.

VLDL-r is distinct from LDL-r in several other ways as well. It has a different tissue distribution, being nearly absent from liver, whereas LDL-r is abundant in liver. In contrast, VLDL-r mRNA is expressed most strongly in skeletal muscle, adipose tissue, heart, brain, and placenta (16, 27). Transcriptional regulation also differs significantly between the two receptors. Elevated intracellular cholesterol downregulates LDL-r expression via the sterol response element SRE-1, an upstream regulatory element shared with HMG-CoA reductase and other enzymes of cholesterol metabolism. While the VLDL-r gene contains an upstream SRE-1 site, this site is non-functional, as demonstrated in vitro by Sukai et al (16). By contrast, cAMP upregulates LDL-r mRNA but downregulates VLDL-r message in cultured choriocarcinoma cells (27). Jokinen et al (24) have demonstrated that VLDL-r mRNA and protein levels in rat skeletal muscle, while unaffected by fasting, were markedly altered by changing thyroid hormone levels, arguing for a physiological role for VLDL-r beyond that of lipoprotein homeostasis.

We have recently examined the distribution of the four known members of the LDL-receptor family in the brain. LRP is localized to activated astrocytes and to many pyramidal neurons, and is present as a component of senile plaques in AD (4). Though the presence of LDL-r in the CNS has been confirmed by Western blot analysis, it does not appear to be restricted to a specific cell type, showing a diffuse pattern of immunoreactivity in the neuropil (4). Megoln, a receptor for both apoE and apoE (15), has been reported to be present in brain only in the ependyma (28). The present work examines the distribution of VLDL-r in brain, its relation to AD neuropathology, and the regional variation in expression of its splice forms.

METHODS

Immunohistochemistry

Brain tissue from control (n=7; mean age 68 years (yr): postmortem interval (PMI) 7 to 26 hour [hr]) and AD patients (n=14; mean age 80 yr; PMI 5 to 27 h) was received at autopsy from the Alzheimer Disease Research Center. All AD cases met Khachaturian (29) and CERAD diagnostic criteria (30) for AD based on examination of Bielschowsky-stained paraffin sections of multiple cortical and subcortical areas. Dissected regions containing hippocampus and adjacent temporal lobe were fixed...
in paraformaldehyde-lysine-metaperiodate for 24 to 36 h, and
transferred to 15% glycerol in Tris-buffered saline, pH 7.4, at
4°C. Occipital lobe was available in 5 of these cases for eva-
uation of neuronal staining. Sections were prepared at 50 μm
on a freezing sledge microtome, and stored in a solution of 15%
glycerol in tris-buffered saline, pH 7.4 at −80°C, until use. Im-
munohistochemistry was performed on floating sections with
standard methods, using biotin-conjugated secondary an-
tibodies, an avidin-biotin-peroxidase amplification stage (Elite
Kit, Vector Laboratories, Inc, Burlingame, CA), and dianino-
benzidine as a chromagen. For experiments requiring double
labeling, a subsequent primary antibody was detected either
with a gold-conjugated secondary antibody and subsequent sil-
ver precipitation enhancement kit (Amersham, Arlington
Heights, IL) or with a Cy3-labeled fluorescent secondary an-
tibody (Jackson Immunoresearch, West Grove, PA). The order
of antigens and chromogens was varied to control for possible
methodological artifacts. Sections processed without primary
antiserum or with primary antisera incubated with excess gener-
ating peptide were used as negative controls and showed no
specific staining. A Nissl counterstain was used to aid in iden-
tification of cytoarchitectural regions. A thioflavine S stain
was also used to identify Alzheimer disease neuropathology.

Antibody R2623 is a rabbit polyclonal antibody raised against a
synthetic peptide, ASYGHTYPAlSIVSTDDDL, which represents the
carboxyl terminus of human VLDL-r. The antibody was affinity
purified over peptide-sepharose (27). It was used at a dilution of 1:100 (1.93 μg/μL) in 1% normal goat
serum. An aliquot of antibody was preabsorbed with excess
generating peptide to establish the specificity of the serum. To
stain plaques in AD brains, an anti-Aβ monoclonal antibody,
10D5 (Athena Neurosciences, San Francisco [31]) was used at
a 1:250 dilution. Anti-apoE polyclonal antibody was purchased
from Medix (Foster City, CA), and diluted 1:500. Anti-glia-
fibrillary acidic protein (GFAP) antibody was obtained from
Amersham (Arlington Heights, IL), and used at a 1:400 dilu-
tion. LN-3 is a mouse monoclonal antibody against Class II
MHC (HLA-DR), and was used as received from ICN Bio-
chemicals (Irvine, CA).

Western Blot Analysis: Plasma membrane–enriched fractions
were prepared from frozen tissue from frontal cortex of a nor-
mal and an AD patient essentially as described for rat liver (32).
Briefly, frozen tissue was thawed rapidly, diced with scissors,
and homogenized in 0.25 M sucrose-10 mM HEPES, pH 7.5.
Sucrose was then added to a final concentration of 1.60 M, and
the suspension overlaid with 0.25 M sucrose-10 mM HEPES,
pH 7.5. The suspension was centrifuged at 70,900 × g for 70
minutes (min), and the plasma membrane fraction suspended
in 50 mM HEPES, pH 7.4 containing 0.5 M NaCl, 0.05% Tween-
20, and 1% Triton X-100. Protein concentrations were deter-
mined by the method of Bradford (33) using BSA as a standard.
Cell extracts were subjected to SDS-PAGE on gradient gels (4–
12%, Novex, San Diego CA) and electrophoretically trans-
ferred to nitrocellulose membranes. Filters were blocked with 50 mM
Tris, 150 mM NaCl, pH 8.0 (TBS) containing 3% nonfat milk for
1 h at 25°C. The membranes were then incubated with 1 μg/
μL anti-VLDL-r IgG (R2623) in TBS containing 3% nonfat
milk, 5 mM CaCl₂, and 0.02% Tween 20 (buffer C) for 1 h at
25°C. After this and between each subsequent step, the filters were washed 3 times with buffer C. The filters were then incubated with a goat anti-rabbit IgG-horse radish peroxidase conjugate for 1 h at 25°C, and the bands visualized with the Renaissance Chemiluminescence kit (DuPont NEN, Boston, MA). Primary antibody absorbed with excess generating peptide was used to demonstrate specificity of the antisera.

**Splice Form Analysis**

The expression of VLDL-r mRNA splice forms was studied in different brain regions and in peripheral organs. In all, 51 regions from 6 control cases and 48 regions from 10 AD cases were analyzed for exon 16 splice forms; 10 regions from 4 control and 16 regions from 11 AD cases were analyzed with respect to exon 4. Regions available varied from case to case, but included dorsolateral frontal cortex, motor/ sensory cortex, thalamus, areas 17 and 18, caudate, putamen, superior temporal cortex, inferior temporal cortex, entorhinal cortex, hippocampus, and cerebellar hemisphere. Peripheral organs from 2 cases (heart, skeletal muscle, liver, and kidney) were available for analysis of exon 16 splice forms, and from 4 cases for exon 4 analysis. RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH), and reverse transcriptase-polymerase chain reaction (RT-PCR) method was used according to the manufacturer's directions (Promega, Madison, WI). 1 µg of total isolated RNA was used to synthesize cDNA. Subsequent PCR was performed in a final volume of 50 µl containing 10 µl cDNA template, 200 mM of each primer, 50 mM dNTPs, 3 mM MgCl₂, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI). The reaction mixture was heated to 95°C for 2 min and followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 56°C for 0.5 min, and elongation at 72°C for 1 min. The PCR products were analyzed by 2% agarose gel with products visualized with ethidium bromide (1 mg/ml). Primers used for analysis of exon 16 variation were: 5′-CTAGTCAACAACCTGATGATG-3′ and 5′-AAGATGCCCCATGCGGCAAA-3′; primers for analysis of exon 4: 5′-CAACAATGGCCAGTGTTTC-3′ and 5′-TGGAGCAGGTTGAACCTGTC-3′.

A Bio-Rad (Hercules, CA) image analysis system was used for quantification of the relative amount of each splice form within brain regions. Gel images were digitized such that band intensities remained within the linear response range of the GelDoc analyzer; for each sample, areas corresponding to the two splice forms were integrated from manually defined peaks on computer-generated intensity profiles. The ratio of larger splice form to smaller splice form was computed for each lane to control for intersample variation in PCR amplification or total mRNA.

**RESULTS**

**Immunoreactivity**

VLDL-r immunoreactivity was found in neurons and microglia in control and AD brains (Fig. 1). Microglia displaying the characteristic resting morphology of small cell bodies with fine ramified processes as well as those of activated morphology, with blunter, thicker processes and an enlarged rounder cell body were immunoreactive, with the latter showing more robust staining. Clusters of activated, VLDL-r-positive microglia were colocalized with Aβ (Fig. 2) and apoE in senile plaques of AD brains. Double immunostaining of VLDL-r with antibody LN-3 immunoreactivity confirmed the identity of these cells as microglia. GFAP-positive astrocytes were not colocalized with VLDL-r immunoreactivity, nor were ependymal cells or blood vessels.

Although neuronal staining for VLDL-r was variable among the cases examined, perhaps due to epitope sensitivity to postmortem conditions or fixation, staining of 2 distinct sets of cells was frequently observed. Dendritic processes of granule cells in the dentate gyrus and pyramidal cells (Fig. 3A, C) in CA4, CA3, presubiculum, subiculum, entorhinal cortex, and layers III and IV of temporal lobe neocortex were immunoreactive in the most extensively stained cases. Most of the cases examined (4 of 6 control and 9 of 13 AD) showed cortical pyramidal cell staining as well as a punctate fiber staining in the molecular layer of the dentate gyrus. In addition, occasional small, finely ramified VLDL-r positive interneurons (Fig. 3B) appeared evenly distributed throughout the cortex and occasionally in white matter. Examination
of immunoreactive neurons at 100x magnification revealed positive staining of dendrites, cell bodies, and axons.

The immunostaining pattern in AD was similar, including expression on resting and activated microglia, a variable population of pyramidal cells, and some interneurons. There were more microglia of activated morphology in AD cortex than in control cases, and these were particularly strongly immunoreactive. An additional change in AD was seen in the molecular layer of the dentate gyrus. In control cases, staining was uniform through the depth of the molecular layer, but in AD cases, staining appeared stronger, particularly in the inner third.

Western Blot Analysis

The specificity of the VLDL-r antibody was examined by immunoblotting of extracts prepared from tissue of a normal and an AD patient. Immunoblotting (Fig. 4) revealed two polypeptides, with apparent molecular weights of 130 and 110 kDa. The 130kDa polypeptide comigrates with purified full-length VLDL-r, while the 110kDa polypeptide likely represents the VLDL-r form lacking the O-linked glycosylation domain (see below). A faint higher molecular weight band is also noted, which likely represents the VLDL-r dimer which has been noted previously (22). No bands were observed after absorption of the primary antibody with excess immunogen.

Splice Form Analysis

RT-PCR analysis around exon 16 of the VLDL-r cDNA revealed two products, approximately 285 and 369 nucleotides in length. These results correspond with the expected lengths of the VLDL-r RNA splice forms lacking and containing the O-linked glycosylation region (16). Amplification around exon 4 similarly revealed two products, of 122 and 251 nucleotides in length, corresponding to the expected lengths of forms lacking and containing a cysteine-rich repeat region of the binding domain (Fig. 5).

Quantitation of splice forms revealed no difference in the ratio of larger to smaller splice forms either across brain regions or with respect to disease group. These splice forms were also examined in various organs. No detectable VLDL-r mRNA was present in liver. The exon 4-containing splice form was found in kidney, heart, and muscle, and both the exon 4-containing and lacking splice forms were present in brain. Both exon 16 splice forms were present in all tissues containing VLDL-r mRNA (Fig. 5).

DISCUSSION

Our results show that, in comparison to the other identified apoE receptors, VLDL-r has a unique distribution of expression in the human brain. VLDL-r is present in microglia and some neurons. LRP, on the other hand, is present in neurons, choroid plexus, and reactive (but not resting) astrocytes (4), while megalin is expressed primarily in ependymal cells (28). LDL-r has been reported to be present diffusely in neuropil (4), and is probably expressed in multiple CNS cell types. These results taken as a whole suggest differential regulation and cell type–specific expression of different members of the LDL receptor gene family in the CNS, possibly reflecting different roles of each in catabolism of molecules.

In AD, VLDL-r is expressed strongly on microglia associated with senile plaques (SPs). LRP stains the plaques
Fig. 5. Splice form analysis of VLDL receptor mRNA in human brain regions. (a) Amplification around exon 4. (b) Amplification around exon 16. FC, frontal cortex; CD, caudate; EC, entorhinal cortex; HF, hippocampus; IT, inferior temporal cortex; CBL, cerebellum; H, heart; M, skeletal muscle; K, kidney; L, liver.

themselves, potentially due to the presence of a secreted form of the LRP receptor bound to the plaque surface (34); LRP-positive reactive astrocytes have also been described in white matter in AD. No plaque-associated staining is seen for LDL-r (4). Earlier studies have revealed the presence of VLDL-r mRNA in THP-1 cells, a human monocytic leukemia cell line (16) and in placental macrophages (27), supporting a role for VLDL-r activity in cells of the monocyte lineage. An intimate association of activated microglia with Aβ deposits has been previously described, and these cells contribute to what has been described as a chronic inflammatory state in the vicinity of SPs (35, 36).

VLDL-r immunostaining was also observed in neurons, in accord with a recent report using a monoclonal VLDL-r antibody (18). Unlike that report, however, we did not observe differences in intensity of neuronal staining between AD and control. There was substantial case-to-case variability in neuronal immunoreactivity in the temporal lobe, likely reflecting variation in perimortem or postmortem course. Authenticity of the neuronal staining is supported by in situ hybridization studies showing VLDL-r mRNA in rat cortical neurons (37). It is not yet known whether the VLDL-r splice forms we describe are differentially expressed in different cell types in the CNS. The biological significance and regulation of receptor expression in neurons is unknown, although we speculate that it may be active in the context of synaptic remodeling or other membrane alterations. It is also possible that, like LRP, VLDL-r mediates neurite outgrowth of apoE treated neurons in vitro (38). VLDL-r might also contribute to internalization of apoE-containing complexes by neurons, supporting an assumption of the hypothesis that apoE interacts with tau (11).

The structural and biochemical similarities between VLDL-r and LRP suggest that the two receptors may serve analogous metabolic roles. This is of interest from the point of view of AD pathophysiology, because apoE-containing lipoproteins are ligands for both receptors. In addition, we have recently demonstrated that LRP is the receptor responsible for internalization and degradation of secreted Kunitz Protease Inhibitor-containing forms of the amyloid precursor protein (also called protease nexin II) (39), and it is possible that soluble APP would also be a VLDL-r ligand. As neuronal receptors for both apoE and (potentially) APP, LRP and VLDL-r might both contribute to apoE-mediated changes in neuronal function.

Based on its specificity for apoE-containing ligands and its primary localization to microglia, a cell type specialized for recognition, internalization, and degradation of macromolecules, VLDL-r is an excellent candidate for a receptor-mediated uptake mechanism for apoE-containing complexes. ApoE is synthesized by astrocytes in the CNS (12). We speculate that, upon its release into the local extracellular space, ApoE binds free lipids and lipophilic macromolecules, rendering them susceptible to uptake via VLDL-r. This "opsonization" of free lipophilic molecules may represent a general constitutive mechanism of lipid clearance in the CNS. The expression of VLDL-r appears to be more robust on "activated" microglia than "resting" microglia, and therefore VLDL-r activity may be additionally influenced by cellular interactions with inflammatory cytokines, complement proteins, or extracellular matrix adhesion molecules.

Aβ/apoE complexes form in vitro (40, 41) and in vivo (42), but the metabolic fate of these complexes is unknown. In AD, inheritance of the apoE ε4 allele is associated with increased deposition of Aβ in a gene-dose dependent manner (4, 43). We had observed that both apoE and LRP immunostain cortical senile plaques, and hypothesized that the increased Aβ burden seen with apoE ε4 was due to impaired clearance of E4-containing apoE/Aβ complexes by LRP(4). Our current observations suggest that another multifunctional apoE receptor, VLDL-r, is also in the immediate vicinity to interact with apoE/Aβ complexes and with senile plaques. Because it is present on activated microglial cells, this receptor may be involved in clearance of apoE/Aβ complexes. If so, we postulate that regulation of VLDL-r activity by inflammatory mediators may provide a signal to increase clearance in the context of brain injury.
ACKNOWLEDGMENTS

We thank the Massachusetts Alzheimer Disease Research Center Brain Bank (E.T. Hedley-Whyte, MD, director) for brain tissue (grant AG05134). We appreciate the excellent technical contributions of Fran Battey.

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J Neuropsychol Exp Neurol. Vol 55, April, 1996

Received October 26, 1995
Revision received February 8, 1996
Accepted February 9, 1996