Loss of Apolipoprotein E Receptor LR11 in Alzheimer Disease

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**Background:** Genetic, epidemiologic, and biochemical evidence suggests that apolipoprotein E, low-density lipoprotein receptors, and lipid metabolism play important roles in sporadic Alzheimer disease (AD).

**Objective:** To identify novel candidate genes associated with sporadic AD.

**Design:** We performed an unbiased microarray screen for genes differentially expressed in lymphoblasts of patients with sporadic AD and prioritized 1 gene product for further characterization in AD brain.

**Setting:** Emory University, Atlanta, Ga.

**Subjects:** Cell lines were used from 14 patients with AD and 9 normal human control subjects.

**Results:** Six genes were differentially expressed in lymphoblasts of 2 independent groups of patients with probable AD and autopsy-proven AD. We hypothesized that 1 of the genes, termed low-density lipoprotein receptor relative with 11 binding repeats (LR11) (reduced 1.8- and 2.5-fold in AD lymphoblasts vs controls), might be associated with sporadic AD on the basis of its function as neuronal apolipoprotein E receptor. We found dramatic and consistent loss of immunocytochemical staining for LR11 in histologically normal-appearing neurons in AD brains. This reduction of LR11 protein was confirmed by quantitative Western blotting ($P = .01$).

**Conclusions:** There is loss of the microarray-derived candidate, LR11, in neurons of AD brains. This study shows that microarray analysis of widely available lymphoblasts derived from patients with AD holds promise as a primary screen for candidate genes associated with AD.
ues. In this study, we used complementary DNA microarrays to screen for genes differentially expressed in lymphoblasts from patients with probable or definite AD. This novel strategy identified changes in 6 transcripts, including the lipoprotein receptor LR11 (LDL receptor relative with 11 binding repeats). On the basis of its function as a neuronal ApoE receptor and its expression in the brain, we hypothesized that LR11 might play a role in sporadic AD. To test this hypothesis, we examined LR11 protein expression in AD brains.

METHODS

SUBJECTS

Lymphoblast lines were obtained from healthy elderly control subjects and patients with AD who were all well characterized via annual assessments in the Alzheimer’s Disease Center at Emory University, Atlanta, Ga. Informed consent was obtained in accordance with the regulations of the institutional review board at Emory University. The diagnosis of probable AD was made according to criteria of the National Institute of Neurological Disorders and Stroke and consensus of 2 experienced clinicians. (Multiple clinicians, including A.I.L. and J.J.L., participated in establishing the consensus diagnosis.) The pathologic diagnosis of definite AD was made by a neuropathologist according to criteria of the Consortium to Establish a Registry for Alzheimer’s Disease. (Multiple neuropathologists, including M.G., were involved.) Cell lines were used from a total of 14 patients with AD and 9 normal human control subjects (Table 1).

LYMHOBLASTS

Patient lymphocytes were immortalized by the Neitzel method22 in the Emory General Clinical Research Center. In brief, the lymphocytes were removed in the buffy coat layer after gradient separation (Histopaque-1077; Sigma-Aldrich Corp, St Louis, Mo). The lymphocytes were then incubated with Epstein-Barr virus (B95-8, American Type Culture Collection, Manassas, Va) to induce transformation, the lymphoblasts were maintained in RPMI-1640 medium supplemented with 15% bovine serum and 110-µg/mL sodium pyruvate, and stocks of cells were stored in liquid nitrogen. The cell lines were thawed and cultured in RPMI-1640 medium supplemented with 15% heat-inactivated fetal bovine serum to a confluency of 80%.

COMPLEMENTARY DNA MICROARRAY SCREEN

Lymphoblast lines from patients with AD and controls were matched for age, sex, and race. As expected, a higher APOE ε4 allele frequency was present in the patients with AD than controls, but this was matched as closely as possible. Two experiments were performed with the use of lymphoblast messenger RNA (mRNA) from patients with clinically diagnosed probable AD, and a group with autopsy-confirmed definite AD. For experiment 1, cell lines from 7 patients with probable AD plus 1 patient with definite AD were compared with those from 8 controls; in experiment 2, 6 definite AD samples were compared with 6 controls. Total RNA was purified from each cell line by means of a kit (RNaseasy Mini kit; Qiagen, Valencia, Calif) and pooled for controls and patients with AD in each experiment. The mRNA was isolated according to a batch protocol (Oligotex; Qiagen) and quantified by spectrophotometry. Complementary DNA probe synthesis, hybridization with human UniGEM V complementary DNA microarrays, and signal analysis were conducted by Incyte Genomics (St Louis, Mo) as described. (Multiple hybridization experiments, including M.G., were involved.) Cell lines were used from a total of 14 patients with AD and 9 normal human control subjects (Table 1).

NORTHERN ANALYSIS

In experiment 1, quantification of mRNA was additionally confirmed by Northern blot hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Northern hybridization analysis was performed with the use of formaldehyde to denature 500 ng of polyA RNA, followed by electrophoresis and transfer to a hybridization transfer membrane (GeneScreen; NEN Research Products, Boston, Mass). An in vitro transcription kit
with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif). Monoclonal antibody (C.J.H., A.I.L., unpublished data, 2000) was used for protein loading by reprobing blots with anti–14-3-3 and software (Perkin-Elmer). Band intensities were normalized for hybridization signal for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is similar in both samples.

(MEGAscript; Ambion, Inc, Austin, Tex) was used for (α-32P)dUTP labeling of RNA probes. The intensity of bands was quantified with a PhosphoImager (Molecular Dynamics, Sunnyvale, Calif).

WESTERN BLOTTING

Tissue samples from frontal cortex of 5 human controls and 6 patients with AD were thawed and homogenized in Tris-EDTA, pH 7.4, plus protease inhibitors (Complete Protease Inhibitors; Hoffman-La Roche, Inc, Nutley, NJ). Protein concentrations were measured with a protein assay kit (BCA; Pierce, Rockford, Ill). Samples were separated across a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred overnight to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, Mass). Blots were blocked in 5% nonfat milk–Tris-buffered saline at room temperature for 30 minutes, then probed overnight at 4°C with a polyclonal antibody to the VPS10 domain, whose specificity has been demonstrated previously, or a monoclonal antibody to the VPS10 domain. Blots were rinsed and incubated for 1 hour at room temperature with secondary antibodies conjugated to horseradish peroxidase (Pierce) and visualized by chemiluminescence (Renaissance; Perkin-Elmer, Boston, Mass). Images were captured and band intensities measured by means of a Kodak Image Station 440cf (Eastman Kodak, New Haven, Conn) and software (Perkin-Elmer). Band intensities were normalized for protein loading by reprobing blots with anti–14-3-3 monoclonal antibody (C.J.H., A.I.L., unpublished data, 2000).

IMMUNOHISTOCHEMISTRY

Blots of frontal cortex from 13 patients with AD and 7 controls were fixed for 24 to 48 hours in 4% paraformaldehyde, then embedded in paraffin or cryoprotected in 30% sucrose and frozen. Paraffin-embedded blocks were cut into 8-µm sections, deparaffinized, and pretreated with pepsin (Biomeda, Foster City, Calif). Frozen blocks were cut into 50-µm sections. Sections were then treated with hydrogen peroxide, washed in Tris buffer, blocked with normal serum, and incubated with anti–LR11 antibodies overnight at 4°C. On day 2, sections were incubated with biotinylated secondary antibody followed by avidin-biotin-peroxidase complex (Vector Elite ABC kit; Vector Laboratories, Burlingame, Calif). Color development was carried out with 3,3′-diaminobenzidine. Control sections incubated without primary antibody showed negligible staining.

RESULTS

PRIMARY MICROARRAY SCREEN IN LYMPHOBLASTS OF PATIENTS WITH PROBABLE AD

To screen for genes differentially expressed in lymphoblasts of patients with AD, we performed 2 independent microarray experiments. In the first experimental set, immortalized lymphoblasts from 7 patients with a clinical diagnosis of probable AD and 1 patient with autopsy-confirmed definite AD were compared with lymphoblasts from a group of 8 age-, sex-, and race-matched control lymphoblast lines (Table 1). Of 7270 genes analyzed, expression of 15 mRNAs was decreased by 1.8-fold or greater, expression of 3 was increased by 1.8-fold or greater, and 7252 mRNAs were below significance threshold (a complete list of differentially expressed genes is available from the authors). To technically confirm the initial microarray results with an independent method, mRNA levels of selected genes were assessed by Northern hybridization in the probable AD and control groups. Hybridization signals for Bcl2-related protein A1 and interferon-γ receptor mRNA were decreased, while hybridization signal for IgG3 mRNA was increased (Figure 1). These results were consistent with the fold changes observed for each of these genes by microarray hybridization (~2.1, ~2.4, and +3.3, respectively). Hybridization signal for the housekeeping gene GAPDH was unchanged.

SECONDARY MICROARRAY SCREEN IN LYMPHOBLASTS OF PATIENTS WITH DEFINITE AD

We sought to validate our initial results and narrow the list of candidate genes by analyzing samples from an independent group of patients with autopsy-confirmed diagnosis of AD. In this second experiment, lymphoblast mRNA from 6 patients with definite AD was compared with lymphoblast mRNA from 6 age-, sex-, and race-matched normal controls (Table 1). In the definite AD group, of 9374 genes analyzed, mRNA expression of 108 was decreased by 1.8-fold or greater, expression of 7 was increased by 1.8-fold or greater, and 9259 mRNAs were below significance threshold (a complete list of differentially expressed genes identified in the microarray screens is available from the authors). Down-regulation of 5 genes and up-regulation of 1 gene was confirmed in both microarray experiments (Table 2). One of the consistently down-regulated genes, LR11, is an ApoE recep-
tor that is predominantly expressed in brain and possesses structural and functional homologies to LDL receptor–related protein, a receptor etiologically linked to AD. On the basis of these considerations, we selected LR11 for further examination in control and AD brains.

**LR11 PROTEIN EXPRESSION IN HUMAN BRAIN**

To establish the potential biological relevance of changes in LR11 gene expression for AD, we examined LR11 in control and AD brains at the level of protein expression. Immunohistochemistry of 13 AD brains and 7 controls showed a remarkable reduction in LR11 expression in AD (Figure 2A and B). In control brains, pyramidal neurons in the frontal cortex showed strongly labeled small cytoplasmic puncta throughout the cell body and the proximal dendrites (Figure 2C). In striking contrast, there was dramatic loss of LR11 staining in pyramidal neurons in AD frontal cortex (Figure 2D). The difference between control and AD brains was remarkably consistent, and marked loss of LR11 staining in pyramidal neurons was found in each of the AD cases examined. In addition to neurons, punctate LR11 staining was also found in glial cells. However, unlike pyramidal neurons, glial staining in frontal cortex was preserved in AD brains (Figure 2E and F). Hematoxylin-counterstained hippocampal dentate granule neurons showed strong LR11 immunoreactivity in controls (G), but very little staining in AD brain (H). Scale: in A and B, bars indicate 100 μm; in C-H, 10 μm.

**Table 2. Identities of Consistently Altered Transcripts in Microarray Screens**

<table>
<thead>
<tr>
<th>Fold Change*</th>
<th>Gene Name Function Accession No.</th>
<th>Probable AD</th>
<th>Definite AD</th>
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<tr>
<td>−1.8</td>
<td>LDL receptor relative with 11 repeats (LR11) LDL receptor Y08110</td>
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<tr>
<td>−2.4</td>
<td>Interferon-γ receptor 1 Interferon receptor J03143</td>
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<tr>
<td>−2.1</td>
<td>Stimulated trans-acting factor (Staf-50) Transcription regulation AA853455</td>
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<tr>
<td>−1.8</td>
<td>Pleckstrin PKC substrate X07743</td>
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</tr>
<tr>
<td>−2.3</td>
<td>Amylo-(1,4-1,6)-transglycosylase 1 Glycogen branching enzyme L07956</td>
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<td></td>
</tr>
<tr>
<td>+1.9</td>
<td>Homo sapiens SNC73 mRNA Immunoglobulin heavy chain AF067420</td>
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</tbody>
</table>

Abbreviations: AD, Alzheimer disease; LDL, low-density lipoprotein; mRNA, messenger RNA; PKC, protein kinase C substrate.

*Change in transcript abundance compared with reference control mRNA.
in neuronal LR11 staining by immunocytochemistry, Western blotting indicated relatively modest reduction in LR11 band intensity in total cortical homogenates. This difference may reflect the contribution of glial LR11, which is retained in AD cortex (Figure 2E and F).

**COMMENT**

Our findings suggest a novel link between LR11 and AD; to our knowledge, this report is the first to identify a candidate disease-associated gene in an unbiased microarray screen of blood from patients with AD. Peripheral cells express genes associated with AD and model some processes involved in pathological changes of AD brains. The structure and function of LR11 as a mosaic ApoE receptor lends biological plausibility to the microarray results, and examination of LR11 in brain strongly supports the hypothesis that it plays a role in AD. In agreement with previous studies, we detected LR11 in widespread populations of neurons in neocortex, limbic cortex, and cerebellum. In AD brains, LR11 immunoreactivity was lost with remarkable consistency. Moreover, LR11 staining was decreased specifically in neurons, but staining was preserved in glia. The loss of immunoreactivity was not simply due to cell loss, as hematoxylin counterstaining showed otherwise healthy-appearing neurons (Figure 2). Most of the AD brains in this study were from patients with late-stage disease. Additional studies of patients with mild AD and mild cognitive impairment will be helpful in determining whether LR11 plays a role in early stages of disease development.

The unique multidomain structure of LR11 suggests potential roles as a cell-surface lipoprotein receptor and as an intracellular sorting receptor. There is a cluster of extracellular ligand binding repeats and a cytoplasmic internalization sequence that are present in all endocytosis competent lipoprotein receptors. In addition, LR11 contains a VPS10 homology domain near the amino terminus and a Golgi-localized, gamma-ear-homology domain, adenosine diphosphate–ribosylation (ARF)–binding protein (GGA) binding domain in the cytoplasmic tail. The VPS10 domains are involved in trafficking from the Golgi to the vacuole in yeast, and GGAs have been shown to mediate trafficking between Golgi and the endosomal-lysosomal system. Given its structural features, LR11 seems to be ideally positioned to interact with AD-associated proteins, and additional studies suggest that LR11 expression may influence levels of Aβ.

This exploratory study suggests that gene expression analysis of widely available lymphoblasts derived from patients with AD holds promise as a primary screen for candidate genes associated with AD. Our current studies, using this approach, identified the brain ApoE receptor, LR11, as an intriguing candidate molecule for sporadic AD. Further studies using larger sample sizes and refined microarray and bioinformatics procedures coupled with mechanistic validation of candidates are warranted.

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**Author Contributions:** Study concept and design: Scherzer, Levey, Lah, and Offe. Acquisition of data: Scherzer, Gearing, Rees, Bujo, Lah, Offe, Fang, and Heilman. Analysis and interpretation of data: Scherzer, Gearing, Schaller, Levey, Lah, and Offe. Drafting of the manuscript: Scherzer, Bujo, Levey, Lah, and Fang. Critical revision of the manuscript for important intellectual content: Scherzer, Gearing, Rees, Schaller, Levey, Lah, Offe, and Heilman. Obtained funding: Levey. Administrative, technical, and material support: Gearing, Rees, Bujo, Levey, Lah, Fang, and Heilman. Study supervision: Levey and Lah.

**REFERENCES**