Decreased Catalytic Activity of the Insulin-degrading Enzyme in Chromosome 10-Linked Alzheimer Disease Families*

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Insulin-degrading enzyme (IDE) is a zinc metalloprotease that degrades the amyloid β-peptide, the key component of Alzheimer disease (AD)-associated senile plaques. We have previously reported evidence for genetic linkage and association of AD on chromosome 10q23–24 in the region harboring the IDE gene. Here we have presented the first functional assessment of IDE in AD families showing the strongest evidence of the genetic linkage. We have examined the catalytic activity and expression of IDE in lymphoblast samples from 12 affected and unaffected members of three chromosome 10-linked AD pedigrees in the National Institute of Mental Health AD Genetics Initiative family sample. We have shown that the catalytic activity of cytosolic IDE to degrade insulin is reduced in affected versus unaffected subjects of these families. Further, we have shown the decrease in activity is not due to reduced IDE expression, suggesting the possible defects in IDE function in these AD families. In attempts to find potential mutations in the IDE gene in these families, we have found no coding region substitutions or alterations in splicing of the canonical exons and exon 15b of IDE. We have also found that total IDE mRNA levels are not significantly different in sporadic AD versus age-matched control brains. Collectively, our data suggest that the genetic linkage of AD in this set of chromosome 10-linked AD families may be the result of systemic defects in IDE activity in the absence of altered IDE expression, further supporting a role for IDE in AD pathogenesis.

Amyloid β-protein (Aβ)2 is the primary component of senile plaques, a pathological hallmark in the brains of patients with Alzheimer disease (AD). Elevated levels of cerebral Aβ have also been observed in AD patients (1, 2), implicating excessive accumulation of Aβ as a key pathogenic event in AD. Unlike early onset autosomal dominant AD, the vast majority of AD cases do not show any clear evidence of Mendelian transmission and predominantly present with late onset AD (LOAD) (onset age >65). However, there is evidence that genetic factors play a significant role in modifying the disease risk/age of onset in the majority of LOAD cases (3, 4). To date, only the e4 allele of the apolipoprotein E gene (APOE) has been firmly established as a LOAD genetic risk factor and has been proposed to be involved in Aβ clearance (5). Cerebral Aβ accumulation has been proposed to greatly influence the age of onset of LOAD and is determined by the amount of Aβ generated versus the amount that is degraded and exported from the brain over one’s lifetime (6, 7).

Several proteases have been identified to degrade Aβ, including neprilysin, plasmin, endothelin-converting enzyme-1 as well as insulin-degrading enzyme (IDE) (EC 3.4.24.56) (1). IDE, also called insulysin, is a zinc metalloprotease that cleaves small polypeptides, many of which share amyloid fibril-forming ability, including insulin, atrial natriuretic peptide, amylin, calcitonin, and Aβ (8, 9). IDE is a major protease to degrade soluble, monomeric Aβ (10, 11) and is localized in the cytoplasm as well as on the cell surface and within mitochondria (11, 12). Recent studies in animal models have demonstrated that knock out of IDE leads to elevated cerebral Aβ levels along with phenotypic characteristics of type 2 diabetes mellitus (13, 14). Conversely, overexpression of IDE attenuates Aβ accumulation in transgenic AD mouse models (15). Finally, diabetes-inducing mutations in IDE in rat are associated with impaired neuronal Aβ catabolism, supporting co-morbidity of AD, and type 2 diabetes mellitus (16). Several studies have suggested genetic linkage and allelic association between LOAD and the IDE/KIF11 region on chromosome 10q in independent samples (17–20). Our recent report on meta-analyses across all published studies (12 published reports for IDE to date) reveals a significant association of IDE with AD (21), confirming our original finding on the association and linkage of IDE to AD (17). However, neither pathogenic nor protective IDE gene mutations/variants have yet been validated in AD patients.

In an effort to localize novel AD genes, our group previously performed a full-genome linkage screen of the National Insti-
In Vitro Aβ Degradation Assay—Lymphoblast samples were disrupted by incubation in hypotonic buffer (50 mM Tris-HCl, pH 7.4) and extrusion through a 22-gauge hypodermic needle three times. The post-nuclear homogenate was centrifuged at 20,000 × g for 20 min to separate cytosolic (supernatant) and membrane (pellet) fractions, and protein concentration was determined by bicinchoninic acid assay (Pierce). Fluorometric quantification of Aβ degradation was performed using FAβB (fluorescein-Aβ(1–40)-Lys(LC-biotin)) as described by Leisering et al. (28). Briefly, 5 mg of protein was incubated with 0.5 mM FAβB for 90 min at 20 °C in a degradation buffer (50 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, 0.05% bovine serum albumin, pH 7.4). The reactions were quenched by adding avidin to a final concentration of 0.5 mM, and fluorescence polarization (485 excitation, 535 emission) was determined on a PerkinElmer Life Sciences Victor2 multilabel plate reader. Reactions were performed in quadruplicate in the presence and absence of 10 μM insulin, a relatively specific inhibitor of IDE activity, and the percentage of hydrolysis was calculated from standards containing no protease or excess recombinant IDE.

RNA Extraction—Total RNA was extracted from lymphoblast samples using the RNeasy kit (Qiagen) as described in the manufacturer’s instructions. The concentration of the total RNA was determined using an NP-1000 spectrophotometer (Nanodrop). For brain samples, a 30–50-mg piece of gray matter from the superior temporal sulcus was dissected under stringent RNase-free conditions from each brain in accordance with previously published methods (29). From each tissue, RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). All samples were controlled for integrity of the 18 and 28 S ribosomal RNAs by microcapillary electrophoresis (not shown) (RNA 6000 Nano Assay, Agilent Technologies), and samples showing degradation were excluded from the study.

Reverse Transcription PCR (RT-PCR)—For the lymphoblast samples, first-strand cDNAs were synthesized from 5 μg of the total RNA using 200 units of Superscript III reverse transcriptase (Invitrogen) and random hexamers as described in the manufacturer’s instructions. To investigate alternatively spliced transcript variants, RT-PCR was carried out using the cDNAs as templates with TaqDNA polymerase (QiaGen) or Turbo Pfu DNA polymerase (Stratagene) and corresponding primers (Table 1). For the brain tissue samples, reverse transcription was carried out on 2 μg of all total RNA samples to generate an equal number of cDNA copies using random hexamers and 200 units of Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions.

Quantitative RT-PCR—All quantitative PCR analyses were performed with iCycler™ (Bio-Rad). To determine IDE mRNA levels in the lymphoblast and brain samples, a 6-carboxyfluorescein-labeled TaqMan probe and a set of PCR primers (forward and reverse primers—TaqMan, Table 1) were syn-
TABLE 1
Primers and a probe used in this study

| Name                          | Sequence                      |
|-------------------------------|-------------------------------|
| IDE TaqMan probe              | 5’-ATGCAACTGTCTTATTGTGCTTGGT-3’ |
| IDE forward primer-TaqMan     | 5’-AGGATCTCAACCACTCTTACAT-3’   |
| IDE reverse primer-TaqMan     | 5’-AGGATCTCAACCACTCTTACAT-3’   |
| IDE 1F                        | 5’-GCCACCTCGAGCTTTGCTGCAT-3’   |
| IDE 5R                        | 5’-CCCAAGCTTGTGCTCACTATT-3’    |
| IDE 6F                        | 5’-CTGCAAGATCCCCTGGCAGAAC-3’   |
| IDE 13R                       | 5’-AGAAGAAGGCCATTAAGGTTC-3’    |
| IDE 14F                       | 5’-GGCTCCCTCGAGCTTTGCTGCAT-3’  |
| IDE 15a-R                     | 5’-GAGGATCTCAACCACTCTTACAT-3’  |
| IDE 15b-R                     | 5’-GAGGATCTCAACCACTCTTACAT-3’  |
| IDE 17R                       | 5’-GCACCTCGAGCTTTGCTGCAT-3’    |
| IDE 19R                       | 5’-AACAGCTTGTGCTCACTATT-3’     |
| IDE 20F                       | 5’-GAAGAAGGCCATTAAGGTTC-3’     |
| IDE 25R                       | 5’-AACTGAGGTGCACTCTGAGAAC-3’   |
| GAPDH forward primer          | 5’-GAGAAGAAGGCCATTAAGGTTC-3’   |
| GAPDH reverse primer          | 5’-ATGAGGATCTCAACCACTCTTACAT-3’ |
| NSE forward primer            | 5’-GCACCTCGAGCTTTGCTGCAT-3’    |
| NSE reverse primer            | 5’-AGGATCTCAACCACTCTTACAT-3’   |

IDE Activity in Chromosome 10-Linked AD Families—
We first assessed IDE activity in the lymphoblast cell lines derived from 12 subjects of three AD families exhibiting particularly strong evidence of genetic linkage to chromosome 10 in the IDE gene region (17, 22). Because insulin possesses a high affinity for IDE and is a major substrate of this peptidase, we employed a sensitive and reliable insulin degradation assay to assess IDE catalytic activity in membrane and cytosolic fractions prepared from lymphoblast samples (Fig. 1A). Peptidolytic activity of cytosolic IDE was significantly decreased in the affected versus unaffected subjects (>50%; p = 0.001) after combining the members of all three families. Additionally, in one family (I), cytosolic activity of IDE was significantly lower in three affected versus three unaffected subjects (p = 0.008). IDE activity was also decreased in the membrane fractions of the affected (versus unaffected) individuals; however, these differences did not reach statistical significance.

To confirm our finding of decreased cytosolic IDE activity particularly in families I and III, affecteds of which exhibited the most robust decreases in IDE activity in the insulin degradation assay, peptidolytic activity of IDE was next assayed using Aβ1–40 as a substrate (Fig. 1B). IDE-mediated degradation of Aβ was decreased in affected versus unaffected subjects in both families. However, this difference did not reach statistical significance at least partly because of the less sensitive nature of this assay (versus the insulin-degrading assay) and lower affinity of IDE for Aβ. Meanwhile, we could not obtain reliable data for the Aβ-degrading ability of membrane IDE, possibly because of the limiting amount of IDE in the membrane fractions and decreased affinity of IDE for Aβ.

IDE Expression in the Chromosome 10-Linked AD Families—
We next assessed IDE expression by performing quantitative RT-PCR on cDNAs generated from lymphoblast cell lines from the same three AD families. The mRNA levels of IDE, normalized to GAPDH, were highly variable across samples and revealed no significant or consistent differences among affected versus unaffected subjects across the families (Fig. 2, A and B, top). The families also exhibited differing trends for IDE mRNA levels in affected versus unaffected individuals; e.g., in family I, affected subjects had lower IDE mRNA levels than the unaffecteds (p = 0.022), whereas an opposite trend was observed in family III (p = 0.020).

Next, IDE protein levels were determined by quantitative Western blot analysis of total protein extract (Fig. 2, A and B, bottom). Relatively high IDE expression in affected versus unaffected subjects of family III was confirmed at the protein level (p = 0.018). However, the affected subjects in family I, who had relatively low IDE message levels, revealed no significant reductions in IDE protein levels. Overall, protein levels of IDE across the families did not exhibit significant or consistent differences according to disease status.

Levels of Alternative IDE Transcripts in the Chromosome 10-Linked AD Families—After establishing that no IDE coding mutations existed in the canonical exons of the IDE gene in these AD families, we next explored the possibility that altered mRNA splicing of IDE may account for the observed reduction.
in IDE activity. First, Northern blot analysis was performed with total RNA extracted from the lymphoblast samples. Two different IDE transcripts were detected (data not shown) that had similar sizes to those previously reported in rat, 3.6 and 5.9 kb (30). Northern blotting using PCR fragments covering three different IDE coding regions (Fig. 3A, (a), (b), and (d)) as probes, detected the two IDE mRNAs of indistinguishable sizes with no other additional bands (data not shown). Recently, Farris et al. (31) detected up to four different Northern blot bands of IDE in different tissues, two of which corresponded in size with the transcripts found in our lymphoblast samples. Based on the previously reported observations, we suspect that the two major IDE messages observed in these lymphoblasts are due to alternative polyadenylation sites. In any event, the two Northern blot bands were coordinately expressed, suggesting no significant differential regulation of the two transcripts among lymphoblasts from these families.

To assess alternative splicing of IDE, we performed RT-PCR on total RNA obtained from each lymphoblast sample using oligonucleotide primer pairs spanning the entire IDE coding region (Fig. 3A). As shown in Fig. 3B, no changes in sizes of PCR products were observed, suggesting no evidence for alternative splicing in the major transcript in these samples. Subsequently, full-length IDE cDNA was produced by RT-PCR using high fidelity DNA polymerase (Pfu, Stratagene), and possible sequence changes in IDE mRNA were investigated more closely by sequencing. Neither exon swapping between similar-sized exons nor nucleotide sequence changes was observed in the major IDE transcript (data not shown).
15 region were produced from each lymphoblast sample. Because 15a and 15b have exactly the same size (145 bp), RT-PCR for the exon 15 region produced only one band (Figs. 3B and 4B), demonstrating that the two exons are spliced into IDE transcripts at the exclusion of each other in the lymphoblast cell lines. To discriminate between the transcripts harboring 15a versus 15b, the same amounts of the amplified DNA fragments were digested with exon-specific restriction enzymes (Fig. 4A). As can be seen in Fig. 4B, small but significant amounts of the amplified PCR fragments were cleaved by Dral, the 15b-specific restriction enzyme, and left uncleaved by the 15a-specific digestion enzyme Nhel in all of the lymphoblast samples. Complete digestion of the PCR fragments by the restriction enzymes was confirmed by digestion of same amount of corresponding PCR fragment amplified with IDE cDNA plastid containing 15a as template (Fig. 4B, exon 15a). This result suggested that the IDE transcript containing 15b in place of 15a was expressed in the lymphoblast cell lines in the affected and the unaffected subjects from these families. Next, the levels of the 15a- and 15b-IDEs were measured by quantitative RT-PCR using exon-specific 3′ primers for 15a and 15b and a common 5′ primer residing in exon 14 (Fig. 4A). The ratio of 15b-IDE versus 15a-IDE was not significantly altered relative to IDE activities in the affected individuals (Fig. 4C).

IDE mRNA Levels in Sporadic AD Brain Samples—Given our observation of variable IDE expression in the lymphoblast samples from the chromosome 10-linked AD families (Fig. 2), we next set out to examine IDE expression in post-mortem brain samples from AD versus control subjects. As brain samples were not available from members of the chromosome 10-linked AD families, we assessed IDE mRNA levels in temporal cortical samples from 24 sporadic AD patients and 11 age-matched controls. Levels of absolute IDE mRNA were highly variable among individual samples within each group, and no significant differences were detected between AD patients and control subjects (data not shown). We also measured the mRNA levels of two controls, GAPDH (a general housekeeping protein) and NSE (a neuronal-specific marker). NSE mRNA levels normalized to GAPDH mRNA levels (NSE/GAPDH) were reduced by 58.7% in the AD brains relative to the control brains (p = 0.031), consistent with a substantial neuronal cell loss (Fig. 5A). Although mRNA levels of IDE normalized to GAPDH were not significantly higher in AD versus control...
IDE in Chromosome 10-Linked AD Families

FIGURE 5. IDE mRNA levels in sporadic AD brain samples. The mRNA levels of IDE, GAPDH, and NSE were measured by quantitative RT-PCR using equal amounts of cDNAs extracted from temporal cortices of age-matched control and AD brains. Relative mRNA levels were calculated by normalization with GAPDH and NSE mRNA levels. A, scatter plot of the relative NSE mRNA levels normalized to GAPDH (NSE/GAPDH). An average of each group is indicated. B, scatter plots of the relative IDE mRNA levels normalized to GAPDH (IDE/GAPDH, left) and NSE (IDE/NSE, right). p values were calculated by two-tailed Student's t test, and asterisks indicate statistically significant values.

brain samples (p = 0.109) (Fig. 5B, left), IDE message levels normalized to NSE mRNA (IDE/NSE) were ~3-fold higher in AD versus control brain samples (p = 0.010) (Fig. 5B, right). In addition, we found that the presence of the APOE-ε4 allele in the AD subjects was not accompanied by statistically significant changes in IDE mRNA levels in this cohort of AD brains (data not shown).

DISCUSSION

This study represents the first functional assessment of the IDE gene, a significant candidate gene for LOAD (21), in AD families exhibiting genetic linkage to the IDE gene region of chromosome 10. We assessed IDE activity in lymphoblast cell lines from three chromosome 10-linked AD families and consistently observed reduced catalytic activity of IDE across affected individuals of all three AD families. Because IDE activity was decreased in both the cytosolic and the membrane fractions of the lymphoblast lines, the differences observed in affected versus unaffected subjects are unlikely to be the result of altered cellular compartmentalization of IDE. Reduced IDE activity in these AD lymphoblast lines was not due to decreased expression of IDE, because levels of IDE mRNA and protein did not differ between affected and unaffected members of these families. Taken together, these findings suggest that genetic linkage of these families to chromosome 10 may be driven by gene defects in IDE leading to dysfunction. Although the pathogenic defects in IDE remain unidentified, our results suggest that the deficit in activity is not due to altered gene expression.

Interestingly, in one family (III), the cytosolic activities and protein levels of IDE were inversely correlated, i.e. the lymphoblast samples from affected subjects with reduced IDE activity exhibited increased IDE expression (Figs. 1A and Fig. 2A). Thus, it is possible that the deficit in IDE activity in affected individuals of this family may lead to compensatory increases in IDE expression.

Although little is known about the regulation of IDE activity, chemical inhibitors known to abolish IDE catalytic activity include the Zn2+ chelator 1,10-phenanthroline, the insulin-binding inhibitor bacitracin, the thiol-blocking agents, p-hydroxy-mercuribenzoate, and N-ethylmaleimide (32). In addition, two heat-stable IDE-interacting proteins have been shown to inhibit its insulin-degrading activity in mouse leukemic splenocytes (33, 34). One of them was identified as ubiquitin, which, upon binding with IDE in a reversible and ATP-independent manner, exhibits a strong inhibitory effect at physiological concentrations. Ubiquitin and IDE are present in both neurofibrillary tangles and senile plaques (35–38), raising the possibility that ubiquitin may regulate IDE activity in these pathological lesions. Allosteric properties of IDE activity have also been elucidated in which binding of certain peptide substrates to one subunit of IDE induces a conformational change that shifts the equilibrium to the more active dimer while also activating the adjacent subunit (39). The active site motif (HXXEH) and a cationic regulatory site both affect the allosteric properties. The cationic regulatory site binds anions, including nucleotide triphosphates (such as ATP), and alters the oligomerization state of the enzyme toward a monomer, which consequently changes the substrate specificity (27).

Interestingly, some peptides, e.g. dynorphin B-9, activate IDE selectively toward AB cleavage but not toward insulin cleavage (39, 40). In addition, IDE activity can be inhibited by phosphorylation (41), free fatty acids, and acyl-coenzyme A thioesters (42). Collectively, these previous findings indicate that IDE activity may be regulated by a complex combination of diverse regulatory factors. Thus, our observation of altered IDE activity in the lymphoblast lines from chromosome 10-linked AD families might also stem from, as of yet, unidentified gene defects impacting IDE regulatory mechanisms. Although no coding sequence changes were found in the major IDE transcript of the affected members of these three families, it is still possible that variants may exist in other, more elusive, alternatively transcribed coding regions of IDE. Along these lines, we investigated the potential contribution of 15b, the only alternatively transcribed exon to date shown to alter IDE activity. We found
neither coding DNA changes in the alternatively spliced 15b nor any evidence for changes in the levels of IDE transcript containing 15b in these AD families (Fig. 4C). Further analyses of the IDE gene will be necessary to determine the molecular mechanism underlying reduced IDE activity in these three chromosome 10-linked families.

Previous studies of IDE expression in AD brain have thus far produced inconsistent results. Although increased intracellular and neuronal immunostaining have been reported in AD brains (35), decreased levels of a carboxyl-terminal fragment of IDE were detected in cytosolic fractions of AD brains by Western blot analysis (43). IDE protein levels measured by enzyme-linked immunosorbent assay were also reported to be increased in the cortical microvessels of AD patients by another group (44). Meanwhile, Cook et al. (45) report a statistically significant reduction in hippocampal IDE expression (protein and mRNA) in AD brains of APOE-ε4 allele carriers as compared with brains of non-APOE-ε4 AD patients and both APOE-ε4-positive and -negative non-demented controls. Using a similar number of brain samples as Cook et al. (45) but from temporal cortex, we did not detect a statistically significant decrease in the IDE expression for our cohort of AD brains (versus controls) when absolute IDE mRNA levels and the relative levels normalized to GAPDH were compared. Expression of GAPDH, which has previously been shown to be similar in AD and control brain (46), was also not significantly different in our AD versus control brain samples (data not shown), validating it as an appropriate control. Although our result would appear to be discrepant with the observations of Cook et al. (45), it should be pointed out that they had measured IDE levels by in situ hybridization and Western blot analysis, whereas we employed quantitative RT-PCR. Moreover, they had analyzed IDE levels in the hippocampus and observed regional differences (i.e. no decrease in CA-1), and we measured IDE levels in temporal cortical tissue. Other studies have also reported temporal and spatial differences in IDE steady-state expression levels, showing that in the hippocampus, IDE protein levels diminish as a function of age, whereas cerebral cortical IDE expression is elevated (47). More in-depth investigation of local IDE expression and activity will be necessary for a better understanding of potentially pathogenic changes in IDE in different brain regions.

Additionally, we found that IDE expression was significantly increased in the AD brain samples when normalized to the neuronal specific message NSE. We also observed a significant decrease in NSE/GAPDH ratio, consistent with neuronal cell loss in AD. Because IDE is known to be expressed predominantly in neurons (11, 35, 45), one possible interpretation of these data is that IDE expression may be increased in certain neuronal populations in AD in temporal cortex, whereas the total number of neurons is decreased as a potential response to elevated Aβ levels. However, we cannot rule out the alternative possibility of increased IDE mRNA levels owing to activated astrocytes or microglia in AD. Cell type-specific IDE expression should be monitored to address this issue.

In summary, we have found that IDE activity is reduced in lymphoblast samples from affected versus unaffected family members of three chromosome 10/IDE-linked AD families. However, reduced IDE activity was not due to decreased IDE expression, suggesting the possibility of systemic functional defects in IDE in these families. Although sequencing of the major and the 15b-IDE transcripts did not reveal any coding region substitutions, further sequence analysis of alternative IDE transcripts may be warranted. Collectively, these data provide the first evidence of a systemic functional defect in IDE in AD patients from chromosome 10-linked families. Although these results provide further support for a role of IDE in AD pathogenesis, further investigation of this peptidase will be necessary to establish the mechanism by which IDE activity is altered in IDE-linked AD families.

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IDE in Chromosome 10-Linked AD Families

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