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Association Studies of Transforming Growth Factor-β1 and Alzheimer's Disease

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Substantial laboratory evidence suggests Transforming Growth Factor-β1 (*TGFB1*) is linked to Alzheimer's Disease (AD) pathology. The purpose of the study was to estimate the genetic association of *TGFB1* with AD while controlling for apolipoprotein E4 allele (*APOE4*) status, the only well-established genetic risk factor for AD. Two study populations were genotyped for the *TGFB1* −509 and +869 single nucleotide polymorphisms (SNPs) that have been associated with TGFB1 levels. Constituting these populations were 203 families from the NIMH AD Genetic Initiative with at least two affected siblings and a normal sibling, and a population of 126 African-American (AA) AD cases versus 93 age matched controls. Results from family-based analyses showed a significant association between the *TGFB1* −509 SNP and AD for the entire set of 203 families ($P = 0.007$), and a subset of these families without a homozygous *APOE4* family member ($P = 0.026$). Results from family-based analyses on the *TGFB1* +869 SNP were not significant in the 203 families. While associations for the main effects of the *TGFB1* +869 and −509 SNP with AD in the AA case-control study were also not significant, results did indicate that *TGFB1* may function as an effect modifier of *APOE4* risk. Specifically, the odds of AD associated with having at least one *APOE4* allele increased in an additive fashion with one or two copies of the higher producer allele when stratified by *TGFB1* −509 genotype and by *TGFB1* +869 genotype. Results support a role for *TGFB1* in AD pathogenesis.

KEY WORDS: cytokines; neuroinflammation; apolipoprotein; *TGFB1* +869 SNP; *TGFB1* −509 SNP

INTRODUCTION

Alzheimer's Disease (AD) is a neurodegenerative disorder with a complex etiology and pathogenesis. It is characterized by progressive memory loss and depletion of cognitive functions. The biological processes stimulating the development of AD pathology are not completely understood. One characteristic of neuropathological autopsy findings is the presence of extracellular plaques consisting of aggregates of amyloid-β (Aβ) peptides, which are the proteolytic cleavage products of the amyloid-β precursor protein (APP) [Neve et al., 2000]. The function of APP is not confirmed, but APP is speculated to support formation and maintenance of synapses in vivo [Mattson et al., 1997; Wang et al., 2005].

A genetic component of AD has long been recognized, since clustering of this disease within families has been observed [Kamboh, 2004]. The only well-established genetic risk factor for AD is the E4 allele of the apolipoprotein (*APOE*) gene. The exact role of the APOE protein in AD pathology is not clear, however it is known that APOE functions as a cholesterol transporter, where APOE rich lipoprotein complexes are one source of cholesterol for membrane synthesis and maintenance in the brain [Lahiri et al., 2004; Poirier, 2005]. These complexes also bind Aβ, however, the APOE4 isoform does not form complexes with Aβ peptides, as well as other two APOE isoforms (*APOE2* and *APOE3*), therefore APOE4 may result in lower efficiency in Aβ clearance [LaDu et al., 1997]. Several studies report AD patients homozygous for the E4 allele account for a fraction (<30%) of all AD cases [Kamboh, 2004].

It is well established that *APOE4* is only predictive of AD onset and its influence varies across populations [Kamboh, 2004]. Therefore, it remains necessary to consider other genes that might influence the development of disease. Transforming Growth Factor-β1 (TGFB1) is a multifunctional cytokine with pro- and anti-inflammatory properties and, in particular, is a key regulator of the brain's responses to injury and inflammation [Mattson et al., 1997; Akiyama et al., 2000]. Within the CNS, TGFB1 is expressed in neurons, astrocytes, and microglia [Mattson et al., 1997; Harris-White et al., 1998]. Studies show increased TGFB1 levels in the cerebral spinal fluid of AD patients versus controls, and TGFB1 is detected in senile plaques [van der Wal et al., 1993; Zetterberg et al., 2004].

Two single nucleotide polymorphisms (SNPs) located in *TGFB1* have been shown to be related to TGFB1 levels. The T allele of the −509 SNP located in the promoter has been associated with higher plasma concentrations of TGFB1 in twins, in a dose-dependent fashion [Grainger et al., 1999]. In addition, Luedecking et al. [2000] reported that the T allele was marginally associated with higher transcription activity than the other allele and that the frequency of the TT genotype was significantly higher in AD patients as compared to controls. The +869 SNP is located in the signal peptide region and results in a leucine → proline substitution affecting the protein's polarity and its export, which could affect protein production [Wood et al., 2000]. Both a clinical osteoporosis study and a hypertension study found associations between the +869 C SNP and higher TGFB1 levels, thus implying that the proline (C) substitution could lead to increased TGFB1 production [Yamada et al., 1998; Suthanthiran et al., 2000].

In order to investigate the association of *TGFB1* with AD, the °509 C → T promoter SNP and the +869 T → C SNP in the signal peptide region were genotyped in a cohort of 203 families with at least two AD affected siblings and one unaffected sibling from the NIMH AD Genetics Initiative sibling dataset [Blacker et al., 2003] and in an expanded Alabama African-American (AA) case-control cohort (N = 219) [Perry et al.,

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2001]. The purpose of this study is to estimate the effects of *TGFB1* SNPs on AD risk singly and in the context of *APOE4* exposure.

MATERIALS AND METHODS

Study Population

Comprising the family study group were 203 families with at least two AD affected siblings (onset $\geq$50 years) with mean age of onset (MAO) 71.0 ± 7.5 years and one unaffected sibling. This group (candidate gene set) is a subset of a larger group of families collected as part of the NIMH AD Genetics Initiative [Blacker et al., 2003] following a standardized protocol utilizing the NINCDS-ADRDA criteria for diagnosis of definite and probable AD [Tierney et al., 1988]. Since parental genotypes are not available for this dataset, a subset of families with two affected siblings and at least one unaffected sibling were necessary for family-based statistical analysis. The candidate gene set was further subdivided into 64 *APOE4*/*APOE4* (E4) families with at least one member who is homozygous for the E4 allele (MAO = 67.6 ± 7.1 years), and 139 non-homozygous E4 (NHE4) families where no individuals possess the E4/E4 genotype (MAO = 72.3 ± 7.1 years). Stratifying on homozygous *APOE4* status rather than carrier status separates families with strong *APOE4* presence from those with no or weak *APOE4* presence.

Comprising the AA case-control group were 126 AD cases and 93 age and race matched controls previously reported on by Perry et al. [2001]. AA AD patients had a MAO of 70.8 ± 7.8 years, while the mean age of the control group was 75.2 ± 7.6 years. Cases were identified from patients seen in the Memory Disorder Clinic at the University of Alabama at Birmingham utilizing the NINCDS-ADRDA criteria mentioned above for diagnosis of definite and probable AD. Additional neurological and laboratory tests were completed to exclude cases of other illnesses that can mimic AD. Unaffected spouses of AD patients and members of AD support groups, who screened negative for a personal and family history of memory problems and scored normal on cognitive screening, comprised the control group.

Determination of *APOE* and *TGFB1* Genotypes

Blood from the NIMH Genetics Initiative families was collected and sent to the NIMH repository at Rutgers where genomic DNA was extracted from lymphocyte cell lines. Isolation of genomic DNA from the AA subjects has been described [Perry et al., 2001].

APOE4 genotyping followed a modified version of the procedure from Hixson and Vernier [1990] [Blacker et al., 1997]. Primers and enzymes for the PCR-RFLP analysis of the −509 and +869 SNPs of *TGFB1* were obtained from Luedecking et al. [2000] and Wood et al. [2000], respectively. All PCRs were performed on MJ thermocyclers (Watertown, MA). Amplification of both SNPs were performed in 25 μl reactions containing 0.5 μM of each primer, 1.5 μM $MgCl_2$, 200 μM of each dNTP, 1U Taq (Promega, Madison, WI) and 100 ng of DNA. Conditions were 30 cycles of 30 sec cycling times, with the annealing temperatures for the −509 and +869 SNPs set at 64 and 58°C, respectively. The microtiter plates used in the PCRs were allowed to cool at 12°C/30 min to reduce condensation. Digestions of the −509 and +869 products were performed with 2 and 3U of enzyme (N.E. Biolabs, Beverly, MA), respectively in 15 μl reactions containing 8 μl of product at 37°C/4 hr. Digested products were separated on 2% SFR agarose gels (Amresco, Solon, OH) and photographed on a Fluor-S imager (Bio-Rad, Hercules, CA). Two independent readers were used for assigning genotypes to reduce errors and clarify any ambiquities.

Statistical Analysis

The family-based association tests implemented in FBAT [Horvath et al., 2001] were used to examine the association of the *TGFB1* SNPs with AD in the candidate gene family set and the E4 and NHE4 subsets. *APOE4* allele dose was controlled for in FBAT in the total candidate gene set by utilizing a difference residual outcome variable from the logistic regression of *APOE4* status on AD [Lunetta et al., 2000].

For the AA population, the case and control groups were tested for Hardy–Weinberg equilibrium (HWE) at the *TGFB1* and *APOE* loci using the chi-square test. Two sample tests for binomial proportions were used to compare genotype frequencies between cases and controls for each locus. Logistic regression was used to investigate the main effects association of the *TGFB1* SNPs with AD controlling for *APOE4* genotype. Mantel–Haenszel methods were applied to examine the association between *APOE4* and AD stratified by *TGFB1* SNP genotype to further explore the possibility of an interaction between APOE4 and TGFB1 that affects disease status [Mantel and Haenszel, 1959].

RESULTS

Results of the family-based association analyses for the −509 (C → T) polymorphism of *TGFB1* show a significant association ($P = 0.007$) with AD for the T allele in the candidate gene set, and also marginal evidence for an association in the NHE4 subset ($P = 0.026$). When controlling for the presence of at least one *APOE4* allele in the candidate gene set in FBAT, the association between AD and the −509 SNP remained highly significant ($P = 0.007$). This association shows the T allele of the −509 (C → T) polymorphism of *TGFB1* is transmitted more often to the affected siblings. Results from family-based analyses on the *TGFB1* +869 SNP did not reveal a significant association with AD (data not shown).

The AA case-control population is in HWE for the *APOE* locus and the *TGFB1* SNPs. Results of baseline comparisons of *APOE* genotype and the *TGFB1* genotypes in cases compared to controls show the frequency of the *APOE4* genotype differs among cases and controls ($P < 0.013$) as expected, but the *TGFB1* genotypes do not (data not shown). The main effects models testing the association of the −509 SNP and +869 SNP genotypes with AD controlling for *APOE4* status do not show an association between *TGFB1* and AD. Analysis of the association of the *TGFB1* −509 genotype with AD stratified by *APOE*4 status (having 0, 1, or 2 *APOE4* alleles) also showed no significant results, as did the same analysis for the *TGFB1* +869 SNP (data not shown).

The association of at least one *APOE4* allele with AD stratified by *TGFB1*−509 or +869 SNP genotype is displayed in Table I. Results suggest the T allele of the −509 SNP and C

TABLE I. Test for the Association of at Least One *APOE4* Allele With AD Stratified by the *TGFB1* −509 or +869 SNP Genotypes in the African-American Case-Control Population

	OR	95% CI
TGFB1 −509 SNP		
CC (n = 115)	2.77	1.28, 6.01
CT (n = 78)	4.02	1.57,10.33
TT (n = 17)	6.00	0.52, 69.75
Mantel–Haenszel combined OR	3.35	1.88, 5.98
TGFB1 +869 SNP		
TT (n = 64)	2.23	0.77, 6.45
CT (n = 92)	3.53	1.49, 8.33
CC (n = 40)	7.43	1.77, 31.04
Mantel–Haenszel combined OR	3.50	1.93, 6.36

allele of the +869 SNP may modify the effects of APOE4 in an additive manner since the odds ratio between AD occurrence and presence of at least one *APOE4* allele increase with each additional copy of the T or C allele. However, these results are only suggestive since the confidence intervals for the odds ratios in the three strata of both SNPs are wide and overlap.

DISCUSSION

A significant association between the −509 *TGFB1* SNP and AD was found for the candidate gene set using the family-based association test implemented in FBAT ($P = 0.007$). The significant association indicates higher transmission of the −509 T allele to affected siblings as compared to unaffected siblings. When controlling for at least one *APOE4* allele in FBAT, the association between the *TGFB1* −509 SNP and AD remained highly significant. The *TGFB1* −509 SNP lies in a negative regulatory element of its promoter, but the T substitution appears to be associated with higher transcription activity and higher plasma levels of TGFB1 [Grainger et al., 1999; Luedecking et al., 2000]. Our results are similar to a Pittsburgh study that found the TT genotype was significantly higher in AD patients versus controls [Luedecking et al., 2000]. However, a larger group of French sporadic patients as compared to age-matched controls [Araria-Goumidi et al., 2002] failed to replicate this association.

Like the *TGFB1* −509 T substitution, the +869 C substitution may also affect TGFB1 production [Wood et al., 2000]; associations of the +869 C substitution with higher TGFB1 levels have been reported [Yamada et al., 1998; Suthanthiran et al., 2000], however, our results from family-based analyses on the +869 SNP with AD failed to find any association.

For the case-control study in the AA population, there were no associations between the *TGFB1* SNPs and AD. However, the association between carrying at least one *APOE4* allele and AD increased when stratified by the *TGFB1* −509 or the +869 SNP genotype. The odds of AD, as determined by *APOE4* carrier status, increase in an additive manner based on the number of *TGFB1* −509 T alleles or +869 C alleles (0, 1, or 2) (Table I). Although these differences are not significant due to the wide confidence intervals, it is notable that all six participants who were homozygous for *APOE4* and the *TGFB1* +869 C SNP have AD. There are no subjects homozygous for both *APOE4* and the *TGFB1* −509 T SNP. Interestingly, these results also show that having no *TGFB1* +869 C alleles dilutes the risk for AD associated with *APOE4* carrier status. The above results for the additive effects from the −509 T allele and the +869 C allele in the presence of at least one *APOE4* allele lend support for the possibility that TGFB1 levels may modify the effects of APOE4 protein in AD. Although small sample size restricts the power of these stratified analyses, the consistency of the results between these two SNPs suggests further investigation of a possible synergism between APOE4 and TGFB1 is warranted.

A model of AD that includes TGFB1 has biologic support. TGFB1 increases APP mRNA and protein expression in brain cell cultures [Gray and Patel, 1993; Monning et al., 1994] and appears to increase Aβ deposition in brain cell cultures and in APP and *TGFB1* transgenic mice, when overexpressed [Harris-White et al., 1998; Buckwalter et al., 2002; Lesne et al., 2003]. In addition, one transgenic mouse study showed astroglial overexpression of TGFB1 produced a strong upmodulation of extracellular matrix (ECM) proteins in the central nervous system (CNS) [Wyss-Coray et al., 1995]. ECM proteins have been identified in Aβ plaques of AD brains where they appear to play a central role in plaque deposition and stabilization [Fillit and Leveugle, 1995]. This model of the contribution of T6FB1 to plaque formation and stabilization is also consistent with the model in atherosclerosis, another risk factor for AD [Forrester, 2004]. The bulk of severe clinical manifestations of atherosclerosis is attributed to disruption of plaques rich in inflammatory macrophages and T cells, that are characterized by a thin fibrous cap with substantial loss in ECM [Libby, 2001]. Transgenic mice with T cell specific inhibition of TGFB1 display plaques with these vulnerable properties of plaque T cell infiltration and loss of ECM production [Gojova et al., 2003].

How does APOE4 fit into this picture? Exposure of cultured neurons to Aβ peptides causes an increase in oxyradical formation and subsequent radical mediated damage to neuron membrane lipids and proteins [Markesbery, 1999]; and the APOE4 isoform has been shown to provide cultured neurons the least antioxidant protection from Aβ generated hydrogen peroxide compared to APOE2 or APOE3 [Miyata and Smith, 1996]. Furthermore, the *TGFB1* promoter contains an element responsive to the transcription factor AP-1 that can be regulated by redox reactions [Iglesias-De La Cruz et al., 2001; Wilmer et al., 2002]. If TGFB1 upregulates APP and Aβ production and deposition, and Aβ induces ROS production, which then upregulates TGFB1 further, the reduced antioxidant efficiency of APOE4 may lead to exacerbation of the AD pathological process. Given this model, it is plausible that TGFB1 may modify the risk for AD associated with the APOE4 isoform by cooperating with Aβ in aggravating the inflammatory process such that it overwhelms the lesser antioxidant capacity of APOE4.

Our results suggest that TGFB1 may be associated with AD independently as results from family-based tests indicate or it may function as an effect modifier of APOE4 risk by contributing to a chronic, inflammatory neuropathologic state as results from the case-control association analyses suggest. Biologically both models are feasible, however, both our samples were underpowered to confirm either model. Therefore, we recommend that *APOE* and *TGFB1* SNPs should be genotyped in family and case/control populations using large sample sizes with sufficient power to distinguish between these two models to confirm our suggestive findings. If either model can be substantiated for TGFB1 in AD, then the discovery of its exact role in this pathologic process from future cell specific laboratory studies and molecular genetic studies, may provide better insight into the role of cytokines in the neuroinflammatory processes underlying AD pathology, and hence lead to new targets for therapy.

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