THE RELEVANCE OF APOLIPOPROTEIN E POLYMORPHISM TO ALZHEIMER'S DISEASE.

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ABSTRACT

The apo E gene located on chromosome 19 in humans is polymorphic. The three apo E isoforms E2, E3, and E4 are coded by three common alleles of the gene. The amyloid plaques in brains of Alzheimer disease (AD) patients are known to contain apo E. There is an increased prevalence of E4 allele in AD patients. apo E exhibits increased binding to a peptide A β deriving from amyloid precursor protein. apo E, the risk factor for late AD disease is unable to prevent formation of paired helical filaments which in turn destabilizes neuronal microtubules.

A variety of molecular techniques are available for apo E genotyping using DNA amplified by the polymerase chain reaction (PCR). The high guanine to cytosine content of apo E is problematic to the extent that the yield of PCR product and hybridization stringency can be compromised. The specificity of diagnosis of late-onset AD can be improved when results of apo E genotyping are evaluated together with clinical criteria.

INTRODUCTION

Apolipoprotein E (apo E) has a major role in lipid metabolism (1). This 34 kilodalton (kd) protein is coded by a single four exon gene, 3.6 kilo base (Kb) in length, that is located on the long arm of chromosome 19. It is a 299 amino acid protein. It is involved in the transport of chylomicrons and very low density lipoproteins (VLDL). The role of apo E is to assist in the redistribution of lipids among cells of various organs. It also serves through interaction with a sub class of high density lipoprotein (HDL₁) to supply lipids to cells for proliferation or membrane repair. The ability of apoE-containing lipoproteins to interact with receptors such as the

low density lipoprotein (LDL) receptor, LDL receptor related protein (LRP), lipolysis-stimulated receptor, and VLDL receptor facilitates the uptake and processing of lipoproteins by the cells, and in turn the utilization of cholesterol for the metabolic needs of the cell. The delivery of excess cholesterol by HDL to the liver for elimination through conversion to bile acids is facilitated by apoE. Indeed apoE released into the interstitial fluid by cholesterol-laden cells can bind avidly to cholesterol-laden HDL.

apo E also plays a neurological role. Indeed, in addition, to the liver, the brain is also a major source of apo E. Synthesis of apo E occurs primarily in the astrocytes (2). The

presence of apoE in the cerebrospinal fluid in contrast to the absence of both LDL and apo B points to a major role for apo E in the transport of lipids in the central nervous system. The dramatic increase in apo E levels after peripheral nerve injury is a prelude to its regeneration.

As a consequence of injury to the nerve, cellular debris is created from axon degeneration and myelin destruction. apo E scavenges cholesterol from the debris and at first delivers it to the macrophages that have been recruited to the site of damage. As the regeneration of nerve begins apo E delivers cholesterol to sprouting neurites which have acquired at their tip LDL receptors for the uptake of cholesterol that is required for membrane synthesis. apo E also provides cholesterol to Schwann cells via LDL receptors on these cells. Thus the process of remyelination of the regenerated axon by Schwann cells is facilitated by apo E.

The ability of apo E to bind heparin and heparin-like glycosaminoglycans that constitute the matrix of the arterial wall suggests a role for it in smooth muscle cell proliferation and differentiation (1).

The fact that apo E containing lipoproteins and LDL can interact with specific sites on the surface of lymphocytes suggests also a role for apo E in immunomodulation (1).

Structure of apo E

The secondary structure of apo E has a predominance of α -helix (62 %). The remainder is made up of 18% random structure, 11% β -turn and 9% β -sheet (1). The random structure

separates the highly ordered amino and carboxy terminal regions. A structural feature important for binding lipid is the amphipathic nature of the α -helix, with one face composed of apolar residues while the other has polar residues. The ability of apo E and other apoproteins to function as surfactants on the surface of lipoprotein particles is attributed to the amphipathic helical structure.

The carboxy terminal region rich in amphipathic α -helices have been implicated in binding lipid (residues 263-286) and in the association of apo E with VLDL. The amino terminal region bears the receptor binding sites (residues 145 to 160). The basic amino acids (arginine, histidine and lysine) of apo E interacts with the acidic amino acids (aspartic and glutamic acids) of the receptor. Indeed single point mutations at residues 142, 145, 146 and 158 causing a single amino acid substitution where a basic amino acid such as arginine or lysine is replaced by a neutral amino acid result in defective binding of apo E to receptor. Indeed in type III hyperlipidemia where there is an accumulation of intermediate density lipoprotein (IDL), a mutation causing replacement of arginine at residue 158 of apo E by cysteine is implicated (1).

apo E Polymorphism

Three common variations in the apo E gene are prevalent in the general population. These alternative forms of genes or alleles are designated as E2, E3 and E4. The most common allele is E3 accounting for approximately 78% of all alleles in the general population (3). The other two alleles, E2 and E4 account for

approximately 7% and 15% respectively in the general population. The prevalence of homozygous genotypes in the Caucasian population is approximately: E3/E3: 59%, E4/E4: 2% and E2/E2: < 1%. The distribution of the heterozygous genotypes in this population are approximately E3/E4: 21%, E2/E3: 11% and E2/E4: 6%.

The three alleles E3, E4 and E2 code for proteins apo E3, apo E4 and apo E2 respectively. Polymorphism at nucleotides 3745 and 3883 in the apo E alleles which code for amino acids at residues 112 and 158 gives rise to apo E isoforms as illustrated in Figure 1. Since apo E3 is considered to be the parent form of the protein resulting from the most common allele E3, apo E2 and E4 are regarded as variants. apo E2 differs from apo E3 in having cysteine instead of arginine at amino acid residue 158. In contrast, apo E4 differs from apo E3 by the incorporation of arginine instead of cysteine at residue 112.

Figure 1. apo E Polymorphism

Due to this polymorphism in homozygotes the apo E phenotypes encountered are E3/3, E2/2 and E4/4. In the heterozygotes the three apo E phenotypes that are encountered are: E3/4, E2/4, and E2/3. Compared to the apo E2 isoform, both apo E3 and apo E4 tend to have a greater affinity for the LDL receptor. Clearance of apo E4 associated lipoproteins from plasma is more efficient when compared to the clearance of either apo E2 or apo E3 (4).

apo E2 as mentioned previously is associated with Type III hyperlipidemia due to a defect in binding to receptor (1).

apo E4, however, is associated with increased plasma cholesterol and LDL levels even though binding to receptor is normal.

The E4 allele is associated with the late-onset familial and sporadic forms of Alzheimer's disease (5).

Association of E4 Allele and Alzheimer's Disease (AD)

Increase in E4 allele number reduces the age of onset of AD. In one study 42 families with late onset of AD was examined (5). The fact that 91% of subjects with genotype E4/4 and 47% of subjects with genotypes E2/4 or E3/4 were affected indicates the importance of the E4 allele in late-onset AD. In contrast only 20% of subjects with one copy of E2 and one of E3 (genotype E2/3) or genotype E3/3 were affected.

The mean age of onset of AD with no E4 allele was 84.3 (standard error of the mean SEM 1.3), and 75.5 (SEM 1.0) with 1 E4 allele. However, the mean age of onset of AD

decreased to 68.4 (SEM 1.2) in individuals with 2 E4 alleles indicating that increase in E4 allele number carries the risk of lowering the age of onset of late onset familial and sporadic forms of AD (5).

E4 and the Pathogenesis of AD

In subjects with E4 allele, extra cellular neuritic plaques representing deposits of amyloid beta (A β) peptide were noted to be more extensive (6). Subjects with two E4 alleles at autopsies show increased beta amyloid staining compared with other AD subjects. (7).

Subjects with E2 allele, however, had less extensive deposition of A β peptide.

Experiments conducted *in vitro* have demonstrated that apoE4 and A β peptide interact readily to form a very stable complex (8).

In contrast apoE3 interacts less readily with A β peptide *in vitro*.

The extensive net work of monofibrils formed between A β -peptide and apoE4 *in vitro*, could *in vivo* account for its impaired clearance by LDL receptor-related protein (LRP) thus enhancing plaque formation (8). Another hallmark of AD is the formation of intracellular neurofibrillary tangles which are paired helical filaments (PHFs) of a modified microtubule - associated axonal phosphoprotein called Tau (9). This microtubule associated phosphoprotein, normally stabilizes neuronal microtubules, and promotes neurite outgrowth.

The stabilized microtubules facilitate cytoplasmic transport. However, when tau is

hyperphosphorylated the ability to interact with microtubules is affected. Hence, neurofibrillary tangles which are PHFs of hyperphosphorylated tau accumulate in the cytoplasm of neurons. *In vitro* experiments have demonstrated the ability of apoE3 to interact with tau to prevent hyperphosphorylation and thus stabilize microtubular structure and function. In contrast, apoE4, the risk factor for late-onset AD is unable to prevent formation of PHFs. As such, PHF-tau deposition destabilizes microtubules, thus affecting rapid axonal transport and contributes to the degeneration of affected nerve cells (9).

There is an inverse relationship between E4 allele copy number and choline acetyl transferase activity in sporadic late onset AD subjects, with a decrease in enzyme activity associated with increased E4 allele copy number (10). The decrease in choline acetyl transferase activity is perhaps related to the loss of cholinergic neurons in the basal forebrain of AD patients (10).

apoE receptors in Brain and Relationship to AD Pathology

Four receptors for apoE are expressed in the brain (11). The LDL receptor while present primarily on astrocytes, may also be expressed on neuronal processes as suggested by the intense staining of the neuropil.

The very low density lipoprotein (VLDL) receptor is primarily expressed on microglial cells.

A receptor expressed in the choroid plexus has been named GP 330.

A multifunctional receptor called LDL

receptor related protein (LRP) is expressed on a subset of neurons, and on reactive and not resting astrocytes. Both the VLDL receptor and GP330 share many of the properties of LRP as compared to the LDL receptor.

LRP is capable of reacting in addition to apoE with six other ligands such as α -2 macroglobulin, tissue plasminogen activator (t-PA), urokinase plasminogen activator, plasminogen activator inhibitor-1 (PAI-1) protease inhibitor, lipoprotein lipase and lactoferrin. Interestingly all the seven LRP ligands are constituents of senile plaques. The presence of LRP on senile plaques in AD Brain and VLDL receptor on microglia would suggest that these receptors could interact with small aggregates of a 39-42 aminoacid peptide called β A4, which is derived from amyloid precursor protein (APP). This interaction takes place possibly through association with apoE or other ligands for the receptors (6). The apoE 4 isoform may be defective in clearing β A4 from the neuropil due to either altered apoE- β A4 interactions or due to lower levels of apoE (6).

Early-Onset Alzheimer's Disease

Mutations in three genes have been implicated in early-onset (less than 60 years of age) AD (6). 70 to 80% of cases of familial early-onset AD have been attributed to a gene on chromosome 14 designated as AD3. Approximately 20% of cases of early-onset familial AD are, perhaps, due to mutations in the gene on chromosome 1 designated as AD4. Only 2 to 3% of cases of early-onset familial AD were due to mutations in the gene coding for the β -amyloid precursor protein on chromosome 21

designated as AD1. Deposition of amyloid in AD brain might conceivably be due to mutations in genes that code for proteins that affect the neuronal processing of the β -amyloid precursor protein. As such, the transport of the β -amyloid precursor protein to the intracellular compartment is favored, thus providing an environment for the formation of the amyloidogenic β -amyloid protein and the deposition of amyloid (6).

Molecular Methods for Assessment of apoE Polymorphism

A variety of molecular methods are available for the detection of apoE polymorphism. Typically DNA is amplified by the polymerase chain reaction (PCR) using primers flanking the two common polymorphic sites present in codons 112 and 158 of apoE gene (see Fig.1). The amplified DNA is subjected to cleavage by restriction enzyme Hha 1, which cleaves between nucleotides indicated by arrow in the sequence:

5' — GCG↓C — 3'

3' — C↑GCG — 5'

The digested fragments are separated by electrophoresis on polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet (U.V) illumination. Bands in the 91, 48 and 35 base pair (bP) region are typical of the E3 allele, while bands in the 91 and 83 bP region identify the E2 allele. The E4 allele is identified by bands in the 73, 48 and 35 bP region. Thus the presence of an 83 bP fragment is specific for the E2 allele, while the 73 bP fragment identifies the E4 allele. Heterozygotes such as E2/E3 can be identified by the presence of

bands in the 91, 48, 35 and 83 bP region. The E3/E4 genotype can be identified by the presence of bands in the 91, 48, 35 and 73 bP region. The high guanine to cytosine (G:C) content of the apoE gene can be problematic resulting in low yield and misproducts if the conditions for the PCR are not optimized. The PCR-RFLP procedure has been modified to permit large-scale screening of nearly hundreds of DNA samples in a day (12).

In addition to the PCR-RFLP procedure other genotyping approaches for the detection of apoE polymorphism have been described.

One approach is to probe the PCR product with allele-specific oligonucleotides (ASO) in dot blot format. The basis for the use of ASO probes is that they are specific for one allele. As such binding to other alleles is prohibited if the probe has a single base mismatch (13). In a technique that was used to genotype DNA extracted from formalin-fixed paraffin-embedded brain tissue, a semi-nested PCR procedure was used followed by hybridization with biotin-labeled ASO probes on a nylon membrane and detection by a chemiluminescent reaction (14). The high G:C content of target DNA can be problematic in terms of non-specific probe hybridization if non-optimized conditions are used.

The basis of another procedure for apoE genotyping takes advantage of the fact that under non-denaturing conditions single-stranded DNA has a folded structure. However, if a mutation is present a change occurs in the folded structure resulting in a change in mobility during polyacrylamide gel electrophoresis performed under non denaturing conditions (13). This

procedure called single strand conformational polymorphism (SSCP) is performed subsequent to PCR and hence is referred to as PCR-SSCP. In a procedure devised for apo E genotyping bands obtained upon electrophoresis of single stranded DNA prepared from the PCR product were examined by silver staining (15). The experimental conditions for PCR SSCP should be carefully controlled since the resolution of bands are dependent on variables such as ionic strength and temperature.

Another genotyping technique called amplification refractory mutation system (ARMS) uses primers mismatched in the first base at the 3' end. In this procedure primers mismatched to target DNA due to a point mutation are not amplified. Essentially in this allele-specific PCR technique the allele-specific primer is synthesized in two forms, one with a mutant and the other with a non-mutant 3' oligonucleotide. Amplification will occur with a non-mutant primer if the mutation is absent, and not with the primer containing the mutated sequence and vice versa (13). The ARMS technique has been utilized for apo E genotyping (16). A refinement of the multiplex arms PCR procedure involves detection of the amplified products by automated capillary gel electrophoresis with measurement of eluant absorbance in the ultraviolet region (258nm) (17).

Finally apo E genotyping can also be accomplished by sequencing of the PCR product (18).

Needless to say that the various steps involved not only in the isolation of DNA but also the analytical steps during and after PCR and

related molecular techniques should be carefully evaluated and optimized prior to the introduction of a genotyping procedure (19).

Clearly, genotyping procedures for the detection of apo E polymorphism are superior to phenotyping procedures which usually involve isoelectric focusing of native or delipidated plasma followed by either immunoblotting or immunofixation to the extent that rare variants with the same charge as one of the common apo E isoforms can interfere (20). Other variables such as the reduced efficiency of transfer of apo E4 isoform during electroimmunoblotting when compared to the transfer of apo E2 and E3 isoforms, and the misclassification of phenotypes due to bands introduced by non enzymatic glycation of apo E in diabetic patients can be problematic (20, 21).

While apo E genotyping is superior to

phenotyping, yet by itself it does not provide sufficient sensitivity or specificity as a diagnostic test for Alzheimer's disease. However, when the results of apo E genotyping are used in conjunction with clinical criteria the specificity of diagnosis is improved (22).

Conclusion

The importance of the apo E4 allele in late onset familial and sporadic form of Alzheimer's disease (AD) is reinforced by the finding of amyloid beta (A β) peptide deposits in brain of AD subjects with E4 allele. Similar correlation with the E4 allele and the hyperphosphorylation of Tau protein has been documented. In this context the identification of patients at risk of developing late onset AD by the application of apo E genotyping techniques together with clinical criteria can hopefully lead to the effective use of therapy.

REFERENCES

1. Mahley, R.W. (1988) Apolipoprotein E: Cholesterol transport protein with expanding role in cell Biology. *Science*. 240, 622-630.
2. Boyles, J.K., Pitas, R.E., Wilson, E., Mahley, R.W. and Taylor, J.M. (1985) Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest* . 76, 1501-1513.
3. Roses, A.D. (1996) Apolipoprotein E and Alzheimer's disease. A rapidly expanding field with medical and epidemiological consequences. *Ann. N.Y. Acad. Sci.* 802 , 50-57.
4. Boerwinkle, E., Visvikis, S., Welsh, D., Steinmetz, J., Hanash, S.M. and Sing, C.F. (1987) The use of measured genotype information in the analysis of quantitative phenotypes in Man. II. The role of apolipoprotein polymorphism in determining levels, variability and covariability of cholesterol, beta lipoprotein and triglycerides in a sample of unrelated individuals. *Am. J. Hum. Genet.* 27, 567-582.
5. Corder, E.H., Saunders, A.M., Strittmatter, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of alzheimer's disease in late onset families. *Science* . 261, 921-923.

6. Hyman, B.T. and Tanzi, R. (1995) Molecular epidemiology of alzheimer's disease. *New Engl J. Med.* 333, 1283-1284.
7. Polvikoski, T., Sulkava, R., Haltia, M., Kainulainen, K., Vuorio, A., Verkkoniemi, A., Niinisto, L., Halonen, P. and Kontula, K. (1995) Apolipoprotein E, Dementia, and cortical deposition of β amyloid protein. *New Engl. J. Med.* 333, 1242-1247.
8. Mahley, R.W., Nathan, B.P. and Pitas, R.E. (1996) Apolipoprotein E structure, function and possible roles in Alzheimer's disease. *Ann. N.Y. Acad. Sci.* 777, 139-145.
9. Goedert, M. (1996) Tau protein and the neurofibrillary pathology of alzheimer's disease. *Ann. N.Y. Acad. Sci.* 777, 121-131.
10. Beffert, U. and Poirier, J. (1996) Apolipoprotein E, Plaques, tangles and cholinergic dysfunction in alzheimer's disease. *Ann. N.Y. Acad. Sci.* 777, 166-174.
11. Hyman, B.T., Gomez-Isla, T., West, H., Briggs, M., Chung, H., Growdon, J.H. and Rebek, G.W. (1996) Clinical and neuro pathological correlates of apolipoprotein E genotype in alzheimer's disease. *Window on molecular epidemiology. Ann. N.Y. Acad. Sci.* 777, 158-165.
12. Bolla, M.K., Haddad, L., Humphries, S.E., Winder, A.F. and Day I.N.M. (1995) High-throughput method for determination of apolipoprotein E genotypes with use of restriction digestion analysis by microplate array diagonal gel electrophoresis. *Clin. Chem.* 41, 1599-1604.
13. Narayanan, S. (1996) Concepts, Principles, and Applications of Selected Molecular Biology techniques in Clinical Biochemistry. *Adv. Clin. Chem.* 32, 1-38.
14. Ballering, L.A.P., Steffens-Nakken, H.M., Esselink, R.A.J., Devos R.A.I., Jansen, Steur. E.N.H. and Vermes, I. (1997) Apolipoprotein E genotyping in patients with neurodegenerative diseases. *Clin. Biochem.* 30, 405-411.
15. Aozaki, R., Kawaguchi, R., Ogasa, U., Hikiji, K., Kubo, N. and Sakurabayashi, I. (1994) Rapid identification of the common apo E isoform genotype using polymerase chain reaction - single strand conformation polymorphism (PCR-SSCP). *Mol. Cell. Probes* 8, 51-54.
16. Wenham, P.R., Newton, C.R. and Price, W.H. (1991) Analysis of apolipoprotein E genotypes by the amplification refractory mutation system. *Clin. Chem.* 37, 241-244.
17. Donohoe, G.G., Salomaki, A., Lehtimaki, T., Pulkki, K. and Kairisto, V. (1999) Rapid identification of apolipoprotein E genotypes by multiplex amplification refractory mutation system PCR and capillary gel electrophoresis. *Clin. Chem.* 45, 143-146.
18. Miettinen, H.E., Korpela, K., Hamalainen, L. and Kontula, K. (1994) Polymorphisms of the apolipoprotein and angiotensin converting enzyme genes in young North Karelian patients with coronary heart disease. *Hum. Genet.* 94, 189-192.

19. Narayanan, S. (1997) Preanalytical and analytical pitfalls in molecular biology techniques. J Clin. Ligand Assay 20, 200-205.
20. Wenham, P.R., Sedky, A. and Spooner, R.J. (1991) Apolipoprotein E phenotyping: a word of caution. Ann. Clin. Biochem. 28, 599-605.
21. Snowden, C., Houlston, R.S., Arif, M.H., Laker, M.F., Humphries, S.E. and Alberti, K.G. (1991) Disparity between apolipoprotein phenotypes and genotypes (as determined by polymerase chain reaction and oligonucleotide probes) in patients with non-insulin-dependent diabetes mellitus. Clin. Chim. Acta 196, 49-58.
22. Mayeux, R., Sanders, A.M., Shea, S., Mirra, S., Evans, D., Roses, A.D., Hyman, B.T., Crain, B., Tang, M.X. and Phelps, C.H. (1998) Utility of apolipoprotein E genotype in the diagnosis of Alzheimer's disease. New Engl. J. Med. 338, 506-511.