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Featured Article

Apolipoprotein E4 inhibits autophagy gene products through direct, specific binding to CLEAR motifs

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Abstract

Introduction: Alzheimer apolipoprotein E (APOE) ε4,4 carriers have earlier disease onset and more protein aggregates than patients with other APOE genotypes. Autophagy opposes aggregation, and important autophagy genes are coordinately regulated by transcription factor EB (TFEB) binding to "coordinated lysosomal expression and regulation" (CLEAR) DNA motifs.

Methods: Autophagic gene expression was assessed in brains of controls and Alzheimer's disease (AD) patients parsed by APOE genotype and in a glioblastoma cell line expressing either ApoE3 or ApoE4. Computational modeling assessed interactions between ApoE and mutated ApoE with CLEAR or modified DNA.

Results: Three TFEB-regulated mRNA transcripts—SQSTM1/p62, MAP1LC3B, and LAMP2—were lower in AD ε4,4 than in AD ε3,3 brains. Computational modeling predicted avid specific binding of ApoE4 to CLEAR motifs. ApoE was found in cellular nuclei, and in vitro binding assays suggest competition between ApoE4 and TFEB at CLEAR sites.

Conclusion: ApoE4-CLEAR interactions may account for suppressed autophagy in APOE ε4,4 carriers and, in this way, contribute to earlier AD onset.

Keywords: Alzheimer's disease; APOE genotype; ApoE protein; autophagy; DNA binding; TFEB; Transcription; Protein aggregation; Molecular-dynamic simulation; EMSA; PLA

1. Introduction

Inheritance of two apolipoprotein E (*APOE*) ε4 alleles (*APOE* ε4,4) is the single greatest genetic risk factor for development of Alzheimer's disease (AD) [1–3], the world's most common neurodegenerative disease [4]. The importance of the risk of having the *APOE* ε4 gene product, that is, the ApoE4 protein, rather than either of the other two possible gene products—ApoE2 or ApoE3—is underscored by the fact that the odds of development of AD in *APOE* ε4,4 carriers are 12 to 15 times that of those carrying either *APOE* ε3,3 or *APOE* ε2,4, and three times that of *APOE* ε3,4 carriers [3]. Sixty percent of all AD patients carry at least one *APOE* ε4 allele [5]. Moreover, relative to their counterparts, who carry one of the five other allelic combinations of the *APOE* gene, Alzheimer patient carriers of *APOE* ε4,4 have conspicuous increases in the defining neuropathological changes of AD, viz., extracellular plaques of amyloid beta (Aβ) [7,8] and intraneuronal paired helical filaments of hyperphosphorylated tau (P-tau) in neurofibrillary tangles [9,10]. This association suggests that the ApoE4 protein, itself, may interfere with autophagic processes so as to favor proteostatic failure and aggregate buildup over

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clearance of unwanted proteins. Failures in proteostasis such as autophagic insufficiency are known to be early and persistent features of Alzheimer pathogenesis [11,12] and appear to be particularly accentuated in the presence of *APOE* ε4 [13]. Studies of autophagy-related failures have demonstrated that defects in retrograde transport [10,14] and lysosomal acidification [15,16] lead to elevations in Alzheimer-like pathology, while transcription factor EB (TFEB)-mediated activation of autophagy in various models ameliorated both Aβ [17,18] and tau pathology [19].

Despite the clear importance of the *APOE* ε4,4 genotype in both AD risk and aggregate density, at present, there is no consensus as to how the presence of ApoE4 proteins may directly or indirectly influence either disease risk or the genesis of early, excessive accumulations of AD-defining aggregates. Therefore, based on current knowledge regarding the importance of ApoE4 in Alzheimer neuropathogenesis, we undertook a more direct approach and investigated the potential of ApoE4 to interfere with autophagy by altering the expression of three essential protein elements of autophagy: sequestosome (p62), LC3B, and LAMP2. These proteins are the products of the binding of TFEB to the coordinated lysosomal expression and regulation (CLEAR) DNA motif [20] for transcription of the genes *SQSTM1* and *MAP1LC3B* [21], and *LAMP2* [22]. The demonstration by Theendakara and his colleagues [23] of possible productive interactions between ApoE and DNA, together with recognition of the importance of TFEB/CLEAR binding in regulation of autophagy in general [21] and of Aβ [17,18] and tau [19], in particular, led us to investigate a new hypothesis, viz., that ApoE4 interferes with TFEB/DNA interactions at point(s) before translation of LCB3, p62, and LAMP2 and in this way accounts, at least in part, for the observed early and persistent elevation of the numbers of plaques and neurofibrillary tangles in brains of *APOE* ε4,4 patient carriers.

2. Methods

2.1. Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were blinded as to the sex and genotype of the patient sources of the samples used.

2.2. Cell lines and culture

T98G cells were obtained from American Type Culture Collection. Stable transformants were generated as described previously by Wang et al. [24]. Recombinant ApoE was prepared under native conditions with the intention of retaining any lipid content present. This precludes harsh approaches necessary to isolate the protein. Concerns about purity, however, are relieved by the comparative difference between the ApoE3 and ApoE4 preparations and by sensitivity of the binding to an antibody recognizing ApoE.

2.3. Cell culture conditions

T98G cells, expressing either ApoE3 or ApoE4, were grown in DMEM (Cat. No: 11995040, Thermo Fisher Scientific, Waltham, MA, USA) supplemented to 10% with fetal bovine serum (Cat. No: 16000044, Thermo Fisher Scientific). For amino-acid starvation, cultures were washed twice with serum and amino-acid free EBSS and incubated in EBSS at 37°C for 3 hours, whereas control ("fed") cells were washed twice with DMEM/FBS and incubated at 37°C for 3 hours in DMEM/FBS.

2.4. Antibodies and reagents

The following commercially available antibodies were used: anti-pan 14-3-3 (sc-629, Santa Cruz Biotechnology), anti-TFEB (ab2636, Abcam), anti-p62 (BD610832, BD Biosciences), anti-actin (ab6276, Abcam), anti-LC3B (NB600-1384, Novus Biologicals), and mouse monoclonal anti-ApoE (1484 273; Boehringer-Mannheim).

2.5. Immunofluorescence

Human hippocampal immunohistochemistry: Samples were acquired from human brain specimens either with pathologically diagnosed Alzheimer's disease (without Parkinson's disease) or age-matched controls (AMCs) from the UAMS brain bank, where they were stored as formalin-fixed, paraffin-embedded tissue. Our tissues were from patients who did not qualify as human subject research according to U.S. Department of Health and Human Services Exemption 4. A total of eight Alzheimer's disease patients and four age-matched control patients were used in TFEB nuclear localization immunofluorescence, using ab2636 at 1:50 dilution. The average age of the patients was 76 years old, with postmortem intervals between 3 and 13 hours, with an average postmortem interval of 5.3 h. Hippocampal tissue blocks were sectioned at 7-μm thickness and mounted on slides and subsequently deparaffinized in xylene, rehydrated in serial dilutions of ethanol to water, and washed with PBS + 0.1% Tween 20. Antigen retrieval was performed in boiling citrate buffer for 30 minutes; slides were blocked in Dako Animal-Free Protein Blocker, then incubated in primary antibody overnight at 4°C, washed, and then incubated in secondary antibody for 1 hour. After washing in PBS, slides were quenched for autofluorescence with 0.1% Sudan Black B in 70% EtOH, washed with water, then treated with DAPI and coverslipped in Prolong Gold Mounting Medium. A total of six images per case were analyzed, with images taken from nonadjacent locations in pyramidal cell layers in CA1. Nuclear localization was assessed by ImageJ, with DAPI used to create a mask for the TFEB channel, and TFEB intensity was divided by nuclear area to control for cell density.