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Research Article [Araştırma Makalesi]

Expression of ASC in post-mortem brain samples of Alzheimer's disease patients: A possible role for ASC in Aβ amyloid formation

[Alzheimer hastalarının post-mortem beyin dokusu örneklerinde ASC ifadesi: Aβ amyloid oluşumunda ASC'nin rolü]

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ABSTRACT

Aim: Amyloid is an extracellular insoluble protein aggregate which accumulates in several tissues in various clinical conditions. The co-localization of ASC, a key molecule in both apoptotic and inflammatory processes, with AA type amyloid fibrils has previously been demonstrated by our group. The aim of this study was to determine whether the distribution of ASC is altered around Aβ deposits and senile plaques in post-mortem brain samples of Alzheimer's disease patients.

Material and Methods: Immunohistochemical and immunofluorescence staining of paraffin-embedded tissues from post-mortem brain samples of 12 Alzheimer's disease patients were performed using anti-ASC and anti-Aβ antibodies. Then we investigated possible ASC- Aβ co-localization in an in-vitro system using COS-7 cells.

Results: Immunohistochemical staining of paraffin-embedded tissues revealed co-localization of ASC protein with Aβ peptide in senile plaques. We further demonstrated the interaction between ASC and Aβ in ASC-YFP and amyloid precursor protein co-transfected COS-7 cells which also showed that specks are located near the intracellular Aβ deposits.

Conclusion: We hypothesize that expression of ASC may be important in the pathogenesis of Aβ amyloid formation and in senile plaque development in predisposed tissues. Further functional studies are required to explore the link between ASC and Aβ amyloid formation.

Key Words: ASC, Aβ, APP, Alzheimer's disease, inflammation, COS-7 cell line.

Conflict of interest: Authors have no conflict of interest.

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ÖZET

Amaç: Amiloid, farklı patolojik durumlarda, farklı dokularda birikebilen çözünmesi zor hücreler arası birikimdir. Daha önce grubumuz tarafından, inflamasyon ve apoptozis mekanizmasında önemli rol oynayan ASC proteini ile AA tipi amiloid fibrillerinin birlikteliği gösterilmiştir. Bu çalışmanın amacı ise ASC proteininin Alzheimer hastalarında görünen Aβ birikimleri ile birlikte olup olmadığının araştırılmasıdır.

Materyal ve Metod: 12 Alzheimer hastasına ait post-mortem beyin dokularında ASC proteini ve Aβ peptidine özgül antikorlarla immünboyama gerçekleştirildi. Ayrıca olası ASC-Aβ birlikteliği in-vitro ortamda COS-7 hücreleri ile incelendi.

Bulgular: Alzheimer hastalarının paraffin blok kesitlerinde yapılan immünboyama çalışmaları sonucunda ASC proteininin senile plak yapıları içerisinde Aβ peptide ile birlikte bulunduğu gösterildi. APP ve ASC-YFP ekspresyon vektörleri ile transfekte edilen COS-7 hücrelerinde, ASC-YFP protein ile hücre içi Aβ birikimlerinin bir arada bulunduğu ortaya çıkarıldı.

Sonuç: Bu çalışmanın sonucuna göre, ASC'nin Aβ tipi amiloid ve senil plak oluşumunda önemli rolü olabileceği düşünülmüştür. Bununla beraber, Amiloid oluşum sürecinde ASC-Aβ ilişkisinin rolünü aydınlatmak için ileri seviyede işlevsel çalışmaların yapılması gerekmektedir.

Anahtar Kelimeler: ASC, Aβ, APP, Alzheimer hastalığı, inflamasyon, COS-7 hücre hattı.

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Introduction

Amyloids are complex tissue deposits which are composed of specific polypeptides and proteoglycans. They accumulate in certain tissues, which causes disruption of the architecture and function, of them [1]. In recent years it has been shown that, amyloid protein aggregates play a crucial role in important human pathologies, including Alzheimer's disease (AD) familial polyneuropathy, type II diabetes, chronic renal disease and rheumatoid arthritis [1-3].

Each type of amyloid is identified over 20 biochemically diverse protein molecule [3]. Although the process of amyloid formation is poorly understood, it can be explained as, these proteins become highly ordered with a predominant β-sheet secondary structure that allows inter-molecule hydrogen bonding, and consequently results in a highly stable product [2].

Despite the diversity of their chemical differences and the associated diseases, the fibriller products of these components share common tinctorial and morphological characteristics [2,4]. Some of the amyloid proteins are; islet amyloid peptide, Aβ protein, and acute phase protein serum amyloid A.

Our group is focused on familial Mediterranean fever (FMF) which is included in the large family of systemic auto-inflammatory diseases. FMF is characterized by recurrent attacks of fever with localized painful inflammation. FMF patients are susceptible to multi-organ deposition of AA type amyloid [5]. The FMF gene (MEFV) is located on the short arm of chromosome 16 and encodes the pyrin protein [6,7]. The biological function of pyrin is not clearly understood, it appears to play an important role in the inflammatory pathways [8]. It is composed of four domains; PYD, two central B-box zinc-fingers, coiled coil and B30.2/rfp/SPRY domains. The pyrin protein is expressed predominantly in neutrophils, monocytes, and eosinophils [9].

Most FMF-causing mutations are located in the B30.2/rfp/SPRY domain at the C-terminal end of pyrin. This domain interacts with Siva [10], caspase-1, pro IL-1B and NALP3, thereby regulating the assembly of the NALP3 inflammasome [11]. Pyrin also associates through a PYD-PYD interaction with apoptosis associated speck like protein containing CARD (ASC) [12]. ASC is an adaptor protein which locates in cytoplasm. It has an N-terminal PYD and C-terminal CARD involved in the activation of caspase-1 [13]. Most of the studies suggest that both pyrin and cryopyrin are capable of assembly independent of inflammasome complexes with ASC and procaspase-1 and activating caspase-1 via ASC oligomerization [14].

In our previous study, we demonstrated that (i) pyrin colocalizes with ASC in speck formation; (ii) both wild type and mutant pyrin increase ASC speck formation; (iii) colchicine prevents speck formation; (iv) specks can survive in the extra cellular space after cell death; (v) ASC is expressed in renal glomeruli of FMF patients with amyloidosis, but not in the control patients. Thus we concluded that ASC expression in renal glomeruli may result in speck formation and that speck from dying cells may persist in the extra cellular space where they have the potential to nucleate amyloid (15). We have shown that ASC is highly expressed in renal glomeruli of FMF patients with amyloidosis. These findings afford the question "Is ASC associated with Aβ protein, which is found in Alzheimer's disease?" which formed the basic hypothesis of this study.

In this study, we showed that ASC co-localizes with extra cellular Aβ deposits in brain samples of Alzheimer's disease patients. This study also revealed that Aβ fibrils locate in the same cellular compartment with ASC in transfected COS-7 cells.

Materials and Methods

Patients

We investigated temporal lobe sections from 12 human autopsy cases with AD. Nine of them were provided by Abcam Company (United Kingdom, Cambridge). Pathological findings of these patients were given in Table-1. These are examined and diagnosed by a licensed pathologist and had ethical approval (Ethic Committee of Hacettepe University, LUT 07/11-13). Three patients were diagnosed according to the clinical protocols which confirmed dementia by fulfilling the DMS-IV criteria and also according to their pathology findings at autopsy which exerted the recommended criteria for the diagnosis of dementia.

Cell Culture and Plasmids

COS-7 (monkey fibroblast cell line) cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. For transfection, cells were plated onto glass cover slips placed in six-well culture plates. Cells were transfected using the FuGENE-HD transfection reagent (Roche).

APP was expressed using the pCEP4 expression vector containing APP cDNA (Invitrogen) and ASC-YFP was kindly provided by Dr. Deborah Gumucio (University of Michigan) which was generated using the pE-YFP vector from Clontech.

Antibodies

Goat anti-ASC polyclonal antibody (Santa Cruz), mouse anti-Aβ monoclonal antibody (6E10 Sigma), rabbit anti-Aβ (Cell Signaling Technology), AF488 rabbit antimouse and AF568 rabbit anti-goat fluorescent secondary antibodies (Molecular Probes Invitrogene) were used in the study.

Congo red staining

All patients' temporal lobe sections stained with Congo Red Kit (Sigma) following the manufacuturer's protocol.

Apple-green birefringence was examined by bright-field microscopy with a polarizing filter (Olympus BX51). Slides of human kidney tissue containing amyloid were processed at the same time as positive controls.

Immunohistochemistry

Immunohistochemical staining was performed with the anti-ASC monoclonal antibody using the streptavidin biotin peroxidase method (DAKO LSAB Kit, Dako, Carpenteria, CA). Anti-Aβ monoclonal antibody and culture fluids of the hybridoma producing anti-ASC monoclonal antibody were used as the primary antibody and tris-buffered saline (TBS) was used instead of MoAb for negative controls. Five µm thick sections of formalin-fixed, paraffin-embedded tissues were cut and deparaffinized. Antigen retrieval was performed by formic acid treatment. Endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide in methanol for 30 min, and then the sections were washed and blocked with 1% bovine serum albumin (BSA) in TBS for one hour. Sections were incubated with purified primary antibodies overnight at 4°C and then with biotinized anti-mouse secondary antibody for 60 min at room temperature. Tris-buffered saline (TBS) was used in place of the primary antibody as the negative control. Background staining in the sections was minimal. After washing three times with TBS, sections were incubated with horseradish-peroxidase conjugate streptavidin for 30 minutes. After washing three times with TBS the staining reaction was developed with 3,3-diaminobenzidine (Sigma Chemical). Counterstaining was performed with hematoxylin. Sections were visualized by using an Olympus BX51 light microscope.

Immunoflourescence

In situ Formalin-fixed, paraffin-embedded tissues deparaffinized, and then the sections were washed and blocked with 0.1% Tween20 in phosphate-buffered saline containing 10% goat serum and 0.01g/mL BSA for one hour. Sections were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 60 min at room temperature. After washing three times with PBS, counterstaining was performed with DAPI.

In vitro COS-7 cells were fixed for 30 minutes in 4% paraformaldehyde in phosphate-buffered saline, permeabilized using 0.2% Triton X-100 in phosphate-buffered saline, and blocked using 0.1% Tween20 in phosphate-buffered saline containing 10% goat serum and 0.01g/mL BSA. Cover slips were incubated with primary antibodies overnight at 4°C and then with secondary antibodies for 60 min at room temperature. Antibody diluent, not containing primary or secondary antibody, were used as negative control. After washing three times with PBS, counterstaining was performed with DAPI. Sections were visualized by using a fluorescence microscope (Leica IM50).

Results

ASC co-localizes with Aβ in senile plaques

Firstly, immunohistochemistry with Aβ (Sigma, 6E10) antibody was performed in all patients' materials. According to this, high amount of senile plaques were detected in all cases (Figure 1a) which was necessary of pathological diagnose of Alzheimer's disease (see also table 1). Addition to this, congophilic angiopathy, which formed around blood vessels, was detected in 8 of patients (Figure 1b). The gold standard amyloid staining, which is congo red, was also performed. Both senile plaques and congophilic angiopathies were appeared as apple-green by polarizing filter (Figure 1c-d)

To investigate whether ASC co-localized with senile plaques, temporal lobe samples from AD patients were analyzed after immunoflourescence. We demonstrated that, in all patients, ASC co-localizes with Aβ in some of the senile plaques, especially in the central amyloid core (Figure.1 e-g). We also showed that, in several cases, ASC co-localizes with Aβ deposits in amyloid angiopathy (Figure.1 h-j).

Intracellular Aβ deposits surround ASC Specks

To examine ASC-Aβ protein-protein interaction in an in-vitro system, we used COS7 cells expressing beta secretases. Cells were co-transfected with ASC-YFP (yellow florescence protein) and Amyloid Precursor Protein (APP). Twenty-four hours after transfection, immunoflorescence staining was performed. The Aβ peptide was labeled with AlexaFluor568-conjugated anti-mouse to detect intracellular Aβ deposits. Random fields of fixed and stained cells were imaged. Using this method, we demonstrated that, in co-transfected cells, intracellular Aβ deposits surround ASC specks (Figure.2 a-c). We also performed this experiment with another Aβ antibody, which is polyclonal and recognizes different part of the Aβ peptide. It was observed that ASC specks and Aβ accumulation occurs at the same cytoplasmic distribution (Figure.2 d-f).

Discussion

Alzheimer's disease is an irreversible, progressive and degenerative disorder that destroys the higher structures of the brain [16]. Prominent neuropathological features of AD are senile plaques, neurofibrillary tangles, synaptic and neuronal loss [16-18]. There is mounting evidence that chronic inflammatory processes play a fundamental role in the progression of neuropathological changes of AD [19-20]. The major players involved in the inflammatory process of AD are thought to be the microglia and astrocytes. The process of the activation of glia is characterized by up-regulation or newly expression of a variety of molecules involved in inflammatory response including cytokines [21], various components of the complement cascade [22-24], proteases and

Table . Pathological findings of the patients

Patient No	Age	Gender	Senile plaque	Congophilic angiopathy	ASC-Aβ co-localization (sp)	ASC-Aβ co-localization (ca)
1	70	M	++++	-	+	-
2	60	M	+++	-	+	-
3	75	M	+++	+	+	+
4	87	M	++	+	++	+
5	80	M	+++	-	+	-
6	83	M	+++	+	+	-
7	85	F	++++	++	+++	+
8	73	F	++	+	+	-
9	82	M	++	+	+	-
10	81	M	+++	+	+	+
11	77	F	+++	+	+	-
12	83	M	+++	-	+	-

(M:male, F:female, sp: senile plaque, ca: congophilic angiopathy)

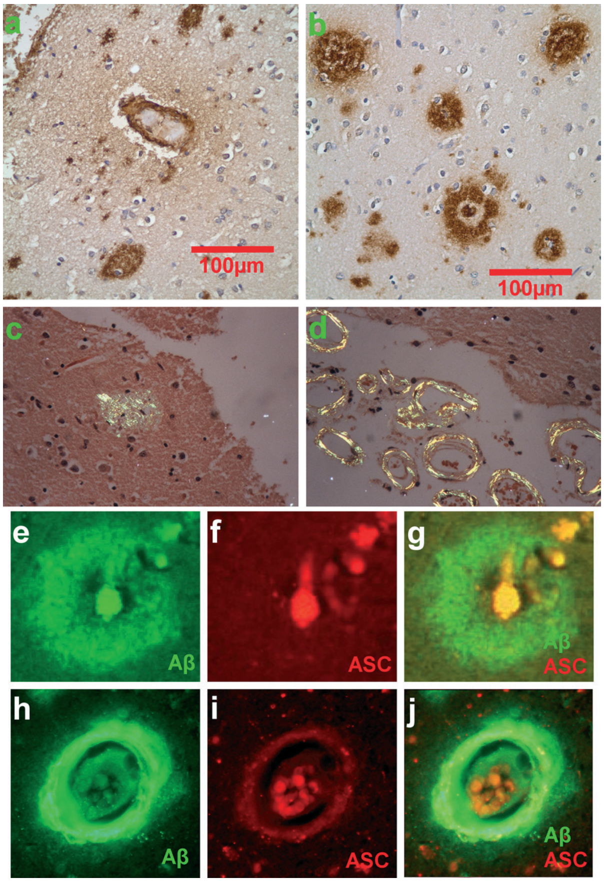

Figure 1. Senile plaques (a) and congophilic angiopathies (b) were visualized by immunohistochemistry with Aβ antibody in Alzheimer's disease patients' temporal lobe slides (bar indicates 100 micrometer). Birefringence of congo red staining under polarized light showed clear amyloid deposition in both senile plaques (c) and congophilic angiopathies (d) (200X). A senile plaque stained with antibody raised against Aβ (green) (e), ASC immunreactivity (red) in a senile plaque amyloid core (f). Merged image showing co-localization (yellow) of ASC with Aβ in a senile plaque amyloid core (g). Congophilic angiopathy stained with antibody raised against Aβ (green) (h), ASC immunreactivity (red) in congophilic angiopathy (i). Merged image showing co-localization (yellow) of ASC (red) with Aβ (green) in a vascular wall (j) (400X).

protease inhibitors [25], and neurotoxic products [26]. The importance of inflammation in the pathogenesis of AD was indirectly confirmed by epidemiological investigations that revealed a decreased incidence of AD in subjects using anti-inflammatory drugs, especially the non-steroidal anti-inflammatory drugs (NSAIDs) [27]. ASC expression has been shown to be up-regulated by inflammation in human neutrophils [28]. Previous report demonstrated that glial cells express ASC but not neurons [29]. However, we observed that many neurons express ASC in AD patients (data not shown). (We did not perform immunostaining of neuron specific marker and ASC together in AD patients' sections) All of these findings indicate that ASC might be up-regulated in the AD patient brains.

Most investigators believe that diffuse plaques evolve into neuritic plaques, accompanied by the crowns of degenerated neurites, and during that process, amorphous deposits of soluble Aβ with an α -helix structure also evolve into fibrillar forms with an insoluble β-plated sheet, and finally form dense amyloid cores placed centrally in the senile plaque [30]. In previous study we have shown that ASC specks are stable in the extracellular space [15] and therefore we have speculated that such specks can also survive in vivo and these bare specks may enhance amyloid core formation of senile plaques.

Our studies have remained an open issue whether ASC is activated in the senile plaque structure and speed up that process or triggered after Aβ amyloid formation in order to induce immune response. The expression pattern of ASC in time lapse manner during amyloid development in a cell based system remains to be defined but we speculate that it may be involved at the beginning of dense amyloid core formation, because ASC itself is an aggaregating protein. Furthermore, it has been shown that in cultured cells under conditions of oxidative stress or proteosome impairment leads to sequestration of the

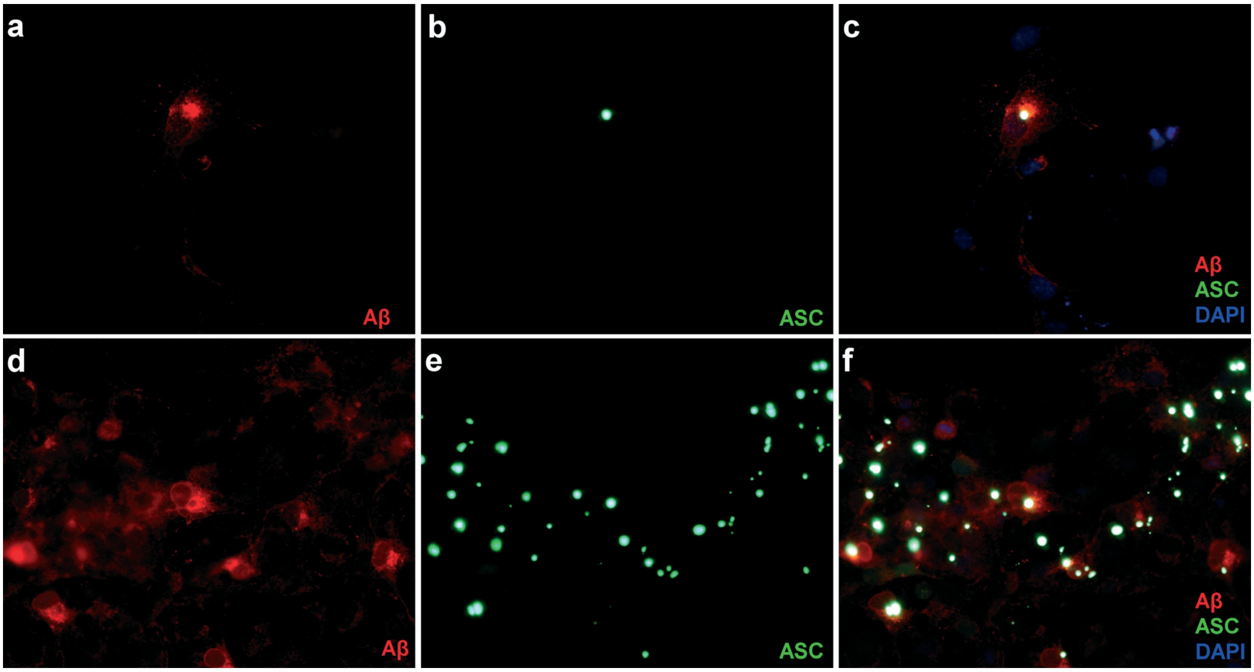

Figure 2. Intracellular Aβ deposit surrounds an ASC speck in COS-7 cells. Intracellular Aβ stained with antibody raised against Aβ (red) (a and d), ASC-YFP (yellow-green) (b and e) and merged image (c and f). Counterstaining was performed by DAPI (blue). Two different Aβ antibodies were used in these experiments (The immunostaining with monoclonal antibody against N-terminal of Aβ peptide was used in a, while the polyclonal antibody was used in d). (a, b and c 200X, d, e and f 400X)

mutant amyloid precursor protein within multicomponent proteinaceous inclusions known as aggrosomes and sequestration is actively mediated by microtubules [31]. In our previous study we showed that ASC specks are located near the microtubule organizing center of cells and microtubule disruption, using a microtubule toxin, reduces speck formation [15]. In this study; staining with Aβ in Aβ-ASC-YFP cotransfected COS7 cells revealed that although the 2 proteins do not directly co-localize, their close cellular organization within the cell showed that ASC-YFP specks usually surrounded by Aβ protein. After all these data, we concluded that expression of ASC may be important in the pathogenesis of Aβ amyloid formation and in senile plaque development in predisposed tissues. However, further functional studies are required to explore the link between ASC and Aβ amyloid formation.

In a recent study consistent with our hypothesis, it has been found that Aβ activated NALP3 inflammasome [32]. The cytoplasmic receptors of the NALP family are central components of the inflammasome that associate with the adaptor protein ASC [13]. They clearly demonstrated that NALP3 inflammasome was required for Aβ- induced activation of caspase-1, the release of mature IL-1β and the secretion of proinflammatory and potentially neurotoxic cytokines and chemokines. They concluded that the NALP3 inflammasome may function as a general sensor for the recognition of peptide or protein aggregates that are involved in the pathogenesis of amyloid diseases [32].

Our previous results about ASC - AA type amyloid co-localization raised the question if inflammatory pathways through ASC have an important effect on amyloid development [15]. This study showing ASC - Aβ co-localization supported the idea that neuroinflammatory changes may occur at early stages in the AD brain and significantly contribute to the pathogenesis of the disease. These findings have suggested that pharmacological intervention targeting the ongoing inflammation may hold therapeutic promise. The information regarding the extent to which inflammatory mediators affect the disease process and if ASC is one of those, how these mediators potentiate their detrimental effects remains to be defined.

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