Apo D and Apo E Levels in Autism Spectrum Disorders

Erman Esnafoglu (eman esnafoglu@yahoo.com.tr)
Ordu Universitesi Tip Fakultesi  https://orcid.org/0000-0001-8685-1153

Selma Cırık
Ordu University Faculty of Medicine: Ordu Universitesi Tip Fakultesi

Research Article

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Abstract

Apo D is an atypical plasma apolipoprotein and a member of the lipocalin protein superfamily. In recent years, Apo D has been identified as an important factor in the pathology of neurodegenerative and neuropsychiatric diseases. Apo D is highly produced in the brain and acts as a multiligand and multifunctional carrier. Apo D binds to and stabilizes Arachidonic acid in the cell membrane and cytosol. Thus, it suppresses inflammation and protects the cell membrane against oxidation. Apo E is important in cholesterol transfer and it has been reported that it may play a role in immune regulation, nerve regeneration, synaptogenesis and neuronal homeostasis. In this study, plasma Apo D and Apo E levels were examined and its possible role in Autism Spectrum Disorders (ASD) pathology was investigated. Thirty-nine subjects with ASD were compared with 30 healthy subjects who typically developed. Accordingly, plasma Apo D and Apo E levels were statistically significantly lower in the ASD group compared to the healthy control group. According to the results of this study, it can be suggested that low levels of Apo D and Apo E may play a role in ASD pathogenesis.

Introduction

Apolipoproteins are lipoprotein proteins and their important functions include; Helping the dissolution of non-polar lipids, participating in the activation of important enzymes in lipoprotein metabolism, providing lipoprotein uptake and destruction by binding to specific receptors on the cell surface (Kelley 1997). In addition to their functions in lipid metabolism, the role of some apolipoproteins such as Apolipoprotein E (Apo E) and Apolipoprotein D (Apo D) in the pathology of various diseases has been emphasized in recent years.

Apo D is a 29 kDa glycoprotein of 169 amino acids. Although classified as apolipoprotein, Apo D is a member of the lipocalin family. In recent years, Apo D has been identified as an important factor in the pathology of neurodegenerative and neuropsychiatric diseases (Rassart et al. 2000; Thomas et al. 2001; 2003a; Mahadik et al. 2002). Apo D is highly produced in the brain and functions as a multiligand and multifunctional carrier (Hu et al. 2001). It has been shown that Apo D can carry small hydrophobic molecules such as arachidonic acid (AA), steroid hormone, and cholesterol during the regulation of metabolism or signal transduction (Rassart et al. 2000; Dassati et al. 2014; Martínez et al. 2013). Apo D stabilizes AA in the cell membrane, and also binds to cytosolic free AA and prevents its normal functions. Thus, it restricts eicosanoid synthesis by removing AA from the cyclooxygenase pathway (Thomas and Yao 2007). Most eicosanoids such as prostaglandins and leukotrienes are mediators of inflammation. Inflammation has been reported to play a role in many psychiatric illnesses. It has also been reported that Apo D can protect the membrane against oxidation. Consistent with these functions, changes in Apo D expression have been reported to be associated with many pathological conditions such as breast cancer, prostate cancer, Parkinson's disease, Alzheimer's disease, schizophrenia, and bipolar disorder (Dassati et al. 2014).
Apo E is a glycoprotein with a molecular weight of ~34 kDa and consisting of 299 amino acids (Rall et al. 1982; Weisgraber et al. 1994). In addition to its classical functions such as transport and uptake of lipids, it has been reported to be associated with cytoskeleton and microtubule remodeling, axon growth, synaptic plasticity and cell migration (Mahley 2016; Liu et al. 2013). Due to its wide-ranging role in neuronal signal transduction pathways, the place of Apo E in the pathology of various neurological diseases has started to attract attention. Studies have shown that Apo E is polymorphic and has many isoforms that can be detected by isoelectric focusing. Analysis of Apo E isoforms reveals two types of Apo E gene polymorphism that are genetically determined and cannot be determined. Genetically determined polymorphism occurs with ε2, ε3 and ε4 alleles. Three isoforms of ApoE have been identified as Apo E2, Apo E3 and Apo E4 (Zannis et al. 1982). The second Apo E gene polymorphism, which cannot be determined genetically, occurs as a result of posttranslational modification of Apo E (Zannis et al 1981). Studies have found that Apo Eε4, a polymorphic variant of Apo E, significantly increases the risk of development and age of onset of Alzheimer’s disease (AD), while the allele Apo Eε2 is negatively associated with AD (Rasmussen 2016; Lavados et al. 2005; Nierenberg et al. 2005; Weisgraber and Mahley 1996; Mahley and Huang 1999). Subsequent studies suggested that Apo E dysfunction may be associated with the pathology of psychiatric diseases such as schizophrenia and mood disorders (Gibbons et al. 2011; Giunco et al. 2009). Apo E’s role in psychiatric diseases is more related to its role in synaptic signaling (Gibbons 2011).

Although Apo D and Apo E, which have important physiological functions other than lipid transfer, have been shown to be associated with neurodegenerative diseases and mood disorders, their relationship with autism spectrum disorders (ASD) has not been previously investigated. Epidemiological studies estimate that the incidence of autism spectrum disorders are increasing and affects approximately 1% of the population (Baird et al. 2006; Baxter et al. 2015). To date, despite much research, the etiology and pathophysiology of ASD are not well understood. In this study, Apo D and Apo E levels in the circulation of ASD patients were examined and the possible role of these lipoproteins in the pathophysiology of ASD was evaluated.

**Methods**

The study was conducted with subjects diagnosed with autism spectrum disorder (ASD) according to DSM 5, among the patients who applied to the Child Psychiatry Outpatient Clinic of Ordu University Training and Research Hospital. The healthy control group was composed of volunteers. After these volunteers were evaluated with routine blood tests, they were evaluated by expert psychiatrists. Accordingly, subjects who did not have any problems in their routine tests and history and who did not have a psychiatric diagnosis were included in the healthy control group. The study was approved by Ordu University Clinical Research Ethics Committee (2018/97). In this study, the exclusion criteria were neurological deficit, obesity (BMI> 25), epilepsy, tumor, and history of systemic disease, and the use of psychotropic drugs in the last 6 months. The subjects and their families were informed in detail about the study and written consent forms were obtained. Then, some plasma obtained from blood samples taken...
during routine blood tests was reserved for this study and stored until measurements were made at -80 degrees.

**Sociodemographic data form**

This form was developed by the researchers and contained information about gender, age, number of siblings, residence, history and order of pregnancy, week of pregnancy, form of birth, birth history, birth weight, birth complications, psychomotor development history, duration of breastfeeding, medical history, family history, medications used, special education status, and body mass index.

**Childhood Autism Rating Scale**

The childhood autism rating scale (CARS) comprises 15 items that are used to generate a total score defining the severity of autism. CARS rates the child on a scale from 1 to 4 in each of 15 areas (relating to people; imitation; emotional response; listening response; body use; object use; adaptation to change; visual response; taste, smell and touch responses; fear or nervousness; verbal communication; nonverbal communication; activity level; level and consistency of intellectual response; and general impressions). A total score of between 30 and 36.5 indicates mild-to-moderate autism, whereas the interval between 37 and 60 denotes severe autism. CARS is scored by observing the child and through interviews with the family (Esnafoglu and Ayyıldız 2017; Schopler et al. 1986; Sucuoglu et al. 1996).

**Apo D Measurement**

Plasma Apo D level was measured by a commercial kit using ELISA method (Cat. No. E-EL-H0469, Elabscience Biotechnology, Wuhan, China). The kit based on sandwich ELISA method, displayed a sensitivity of 0.38 ng/ml and the detection range was 0.63-40 ng/ml. According to kit protocol, the samples and standards were added to the wells pre-coated with antibody and then the manufacturer’s instructions were followed. After the enzyme-substrate reaction was terminated, the optical density was measured spectrophotometrically at a wavelength of 450 nm (BioTek, ELx800 brand REF ELX508 SN1310149). Apo D levels in the samples was calculated using the standard curve and the results were presented as µg/ml.

**Apo E Measurement**

A commercial sandwich-ELISA kit was used to measure plasma Apo E level (Cat. No. E-EL-H0470, Elabscience Biotechnology, Wuhan, China). The kit showed a sensitivity of 14.06 ng/ml and the detection range was 23.44-1500 ng/ml. The measurement was carried out in accordance with the kit protocol. Briefly, the samples and standards were added to the wells pre-coated with human Apo E antibody and incubated for 90 minutes at 37°C. Then, the biotinylated Apo E antibody was added to each well and incubated for 60 minutes at 37°C. After the washing process, Avidin-Horseradish Peroxidase conjugate was added to each well and incubated for 30 minutes at 37°C. At the end of the incubation, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added and after a 15 min incubation period the enzyme-substrate reaction was terminated by the addition of a stop solution. The absorbance of resulting
yellow product was measured spectrophotometrically at a wavelength of 450 nm (BioTek, ELx800 brand REF ELX508 SN1310149). Concentration of Apo E in the samples was calculated using the standard curve and the results were presented as µg/ml.

**Statistical analyzes**

Statistical analyzes were made using the SPSS 22 software program. Normally distributed data were presented as mean ± standard deviation and those not showing normal distribution as median (minimum-maximum). It was examined whether the numerical values were normally distributed using the Sapiro Wilk test. Chi-square test was used for comparison of categorical variables. Student-t test was used for comparison of BMI, Apo D and Apo E numerical values between the groups, as they showed normal distribution. Since the age distribution was not normally distributed, the Mann Whitney U test was used. Spearman and Pearson tests were used in correlation analysis.

**Results**

There were no statistically significant differences between the groups in terms of age, gender, and BMI. The mean CARS score in the ASD group was 49.05 ± 5.53. Accordingly, disease severity was evaluated as severe in the ASD group. Apo D and E levels were found to be significantly lower in the ASD group (Table 1, Figs. 1 and 2). According to the correlation analysis, no significant correlations were found between Apo D and E levels and CARS score, age and BMI. The results found are shown in Table 1.
Table 1
Characteristics of the groups and Apo D and E values

|                  | ASD group (n = 39) | Healthy control group (n = 30) | P value |
|------------------|-------------------|-------------------------------|---------|
| Gender (F/M)     | 6/33              | 7/23                          | 0.298¹  |
| Age (median)     | 6.5 (3–13)        | 6.0 (3.5–14)                  | 0.918²  |
| BMI              | 18.52 ± 2.89      | 16.71 ± 1.85                  | 0.004³  |
| CARS             | 49.05 ± 5.53      | -                             | -       |
| Apo D            | 1.26 ± 0.19       | 1.39 ± 0.18                   | 0.008³  |
| Apo E            | 0.91 ± 0.37       | 1.22 ± 0.34                   | 0.001³  |

Footnote: BMI = Body Mass Index; CARS = Childhood Autism Rating Score;
¹ = Khi-square test
² = Mann Whitney-U test
³ = Student-t test

Discussion

Autism spectrum disorder, which manifests itself with delay or deviation in social relationship, communication and cognitive development, is a lifelong neurodevelopmental disorder that begins in the early stages of life. In this disorder, there are significant difficulties in social interaction and communication, unusual behavior and interests (Steyaert and Marche 2008). Although the pathophysiology of autism has not been clearly explained, it is thought to be a multifactorial disorder that occurs with the interaction of many neurological, immunological, environmental and genetic factors (Steyaert and Marche 2008; Muhle et al. 2004, Lai et al. 2014; Mandy et al. 2016). There is increasing evidence that lipid metabolism is impaired in autism etiology (Tamiji and Crawford 2010; Tierney et al. 2006). In this study, plasma apo D and apo E levels of children and adolescents with ASD were examined for the first time according to our best knowledge.

Apo D is a soluble carrier protein that mediates the transport of steroid hormones such as progesterone and pregnenolone, as well as lipophilic molecules such as cholesterol and arachidonic acid (Rassart et al. 2000; Dassati et al. 2014). Recent data show that ApoD is not only a lipophilic molecule carrier, but also controls the fate of these ligands by altering their stability and oxidation state. It is particularly interesting that Apo D, which is expressed in neurons and glial cells in the central and peripheral nervous system, binds to arachidonic acid and its derivatives, which play a central role in normal brain functions (Hu et al.
Apo D has been shown to act as a catalyst for the reduction of peroxideed eicosanoids and reduce lipid peroxidation in the brain (Thomas et al. 2003b). It has been shown that the Apo D gene in the human brain is upregulated with aging (Perdomo and Dong 2009; Muffat and Walker 2010).

In addition, it has been found that Apo D levels in the nervous system increase in many neurological diseases such as Alzheimer's disease, schizophrenia, and stroke (Dassati et al. 2014). There is increasing evidence that Apo D has a neuroprotective role due to its antioxidant and anti-inflammatory activities. Apo D is an evolutionarily conserved protein and is considered an anti-stress protein because it is induced by oxidative stress and inflammation, and is seen as an effective therapeutic agent against various neuropathologies and even against aging (Dassati et al. 2014). In this study we conducted with ASD patients, it was observed that the plasma apo D level was statistically significantly lower compared to the control group. Considering its antioxidant and anti-inflammatory effects, apo D deficiency may have a role in the pathophysiology of ASD.

Apo E, found in all plasma lipoproteins, accounts for approximately 10–20% of VLDL and approximately 1–2% of HDL. Critical for cholesterol transport and lipoprotein particle metabolism, Apo E also plays a role in immune regulation, nerve regeneration, lipolytic enzyme activation, synaptogenesis. Apo E is synthesized in most organs and the second largest production site in the body is the brain (Liu et al. 2013; Mahley 2016; Rall et al. 1982; Weisgraber 1994). Lipoproteins containing Apo E stimulate both axonal growth and neuronal survival by binding LDL receptors in neurons (Vance and Hayashi 2010; Cedazo-Mínguez 2007). Both Apo E and cholesterol are very important in the protection of myelin sheath and neuronal synapses. In our study, the change in plasma Apo E level in ASD patients was examined and it was found that plasma Apo E concentration was statistically significantly lower in ASD children compared to healthy children. In accordance with other studies showing the relationship of Apo E with psychiatric diseases such as schizophrenia and mood disorders, it is observed that Apo E contributes to ASD pathology.

**Conclusion**

In this study, it was determined that the levels of Apo D and Apo E in the plasma of ASD patients were significantly decreased. Considering the antioxidant and anti-inflammatory effects of Apo D and the role of Apo E in axonal growth and neuronal survival, the decrease in these proteins may play an important role in the pathophysiology of ASD. Approaches that will cause an increase in Apo D and Apo E levels can provide positive improvements in these patients. Yet whether the decrease in circulation reflects the decrease in brain tissue is unknown. This issue should be examined in more detail with both clinical and experimental studies.

**Declarations**

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**Author contributions**

Conceived and designed the experiment: EE. Performed the experiments: EE, SC. Analyzed the data: EE, SC. Wrote the paper: EE, SC.

**Disclosure of conflicts of interest**

EE and SC report no conflicts of interest.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures
Figure 2
Apo E distribution in ASD and healthy control group