

Stress Sensitivity in Patients with Atopic Dermatitis in Relation to the Translocator Protein 18 kDa (TSPO)

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Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by pruritic and eczematous skin lesions and dermatitis that worsens under stressful conditions. However, the relation of these symptoms to an individual's stress sensitivity is not well understood. On the other hand, expression of the translocator protein (18 kDa) (TSPO), formerly known as the peripheral-type benzodiazepine receptor, has been used as a biological marker of trait anxiety and stress sensitivity. The present study was designed to address this issue by examining TSPO in patients with AD. Fifty-two patients with AD (30 male and 22 female) and 163 healthy volunteers (89 male and 74 female) participated in this study. State-Trait Anxiety Inventory (STAI) scores were significantly higher in patients with AD, especially male patients, than in healthy subjects. The expression of platelet TSPO, as determined with a binding assay with [³H] PK11195, was also significantly higher in patients with AD, indicating that AD is a stress-responsive disease. In genomic analysis using lymphocytes, a single-nucleotide polymorphism of the human TSPO gene at exon 4 (485G>A), which is presumably associated with an individual's stress sensitivity, showed significantly lower frequencies of G/G and higher frequencies of G/A in patients with AD than in healthy subjects. The severity of AD, as determined with the Scoring of Atopic Dermatitis index, was correlated with TSPO expression in male patients with the G/A phenotype. In conclusion, the present study provides new evidence that variation in the TSPO gene affects susceptibility to AD.

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Key words: atopic dermatitis, stress sensitivity, translocator protein 18 kDa (TSPO), genomic analysis

Introduction

Atopic dermatitis (AD) is a chronic relapsing

inflammatory skin disease of persons with the predisposing factor of atopy. In 2006, mutations in the gene for the production of filaggrin were found to strongly increase the risk of AD¹. Genetic

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mutations in filaggrin often reduce the barrier function of skin, elevate immunoglobulin E levels, and lead to the pathogenesis of chronic dermatitis².

Many patients with AD live under stressful conditions, in which intense unavoidable itching disturbs their sleep and markedly reduces their quality of life³. Repeated scratching can cause erythema, pigmentation, and lichenification. Due to these cosmetic problems, the patients are exposed to psychological stress as well as physical stress. Frequent scratching as a means of escaping the intolerable stress worsens the pruritic and eczematous skin lesions.

AD can lead to psychological disturbances, such as stigmatization, social isolation, and discrimination⁴. Patients with AD have been reported to exhibit anxiety, depression, and emotional excitability^{5,6}. Psychological stress and symptoms of AD appear to form a vicious cycle⁷. It remains unclear, however, how stress affects AD.

On the other hand, there has been a growing interest in *the translocator protein (18 kDa)* (TSPO), formerly known as *the peripheral-type benzodiazepine receptor (PBR)*, in the subjects of steroidogenesis, apoptosis, and immunomodulation⁸⁻¹⁰. The TSPO is involved in the regulation of several major stress systems, *i.e.*, the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system, the renin-angiotensin axis, and the neuroendocrine-immune axis⁸.

Our previous studies have found that the expression of TSPO on platelets is significantly correlated with the trait anxiety score in healthy human subjects¹¹. The evidence for TSPO as a promising biological marker of stress has prompted us to investigate the stress response of TSPO at the genomic level.

A 485G>A single nucleotide polymorphism (SNP) in a coding region of exon 4 of the TSPO gene was found to affect susceptibility to panic disorder (PD)¹². Before the onset of PD, individuals with the G/G genotype showed high anxiety sensitivity and an increase in TSPO. Our study suggests that individuals with the G/G genotype are at increased risk for stress-related disorders.

The present study was designed to examine how

the symptoms of AD are related to individual's stress sensitivities by analyzing the density of platelet TSPO together with the genetic variation of TSPO.

Materials and Methods

Subjects

Fifty-two patients with AD (30 male and 22 female) and 163 healthy volunteers (89 males and 74 female) participated in this study. The participants were given the State-Trait Anxiety Inventory (STAI), a self-reported measure of anxiety. For patients with AD, the Scoring of Atopic Dermatitis (SCORAD) index was performed. The SCORAD index is a well-established severity-scoring tool for AD which is widely used in dermatology. The SCORAD index consists of the interpretation of the extent of the disorder (A: according to the rule of nines; score 0–20), the intensity composed of 6 items (B: erythema, edema/papules, effect of scratching, oozing/crust formation, lichenification and dryness; score 0–63; each item has 4 grades: 0, 1, 2, and 3), and symptoms (C: itch, sleeplessness; score 0–20). All subjects were fully informed about the nature of the study and gave their written consent. This study was approved by the Ethics Committees of Nippon Medical School and Tokyo Metropolitan Institute of Medical Science.

Preparation of Platelet Membranes

Blood samples (20 mL) were collected, and platelets were isolated with our standard procedures¹¹. In brief, blood samples (20 mL) were obtained from the subjects in the morning between 9:00 a.m. and 10:00 a.m. The samples were collected in plastic-walled, evacuated blood collection tubes (Venoject II, Terumo Corp., Tokyo, Japan) and spun twice at 180 x g for 15 minutes at 4°C. Platelet-rich plasma was collected and spun at 1,500 x g for 15 minutes at 4°C. The platelet-containing pellet was frozen at –80°C.

Before the binding assay, the samples were thawed, and each pellet was homogenized in 10 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.4) in a homogenizer (Polytron PT-10, Thermo Fisher

Scientific, Inc., Waltham, MA, USA). The homogenate was then centrifuged at 49,000 x g for 15 minutes at 4°C, and the pellet was suspended in 100 volumes of Tris-HCl buffer.

The platelet membranes were finally adjusted to 0.1 mg protein/mL with assay buffer (50 mM Tris-HCl, pH 7.4). The protein content was determined with the Lowry technique.

Binding Assay

The binding of [³H] PK 11195, a specific ligand of TSPO, to platelets was assayed with a method described previously¹³. Tissue (0.8 mL, 0.08 mg protein) was incubated with a radioligand (0.1 mL) and a cold ligand (or assay buffer; 0.1 mL) in an incubation volume of 1 mL (0°C–4°C) for 60 minutes. The reaction was terminated by rapid filtration over GF/B glass microfiber filters (FP-100, Whatman, GE Healthcare, Little Chalfont, UK) that had been soaked in poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO, USA) using a cell harvester (M-24, Brandel, Gaithersburg, MD, USA), with 5 washes with 5 mL of ice-cold buffer.

The specific binding of [³H] PK 11195 was defined as the difference in binding obtained in the presence and the absence of PK 11195 (10 μM, Research Biochemicals International, Natick, MA, USA). The radioactivity retained by the filters placed in a 24-well microplate (PicoPlate-24, PerkinElmer, Inc., Waltham, MA, USA) was measured with a microplate scintillation counter (Top Count, Packard Instrument Co., Meriden, CT, USA), using 500 μL of a scintillant (MicroScint-20, Packard Instrument Co.). [³H] PK 11195 (86.0 Ci/mmol) was purchased from Daiichi Pure Chemical Company (Tokyo, Japan).

The dissociation constant (K_d) and the receptor density (B_{max}) were determined with least-squares regression. Unless otherwise stated, the statistical data are presented as the mean and S.D.

Genomic Analysis

The 485G>A polymorphism of the TSPO gene was examined as described previously¹². Genomic DNA was prepared from peripheral blood lymphocytes with a DNA extraction kit (Stratagene, La Jolla, CA, USA). The fragments including exon 4

of the TSPO gene were amplified with the polymerase chain reaction (PCR), and direct sequencing was performed. Sequence variations of the TSPO gene were analyzed within exon 4.

The PCR amplifications were performed in a 20-μL reaction mixture containing 100 ng of genomic DNA, 15 pM of each primer, 1.5 mM of MgCl₂, and 1 U Ex Taq polymerase (Takara, Tokyo, Japan).

The coding region in exon 4 of the TSPO gene was screened with direct sequencing, using the primer sets. Sequencing was performed on both strands with a sequencing kit (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA) and a sequencer (ABI 3700, Applied Biosystems).

The SNPs were scored with custom genotyping products (TaqMan Assays-by-Design SNP Genotyping Service, Applied Biosystems) based on the TaqMan assay method¹⁴. Genotypes were determined with a sequence detection system instrument (ABI 7900, Applied Biosystems) and analysis software (SDS v2.0, Applied Biosystems).

Statistical Analysis

Pearson product-moment correlation and analysis of variance were used to identify associations among B_{max} values and STAI scores. The allelic distributions were compared between patients and control subjects by means of chi-square statistics and Fisher's exact test. All differences were considered significant at p<0.05. Statistical analysis was performed with the Prism software program (version 4.0) for Macintosh (GraphPad Software, San Diego, CA, USA).

Results

The subjects were 52 patients with AD (30 male and 22 female) and 163 healthy volunteers (89 male and 74 female). The STAI scores were significantly higher in patients with AD, especially male patients, than in healthy subjects (**Fig. 1**). In male patients, both state and trait anxiety scores were significantly higher, whereas in female patients, only trait anxiety scores were significantly higher.

The expression of platelet TSPO, as determined

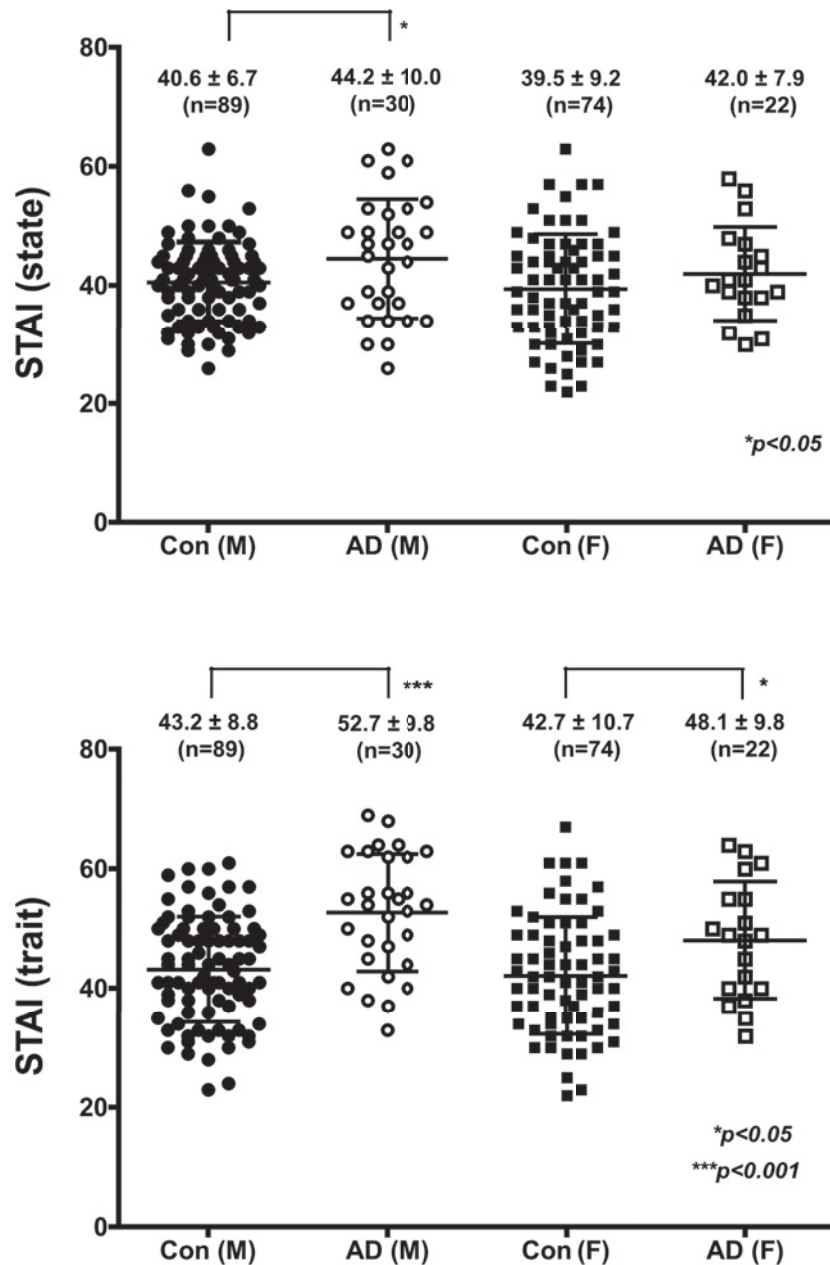


Fig. 1 Comparison of STAI scores of patients with atopic dermatitis and control subjects
 Scores of the state anxiety (upper) and the trait anxiety (lower) are further compared between male (M) and female (F) subjects. There were significant differences in either state or trait anxiety between patients with atopic dermatitis (AD) and control subjects (Con), except for state anxiety in female subjects. The data are presented as means and S.D.

with a binding assay with [³H] PK11195 in terms of B_{max}, was also significantly higher in patients with AD than in healthy control subjects. The increase was greater in male patients (by 62% on average) than in female patients (by 22% on average).

Genomic analysis of the 485G>A polymorphism of

the human TSPO gene in exon 4 showed, contrary to our expectation, that the G/G genotype was less frequent and the G/A and possibly A/A genotypes were more frequent in patients with AD than in control subjects (Table 1). The difference in the frequency distribution was significant in male

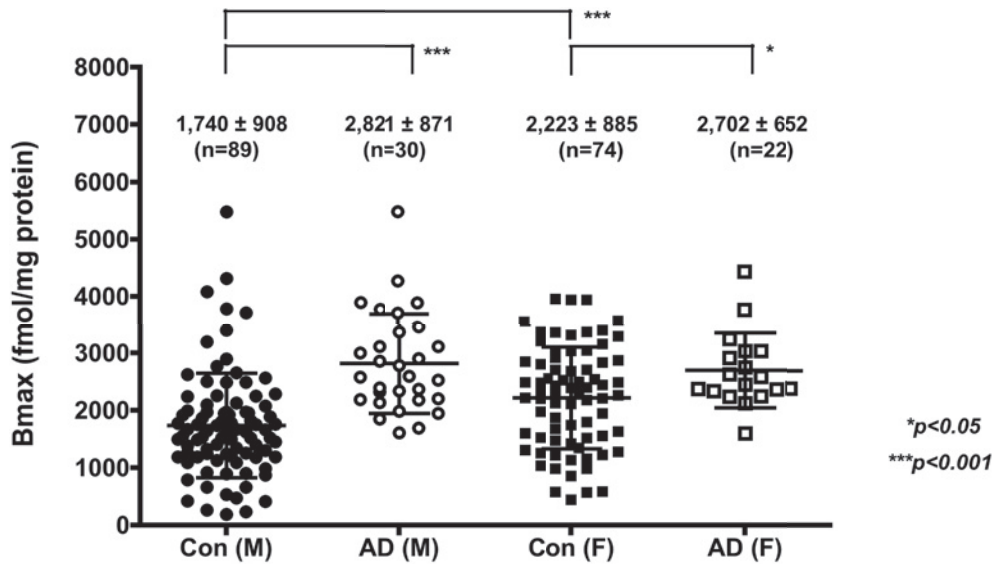


Fig. 2 Comparison of the platelet TSPO expression (Bmax) of patients with atopic dermatitis with control subjects
 The Bmax values are further compared between male (M) and female (F) subjects. There were significant differences between patients with atopic dermatitis (AD) and control subjects (Con) in both male and female subjects. The data are presented as means and S.D.

Table 1 Genotypic and allelic distribution of the 485G>A polymorphism of the TSPO gene in patients with atopic dermatitis and control subjects

	N	Genotype (%)			Allele (%)	
		G/G	G/A	A/A	G	A
Atopic Dermatitis	52	23 (44.2)	26 (50.0)	3 (5.8)	72 (69.2)	32 (30.8)
Control	163	99 (60.7)	53 (32.5)	11 (6.8)	251 (77.0)	75 (23.0)
Atopic Dermatitis (M)	30	9 (30.0)	18 (60.0)	3 (10.0)	36 (60.0)	24 (40.0)
Control (M)	89	55 (61.8)	29 (32.6)	5 (5.6)	139 (78.1)	39 (21.9)
Atopic Dermatitis (F)	22	14 (63.6)	8 (36.4)	0 (0.0)	36 (81.8)	8 (18.2)
Control (F)	74	44 (59.5)	24 (32.4)	6 (8.1)	112 (75.7)	36 (24.3)

*P=0.0104 (genotype), 0.0105 (allele)

There was a significant difference between male patients with atopic dermatitis patients and male control subjects.

subjects, but not in female subjects.

The severity of AD, as determined with the SCORAD index, appeared to be associated with TSPO expression (Fig. 3). Male patients showed a positive correlation between AD severity and TSPO expression, in which the relation was significant for the G/A genotype. In contrast, female patients showed no such correlations.

Discussion

In the present study, we found that AD symptoms are related to an individual's stress response, as reflected by the expression and genetic variation of TSPO. To the best of our knowledge, this is the first study showing a definite correlation between AD and TSPO.

Patients with AD, especially male patients, had significantly higher STAI scores, The expression of

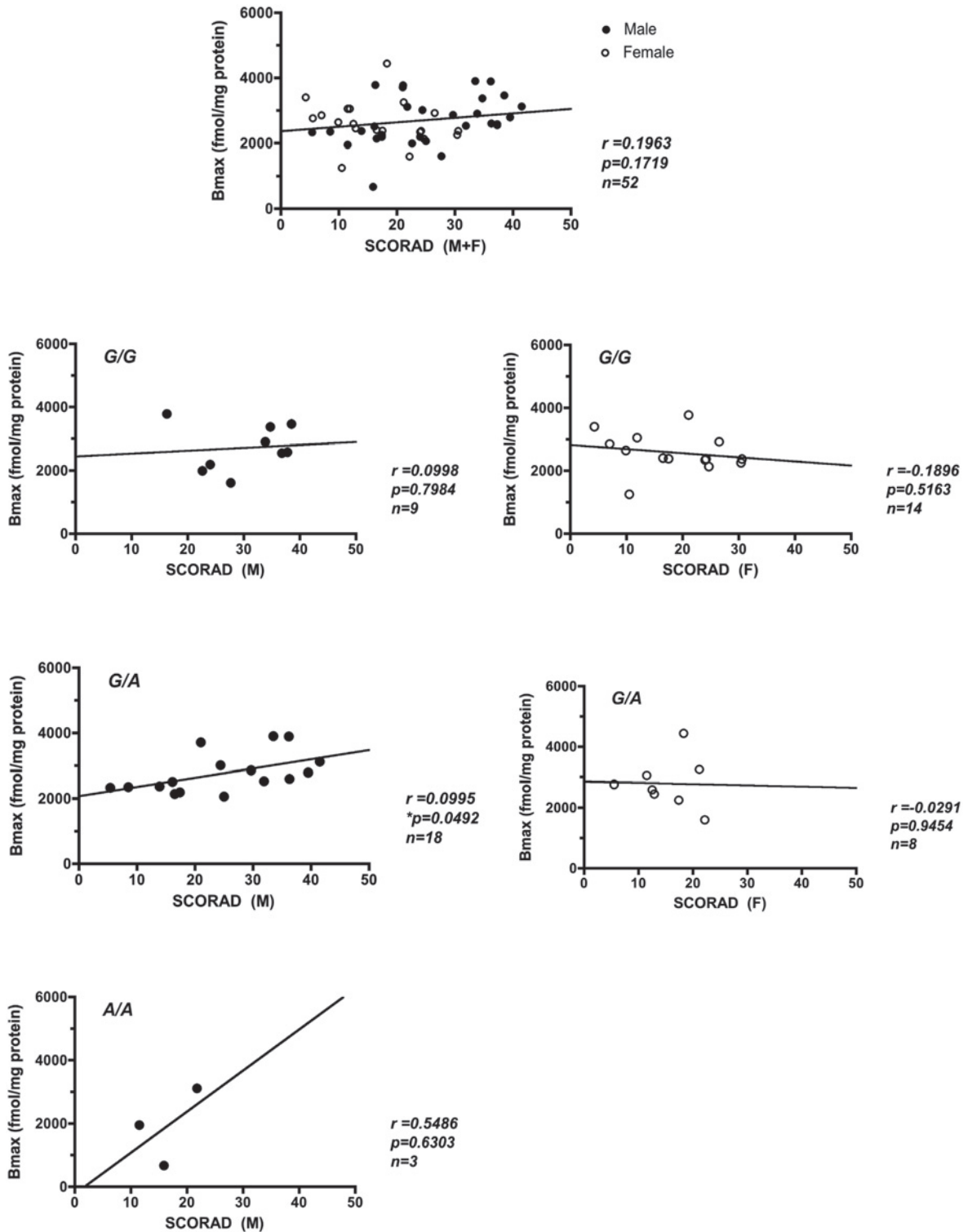


Fig. 3 Relation of Scoring of Atopic Dermatitis index to TSPO expression in atopic dermatitis. The graph at the top shows the relation of the Scoring of Atopic Dermatitis (SCORAD) index and TSPO expression (Bmax) in both male and female patients. The graphs in the second and third rows and at the bottom show patients with G/G, G/A, and A/A genotypes, respectively. The graphs on the left side show male subjects, and those on the right side show female subjects. Male patients showed a positive correlation, in which the relation was significant for the G/A genotype. In contrast, female patients showed no such correlations.

platelet TSPO was also significantly higher in patients with AD, indicating that AD is a stress-responsive disease as reported previously^{4,5}.

Genomic analysis with lymphocytes for an SNP of the human TSPO gene in exon 4 (485G>A), in which the G/G genotype is presumably associated with individual's stress sensitivity¹², showed, contrary to our expectations, significantly lower and higher frequencies of the G/G and G/A genotypes, respectively, in male patients with AD, but not in female patients.

The severity of AD, as determined with the SCORAD index, was correlated with TSPO expression in male patients but not in female patients. The sex difference remains to be clarified, but a possible explanation is that hormonal changes during the menstrual cycle in female patients affects the function of TSPO.

Structure and Function of TSPO

The involvement of TSPO in steroidogenesis, apoptosis, and immunomodulation has attracted much interest⁸⁻¹⁰. TSPO may constitute a biomarker of brain inflammation and reactive gliosis and has been used as a therapeutic target in neurological and psychiatric disorders¹⁵.

TSPO appears to be a heteromeric complex of at least 3 different subunits, including an isoquinoline-binding subunit (18 kDa), a voltage-dependent anion channel (VDAC, 32 kDa), and an adenine nucleotide translocase (ANT, 30 kDa)¹⁵. Isoquinolines, such as PK 11195, that bind specifically to TSPO interact specifically with the 18-kDa subunit, whereas TSPO-specific benzodiazepines, such as Ro 5-4864, bind to a site consisting of both VDAC and the 18-kDa subunits.

The 18-kDa subunit, recently referred to as "translocator protein (18 kDa)"¹⁰, is involved in the regulation of cholesterol transport from the outer to the inner mitochondrial membrane (the rate-determining step in steroidogenesis). The protein is highly expressed in steroidogenic tissues. In the brain, the 18-kDa protein is primarily expressed in ependymal and glial cells.

The 18-kDa subunit is a small, highly conserved protein that is predominantly localized on the outer

membrane of mitochondria, where it is associated with VDAC and ANT to form the mitochondrial permeability transition pore (MPTP)¹⁶. Consistent with its localization in the outer mitochondrial membrane and its association with the MPTP, the 18-kDa subunit plays a role in the regulation of apoptosis.

The full-length complementary DNA for the 18-kDa subunit has been cloned from humans⁸. The human TSPO gene (approximately 13 kbp) is located in the 22q13.31 band on chromosome 22 and is composed of 4 exons, with the first exon and half of the fourth exon being untranslated. The protein domain of exon 2 has been linked to the isoquinoline-binding site and part of the TSPO-specific benzodiazepine-binding site. In addition, a cholesterol-interaction site has been characterized at the carboxyl end of the protein.

The 18-kDa TSPO subunit is highly conserved in the 4 species cloned (*i.e.*, human, cow, rat, and mouse). As far as exons 2-4 are concerned, this gene is well conserved even in bacteria, implying that the functions of this gene must be fundamental for the cell⁸.

Stress Response of TSPO

The TSPO is involved in the regulation of several major stress systems: 1) the hypothalamic-pituitary-adrenal axis, 2) the sympathetic nervous system, 3) the renin-angiotensin axis, and 4) the neuroendocrine-immune axis⁸.

The sensitivities of TSPO to stress have been demonstrated in both animal and human studies⁸. Drugan *et al* (1986), using inescapable tail shocks in an animal model of stress, were the first to show the involvement of TSPO in the physiological response to stress¹⁷. Exposure of rats to 5 shocks induced a significant increase in the expression of renal TSPO.

Studies in humans have also shown that the expression of TSPO in platelets is sensitive to stress and anxiety. Increased platelet TSPO density was detected in resident physicians exposed to "examination stress." However, changes in the expression of TSPO were not accompanied by changes in "stress hormones" (cortisol, prolactin, and growth hormone), which can be explained either by

the fact that the study dealt with prolonged stress or by the fact that the hormonal peak release occurred earlier, during the examination. In conclusion, alterations in the expression of TSPO seem to be a sensitive indicator of stress.

Polymorphism of the TSPO Gene in Relation to Stress

In our recent studies, expression of platelet TSPO was significantly correlated with the trait anxiety score in healthy human subjects¹¹. The evidence for TSPO as a promising biological marker of stress has prompted us to investigate the stress response of TSPO at the genomic level¹².

Nakamura *et al* (2006) have detected a novel missense variant in exon 4 of the TSPO gene, derived from the nucleotide transition in codon 162 (CGT → CAT: 485G>A), resulting in an arginine-to-histidine (Arg → His) change. Genotypic and allelic analyses of the 485G>A polymorphism have revealed significant differences between the patients with PD and healthy control subjects¹².

Unlike the present study, the study of Nakamura *et al* found that the G/G genotype was significantly more frequent in subjects with PD than in control subjects. Patients with PD had a nearly twofold higher rate of the G allele than did control subjects. Before the onset of PD, individuals with the G/G genotype showed high anxiety sensitivity and an increase in TSPO. These results suggest that individuals with the G/G genotype are at increased risk for PD.

The present study of the 485G>A polymorphism of patients with AD stands in contrast to the previous study in patients with PD. The STAI and TSPO expression suggest that patients with AD are under stress. Therefore, the G/G genotype is likely responsible for the pathogenesis of AD. If this is the case, then the G/G genotype should be more frequent in subjects with AD. Contrary to this expectation, the G/G genotype was less frequently observed, and the G/A and possibly A/A genotypes were more frequently observed in patients with AD. To explain this result, factors other than stress sensitivity should be considered.

Immune Factors in Relation to AD and TSPO

Because various immune and nonimmune factors participate in the pathogenesis of AD¹⁸⁻²⁰, anxiety and stress sensitivity cannot be attributed solely to increased disease activity. The most likely psychological substrate in anxiogenic stimuli is nerve growth factor (NGF)^{21,22}. In addition, NGF is considered an important mediator lowering the itching threshold. The epidermis of lesions from patients with AD shows a higher expression of NGF than does the epidermis of healthy control subjects²³.

Genes associated with skin-barrier formation and adaptive immunity have been implicated in the development of AD. For example, filaggrin is essential for the maintenance of the skin-barrier function. Genetic mutations in filaggrin are significantly associated with the risk of AD and elevated immunoglobulin E levels².

On the other hand, TSPO is an attractive drug target for controlling inflammation. For example, administration of the TSPO ligand etifoxine modulates macrophage activation and blunts the production of inflammatory cytokines after peripheral nerve injury. This anti-inflammatory effect of etifoxine likely involves TSPO because the selective TSPO ligands PK 11195 and Ro5-4864 have also been shown to inhibit inflammatory responses^{15,24}.

The G allele in the 485G>A polymorphism of the TSPO gene might facilitate such an anti-inflammatory effect, and, accordingly, the G/G genotype would be less frequently observed in patients with AD. The relation of the phenotype variation of TSPO (Arg162His) to the anti-inflammatory effect remains to be clarified.

In conclusion, the present study provides new evidence that variation in the TSPO gene affects susceptibility to AD.

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