first report demonstrating decreased serum levels of BDNF in adult autistic patients, however, our data is inconsistent with previous reports (Miyazaki et al., 2004; Connolly et al., 2006). One possible reason for this discrepancy may be the difference in the age range of the subjects: 3 to 27 years (Miyazaki's study) and 5.9±3.9 years (Connolly's study) vs. 18 to 26 years in the present study. This may be significant since BDNF levels in the blood of rats (Karege et al., 2002) and healthy human subjects (Nelson et al., 2006) have been found to be significantly affected by age. Furthermore, serum BDNF levels of normal controls in our study were higher than those of other reports (Shimizu et al., 2003; Lommatzsch et al., 2005). At present, the reasons for this discrepancy are unknown. Recently, Lommatzsch et al. (2005) demonstrated a negative correlation between plasma BDNF levels and age. Therefore, a possibility for this discrepancy may be the difference in the age range of the subjects. Another possibility for this discrepancy may be the difference of methodological differences (e.g., time of sample collection, preparation of serum). Furthermore, there is a positive correlation between serum BDNF levels and cortical BDNF levels that continues from early maturation throughout the aging process (Karege et al., 2002). Taken together, it is likely that decreased levels of BDNF occur in the brain of autistic patients.

Accumulating evidence suggest the role of immune system in the pathophysiology of autism (Belmonte et al., 2004; Cohly and Panja 2005). Recently, it has been reported that IgG and IgM BDNF autoantibodies were elevated in children with autism (Connolly et al., 2006). Furthermore, BDNF is also produced by activated T-cells, B-cells, and monocytes (Kerschensteiner et al., 1999). Based on the role of immune system in the pathophysiology of autism, these findings suggest the unrecognized interaction between the immune system and BDNF in autism. Further studies underlying the role of BDNF in immune system will be necessary to examine the role of BDNF in the pathophysiology of autism.

It has been reported that social isolation (8 weeks) selectively reduced the BDNF levels in rat hippocampus whereas plasma corticosterone levels were not altered (Scaccianoce et al., 2006), suggesting that BDNF levels is responsive to psychological state (Hashimoto et al., 2004). Furthermore, we reported that serum BDNF levels in drug nave patients with major depressive disorders recovered to basal levels after antidepressant treatment (Shimizu et al., 2003). Therefore, it may be important to take the psychological states in subjects into consideration to unravel the role of BDNF in the pathophysiology of autism.

Given the critical role of BDNF in brain development, our findings lead us to the hypothesis that decreased levels of BDNF in the brain may contribute to the pathophysiology of autism. It is therefore of great interest to measure serum BDNF levels in children with and without autism in order to determine the role of BDNF as a serological marker in children who will go on to develop an autistic disorder.

5. Conclusions

The present study suggests that serum BDNF may be a biological marker for autism, and that reduced BDNF levels might play a role in the pathophysiology of this disorder. In the future, we hope to gain a more complete understanding of the role of the BDNF–TrkB pathway in the pathophysiology of autism in order to provide new perspectives on its treatment.

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Decreased serum levels of transforming growth factor-β1 in patients with autism

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Abstract

Background: The neurobiological basis for autism remains poorly understood. Given the key role of transforming growth factor-β1 (TGF-β1) in brain development, we hypothesized that TGF-β1 plays a role in the pathophysiology of autism. In this study, we studied whether serum levels of TGF-β1 are altered in patients with autism.
Methods: We measured serum levels of TGF-β1 in 19 male adult patients with autism and 21 age-matched male healthy subjects using enzyme-linked immunosorbent assay (ELISA).
Results: The serum levels (7.34±5.21 ng/mL (mean±S.D.)) of TGF-β1 in the patients with autism were significantly ($z=-5.106$, $p<0.001$) lower than those (14.48±1.64 ng/mL (mean±S.D.)) of normal controls. However, there were no marked or significant correlations between serum TGF-β1 levels and other clinical variables, including Autism Diagnostic Interview-Revised (ADI-R) scores, Yale-Brown Obsessive–Compulsive Scale (Y-BOCS), aggression, Theory of Mind, and Intellectual Quotient (IQ) in patients.
Conclusions: These findings suggest that decreased levels of TGF-β1 may be implicated in the pathophysiology of autism.

Keywords: Autism; Growth factor; Human serum; Neurodevelopmental disorder

1. Introduction

Autism is a neurodevelopmental disorder resulting in pervasive abnormalities in social interaction and communication, repetitive behaviors and restricted interests. However, the precise mechanisms underlying the pathophysiology of this disorder remain to be determined (Volkmar and Pauls, 2003; Baron-Cohen and Belmonte, 2005; Levitt et al., 2004).

Transforming growth factor betas (TGF-βs) are known as multifunctional growth factors, which participate in the regulation of key events of development, disease, and tissue repair (Böttner et al., 2000; Buisson et al., 2003; Gomes et al., 2005). TGF-β family is represented by a small group of multiple functional cytokines, consisting of three isoforms: TGF-β1, TGF-β2 and TGF-β3. Immunohistochemical and *in situ* hybridization studies have provided evidence for the widespread distribution of immunoreactive TGF-β2 and TGF-β3 and sites of their synthesis in the developing and adult central nervous system (CNS) and peripheral nervous system (PNS). These aspects appear to be of importance: (1) the virtual ubiquity of

Abbreviations: ADI-R, Autism Diagnostic Interview-Revised; AQ, Aggression questionnaire; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; IQ, intellectual quotient; PNS, peripheral nervous system; S.D., standard deviation; TGF-β1, transforming growth factor-β1; Y-BOCS, Yale-Brown Obsessive–Compulsive Scale.

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TGF-β in all areas of the CNS as well as in the PNS, (2) consistent coexpression of TGF-β2 and TGF-β3 in neurons, astroglia, and Schwann cells, and (3) the almost complete lack or low levels, respectively, of TGF-β1 in the unlesioned nervous system (Böttner et al., 2000). Within the brain, these three isoforms are produced by both glial and neuronal cells (Gomes et al., 2005). Among these isoforms, TGF-β1 is a potent immunosuppressive cytokine that can be expressed by virtually all cells of the body. Accumulating evidence suggest that TGF-β1 has emerged as a crucial regulator of nervous system physiology, although this cytokine has been widely considered an injury-related cytokine (Gomes et al., 2005). First, TGF-β1 and its receptor are expressed in the developing nervous system, suggesting the role of TGF-β1 in brain development (Böttner et al., 2000; Gomes et al., 2005). Second, the knock-out mice for TGF-β1 gene show severe impairment in cortical development with widespread increased neuronal cell death and microgliosis (Brionne et al., 2003). Third, in adult neural stem and progenitor cell cultures and after intracerebroventricular infusion, TGF-β1 induced a long-lasting inhibition of neural stem and progenitor cell proliferation and a reduction in neurogenesis, suggesting the potential implications for neurogenesis in a variety of TGF-β1 associated CNS diseases and pathologic conditions (Wachs et al., 2006). However, no studies demonstrating on the role of TGF-β1 in autism have been reported.

Considering the key role of TGF-β1 in brain development (Gomes et al., 2005), it is of great interest to study the role of TGF-β1 in the pathophysiology of autism. The purpose of the present study is to examine whether serum levels of TGF-β1 in autistic patients are altered as compared to age-matched normal controls. Furthermore, we also examined relationships between serum TGF-β1 levels and clinical variables in autistic patients.

2. Methods

2.1. Subjects

Nineteen male autistic subjects (23.4±2.6 years (mean±S.D.), 18–28 years (range)) and twenty-one age-matched male healthy control subjects (22.7±2.3 years (mean±S.D.), 18–26 years (range)) were included in this study (Table 1). All participants for both groups were Japanese. The autistic subjects were recruited through advocacy groups in Nagoya and Hamamatsu cities, which are located in the middle of the mainland of Japan. For diagnosis of autism, the recruited individuals were initially assessed according to the Diagnostic and Statistical Manual of Mental Disorders, Forth Edition (DSM-IV) (American Psychiatric Association, 1994), followed by assessment using the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994) by clinicians. Participants were excluded from the study, if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive–compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. All the autistic subjects were drug naive or free of psychoactive medications for at least 6 months: the majority of autistic participants have never previously received psychoactive medications, and the minority participants had been given sedatives more than 6 months before this study. Healthy controls were recruited from Hamamatsu City by advertisement. All control group participants underwent a comprehensive assessment of medical history to eliminate individuals with any neurological or other medical disorders. The Structured Clinical Interview for the DSM-IV (SCID) was also conducted to scrutinize any personal or familial history of past or present mental illness. None of the comparison subjects initially recruited was found to fulfill these exclusion procedures. This study received approval from the ethics committee of the Hamamatsu University School of Medicine. After the participants were given a complete description of the study, written informed consent was obtained from all subjects before they entered the study.

Table 1
Clinical characteristics of subjects

Characteristic	Control (n=21)	Autism (n=19)
Age, year	23.4±2.61 (18–26)	22.7±2.26 (18–28)
Gestational age at birth, week		38.63±1.54 (37–41)
ADI-R		
Domain A score		22.05±4.82 (14–29)
Domain BV score		15.69±4.82 (6–21)
Domain C score		5.16±1.74 (3–10)
Domain D score		2.94±1.06 (1–5)
Y-BOCS		10.76±5.08 (2–26)
Obsession		6.24±3.09 (1–14)
Compulsion		4.65±3.50 (0–14)
AQ—aggression		51.53±11.98 (33–69)
Theory of mind—Faux Pas Test		22.37±9.21 (3–34)
IQ		
Full scale IQ		97.65±20.65 (59–140)
Verbal IQ		96.00±20.11 (53–131)
Performance IQ		100.6±19.05 (40–137)

Values are expressed as mean±S.D. (range).
ADI-R: Autism Diagnostic Interview-Revised, Y-BOCS: Yale-Brown Obsessive–Compulsive Scale, AQ: Aggression Questionnaire, IQ: Intellectual Quotient.

2.2. Psychological measures

ADI-R is a semi-especially-formulated structured psychiatric interview with a parent, especially a mother, which is administered to the parent. It is used to confirm diagnosis and also to evaluate the core symptoms of autism. ADI-R is based on three separate scores. ADI-R domain score A quantifies impairment in social interaction (range of score: 0–32), domain score BV quantifies impairment in communication (range of score: 0–26), and domain score C quantifies restricted, repetitive, and stereotyped patterns of behavior and interests (range of score: 0–16). Higher scores on each indicate worse performance. ADI-R domain D corresponds to age of onset criterion for autistic disorder. If the score is 1 or higher, the subject is quite likely to have the age of onset earlier than or equal to 3 years old. All of the subjects with autism have age of onset no later than 3 years old since none had ADI-R domain D score lower than 1 (Table 1).

Obsessional/repetitive behavior was rated using the Yale-Brown Obsessive–Compulsive Scale (Y-BOCS) (Goodman

et al., 1989a,b); additional aggression symptoms were also assessed using the Aggression Questionnaire (AQ) (Buss and Perry, 1992). We used a Faux Pas Test to evaluate the function of "Theory of Mind" (*mentalizing*) (Baron-Cohen et al., 1999: Stone et al., 2003). The performance of individuals with autism on the *Faux Pas Test* is an experimental demonstration of their theory-of-mind deficit at a higher level. There were 40 points possible for *Faux Pas*—related questions about 10 stories (range: 0–40, 1 point for each question).

2.3. Procedures

The serum samples of autistic patients and normal comparison subjects were collected during 11:00–noon, and stored at −80 °C until assay. The serum levels of TGF-β1 were measured using TGF-β1 ELISA Kit (R&D Systems, Inc., Minneapolis, MN), which involved a sandwich enzyme-linked immunosorbent assay (ELISA) using anti-TGF-β1 monoclonal antibody and one enzyme-linked polyclonal antibody for TGF-β1. The assay was performed according to the supplier's recommendation. The calibrator consisted of recombinant human TGF-β1. All samples were measured in duplicates and respective mean value was calculated.

2.4. Statistical analysis

The data were presented as the mean±standard deviation (S.D.). The data were analyzed using a Mann–Whitney *U*-test. Evaluation of relationships between levels of TGF-β1 and clinical variables among patients with autism was determined by Pearson correlations or Spearman correlations. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

The serum levels (7.34±5.21 ng/mL (mean±S.D.)) of TGF-β1 in the patients with autism were significantly ($z=-5.106$, $p<0.001$) lower than those (14.48±1.64 ng/mL (mean±S.D.)) of normal controls (Fig. 1).

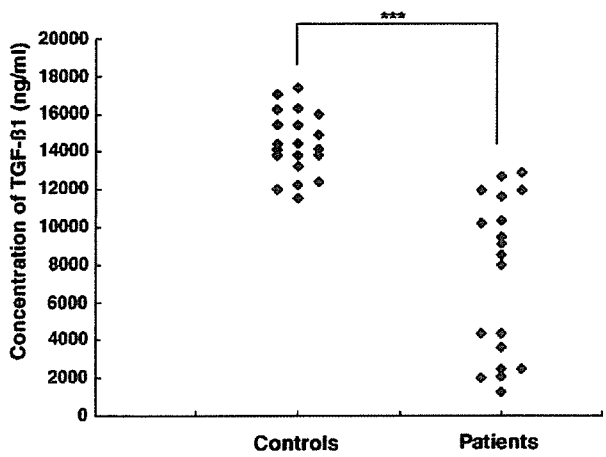

Fig. 1. The serum levels of TGF-β1 in autistic patients and normal controls. The serum levels of TGF-β1 in autistic patients ($n=19$) were significantly lower than those of normal controls ($n=21$). ***$p<0.001$ (Mann–Whitney *U*-test).

We then examined the correlations between serum TGF-β1 levels and clinical variables among patients with autism. There was a trend toward a positive correlation ($r=0.400$, $p=0.089$) between serum TGF-β1 levels and gestational age at birth in patients. However, there were no marked or significant correlations between serum TGF-β1 levels and other clinical variables, including ADI-R scores, Y-BOCS, aggression, Theory of Mind, and IQ.

4. Discussion

The finding of the present study is that serum levels of TGF-β1 in the patients with autism are significantly lower than those of age-matched normal healthy controls. To the best of our knowledge, this is the first report demonstrating the decreased serum levels of TGF-β1 in autistic patients. It remains unclear whether serum TGF-1β levels reflect the levels of TGF-1β in the brain since it is shown that TGF-β1 does not cross the intact blood–brain barrier (BBB) whereas TGF-β1 can cross the disrupted BBB (Kastin et al., 2003). Therefore, it may be of great interest to study whether cerebrospinal fluid (CSF) levels of TGF-1β are altered in autistic patients. Given the key role of TGF-β1 in brain development (Gomes et al., 2005), our findings lead us to the hypothesis that decreased levels of TGF-β1 may be implicated in the pathophysiology of autism although the result does not necessarily indicate either causation or its direction. Furthermore, it is also of interest to measure serum levels of TGF-β1 in children with and without autism in order to determine the role of TGF-β1 as a serological marker for children who will go on to develop autistic disorder.

A recent immunohistochemical study and ELISA assay using postmortem brain samples showed that a number of cytokines, and growth factors including TGF-β1 were altered in the middle frontal gyrus, anterior cingulated gyrus, and cerebellum in the patients with autism, although there was no difference in CSF levels of TGF-β1 between two groups (Vargas et al., 2005). These findings suggest that innate neuroimmune reactions play a pathogenic role in an undefined proportion of autistic patients (Vargas et al., 2005). Taken together, our findings suggest that TGF-β1 may play a role in the pathophysiology of autism but further work is necessary to study its precise role and how specifically TGF-β1 is linked to core autism. Given the sample size of the groups, the present results may not have the statistical power. Therefore, further studies using a large sample will be necessary.

Accumulating evidence suggests that brain-derived neurotrophic factor (BDNF) plays a role in the pathophysiology of psychiatric disorders, including schizophrenia, mood disorders, and eating disorders (Hashimoto et al., 2004, 2005a,b). It has been demonstrated that serum levels of BDNF were significantly correlated with serum levels of TGF-β1, suggesting that BDNF and TGF-β1 could be anatomically and functionally related in the human blood (Lommatzsch et al., 2005). Interestingly, we recently reported that serum levels of BDNF in patients with autism were significantly lower than those of

age-matched normal controls, suggesting that reduced BDNF levels may be implicated in the pathophysiology of autism (Hashimoto et al., 2007). In addition, we found a positive correlation ($r=0.738$, $p<0.001$) between serum BDNF levels and serum TGF-β1 levels in the same sample of patients, suggesting the possible relationship of BDNF and TGF-β1 in patients with autism. It has been reported that TGF-β1 enhances expression of BDNF and its receptor TrkB in neurons from rat cerebral cortex (Sometani et al., 2001), suggesting that BDNF may require TGF-β1 as a cofactor to exert its neurotrophic activities. Taken together, it is likely that decreased levels of TGF-β1 and BDNF may be implicated in the pathophysiology of autism although the precise mechanism(s) underlying the functional role of BDNF and TGF-β1 in the autism are currently unclear.

5. Conclusion

Our findings suggest that reduced levels of TGF-β1 might be implicated in the pathophysiology of autism. Further studies on the potential mechanisms and physiological implications of reduced TGF-β1 levels in autism will be necessary in the future.

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Decreased Serum Levels of Epidermal Growth Factor in Adult Subjects with High-Functioning Autism

Katsuaki Suzuki, Kenji Hashimoto, Yasuhide Iwata, Kazuhiko Nakamura, Masatsugu Tsujii, Kenji J. Tsuchiya, Yoshimoto Sekine, Shiro Suda, Genichi Sugihara, Hideo Matsuzaki, Toshiro Sugiyama, Masayoshi Kawai, Yoshio Minabe, Nori Takei, and Norio Mori

Background: *The neurobiological basis for autism remains poorly understood. Given the role of growth factors in brain development, we hypothesized that epidermal growth factor (EGF) may play a role in the pathophysiology of autism. In this study, we examined whether serum levels of EGF are altered in adult subjects with high-functioning autism.*

Methods: *We measured serum levels of EGF in the 17 male subjects with high-functioning autism and 18 age-matched healthy male subjects.*

Results: *The serum levels of EGF in the subjects with high-functioning autism (72.4 ± 102.8 pg/mL [mean ± SD]) were significantly lower (Mann-Whitney U = 22.0, $p < .001$) than those of normal control subjects (322.3 ± 122.0 pg/mL [mean ± SD]). However, there were no correlations between serum EGF levels and clinical variables in the subjects with autism.*

Conclusions: *This study suggests that decreased levels of EGF might be implicated in the pathophysiology of high-functioning autism.*

Key Words: Autism, developmental disorders, ELISA, epidermal growth factor, growth factors, human blood

Autism is a neurodevelopmental disorder resulting in pervasive abnormalities in social interaction and communication, repetitive behaviors, and restricted interests. However, the precise mechanisms underlying the pathophysiology of this disorder remains to be determined (Volkmar and Pauls 2003; Rubenstein and Merzenich 2003; Belmonte et al 2004; Polleux and Lauder 2004; Levitt et al 2004; Baron-Cohen and Belmonte 2005; Cohly and Panja 2005).

Epidermal growth factor (EGF) is a member of the EGF family of growth factors, and this growth factor binds to the epidermal growth factor receptor (EGFR) with high affinity. Epidermal growth factor is detected in the majority of neurons and in maturing astrocytes in the developing and adult brain of humans and different species of animals (Ferrer et al 1996; Xian and Zhou 1999). Recently, it has been demonstrated that levels of EGF are significantly decreased in the postmortem brains of schizophrenia (also a neurodevelopmental disorder) and that serum EGF levels in both the medicated and drug-free patients with schizophrenia are also markedly reduced as compared with normal control subjects (Futamura et al 2002). However, a recent study using a large sample revealed no changes of serum EGF levels in drug-naive patients or medicated patients with schizophrenia (Hashimoto et al 2005).

At present, no studies demonstrating alterations in the EGF in autism have been reported. Considering the role of EGF in brain development, it is of interest to study the role of EGF in the pathophysiology of autism. The purpose of the present study is to examine whether serum levels of EGF in adult subjects with high-functioning autism are altered as compared with age-matched normal control subjects. Furthermore, we also examined any relationship between serum levels of EGF and clinical variables in autistic patients.

Methods and Materials

This study received approval from the ethics committee of the Hamamatsu University School of Medicine. After the participants were given a complete description of the study, written informed consent was obtained from all subjects before they entered the study.

Seventeen male autistic subjects (23.1 ± 2.52 years [mean ± SD], 19–28 years [range]) and 18 age-matched male healthy control subjects (23.0 ± 2.03 years [mean ± SD], 20–26 years [range]) were included in this study (Table 1). All participants for both groups were Japanese. The autistic subjects were recruited through advocacy groups in Nagoya and Hamamatsu, which are cities located in the middle of the mainland of Japan. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al 1994), Japanese version. One of the authors (K.J.T.), having established reliability of diagnosing autism with the authors, conducted the interview for all subjects. Then, based on the results, a DSM-IV (American Psychiatric Association 1994) diagnosis of autistic disorder was made for all subjects. We also conducted the Wechsler Adult Intelligence Scale-Revised (WAIS-R) to exclude subjects with a full-scale intelligence quotient (IQ) of less than 70, resulting in a group of 17 subjects with high-functioning autism. Participants were excluded from the study if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive-compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. All the autistic subjects were drug naive or had been free of psychoactive medications for at least 6 months: the majority of autistic participants ($n = 12$) had never previously received psychoactive medications and a minority ($n = 5$) of participants had been given sedatives more than 6 months before this study. Healthy control subjects were recruited from Hamamatsu City by advertisement. All control group participants underwent a comprehensive assessment of their medical history to eliminate individuals with any neurological or other medical disorders. The Structured Clinical Interview for DSM-IV (SCID) was also conducted to scrutinize any personal or family history of

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Table 1. Clinical Characteristics of Normal Control Subjects and Subjects with High-Functioning Autism

Group (*n*)	Control Subjects (18)	High-Functioning Autism (17)
Age (Year)	23.0 ± 2.03 (20–26)	23.1 ± 2.52 (19–28)
EGF (pg/mL)	322.3 ± 122.0 (120.7–539.8)	72.4 ± 102.8 (.10–320.2)[b]
ADI-R		
Domain A Score		22.5 ± 4.8 (14–29)
Domain BV Score		16.5 ± 3.8 (9–22)
Domain C Score		5.3 ± 1.8 (3–10)
Domain D Score		3.7 ± 1.1 (1–5)
Y-BOCS (Total Score)		11.2 ± 5.6 (2–26)
Hamilton Depression Scale Score		2.4 ± 3.7 (0–15)
Hamilton Anxiety Scale Score		4.1 ± 3.3 (0–11)
AQ Total Score		50.6 ± 12.7 (34–69)
Faux Pas Test-Theory of Mind		23.4 ± 8.8 (3–34)
WAIS-R (Full-Scale IQ)		98.9 ± 18.9 (71–140)
Gestational Age (Week)[a]		38.8 ± 1.7 (34–41)
Birth Weight (g)[a]		3382 ± 502 (2376–4148)
Head Circumference at Birth (cm)[a]		34.0 ± 2.3 (29.2–37.6)

Values are expressed as mean ± SD (range).

EGF, epidermal growth factor; ADI-R, Autism Diagnostic Interview-Revised; Y-BOCS, Yale-Brown Obsessive Compulsive Scale; AQ, Aggression Questionnaire; WAIS-R, Wechsler Adult Intelligence Scale-Revised.

[a]One subject had no available information.

[b]$p < .001$ as compared with control (Mann-Whitney *U* test).

past or present mental illness. None of the comparison subjects initially recruited was found to fulfill these exclusion procedures.

Serum samples of autistic patients and normal comparison subjects were collected during 11:00 AM to noon and stored at −80°C until assay. Serum levels of EGF were measured using EGF ELISA Kit (R&D Systems, Inc., Minneapolis, Minnesota). The data were presented as the mean ± SD. The data were analyzed using the Mann-Whitney *U* test. Among patients with autism, relationships between serum EGF levels and clinical variables were determined by Pearson or Spearman correlations. A *p* value of less than .05 was considered to be statistically significant.

Results

The serum levels of EGF in the subjects with high-functioning autism (72.4 ± 102.8 pg/mL [mean ± SD]) were significantly lower (Mann-Whitney $U = 22$, $p < .001$) than those of normal control subjects (322.3 ± 122.0 pg/mL [mean ± SD]) (Table 1 and Figure 1). We then examined the correlations between serum EGF levels and clinical variables among subjects with autism. There were no marked or significant correlations between serum EGF levels and clinical variables, including ADI-R scores, Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) scores, Hamilton Depression Scale scores, Hamilton Anxiety Scale scores, aggression, Faux Pas Test-Theory of Mind scores, WAIS-R scores, gestational age, birth weight, and head circumference at birth (Table 1).

Discussion

The finding of the present study is that serum levels of EGF in the adult subjects with high-functioning autism were significantly lower than those of age-matched normal healthy control subjects. To the best of our knowledge, this is the first report demonstrating the decreased serum levels of EGF in autism. It has been reported that ^{125}I-labeled epidermal growth factor (^{125}I-EGF) crosses the blood-brain barrier (BBB) rapidly and that the fast rate of influx is significantly decreased by co-administration of nonradiolabeled EGF, suggesting that a rapid, saturable, and unidirectional transport system on the BBB enables EGF to enter the brain (Pan and Kastin 1999). Therefore, it is likely that decreased levels of EGF may occur in the brain of autistic patients.

Accumulating evidence suggests the role of immune signaling in the pathophysiology of autism (Belmonte et al 2004; Cohly and Panja 2005). It has been reported that excessive production of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 receptor antagonist (IL-1RA) with stimulation of endotoxin lipopolysaccharide is shown in children with autism spectrum disorders (Croonenberghs et al 2002; Jyonouchi et al 2002, 2005), suggesting that increased production of proinflammatory cytokines could play a role in the pathophysiology of autism. A recent study using postmortem brain samples showed abnormality of proinflammatory and anti-inflammatory cytokines and growth factors in the brains with autism, suggesting that innate neuroimmune reactions play a pathogenic role in an underlined proportion of autistic patients (Vargas et al 2005). Taken together, it is also likely that abnormality of growth factors, including EGF, may be implicated in the mechanisms associated with neural dysfunction in autism, although further studies using a large sample will be necessary.

Recently, we reported that serum levels of brain-derived neurotrophic factor (BDNF) in adult subjects with autism were significantly lower than those of age-matched normal control subjects (Hashimoto et al, in press), inconsistent with previous reports (Nelson et al 2001; Connolly et al 2006). One possible reason for this discrepancy may be the difference in the age range of the subjects: neonatal blood in the Nelson et al (2001) study and 5.9 ± 3.9 years in the Connolly et al (2006) study versus 18 to 26 years in the (Hashimoto et al, in press) study. This may be significant, since BDNF levels in the blood of human subjects have been found to be significantly affected by age (Nelson et al 2006). In contrast, the cerebrospinal fluid (CSF) levels of nerve growth factor (NGF) in children with infantile autism were not altered, whereas CSF levels of NGF were decreased in children with Rett syndrome, suggesting that CSF levels of NGF could be used as a biochemical marker for differentiation of patients with autism from those with Rett syndrome (Riikonen and Vanhala 1999). Taken together, these

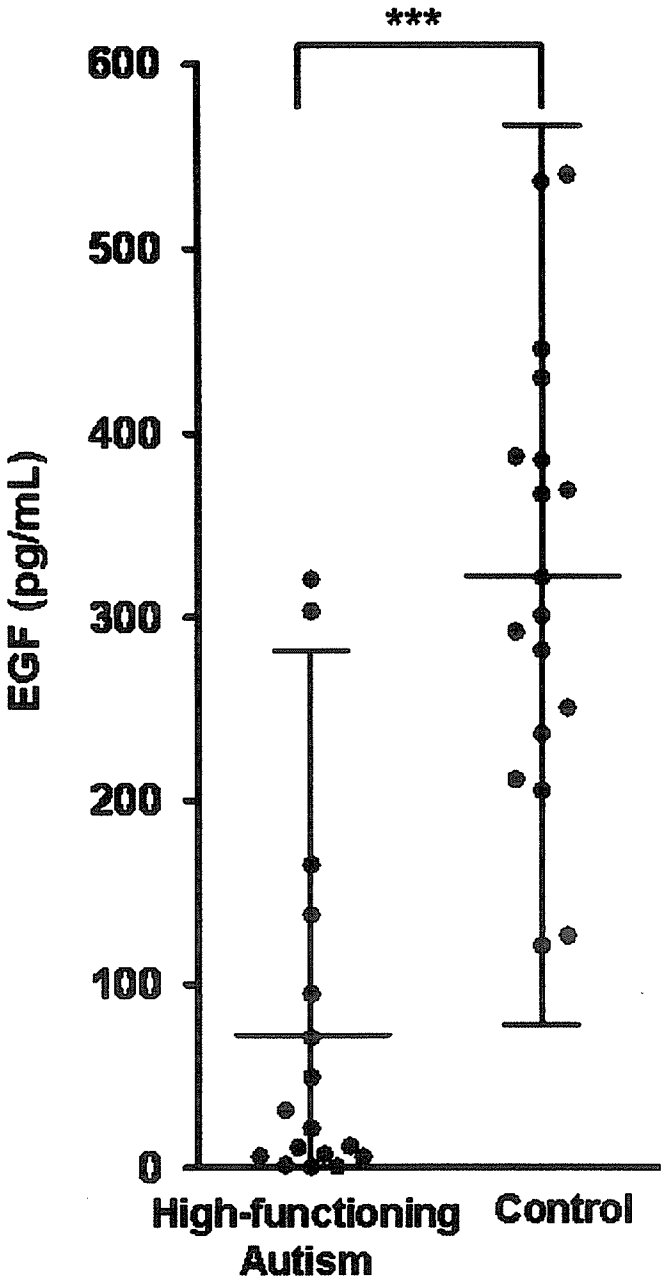

Figure 1. The serum levels of EGF in subjects with high-functioning autism and normal control subjects. The serum levels of EGF in subjects with autism (n = 17) were significantly lower than those of normal control subjects (n = 18). The bars show the mean ± 2 SD. ***$p < .001$ (Mann-Whitney U test). EGF, epidermal growth factor.

findings suggest that reduced levels of growth factors and/or neurotrophic factors, including EGF and BDNF, may play a role in the pathophysiology of autism.

Given the key role of EGF in brain development, our findings lead us to the hypothesis that decreased levels of EGF may be implicated in the pathophysiology of high-functioning autism. It is, therefore, of interest to measure serum levels of EGF in children with and without autism to determine the role of this growth factor as a serological marker for children who will go on to develop autistic disorder.

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Decreased serum levels of hepatocyte growth factor in male adults with high-functioning autism

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Abstract

Background: The mechanisms underlying the pathophysiology of autism are currently unknown. Given the role of hepatocyte growth factor (HGF) in brain development, we hypothesized that HGF plays a role in the pathophysiology of autism. In this study, we studied whether serum HGF levels are altered in subjects with high-functioning autism.
Methods: Using an enzyme-linked immunosorbent assay (ELISA), we measured serum levels of HGF in 17 male adults with high-functioning autism and age-matched 18 male healthy subjects.
Results: The serum levels (503.5±160.5 pg/mL (mean±SD)) of HGF in the subjects with high-functioning autism were significantly (Mann–Whitney $U=34.0$, $p<0.001$) lower than those (817.6±232.4 pg/mL (mean±SD)) of control subjects. However, there were no correlations between serum HGF levels and clinical variables in the patients.
Conclusions: This study suggests that reduced HGF levels may play a role in the pathophysiology of high-functioning autism.

Keywords: Autism; Developmental disorders; ELISA; Hepatocyte growth factor; Human blood

1. Introduction

Autism is a neurodevelopmental disorder characterized by severe and sustained impairment in social interaction, deviance in communication, and patterns of behavior and interest that are restricted, stereotyped, or both. However, the precise mechanisms underlying the pathophysiology of this disorder remain to be determined (Volkmar and Pauls, 2003; Levitt et al., 2004; Baron-Cohen and Belmonte, 2005).

Hepatocyte growth factor (HGF) is a polypeptide growth factor which acts by binding to the MET tyrosine kinase receptor. HGF influences the growth, motility and morphogenesis of various epithelial and endothelial cells and functions as a trophic factor for organ regeneration (Maina and Klein, 1999; Levitt et al., 2004). Accumulating evidence suggest that HGF and its receptor MET play a role in neuronal cell development (Maina and Klein, 1999; Levitt et al., 2004). First, HGF and its receptor MET are widely expressed in the developing and mature mouse brain, with expression beginning as early as embryonic day 12 (E12) and E13, respectively (Achim et al., 1997; Maina and Klein, 1999). Second, HGF promotes the migration

Abbreviations: ADI-R, Autism Diagnostic Interview-Revised; ELISA, enzyme-linked immunosorbent assay; GABA, γ-aminobutyric acid; HGF, hepatocyte growth factor; IQ, Intelligence Quotient; MCHH, Mother and Child Health Handbook; uPAR, urokinase plasminogen activator receptor; Y-BOCS, Yale–Brown Obsessive Compulsive Scale.

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of cortical interneurons from the ventral to the dorsal telencephalon in rodents (Powell et al., 2001), and HGF-MET signaling systems are implicated in regulating the proliferation and differentiation of cerebellar granule cells (Ieraci et al., 2002). Furthermore, HGF plays a role in regulating the morphology of cortical pyramidal dendrites in the early postnatal period, and endogenous levels of HGF are necessary for the normal development of these neurons (Gutierrez et al., 2004). Taken together, these findings suggest that HGF may be a candidate for mediating interneuron development *in vivo* (Levitt et al., 2004).

Given the key role of HGF in brain development, it is of great interest to study the role of HGF in the pathophysiology of autism. In this study, we therefore studied whether serum HGF levels in subjects with high-functioning autism are altered as compared with age-matched healthy controls. Furthermore, we also examined the relationship between serum HGF levels and clinical symptoms in subjects with autism.

2. Methods

2.1. Subjects

This study was approved by the Ethics Committee of the Hamamatsu University School of Medicine. After the participants were given a complete description of the study, written informed consent was obtained from all subjects before they entered the study.

One of the authors (M.T.) coordinates a self-help group for subjects with autism and their families, "Asupe-erude-no-kai" (the Association for Asperger Syndrome and Learning Disorders), in Nagoya, Japan. Seventeen male subjects with high-functioning autism were recruited from this group and enrolled in this study. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 1994), Japanese version. One of the authors (K.J.T.) having established reliability of diagnosing autism with the authors conducted the interview for all subjects, and then, based on the results, a DSM-IV (American Psychiatric Association, 1994) diagnosis of Autistic Disorder was made for all subjects. ADI-R is a semi-specially-formulated structured psychiatric interview with a parent, especially a mother, which is administered to the parent. It is used to confirm diagnosis and also to evaluate the core symptoms of autism. ADI-R is based on three separate scores. ADI-R domain score A quantifies impairment in social interaction (the range of score: 0–32), domain score BV quantifies impairment in communication (the range of score: 0–26), and domain score C quantifies restricted, repetitive, and stereotyped patterns of behavior and interests (the range of score: 0–16). Higher scores on each indicate worse performance. The cut-off scores of domain score A, domain score BV, and domain score C are 10, 8, and 3, respectively. All of the subjects with autism have scores above the cut-off scores. ADI-R domain D corresponds to age of onset criterion for autistic disorder. If the score is 1 or higher, the subject is quite likely to have the age of onset prior to 3 year old. All of the subjects with autism have age of onset no later than 3 year old since none has ADI-R domain D score lower than 1 (Table 1).

Table 1
Clinical characteristics of normal controls and subjects with high-functioning autism

Group (n)	Control (18)	High-functioning autism (17)
Age (year)	23.0±2.03 (20–26)	23.1±2.52 (19–28)
HGF (pg/mL)	817.6±232.4 (552–1243)	503.5±160.5 (302–841)***
ADI-R		
Domain A score, social		22.5±4.8 (14–29)
Domain BV score, communication		16.5±3.8 (9–22)
Domain C score, stereotype		5.3±1.8 (3–10)
Domain D score, age of onset		3.7±1.1 (1–5)
Y-BOCS (total score)		11.2±5.6 (2–26)
Hamilton Depression Scale score		2.4±3.7 (0–15)
Hamilton Anxiety Scale score		4.1±3.3 (0–11)
AQ total score		50.6±12.7 (34–69)
Faux Pas test — Theory of Mind		23.4±8.8 (3–34)
WAIS-R (full-scale IQ)		98.9±18.9 (71–140)
Gestational age (week)[a]		38.8±1.7 (34–41)
Birth weight (g)[a]		3382±502 (2376–4148)
Head circumference at birth (cm)[a]		34.0±2.3 (29.2–37.6)

ADI-R: Autism Diagnostic Interview-Revised, Y-BOCS: Yale–Brown Obsessive Compulsive Scale, AQ: Aggression Questionnaire, WAIS-R: Wechsler Adult Intelligence Scale-Revised.
Values are expressed as mean±SD (range).
*** $p<0.001$ as compared to control (the Mann-Whitney U test).
[a] One subject had no available information.

We also conducted the WAIS-R to exclude subjects with a full-scale Intelligence Quotient (IQ) of less than 70, resulting in a group of 17 subjects with high-functioning autism. Participants were excluded from the study, if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive–compulsive disorder, affective disorders, or any additional psychiatric or neurological diagnoses. All the subjects with autism were drug naive or free of psychoactive medications for at least 6 months: the majority of participants with autism have never previously received psychoactive medications, and the minority participants had been given sedatives more than 6 months before this study. Eighteen subjects who had no developmental delay and no history of psychiatric disorders or treatment joined our study as controls. All control group participants underwent a comprehensive assessment of medical history to eliminate individuals with any neurological or other medical disorders. None of the comparison subjects initially recruited was found to fulfill these exclusion procedures. There was no significant age difference between the control subjects and the subjects diagnosed with high-functioning autism (Table 1). All participants for both groups were Japanese.

2.2. Psychological measures

In addition to the IQ and ADI-R assessments, we adopted the Yale–Brown Obsessive Compulsive Scale (Y-BOCS) (Goodman

et al., 1989a,b), the Aggression Questionnaire (Buss and Perry, 1992), the Faux Pas test (Baron-Cohen et al., 1999; Stone et al., 2003), the Hamilton Depression Scale (HAM-D) (Hamilton, 1960), and the Hamilton Anxiety Scale (HAM-A) (Hamilton, 1969) to evaluate clinical and psychological correlates of the subjects with high-functioning autism. To extract early developmental factors, we made use of the Mother and Child Health Handbook (MCHH). The MCHH is a special notebook provided to each pregnant woman in Japan for recording the child's medical information throughout pregnancy, delivery, and early development. Subjects with high-functioning autism were asked to submit the MCHH, which was used to collect information on gestational week, birth weight and head circumference at birth.

2.3. Measurement of serum HGF levels

Serum samples of subjects with autism and normal comparison subjects were collected during 11:00-noon, and stored at −80 °C until assay. Serum HGF levels were measured using HGF enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, Inc., Minneapolis, MN). The assay was performed according to the supplier's recommendation. The calibrator consisted of recombinant human HGF. All samples were measured in duplicates and respective mean value was calculated.

2.4. Statistical analysis

The data were presented as the mean±standard deviation (SD). The data were analyzed using the Mann–Whitney U test. The relationship between HGF levels and clinical variables among subjects with autism was determined by Spearman correlations. A p value of less than 0.05 was considered to be statistically significant.

3. Results

The serum levels (503.5±160.5 pg/mL) of HGF in the subjects with high-functioning autism were significantly (Mann–Whitney $U=34.0, p<0.001$) lower than those (817.6±232.4 pg/mL) of normal controls (Table 1 and Fig. 1). However, there were no marked or significant correlations between serum HGF levels and clinical variables, including ADI-R subscale scores, Y-BOCS total score, Hamilton Depression Scale score, Hamilton Anxiety Scale score, Aggression Questionnaire total score, Faux Pas test score, Full-scale IQ, gestational age, birth weight, and head circumference at birth (Table 1).

4. Discussion

In this study, we found that serum HGF levels in subjects with high-functioning autism were significantly lower than those of age-matched normal controls. To the best of our knowledge, this is the first report demonstrating the decreased serum levels of HGF in subjects with autism. Although it remains unclear whether serum HGF levels reflect the levels of HGF in the brain, it is possible that decreased levels of HGF may occur in the brain of subjects with high-functioning autism since exogenous HGF prevented neuronal cell death in the hippocampal CA1 region after transient global ischemia (Miyazawa et al., 1998). Recently, it has been reported that cerebrospinal fluid levels of HGF in subjects with autism (aged 3–10 years, $n=6$) are significantly higher than those of normal control (aged 12–45 years, $n=9$) (Vargas et al., 2005). However, age of subjects enrolled in that paper was not matched to normal controls (Vargas et al., 2005), indicating that further studies using age-matched large samples are necessary. Given the key role of HGF in brain development (Maina and Klein, 1999; Levitt et al., 2004), our findings lead us to the hypothesis that decreased levels of HGF in the brain may contribute to the pathophysiology of autism. In this study, serum HGF levels between autistic subjects and controls were overlapped. Furthermore, we did not find any correlations between serum HGF levels and clinical variables in the autistic subjects, suggesting that serum HGF levels may be a trait marker, not a state marker, in subjects with autism. Nonetheless, it may be of interest to measure serum HGF levels in children with and without autism in order to determine the role of HGF as a serological marker in children who will go on to develop an autistic disorder. In addition, the mechanism by which decreased HGF levels play a role in the pathophysiology of autism remains to be established.

Subjects with autism are at greater risk for developing seizure disorders, particularly in adolescence (Tuchman and Rapin, 2002; Volkmar and Pauls, 2003). Multiple lines of evidence suggest that seizure is caused by imbalance excitatory and inhibitory

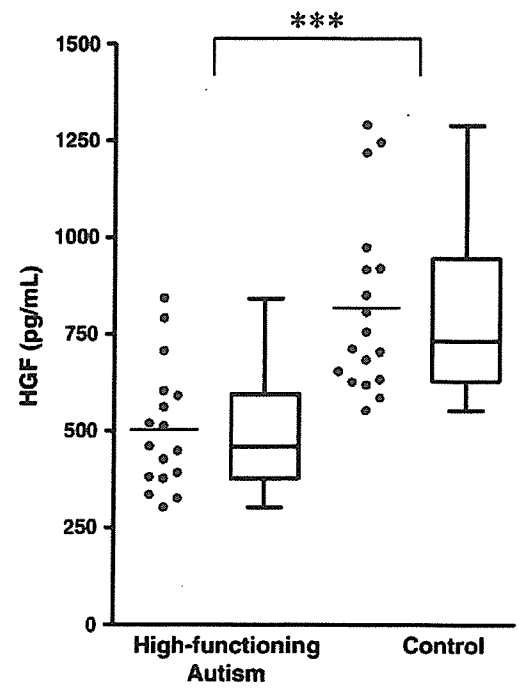

Fig. 1. The serum levels of HGF in normal controls and subjects with high-functioning autism. The serum levels of HGF in subjects with autism ($n=17$) were significantly lower than those of normal controls ($n=18$). The bar in the dot plot shows the mean values. The boxes represent the median, the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits, respectively). ***$p<0.001$ (Mann–Whitney U test).

neurotransmitter systems in brain, and that γ-aminobutyric acid (GABA) interneurons play a critical role in maintaining this balance (Levitt et al., 2004). The pathophysiology in autism is currently unknown, but there is interesting convergence of data to suggest a regional disruption of interneuron development (Levitt et al., 2004). Mice with a targeted mutation of the gene encoding urokinase plasminogen activator receptor (uPAR), a key component in HGF activation and function, have decreased levels of HGF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages (Powell et al., 2003). Behavioral test of *uPAR* (−/−) mice showed increased anxiety in three paradigms (open field, light–dark exploration, and the elevated plus maze), and spontaneous myoclonic seizures and a greater susceptibility to pharmacologically induced convulsion (Powell et al., 2003). Furthermore, the mutation of *uPAR* gene results in interneuron loss and behaviors similar to human epilepsy, autism and anxiety disorders (Powell et al., 2003; Levitt et al., 2004). These findings suggest that disruption of the HGF-MET signaling systems results in complex alterations in GABAergic interneuron development in the forebrain (Levitt et al., 2004). Taken together, it is likely that decreased HGF levels may be implicated in the high rates of seizure disorder in autism although further studies on the role of HGF-MET signaling systems on the high rate of seizure in autism are required for investigation of its pathological roles in autism.

5. Conclusion

In conclusion, this study suggests that reduced HGF levels might be implicated in the pathophysiology of high-functioning autism. Further studies on the molecular and cellular implications of HGF in the pathophysiology of autism will be necessary.

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Genetic analyses of the brain-derived neurotrophic factor (BDNF) gene in autism

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Abstract

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. Brain-derived neurotrophic factor (BDNF) plays a critical role in the pathogenesis of autism. In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study (the Autism Genetic Resource Exchange). We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects. In the TDT of autism trios, the SNP haplotype combinations showed significant associations in the autism group. BDNF expression in the drug-naïve autistic group was found to be significantly higher than in the control group. We suggest that BDNF has a possible role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system.

Keywords: Autism; Brain-derived neurotrophic factor; Serotonin transporter; A trios-based association; Peripheral blood lymphocytes

Autism is a severe neurodevelopmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that are detectable in early childhood. The serotonergic system has been found to be developmentally dysregulated in autism; factors that regulate serotonergic neuronal development and serotonin metabolism might have a crucial role in the pathophysiology of autistic disorders caused by the dysfunction of the serotonergic system [1]. Specifically, altered developmental dynamics of serotonin synthesis [2,3] and increases in whole blood serotonin levels have been reported in autistic individuals [4,5]. Effective medications for treating autistic symptoms include drugs that have an impact on the serotonergic system, such as the serotonin receptor antagonists (e.g. Risperidone) and serotonin depleting agents (e.g. Fenfluramine) [6–8].

Multiple lines of evidence suggest that brain-derived neurotrophic factor (BDNF) plays a critical role in the serotonergic function. In the rat brain, BDNF has been found to promote the survival and sprouting of serotonergic axons [9] and the axonal growth of injured serotonergic neurons

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[10,11]. In vitro and in vivo studies support a regulatory role of BDNF in the survival and maturation of serotonergic neurons [12,13]; BDNF has also been shown to modulate serotonergic neurotransmission in vitro [14]. In addition, BDNF administration has been found to increase the synthesis and/or turnover of serotonin in vivo [15–18].

BDNF has a detrimental effect on the aforementioned processes, and has been implicated in the pathogenesis of neurodevelopmental disorders like autism. Specifically, elevated BDNF expression has been observed in the brain [19], blood [20] and serum [21,22] of autistic individuals, compared to healthy controls. Recently, we found that the serum levels of BDNF in patients with autism were significantly lower than those of normal controls [23].

In this study, we examined the SNP- and haplotypic-association of BDNF with autism in a trios-based association study. We also examined the expression of BDNF mRNA in the peripheral blood lymphocytes of drug-naïve autism patients and control subjects, since lymphocytes are now considered to be a convenient and accessible alternative to brain samples for use in biochemical and genetic investigations of the functions of the central nervous system [24].

Materials and methods

Association study

Subjects. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine.

DNA samples from trios families recruited to the Autism Genetic Resource Exchange (AGRE; http://www.agre.org) were used for this study [25]. We selected trios families, with male offspring scored for autism; additional selection criteria required that (i) there be no possible non-idiopathic autism flag and (ii) all the trios be Whites. Two sets of samples were used in this study; the first set consisted of 104 high-functioning autism (HFA) trios, with the affected offspring having an intelligence quotient (IQ) > 70, whereas, the second set consisted of randomly chosen trios with no IQ information.

Genotyping. The genomic structure of BDNF is based on the UCSC May 2004 draft assembly of the human genome (http://www.genome.ucsc.edu). The BDNF gene is located in 11p14, spanning a genomic stretch of 66.8 kb (mRNA 1580 bases). SNPs were selected based on information from the National Centre for Biotechnology Information (NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP), The SNP Consortium (TSC: http://www.snp.cshl.org) and the International HapMap Project (http://www.hapmap.org). On the basis of their genomic locations and the minor allele frequencies (MAF) in the Caucasian population, 25 SNPs were chosen for our analysis in order to span the BDNF gene as evenly as possible. Assay-on-demand/Assay-by-design SNP genotyping products (Applied Biosystems, Foster City, CA, USA) were used to score the SNPs based on the TaqMan assay method [26]. Genotypes were determined using the ABI 7900 Sequence Detection System (SDS) (Applied Biosystems) and analyzed using SDS v2.0 software (Applied Biosystems). The SNPs used in the study and their locations are shown in Table 1.

Statistical analysis. PedCheck program v1.1 (http://www.watson.hgen.pitt.edu) was used to identify and eliminate all Mendelian inconsistencies in the trios data set. Markers were tested for association by the conventional transmission disequilibrium test (TDT) using the TDTPHASE program of the UNPHASED software package v2.403 (http://www.hgmp.mrc.ac.uk). All of the three-, four-, and five-marker haplotypes were tested for association in a sliding window across the locus. The option 'drop rare haplotypes' was used to restrict the analysis of haplotypes with a frequency <3%. Pair-wise linkage disequilibrium (LD) between the various markers, based on D' (linkage disequilibrium coefficient) values [27], was estimated using the Haploview software v3.2 (http://www.broad.mit.edu/mpg/haploview); an LD plot was also constructed using this software.

Gene expression analysis

Lymphocyte RNA. The study was approved by the Ethics Committee of the Hamamatsu University School of Medicine. Written informed consent was obtained from each participant after having been provided an explanation of the study procedures. We obtained blood samples from 11 drug-naïve autism patients (age 21.4 ± 2.31 years [mean ± SD]) and 13 age-matched (22.3 ± 1.93) healthy controls. All the patients and control subjects were males, and were of Japanese origin.

The autism patients were diagnosed according to the Autism Diagnostic Interview-Revised (ADI-R) by trained and certified psychiatrists (K.T.,A.S.) [28]. All of the patients met the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria (American Psychiatric Association, 1994) [29] and International Classification of Diseases, 10th Revision (ICD-10; World Health Organization, 1992) [30], criteria for autism. The patients underwent screening, and were excluded if they had any major medical- or psychiatric-conditions; they had been drug-naïve for at least 6 months. Comorbid anxiety and depressive symptoms were assessed using the Hamilton Anxiety Rating Scale (HAM-A) [31] and the Hamilton Depression rating scale (HAM-D) [32], respectively. Obsessional/repetitive behaviors were clinically rated using the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [33,34]. Additional aggression symptoms were also assessed using the aggression questionnaire (AQ) [35]. A faux pas detection task was used to measure theory of mind (ToM) [36,37]. All of the evaluations were conducted by a trained research psychiatrist (K.N.).

All of the controls were free of medications, and underwent screening to exclude neurological-, developmental-, or psychiatric-disorders and mental retardation; none of them met any of the relevant criteria of DSM-IV.

Peripheral blood (20 ml) was drawn from the cubital vein into EDTA-containing plastic syringes. Lymphocytes were isolated from blood samples by the Ficoll-Paque gradient method, and total RNA was extracted using RNAzolB reagent (Sawady, Tokyo, Japan) according to the manufacturer's instructions. RNA samples were quantified by analyzing the absorbance at 260 nm in a UV-spectrophotometer. Complementary DNA (cDNA) was synthesized by first strand reverse transcriptase reaction (RT) using Random Primer and M-MLV reverse transcriptase (Invitrogen, CA, USA).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR): Real-time qRT-PCR analysis was performed using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). TaqMan primer/probes for BDNF and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand™ gene expression products Hs00156058 and Hs99999905, respectively). All reactions were performed in duplicate according to the manufacturer's protocol. A comparative threshold cycle (C_T) method validation experiment was done to check whether the efficiencies of the target and reference amplifications were approximately equal (the slope of the log input amount versus $\Delta C_T < 0.1$). One sample was randomly chosen as a calibrator, and was amplified in each plate to correct for experimental differences among consecutive PCR runs. The amounts of BDNF mRNA were normalized to the endogenous reference, and were expressed relative to the calibrator as $2^{-\Delta\Delta C_T}$ (comparative C_T method).

Statistical analysis. Statistical calculations were performed using SPSS statistical package, version 11.0.1 (SPSS Co. Ltd., Tokyo, Japan) and GraphPad Prism, version 4.00 (GraphPad Software, San Diego, CA, USA). The difference in BDNF expression between the groups was analyzed using the t-test. The correlation between various clinical features and BDNF expression was examined using Pearson's correlation coefficient.

Table 1
Single SNP TDT results of BDNF SNPs

Marker	dbSNP ID	Variation[a]	Location (NCBI B34)	Minor allele frequency[b]	HFA trios (IQ > 70)		Random trios	
					T (%)[c]	*p*-value	T (%)[c]	*p*-value
SNP01	rs1491851	A/G	27717072	0.459	47.77	0.4	50.72	0.753
SNP02	rs727155	G/A	27714758	0.044	50.25	0.586	50	1
SNP03	rs1491850	A/G	27714034	0.436	47.66	0.226	50.6	0.74
SNP04	rs908867	G/A	27710073	0.093	49.73	0.75	50.47	0.479
SNP05	rs12273363	A/G	27709168	0.166	50.14	0.893	50.4	0.658
SNP06	rs11030121	G/A	27700516	0.266	49.92	0.961	50.26	0.832
SNP07	rs7934165	C/T	27696292	0.473	52.91	0.239	49.68	0.869
SNP08	rs2030324	T/C	27691224	0.473	52.68	0.281	49.68	0.869
SNP09	rs988748	C/G	27689054	0.264	52.69	0.567	50.18	0.887
SNP10	rs2049046	A/T	27688084	0.458	53.3	0.17	49.29	0.708
SNP11	rs7127507	A/G	27679193	0.28	49.65	0.83	50.59	0.646
SNP12	rs7103411	A/G	27664434	0.264	49.21	0.545	50.03	0.98
SNP13	rs2049045	C/G	27658550	0.226	51.88	0.719	50.18	0.869
SNP14	rs1401635	C/G	27658300	0.268	52.73	0.495	50.61	0.625
SNP15	rs11030104	T/C	27648826	0.256	49.41	0.642	50.37	0.759
SNP16	rs6265 (V66M)	G/A	27644225	0.226	49.7	0.799	49.94	0.957
SNP17	rs7124442	A/G	27641350	0.27	48.97	0.51	50	1
SNP18	rs1519480	A/G	27640021	0.274	49.07	0.554	50.23	0.854
SNP19	rs4923463	T/C	27636809	0.259	49.38	0.624	50.37	0.76
SNP20	rs2203877	A/G	27635219	0.461	53.59	0.141	49.22	0.68
SNP21	rs10501087	A/G	27634417	0.259	49.38	0.624	50.37	0.76
SNP22	rs1519479	G/A	27631840	0.468	54.27	0.092	49.28	0.709
SNP23	rs925946	C/A	27631511	0.263	48.16	0.212	50.37	0.76
SNP24	rs11030096	T/C	27629852	0.461	54.19	0.092	49.29	0.708
SNP25	rs4923461	A/G	27621219	0.256	49.41	0.642	50.14	0.906

HFA, high-functioning autism; T, transmitted.
[a] Common allele is listed first.
[b] Based on the parental genotypes of random trios.
[c] T percentage of common allele is listed.

Results

Association study

Single SNP TDT

TDT was done separately for the HFA trios and for the random trios; the results are shown in Table 1. None of the SNPs showed a significant association in the HFA trios or random trios.

Haplotype TDT

The results of haplotype TDT for HFA- and random trios are shown in Table 2. The three-SNP haplotype combination of SNP04-SNP05-SNP06 ($p = 0.017$), the four-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07 ($p = 0.02$) and the five-SNP haplotype combination of SNP04-SNP05-SNP06-SNP07-SNP08 ($p = 0.02$) showed significant associations in the random group; however, the global values were not significant. None of the three-, four- or five-SNP haplotypes showed significant association in the HFA trios.

LD analysis

LD analysis identified a single haplotype block across the BDNF gene, comprising SNPs 03–25 (Fig. 1).

Lymphocyte gene expression analysis: BDNF expression in the drug-naïve autistic (0.094 ± 0.1 [mean $\pm$ SD]) group was found to be significantly higher than in the control (0.034 ± 0.02) group ($t = -2.2$; $df = 22$; $p = 0.039$) (Fig. 2). No significant correlation was observed between any of the clinical features and BDNF expression in the autistic group (Table 3).

Discussion

In the present study, we reported the haplotypic association of BDNF with autism; three-, four-, and five-SNP haplotypes consisting of SNP04 (rs908867), SNP05 (rs12273363) and SNP06 (rs11030121) showed significant associations with random trios. Furthermore, we found that BDNF expression in the drug-naïve autism group was significantly higher than in the control group. To the best of our knowledge, this is the first report demonstrating an association and increased BDNF expression in drug-naïve autism subjects.

The BDNF Val66Met polymorphism (SNP16 in this study) has been reported to be associated with obsessive-compulsive disorder [38], attention deficit hyperactivity disorder [39] and anxiety-related personality traits [40]; this SNP has also been suggested to have a role in the

Table 2
Three-, four- and five-SNP haplotype analysis of BDNF

Three-SNP[a]	*p*-value[b]		Four-SNP[a]	*p*-value[b]		Five- SNP[a]	*p*-value[b]	
	HFA trios	Random trios		HFA trios	Random trios		HFA trios	Random trios
1-2-3	0.151	0.863	1-2-3-4	0.151	0.947	1-2-3-4-5	0.197	0.931
2-3-4	0.658	0.698	2-3-4-5	0.73	0.8	2-3-4-5-6	0.67	0.887
3-4-5	0.499	0.822	3-4-5-6	0.622	0.068	3-4-5-6-7	0.531	0.071
4-5-6	0.698	***0.017***	4-5-6-7	0.576	***0.02***	4-5-6-7-8	0.611	***0.02***
5-6-7	0.721	0.937	5-6-7-8	0.766	0.937	5-6-7-8-9	0.833	0.914
6-7-8	0.636	0.98	6-7-8-9	0.717	0.987	6-7-8-9-10	0.52	0.968
7-8-9	0.635	0.987	7-8-9-10	0.47	0.968	7-8-9-10-11	0.52	0.956
8-9-10	0.49	0.968	8-9-10-11	0.539	0.956	8-9-10-11-12	0.539	0.965
9-10-11	0.634	0.918	9-10-11-12	0.634	0.932	9-10-11-12-13	0.752	0.996
10-11-12	0.634	0.932	10-11-12-13	0.752	0.996	10-11-12-13-14	0.565	0.975
11-12-13	0.702	0.985	11-12-13-14	0.53	0.958	11-12-13-14-15	0.547	0.963
12-13-14	0.538	0.948	12-13-14-15	0.586	0.95	12-13-14-15-16	0.612	0.907
13-14-15	0.681	0.933	13-14-15-16	0.659	0.892	13-14-15-16-17	0.193	0.943
14-15-16	0.686	0.89	14-15-16-17	0.201	0.94	14-15-16-17-18	0.234	0.922
15-16-17	0.555	0.966	15-16-17-18	0.601	0.958	15-16-17-18-19	0.596	0.958
16-17-18	0.582	0.998	16-17-18-19	0.596	0.964	16-17-18-19-20	0.61	0.966
17-18-19	0.43	0.931	17-18-19-20	0.444	0.92	17-18-19-20-21	0.444	0.92
18-19-20	0.4	0.916	18-19-20-21	0.4	0.916	18-19-20-21-22	0.447	0.916
19-20-21	0.352	0.912	19-20-21-22	0.41	0.912	19-20-21-22-23	0.42	0.822
20-21-22	0.41	0.912	20-21-22-23	0.42	0.822	20-21-22-23-24	0.42	0.822
21-22-23	0.32	0.829	21-22-23-24	0.314	0.829	21-22-23-24-25	0.314	0.845
22-23-24	0.253	0.926	22-23-24-25	0.314	0.985			
23-24-25	0.305	0.985						

[a] Based on all possible haplotypes for each combination of SNPs.
[b] Computed on the basis of likelihood ratio test. Significant *p*-values (<0.05) are indicated in bold italics.

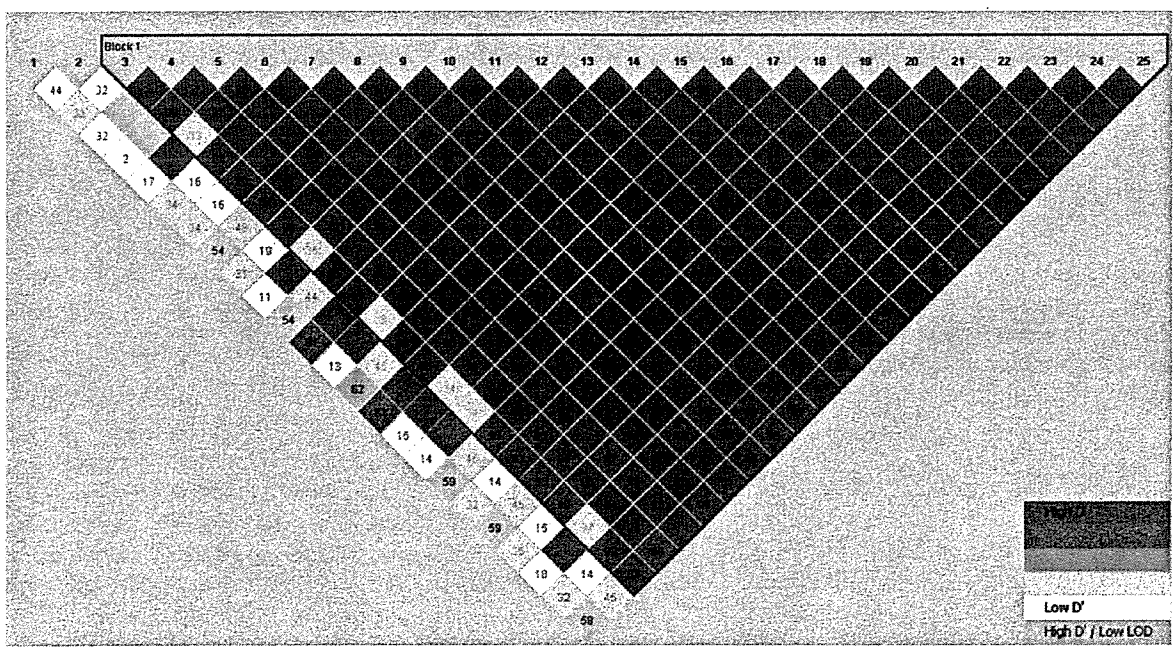

Fig. 1. Haplotype block structure of BDNF based on *D'* values calculated from 148 random trios.

hippocampal and prefrontal cortex functions involved in human memory and learning [41,42]. However, we did not observe any significant association of Val66Met with autism.

Several lines of evidence suggest that BDNF hyperactivity can be deleterious to the neurodevelopmental process, and it has been implicated in the pathophysiology of neurodevelopmental disorders like autism. Specifically, Nelson et al. [20] reported higher BDNF levels in the archived samples of neonatal blood from autistic children compared to normal controls. This finding was further supported by observations of higher concentrations of BDNF in the

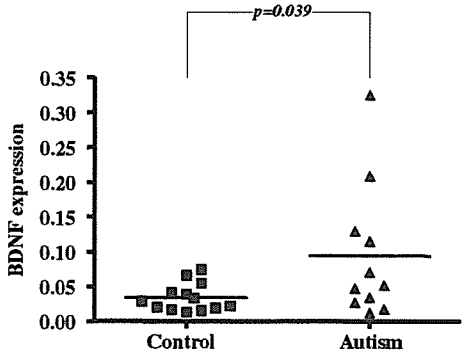

Fig. 2. *t*-test comparison of BDNF mRNA levels in the lymphocytes from control subjects and drug-naïve autism patients. Horizontal bars indicate means. A significant difference in BDNF expression was observed between the two groups ($p = 0.039$).

Table 3
Correlation between clinical features and lymphocyte BDNF expression in the autism group

Clinical feature	Pearson (*r*)	*p*-value
HAM-A	−0.199	0.581
HAM-D	−0.223	0.536
Y-BOCS	−0.141	0.699
Obsession	−0.223	0.536
Compulsion	−0.046	0.899
Aggression questionnaire	0.185	0.610
Faux pas test	0.231	0.530

basal forebrain [19] and in the serum [21,22] of autistic patients compared to healthy controls. In this present study, enhanced BDNF m-RNA expression in the lymphocytes was observed in the drug-naïve autistic group compared to the control group. Therefore, BDNF hyperactivity could result in disruption of the normal developmental program in the brain, leading to abnormalities like overgrowth of brain- and neuronal-tissues, which has been observed in autistic individuals [43–47].

There was no significant correlation between BDNF expression and any of the clinical features of the autistic group. Hence, it may be suggested that elevated BDNF expression is indicative of the disease state per se, and is not dependent on the clinical features of the disease.

Since BDNF has a proven role in regulating the structural [9–13] and functional aspects [14–18] of serotonergic neurons, its hyperactivity might cause dysfunction of the serotonergic system. In a B lymphoblast model, which had several molecular and functional similarities to serotonergic neurons, BDNF treatment was found to decrease serotonin uptake by serotonin transporters, thereby increasing extracellular serotonin levels [48].

Given the critical role of BDNF in brain development, our findings lead us to the hypothesis that enhanced levels of BDNF may contribute to the pathophysiology of autism. It is therefore of great interest to measure BDNF levels in children with autism in order to determine the role of BDNF as a serological marker in children who will go on to develop an autistic disorder.

In conclusion, we suggest that BDNF hyperactivity may play a role in the pathogenesis of autism through its neurotrophic effects on the serotonergic system. Moreover, this is the first report of a genetic association between BDNF and autism; however, replication of these findings and further studies of the functional impact of BDNF in autism are warranted.

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Decreased Serum Levels of Platelet-Endothelial Adhesion Molecule (PECAM-1) in Subjects with High-Functioning Autism: A Negative Correlation with Head Circumference at Birth

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Background: Accumulating evidence suggests that the immune system plays a role in the pathophysiology of autism, and that the adhesion molecules play an important role in the process of inflammation. This study was undertaken to determine whether serum levels of the adhesion molecules in subjects with high-functioning autism are altered as compared with those of normal controls.

Methods: Seventeen male subjects with high-functioning autism and 22 male age-matched unrelated healthy control subjects were enrolled. Serum levels of the soluble forms of platelet-endothelial adhesion molecule (PECAM-1), intracellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1) were measured.

Results: Levels of PECAM-1, but not ICAM-1, in the subjects with autism were significantly lower than those of control subjects. VCAM-1 showed a weak trend for a lowered level. There was a negative correlation between serum levels of PECAM-1 and head circumference at birth in the autistic subjects.

Conclusions: These results suggest that PECAM-1 plays a role in the pathophysiology of high-functioning autism.

Key Words: Adhesion molecules, developmental disorders, ELISA, head circumference, high-functioning autism, human blood

Autism is a developmental disorder that is characterized by severe impairment in social interaction and communication and by the presence of stereotyped behaviors. The mechanisms underlying the pathophysiology of this disorder remain to be determined (Baron-Cohen and Belmonte 2005; Volkmar and Pauls 2003). However, accumulating evidence suggests that the immune system plays a role in the pathophysiology of autism (Cohly and Panja 2005; Krause *et al.* 2002; Pardo *et al.* 2005).

Adhesion molecules are localized both on the membranes of activated platelets and leukocytes and on the vascular endothelium. They mediate the binding of leukocytes to the blood vessel wall, which is the main step in the process of inflammation (Blankenberg *et al.* 2003; Lee and Benveniste 1999). Intracellular adhesion molecule ICAM-1 is widely expressed at a basal level and can be up-regulated by pro-inflammatory cytokines in leukocytes and endothelial cells. The vascular cell adhesion molecule VCAM-1 is transcriptionally induced on endothelial cells but can also be expressed by other cell types, such as macrophages, myoblasts, and dendritic cells. The platelet-endothelial adhesion molecule PECAM-1 is particularly dense at the junctions between endothelial cells, where it mainly participates in homophilic binding between adjacent cells. Circulating levels of soluble-forms of adhesion molecules have been shown to be increased in various inflammation-related diseases, such as atherosclerosis and multiple sclerosis (Blankenberg *et al.* 2003), although it is not known whether adhesion molecules play roles in the pathophysiology of other disorders occurring in central nervous system (CNS).

Considering the key role played by adhesion molecules in immune responses in the CNS (Blankenberg *et al.* 2003; Lee and Benveniste 1999), it would be of interest to study the role of adhesion molecules in the pathophysiology of autism. The purpose of this study was to examine whether serum levels of ICAM-1, VCAM-1, and PECAM-1 in subjects with high-functioning autism would be altered as compared to those in age-matched normal controls. In addition, we examined the relationships between serum levels of adhesion molecules and clinical variables in autistic subjects.

Methods and Materials

One of the authors (MT) coordinates a self-help group for subjects with autism and their families, "Asupe-erude-no-kai" (the Association for Asperger Syndrome and Learning Disorders), in Nagoya, Japan. Seventeen male subjects with high-functioning autism were recruited from this group and enrolled in this study. The diagnosis of autism was made on the basis of the Autism Diagnostic Interview-Revised (ADI-R)(Lord *et al.* 1994), Japanese version. One of the authors (KJT) having established reliability of diagnosing autism, conducted the interview for all subjects, and then, based on the results, a DSM-IV (American Psychiatric Association 1994) diagnosis of autistic disorder was made for all subjects. Five of the authors (KJT, YI, YS, GS, MK) confirmed,

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