Virus	Strain	518	530	540	550			579
Measles	Edmonston	RGR	CNKKGEQVGM	SRPGLKPDLT	GTSKSYVRSL			
	Toyoshima	***	**********	**********	**********			
	Nagahata	***	**********	**********	**********			
	Masusako	***	**********	**********	**********			
SSPE	Osaka-1 Fr/V	***	***N******	***DPN**P*	*********R	RSSTTLETQM	SHKSPLRHQA	TTASSTKPT
	Osaka-1 Fr/H	***	***N******	***DPN**P*	*********R	RSSTTLETQM	SHKSPLRHQA	TTASSTKPT
	Osaka-1 Oc/V	***	***N******	***DPN**P*	*********R	RSSTTLETQM	SHKSPLRHQA	TTASSTKPT
	Osaka-2 Fr/V	***	*********I	****P***P*	**P**HA***			
	Osaka-2-Fr/B	***	**********	****P***P*	**P**HA***			
	Osaka-3 Bs/V	***	****E*****	***D******	*****			
	Osaka-3 Bs/B	***	****E*****	***D******	*****			
	Osaka-3 Oc/V	***	****E*****	***D******	*****			
	Biken	***	******					
	Yamagata-1	***	***RENKLVC	QDQA				
	IP-3-Ca	***	******					
	Patient C	***	**********	**********	**********			

Fig. 2. Deduced amino acid sequence of the carboxyl terminal region (the cytoplasmic tail) of the F protein of the measles and SSPE viruses. Asterisks indicate amino acids identical to the corresponding residues of the Edmonston strain. Positions relative to the second AUG codon of the open reading frame of the F protein are indicated at the top. The F protein sequences of the Biken, IP-3-Ca, and Yamagata-1 strains of SSPE viruses were from Watanabe et al. (1995), Cattaneo et al. (1989) and Komase et al. (1990), respectively.

tivity. There was no common amino acid substitution found only among the three SSPE strains, and the substitutions were scattered in the extracellular domain of the protein.

Concerning the cytoplasmic domain of the F protein, however, all three strains of SSPE virus were markedly affected, whereas the extent and the mode of alteration differed among the strains (Fig. 2). The predicted F protein sequences of the three sibling viruses of the Osaka-1 strain showed six nonconservative amino acid substitutions, in addition to elongation of the C-terminus by 29 residues. In contrast, C-terminal truncation with two nonconservative substitutions was found in the sequences of the three sibling viruses of the Osaka-3 strains. The Osaka-2 Fr/V and Osaka-2 Fr/B sibling viruses of the Osaka-2 strain had six and five amino acid substitutions, respectively, showing neither elongation nor truncation.

The common feature among the F genes of the SSPE viruses was the overall conservation of the F protein-coding capacity despite the predicted numerous amino acid substitutions. This contrasts with the mutation of the M gene of SSPE viruses, which severely affected the expression of the M protein (Cattaneo et al., 1986, 1987, 1988a,b; Ayata et al., 1989, 1998a,b; Seto et al., 1999). Biased hypermutation, which was commonly found in the M genes of the SSPE virus Osaka-1, -2, and -3 strains (Ayata et al., 1998a), was not obvious in the F genes of the same strains. A subtilisin-related protease cleavage site at amino acid residues 108–112 and the hydrophobic region located at amino acid residues 113–136, which has been postulated to play an important role in fusion activity (Richardson et al., 1986), was perfectly conserved. Cysteine residues used for the disulfide bond formation at amino acid residues 68 and 195 (Griffin and Bellini, 1996) were also conserved. There was no amino acid substitution at the potential sites of glycosylation (at amino acid residues 29, 61, and 67) or of palmitoylation (at amino acid residues 503, 515, and 521) (Caballero et al., 1998). An A to G transition generated an arginine to glycine substitution at residue 70, which was found in two of the three sibling viruses of the Osaka-3 strain, might be significant for viral survival in the brain based on the fact that amino acid residue 70 plays a role in the dominant antigenic site of the F protein (Fayolle et al., 1999). In addition, no common substitutions in the F protein sequences were found

among the four SSPE viruses including the Biken strain (Watanabe et al., 1995), an SSPE virus closely related to the Osaka-1 and -3 strains (Ayata et al., 1998b).

When the predicted amino acid sequences of the F proteins were compared in order to consider the evolutionary relationship between the Osaka-3 Bs/V (or the Osaka-3 Bs/B) and the Osaka-3 Oc/V sibling viruses, a newly created premature termination and eight substitutions, for 14 and 14 amino acid differences between the Nagahata strain and the Osaka-3 Bs/V, and between the Nagahata strain and the Osaka-3 Oc/V, respectively (Table 2), were common to the two sibling viruses. Six and six amino acid substitutions independently occurred in the Osaka-3 Bs/V and the Osaka-3 Oc/V sibling viruses, respectively. Therefore, it is likely that the Osaka-3 Bs/V and the Osaka-3 Oc/V viruses emerged separately, according to the progression of SSPE, from an intermediate ancestor that contained the common substitutions.

As previously indicated by Schmid et al. (1992), it is noteworthy that the cytoplasmic tail of the F protein was found to be largely affected in all three strains of SSPE viruses, whereas the extent and the mode of alteration were different among the strains. These variations might have been the result of selectivity for persistence in the brain. Thirty-three residues of the cytoplasmic tail of the F protein were identical among the known wild-type MVs, whereas the predicted F protein of three sibling viruses of the Osaka-1 strain had six nonconservative amino acid substitutions in this region. Further, a U–C transition at the authentic termination codon for the F protein of MV resulted in elongation of the C-terminus by 29 residues. Similar elongation of the cytoplasmic tail was found in the brain of SSPE patient P (Schmid et al., 1992). In contrast, in the sequence of the Osaka-3 strain, a U–G transversion generated a premature termination codon and resulted in a truncation of the C-terminus by five residues, in addition to two nonconservative substitutions. Truncations of the cytoplasmic tail were very common, and were found in the brains of SSPE patients A, B, F, G, O, R, and S (Schmid et al., 1992), as well as in the F protein of the Biken (Watanabe et al., 1995) and the IP-3-Ca strains (Cattaneo et al., 1989). The C-terminus of the Yamagata-1 strain was also truncated and had an altered reading frame (Komase et al., 1990). Although the termination codon conserved for the F protein of the Osaka-2 strain was similar to that found in the brains of SSPE patients D and K (Schmid et al., 1992), five and six substitutions were predicted in the F protein of the sibling viruses of Osaka-2 Fr/B and Osaka-2 Fr/V, respectively. These changes could greatly alter the primary and the secondary structure of the cytoplasmic tail of the F protein, and would affect possible interaction with other proteins such as M, H, and N to alter the virus assembly within membrane rafts, virus release (Naim et al., 2000; Vincent et al., 2000), and fusogenic activity. Cathomen et al. (1998b) reported that the recombinant MV with altered cytoplasmic tails of the envelope glycoproteins produced a larger syncytium than that of its parental MV. In addition, M-less MV showed enhanced syncytium forming ability and restricted virus production (Cathomen et al., 1998a), suggesting that F and H proteins need to interact M protein at the cell membrane. Since all of our SSPE strains are defective M protein expression, both acquired characters of M protein defectiveness and altered cytoplasmic tail of the F protein might be much of significance in developing of MV into SSPE virus in patient brains. The reason why the F genes with a highly mutated cytoplasmic tail are selected in the brains of SSPE patients is not fully understood. An exception was the F protein analyzed directly from a cloned gene from SSPE patient C, a patient with measles inclusion body encephalitis (MIBE), in which the cytoplasmic tail was not altered (Cattaneo et al., 1989). Although the characteristics of the viruses isolated from SSPE and MIBE patients might be similar (Roos et al., 1981; Ohuchi et al., 1987), the mechanisms of the emergence or the evolution of the virus might be different. It is of great interest that the recombinant virus with an SSPE virus-like cytoplasmic tail of the F protein penetrated more deeply into brain parenchyma but demonstrated rather reduced neurovirulence to mice when it was inoculated into the brains of CD46 transgenic mice (Cathomen et al., 1998b). Studies

of mutations commonly found in SSPE viruses using reverse genetics technology will provide further evidence to clarify our understanding of the molecular mechanisms of MV persistence in the brain.

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ORIGINAL ARTICLE

Akemi Tanaka · Masatsugu Kimura
Hoàng Thi Ngoc Lan · Natsuko Takaura
Tunekazu Yamano

Molecular analysis of the α-N-acetylglucosaminidase gene in seven Japanese patients from six unrelated families with mucopolysaccharidosis IIIB (Sanfilippo type B), including two novel mutations

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Abstract Molecular analysis of the α-N-acetylglucosaminidase gene in seven Japanese patients with Sanfilippo syndrome type B from six unrelated families was carried out, and six disease-causing mutations were found. The parents of Patient 2 had a consanguinous marriage, but other families did not have any record of consanguinity. Two families were from Okinawa Island, where more patients with Sanfilippo syndrome were found than in other areas in Japan. Patients 1 and 6 showed the most severe phenotype with rapid progression. Patients 2, 5, and 7 were moderate. Patients 3 and 4 (sib cases) showed an attenuated form compared with other patients. Patients 1, 2, and 6 were homozygous for R482W, R565W, and R565P, respectively. Patients 3 and 4 were compound heterozygous for F314L and R565P. Patient 5 had delTG2171–2172 in exon 6 in one allele, and the other allele was unknown. Patient 7 was compound heterozygous for V241M and R482W. The family of Patients 3 and 4 and that of Patient 6 are unrelated, although both families are from Okinawa Island, and the patients have the same mutation, R565P; thus, R565P might be a common mutation in the Okinawa district. F314L and V241M are novel mutations.

Key words Sanfilippo syndrome type B · Mucopolysaccharidosis type IIIB · α-N-acetylglucosaminidase · Molecular analysis · Japanese patients · Okinawa district

A. Tanaka (✉) · H.T.N. Lan · N. Takaura · T. Yamano
Department of Pediatrics, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
Tel. +81-6-6645-3816; Fax +81-6-6636-8737
e-mail: akemi-chan@med.osaka-cu.ac.jp

M. Kimura
Radioisotope Center, Osaka City University Graduate School of Medicine, Osaka, Japan

Introduction

Sanfilippo syndrome, mucopolysaccharidosis type III, is characterized by severe central nervous system degeneration, but shows only mild somatic disease. Such disproportionate involvement of the central nervous system is unique among mucopolysaccharidoses. Patients with Sanfilippo syndrome are classified into four types based on deficient enzymes: heparan N-sulfatase (type A), α-N-acetylglucosaminidase (type B), acetyl CoA: α-glucosaminide acetyltransferase (type C), and N-acetylglucosaminide 6-sulfatase (type D) (Neufeld and Muenzer 2001). Type A is the most common subtype, and type B is the second most common among Sanfilippo syndrome patients in northern Europe (OMIM #252900 and #252920) and in Australia (type A:type B = 1.9:1) (Weber et al. 1999). However, among Japanese patients, type B is more frequent than type A (type A:type B = 1:1.2) according to the registered members of the Japanese Society of the Patients and the Families with Mucopolysaccharidoses.

The α-N-acetylglucosaminidase gene has been isolated and characterized (Zhao et al. 1996; Weber et al. 1996), and many disease-causing mutations have been reported (Bunge et al. 1999; Coll et al. 2001; Emre et al. 2002). Type B is reported to be phenotypically heterogenous with severe and mild forms (Zhao et al. 1998; Weber et al. 1999) compared with type A, although most of the disease-causing mutations reported so far have been for the severe phenotype.

This is the first report of mutation analysis of the α-N-acetylglucosaminidase gene in Japanese patients with Sanfilippo type B.

Patients and methods

Patients

Patient 1 was an 8-year-old boy. He showed speech delay at the age of 2 years, and the diagnosis of Sanfilippo type B

was made when he was 4 years old. He showed coarse facial features, joint stiffness, and hepatomegaly. He deteriorated rapidly: He could speak only one-word sentences when he was 3 years old, he could not speak at all when he was 5 years old, and he could not walk at the age of 6 years.

Patient 2 was a male patient who died of respiratory infection at the age of 26 years. He was noted to have hyperactivity and speech delay at the age of 3 years. He was diagnosed with Sanfilippo type B at the age of 4 years. He showed coarse facial features and joint stiffness. He could not talk at the age of 9 years, seizures appeared when he was 13 years old, he became bedridden at the age of 15 years, and nasal tube feeding was instituted at the age of 19 years.

Patients 3 and 4 were siblings aged 20 and 18 years, respectively. Neither patient showed abnormal facial features. Patient 3, a brother, was noted to have delay of speech at the age of 3 years, and showed hyperactivity at the age of 5 years. He could talk and walk until the age of 8 and 14 years, respectively. Seizures appeared at the age of 10 years. Patient 4, a sister, was noted to have hyperactivity at the age of 6 years. She could not talk when she was 14 years old, and sleep disorders appeared at the age of 16 years. She could walk and had no seizures at the age of 18 years.

Patient 5 was a 9-year-old boy. He showed coarse facial features and developmental delay at the age of 3 years, and showed hyperactivity at the age of 6 years. He could walk but could not talk at the age of 9 years.

Patient 6 was a 5-year-old girl. She was noted to have speech delay and hyperactivity at the age of 2 and 3 years, respectively. She showed coarse facial features. She could speak one-word sentences at the age of 5 years.

Patient 7 was a 5-year-old boy. He had a large skull and hepatomegaly, and showed hypertrichosis. He showed a delay of speech but no hyperactivity at the age of 5 years.

The parents of Patient 2 had a consanguinous marriage, but other families did not have any record of consanguinity.

α-N-acetylglucosaminidase assay

The activity of α-N-acetylglucosaminidase was analyzed in cultured skin fibroblasts (Patients 1, 3, 4, and 6) or in lymphocytes (Patients 2, 5, and 7) by the method described previously (Marsh and Fenson 1985) using the fluorogenic substrate of 4-methylumbelliferyl-α-N-acetylglucosaminide (Sigma-Aldrich, Tokyo, Japan).

Molecular analysis

Cultured fibroblasts were used in Patients 1, 3, 4, and 6, and peripheral white blood cells were used in Patients 2, 5, and 7. Genomic DNA was extracted by the standard method. Each exon of the α-N-acetylglucosaminidase gene was amplified by polymerase chain reaction (PCR) according to the method of Schmidtchen et al. (1998) with some modifications. PCR products were sequenced by the direct sequencing method using a capillary sequencer ABI PRISM 310 Genetic Analyzer (Perkin Elmer Japan/ABI, Chiba, Japan) with a dRhodamin Terminator Cycle Sequencing Kit from the same company.

Table 1. α-N-acetylglucosaminidase activity in six patients with MPS IIIB

Subject	Activity (nmol/mg/h)	
	Fibroblasts	Lymphocytes
Normal control (n)	9.02 ± 2.29 (n = 6)	3.01 ± 0.90 (n = 6)
Patient 1	0.595	NA
Patient 2[a]	NA	0.673
Patient 3[b,c]	0.734	NA
Patient 4[b,c]	0.854	NA
Patient 5	NA	0.449
Patient 6[c]	0.169	NA
Patient 7	NA	0.109

MPS, Mucopolysaccharidoses; NA, not available
[a] Parents are cousins
[b] Siblings
[c] From Okinawa district

Results

The activity of α-N-acetylglucosaminidase in each patient as analyzed in cultured skin fibroblasts or peripheral blood lymphocytes is shown in Table 1. No significant difference of enzyme activity among the patients was seen. The results of the molecular analysis are summarized in Table 2. Patients 1, 2, and 6 were homozygous for R482W (CGG→TGG; exon 6), R565W (CGG→TGG; exon 6), and R565P (CGG→CCG; exon 6), respectively. Patients 3 and 4 were compound heterozygous for F314L (TTC→TTG; exon 5) and R565P (CGG→CCG; exon 6). Patient 5 had a two-nucleotide deletion in exon 6 (2171–72 TG) in one allele, and the mutation of the other allele was unknown. Patient 7 had V241M (GTG→ATG; exon 4) in one allele, and R482W (CGG→TGG; exon 6) in the other. Four polymorphisms were found in the noncoding region. They included g1352insC (5′ end of the gene; 14/14 alleles), a2259A→C (intron 1; 10/14 alleles), g2304insA (intron 1; 14/14 alleles), and g2739G→C (intron 2; 12/14 alleles).

Discussion

In patients with Sanfilippo syndrome, type B occurs more frequently than type A (type A:type B = 1:1.2) among the Japanese, according to the registered members in the Japanese Society of the Patients and the Families with Mucopolysaccharidoses. Moreover, type B occurs more frequently in the Okinawa district in Japan. We examined molecular defects of the α-N-acetylglucosaminidase gene in seven patients with Sanfilippo type B from six unrelated families, including two families from Okinawa. We found six distinct disease-causing mutations including five missense mutations and one two-base deletion.

The mutations R482W, R565W, R565P, and delTG2171–2172 have been reported previously (Weber et al. 1999; Bunge et al. 1999; Coll et al. 2001) in patients from other ethnic groups with severe phenotypes of type B. The mutations R482W and R565W were caused by a C-to-T transi-

Table 2. Mutations and polymorphisms in the α-N-acetylglucosaminidase gene

Subject	Mutation	Polymorphism (frequency in normal individuals) g1352insC (14/14)	a2259A→C (8/14)	g2304insA (14/14)	g2739G→C (11/14)
Patient 1					
Allele 1	R482W	(+)	(+)	(+)	(+)
Allele 2	R482W	(+)	(+)	(+)	(+)
Patient 2					
Allele 1	R565W	(+)	(−)	(+)	(+)
Allele 2	R565W	(+)	(−)	(+)	(+)
Patient 3					
Allele 1	R565P	(+)	(+)	(+)	(+)
Allele 2	F314L	(+)	(+)	(+)	(+)
Patient 4					
Allele 1	R565P	(+)	(+)	(+)	(+)
Allele 2	F314L	(+)	(+)	(+)	(+)
Patient 5					
Allele 1	delTG2171–2172	(+)	(−)	(+)	(−)
Allele 2	Unknown	(+)	(−)	(+)	(−)
Patient 6					
Allele 1	R565P	(+)	(+)	(+)	(+)
Allele 2	R565P	(+)	(+)	(+)	(+)
Patient 7					
Allele 1	V241M	(+)	(+)	(+)	(+)
Allele 2	R482W	(+)	(+)	(+)	(+)

tion at a CpG hot spot. R482W has been reported in a Turkish patient with the severe phenotype as a homozygote (Bunge et al. 1999). Weber et al. (1999) reported that R565W accounted for 6% of mutant alleles in Australian patients with Sanfilippo type B. Each of R565P and delTG2171–2172 has been found in 1 allele of 50 mutant alleles among Australian patients (Weber et al. 1999).

R565P was found in two unrelated families from the Okinawa district, and the parents of Patient 6, who was homozygous for R565P, had no record of consanguinity, although both parents come from Okinawa. This mutation was a nucleotide substitution from CGG to CCG, which was neither a CpA nor a TpG change although it was at a CpG site. Moreover, four polymorphisms studied in this report were completely the same in these three alleles carrying R565P. Thus, R565P might be a common mutation in Okinawa. However, these polymorphisms did not necessarily indicate that R565P in these patients originated from a common ancestor, because each polymorphism was frequent in the Japanese population.

F314L and V241M are novel mutations. F314L may cause an attenuated phenotype in Patients 3 and 4, because the other allele in these patients was R565P, which was known to cause a severe Sanfilippo disease. Patient 6 was homozygous for R565P and showed a severe phenotype. Although a wide clinical spectrum and allelic heterogeneity of Sanfilippo type B has been reported, only a few mutations were shown to cause attenuated clinical forms so far. For example, R643C was shown by a clinical survey in Dutch patients to cause the attenuated phenotype (Weber et al. 1999), and F48L was shown to have significant residual enzyme activity by in vitro gene expression study (Yogalingam et al. 2000).

Four polymorphisms were found. Two of them, g2304insA and g2739G→C, were also reported in non-Japanese individuals (Zhao et al. 1998; Tessitore et al. 2000). The frequencies of g2304insA and g2739G→C were 14/14 and 11/14 in the Japanese, and 2/36 and 20/36 in non-Japanese (Zhao et al. 1998), respectively. Two other polymorphisms, g1352insC (14/14) and a2259A→C (8/14), were frequently in the Japanese, and have not been reported in non-Japanese; thus, these polymorphisms might have originated in Japan.

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Trophic Effect of Multiple Growth Factors in Amniotic Fluid or Human Milk on Cultured Human Fetal Small Intestinal Cells

*Chie Hirai, *Hiroyuki Ichiba, *Mika Saito, *Haruo Shintaku, *Tsunekazu Yamano, and †Satoshi Kusuda

**Department of Pediatrics, Osaka City University Graduate School of Medicine, and †Department of Neonatology, Osaka City General Hospital, Osaka, Japan*

ABSTRACT

Objectives: To evaluate the role of growth factors in amniotic fluid and in human milk on gastrointestinal adaptation of the fetus and very low-birth-weight infants, the effects of these fluids and multiple growth factors were investigated in a human fetal small intestinal cell line (FHs 74 Int).

Methods: After FHs 74 Int cells were incubated with amniotic fluid, human milk, or recombinant growth factors, growth-promoting activity was measured by [^{3}H]-thymidine incorporation into cells.

Results: Incubating cells with amniotic fluid or human milk promoted growth dose dependently. Genistein almost completely inhibited growth-promoting activity in amniotic fluid $P = 0.002$), and growth was partially inhibited by antibodies against epidermal growth factor (EGF) ($P = 0.047$), insulin-like growth factor-1 (IGF-1, $P = 0.047$), or fibroblast growth factor (FGF, $P = 0.014$). This activity in human milk was inhibited almost completely by genistein ($P < 0.0001$) and partially inhibited by antibodies against EGF ($P = 0.036$), IGF-1 ($P = 0.009$), FGF ($P = 0.004$), hepatocyte growth factor (HGF, $P = 0.001$), or transforming growth factor-α (TGF-α, $P = 0.001$). Although recombinant EGF, IGF-1, FGF, HGF, and TGF-α elicited a synergistic trophic response on cultured cells, the response was much less than with amniotic fluid or with human milk.

Conclusion: In aminiotic fluid and in human milk, EGF, IGF-1, FGF, HGF, and TGF-α have a strong trophic effect on immature intestinal cells and may be involved in perinatal gastrointestinal adaptation. *JPGN 34:524–528, 2002.* **Key Words:** Intestinal cells—Amniotic fluid—Human milk—Trophic factors—Functional maturation.

Growth factors such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF) have a trophic effect on the fetal and neonatal gastrointestinal tract (1), promoting proliferation and differentiation of fetal and neonatal gastrointestinal cells. In our previous study, we established a bioassay for growth factors in human milk using a human fetal small intestinal cell line, reporting that human milk had a growth-promoting effect on the cells and also demonstrating that EGF in human milk contributed to this activity (2). These growth factors also have been identified in amniotic fluid (3–5). Other investigations that used various cell lines indicated that growth factors in amniotic fluid might promote proliferation of fetal intestinal cells (6,7). However, the single-factor models used in these studies represented a limited range of in vivo interactions (2,6–8). Thus little is known about the combined actions of multiple growth factors in human milk or in amniotic fluid on the immature gastrointestinal tract (9).

Very low-birth-weight (VLBW) infants who receive early enteral feeding with human milk have been reported to require fewer days to reach full enteral feeding, to have greater gains in weight and head circumference, and to experience fewer gastrointestinal complications than formula-fed infants (10–14). Although gut hormones such as gastrin probably are involved, precise mechanisms are unclear. Suspecting that growth factors in human milk could contribute to these beneficial effects, we tested amniotic fluid, human milk, and various growth factors contained in these fluids on a human fetal

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Address correspondence and reprint requests to Dr. Hiroyuki Ichiba, Department of Pediatrics, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abenoku, Osaka 545-8586, Japan (e-mail: h-ichiba@med.osaka-cu.ac.jp).

small intestinal cell line that represented a suitable model of intestinal cells in the fetus and VLBW infant.

METHODS

A human fetal small intestinal cell line, FHs 74 Int (15–17), was purchased from the American Type Culture Collection (line number CCL241 ATCC, Bethesda, MD, U.S.A.). Its morphology is epithelial-like, the viability is approximately 93%, and EGF (30 ng/ml) reduces the doubling time from 168 to 65 hours. Dulbecco minimal essential medium and fetal calf serum were obtained from Nissui (Tokyo, Japan). [^{3}H]-thymidine (4.0 Ci/mmol) was purchased from ICN Biomedicals Inc. (Costa Mesa, Ca, U.S.A.). A radioimmunoassay kit for EGF was purchased from Amersham (Amersham Place, United Kingdom). Genistein and dimethyl sulfoxide were purchased from Calbiochem (Tokyo, Japan). Recombinant human TGF-α was purchased from Wako Pure Chemical (Osaka, Japan), whereas recombinant human EGF, HGF, fibroblast growth factor (FGF), anti-human EGF, HGF, and IGF-2 were purchased from Genzyme/Techne (Minneapolis, MN, U.S.A.). Recombinant human IGF-1 was purchased from SIGMA (S. Louis, MO, U.S.A.). Antibodies against human IGF-1, TGF-α, FGF, and vascular endothelial growth factor (VEGF) were purchased from Pepro Tech (London, United Kingdom).

All donors of amniotic fluid and human milk samples gave informed consent. Thirty samples of amniotic fluid were obtained at delivery. Twelve samples were from the mothers of full-term infants (37 to 42 weeks of gestation), and 18 were from mothers of preterm infants (25 to 36 weeks). Five samples of manually expressed breast milk were obtained from five mothers of full-term infants. No maternal disease was documented during pregnancy. Bloody or stained amniotic fluid was excluded. Samples were centrifuged at 18,000*g* for 10 minutes at 4°C, and supernatant was frozen and stored at −80°C until subsequent thawing for assays. Epidermal growth factor concentrations were measured using the RIA kit from Amersham (Amersham Place, United Kingdom).

For bioassay of growth-promoting activity, a uniform number of human fetal small intestinal cells were added to Costar 96-well plates and cultured with 200 μL of Dulbecco minimal essential medium containing 10% (vol/vol) fetal calf serum, for 10 to 14 days. All measurements were performed on confluent monolayers. Cells were cultured with 200 μL of serum-free Dulbecco minimal essential medium for 10 hours and then with 20 μL of control fluid, sample fluids, or various concentrations of growth factors added to each well for 24 hours, followed by 2 hours of incubation with [^{3}H]-thymidine (1 μCi/well). Cells were collected on a cell harvester and deposited on discs of filter paper. Radioactivity then was measured with a scintillation counter. Radioactivity was observed and defined as the measure of cell proliferation (growth).

Genistein, an inhibitor of tyrosine kinase (18), was dissolved in dimethyl sulfoxide and used at a final concentration of 50 μg/mL. Cells were preincubated with this inhibitor for 1 hour and then washed free of inhibitor before adding test samples. To study blocking of several antibodies against growth factors, cells were incubated for 24 hours with antibodies against EGF, IGF-1, IGF-2, FGF, HGF, TGF-α, or VEGF (5 μg/mL, respectively) together with fluid samples.

The significance of differences between means was determined using the Student *t* test, and correlations were determined using the Pearson test. Statistical significance was set at *P* less than 0.05.

RESULTS

Figures 1 and 2 show [^{3}H]-thymidine incorporation into cultured small intestinal cells incubated with various concentrations of amniotic fluid or human milk. With both fluids, the growth increment beyond control cultures was dose dependent, reaching a maximum at 40% concentration for amniotic fluid and 5% for milk. Accordingly, we used 40% amniotic fluid and 5% human milk in subsequent assays.

Figure 3 shows EGF concentration in amniotic fluid at each gestational age. The concentration of EGF in preterm amniotic fluid obtained before 30 weeks of gestation was significantly lower than in fluid obtained subsequently. However, no significant difference was observed among gestational ages in growth-promoting activity in amniotic fluid (Fig. 4).

Genistein inhibited growth-promoting activity in amniotic fluid by 96% (mean) in cultured small intestinal cells ($P = 0.002$). This activity in amniotic fluid also was partially inhibited by antibodies against EGF (40%, $P = 0.047$), IGF-1 (38%, $P = 0.047$), or FGF (58%, $P = 0.014$, Fig. 5). Genistein inhibited cell culture growth-promoting activity in human milk by a mean of 98% ($P < 0.0001$), and activity was partially inhibited by antibodies against EGF (22%, $P = 0.036$), IGF-1 (40%, $P = 0.009$), FGF (75%, $P = 0.004$), HGF (56%, $P = 0.001$), or TGF-α (73%, $P = 0.001$, Fig. 6).

When cultured human small intestinal cells were incubated with EGF, IGF-1, FGF, HGF, or TGF-α, [^{3}H]-thymidine incorporation into cells increased in a dose-dependent manner (Fig. 7). Although EGF, IGF-1, FGF, HGF, or TGF-α had a trophic response on the cells, the response was much lower than that for amniotic fluid or human milk. When EGF, IGF-1 and FGF; or EGF, IGF-1, FGF, HGF, and TGF-α were added in combination, the response was greater than that for each growth factor alone. However, the synergistic response of these growth

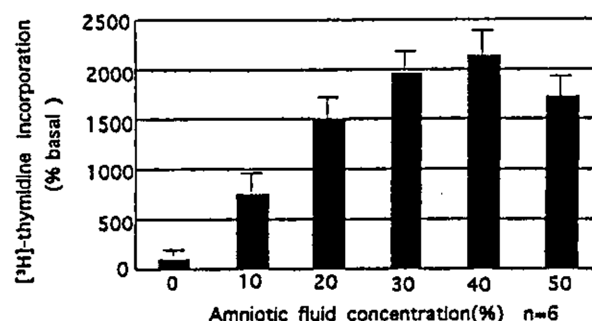

FIG. 1. Incorporation of [^{3}H]-thymidine into cultured human fetal small intestinal cells incubated with amniotic fluid. Bars show means ± SD.

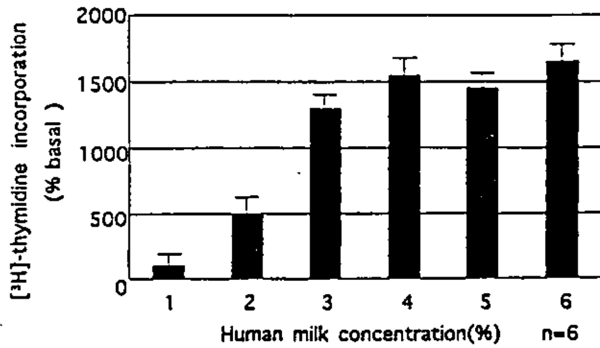

FIG. 2. Incorporation of [^{3}H]-thymidine into cultured human fetal small intestinal cells incubated with human milk. Bars show means ± SD.

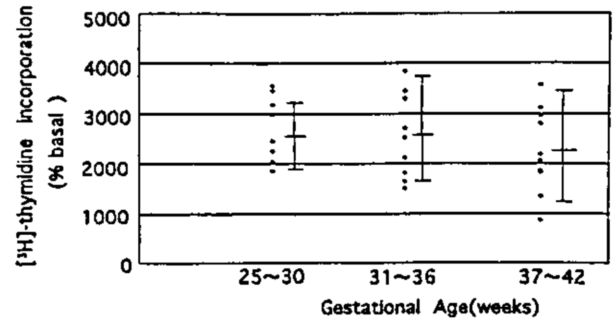

FIG. 4. Incorporation of [^{3}H]-thymidine into cultured human fetal small intestinal cells incubated with 40% amniotic fluid obtained at various gestational ages. Results shown are means ± SD. No significant difference was observed among gestational ages.

factors was lower than that for amniotic fluid or human milk (Table 1).

DISCUSSION

To clarify the role of growth factors in amniotic fluid and human milk in the gastrointestinal adaptation of the fetus and the VLBW infant, effects of these fluids and multiple growth factors were investigated in a human fetal small intestinal cell line (FHs 74). Because this cell line is derived from the normal human fetus, because its morphology is epithelial-like, and because EGF reduces the doubling time, this cell line is a suitable model of intestinal cells in the fetus and VLBW infant.

After incubation of cultured human fetal small intestinal cells with amniotic fluid, [^{3}H]-thymidine incorporation showed a dose-dependent increase. This result indicated that amniotic fluid possessed growth-promoting activity in cultured human fetal small intestinal cells. Mulvilli et al. (19,20) reported that esophageal ligation of the fetal rabbit resulted in significant inhibition of gastrointestinal tract growth and gastric function that was reversed by intragastric infusion of amniotic fluid. These results suggest that amniotic fluid may promote gastrointestinal tract growth in the human fetus.

After incubation of fetal intestinal cells with human milk, [^{3}H]-thymidine incorporation into the cells showed dose-dependent increases, indicating that human milk had growth-promoting activity in these cells. Evidence suggests that the fat compartment of human milk provides growth factors and receptors through its milk fat globule/membrane (21). We studied the effects of the aqueous, nonfat, and noncellular components of human milk, and investigated whether, if these components were studied as whole milk, perhaps greater cellular proliferation and, therefore, trophic effects would be attributable to human milk. Small intestinal weight and protein and DNA content in puppies fed canine breast milk were significantly greater than in puppies fed formula (22). Permeability of the small intestine was lower and gastrointestinal tolerance and intestinal lactase activity were greater in preterm infants fed human milk than in those

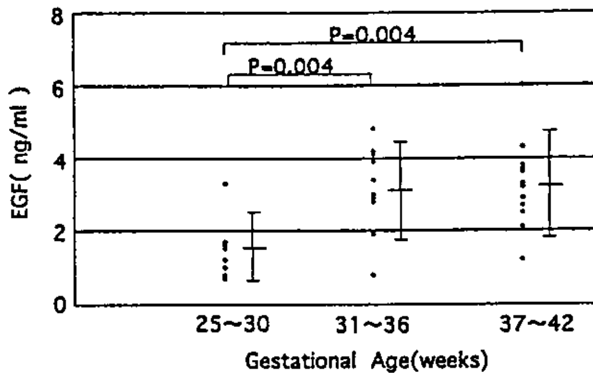

FIG. 3. Epidermal growth factor (EGF) concentration in amniotic fluid at various gestational ages, measured by radioimmunoassay. Results shown are means ± SD.

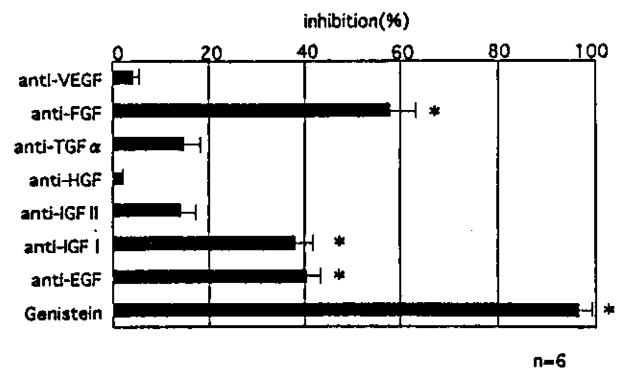

FIG. 5. Growth factor activity blocking by genistein or antibody in amniotic fluid. Human fetal small intestinal cells were incubated with genistein or with one of several antibodies against growth factors. Growth-promoting activity in amniotic fluid showed 96% inhibition by genistein ($P = 0.002$) and partial inhibition by antibodies against epidermal growth factor (EGF) ($P = 0.047$), insulinlike growth factor-1 (IGF-1) ($P = 0.047$), or fibroblast growth factor (FGF) (58%, $P = 0.014$). Asterisks indicate significant inhibition. Bars show means ± SD.

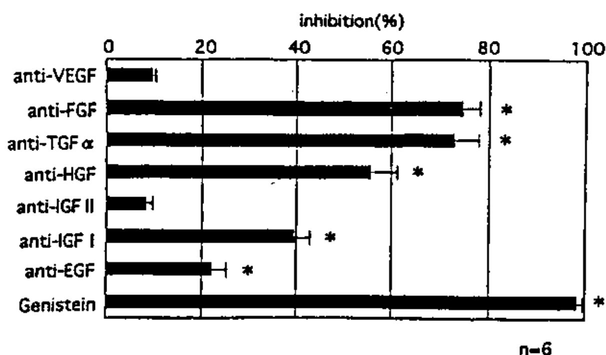

FIG. 6. Blocking by genistein or antibody of growth factor activity in human milk. Human fetal small intestinal cells were incubated with genistein or with one of several antibodies against growth factors. Growth-promoting activity in human milk showed 98% inhibition by genistein (P <0.0001) and partial inhibition by antibodies against epidermal growth factor (EGF) (P = 0.036), insulinlike growth factor-1 (IGF-1) (P = 0.009), fibroblast growth factor (FGF) (P = .004), hepatocyte growth factor (HGF) (P = 0.001), or transforming growth factor-α (TGF-α) (P = 0.001). Asterisks indicate significant inhibition. Bars show means ± SD.

fed formula (13,14). These results suggest that human milk may promote gastrointestinal tract growth and function in newborns.

No significant differences in growth-promoting activity of amniotic fluid in cultured small intestinal cells were seen among a wide range of gestational ages. Because EGF concentration in amniotic fluid obtained before 30 weeks of gestation was lower than the concentration in fluid obtained subsequently, additional factors may contribute to growth- promoting activity in amniotic fluid. Incubation with a tyrosine kinase inhibitor, genistein, almost completely abolished growth-promoting activities in amniotic fluid or human milk, suggesting that these activities are mediated by receptors functionally linked to tyrosine kinase. Blocking activity by antibodies against growth factors suggested that EGF, IGF-1, and FGF contribute to activity in amniotic fluid, whereas

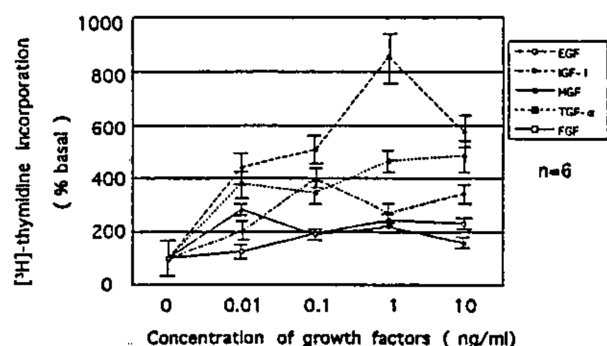

FIG. 7. Incorporation of [^{3}H]-thymidine into cultured human fetal small intestinal cells incubated with recombinant human epidermal growth factor (EGF), insulinlike growth factor-1 (IGF-1), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), or transforming growth factor-α (TGF-α). The results shown are means ± SD.

TABLE 1. *Effect of amniotic fluid, human milk, or recombinant human growth factors on [^{3}H]-thymidine incorporation in human fetal small intestinal cells*

Addition	[^{3}H]-Thymidine incorporation (% basal, n = 6)	P*	P†
Amniotic fluid	2161 ± 383		
Human milk	1452 ± 275		
EGF (1 ng/mL)	635 ± 86	<0.001	<0.001
IGF-1 (1 ng/mL)	272 ± 42	<0.001	<0.001
FGF (1 ng/mL)	537 ± 140	<0.001	<0.001
HGF (1 ng/mL)	229 ± 37	<0.001	<0.001
TGF-α (1 ng/mL)	416 ± 78	<0.001	<0.001
EGF + IGF-1 + FGF (1 ng/mL, respectively)	943 ± 173	<0.001	0.003
EGF + IGF-1 + FGF + HGF + TGF-α (1 ng/mL, respectively)	1034 ± 175	<0.001	0.005

EGF, epidermal growth factor; FGF, fibroblast growth facter; HGF, human growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor.
* Compared with amniotic fluid.
† Compared with human milk.

EGF, IGF-1, FGF, HGF, and TGF-α contribute to activity in human milk. Although EGF has been considered the main growth factor in amniotic fluid and human milk (20,23), our study suggests that other growth factors such as IGF-1, FGF, HGF, and TGF-α are comparably active. Because the trophic effect of each recombinant growth factor or the synergistic effect of these growth factors were much lower than the effects of amniotic fluid or of human milk, unknown factors in these fluids may be important. Therefore, intestinal cell growth-promoting activity equal to that of amniotic fluid or human milk cannot yet be duplicated artificially, even with recombinant growth factors.

This is the first investigation of the combined action of multiple growth factors in amniotic fluid and human milk on cultured human small intestinal cells in which the two fluids were compared. Growth-promoting activity of amniotic fluid on these cells proved equal to that of human milk. The growth factors in amniotic fluid apparently promote proliferation of intestinal cells in utero, whereas the growth factors in human milk promote proliferation of neonatal intestinal cells. These responses are important for the gastrointestinal tract as it adapts to postnatal requirements.

Very low-birth-weight infants, born in the second trimester, have had reduced exposure to the growth factors in amniotic fluid. This may cause gastrointestinal immaturity and lead to complications. However, VLBW infants fed human milk had fewer gastrointestinal complications (24). More specifically, early initiation of enteral feeding with human milk reduced such complications (10). Growth factors in human milk may act on the gastrointestinal tract of VLBW infants to favor structural and functional maturation in lieu of the growth factors no

longer supplied by amniotic fluid. Therefore, enteral feeding of VLBW infants with human milk should be initiated as soon as possible after birth.

In conclusion, EGF, IGF-I, FGF, HGF, and TGF-α in amniotic fluid or in human milk have a trophic effect on immature intestinal cells and may be important in perinatal adaptation of the gastrointestinal tract. Unknown factors in these fluids also may participate, and this issue requires further investigation.

Acknowledgments: The authors thank Professor S. Ogita and Dr. Y. Nakai for collecting amniotic fluid samples.

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Decreased plasma tetrahydrobiopterin in pregnant women is caused by impaired 6-pyruvoyl tetrahydropterin synthase activity

DAISUKE TACHIBANA[1], HIROSHI FUKUMASU[3], HARUO SHINTAKU[2], YASUKO FUKUMASU[4], SEIICHI YAMAMASU[1], OSAMU ISHIKO[1], TSUNEKAZU YAMANO[2] and SACHIO OGITA[1]

Departments of [1]Obstetrics and Gynecology and [2]Pediatrics, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585; [3]Department of Obstetrics and Gynecology, Sumiyoshi Municipal Hospital, 1-2-16 Higashikagaya, Suminoe-ku, Osaka 559-0012; [4]Department of Obstetrics and Gynecology, Juso Municipal Hospital, 2-3-7 Jusohigashi, Yodogawa-ku, Osaka 532-0023, Japan

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Abstract. Tetrahydrobiopterin (BH_4) is an essential cofactor of nitric oxide synthase as well as a cofactor of aromatic amino acid hydroxylases. However, its role in pregnancy is not yet understood. We evaluated the concentrations of BH_4 throughout normal pregnancy and puerperium, and compared them with those of non-pregnant women by measuring its oxidation product biopterin. In addition, we also measured 6-pyruvoyl tetrahydropterin synthase (PTPS) activities, the rate-limiting enzyme in synthesizing BH_4, in pregnant women at the 30th gestational week and non-pregnant women. Although the urinary biopterin levels did not remarkably change, plasma biopterin levels significantly decreased from the 10th gestational week to the 1st day of postpartum compared with those of non-pregnant women. There was no significant difference in PTPS activities between pregnant and non-pregnant women. However, the proportion of reticulocytes, which have been shown to possess high PTPS activity, is significantly higher in pregnant women than in non-pregnant women. Our results suggest that decreased plasma BH_4 levels in pregnancy is caused by impaired PTPS activity.

Introduction

The roles of nitric oxide in pregnancy have been shown to regulate vascular tone to fit with the hemodynamic changes characterizing pregnancy and to modulate uterine contractility (1-3). The activity of nitric oxide synthase is dependent on the availability of cofactors including tetrahydrobiopterin (BH_4) (4). The precise role of BH_4 is not yet known. However, it could be important in maintaining nitric oxide synthase in an active configuration, or may have a regulatory redox role (5,6).

The biosynthesis of BH_4 starts from GTP, which is converted to 7,8-dihydroneopterin. This key intermediate in BH_4 biosynthesis is then further metabolized to BH_4 in a series of steps. In these steps, 6-pyruvoyl tetrahydropterin synthase (PTPS) serves as a rate-limiting enzyme (7). Biopterin and neopterin are oxidation products of BH_4 and 7,8-dihydroneopterin, and are detectable due to their intense fluorescence. The measurements of biopterin and neopterin can be used to study changes in BH_4 metabolism (8,9).

BH_4 may be important in supplying an adequate reserve of nitric oxide needed to maintain normal pregnancy. However at present, there is no such report of the role of BH_4 in pregnancy including its concentration. This study shows BH_4 concentration changes throughout pregnancy and compares them with those of non-pregnant women. We also investigated PTPS activity in erythrocytes and the proportion of reticulocytes, which have been shown to possess high PTPS activity (10), in pregnant and non-pregnant women.

Correspondence to: Dr Daisuke Tachibana, Department of Obstetrics and Gynecology, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan
E-mail: daisuke-t@med.osaka-cu.ac.jp

Key words: tetrahydrobiopterin, pregnancy, biopterin, neopterin, 6-pyruvoyl tetrahydropterin synthase

Materials and methods

Subjects. After obtaining informed consent, blood and urinary samples were collected. Gestational age was determined based on the crown-rump length of the fetuses measured by ultrasonography between the 8th and 10th weeks of gestation. None of the pregnant women were in labor. The plasma of 40 healthy pregnant women with singleton pregnancies, 30 healthy women who had completed spontaneous labor, and 15 healthy non-pregnant women of corresponding age were analyzed. Of the 40 healthy pregnant women, there were 10 women for each 10th gestational week. Of the 30 healthy women who had completed spontaneous labor, there were 10 on the 1st day, 10 on the 5th day and 10 on the 30th day of postpartum. Their postpartum days were all normal. The urine of 60 healthy pregnant women with singleton pregnancies, 30 healthy women after spontaneous labor and 10 healthy non-pregnant women of corresponding age were analyzed. Of the 60 healthy pregnant women, there were 15 women for each 10th gestational week. Of the 30 healthy women who had completed spontaneous

labor, there were 15 on the 5th day and 15 on the 30th day of postpartum. Their postpartum days were normal.

Blood and urinary sampling. The plasma and erythrocytes of heparinized blood were separated by centrifugation. The plasma was frozen until further analysis. Erythrocytes were diluted 2-4 times with 0.2 M Tris-HCl buffer (pH 7.4) and then frozen. Spot urinary samples were also collected and frozen until processing.

Determination of biopterin and neopterin concentrations by high performance liquid chromatography. Plasma and urinary concentrations of biopterin and neopterin were measured by high performance liquid chromatography (HPLC) with fluorimetric detection (11,12). In brief, 10 µl of 30% w/v trichloroacetic acid solution was added to 100 µl of plasma and urine, and this mixture was centrifuged. A 10 µl volume of 1% iodine solution was added to 50 µl of the supernatant and the mixture was kept at room temperature for 60 min. The excess iodine solution was destroyed by adding 15 µl of 1% aq. ascorbic acid. The biopterin and neopterin concentrations of the supernatant were then measured. To take physiological alterations of urine concentration into account, the concentration of urinary creatinine was measured.

Assay for PTPS activity in erythrocytes. The PTPS activity in erythrocytes was measured as described previously (13). Erythrocytes were hemolysed by freezing and thawing. After centrifugation, a portion of the hemolysate supernatant was bubbled with carbon monoxide gas for 1 min. The standard assay mixture with a final volume of 100 µl contained the following components: 0.1 M Tris-HCl buffer (pH 7.4), 10 mM magnesium chloride, 30 µM dihydroneopterin triphosphate, 1 mM NADPH, 1 mM NADH, approximately 5 mU sepiapterin reductase, approximately 200 mU dihydropteridine reductase, and 50 µl hemolysate. The reaction mixture without hemolysate served as the control. The biopterin concentration of the reaction mixture before incubation was used as the zero-time blank derived from the hemolysate. The reaction was started by the addition of the substrate. After incubation at 37°C for 90 min in the dark, the reaction was terminated by the addition of 10 µl of 30% w/v trichloroacetic acid solution and the mixture was then centrifuged. Next, the biopterin concentration was measured. One unit of PTPS activity produced 1 µmol of biopterin per min under the assay conditions described. The specific activity of PTPS was indicated as International Units per gram hemoglobin.

Measurement of reticulocytes. Reticulocytes were measured using the flow cytometry method (Sysmex R-3000).

Statistical analysis. Data was statistically analyzed using the Kruskal-Wallis test (multiple comparison). $p<0.05$ was considered statistically significant.

Results

The plasma concentrations of neopterin and biopterin are shown in Fig. 1. Biopterin levels decreased significantly in the 10th gestational week compared with non-pregnancy (15.14±3.99 vs. 21.71±4.87 nmol/l, respectively, mean ± SD, $p<0.05$), and these decreases continued until the 1st day of postpartum. Neopterin concentration increased as gestational weeks progressed and was significantly higher in the 40th gestational week (34.35±6.76 nmol/l) and in the 1st day of postpartum (38.84±9.03 nmol/l) in comparison to non-pregnant women (20.27±7.53 nmol/l).

The urinary concentrations of neopterin and biopterin are shown in Fig. 2. We did not find significant changes in urinary biopterin concentrations. However, these concentrations were slightly lower for pregnant than for non-pregnant women (0.677±0.401 vs 0.731±0.268 mmol/mol creatinine, respectively). Similar to plasma, urinary neopterin concentrations increased as gestational weeks progressed and were significantly higher in the 5th day of postpartum

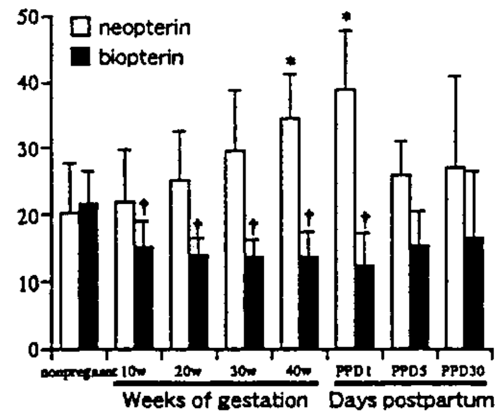

Figure 1. The plasma concentrations of neopterin and biopterin (nmol/l) in healthy non-pregnant women, normal pregnant women and puerperal women. *p<0.05; †p<0.05 vs non-pregnant women. Error bars correspond to SD.

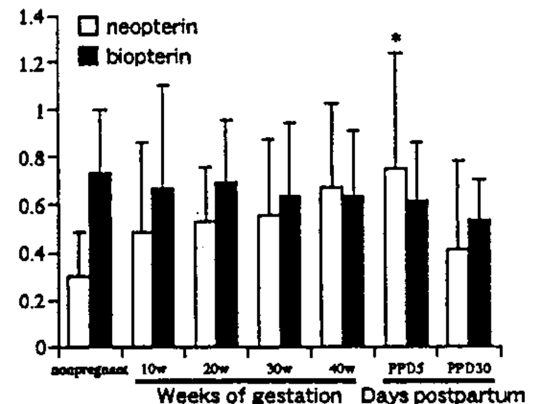

Figure 2. The urinary concentrations of neopterin and biopterin (mmol/mol creatinine) in healthy non-pregnant women, normal pregnant women and puerperal women. *p<0.05 vs non-pregnant women. Error bars correspond to SD.

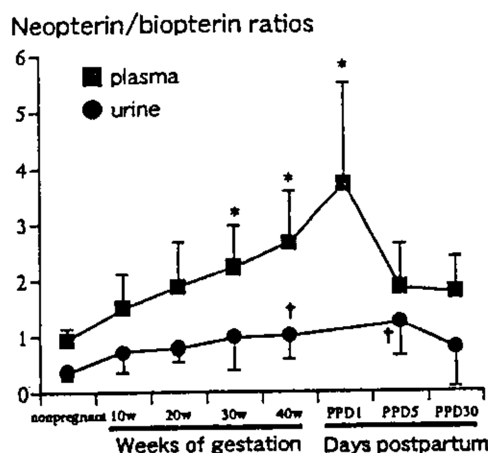

Figure 3. The neopterin/biopterin ratios in healthy non-pregnant women, normal pregnant women and puerperal women. *$p<0.05$; †$p<0.05$ vs non-pregnant women. Error bars correspond to SD.

Table I. PTPS activity in erythrocytes and the proportion of reticulocytes.

	PTPS activity (μU/gHb)	Reticulocytes (‰)
Pregnant	25.5±6.3	18.1±5.7
CTL	25.3±6.3	10.8±5.5
Significance	p=0.7039	p=0.0149

PTPS activities in erythrocytes and the proportion of reticulocytes in normal pregnant women and healthy non-pregnant women. Pregnant, normal pregnant women in their 30th gestational week (n=20); CTL, healthy non-pregnant women (n=20). The results are expressed as mean ± SD.

(0.717±0.588 mmol/mol creatinine) compared to that of non-pregnant women (0.302±0.183 mmol/mol creatinine).

The changes in the ratio of neopterin/biopterin levels in plasma and urine are shown in Fig. 3. Plasma neopterin/biopterin ratios for pregnant and postpartum women were higher than those for non-pregnant women. There were significant increases in the plasma neopterin/biopterin ratios in the 30th (2.214±0.741) and 40th gestational week (2.650±0.909) and in the 1st day of postpartum (3.699±1.776) in comparison to non-pregnant women (0.924±0.211). Urinary neopterin/biopterin ratios for pregnant and postpartum women were also higher than those for non-pregnant women. A significant increase in the urinary neopterin/biopterin ratio were observed in the 40th gestational week (0.991±0.412) and in the 5th day of postpartum (1.114±0.661) comparing to those of non-pregnant women (0.394±0.141).

Table I shows the PTPS activity of erythrocytes and the proportion of reticulocytes in pregnant women in their 30th gestational week and in non-pregnant women. There was no significant difference in PTPS activity between the two groups (25.5±6.3 vs. 25.3±6.3 μU/gHb, pregnant and non-pregnant respectively), although the proportion of reticulocytes in pregnant women (18.1±5.70‰) was significantly higher than in non-pregnant women (10.8±5.50‰).

Discussion

In healthy pregnant women, plasma biopterin levels decreased compared with those of non-pregnant women. The phenomenon of decreased plasma biopterin levels accompanied by elevations in plasma neopterin levels suggests the impaired PTPS activity, which is a rate-limiting enzyme to convert 7,8-dihydroneopterin triphosphate to BH_4 (14). Therefore we examined PTPS activities in both pregnant and non-pregnant women. However, there was no significant difference between the two groups. Shintaku *et al* reported elevated PTPS activity in erythrocytes, which contain a higher proportion of reticulocytes (10). Thus, we also measured the proportion of reticulocytes in both groups. Although a higher proportion of reticulocytes was observed in pregnant women, PTPS activities were not different from those in non-pregnant women. These findings suggest a possible decrease of PTPS activity during pregnancy.

Some investigators who reported elevated neopterin levels during pregnancy concluded that elevated neopterin levels might be related to the immune activation phenomena (15-17). However, they did not comment on BH_4 metabolism, which we thought was important. Therefore, we propose that if an elevation in neopterin level was observed, BH_4 metabolism should be taken into consideration.

Physiological hemodilution usually occurs during normal pregnancy. For example, the hematocrit of normal pregnant women in the 30th gestational week is about 78.5% of non-pregnant women (data is not shown). However, our findings that the plasma biopterin levels of pregnant women at the 30th gestational week is about 63.1% of non-pregnant women suggest that observed decreases in plasma biopterin levels are not only due to physiological hemodilution.

Fuith *et al* reported the elevation of urinary neopterin levels in normal pregnancies (16). Therefore, we also measured urinary neopterin and biopterin levels. Compared with those of non-pregnant women, we did not find any significant changes of urinary neopterin and biopterin levels in pregnant women except for an elevated neopterin level on 5th day of postpartum. In our results, the urinary neopterin and biopterin level distribution ranged wider than the results previously reported.

In conclusion, we presented the concentration levels and changes of BH_4 throughout pregnancy. Whether the decrease of plasma biopterin levels during pregnancy is needed for the adjustment to pregnancy or whether the decrease is due to an impairment in BH_4 synthesis is remained to be examined. However, the BH_4 metabolism in pregnancy should be distinguished from that in non-pregnancy. Although the role of BH_4 in pregnancy is not yet known, we believe our findings will help to understand the pathophysiology of such diseases as preeclampsia and intrauterine growth restriction.

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脳波・筋電図の臨床

脳磁図で推定したてんかん焦点と発作症状との関連性

Correlation of seizure semiology to the epileptic foci estimated by MEG

服部 英司*1 HATTORI Hideji
蔦田 強司*3 TSUTADA Tsuyoshi
露口 尚弘*1 TSUYUGUCHI Naohiro
下川原正博*5 SHIMOGAWARA Masahiro
春田 康博*6 HARUTA Yasuhiro
山野 恒一*2 YAMANO Tsunekazu

脳磁図を用いて推定したてんかん焦点と発作症状との関連性について検討した.
1）脳磁図で焦点の求まった患者で発作症状に焦点性のある患者は約半数であった.
2）前頭葉に焦点のある患者では運動徴候を示す割合が高かった.
3）感覚徴候は頭頂葉，後頭葉に焦点のある患者に多かった.
4）連合野に焦点の推定された患者では脳葉に特徴的な発作症状に乏しかった.
脳磁図により推定された焦点の部位と発作症状には，一定の関連性が認められた.

KEY WORDS 脳磁図，発作症状，てんかん焦点，てんかん源性

はじめに

てんかん焦点を正確に同定することは，てんかん患者の診断ならびに治療に極めて重要なことである．通常の頭皮上脳波検査（EEG）は空間分解能で劣るため，外科治療の対象患者では硬膜外脳波や深部脳波などの侵襲的方法によりてんかん焦点を同定している．一方，大部分のてんかん患者では，非侵襲的な方法のみでてんかん焦点の同定を行う必要がある．脳磁図（Magnetoencephalography, MEG）はてんかん発作波の電流源（ダイポール）の位置をMRI上に数ミリの誤差で表すことができる．MEGは主として発作間歇時の測定となるため，ダイポールが示す焦点はてんかんのirritative zoneであり，必ずしもepileptogenic zoneに一致しないことが問題とされてきた．しかしながら，MEGと頭蓋内脳波記録や外科治療の結果との最近の比較研究は，MEGにより推定された焦点はほぼepileptogenic zoneに一致することを証明した[1-3]．そこで，一般的なてんかん症例を対象にMEGにより推定した焦点部位をもとに，各部位における発作症状について検討した．

対象と方法

対象は大阪市立大学MEG室でMEGを測定し焦点を推定できた患者で，発作症状の聴取できた33人である．MEG測定にあたっては，本人もしくは両親に測定の目的等について説明し同意を得た．年齢は1～21歳，男性13人，女性20人であった．てんかん型は臨床経過や脳波所見，画像診断所見

大阪市立大学医学部小児科学講座 *1講師 *2教授 *3老年科・神経内科 講師 *4脳神経外科 講師 *5金沢工業大学先端電子技術応用研究所 *6横河電機株式会社航機事業部
Address/HATTORI H：Dept. of Pediatrics, Osaka City University, School of Medicine, OSAKA 545-8586

から総合的に診断した．

MEGの測定は，一次微分型グラディオメーターを160チャンネル配置した横置き全頭型脳磁計（横河電機製）を用いて磁気シールドルーム内にて行った．脳磁はバンドパスフィルター：3～100もしくは200Hz，サンプリング周波数：250もしくは1,000Hz，記録時間10もしくは2分間で測定した．発作波の出現頻度に応じて，数セットの測定を行った．同時に10-20法による基準電極法で12チャンネルの脳波と1チャンネルの心電図を測定し，同じ時系列でMEGとともにハードディスクにデジタル信号として記録した．ダイポールは脳波上の棘波を参考にして，等磁場曲線がダイポールパターンを呈する部位で，球モデルを用いて推定し，別に撮像したMRI上にスーパーインポーズした．ダイポールの位置が脳内トポグラフィーのどの領域にあるかをBroadmanの皮質領域に基づいて検討した．ダイポールの限局性については，ダイポールの方向も一定で限局した部位に推定された場合を「1」，ほぼ限局された部位に推定されたが方向性の不定な場合を「2」，やや広範囲な領域に推定された場合を「3」とした．

結　　果

ダイポール推定の結果は，前頭葉に焦点の推定された症例8例，頭頂葉に12例，後頭葉に9例，側頭葉に5例であった．なお2ヵ所に焦点の推定された症例は両方の部位で検討対象としたので合計数は対象症例総数を上回る．

1．前頭葉に焦点の推定された症例（表1）

9例のうち7例で運動徴候を示し，運動徴候を伴う割合が他の部位より高かった．運動野（area 4）にダイポールの推定された6例（症例3-8）の中で3例（症例3，5，6）は顔面の運動徴候を呈していた．症例4は右に焦点が推定され左方向への向反発作を示した．症例7はシルビウス溝付近に推定され発声を伴う単純部分発作であったが側方性は不明瞭であった．残りの1例（症例8）は前頭葉と側頭葉の2カ所に焦点が推定され，強制思考を呈する複雑部分発作で，運動徴候を認めず前部前頭極の症状を示していた[1]．症例1は発作症状に焦点性を認めなかった．症例2も焦点性の運動徴候を示したが焦点部位と同側の運動徴候を示し側方性は一致しなかった．本例は新生児期の脳内出血のため片側脳の障害が著明であり可塑的な変化が起こっていると考えられた．

2．頭頂葉に焦点の推定された症例（表2）

感覚野にダイポールの推定された6例のうち2例（症例13，16）は対応する感覚症状から始まり，運動徴候に進展する単純部分発作を示した．しかし，ほかの4例（症例10，11，14，17）では，意識減損が主で体性感覚症状は示さなかった．症例17は発作開始時より全般性の運動徴候（脱力，間代発作）を示した．ほかの症例は頭頂連合野に焦点が推定され，主として運動徴候を示していた．発作症状の左右差を認めた症例が約半分の6例で，ダイポールの焦点との側方性に関しては全例一致していた．

3．後頭葉に焦点の推定された症例（表3）

9症例で後頭部にダイポールが推定された．推定された焦点部位は一次視覚野よりも視覚野の周辺領域，連合領域が多かった．発作症状として，嘔吐など内臓感覚症状が3例，眼球運動徴候が2例に認められ，運動徴候を6例に認めた，意識減損のみの例が2例にみられた．症例2では，前頭葉から伝播した焦点が視覚野に推定され，運動徴候に続いて陰性視覚症状を呈した．

4．側頭葉に焦点の推定された症例（表4）

意識減損，意識変容や自動症など，複雑部分発作を呈する症例が多かった．また，顔色不良などの自律神経症状を示す例が2例に認められた．症例30はMRI所見から内側側頭葉てんかんと考えられた症例である．

表1　前頭葉に焦点の推定された症例

症例	性	年齢	てんかん型	発作症状	ダイポール		
					部位	Broadman	限局性
1	F	2	SPE	(1) 全身性強直発作，左右差なし． (2) 首を前にカクッとする発作．時に失立．	F (Lt)：前頭極〜帯状回	9, 32	3
2	F	21	West → SPE	左上肢の間代痙攣．意識は保たれる．発作時に右側は見えるが左は見えないことがあった．	F (Lt)：運動前野→PO (Lt)：後頭眼野	6, 19	1
3	F	6	BECT	睡眠中に歯をカチカチ（右顔面の spasms)	F (Lt)：シルビウス，運動野（顔面）	4'	1
4	F	5	FLE	左へ眼球，体を回転させる．意識あり．	F (Rt)：運動野	4	2
5	M	11	SPE	嘔気，嘔吐→意識減損，顔面の硬直，歪み	F (Rt)：運動野（顔面）	4	1
6	F	8	BECT?	睡眠中にしゃっくり様の動き→目を見開き，左手の間代発作．流涎（＋）．	F (Rt)：運動野（顔面）	4	1
7	F	10	BECT?	早朝，カッカッという発声に続き横向きに手を前方に突っ張る．流涎（＋）．意識あり．	F (Rt)：シルビウス	4	1
8	M	12	TLE?	急にボーとして強制思考を伴う．	F (Lt)：運動野（顔面，手）， T (Rt)：シルビウス	4, 22	1
20	M	1	EIEE → SPE	(1) 呼吸が不整→眼球開眼，訴えるようにアーッと発声→左上肢を強直させる． (2) 発声しながら左上肢強直．	P〜F (Rt)	39	3

SPE：症候性局在関連性てんかん，IPE：特発性/潜因性局 在関連性てんかん
PE ：局在関連性てんかん，FLE：前頭葉てんかん，PLE：頭頂葉てんかん
OLE：後頭葉てんかん，TLE：側頭葉てんかん，SGE：症候 性全般てんかん