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Deficiency of α-Dystroglycan in Muscle–Eye–Brain Disease

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α-Dystroglycan is a component of the dystrophin-glycoprotein-complex, which is the major mechanism of attachment between the cytoskeleton and the extracellular matrix. Muscle–eye–brain disease (MEB) is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities and lissencephaly. We recently found that MEB is caused by mutations in the protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) gene. POMGnT1 is a glycosylation enzyme that participates in the synthesis of *O*-mannosyl glycan, a modification that is rare in mammals but is known to be a laminin-binding ligand of α-dystroglycan. Here we report a selective deficiency of α-dystroglycan in MEB patients. This finding suggests that α-dystroglycan is a potential target of POMGnT1 and that altered glycosylation of α-dystroglycan may play a critical role in the pathomechanism of MEB and some forms of muscular dystrophy. © 2002 Elsevier Science (USA)

Key Words: **muscle–eye–brain disease; muscular dystrophy; dystroglycan; glycosylation; POMGnT1; neuronal migration disorder.**

Muscle–eye–brain disease (MEB: MIM 253280) is an autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and brain malformation (type II lissencephaly). Patients with MEB show congenital muscular dystrophy, severe congenital myopia, congenital glaucoma, pallor of the optic discs, retinal hypoplasia, mental retardation, hydrocephalus, abnormal electroencephalograms and myoclonic jerks. All infants with MEB are floppy with generalized muscle weakness, including facial and neck muscles, from birth. Muscle biopsies show dystrophic changes, and brain MRIs reveal pachygyria-type cortical neuronal migration disorder, flat brainstem and cerebellar hypoplasia (1). Very recently, we showed that MEB is caused by mutations in the protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) gene on chromosome 1q33, and we suggested that interference in *O*-mannosyl glycosylation is a new pathomechanism for muscular dystrophy as well as neuronal migration disorder (2).

In eukaryotes, proteins are frequently modified by *N*-glycosylation as well as by *O*-glycosylation (3). While Ser and Thr residues of yeast and fungal proteins are primarily modified with *O*-mannosyl glycans, this modification is rare in mammals, occurring in a limited number of brain, nerve, and skeletal muscle glycoproteins (4). While the role of *O*-mannosylation is not clear, we have demonstrated that sialyl *O*-mannosyl glycan is a laminin-binding ligand of α-dystroglycan (5). α-Dystroglycan is a component of the dystrophin-glycoprotein-complex (DGC), the primary mechanism of attachment between the cytoskeleton and the extracellular matrix.

MEB shares phenotypic similarities with Fukuyama-type congenital muscular dystrophy (FCMD) and Walker-Warburg syndrome, including the unusual combination of muscle, eye, and brain abnormalities. We previously identified the gene responsible for FCMD, which encodes the fukutin protein (6). The function of fukutin is not yet clarified; however, sequence analysis predicts it to be an enzyme that modifies cell-surface glycoproteins or glycolipids (7). Recently, a selective deficiency of highly glycosylated

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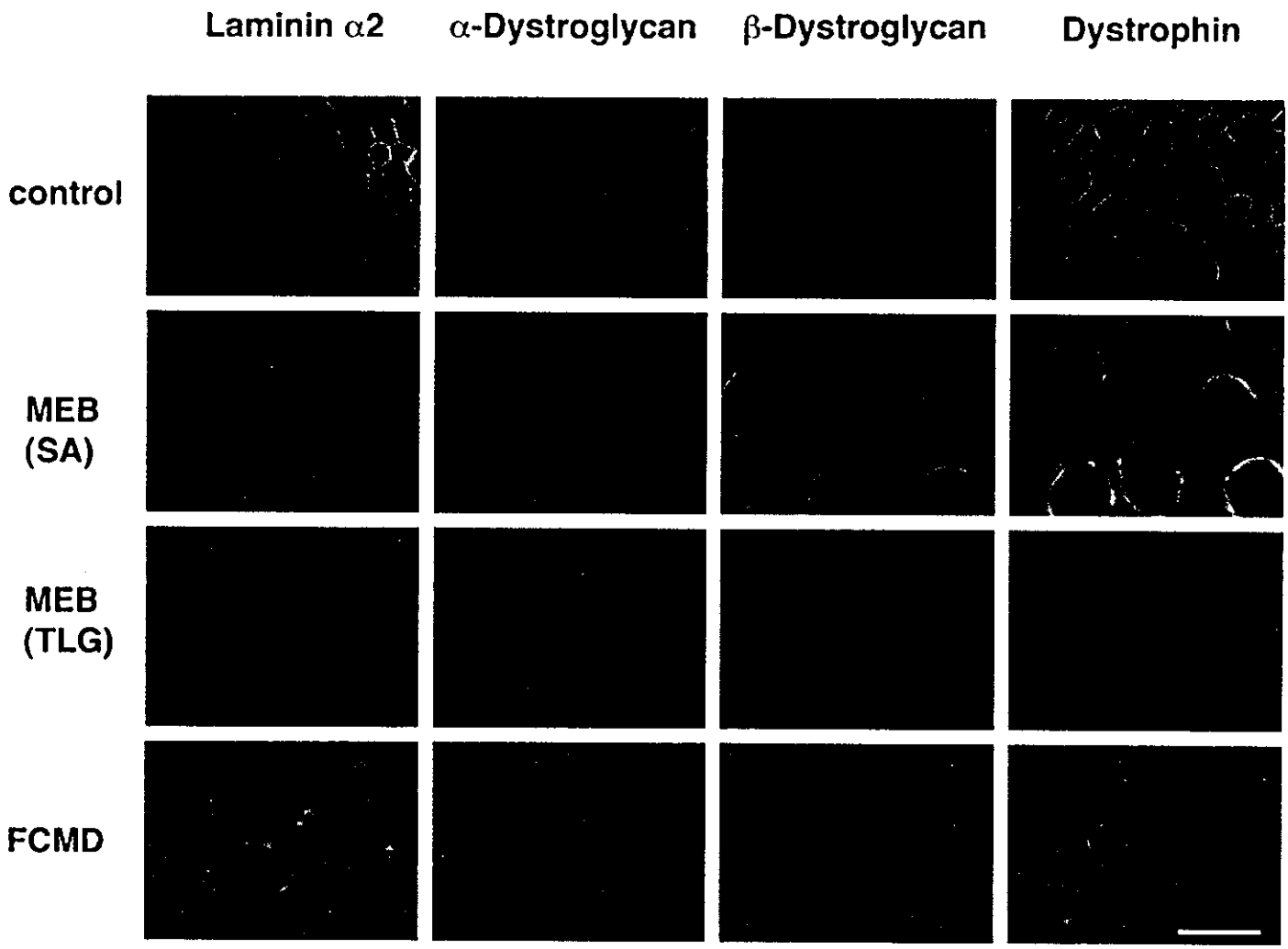

FIG. 1. Immunohistochemical analysis of MEB skeletal muscle. Frozen sections of skeletal muscle from a control individual, muscle-eye-brain (MEB) disease patients and a Fukuyama-type congenital muscular dystrophy (FCMD) patient immunostained with anti laminin α2, α-dystroglycan (VIA4-1 for an MEB patient SA, a FCMD patient and a control, or sheep antiserum for an MEB patient TLG), β-dystroglycan, and dystrophin antibodies. Bar = 50 μm.

α-dystroglycan was found on the surface membranes of skeletal and cardiac muscle fibers in FCMD patients (8). A profound reduction in expression and reduced molecular weight of α-dystroglycan was also shown in patients with a severe form of congenital muscular dystrophy, MDC1C. MDC1C is characterized by disease onset in the first few weeks of life and severe phenotypic effects, including inability to walk and muscle hypertrophy but normal brain structure and function. These patients harbored mutations in the gene encoding fukutin-related-protein (FKRP), a predicted homologue of fukutin (9). In addition, deletion of a glycosyltransferase-like protein gene, *Large*, was found to be the disease causing mutation in the myodystrophy (*myd*) mouse, which also shows markedly reduced glycosylation of α-dystroglycan (10). Together, these findings suggest that altered post-translational modification of α-dystroglycan may cause some forms of muscular dystrophy, although the mechanism by which this occurs is still unclear. In the current study, we examined skeletal muscle tissue samples from MEB patients to evaluate the state of the DGC and to search for proteins that are modified by POMGnT1.

MATERIALS AND METHODS

Clinical materials. We investigated biopsied skeletal muscle tissue from a Turkish MEB patient SA (age 6 years), a French MEB patient TLG (age unknown), an FCMD patient (age 8 months) as a disease control, and an unaffected subject (age 7 months). Patient SA carried a homozygous G-to-A substitution in intron 17 of the POMGnT1 gene. This mutation changed the conserved GT splicing donor sequence to AT, resulting in read-through of the intronic sequence and introduction of a premature termination codon. Patient TLG is a compound heterozygote who carries a C1572G transversion in exon 17 (Pro493Arg) and a 1-bp deletion at base 1970 in exon 21 (frameshift and premature termination at codon 633) (2).

Immunohistochemistry. Immunodetection was performed using a mouse monoclonal anti-α-dystroglycan antibody (clone VIA4-1, Upstate Biotechnology), affinity-purified sheep antiserum directed

against a 20-amino-acid C-terminal sequence of chick α-dystroglycan (11), monoclonal anti-β-dystroglycan (clone 8D5) and anti-dystrophin (clone 6C5) antibodies (Novocastra Laboratories), and a monoclonal anti-laminin α2 chain antibody (clone 5H2, GibcoBRL). Frozen sections (8 μm) from biopsied skeletal muscle tissue were preincubated with PBS containing 5% bovine serum albumin at pH 7.4, then incubated with primary antibodies overnight at 4°C. After incubation with secondary antibodies for 1 h at room temperature, sections were examined by fluorescence microscopy.

Immunoblotting. Biopsied skeletal muscle tissue was homogenized in 10× volume of homogenization buffer (PBS containing 0.2% Triton X-100 and protease inhibitors). After incubation for 10 min at 4°C, protein extracts were centrifuged for 10 min at 4°C and 15,000*g*. The resulting supernatants were rotated overnight at 4°C with an equal volume of wheat germ agglutinin (WGA)-Sepharose 6MB (Amersham Pharmacia) that was pre-equilibrated with 5 volumes of homogenization buffer. The affinity beads were then washed and boiled for 3 min in sample buffer containing 50 mM Tris HCl, 2% SDS, glycerol, and 2-mercaptoethanol.

Extracted samples were separated electrophoretically on 7% (for α-dystroglycan) and 10% (for β-dystroglycan) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore). Blots were probed using anti-α-dystroglycan (clone VIA4-1) and anti-β-dystroglycan (clone 8D5) antibodies. Signal was detected with a peroxidase-conjugated rabbit anti-mouse secondary antibody (DAKO), using SuperSignal CL-HRP (Pierce) as the chemiluminescent substrate.

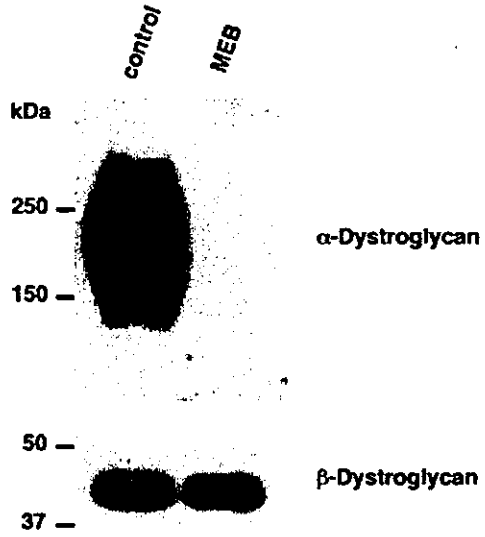

FIG. 2. Immunoblot analysis of skeletal muscle protein extracts from control and muscle-eye-brain (MEB) disease individuals, purified with wheat germ agglutinin-Sepharose. A complete loss of α-dystroglycan (VIA4-1) was observed in the MEB patient SA, although β-dystroglycan expression was normal.

RESULTS AND DISCUSSION

In our search for proteins with sugar chains modified by POMGnT1, we examined the expression of α-dystroglycan in muscle tissue from patients with MEB. The immunoreaction to anti-α-dystroglycan antibody (VIA4-1 except for the MEB patient TLG) was barely detectable on the surface membranes of skeletal muscle fibers in MEB patients relative to the control (Fig. 1). On the other hand, immunoreactivity of laminin α2 chain, β-dystroglycan and dystrophin proteins were similar between MEB patients and the control.

For immunoblotting, we attempted to concentrate α-dystroglycan from skeletal muscle tissue. The chromatography was performed with WGA-Sepharose, a lectin that has previously been shown to bind α-dystroglycan (12). Immunoblot analysis of protein extracts from muscle tissue gave consistent results. The muscle specimen from the MEB patient SA showed a lack of α-dystroglycan expression in the WGA fraction using the VIA4-1 antibody, although normal levels of β-dystroglycan were observed (Fig. 2). Altered glycosylation of α-dystroglycan may affect its binding ability to WGA-Sepharose; however, no immunoreactive band for α-dystroglycan (VIA4-1) was observed in the unbound fraction (data not shown). The sheep α-dystroglycan antibody gave no signal in any of the samples.

The two subunits of dystroglycan, α-dystroglycan and β-dystroglycan, are generated by post-translational cleavage of the proprotein, which is encoded by a single gene (13). α-Dystroglycan is widely expressed and considered to be heavily glycosylated, but its glycosylation pattern varies in a tissue-specific and developmental stage-specific manner (14, 15). Since α-dystroglycan is known to contain *O*-mannosyl glycan (5), and the monoclonal antibody VIA4-1 is thought to recognize a carbohydrate epitope of α-dystroglycan (16, 17), our findings suggest that α-dystroglycan may be one of the target proteins that is post-translationally modified by POMGnT1.

The DGC is the primary structure enabling attachment between the cytoskeleton and the extracellular matrix in skeletal muscle, and disruptions of this linkage are known to cause various muscular dystrophies (18). However, MEB was found to be caused by a defect in a glycosylation enzyme rather than in the DGC itself. This defect results in altered modification of α-dystroglycan, which has also been observed in FCMD, MDC1C, and the *myd* mouse, although the type of change in glycosylation occurring in these three cases remains to be elucidated.

Our current results support the growing body of evidence that some muscular dystrophies may be caused by abnormal glycosylation of α-dystroglycan, which acts as a linker between the cytoskeleton and the extracellular matrix. We suggest that the abnormal glycosylation of α-dystroglycan may be the basis for a novel pathogenic mechanism for some muscular dystrophies.

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Case report

Fukuyama-type congenital muscular dystrophy: a case report in the Japanese population living in Brazil

Zanoteli E, Rocha JCC, Narumia LK, Fireman MAT, Moura LS, Oliveira ASB, Gabbai AA, Fukuda Y, Kinoshita M, Toda T. Fukuyama-type congenital muscular dystrophy: a case report in the Japanese population living in Brazil.
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Introduction – We present herein clinical, histological and magnetic resonance imaging (MRI) findings in a patient with Fukuyama-type congenital muscular dystrophy (FCMD). He is the first case report in the Japanese population living in Brazil. *Case report* – The child presented with neonatal hypotonia, delayed motor abilities and speech, seizures, cerebral and cerebellar gyrus abnormalities with signal intensity change in the white matter by MRI, high serum level of creatinephosphokinase (CK), and dystrophic skeletal muscle with normal merosin, α-sarcoglycan and dystrophin expression. The fukutin gene study showed one founder 3-kb retrotransposal insertion in the 3′-non-coding region, and in the other allele no mutation was detected after screening all exons and flanking introns by sequencing. *Discussion* – This case report emphasizes the importance to consider FCMD in Japanese people living in other countries.

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Key words: Fukuyama disease; fukutin gene; congenital muscular dystrophy; brain dysplasia; skeletal muscle

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Fukuyama-type congenital muscular dystrophy (FCMD) is an autosomal-recessive disorder manifesting by brain malformation, principally cerebral and cerebellar cortical dysplasia, and by primary dystrophic changes in skeletal muscle (1, 2). It is recognized almost exclusively in Japanese children; being the second most common muscular dystrophy in Japan (incidence: 0.7–1.2/10,000) (2). A few families of non-Japanese origin with 'FCMD-like phenotype' have been reported (3). The patients manifest generalized muscle weakness and hypotonia from early infancy and the peak motor function usually consists only of unassisted sitting or sliding on the buttocks (2).

The gene responsible for FCMD, identified on the chromosome 9q31, encodes a 461-amino-acid protein that was termed fukutin (4–6). Kobayashi et al. (6) suggested that fukutin may be located in the extracellular matrix, where it interacts with and reinforces a large complex encompassing the outside and inside of muscle membranes. However, Sasaki et al. (7) suggested that fukutin may influence neuronal migration itself rather than formation of the basement membrane. It has been showed that about 87% of FCMD-bearing chromosomes had been derived from a single ancestral founder, whose mutation consisted of a 3-kb retrotransposal insertion in the 3′-non-coding region of the fukutin gene (6, 8, 9). The founder mutation is assumed to have been introduced into the Japanese population 100 generations ago (2000–2500 years) (6, 10). FCMD was the first human disease known to be caused primarily by ancient retrotransposal integration.

Brazil has one of the largest Japanese populations in the world outside of Japan (estimated in 1,250,000 people) and most live in the city of São Paulo. The Japanese people migrated to Brazil at the beginning of the 20th century. It would thus be expected that the founder insertion should be spread out in Brazil. However, in Brazil there is only one report of a clinically suggestive FCMD patient (11). In that case the cranial computed tomography imaging was not consistent with FCMD, and the authors did not inquire if the patient had a Japanese descent. In this work we present the first FCMD patient, with a founder insertion in the fukutin gene, in the Japanese population living in Brazil.

Case report

The 2-year-old boy was seen at the Neuromuscular Section in June 1999, because of intense hypotonia and delayed motor functions. He was the first child of a non-consanguineous couple and was born in Japan when the parents were visiting that country. His mother is of Japanese descent and his father is of both Japanese and Brazilian descent. The mother had a normal pregnancy and the child had a mild perinatal hypoxia, with neonatal hypotonia, poor cry and limb mobility. Birth weight was 2400 g. A mild motor function improvement had been observed in the previous months and he could crawl and sit without support (Fig. 1). He could not stand up even with support. Upon examination he was alert, interested in his surroundings and co-operative, trying to learn what was asked of him. He could speak only one or two words, not forming complete sentences. There were a general hypotonia, areflexia, and mild tendon retractions of knee and elbow. The weakness was diffuse affecting predominantly the neck and the proximal portions of limbs. He did not present dysphagia and his facial aspect was normal. Ocular mobility and ophthalmological examinations were normal. He had had three febrile seizures associated with pneumonia. The last two occurred despite using phenobarbital. The eletroencephalography was normal. The serum creatinephosphokinase (CK) level was 8518 (normal < 160 U/l).

The magnetic resonance imaging (MRI) done at the age of 5 months showed a bilateral fronto-temporo-parietal and cerebellar pachygyria, asymmetrical dilatation of the lateral ventricles, cortical atrophy and signal density abnormality in the cerebral and cerebellar white matter (Fig. 2A–C).

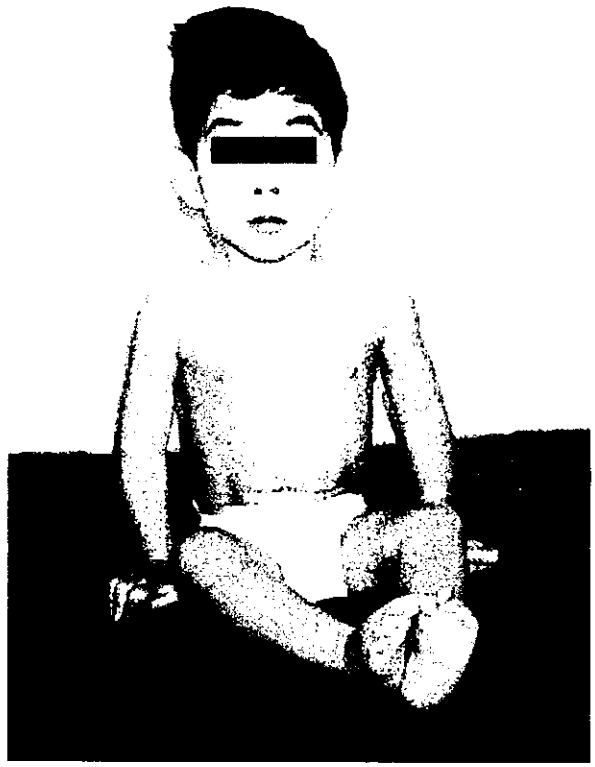

Figure 1. The FCMD boy with a typical clinical phenotype.

Deltoid muscle biopsy at the age of 7 months was processed as previously described (12) and showed an intense connective tissue infiltration, fiber type variability, and many rounded atrophic fibers without specific internal structural changes (Fig. 3A). There were many oxidative activity abnormalities such as moth-eaten and whorl fibers. Myosin ATPase stains revealed 100% of type 1 fiber predominance. Inflammation or fiber necrosis was not observed. The immunohistochemical study showed normal expression of α2-laminin chain (Novocastra, 300 kD), C- and N-dystrophin (Novocastra) and α-sarcoglycan (adhalin) (Novocastra) (Fig. 3B,C).

Genomic DNA was isolated from peripheral blood using a high-salt precipitation method. The FCMD gene was analyzed at the Division of Functional Genomics of Osaka University Graduate School of Medicine (Prof T. Toda) using probes and a protocol previously described (9). The patient had one founder 3-kb retrotransposal insertion in the 3′-non-coding region (Fig. 4). In the other allele no point mutation was detected after screening all exons and flanking introns of the FCMD gene by sequencing. The FCMD gene analysis of the parents was not done.

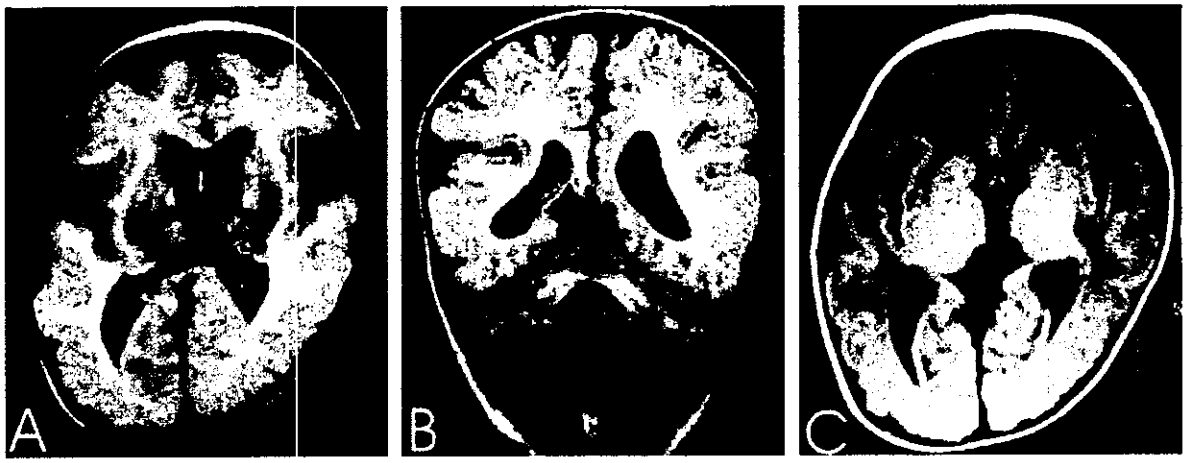

Figure 2. Brain magnetic resonance imaging showing, in (A) and (B), bilateral fronto-temporo-parietal and cerebellar pachygyria, asymmetrical dilatation of the lateral ventricles, cortical atrophy and high density in the cerebral and cerebellar white matter area (FLAIR sequence: TR = 11,000/TE = 140/TI = 2500); and in (C) low density in the cerebral white matter area (T1-weighted image: TR = 550/TE = 15).

Discussion

We present herein a child with clinical, histological and MRI characteristics of FCMD (1, 2). The clinical spectrum of FCMD is broad and the patients have been classified into three groups according to maximum motor abilities: (A) patients with the typical phenotype are able to sit unassisted or to slide on the buttocks; (B) patients with a mild phenotype can stand or walk with or without support; and (C) patients with a severe phenotype are able to sit only with support or have no head control (2). Our patient had a typical phenotype. The motor function had slowly improved over time after the neonatal period; and he did not have significant tendon retractions, which are frequently noted in FCMD patients. Although he had a relatively normal cognition, he could not form complete sentences. Speech difficulties are detected in all cases of FCMD. Seizures have been described in about half of the patients (13). Our patient presented with febrile seizures with poor medication control. Ophthalmological changes such as optic atrophy, myopia, retinal detachment and abnormal eye movements have also been described in FCMD patients (2). These abnormalities were not found in our case. The main MRI changes in our case were cerebral and cerebellum gyrus abnormalities, enlargement of ventricles and signal abnormalities of white matter of the periventricular area. These changes are characteristically described in FCMD patients (2).

Skeletal muscle that disclosed congenital muscular dystrophy (14) was characterized by a dystrophic aspect with intense connective tissue infiltration, fiber size variability, muscle fibers rounded, internal disarrangement of architecture of fibers without specific abnormalities. The expression of merosin, dystrophin and α-sarcoglycan was normal in this case, as has usually been described in FCMD patients.

The study of the FCMD gene in our patient detected one founder 3 kb retrotransposal insertion on one allele. Most FCMD patients (about 75%) are homozygous for the 3 kb insertion (9). Patients with one founder insertion usually have another point mutation on the other allele (9). However, routine sequencing of the FCMD gene did not detect any other mutation in our patient. Normal individuals, other than carriers, have no insertion; hence, this patient most probably has FCMD (9). Mutations may be difficult to be found by routine sequencing (e.g. in the promoter region or introns), and this can explain the fact that we did not find one in our case.

Kondo-Iida et al. (9) showed that the frequency of severe phenotype is significantly higher among probands who were compound heterozygotes carrying a point mutation on one allele and the founder mutation on the other than it is among probands who were homozygous for the founder insertion. Remarkably, no FCMD patients have been described with non-founder mutations on both alleles of the gene, suggesting that such cases might be embryonic-lethal (9). This could explain why few FCMD cases are reported in non-Japanese populations (9). Using a haplotype-phenotype correlation, Saito et al. (15) confirmed that severe FCMD patients appeared to be compound heterozygotes for the founder insertion and another mutation. Interestingly, although our case had only one founder insertion, he had a better

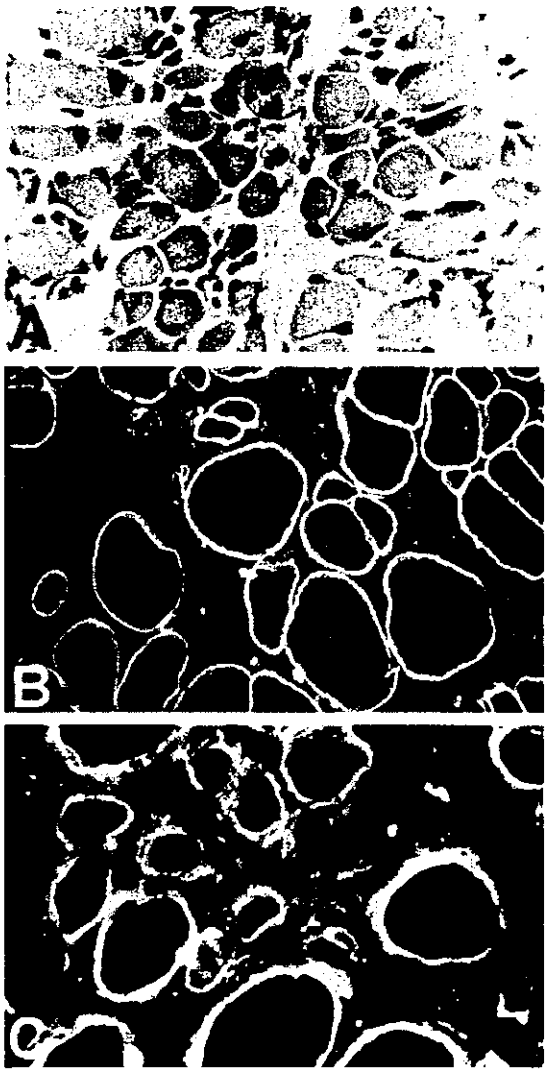

Figure 3. (A) Dystrophic aspect of skeletal muscle tissue (HE ×400). Normal expression of dystrophin (B) and merosin (C) by immunofluorescense analysis.

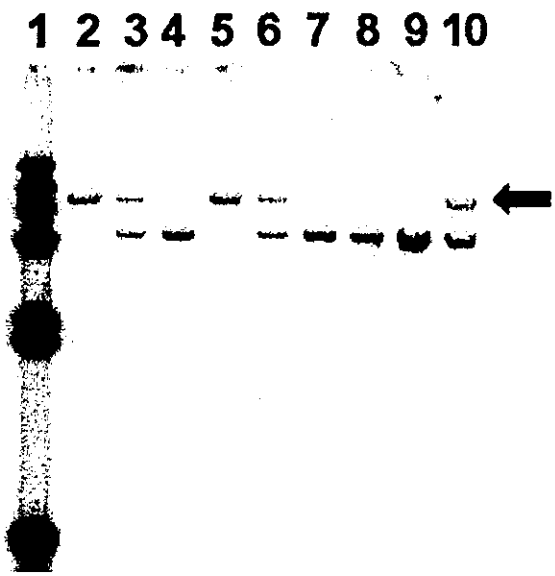

Figure 4. PvuII digested Southern blot hybridized with a fragment probe, E6f3, that detects the 3 kb retrotransposal insertion (arrow) in FCMD. Lane 1: molecular weight marker (lambda DNA/HindIII), 2: FCMD, 3: 2's mother, 4: non-FCMD, 5: FCMD, 6: 5's mother, 7–9: control, 10: present patient.

phenotype than is usually observed in the compound heterozygotes patients. The other mutation in compound heterozygotes is in most of the patients a nonsense or frameshift mutation (9). Chromosomes carrying the 3 kb insertion may merely produce a lower level of mature fukutin than normal and generate a relatively mild FCMD phenotype, as opposed to mutations (nonsense or frameshift) that cause serious structural changes in fukutin (9). Our patient has most probably a mutation (not detected) that has little effect on protein function.

By applying two methods for the study of linkage disequilibrium between flanking polymorphic markers and the disease locus, and of its decay over time, Colombo et al. (10) calculated the age of the insertion mutation causing FCMD in the Japanese population to be approximately 102 generations or slightly less. The estimated age dates the most recent common ancestor of the mutation-bearing chromosomes back to the time (or a few centuries before) the Yayoi people began migrating to Japan from the Korean peninsula (10). A FCMD carrier with a single FCMD mutation has spread throughout Japan.

We think that the main cause for the paucity of FCMD patients described in Brazil is a misdiagnosis. Another possibility may be because of the sizable racial intermingling in Brazil. The intermingling would 'dilute' the founder mutation diminishing the incidence of the disease that, when present, would most of the time be a compound heterozygote. Being severe there would be a high probability of early death without a proper diagnosis. A study of the prevalence of the founder mutation in Japanese people outside of Japan, including Brazil, would be crucial to understand the paucity of FCMD described outside of Japan. Kondo-Iida et al. (9) showed that compound heterozygotes patients can present a Walker–Warburg syndrome-like phenotype; and this situation has been described more frequently in Brazil (16, 17), but those cases did not present a Japanese descent.

This report emphasizes the importance of considering FCMD in Japanese people living outside of Japan. In those, the miscegenation can produce

a much severe phenotype because of a compound heterozygosity for the founder insertion.

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Isolation and Characterization of the Mouse Ortholog of the Fukuyama-type Congenital Muscular Dystrophy Gene

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Fukuyama-type congenital muscular dystrophy (FCMD) is a severe autosomal-recessive muscular dystrophy accompanied by brain malformation. Previously, we identified the gene responsible for FCMD through positional cloning. Here we report the isolation of its murine ortholog, *Fcmd*. The predicted amino acid sequence of murine fukutin protein encoded by *Fcmd* is 90% identical to that of its human counterpart. Radiation hybrid mapping localized the gene to 2.02 cR telomeric to *D4Mit272* on chromosome 4. Northern blot analysis revealed ubiquitous expression of *Fcmd* in adult mouse tissues. Through *in situ* hybridization, we observed a wide distribution of *Fcmd* expression throughout embryonic development, most predominantly in the central and peripheral nervous systems. We also detected high *Fcmd* expression in the ventricular zone of proliferating neurons at 13.5 days post-coitum. Brain malformation in FCMD patients is thought to result from defective neuronal migration. Our data suggest that neuronally expressed *Fcmd* is likely to be important in the development of normal brain structure.

Key Words: Fukuyama-type congenital muscular dystrophy, *FCMD*, fukutin, mouse, cDNA, neuronal migration, brain development, alternative splicing

INTRODUCTION

Fukuyama-type congenital muscular dystrophy (FCMD; MIM 253800) is an autosomal-recessive disorder characterized by primary dystrophic alterations in skeletal muscle and brain malformation [1]. Within the Japanese population, FCMD is the second most common muscular dystrophy and one of the most prevalent autosomal-recessive diseases. FCMD patients manifest generalized muscle weakness and hypotonia from early infancy, and most never achieve the ability to walk. In addition, the patients are mentally retarded and some experience seizures. The predominant brain anomalies in FCMD are polymicrogyria, pachygyria, and agyria. These are the result of cortical dysplasia, which is characterized by the massive penetration of neurons into the extracortical glial layer through breaches in the glia limitans and basal lamina [2–4].

We earlier identified the gene responsible for FCMD through positional cloning [5]. The *FCMD* gene encodes a novel 461-amino-acid protein, termed fukutin, of unknown function. Most patients with FCMD carry a 3-kb retrotransposal insertion into the 3′-untranslated region of the *FCMD* gene. In patients carrying this insertion, *FCMD* mRNA is nearly undetectable.

Here we report the isolation, chromosomal mapping, and expression profile of the mouse *Fcmd* gene. Some areas of *Fcmd* expression in the mouse embryo and neonate overlap with regions that exhibit abnormalities in FCMD patients. The mapping of *Fcmd* to mouse chromosome 4 and the evaluation of its expression will be important in understanding the genetic basis of FCMD.

RESULTS

Isolation of the Mouse *Fcmd* cDNA

To clone the murine homolog of *FCMD*, we screened a mouse brain cDNA library using a human *FCMD* cDNA as a probe. This screen produced two independent cDNA

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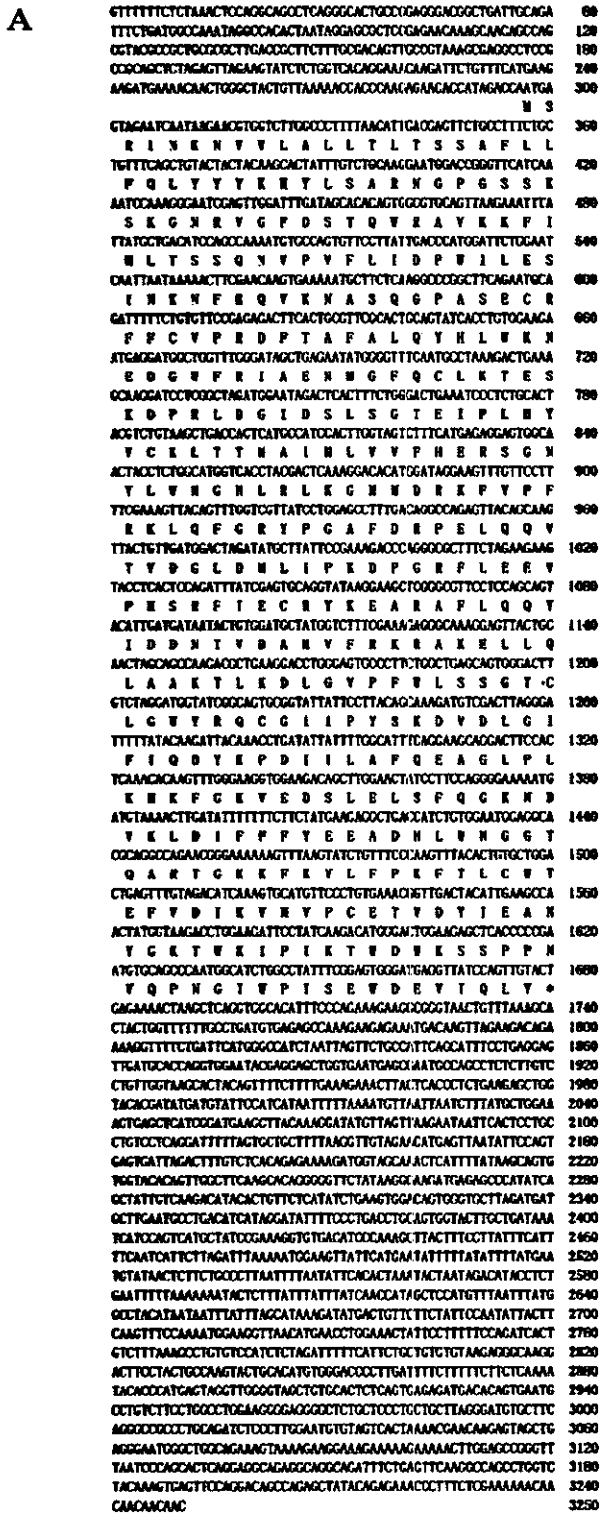

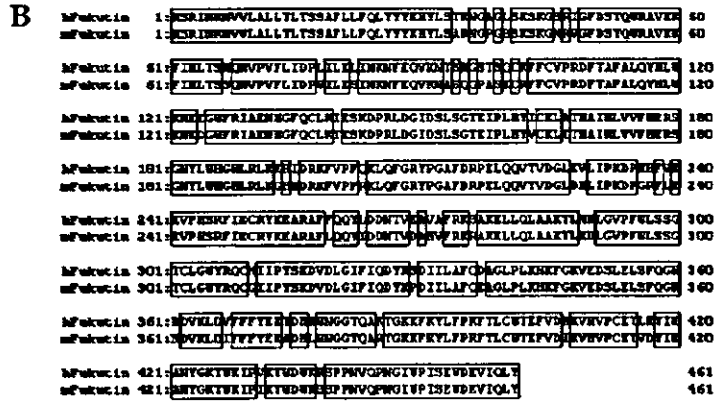

FIG. 1. Isolation of the mouse *Fcmd* cDNA. (A) Nucleotide and predicted amino acid sequences of murine *Fcmd*. (B) Alignment of deduced amino acid sequences of human (h) and murine (m) fukutin.

clones. One clone (pBSmFCMD-1) carried a 3250-bp insert containing the entire region encoding fukutin protein (Fig. 1A). The other clone harbored a 3068-bp insert that is identical to nucleotides from 183 to 3250 of pBSmFCMD-1. Sequence analysis and alignment showed 90% identity between the predicted amino acid sequence of murine fukutin protein and human fukutin [5] (Fig. 1B).

Chromosomal Mapping of Mouse *Fcmd*

We determined the chromosomal location of murine *Fcmd* by radiation hybrid (RH) mapping. Data obtained from the 100 RH clones in the T31 RH panel were: 01100 00000 10112 00000 01000 01010 00201 01100 00002 00202 01000 00100 20010 10021 10000 00111 00020 00000 01100 02201. Data analysis by the Whitehead Institute RH Mapping Server indicated that the *Fcmd* gene is located 2.02 cR telomeric to *D4Mit272* on mouse chromosome 4.

Fcmd Expression in Adult and Embryonic Tissues

We examined the expression profile of murine *Fcmd* using northern blots prepared from adult and embryo tissues. The *Fcmd* probe detected a 6.5-kb band in all tissues examined (Fig. 2A). A 4-kb transcript was also present in testis. *Fcmd* mRNA was already detectable in the 7-day embryo, and its expression persisted through late embryonic development (Fig. 2B). During the course of making *Fcmd* gene mutant mice, we examined *Fcmd* transcripts by RT-PCR in AB2.2 prime embryonic stem cells using primers amplifying different exons to assess the effects of mutation in the genome on *Fcmd* gene expression, and found a possible extra transcript (unpublished data). RT-PCR analysis of RNA prepared from AB2.2 cells (data not shown) and from adult mouse brain (Fig. 3A) using primers 5′-TCCGCCGCAGCTCTAGAGTTA-3′ and 5′-CACATTTTGGCTGGATGTCAGCATAATA-3′ revealed an extra band (~ 470 bp) in addition to the expected 330-bp band. No band was detected in the RT(-) control sample (Fig. 3A), excluding the possibility that this extra band is due to contamination of genomic DNA. This extra band was also present in all mouse tissues examined (Fig. 3B). Sequence analysis showed that this product harbors an additional 138-bp sequence within the region coding for fukutin protein (Fig. 3C). This transcript is unlikely to encode a mature fukutin protein, however, because of the presence of an in-frame stop codon within the 138-bp sequence (Fig. 3C).

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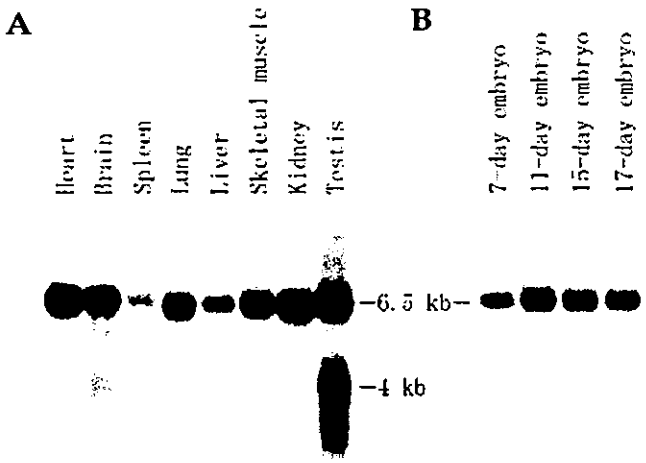

FIG. 2. Expression of the mouse *Fcmd* gene as determined by northern blotting. Mouse MTN (A) and mouse embryo MTN (B) blots (Clontech) were hybridized with a ^{32}P-labeled mouse *Fcmd* probe.

In Situ Hybridization Analysis of *Fcmd* Expression

We conducted tissue *in situ* hybridization analysis by probing sections of normal mouse embryos from days 9.5 to 15 postcoitum (p.c.), newborn brains, and adult brains with a mouse *Fcmd* cRNA. At 9.5 days p.c., *Fcmd* mRNA transcripts were present in the neuroepithelium of the forebrain and hindbrain (Fig. 4A). The sense control probe showed only background levels of signal (Fig. 4K). *Fcmd* transcripts appeared in the forebrain, midbrain, hindbrain, neural tube, and spinal ganglia at 11.5 days p.c. (Fig. 4B). At 12.5 days p.c., expression was detected in the brain, spinal cord, spinal ganglia, and trigeminal ganglia (Fig. 4C), with higher levels observed in the ventral horns of the spinal cord (Fig. 4D). At 13.5 days p.c., expression was seen in the brain neuroepithelium, with a higher level of signal in the ventricular zone of proliferating neurons (Fig. 4E). By 15 days p.c., *Fcmd* expression was seen in the cerebellum, cortex, tectum, thalamus, and spinal cord (Fig. 4F). At birth, transcript levels had decreased relative to prenatal stages, but transcripts were still present at low levels in the cortex, hippocampus, thalamus, pons, tectum, cerebellum, and medulla (Figs. 4G and 4H). In the adult brain, *Fcmd* expression was present in the dentate gyrus and horn of Ammon cells of the hippocampus, and in the cortex (Fig. 4I). In the cerebellum, expression was seen in the Purkinje cell layer (Fig. 4J). These observations indicate that *Fcmd* is expressed in a widespread fashion but at higher levels in the central and peripheral nervous systems.

DISCUSSION

FCMD is a disorder of neuronal migration, and protrusion of neuroglial tissues through breaches in the glia limitans–basal lamina complex is a probable cause of the observed cortical dysplasia in FCMD patients [2–4]. Therefore, it is suspected that altered fukutin function might affect neuronal migration during corticogenesis. The role of fukutin is currently unknown, although detailed sequence analysis suggests that it may function as an enzyme to modify cell-surface glycoproteins or glycolipids [6]. Several lines of evidence support this prediction. First, an abnormal, immature ganglioside pattern has been seen in the cerebral gray and white matter of FCMD patients [7]. Second, a selective deficiency in highly glycosylated α-dystroglycan on the surface membranes of skeletal and cardiac muscle fibers has recently been found in patients with FCMD [8]. Third, the fukutin-related protein (FKRP) gene was identified as a causative gene for another type of congenital muscular dystrophy, MDC1C, which also

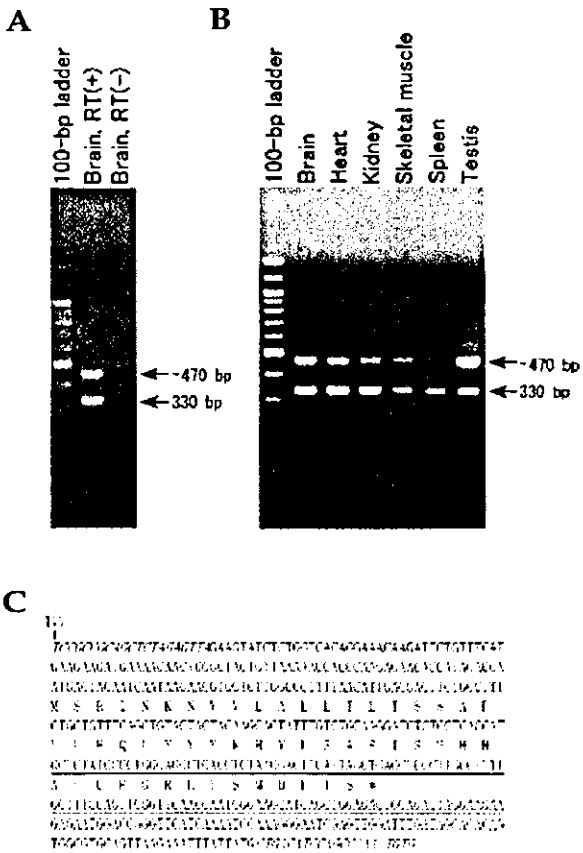

FIG. 3. An alternative transcript of the mouse *Fcmd* gene. (A) Total RNA from adult mouse brain was treated with DNase to remove traces of genomic DNA contamination and was subjected to RT-PCR. Mouse *Fcmd* cDNAs amplified by RT-PCR were analyzed by 2% agarose gel electrophoresis. PCR was also carried out on total RNA without RT (RT(-)) as a negative control. The 330-bp PCR product expected from mouse *Fcmd* sequence shown in Figure 1 and an extra ~ 470-bp PCR product are indicated by arrows. (B) Mouse *Fcmd* cDNAs were amplified from mouse Marathon-Ready cDNA (Clontech) by PCR and analyzed by 2% agarose gel electrophoresis. (C) The nucleotide and predicted amino acid sequences of an alternative mouse *Fcmd* transcript. The nucleotide sequence of the ~ 470-bp RT-PCR product derived from AB2.2 embryonic stem cells was determined. An additional 138-bp nucleotide sequence that is not present in the mouse *Fcmd* sequence shown in Figure 1 is underlined. Mouse *Fcmd* sequences used to design primers for these PCR analyses are italicized.

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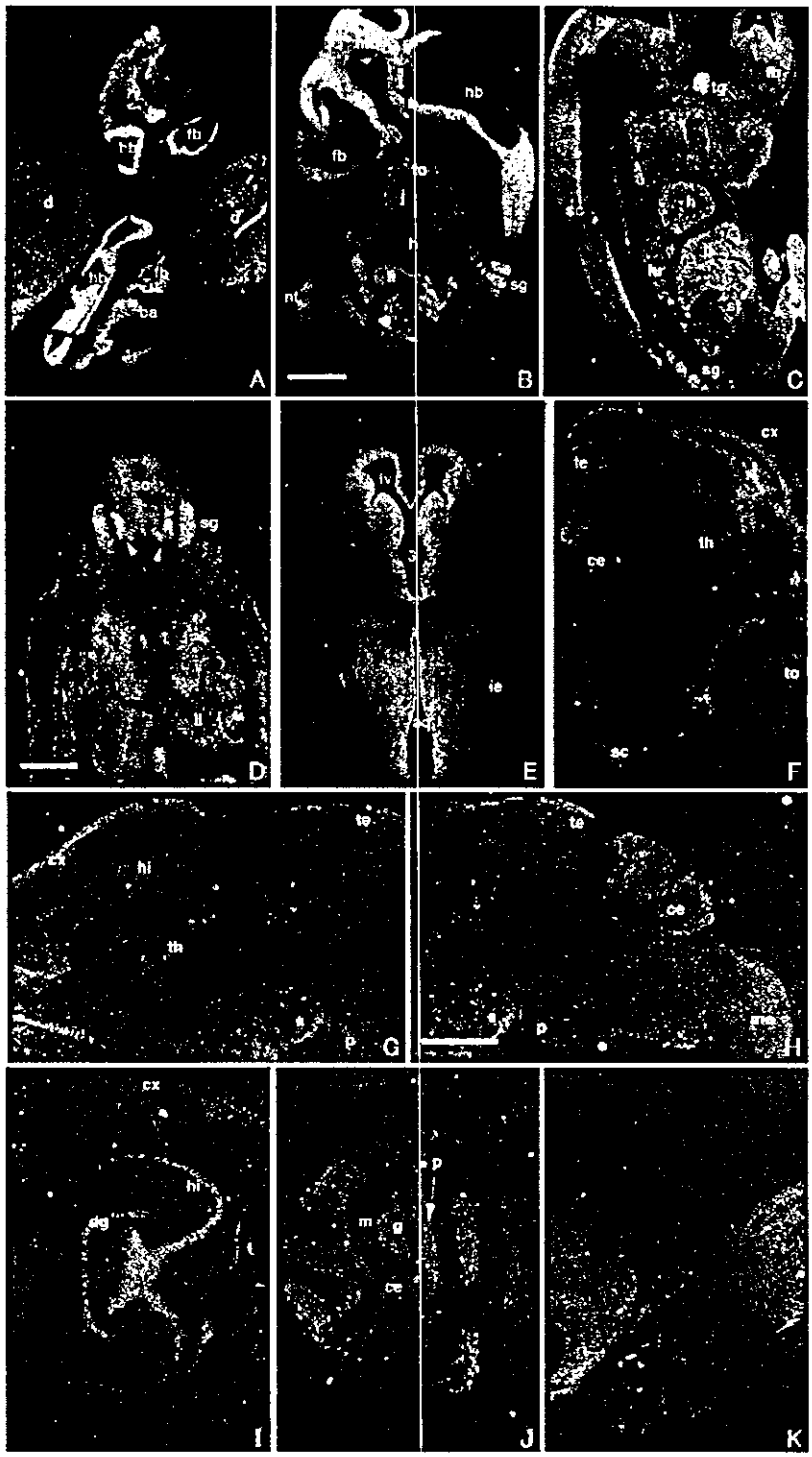

FIG. 4. Tissue *in situ* hybridization of murine *Fcmd* on mouse tissues. (A) Sagittal and oblique section, 9.5 days p.c. (B) Sagittal section, 11.5 days. (C) Sagittal section, 12.5 days. (D) Frontal section, 12.5 days. Arrows indicate ventral horns of the spinal cord. (E) Transverse section, 13.5 days. (F) Sagittal section, 15 days. (G, H) Neonatal sagittal sections. (I) Adult hippocampus sagittal section. (J) Adult cerebellum sagittal section. An arrow indicates the Purkinje cell layer. (K) Sense control probe hybridized to a serial section adjacent to that in (A) to show the background level of the signal. Fb, Forebrain; hb, hindbrain; ba, branchial arch; d, deciduas; mb, midbrain; h, heart; j, jaw; li, liver; nt, neural tube; sg, spinal ganglia; to, tongue; hl, handling; lu, lung; sc, spinal cord; st, stomach; tg, trigeminal ganglia; lv, lateral ventricle; 3, third ventricle; ie, inner ear; aq, aqueduct of sylvius; cx, cortex; te, tectum; ce, cerebellum; th, thalamus; hi, hippocampus; p, pons; mo, medulla; P, Purkinje cell layer; m, molecular layer of the cerebellum; g, granular layer of the cerebellum; dg, dentate gyrus. Scale bars: (G), (H) 1 mm; (A–C), (E), (F), and (I–K) 800 μm; (D) 400 μm.

involves abnormal glycosylation of α-dystroglycan [9]. Fourth, recent findings have demonstrated the involvement of glycosyltransferase genes in other autosomal-recessive muscular dystrophies that show neuronal phenotypes both in human [10] and mouse [11]. These observations strongly suggest an enzymatic function of fukutin, although specific molecular targets of fukutin in the migration process remain to be identified.

The pattern and timing of *Fcmd* expression in the CNS (Figs. 2 and 4), suggest a general involvement of this gene in early development. Histopathological studies have revealed the presence of cerebral polymicrogyria during the mid-fetal period [2,12], when prospective cortical neurons are migrating from the ventricular zone to the cortical plate. In this study, we detected a higher level of *Fcmd* expression in the ventricular zone of proliferating neurons at 13.5 days p.c. (Fig. 4E). In the cerebellum, *Fcmd* expression is widely distributed at 15 days p.c. and at birth; however, its expression is restricted to the Purkinje cell layer in the adult brain. Together, these results imply that fukutin may be required for proper migration of neurons.

In this study we have isolated the mouse *Fcmd* cDNA and have found its alternatively spliced variant. We also characterized the expression profile of the murine *Fcmd*. Determination of the complete genomic structure of murine *Fcmd* will further help to understand the transcriptional and post-transcriptional regulation of *Fcmd*. We have mapped the murine *Fcmd* to mouse chromosome 4 at 2.02 cR telomeric to *D4Mit272*. This region is syntenic to human 9q22.3–q31. Considering that its human ortholog was placed at 9q31 [13–15], it is likely that no chromosomal rearrangements have occurred in this region between human and mouse. To date, no mouse mutant has been mapped to this region. Establishment of mouse models carrying genetic alterations in *Fcmd* will further clarify the molecular mechanisms underlying phenotypes associated with *Fcmd* defects.

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Materials and Methods

Isolation of mouse Fcmd cDNA clones. A 175-bp fragment surrounding the translational start site of human *FCMD* gene was PCR amplified using primers 5′-CCTTTCCAAATCCAAAAAGATG-3′ and 5′-TTGTTGATAAATAGTGCTTGTAG-3′. This fragment was used as a probe to screen a mouse brain cDNA library prepared from 19-week-old female C57BL/6 mice using the Uni-ZAP XR vector (Stratagene; cat. no. 937314). cDNA fragments of positive phages were cloned into the pBluescript SK(−) phagemid vector (Stratagene) by *in vivo* excision. Insert sizes were measured by digestion with *Not*I and *Xho*I, followed by separation on a 0.8% (wt/vol) agarose gel. The pBSmFCMD-1 clone contained the largest insert (3250 bp).

Radiation hybrid mapping. Chromosomal mapping was accomplished by PCR screening of the T31 RH panel (Research Genetics) according to the manufacturer's protocol, using the primers 5′-AAGTATCTCTGGTCACAGGAAAC-3′ (P1) and 5′-CCTTGCAGACAAATAGTGCTTGT-3′ (P2), which were designed to amplify the region surrounding the putative translational start site of the mouse *Fcmd* gene. The amplification profile consisted of 40 cycles of annealing for 30 seconds at 62°C and extension for 30 seconds at 72°C. A 203-bp product was visualized on an ethidium bromide–stained agarose gel. Data were submitted to the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research Radiation Hybrid Mapping Program (http://www-genome.wi.mit.edu/cgi-bin/mouse_rh/rhmap-auto/rhmapper.cgi).

Northern blot analysis. A mouse *Fcmd* cDNA probe was prepared by PCR using P1/P2 primer set. Gene expression was evaluated using mouse multiple-tissue northern (MTN) and mouse embryo MTN blots (Clontech) according to the manufacturer's protocol.

RNA preparation and RT-PCR analysis. Total RNA from AB2.2 prime embryonic stem cells (Lexicon Genetics Inc.) and 3-month-old C57BL/6 mouse brain was isolated using Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. After DNase treatment, reverse transcription was carried out with 1.0 μg of total RNA as template using $pd(N)_6$ random hexamer (Amersham Biosciences) and Superscript II reverse transcriptase (Invitrogen). PCR was done using *Taq* DNA polymerase with primers 5′-TCCGCCGCAGCTCTAGAGTTA-3′ and 5′-CACATTTTGGCTGGATGTCAGCATAATA-3′. PCR was also carried out using mouse brain, heart, kidney, skeletal muscle, spleen, and testis Marathon-Ready cDNA (Clontech) as templates.

Tissue in situ hybridization. Fixation and embedding of BALB/c and C57BL/6XDBA/2 embryos, fetuses, and postnatal brains is detailed in Lyons *et al.* [16]. Briefly, embryos were fixed in 4% paraformaldehyde in PBS overnight, dehydrated, and infiltrated with paraffin. Serial sections of 5–7 μm were mounted per slide, deparaffinized in xylene, rehydrated, and post-fixed. Sections were digested with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed, and dehydrated. An *Eco*RI-*Hind*III fragment of pBSmFCMD-1 (nt 1–2063) was subcloned into the *Eco*RI and *Hind*III sites of pBluescript II SK(−) (Stratagene) and used to prepare cRNA probes. The plasmid was linearized with *Not*I, and T7 polymerase was used to generate antisense cRNA. The plasmid was linearized with *Xho*I, and T3 polymerase was used to generate the sense control cRNA. The cRNA transcripts were synthesized according to the manufacturer's protocol (Stratagene) and labeled with ^{35}S-UTP (> 1000 Ci/mmol; Amersham Pharmacia Biotech). cRNA transcripts were subjected to alkaline hydrolysis to give a mean size of 70 bases for efficient hybridization. Sections were hybridized overnight at 52°C in 50% (vol/vol) deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM $NaPO_4$, 10% (wt/vol) dextran sulfate, 1× Denhardt's, 50 μg/ml total yeast RNA, and 50,000–75,000 c.p.m./μl ^{35}S-labeled cRNA probe. Slides were subjected to stringent washes at 65°C in 50% (vol/vol) formamide, 2× SSC, 10 mM dithiothreitol, and rinsed in PBS before treatment with 20 μg/ml RNase A at 37°C for 30 minutes. Following washes in 2× SSC and 0.1× SSC for 10 minutes at 37°C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 1–2 weeks in light-tight boxes with desiccant at 4°C. Photographic development was carried out in Kodak D-19. Slides were counterstained lightly with toluidine blue and analyzed using light- and darkfield optics of a Zeiss Axiophot microscope.

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Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession numbers AB077383 and AB077384.

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Histamine-induced IL-6 and IL-8 production are differentially modulated by IFN-γ and IL-4 in human keratinocytes

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Abstract

It is known that large amounts of histamine are stored in mast cells located in the superficial dermis of the skin and can be released upon appropriate stimulation. However, the effects of histamine on keratinocyte function have not been well characterized. We therefore examined the capacity of histamine to modulate the production of interleukin (IL)-6 and IL-8 by keratinocytes. We found that histamine significantly augmented the production of IL-6 and IL-8 in a dose- and time-dependent manner. The enhancing effects of histamine were completely inhibited by a potent H1 receptor (H1R) antagonist, emedastine difumarate. Pyrilamine (a much weaker H1R antagonist) and cimetidine (an H2R antagonist) only partially inhibited the enhancing effects of histamine. The histamine-induced up-regulation of IL-6 and IL-8 production, however, was completely abrogated by a combination of pyrilamine and cimetidine. The IL-6 production was significantly enhanced by interferon (IFN)-γ. Interestingly, IFN-γ and IL-4 both significantly augmented the histamine-induced IL-6 production. On the other hand, the production of IL-8 was inhibited by IFN-γ, and IFN-γ and IL-4 both completely abrogated the histamine-induced IL-8 production. These results suggest that the histamine-induced IL-6 production and IL-8 production are differentially regulated by IFN-γ and IL-4. Histamine may be an important modulator of cytokine production in epidermal milieu. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Histamine; Mast cells; Keratinocyte

1. Introduction

Histamine, first discovered in 1910 [1,2], was initially identified as a potent vasoactive substance and is now known to be an important mediator in immediate-type hypersensitivity and inflammatory

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dermatoses, such as urticaria, atopic dermatitis and psoriasis [3].

Keratinocytes actively participate in the initiation and/or regulation of cutaneous inflammatory and immune responses. They can synthesize and release an array of pro-inflammatory cytokines and chemokines such as IL-6 and IL-8 [4]. IL-6 is a multifunctional cytokine that is an important mediator in the host response to injury and infection [5]. It stimulates the production of acute-phase proteins by the liver, enhances B- and T-cell activation and proliferation [6], and stimulates keratinocyte proliferation [7,8]. IL-8, a member of the CXC chemokine family, is a potent chemotactic factor for neutrophils and T cells [4].

In the skin, histamine is produced from histidine by histamine decarboxylase and degraded to methylhistamine by histamine methyltransferase [9]. Histamine is released from mast cells and keratinocytes in inflammatory skin diseases [10–12]. The interstitial concentration of histamine in the normal dermis seems to be 10^{-8}–10^{-7} M [13,14]. However, the effects of histamine on keratinocyte function are not well known. In the present study, we examined the capacity of histamine to modulate the production of IL-6 and IL-8 by keratinocytes.

2. Materials and methods

2.1. Reagents

Histamine, pyrilamine and cimetidine were obtained from Sigma Chemical Co. (St. Louis, M0, USA). Recombinant human IL-4 and IFN-γ were obtained from Genzyme Corp. (Boston, MA, USA). Emedastine difumarate was obtained from Kowa Pharmaceutical Co, Nagoya, Japan. Pyrilamine and emedastine difumarate are H1R antagonists. Emedastine difumarate has a much stronger capacity to bind to H1R. Cimetidine is a strong H2R antagonist.

2.2. Cells and cell culture

The spontaneously immortalized, nontumorigenic human skin keratinocyte cell line HaCaT (kindly provided by Dr N.E. Fusenig, DKFZ Heidelberg) was used for the experiments. Cells were maintained in DMEM with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and were grown in a 250-ml tissue culture flask in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Normal human keratinocytes (NHKs) from neonatal foreskin (Kurabou, Osaka) were grown initially in keratinocyte basal medium (HuMedia-KB2, Kurabou) containing 0.15 mM calcium supplemented with 0.1 ng/ml epidermal growth factor, 0.4% bovine pituitary extract, 50 μg/ml gentamycine, and 50 ng/ml amphotericine B. No serum was added. HaCaT cells or NHKs were harvested by trypsinization and plated at a density of 4×10^5 cells per well into 24-well plates for cytokine measurement or 1×10^6 cells per well into 6-well plates for mRNA analysis.

2.3. Treatment of keratinocytes with histamine, pyrilamine, cimetidine, IFN-γ and IL-4

HaCaT cells or NHKs were plated at a density of 4×10^5 cells per well into 24-well plates and cultured for 24 h. After the medium had been changed to a fresh medium, the cells were treated with various concentrations of histamine (from 10^{-7} to 10^{-4} M). Supernatant was collected 1–48 h later, and concentrations of IL-6 and IL-8 were measured. In order to investigate the type of histamine receptors involved, keratinocytes were incubated with 10^{-5} M histamine in the presence and absence of pyrilamine (10^{-6}–10^{-5} M), emedastine difumarate (10^{-9}–10^{-8} M), cimetidine (10^{-6}–10^{-5}M) or pyrilamine + cimetidine. In some experiments, HaCaT cells were stimulated by incubation with IFN-γ (10–1000 U/ml) or IL-4 (1–100 ng/ml) in the presence or absence of 10^{-5} M of histamine for 24 h.

2.4. Measurement of IL-6 and IL-8 concentrations

Concentrations of IL-6 and IL-8 in the supernatant were measured using ELISA kits (Genzyme-Techne, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.5. RNA isolation and analysis

Total RNA was isolated from cells using RNA isolation kits (Gentra Systems, Minneapolis, MN, USA). Specific mRNA expression was analyzed using an RNA PCR kit (Takara Shuzo Co, Shiga, Japan). The cycling conditions were 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min for a total of 30 cycles, completed by incubation at 72 °C for 7 min.

The sequences of primers used were as follows: H1R, 5′ primer 5′-TGAGCACTATCTGCTTGGTCA-3′, 3′ primer 5′-TGCATGAAGTGATTCCAGCCT-3′ [15]; and H2R, 5′ primer 5′-TCATCACCGTTGCTGGCAATG-3′, 3′ primer 5′-TTAAGAATGGAGGCTGTGCAG-3′ [16]. The expected sizes were 409 bp for the H1R and 234 bp for the H2R. RT-PCR products were electrophoresed on 2.0% agarose gels and visualized by ethidium bromide staining over a transilluminator.

3. Results

3.1. Effects of histamine on IL-6 and IL-8 production in human keratinocytes

Production of IL-6 and IL-8 by keratinocytes was quantified at 24 h in the presence or absence of 10^{-7}–10^{-4} M of histamine. Histamine enhanced the IL-6 and IL-8 production in a dose-dependent manner both in the HaCaT cells and NHKs. Histamine at a concentration of 10^{-6}–10^{-4} M significantly up-regulated the production of IL-6 and IL-8 by HaCaT cells and NHKs (Table 1). The keratinocytes were then incubated with medium only or with 10^{-5} M of histamine for 1–48 h, and the time course of the cytokine production was examined. Histamine enhanced the production of IL-6 and IL-8 in a time-dependent manner (data not shown). The up-regulatory effect of histamine was clearly evident after 24 and 48 h of culture (data not shown). As the spontaneous and histamine-induced cytokine production was higher in the HaCaT cells than in the NHKs and the enhancing effects of histamine were similarly observed both in the HaCaT cells and NHKs, HaCaT cells were mainly used in the following experiments.

3.2. Inhibition of the histamine-induced IL-6 and IL-8 production by H1 and H2 receptor antagonists

In order to determine the involvement of H1R or H2R in the augmentation of IL-6 and IL-8 production by histamine, we first examined the expression of mRNAs for H1R and H2R by RT-PCR. Total RNA was extracted from HaCaT cells and analyzed by RT-PCR as described in Section 2. Products of the expected size for H1R and H2R mRNAs were clearly identified, as has been reported previously [17].

We next investigated whether emedastine difumarate, pyrilamine or cimetidine interferes with the enhancing effects of histamine on cytokine production. Keratinocytes were incubated with 10^{-9}–10^{-8} M of emedastine difumarate, 10^{-6}–10^{-5} M of pyrilamine or 10^{-6}–10^{-5} M of cime-

Table 1
Histamine dose-dependently stimulated the IL-6 and IL-8 secretion by HaCaT cells and NHKs. Representative data of fiveexperiments

		Control	Histamine			
			10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M
HaCaT cells	IL-6 (pg/ml ± S.D.)	53.8 ± 0.8	68.5 ± 3.1*	92.3 ± 1.0*	92.6 ± 2.2*	90.1 ± 2.8*
	IL-8 (pg/ml ± S.D.)	569.2 ± 92.1	781.8 ± 190.1	962.6 ± 161.2*	936.5 ± 20.9*	897.7 ± 74.8*
NHKs	IL-6 (pg/ml ± S.D.)	8.1 ± 0.4	7.9 ± 0.5	11.9 ± 0.5*	11.1 ± 0.1*	10.6 ± 1.1*
	IL-8 (pg/ml ± S.D.)	38.7 ± 14.1	71.8 ± 29.7	72.7 ± 12.2*	71.2 ± 14.4*	86.5 ± 3.7*

*$P < 0.05$ compared with unstimulated control.

Table 2
Percentage inhibition by H1R and H2R antagonists of the secretion of IL-6 and IL-8 from HaCaT cells in response to stimulation by histamine

	IL-6 (%)	IL-8 (%)
Emedastine 10^{-8} M (H1R antagonist)	100*	100
Pyrilamine 10^{-5} M (H1R antagonist)	66.8	63.3
Cimetidine 10^{-5} M (H2R antagonist)	48.7	52.2
Pyrilamine 10^{-5} M + Cimetidine 10^{-5} M	100	100

Mean percentage inhibition ($n = 3$).

tidine in the presence of histamine (10^{-5} M) for 24 h. As shown in Table 1, emedastine difumarate (a strong H1R antagonist) completely inhibited the histamine-induced up-regulation of IL-6 and IL-8 production. The inhibitory effect of pyrilamine (a weaker H1R antagonist) on the histamine-induced IL-6 and IL-8 production was only partial. Cimetidine, an H2R antagonist, also partially inhibited the effects of histamine. Interestingly, pyrilamine in combination with cimetidine completely abrogated the histamine-induced up-regulation of cytokine production (Table 2). Since the viability of keratinocytes was not affected, the inhibitory effects were not attributable to the cytotoxicities of these drugs.

3.3. Effects of IFN-γ and IL-4 on the histamine-induced cytokine production by keratinocytes

We next examined whether IFN-γ and IL-4 affect the histamine-induced IL-6 and IL-8 production. HaCaT cells were incubated with IFN-γ or IL-4 in the presence or absence of histamine (10^{-5}M) for 24 h. As shown in Fig. 1, the spontaneous production of IL-6 was significantly and dose-dependently up-regulated by IFN-γ (Fig. 1A). IL-4 slightly enhanced the spontaneous IL-6 production at concentrations of 1 or 10 ng/ml (Fig. 2A). Moreover, the histamine-induced IL-6 production was significantly augmented by both IFN-γ and IL-4 (Figs. 1A and 2A).

In contrast, the spontaneous production of IL-8 was dose-dependently inhibited by both IFN-γ and IL-4. The histamine-induced IL-8 up-regulation was also significantly down-regulated by both IFN-γ and IL-4 (Figs. 1B and 2B).

4. Discussion

We have examined the effects of histamine on the production of IL-6 and IL-8 by keratinocytes. Histamine significantly augmented the production of IL-6 and IL-8 in a dose- and time-dependent manner. The production of IL-6 and IL-8 by HaCaT cells was about 10-times greater than that by NHKs, partly because the immortalized HaCaT cells may be more metabolically active than NHKs. Keratinocytes expressed both H1R and H2R mRNAs. The enhancing effects of histamine were completely inhibited by a potent H1R antagonist, emedastine difumarate. Pyrilamine (a much weaker H1R antagonist) and cimetidine (an H2R antagonist) only partially inhibited the enhancing effects of histamine. However, the production of IL-6 and IL-8 was completely abrogated by the combination of pyrilamine and cimetidine. These results suggest that H1R signaling is more dominant than H2R signaling in inducing IL-6 and IL-8 production in keratinocytes.

It has been reported that histamine enhances the production of IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human conjunctival epithelial cells [18,19]. The production of IL-8 is also augmented in human bronchial epithelial cells by histamine [20]. As in these human epithelial cells, histamine was found to stimulate the production of IL-6 and IL-8 in human keratinocytes in the present study. Shinoda et al. examined the effect of histamine on production of IL-6 and IL-1α in keratinocytes induced by ultraviolet (UV) B irradiation [17]. They found that histamine, together with UVB, synergistically enhanced IL-6 production. In contrast, it was found that histamine had no effect on IL-1α production by keratinocytes [17]. As was observed in the present study, they also found that the effect of histamine was completely

blocked by an H1R antagonist and was partially inhibited by an H2R antagonist [17].

IL-8 is a potent neutrophilic chemoattractant [21], and IL-6 directly induces the proliferation of keratinocytes, resulting in epidermal acanthosis [7]. The fact that histamine enhances IL-6 and IL-8 production is very interesting when considering the pathomechanism of chronic inflammatory skin disorders such as psoriasis.

Histopathologically, psoriasis is characterized by regular acanthosis, intraepidermal neutrophilic accumulation, and dermal infiltration of activated T cells, monocytes and mast cells [22]. IL-6 and IL-8 have been demonstrated to be expressed in high levels in psoriatic skin [7,23,24]. Moreover, intralesional histamine concentrations have been reported to be increased in the lesional skin of psoriasis [14]. Thus, the histamine-induced IL-6 and IL-8 upregulation may further contribute to the lesional acanthosis and neutrophilic infiltration in psoriasis. Of note is that long-term treatment with a high dose of an H2R antagonist, ranitidine, improved psoriasis [25,26]. Further studies are needed to clarify the role of H1R and H2R in skin diseases.

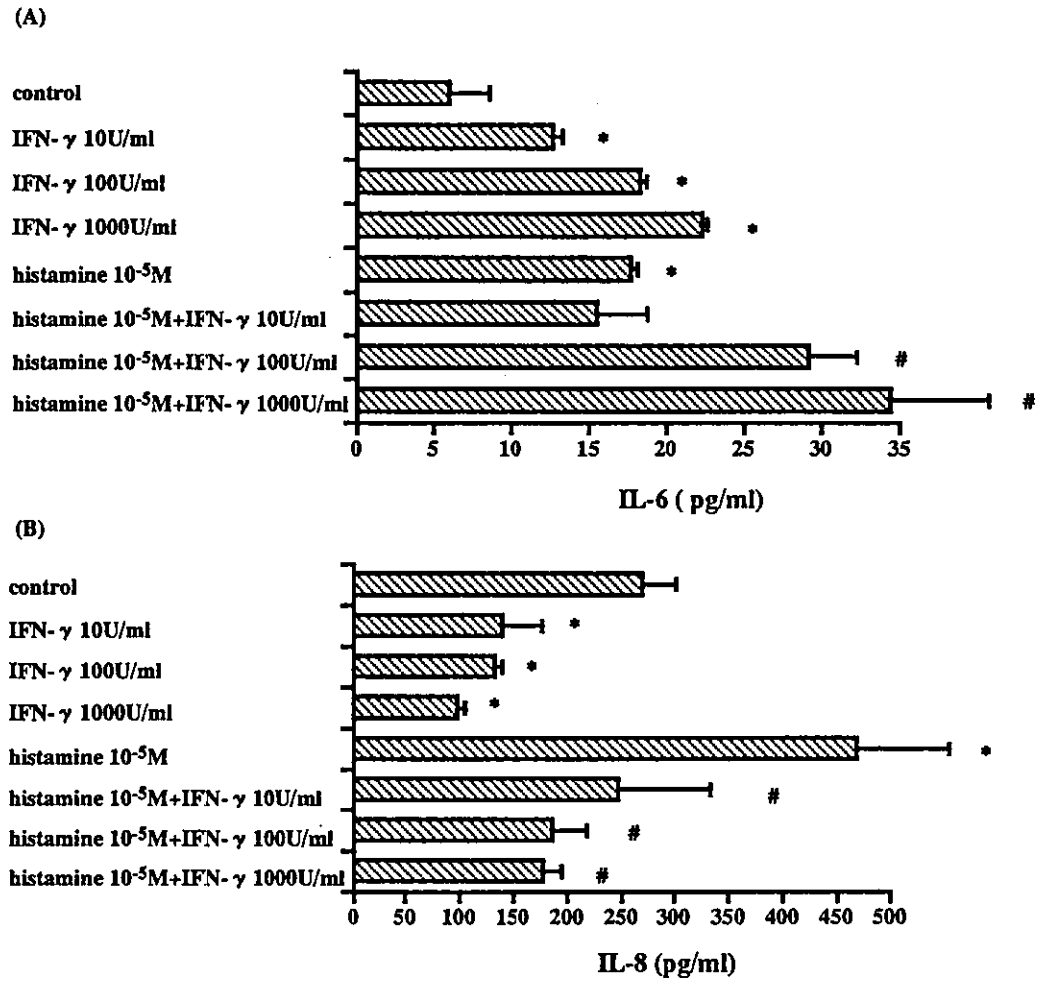

Fig. 1. Effects of histamine (10^{-5} M) and IFN-γ (10 –1000 U/ml) on IL-6(A) and IL-8(B) production by HaCaT cells. Representative data of three experiments. $^{*}P < 0.05$ compared with the spontaneous production (control). $\#\ P < 0.05$ compared with the histamine-induced production.