Colorado, USA.

H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

［ II ］研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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［ III ］研究成果の刊行物・別刷

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PLoS one

Subtypes of Familial Hemophagocytic Lymphohistiocytosis in Japan Based on Genetic and Functional Analyses of Cytotoxic T Lymphocytes

Kozo Nagai[1,2], Ken Yamamoto[3], Hiroshi Fujiwara[1,4], Jun An[1], Toshiki Ochi[1], Koichiro Suemori[1], Takahiro Yasumi[5], Hisamichi Tauchi[2], Katsuyoshi Koh[6], Maho Sato[7], Akira Morimoto[8], Toshio Heike[5], Eiichi Ishii[2]*, Masaki Yasukawa[1,4]*

1 Department of Bioregulatory Medicine, Ehime University Graduate School of Medicine, Ehime, Japan, **2** Department of Pediatrics, Ehime University Graduate School of Medicine, Ehime, Japan, **3** Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, **4** Proteo-Medicine Research Center, Ehime University, Ehime, Japan, **5** Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **6** Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **7** Department of Pediatrics, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan, **8** Department of Pediatrics, School of Medicine, Jichi Medical University, Tochigi, Japan

Abstract

Background: Familial hemophagocytic lymphohistiocytosis (FHL) is a rare disease of infancy or early childhood. To clarify the incidence and subtypes of FHL in Japan, we performed genetic and functional analyses of cytotoxic T lymphocytes (CTLs) in Japanese patients with FHL.

Design and Methods: Among the Japanese children with hemophagocytic lymphohistiocytosis (HLH) registered at our laboratory, those with more than one of the following findings were eligible for study entry under a diagnosis of FHL: positive for known genetic mutations, a family history of HLH, and impaired CTL-mediated cytotoxicity. Mutations of the newly identified causative gene for FHL5, *STXBP2*, and the cytotoxicity and degranulation activity of CTLs in FHL patients, were analyzed.

Results: Among 31 FHL patients who satisfied the above criteria, *PRF1* mutation was detected in 17 (FHL2) and *UNC13D* mutation was in 10 (FHL3). In 2 other patients, 3 novel mutations of *STXBP2* gene were confirmed (FHL5). Finally, the remaining 2 were classified as having FHL with unknown genetic mutations. In all FHL patients, CTL-mediated cytotoxicity was low or deficient, and degranulation activity was also low or absent except FHL2 patients. In 2 patients with unknown genetic mutations, the cytotoxicity and degranulation activity of CTLs appeared to be deficient in one patient and moderately impaired in the other.

Conclusions: FHL can be diagnosed and classified on the basis of CTL-mediated cytotoxicity, degranulation activity, and genetic analysis. Based on the data obtained from functional analysis of CTLs, other unknown gene(s) responsible for FHL remain to be identified.

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* E-mail: yasukawa@m.ehime-u.ac.jp (MY); ishiei@m.ehime-u.ac.jp (EI)

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is characterized by fever and hepatosplenomegaly associated with pancytopenia [1–3]. Histologically, infiltration of lymphocytes and histiocytes with hemophagocytic activity is evident in the reticuloendothelial system, bone marrow, and central nervous system [4]. HLH can be classified as either primary or secondary [5]. Primary HLH, also known as familial hemophagocytic lymphohistiocytosis (FHL), is inherited as an autosomal recessive disorder that usually arises during infancy.

The pathogenesis of FHL has been considered to involve dysfunction of cytotoxic T lymphocyte (CTL) activity, leading to excessive production of inflammatory cytokines and macrophage activation [6]. The genetic mutations responsible for FHL have been identified by various methods. Linkage analysis has indicated two possible loci: FHL1 (MIM 603552) in 9q21.3-22, and FHL2 (MIM 603553) in 10q21-22 [7,8]. In 1999, a mutation in the *perforin* gene (*PRF1*) was identified as the cause of FHL2 [9–12]. Further genetic mutations of the *Munc13-4* gene (*UNC13D*) mapped to 17q25 (the cause of FHL3, MIM 608898) and the *syntaxin11* gene (*STX11*) mapped to 6q24 (the cause of FHL4, MIM 603552) were subsequently identified [13–15]. These mutations affect proteins involved in the transport and membrane fusion, or exocytosis, of perforin contained in cytoplasmic

granules. Recently, mutations of the *Munc18-2* gene (*STXBP2*), located in 19q, were detected as a cause of FHL5 [16,17]. Munc18-2 regulates intracellular trafficking and controls the soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) complex.

The molecular mechanisms underlying vesicular membrane trafficking and regulation of exocytosis have been clarified in recent years. The final step of vesicle transport is mediated by a bridge between a vesicle and its target membrane through formation of a ternary complex between a vesicle-SNARE (v-SNARE), such as a VAMP, and a target membrane-SNARE (t-SNARE), such as a syntaxin11 or a member of SNAP23/25/29 [18]. The SNARE complex is composed of three molecules: VAMP, syntaxin and SNAP23/25/29. Syntaxin11, in association with SNAP23, localizes to the endosome and trans-Golgi network [19]; however, the precise biological functions of the SNARE system are still poorly understood. Recent evidence suggests that members of the SNARE family mediate fusion of cytotoxic granules with the surface of CTLs. Syntaxin11, SNAP23 and VAMP7 are prime candidates for functioning as SNAREs in this fusion event [20].

It has been considered that clarification of the molecular abnormalities in FHL might shed light on the mechanisms of CTL-mediated cytotoxicity. Accordingly, we have been studying the functional abnormalities of CTLs in Japanese patients with FHL [21]. Our previous studies have shown that the FHL2 and FHL3 subtypes account for 20–25% of all FHL cases, respectively, whereas no FHL4 subtype exists; therefore, 45–50% of FHL cases in Japan harbor still unknown genetic mutations [21,22]. However, secondary HLH could be involved in patients with unknown genetic mutations, because both FHL and secondary HLH share similar clinical and laboratory characteristics. Therefore, in the present study aimed at clarifying the incidence and subtypes of FHL in Japanese children by genetic and functional analyses of CTLs, only patients positive for known genetic mutations, a positive family history of HLH, or impaired natural killer (NK)/CTL-mediated cytotoxicity were diagnosed definitively as having FHL.

Materials and Methods

Patients

A total of 87 Japanese children aged <15 years diagnosed as having HLH based on the diagnostic criteria of the Histiocyte Society [23] were registered at our laboratory between January 1994 and December 2009. Among them, 40 were excluded from analysis because they were diagnosed as having secondary HLH, or their parents did not provide permission for use of clinical samples. None of the patients had Chediak-Higashi syndrome, Griscelli syndrome, or Hermansky-Pudlak syndrome type 2, based on clinical and laboratory findings, including albinism or the presence of gigantic granules in lymphocytes or granulocytes. A final total of 31 patients, who met the diagnostic criteria for FHL, and for whom documented informed consent had been obtained in accordance with the Declaration of Helsinki, were entered into the study.

Genetic analysis of the *STXBP2* gene

For the detection of *STXBP2* mutations, genomic DNA was isolated from a T-cell line established from each patient. Genomic DNA (5 ng) was subjected to PCR using the primers listed in Table S1. These primer sets were designed to amplify 19 exons including the 5′-untranslated region and the coding regions with the exon-intron boundaries of *STXBP2*. The PCR products were treated with ExoSAP-IT (GE Healthcare Bio-Sciences, Little Chalfont, England) by incubation at 37°C for 15 minutes to inactivate the free primers and dNTPs, and then subjected to sequencing reactions using forward or reverse primers and BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA). The DNA fragments were purified using Magnesil (Promega, Madison, WI), and sequencing was carried out with an ABI 3730 Genetic Analyzer (Applied Biosystems). Sample sequences were aligned to reference sequences obtained from the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/index.html) using the ClustalW program in order to identify nucleotide changes. Mutations were numbered according to GenBank Reference Sequence NM_001127396.1; additionally, the A of the ATG initiator codon was defined as nucleotide +1. To identify splicing variants generated by c.88-1g>a mutation of *STXBP2*, total RNA was extracted from each patient's T-cell line and reverse transcriptase PCR (RT-PCR) was performed using the forward primer on exon 1 (5′-TTGGGACACACCCGGAAG-3′) and the reverse primer on exon 5 (5′-AAGAAGATATGGGCCGCTTT-3′). The PCR products were directly sequenced using the forward primer, as described above.

Western blot analysis of MUNC18-2 protein

Expression of Munc18-2 protein encoded by *STXBP2* in T-cell lines established from FHL patients and a healthy individual was analyzed by Western blotting. CTLs were harvested after 5 days of stimulation with allogeneic LCL cells. Cell lysates were then prepared by extraction with 1% NP-40, and the extracts (10 μg per lane) were analyzed by Western blotting with anti-Munc18-2 rabbit polyclonal antibody (LifeSpan BioSciences, Seattle, WA). Horseradish peroxidase-labeled anti-rabbit IgG polyclonal antibody was used as the secondary antibody with detection by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

Establishment of alloantigen-specific CTL lines

Alloantigen-specific $CD8^+$ CTL lines were generated as described previously [24,25]. Briefly, peripheral blood mononuclear cells (PBMCs) were obtained from FHL patients and unrelated healthy individuals. These cells were co-cultured with a mitomycin C (MMC)-treated B-lymphoblastoid cell line (B-LCL) established from an HLA-mismatched individual (KI-LCL). Using cell-isolation immunomagnetic beads (MACS beads) (Miltenyi Biotec, Auburn, CA), $CD8^+$ T lymphocytes were isolated from PBMCs that had been stimulated with KI-LCL cells for 6 days. $CD8^+$ T lymphocytes, cultured in RPMI 1640 medium supplemented with 10% human serum and 10 IU/ml interleukin-2 (Roche, Mannheim, Germany), were stimulated with MMC-treated KI-LCL cells 3 times at 1-week intervals; subsequently, these lymphocytes were used as $CD8^+$ alloantigen-specific CTL lines. The alloantigen specificity of the CTL lines was determined by assay of interferon-γ (IFN-γ) production in response to stimulation with KI-LCL cells, as described previously [24,25]. Briefly, 1×10^5 T lymphocytes were co-cultured with or without 1×10^5 MMC-treated B-LCL cells in 0.2 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) in a flat-bottomed 96-well plate. In some experiments, an anti-HLA class I monoclonal antibody (w6/32; American Type Culture Collection, Manassas, VA) was added to wells at an optimal concentration. After 24 hours, the supernatant was collected from each well and assayed for production of IFN-γ using an enzyme-linked immunosorbent assay (ELISA; ENDOGEN, Woburn, MA).

Analysis of CTL-mediated cytotoxicity

The cytotoxic activity of CTLs was measured by a standard ^{51}Cr-release assay, as described previously [21]. Briefly, alloantigen-specific CTLs were incubated with ^{51}Cr-labeled allogeneic