doi:10.1111/j.1600-0609.2009.01300.x

CLINICAL PICTURE

Diffuse large B-cell lymphoma presenting with osteolytic lesions in the bilateral femur

A 14-yr-old boy presented to our hospital with a 5-month history of bilateral knee pain. He had no history of local trauma. Physical examination and hematological data on admission were unremarkable except for an increase in serum level of C-reactive protein to 5.05 mg/dL and lactate dehydrogenase to 379 U/L. Two-dimension computed tomography showed osteolytic lesions in the distal part of the bilateral femur (panel A: right, B: left). In addition, magnetic resonance imaging revealed multifocal lesions of marrow replacement involving the proximal part of the bilateral tibia (panel C). Then a histological examination showed diffuse large B-cell lymphoma, and he received chemotherapy combined with rituximab. These images played an important role in the diagnosis.

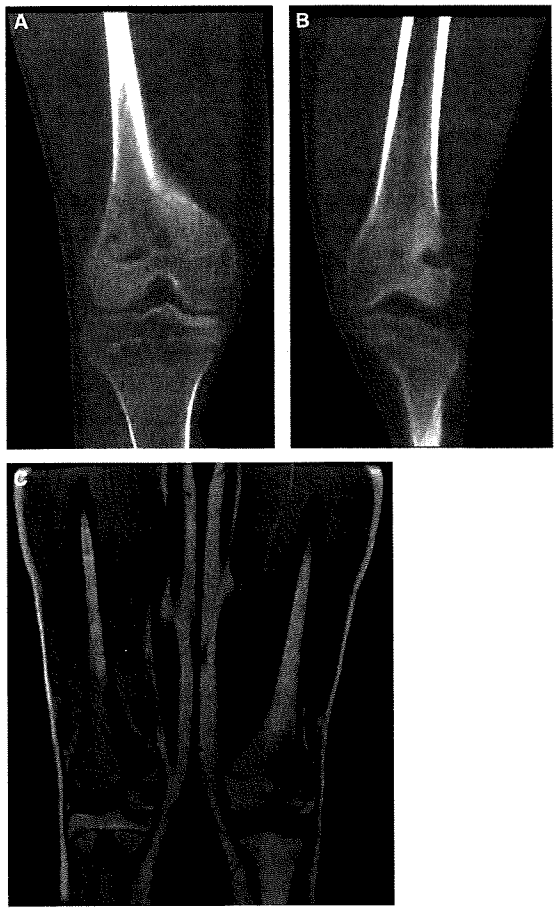

Figure 1 (A,B) Two-dimension computed tomography. (C) T1-weighted magnetic resonance imaging.

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Brain & Development 32 (2010) 169

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Letter to the Editor

Prediction of the pathogenesis of the mutation in MeCP2 C-terminal domain

We read with the great interest the recent contribution by Campos et al. in the Brain and Development [1]. They described a male with mental retardation with a mutation in MeCP2 and considered the p. P405L variant a disease-causing mutation [1]. The authors described that the potential of the programs they used to interpret missense variants was validated by Chan et al [2]. However, Chan et al. described several problems that the predictions have, especially for MeCP2.

First, the predictions should be subject to false positive values of high conservation, which could lower the specificity and the positive predictive value [2]. An important requirement is that large sequence databases must be used in order for computational predictions to be valid [2]. Each evolutionary database should contain three times the number of variants as the gene has codons [3–5]. The available alignments for MeCP2 did not exceed this threshold [2]. Therefore the size of the alignment affected the prediction, even when absolute conservation at a codon was observed in the MeCP2 variants [2]. This false positive conservation emphasizes the importance of compiling an evolutionary database of sufficient size to maximize the predictive value.

Another important issue to be considered is the quality of the alignment. The alignment should contain only true orthologs. A single query sequence let the SIFT program search for and align other sequences [2]. According to the authors [2], the actual alignment generated by SIFT had more sequences (62 sequences) including duplicate sequences from the same species and partial sequences, while the authors recommended the curated alignment (8 sequences). In their study, SIFT performed worse when using the uncurated alignment with a single query sequence. Thus, careful bioinformatics curation which considers gene phylogeny is required for optimal results.

The attempt by Campos et al. would be suggestive or useful, but users of the program should be careful with their conclusion, especially when using a limited dataset or a single query.

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doi:10.1016/j.braindev.2009.04.003

Pediatrics International (2009) **51**, 41–47 doi: 10.1111/j.1442-200X.2008.02652.x

Original Article

Psychological status of patients with mucopolysaccharidosis type II and their parents

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Abstract ***Background***: The aim of the present study was to delineate the psychological status of 10 patients with the attenuated phenotype of mucopolysaccharidosis type II (MPS-II) and their parents (six fathers and five mothers) for the improvement of clinical management.

Methods: Intellectual ability was evaluated using the Wechsler Intelligence Scale. Activities of daily living (ADL) was assessed using the Functional Independence Measure. The personality and psychiatric aspects were analyzed using the Yatabe–Guilford Personality test (Y-G test) and the Tree-Drawing Test. Mental health was assessed using the General Health Questionnaire 60 (GHQ-60) and State–Trait Anxiety Inventory (STAI).

Results: Intellectual background, measured with full-scale, verbal and performance IQ, were 72.8, 76.1 and 79.3, respectively. Nine of 10 patients were not judged as having neurosis and a psychotic tendency with the Y-G test. In the tree-drawing test, many patients drew a tree without ground, suggesting that they have difficulties in making relationships with surrounding people and the community. The child patient with a psychosis pattern on the Y-G test, drew a bizarre tree, suggesting psychological problems. GHQ-60 and STAI survey indicated that the patients and their parents had higher levels of anxiety. A significant negative correlation between GHQ-60 score and ADL ($R = -0.77$) was identified, suggesting that the psychological status may worsen as ADL decreases.

Conclusions: Patients with MPS-II and their parents had higher risks for mental problems. Understanding psychological status is essential when providing genetic counseling or therapeutic intervention.

Key words attenuated phenotype, IQ, mucopolysaccharidosis type II, psychological examination, psychological problem.

The mucopolysaccharidoses (MPS) are a range of lysosomal storage disorders resulting from deficiency of enzymes catalyzing the stepwise degradation process of glycosaminoglycans.[1] Non-degraded glycosaminoglycans are stored in lysosomes, and their excessive storage leads to progressive cell damage in MPS. There are 11 known enzyme deficiencies that give rise to seven distinct MPS types. MPS patients share many clinical features including short stature, characteristic face and posture, multiple joint contractures, coarse skin and hair, cardiovascular disorders, respiratory difficulties, hepatosplenomegaly, hearing disorders and central nervous involvement.[1]

Mucopolysaccharidosis type II, also known as Hunter disease, is an X-linked recessive disorder caused by a deficiency of iduronate-2-sulfatase (I2S). A mutation in *I2S* gene is the primary cause of the disease, and I2S deficiency leads to the accumulation of dermatan sulfate and heparan sulfate in the body as well as increased excretion of these substances into the urine.[1] More than half of the MPS patients in Japan have MPS-II.[2] Two forms of MPS-II have been categorized based on absence or presence of progressive intellectual deterioration.[3–4] Residual activity of I2S or the amount of excreted glycosaminoglycans does not predict the severity of the disease, and molecular analysis of genetic mutations has not clearly elucidated the mechanisms of genotype–phenotype correlations.[5] The severe form of MPS-II usually involves significant neurological and somatic involvement from the early infantile period, and patients usually do not survive their second decade of life.[3–4] Patients with the attenuated form gradually develop somatic manifestations in the late infantile period, and can survive to middle age, although the attenuated form includes patients with various degrees of severity.[4] Patients with the attenuated form usually manifest no or subtle central nervous disorders; but somatic manifestations, including short stature, typical facial expression, joint contracture, walking difficulty, hearing disorders, decreased visual acuity due to retinopathy, respiratory problems, and heart failure due to valvular diseases, are usually severe enough in adulthood, and may lead to psychological problems or inappropriate adaptation to society. Patients may stay at home even if they have the ability to work, or cannot go to school because of their psychological problems, and it may be difficult to make friends. Patients sometimes confess that they fear others looking at them, feel guilty about their parents, and have anxiety for the future, making friends,

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Received 14 September 2007; revised 9 December 2007; accepted 27 December 2007; published online 9 July 2008.

Journal of Pediatric Surgery (2009) 44, E37–E39

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Journal of
Pediatric
Surgery

www.elsevier.com/locate/jpedsurg

Treatment with OK-432 for persistent congenital chylothorax in newborn infants resistant to octreotide

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Received 5 September 2008; revised 4 December 2008; accepted 5 December 2008

Key words:
Chylothorax;
Octreotide;
OK-432;
Alternative therapy;
Neonatal;
Pleurodesis

Abstract *Chylothorax* is a relatively uncommon condition defined as an abnormal collection of lymphatic fluid within the pleural space. We are reporting the use of OK-432 for treatment of prolonged idiopathic congenital chylothorax in 2 newborn infants who failed to respond to conservative medical therapy, including octreotide injection.

Chylothorax is a relatively uncommon condition defined as an abnormal collection of lymphatic fluid within the pleural space. Recent studies have reported the incidence of congenital chylothorax as ranging from 1 in 1000 to 1 in 15,000 pregnancies [1]. The diagnosis of chylothorax is typically established via fluid analysis and includes a triglyceride content of 1.1 mmol/L or more and a total cell count of 1000 cells/mm^3 or more or 80% lymphocytes or more [2].

Treatment of chylothorax has traditionally been nonoperative. Although no strict guidelines exist, most authors have recommended one or several attempts to drain the pleural fluid using thoracentesis or chest tube drainage [3]. Recent reports have suggested alternative medical therapies involving the administration of octreotide or pleurodesis with OK-432 to infants with persistent chylothorax. Octreotide (a somatostatin analog) is thought to have an effect at the vascular somatostatin receptor level, which results in decreased chyle production. On the other hand, OK-432, a lyophilized preparation of a low-virulence strain of group A *Streptococcus pyogenes* of human origin, inactivated by heating with penicillin G (Picibanil), has been developed as a biologic modifier because of its immunostimulating activity [4]. Intrapleural instillation of OK-432 can succeed in generating adherence and decrease pleural effusion [5]. Recently, the successful treatment of neonatal and prenatal chylothorax by intrapleural instillation of OK-432 has been reported [6]. We report the successful use of OK-432 for persistent congenital chylothorax resistant to octreotide.

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0022-3468/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2008.12.008

Int J Hematol (2009) 90:611–615
DOI 10.1007/s12185-009-0442-y

CASE REPORT

Flow cytometric analysis of skin blister fluid induced by mosquito bites in a patient with chronic active Epstein–Barr virus infection

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Received: 18 September 2009 / Revised: 13 October 2009 / Accepted: 21 October 2009 / Published online: 14 November 2009

Abstract In chronic active Epstein–Barr virus (EBV) infection (CAEBV), ectopic EBV infection has been described in T or natural killer (NK) cells. NK cell-type infection (NK-CAEBV) is characterized by large granular lymphocytosis, high IgE levels and unusual reactions to mosquito bites, including severe local skin reactions, fever and liver dysfunction. However, the mechanisms underlying these reactions remain undetermined. Herein, we describe a patient with NK-CAEBV whose blister fluid after mosquito bites was analyzed. The patient exhibited significant increases in the percentage of $CD56^+$ NK cells in the fluid compared with a simple mosquito allergy, in which the majority of infiltrated cells were $CD203c^+$ cells, indicating basophils and/or mast cells. His fluid also contained $CD203c^+$ cells, and his circulating basophils were activated by mosquito extracts in vitro. These results suggest that $CD203c^+$ cells as well as NK cells may play pathogenic roles in the severe skin reactions to mosquito bites in NK-CAEBV.

Keywords Chronic active Epstein–Barr virus infection · NK cells · Mosquito bites · Skin blister · CD203c

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1 Introduction

Epstein–Barr virus (EBV) is a ubiquitous herpes virus that infects the majority of the world's population before adulthood [1]. Primary EBV infection is usually inapparent, but occasionally presents as acute infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [1]. After acute infection, EBV persists in B cells for the lifetime of the seropositive normal host. EBV infection has been also linked with a variety of malignancies, as well as lymphoproliferative disorders of T and natural killer (NK) cells, including hemophagocytic lymphohistiocytosis, chronic active EBV infection (CAEBV) and nasal-type lymphomas [2]. In these cases, T or NK cells are the cellular targets of EBV infection, and the pathogenic roles of ectopic EBV infection have been described [3].

CAEBV is characterized by chronic and recurrent infectious mononucleosis-like symptoms and extremely high viral loads in peripheral blood [4]. Based on the cellular targets of EBV, CAEBV is largely divided into two clinically distinct subtypes; T cell-type and NK cell-type infections [5]. T cell-type infection is associated with fever, high titers of anti-EBV antibodies and higher mortality, whereas NK cell-type infection is characterized by large granular lymphocytosis, elevated serum IgE levels and unusual reactions to mosquito bites. In the latter subtype, affected subjects may present with severe local skin reactions including large erythematous swellings, blister formation and necrotic ulcerations in addition to systemic symptoms, such as fever, lymphadenopathy and liver dysfunction after exposures to mosquito bites. This condition is so called "hypersensitivity to mosquito bites"; however, the immunological mechanism leading to the severe reactions that are quite different from those of

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Rheumatology 2009;48:1168–1169
doi:10.1093/rheumatology/kep159
Advance Access publication 23 June 2009

Catastrophic anti-phospholipid syndrome associated with *Escherichia coli* O157 infection

Sir, Catastrophic APS (CAPS) is a term applied to aPL-mediated disorder in which multiple thrombi of small vessels affect the viscera over a relatively short period [1–3]. CAPS is rare in children and may be confused with haemolytic-uraemic syndrome (HUS)/thrombotic thrombocytopenic purpura (TTP) because of similar clinical manifestations. More importantly, it will be easily overlooked if associated with an acute episode of HUS/TTP itself.

We describe the case of a 9-year-old girl with haemorrhagic colitis caused by *Escherichia coli* O157 who developed CAPS during the course of her disease.

A 9-year-old girl with a 4-day history of fever, bloody diarrhoea and abdominal pain was transferred to our department because of suspected HUS after continued elevation of urea and creatinine levels despite adequate resuscitation. On examination, she had no fever, her heart rate was 88 b.p.m., and her blood pressure was 80/40 mmHg. She had no purpura or oedema. Her consciousness was clear, and neurological examination was normal. Laboratory examinations showed increased CRP levels, 3.2 mg/dl; white blood cell count, 13.52×10^9/l; haemoglobin, 12.3 g/dl; platelet count, 40×10^9/l; signs of haemolysis with positive fragmented red cells in the blood smear; and elevated lactate dehydrogenase levels, 3474 IU/l. Liver and kidney function tests showed elevated urea (47 mg/dl), creatinine (3.87 mg/dl), aspartate aminotransferase (281 IU/l) and bilirubin (3.2 mg/dl) levels. Coagulation tests showed prolonged prothrombin time (15.1 s), increased fibrinogen degradation products (32.3 μg/ml) and increased D-dimer levels (10.5 μg/ml). Activated partial thromboplastin time and fibrinogen were normal. Stool culture and toxin assays for Shiga-like toxin were negative but O157 LPS antibody was positive, thus confirming infection with enterohaemorrhagic *E. coli*. The patient

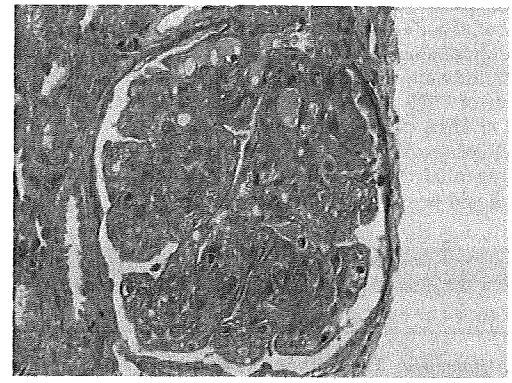

Fig. 1. Histological findings of kidney biopsy show a glomerulus with prominent endothelial swelling, reduced capillary lumen and mesangiolysis (periodic acid Schiff original magnification × 200).

rapidly progressed to anuria and required placement of a dialysis catheter and initiation of haemodialysis. Haemodialysis improved her renal function; it was stopped on the ninth day after admission. Immunoserological testing on the 10th day after admission showed a positive level of IgG aCL (14.2; <10 U/ml; ELISA), IgG phosphatidylserine-dependent prothrombin antibody (aPS/PT) (25; <10 U/ml; ELISA) and IgM aPS/PT (37; <10 U/ml; ELISA). IgG β2-glycoprotein I (β2GPI)-dependent aCLs and lupus anti-coagulant were negative. ANA was positive (30.7; index <20; ELISA). Other autoantibody tests, including RF, anti-ssDNA antibody, anti-dsDNA antibody and MPO-ANCA, were negative. The plasma level of ADAMTS-13 activity was slightly decreased to 15%, but the von Willebrand factor multimeric pattern was normal. A kidney biopsy performed on the 19th day after admission showed a typical histopathological picture of thrombotic microangiopathy (TMA) and no immune deposits (Fig. 1). Brain MRIs were normal. Radioisotope venography revealed left deep-vein thrombosis. The patient fulfilled the classification criteria for CAPS [3]. She received anti-coagulation therapy and was in clinical remission for 16 months.

Escherichia coli O157 is one of the most popular pathogens causing HUS. However, enteropathogenic *E. coli* has not been recognized as an infectious agent that triggers CAPS. To our knowledge, this is the first report of CAPS caused by *E. coli* O157 infection.

There are reports of a high frequency of aCL in children with diarrhoea-associated HUS [4, 5]. Ardiles *et al.* [4] found a positive aCL in 59% (10/17) of the patients with classic HUS, without correlation with clinical variables. Furthermore, Te Loo *et al.* [5] reported a significant increase in aCL levels during the acute phase of diarrhoea-associated HUS; IgM aCL was positive in 60% and IgG aCL in 41% of patients. A new subset of APS, termed microangiopathic APS (MAPS), was recently proposed. MAPS comprises those patients with TMA such as HUS/TTP, haemolysis, elevated liver enzymes, and low platelets syndrome and demonstrable aPL [6]. The aPL detected in this group of patients may be generated by endothelial damage or preceding infection [7]. Some of these non-pathogenic aPL may be rendered pathogenic by unknown factors in these conditions. However, we hypothesize that in some patients with HUS/TTP, the circulating aPL may contribute as a concomitant prothrombotic risk factor to the microvascular thrombotic process. The pathogenic role of aPL in clinical conditions of patients with TMA remains controversial; however, aPL should be examined in patients with TMA because anti-coagulant treatment is necessary in APS, particularly in CAPS.

aPS/PT was highly prevalent in patients with APS compared with patients with other diseases, and the detection of aPS/PT

strongly correlated with clinical manifestations of APS [8, 9]. The specificity of aPS/PT for APS diagnosis is as high as that of aCL/β2GPI. Additional and prospective studies on aPS/PT are needed to establish the clinical relevance; aPS/PT is a useful tool for better recognition of APS. aPS/PT as well as aCL/β2GPI should be examined in TMA patients with positive aCL.

In conclusion, CAPS is rare in children but should be included in the differential diagnosis of HUS/TTP, and aPL, including aPS/PT, should be examined in patients with HUS/TTP.

Rheumatology key message

- CAPS should be included in the differential diagnosis of D+HUS.

Disclosure statement: The authors have declared no conflicts of interest.

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Accepted 15 May 2009

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Rheumatology 2009;48:1169–1170
doi:10.1093/rheumatology/kep172
Advance Access publication 23 June 2009

Aortic aneurysm in MAGIC syndrome successfully managed with combined anti-TNF-α and stent grafting

Sir, A 48-year-old woman with MAGIC (Mouth And Genital Ulcers with Inflamed Cartilages) syndrome {Behçet's disease (BD) and relapsing polychondritis [1]} consulted our medical department in 2000 following development of an acute left cochleovestibular syndrome. She was discovered to have mixed type II cryoglobulinaemia and hepatitis C and responded fully to anti-viral therapy. Severe recurrent bipolar aphthosis, erythema nodosum and oligoarthritis first appeared in 2001, and were treated with an anti-malarial drug. In 2004, bilateral auricular and nasal chondritis was diagnosed, associated with lumbar inflammatory pain and bipolar aphthosis. MAGIC syndrome was diagnosed. Colchicine (1 mg/day) was prescribed, but replaced by SSZ (2 g/day) because of digestive intolerance. In 2005, right cochleovestibular syndrome developed, which responded to intravenous and then oral corticosteroids. Due to persistent biological inflammatory parameters, MTX (15 mg/week) was added to oral prednisone. Five months after discharge, she complained of persistent arthralgias and MTX was switched to AZA (150 mg/day), which obtained disease remission for almost 1 year, when she complained of heart palpitations, thoracic pain and repeated fainting episodes. A biological inflammatory syndrome was found: CRP 80 mg/l; fibrinogen 6 g/l. Echocardiography and pulmonary scintigraphy were normal. Thoracic CT angiograms revealed the presence of an aortic arch aneurysm, 4.4 cm in diameter, without signs of rupture. The positron emission tomography scan showed hyperfixation in the aortic arch and thoracic ascending aorta, suggestive of large-vessel vasculitis. Anti-TNF-α (infliximab, 3 mg/kg) was added to her regimen. Because biological inflammation persisted (CRP 55 mg/l; fibrinogen 5.5 g/l), infliximab was increased to 5 mg/kg at the fourth perfusion and monthly intravenous corticosteroids (15 mg/kg) were added. On this regimen, CRP and fibrinogen values normalized. After the seventh infliximab perfusion, endovascular stent-grafting (Valiant TC3026C150X, Medtronic Europe, Tolochenaz, Switzerland) was performed. Infliximab infusions were spaced every 8 weeks and monthly intravenous corticosteroids were replaced by oral prednisone (10 mg/day). CT angiographic control of the stent showed a thrombosed aneurysm with no aortic wall thickening. The patient has remained asymptomatic on the same maintenance regimen for >2 years of follow-up.

To date, 19 cases of MAGIC syndrome have been reported [1–6]. Four of them, all women (mean age 32.5 years), three of whom (Table 1, Cases A–C) were taking immunosuppressant(s), developed a symptomatic aortic aneurysm involving the thoracic aorta [3–6]. Aneurysms seem to be a common complication of MAGIC syndrome (21.1%), even under immunosuppressants, and can require emergency surgery [3–6].

Vascular involvement occurs in 25–35% of BD patients [7, 8]. Aortic aneurysm is well described but uncommon and its optimal treatment, with immunosuppressant approaches [9] and surgical treatments [10], remains to be defined. Importantly, potential vein involvement means veins cannot be used as autograft replacement material. Endovascular graft-stenting successfully treated BD-associated vascular aneurysms in 20/21 reported patients [11, 12]. Ishikawa *et al.* [13] described abdominal aortic aneurysm progression 5 months after stent-graft placement that required prosthesis replacement.

In relapsing polychondritis patients, aneurysms develop in 5–7% of the patients and are multiple in 50% of them [14], usually involving the ascending aorta, and can cause aortic insufficiency. Moreover, they can occur during remission and under immunosuppressants. Aortic involvement is generally asymptomatic, and can be revealed by sudden rupture of the arterial wall.

Our patient, also a woman with MAGIC syndrome, developed a thoracic aortic aneurysm while taking AZA and oral corticosteroids. Unlike Case C, whose aneurysm occurred 2 years after starting anti-TNF-α [5], our patient achieved clinical and biological remission on monthly intravenous corticosteroids and infliximab, followed by infliximab and oral prednisone (10 mg/day) maintenance therapy. Once this remission was obtained, multidisciplinary consensus, in accordance with the patient's

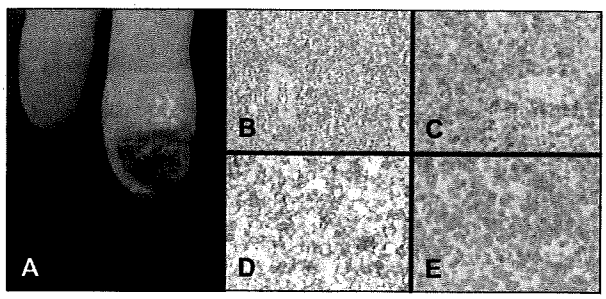

Figure 1. A) Crusted lesion of the fingertip with pyogenic granuloma appearance. **B)** Diffuse lymphomatous infiltrate and vessel proliferation, without angiocentric growth pattern. HE×200. **C)** CD3 cytoplasmic staining. **D)** Immunohistochemical staining strongly positive with CD56. **E)** Granular positivity for TIA-1.

Extranodal NK/T cell lymphoma is an uncommon disease that accounts for less than 3% of cutaneous lymphomas. Although it is more prevalent in Asian and South American middle-aged adults, our patient was Caucasian and younger than usually reported. The skin is the second most common site of involvement after the nasal cavity and its involvement may be a primary or secondary manifestation [1, 3]. Our patient initially had a cutaneous presentation and the rhynopharyngeal involvement was subsequently discovered, but he did not present bleeding, sinusitis, rhinitis or other symptoms due to the obstruction of the nasal cavity. Histologically, NK-cell lymphomas show a broad cytological spectrum with atypical cells. Infiltration and destruction of the vessel wall and fibrinoid changes within the blood vessels are usually seen. The disease is associated with Epstein-Barr virus in 80-100% of cases [4, 5]. However, extranasal lesions are usually EBV-negative in white patients.

Due to the sporadic incidence of this disease, no standard treatment has been established and the response to therapy is variable. Chemotherapy, in association with radiotherapy, has shown effectiveness, although the relapse rate is very high and many cases are resistant to multiagent chemotherapy [6]. NK/T-cell lymphomas follow an aggressive course and have a poor prognosis, particularly in those patients with disease outside the nasal cavity [6].

Our case had an unusual presentation, pyogenic granuloma-like on the fingertip without nasal cavity involvement at first, and not associated with EBV infection. Since the presentation on the fingertip allowed us to make the diagnosis, treatment was started prior to the nasal mass biopsy so we do not know exactly the nature of the mass, but it is supposed not to be a lymphoma due to its rapid resolution and unspecific histopathology. ■

Acknowledgements. *The authors assure the editor that there is neither conflict of interest nor financial implications.*

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Adult-onset Still's disease with a cellulitis-like eruption

Adult-onset Still's disease (AOSD) is a systemic inflammatory disorder of unknown etiology characterized by a high spiking fever, skin rash, and either arthralgia or arthritis. An evanescent salmon-colored rash is considered to be a major diagnostic criterion [1-3]. We report an unusual case of AOSD presenting with cellulitis-like persistent erythema on the thigh.

A 38-year-old Japanese woman was admitted to our hospital with a 5-day history of a high spiking fever, sore throat, a large tender erythema on the left thigh, lymphadenopathy in the left inguinal region, and evanescent slightly-pruritic macular eruptions on the extremities. Nine months prior to examination, small urticarial eruptions had appeared on the extremities and were controlled with oral antihistamines and topical corticosteroids. Examination showed a large indurated brownish erythema and small urticarial red macules on the left thigh *(figure 1A)*. The large brownish erythema persisted and the urticarial eruptions lasted for several hours. There was no hepatosplenomegaly. Histological examination of the cellulitis-like erythema of the left thigh showed a perivascular infiltration of lymphocytes and neutrophils in the upper dermis *(figure 1B)*. Laboratory analysis revealed a leukocyte count of 7,500/mm^3 (6,000/mm^3 neutrophils), C-reactive protein 5.11 mg/dL, and a normal level of ferritin. Liver and kidney function tests were unremarkable. Repeated blood cultures, throat swab, and urinalysis indicated no bacterial infections. Laboratory tests including chest

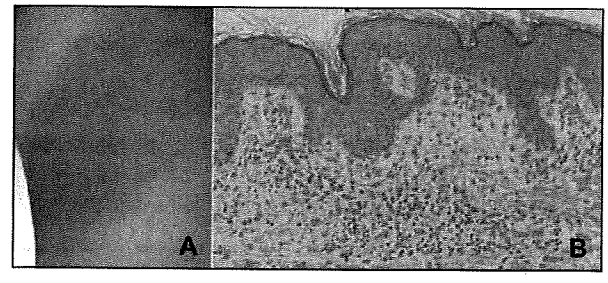

Figure 1. A) A large indurated brownish erythema with small urticarial macular eruptions on the left thigh. **B)** A biopsy specimen of the erythema on the left thigh showed perivascular infiltration of lymphocytes and neutrophils in the upper epidermis. (H & E, original magnification × 100).

doi: 10.1684/ejd.2008.0554

radiograph, abdominal and pelvic computed tomography scans were unremarkable. From these findings, diagnoses of cellulitis and urticaria were made. Intravenous cefazolin sodium and oral antihistamines, however, did not resolve the symptoms. On the 5th day of admission, arthralgia of the wrists and knees and stiffness of both arms and hands appeared. Non-steroidal anti-inflammatory drugs were ineffective and these symptoms lasted for more than two weeks. Moreover, small macular eruptions occurred typically in the evening during febrile attacks. Rheumatoid factor, antinuclear antibody, and other autoantibodies were negative. Serum ferritin level was increased to 1,305 ng/mL. Infectious, neoplastic, and rheumatoid disorders were therefore excluded and the patient met the diagnostic criteria of AOSD [1]. Treatment with oral prednisolone 30 mg daily resulted in resolution of the clinical symptoms. However, fever, arthralgia, and urticarial eruption recurred during the tapering of prednisolone. The addition of oral methotrexate 6 mg weekly and pulsed corticosteroid therapy controlled the symptoms. Over the next year, the dose of prednisolone was tapered to 6 mg daily with satisfactory control, except for the occasional appearance of evanescent small erythema on the left thigh.

The rash of AOSD is characteristically a salmon-colored macular or maculopapular non- or mildly pruritic eruption on the trunk and limbs, usually appearing during fever. The typical rash is of great diagnostic value, especially in cases without arthritis, providing a major diagnostic criterion with high sensitivity and specificity [1]. In the present case, the prominent initial symptoms of fever, persistent tender erythema on the left thigh, and regional lymphadenopathy suggested the diagnosis of cellulitis, although a small macular eruption resembling Still's rash was also present. The cellulitis-like eruption may be associated with AOSD because it did not respond to antibiotics and resolved with corticosteroid treatment. In addition, the recurrent appearance of evanescent macular eruptions only on the left thigh where the cellulitis-like eruption existed suggests the association of both eruptions. Moreover, histological analysis of the cellulitis-like eruption was non-specific but consistent with that of typical Still's rash [4]. Recently, there have been several case reports of AOSD presenting with nonevanescent eruptions, including widespread persistent papules and plaques [5, 6]. To our knowledge, the cellulitis-like lesion of our patient has not been previously described. In conclusion, we should add a cellulitis-like skin lesion to the atypical cutaneous manifestations of AOSD, to reduce the potential diagnostic delay. ■

Acknowledgements. Conflict of interest: none. Financial support: none.

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Apocrine hidrocystoma on the finger

Apocrine hidrocystoma, also called apocrine cystadenoma, is a benign tumor usually presenting as a solitary translucent nodule of cystic consistency. The tumor arises from apocrine sweat glands. The usual locations of this tumor are the face, head, neck, and upper torso, however, it is extremely rare for apocrine hidrocystoma to develop on a finger.

A 35-year-old man presented with a deep-purple, painless cystic nodule, measuring 10 mm diameter, on the ulnar surface of his left fifth finger *(figure 1A)*. The lesion had been present about 10 years and had gradually grown. There was no history of injury. The patient did not have any other cutaneous lesion. Dermoscopic examination showed a bluish subcutaneous spot *(figure 1B)*. Puncture of this nodule resulted in the discharge of a dark red serous fluid. Histologically, the dermis contained a unilocular cyst. It did not connect to a hair follicle or a sweat gland *(figure 1C)*. The cyst was lined with eosinophilic cuboidal or columnar cells that demonstrated decapitation secretion. There were partial papillary excrescences of the cyst lining into the cavity *(figure 1D)*. Periodic acid-Schiff (PAS)-positive, diastase-resistant granules were identified in the secretory cells. The immunohistochemical staining for carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), and gross cystic disease fluid protein-15 (GCDFP-15) were positive. Taken together, this tumor was concluded to be an apocrine hidrocystoma.

Apocrine hidrocystoma was first described in 1964 by Mehregan [1]. The tumor is produced by the cystic proliferation of apocrine secretory glands. This tumor is not uncommon, occurring in adult life in no particular age group, with males and females equally affected. Histologically, apocrine hidrocystoma is differentiated from eccrine hidrocystoma by the presence of secretory cells showing decapitation secretion. The secretory cells in apocrine hidrocystoma also have PAS positive and diastase-resistant granules. In immunohistochemical analysis, S-100 positivity can be found in apocrine tumors [2], but CEA and GCDFP-15 are expressed both in eccrine and apocrine sweat glands [3]. Then, the existence of decapitation secretion is more relevant for the diagnosis of apocrine tumors.

It is unique and interesting for our case that the tumor arose from a finger. Since De Fontaine *et al.* described the first case of apocrine hidrocystoma occurring on a finger [4], to our knowledge, there have been only three reported cases of this tumor on a finger [5, 6]. In the previous report analyzing 167 cases of Japanese cases of apocrine hidrocystoma, 73.7% of cases were found on the face and scalp, and only 9.0% was found in apocrine gland-bearing lesions [7].

The reason for the unusual location of our case is unclear, but it might be explained by the development of the apocrine glands. Apocrine sweat glands stem from primary epithelial germs, and they exist in every hair follicle of a

doi: 10.1684/ejd.2008.0555

ARTHRITIS & RHEUMATISM
Vol. 60, No. 1, January 2009, pp 242–250
DOI 10.1002/art.24134

Role of the *NOD2* Genotype in the Clinical Phenotype of Blau Syndrome and Early-Onset Sarcoidosis

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Objective. **Blau syndrome and its sporadic counterpart, early-onset sarcoidosis (EOS), share a phenotype featuring the symptom triad of skin rash, arthritis, and uveitis. This systemic inflammatory granulomatosis is associated with mutations in the *NOD2* gene. The aim of this study was to describe the clinical manifestations of Blau syndrome/EOS in Japanese patients and to determine whether the *NOD2* genotype and its associated basal NF-κB activity predict the Blau syndrome/EOS clinical phenotype.**

Methods. **Twenty Japanese patients with Blau syndrome/EOS and *NOD2* mutations were recruited. Mutated *NOD2* was categorized based on its basal NF-κB activity, which was defined as the ratio of NF-κB activity without a *NOD2* ligand, muramyldipeptide, to NF-κB activity with muramyldipeptide.**

Results. **All 9 mutations, including E383G, a novel mutation that was identified in 20 patients with Blau syndrome/EOS, were detected in the centrally located NOD region and were associated with ligand-independent NF-κB activation. The median age of the patients at disease onset was 14 months, although in 2 patients in Blau syndrome families (with mutations R334W and E383G, respectively) the age at onset was 5 years or older. Most patients with Blau syndrome/EOS had the triad of skin, joint, and ocular symptoms, the onset of which was in this order. Clinical manifestations varied even among familial cases and patients with the same mutations. There was no clear relationship between the clinical phenotype and basal NF-κB activity due to mutated *NOD2*. However, when attention was focused on the 2 most frequent mutations, R334W and R334Q, R334W tended to cause more obvious visual impairment.**

Conclusion. ***NOD2*** **genotyping may help predict disease progression in patients with Blau syndrome/EOS.**

Sarcoidosis is a systemic inflammatory disease with unknown etiology, but it can be clinically characterized by swelling of the bilateral hilar lymph nodes and histologically defined by the presence of noncaseating epithelioid cell granulomas. A special subtype called early-onset sarcoidosis (EOS; MIM no. 609464) occurs in children younger than 4 years of age and is characterized by a distinct triad of skin, joint, and eye disorders without apparent pulmonary involvement (1). An autosomal-dominant disease with clinical manifestations similar to those of EOS has been recognized as Blau syndrome (MIM no. 186580) (2,3). The gene responsible for Blau syndrome has been mapped close to the inflammatory bowel disease 1 (*IBD1*) locus by linkage analysis (4), and later the nucleotide-binding oligomerization domain 2 gene (*NOD2*) was identified by Miceli-Richard et al to be responsible for Blau syndrome (5). In the study by Miceli-Richard et al, 2 European patients with EOS had no mutation in *NOD2*; therefore, it remained

Supported by the Ministry of Education, Science, Sports, and Culture, Japan.

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Submitted for publication March 19, 2008; accepted in revised form September 5, 2008.

controversial whether Blau syndrome and EOS have the same etiology.

In 2004, we encountered a 27-year-old Japanese man with multiple lichenoid papules. He was almost blind, exhibited camptodactyly, and had a continuous low-grade fever. This case of sporadic systemic granulomatosis with clinical features of EOS showed the same *NOD2* mutation, the arginine-to-tryptophan substitution at amino acid 334 (R334W), as that detected in Blau syndrome (6). Therefore, we expanded this report (6) and retrospectively examined cases of EOS in Japan and observed that 9 of 10 patients with EOS had *NOD2* mutations (7). Until recently, other investigators have also confirmed that Blau syndrome and EOS are clinically and genetically identical across various ethnic groups (8–10).

NOD2 activates NF-κB after recognizing a signal from a bacterial cell wall component, muramyldipeptide, in the cytoplasm of monocytes, and thus can work as an intracellular sensor of bacteria (11,12). *NOD2* has a tripartite domain structure consisting of 2 amino-terminal domains (termed caspase activation and recruitment domains) that are composed of protein–protein interaction cassettes, 1 centrally located NOD, and carboxy-terminal leucine-rich repeats (LRRs) (13). Using assays of NF-κB activity, an impaired ligand-dependent response was demonstrated for 3 Crohn's disease–associated mutations located in *NOD2* LRRs (14,15), whereas enhanced ligand-independent NF-κB activity was demonstrated for *NOD2* alleles associated with Blau syndrome and EOS (5,7,16). However, it remains unknown how increased basal NF-κB activity derived from gain-of-function mutations in *NOD2* affects the pathogenesis of Blau syndrome/EOS and whether a genotype–phenotype correlation exists between the clinical manifestations or onset of Blau syndrome/EOS and *NOD2* mutations.

Because Blau syndrome/EOS is so rare, very few reports are in the literature. Therefore, it was worthwhile to conduct a nationwide survey limited to patients with a specific ethnic background, such as Japanese patients. In this study, we precisely documented the clinical manifestations in a cohort of Japanese patients with Blau syndrome/EOS and *NOD2* mutations, including 9 previously reported cases (7), and explored the genotype–phenotype correlation to the basal NF-κB activity associated with each mutation, especially focusing on the correlation of visual impairment with the most frequent mutations, R334W and R334Q.

PATIENTS AND METHODS

Patients and clinical information. Among patients with clinically diagnosed Blau syndrome/EOS, the 20 patients with *NOD2* mutations were included in this study (7,17–20). None of these mutations were identical to the reported single-nucleotide polymorphisms (SNPs) of *NOD2*, nor were they detected in 100 Japanese healthy volunteers. Clinical information and patient histories were collected from medical records and by direct interviews of the patients and their attending physicians. The presence of each symptom was established as follows: a) persistent or repeated transient skin lesions without definite cause were determined, b) persistent or repeated transient arthritis without definite cause was determined, c) uveitis was diagnosed by an ophthalmologist, and d) remittent or intermittent fever without definite cause was determined under close examination at the time of hospital admission. The age at disease onset was defined as the age of the patient when any of the above-mentioned symptoms appeared.

Clinical evaluation was performed primarily when individual symptoms first appeared that were hardly affected by treatment or disease duration. The severity of visual impairment was assessed in accordance with the World Health Organization definition (21). Briefly, moderate visual impairment was defined as visual acuity between 6/18 and 3/60, and severe visual impairment was defined as acuity of 3/60 or less in the better eye with best correction, as previously described (9). Written informed consent was obtained from the patients and their families, and the study protocol was in accordance with the guidelines of the Institutional Review Board of Kyoto University Hospital.

Genetics analysis. Genomic DNA was extracted from the peripheral blood of the patients, and sequencing of all exons and exon–intron junctions of *NOD2* was performed as previously described (7).

Generation of *NOD2* mutants and NF-κB luciferase assay. Expression plasmids of *NOD2* and its mutants were subcloned into the p3xFLAG-CMV vector, as previously described (7). Blau syndrome/EOS–associated mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described previously (7). The ability of each construct to induce NF-κB activity was assessed by dual luciferase reporter assay in HEK 293 human embryonic kidney cells, as previously described (7).

Other analyses. We determined the age at the time of this survey, the age at onset of each symptom, and the *NOD2* genotype for all patients as well as the distribution of age at disease onset. Next, we analyzed the relationship between age at disease/symptom onset and basal NF-κB activity due to mutated *NOD2*. Basal NF-κB activity was defined as the ratio of NF-κB reporter activity without muramyldipeptide to NF-κB reporter activity with muramyldipeptide, as determined using the in vitro NF-κB luciferase assay described above. The activity was arbitrarily categorized as low (<0.3), moderate (0.3–0.5), and high (>0.5). Finally, we analyzed the relationship between visual impairment (normal, moderate, severe) and basal NF-κB activity (low, moderate, high) due to individual mutated *NOD2* genes, particularly the 2 most frequent mutations, R334W and R334Q. We did not perform statistical analysis because of the limited number of patients.

Table 1. Demographic and clinical characteristics of the patients with Blau syndrome/early-onset sarcoidosis*

Patient/ age/sex	Genotype	Fever		Skin rash		Arthritis		Uveitis		Visual acuity		Ref.
		Age at onset	Type	Age at onset	Type	Age at onset	Type	Age at onset	Type	OD	OS	
1/15/F†	E383G	2 yr 3 mo	Int	8 mo	LP/SE/EN	3 yr	Poly	11 yr	A/P	20/50	20/67	
2/48/F†	E383G	5 yr	Per	5 yr	LP/SE/EN	11 yr	Poly	11 yr	A/P	HM	Null	
3/36/F	H496L	–	–	1 yr	LP/SE	3 yr	Poly	5 yr	A/P	20/20	20/20	7
4/16/M	R334Q	1 yr 8 mo	Int	6 mo	LP/SE	1 yr 8 mo	Poly	1 yr 10 mo	A/P	20/22	20/22	
5/19/M	R334Q	2 yr 7 mo	Per	1 yr 4 mo	LP/SE/EN	10 mo	Poly	5 yr	A/P	20/50	20/20	17
6/8/F	R334Q	–	–	–	–	3 yr	Poly	–	–	20/20	20/20	
7/8/M	T605P	–	–	7 mo	LP/SE	1 yr 6 mo	Poly	3 yr 3 mo	A/P	20/25	20/50	7
8/18/F	D382E	–	–	3 yr 4 mo	LP/SE	4 yr	Poly	5 yr 4 mo	A/P	20/20	20/25	7, 18
9/13/M	R334W	8 mo	Per	1 yr 3 mo	LP/SE/EN	8 mo	Poly	1 yr 8 mo	A/P	20/29	20/33	
10/32/M	R334W	2 yr	Int	2 yr	LP/SE	1 yr 3 mo	Poly	6 yr	A/P	Blind, 20 yr	Blind, 20 yr	6, 7
11/21/F	R334W	2 yr 1 mo	Per	2 yr 1 mo	LP/SE	6 yr	Poly	4 yr	A/P	20/670	20/330	7, 19
12/33/M	R334W	–	–	2 yr	LP/SE	–	–	13 yr	A/P	20/29	20/20	7
13/31/F	R334W	–	–	2 yr 6 mo	LP/SE	8 yr	Poly	3 yr 6 mo	A/P	20/100	20/200	7
14/10/F†	R334W	1 yr	Per	1 yr	LP/SE	1 yr	Poly	2 yr	A/P	20/40	Null	
15/46/F†	R334W	–	–	44 yr	LP/SE	8 yr	Poly	3 yr	A/P	Blind, 28 yr	Blind, 28 yr	
16/16/M†	R334W	–	–	6 yr	SE	1 yr	Oligo	6 yr	A/P	20/13	20/13	20
17/18/F†	R334W	–	–	12 yr	SE	8 yr	Oligo	12 yr	A/P	20/40	20/25	20
18/8/M	M513T	2 yr 10 mo	Int	2 yr 8 mo	SE	2 yr 9 mo	Poly	2 yr 11 mo	A	20/17	20/17	7
19/15/F	N670K	1 yr 8 mo	Int	5 mo	LP/SE/EN	1 yr 8 mo	Poly	3 yr	A/P	20/200	20/200	7
20/7/M	C495Y	1 yr	Int	1 yr	LP/SE	1 yr	Poly	–		20/20	20/20	

* Patient 5 also had left ventricular dysfunction and pulmonary hemorrhage due to bronchial granuloma. Patient 10 also had interstitial pneumonia. Patient 11 also had hepatosplenomegaly and parotid swelling. Patient 18 also had renal calcification. OD = right eye; OS = left eye; yr = years; mo = months; Int = intermittent; LP = multiple lichenoid papules; SE = scaly erythematous plaques; EN = erythema nodosum–like lesion; Poly = polyarticular; A = anterior; P = posterior; Per = persistent; HM = hand motion; Oligo = oligoarticular.
† Familial case.

RESULTS

Genotype and basal NF-κB activity. The study population comprised 9 male patients and 11 female patients, with a median age of 17 years (range 7–48 years) and a median disease duration of 15 years (range 5–43 years). Fourteen of these 20 cases were sporadic (EOS), and 6 were familial (Blau syndrome). The familial cases were in 3 unrelated families; 2 families (patients 14 and 15 and patients 16 and 17, respectively) had Blau syndrome/EOS symptoms in 2 generations, and 1 family (patients 1 and 2) had Blau syndrome/EOS symptoms in 3 generations. The most frequent heterozygous mutation of *NOD2* was R334W (1000C>T), which was recognized in 2 familial and 5 sporadic cases (total of 9 cases), followed by R334Q (1001G>A) in 3 sporadic cases, and E383G (1148A>G, a novel amino acid substitution) in 2 familial cases (in 1 family). H496L (1487A>T), T605P (1813A>C), D382E (1146C>G), M513T (1538T>C), N670K (2010C>A), and C495Y (1484G>A) were detected in 1 sporadic case each (Table 1).

Nine mutations were identified in the centrally located NOD region (Figure 1a) and were associated with increased basal NF-κB activity in the absence of muramyldipeptide (Figure 1b), which is consistent with the finding of a previous study on Blau syndrome/EOS–associated *NOD2* mutations (16). We also confirmed that 100 healthy control subjects and their genotyped asymptomatic relatives did not have these amino acid substitutions. Therefore, we concluded that these *NOD2* mutations (amino acid substitutions) detected in patients with Blau syndrome/EOS were not SNPs but rather were disease-causing mutations.

Disease onset. The defining characteristic of EOS is its onset in children younger than age 4 years (1). In the present study, despite the median age at disease onset of 14 months, the first clinical symptoms developed at age 5 years or older in 2 patients (patients 2 and 17, who were members of different Blau syndrome families) with the E383G mutation and the R334W mutation, respectively (Table 2). In patient 2, skin rash developed at age 5 years; in patient 17, arthritis developed at age 8 years (Table 1).

The earliest presenting symptom was skin rash in 13 patients (65%), arthritis in 8 patients (40%), and ocular symptoms in 1 patient (patient 15, who had familial Blau syndrome with the R334W mutation) (Table 1). Approximately 95%, 95%, and 90% of pa-

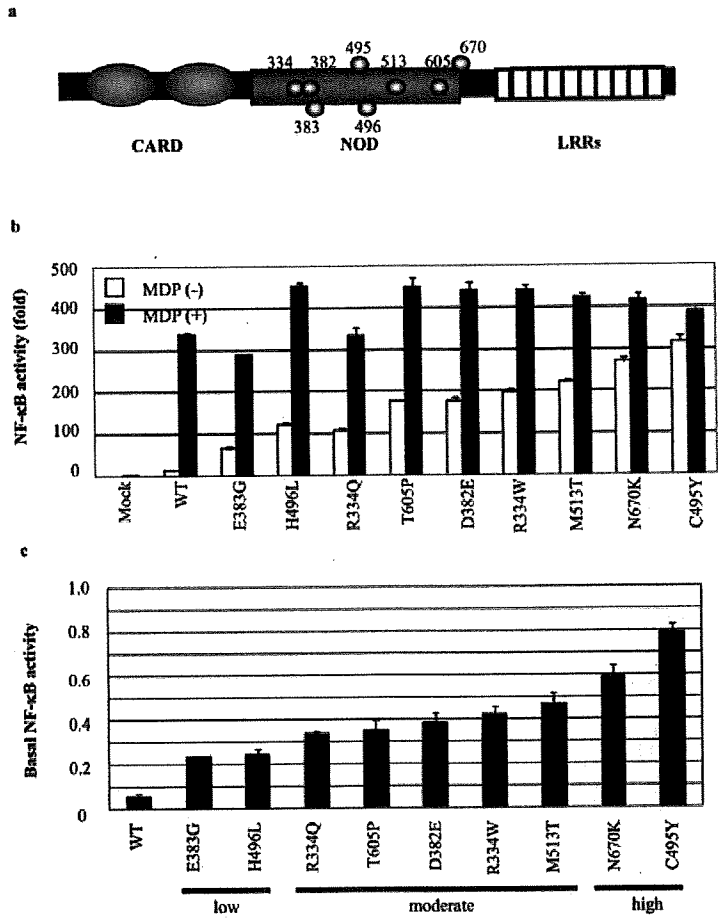

Figure 1. Biologic effects of *NOD2* mutants discovered in patients with Blau syndrome/early-onset sarcoidosis (EOS). **a,** Schematic presentation of NOD-2 protein. Numbers indicate the positions of mutated amino acid residues identified in our cohort. **b,** Increased basal NF-κB activity due to different mutated *NOD2* genes in patients with Blau syndrome/EOS. HEK 293T cells were cotransfected with a *NOD2* mutant together with the NF-κB reporter plasmid and internal control plasmid, and NF-κB reporter activity was measured after 12 hours of incubation with or without muramyldipeptide (MDP; 5 μg/ml). Mock vector and wild-type (WT) *NOD2* were used as controls. Bars show the mean and SD of normalized data (mock without muramyldipeptide = 1) from triplicate cultures. Results are representative of 3 independent experiments. **c,** Basal NF-κB activity due to mutated *NOD2* in patients with Blau syndrome/EOS. Bars show the mean and SD results from 3 independent experiments. CARD = caspase activation and recruitment domain; LRRs = leucine-rich repeats.

tients, respectively, had skin, joint, and ocular symptoms. Consistent with the previous report (1), a triad of skin, joint, and ocular symptoms developed (in this order) in many patients with Blau syndrome/EOS. The median age at onset of rash, arthritis, and uveitis was 24 months, 33 months, and 4.5 years, respectively (Table 2).

The triad of symptoms. All except 1 patient (patient 6 [with the R334Q mutation]) had skin manifestations. Consistent with a previous report (22), the most frequent skin symptom was scaly erythematous plaques with multiple lichenoid papules. Several patients (patients 1 and 2 with the E383G mutation, patient 5

Table 2. Age of the patients at the onset of disease and symptoms*

Age, years	Disease onset (n = 20)	Symptom onset			
		Fever (n = 11)	Rash (n = 19)	Arthritis (n = 19)	Uveitis (n = 18)
0	6 (30)	1 (9)	4 (21)	2 (11)	0 (0)
1	5 (25)	4 (36)	5 (26)	7 (37)	2 (11)
2	4 (20)	5 (45)	5 (26)	1 (5)	2 (11)
3	3 (15)	0 (0)	1 (5)	3 (16)	4 (22)
4	0 (0)	0 (0)	0 (0)	1 (5)	1 (6)
≥5	2 (10)	1 (9)	4 (21)	5 (26)	8 (44)

* Values are the number (%). The median age at disease onset was 1 year 2 months; the median age at onset of fever and rash was 2 years; the median age at onset of arthritis was 2 years 9 months; the median age at onset of uveitis was 4 years 6 months.

with the R334Q mutation, patient 9 with the R334W mutation, and patient 19 with the N670K mutation) had erythema nodosum–like lesions on their lower limbs in addition to solid lichenoid eruptions. Notably, 3 patients (patients 16 and 17 with the R334W mutation and patient 18 with the M513T mutation) showed only scaly erythematous plaques without lichenoid papules (Table 1).

All except 1 patient (patient 12 with the R334W mutation) had joint lesions (polyarticular arthritis in 17 patients and oligoarticular arthritis in 2 [patients 16 and 17]) (Table 1). Both patients with oligoarticular arthritis, who had familial Blau syndrome with the R334W mutation, had camptodactyly without obvious synovial cysts. Camptodactyly with synovial cysts is frequently described as a typical joint sign in patients with Blau syndrome/EOS (10). A consequence of arthritis was the use of a wheelchair for daily mobility in 2 patients (patient 5 with the R334Q mutation and patient 10 with the R334W mutation).

All except 2 patients (patient 6 with the R334Q mutation who also lacked skin eruptions and patient 20 with the C495Y mutation) had ocular lesions. The lesions were bilateral, although visual acuity was asymmetric, as in previous studies (22,23). Moreover, 17 (89%) of all 18 patients with ocular lesions had panuveitis, while only 1 patient (patient 18 with mutation M513T) had anterior uveitis, which demonstrated the predominance of panuveitis over anterior uveitis. Ocular symptoms were the last of the triad to develop in 15 of the 18 patients and the first to develop in only 1 patient (patient 15 with mutation R334W).

Clinical features other than the triad of symptoms. It is noteworthy that 11 patients (55%) experienced fever at a median age of 24 months, almost simultaneously with skin and/or joint symptoms (Table 1). Five patients had persistent fever reaching 38–40°C, and 6 patients had intermittent fever. In particular, in 1 patient (patient 9 with mutation R334W) the disease developed with intermittent fever (which then became persistent fever over the next 6 months) and finger joint swelling. In only 1 previous report (10), fever is mentioned as a clinical symptom of Blau syndrome/EOS, although there are some case reports in which fever was present at disease onset (24).

Four patients had involvement of organs other than the skin, joints, and eyes (Table 1). Two patients had pulmonary lesions (interstitial pneumonitis in patient 10 with the R334W mutation and bronchial granuloma in patient 5 with the R334Q mutation). Bilateral hilar lymph nodes, which are identified by chest radiography and/or computed tomographic scanning, were not observed in any patient. Patient 11 with the R334W mutation exhibited hepatosplenomegaly and parotid swelling (19), and patient 18 with the M513T mutation exhibited renal calcification. No cases of large-vessel vasculitis were observed in this cohort, even though vasculitis has been reported in patients with EOS (25–27).

Triggering factors. BCG vaccination was associated with the onset of disease (i.e., development of multiple papules on the extremities) in 2 patients, although no apparent infection or vaccination was clearly documented in other patients of our cohort. In 1 patient (patient 7 with mutation T605P) who had papules on the extremities, the spread of papules was from the site of BCG vaccination. In the other patient (patient 1 with mutation E383G), Gianotti disease was initially diagnosed, but a close review of her medical history later indicated that her multiple papules were a symptom of Blau syndrome/EOS.

Relationship between the onset of disease/symptoms and basal NF-κB activity due to mutated *NOD2*. Because disease duration and treatment varied among patients, we focused on the onset of disease and of each clinical symptom (i.e., fever, rash, arthritis, and uveitis). We evaluated the relationship between age at the onset of disease/symptoms and basal NF-κB activity due to mutated *NOD2* (defined as the ratio of NF-κB activity without a *NOD2* ligand, muramyldipeptide, to NF-κB activity with muramyldipeptide for each mutated *NOD2*). The calculated basal NF-κB activity ranged from 0.23 to 0.79 (mean 0.42) for mutated *NOD2* and was 0.05 for wild-type *NOD2* (Figure 1c).

Because the number of patients with each *NOD2* mutation was limited, we arbitrarily categorized basal NF-κB activity as low (<0.3), moderate (0.3–0.5), and high (>0.5). According to these criteria, mutations E383G and H496L were associated with low activity; mutations R334Q, T605P, D382E, R334W, and M513T were associated with moderate activity; and mutations

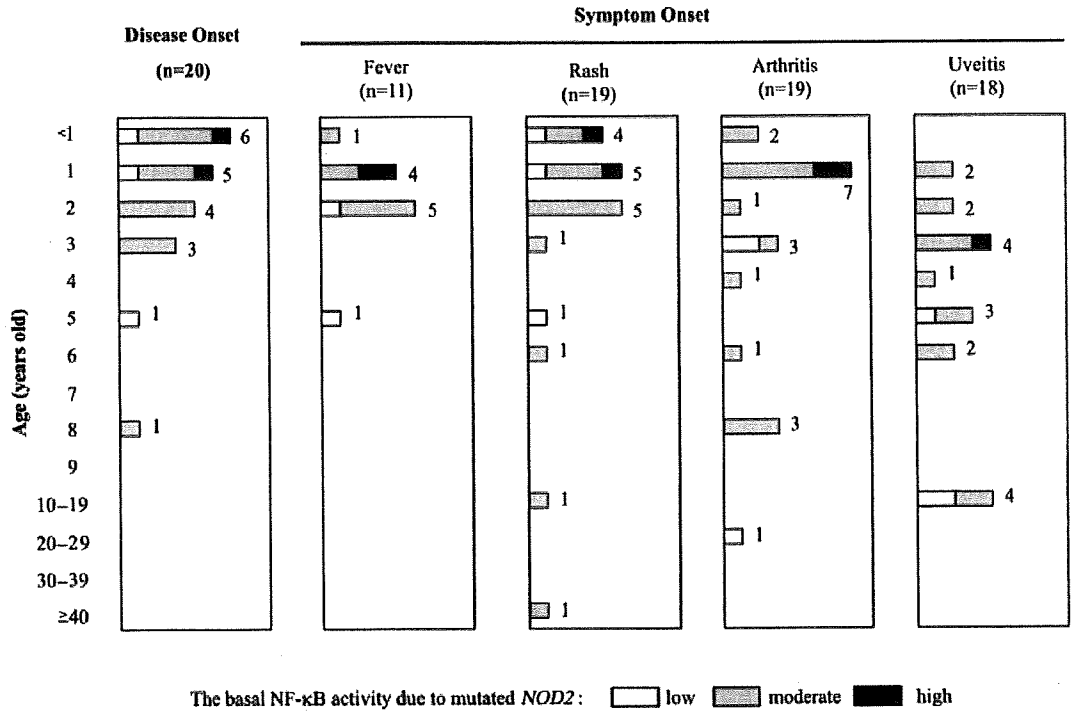

Figure 2. Relationship between age at disease or symptom onset and basal NF-κB activity due to mutated *NOD2*. Among the 9 patients without fever, 8 had moderate and 1 had low basal NF-κB activity. One patient without rash had moderate basal NF-κB activity, and 1 patient without arthritis had moderate basal NF-κB activity. Of 2 patients without uveitis, 1 had high and the other had moderate basal NF-κB activity.

N670K and C495Y were associated with high basal NF-κB activity. Our limited number of patients was insufficient to detect a correlation between the defined basal NF-κB activity and the onset of disease, fever, rash, arthritis, and uveitis (Figure 2). Notably, the age at onset of symptoms varied markedly between patients with the same R334W mutation, even in familial cases (Table 1).

Relationship between visual impairment and basal NF-κB activity due to mutated *NOD2*. The most relevant morbidity associated with Blau syndrome/EOS is ocular involvement, which is usually refractory to conventional treatment. Thus, we next explored the relationship between visual impairment and basal NF-κB activity. There was no clear correlation when the analysis included all recruited patients (Table 3). When we focused on the most frequent genotypes R334Q and R334W, between-genotype differences in visual impairment were observed (Table 4). Basal NF-κB activity was higher in patients with the R334W mutation than in those with the R334Q mutation (Figure 1c). None of the 3 patients with the R334Q mutation had visual impairments, while 4 of 9 patients with the R334W mutation

Table 3. Correlation between visual impairment and basal NF-κB activity*

	Visual impairment			Disease duration, median (range) years
	Normal	Moderate	Severe	
Basal NF-κB activity				
Low	2	0	1	35 (15–43)
Moderate	11	2	2	15 (5–43)
High	1	1	0	10.5 (6–15)

* Except where indicated otherwise, values are the number of patients.

Table 4. Correlation between visual impairment and the 2 most frequent genotypes*

	Visual impairment			Disease duration, median (range) years
	Normal	Moderate	Severe	
Present study				
R334Q	3	0	0	15 (5–19)
R334W	5	2	2	19 (9–43)
Previous study (9)				
R334Q	8	0	0	12 (3–26)
R334W	8	2	1	16 (5–44)

* Except where indicated otherwise, values are the number of patients.

had visual impairments. This result suggests that patients with the R334W mutation were more likely to have visual impairments than were those with the R334Q mutation (Table 4).

DISCUSSION

Blau syndrome/EOS is a rare systemic granulomatosis that has been associated with *NOD2*. In this study, patients with Blau syndrome/EOS and *NOD2* mutations were retrospectively recruited nationwide in Japan, to determine whether the *NOD2* genotype and its functional abnormality predict the Blau syndrome/EOS clinical phenotype. This study is the first to investigate the correlation between the *NOD2* genotype and its functional abnormality and the Blau syndrome/EOS clinical phenotype. Our findings suggest that *NOD2* genotyping may help predict disease progression in patients with Blau syndrome/EOS, although the clinical severity of Blau syndrome/EOS was not clearly associated with basal NF-κB activity due to mutated *NOD2* among the limited number of patients we studied.

The classic Blau syndrome/EOS symptom triad is skin rash, arthritis, and uveitis. Corresponding clinical manifestations include widespread erythematous papules, polyarthritis with boggy synovial swellings, and panuveitis (1,9,10,23), which were also identified in the present study. Rose et al described 2 patients who also had 1 episode of erythema nodosum–like lesions during the course of the disease (9). In our cohort, 5 patients had erythema nodosum–like lesions, suggesting that this should be recognized as one of the skin manifestations associated with Blau syndrome/EOS.

In the current study, 55% of the patients had fever, which always accompanied at least 1 symptom of the classic triad. Arostegui et al also reported that 50% of their cohort had recurrent or persistent fever (10). These findings suggest that fever is one of the important symptoms of Blau syndrome/EOS and is the reason why Blau syndrome/EOS is misdiagnosed as systemic-onset juvenile idiopathic arthritis (JIA). In fact, patient 11 in our study (who had the R334W mutation) experienced persistent fever reaching 40°C and received aggressive immunosuppressive therapy, because systemic-onset JIA was initially diagnosed. This case alerts us to the possibility that patients with Blau syndrome/EOS can sometimes have fever, and that Blau syndrome/EOS can resemble systemic-onset JIA.

Bilateral hilar lymph nodes, which are often seen in adult sarcoidosis, are not observed in Blau syndrome/EOS, but this does not mean that pulmonary lesions do not occur in patients with Blau syndrome/EOS. In fact, 2 patients (patient 5 [with the R334Q mutation] and patient 10 [with the R334W mutation]) had pulmonary lesions; in particular, patient 10 had the first reported case of sporadic EOS in association with the *NOD2* mutation (6). Another case of Blau syndrome/EOS with pulmonary lesions and interstitial pneumonitis, but not bilateral hilar lymph nodes, has also been reported (28). These findings suggest the importance of following up patients with Blau syndrome/EOS to check for not only the classic triad of symptoms but also other abnormalities, including pulmonary lesions.

Blau syndrome/EOS, which usually occurs in children younger than age 4 years, developed at 5 years and 8 years, respectively, in 2 patients in the present study (patient 2 [with the E383G mutation] and patient 17 [with the R334W mutation]). Because both of these patients had a family history of skin rash/arthritis/uveitis, they had been closely monitored by their parents as well as by their physicians. Therefore, it is unlikely that any symptoms that occurred when the patients were younger than 4 years of age were overlooked in these 2 cases. In the literature, there is 1 case of Blau syndrome in which skin rash, persistent fever, and camptodactyly started to develop at age 18 years (10). These findings indicate that the onset of Blau syndrome/EOS can be at age 5 years or older, and that disease onset in a patient younger than 4 years should not be considered requisite for a diagnosis of Blau syndrome/EOS.

In our cohort, the age at disease/symptom onset, organ involvement, and severity of Blau syndrome/EOS varied substantially even within affected families and between individuals with the same *NOD2* mutation (e.g., R334W). In other genetic disorders, identical mutations have been associated with phenotypic variation in unrelated individuals, within a family, and even in monozygotic twins (29). Phenotypic variation in Blau syndrome/EOS has been reported in monozygotic twins; therefore, nongenetic factors such as environmental conditions and/or infectious agents might be involved in phenotypic variation (24). Interestingly, in 2 of our cases, BCG vaccination was an obvious triggering factor. In addition, a previous report noted that cutaneous lesions first arose after BCG vaccination in a patient with Blau syndrome/EOS (30). The BCG vaccine contains muramyldipeptide, a ligand for NOD-2 protein (11,12), which is interesting from a pathophysiologic point of view. However, BCG vaccination did not always cause the onset of disease in patients with Blau syndrome/EOS, because most patients in our cohort were vaccinated with BCG according to the immunization protocol used in areas of

Japan where the risk for tuberculosis was high. An unknown endogenous ligand for NOD-2 could influence disease onset and/or progression, similar to uric acid as an endogenous cryopyrin/NLRP3 ligand (31). The potential roles of endogenous ligands, pathogen-associated molecular patterns, and/or danger-associated molecular patterns in disease pathogenesis remain to be elucidated.

Although increased basal NF-κB activity due to mutated *NOD2* has been proposed as an etiology of Blau syndrome/EOS, how such activity causes the characteristic symptoms remains unclear. We hypothesized that if increased basal NF-κB activity is the key to the pathophysiology of this disease, it should be related to disease severity or disease progression. Unfortunately, there was no clear correlation between basal NF-κB activity and the onset of disease/symptoms. However, patients with mutated *NOD2* and low basal NF-κB activity tended to experience complications, e.g., arthritis and uveitis, at a later age. This finding raises the possibility that basal NF-κB activity may affect disease progression rather than disease onset. Given that NOD-2 protein signals through MAPK/ERK as well as the NF-κB pathway (32), the possibility cannot be excluded that the MAPK/ERK activation potential of each *NOD2* genotype might also be correlated with disease severity or progression.

From the perspective of quality of life, the ocular manifestations of Blau syndrome/EOS require the closest attention (33). In a previous study, one-third of patients with Blau syndrome/EOS and *NOD2* mutations had a poor or extremely poor visual outcome, and the progression of visual field loss was independent of the particular *NOD2* mutant and was not associated with disease duration (9). In our cohort, however, patients with the R334W mutation experienced more visual impairment than did patients with the R334Q mutation, although 4 patients with the R334W mutation were from 2 families (patients 14 and 15 and patients 16 and 17, respectively). Therefore, familial genetic and environmental factors could easily influence the phenotype. Thus, in order not to favor our hypothesis, we excluded patients 15 and 17 from the analysis, and the trend was still evident. This observation was consistent with the findings of Rose et al (9), although those investigators did not address this issue. These findings suggest that *NOD2* genotyping could help predict the course of eye disease in patients with Blau syndrome/EOS, especially those with the R334Q mutation or the R334W mutation.

The relationship between visual impairment and basal NF-κB activity also remains a matter for discussion. Our data showed that visual impairments were more severe in patients with the R334W mutation than in those with the R334Q mutation, which seems to be consistent with the hypothesis that higher basal NF-κB activity causes more severe disease or more disease progression. However, no ocular symptoms have developed during the 6 years since disease onset in patient 20 (with the C495Y mutation and the highest basal NF-κB activity in our cohort), although ocular symptoms developed in another patient with the same genotype (10). Also, in patient 2, who had the E383G mutation and the lowest basal NF-κB activity, severe visual impairment occurred when she was in her late twenties. These findings contradict our hypothesis that *NOD2* genotypes with higher basal NF-κB activity are associated with severe disease. However, Blau syndrome/EOS was promptly diagnosed in patient 20 with the C495Y mutation, who luckily was under the care of the same pediatric rheumatologist who treated patient 19 (who had the N670K mutation) and was treated with systemic steroid therapy. Patient 2 (who had the E383G mutation) subsequently received inappropriate immunosuppressive therapy, because the patient refused steroid treatment. Furthermore, patient 10 (with the R334W mutation), who had no obvious systemic inflammatory findings and did not receive systemic steroid therapy, became blind at 20 years of age. These findings raise the possibility that the extent of visual impairment could be modified by therapy.

Finally, we were not able to prove a link between the clinical severity of Blau syndrome/EOS and basal NF-κB activity in the whole cohort, possibly because of the restricted number of patients and because of the differences in treatment among patients. Therefore, a prospective study involving a sufficient number of patients to allow analysis of each genotype–phenotype correlation would be required to test our hypothesis. Given that there is no standard treatment protocol for Blau syndrome/EOS, some predictors of disease progression, especially progression of visual impairment, would have great benefit for clinicians. We observed a difference in the development of visual impairment only between patients with the R334W mutation and those with the R334Q mutation, which provides a clue that predicts the development of visual impairment in patients with the R334W and R334Q mutations. We also believe that understanding the mechanisms of how *NOD2* acts in disease pathogenesis should help in discovering therapeutic targets for the treatment of Blau syndrome/EOS.

ACKNOWLEDGMENTS

We appreciate the invaluable assistance of the following physicians, who kindly provided materials and allowed us to study their patients: Drs. Sonoko Nagai, Takenosuke Yuasa, Akira Manki, Yoshihiko Sakurai, Mitsuru Nakajima, Hiroko Kobayashi, Ikuma Fujiwara, Hiroyuki Tsutsumi, Shuji Takei, Kumiko Nakao, Yoshikazu Otsubo, Kouichi Ohta, Kazunaga Agematsu, Hiroaki Azukisawa, Hiroyuki Murota, and Kenji Katamura.

AUTHOR CONTRIBUTIONS

Dr. Nishikomori had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Okafuji, Nishikomori, Heike, Miyachi, Nakahata.
Acquisition of data. Okafuji, Fujisawa, Saito, Yoshioka, Kawai, Sakai, Tanizaki.
Analysis and interpretation of data. Okafuji, Nishikomori.
Manuscript preparation. Okafuji, Nishikomori, Kanazawa, Kambe.
Statistical analysis. Yamazaki.

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Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells

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ABSTRACT **Satellite cells are myogenic stem cells responsible for the postnatal regeneration of skeletal muscle. Here we report the successful *in vitro* induction of Pax7-positive satellite-like cells from mouse embryonic stem (mES) cells. Embryoid bodies were generated from mES cells and cultured on Matrigel-coated dishes with Dulbecco's modified Eagle medium containing fetal bovine serum and horse serum. Pax7-positive satellite-like cells were enriched by fluorescence-activated cell sorting using a novel anti-satellite cell antibody, SM/C-2.6. SM/C-2.6-positive cells efficiently differentiate into skeletal muscle fibers both *in vitro* and *in vivo*. Furthermore, the cells demonstrate satellite cell characteristics such as extensive self-renewal capacity in subsequent muscle injury model, long-term engraftment up to 24 wk, and the ability to be secondarily transplanted with remarkably high engraftment efficiency compared to myoblast transplantation. This is the first report of transplantable, functional satellite-like cells derived from mES cells and will provide a foundation for new therapies for degenerative muscle disorders.—Chang, H., Yoshimoto, M., Umeda, K., Iwasa, T., Mizuno, Y., Fukada, S., Yamamoto, H., Motohashi, N., Yuko-Miyagoe-Suzuki, Takeda, S., Heike, T., Nakahata, T. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. *FASEB J.* 23, 1907–1919 (2009)**

Key Words: long-term engraftment · secondary transplantation · high engraftment efficiency · self-renewal

DUCHENNE MUSCULAR DYSTROPHY (DMD; ref. 1) is a progressive, lethal muscular disorder (2) with no effective cure despite extensive research efforts. DMD results from mutations in the X-linked *dystrophin* gene (3). Dystrophin and its associated proteins function to link the intracellular actin cytoskeleton of muscle fibers to laminin in the extracellular matrix (4), thereby protecting myofibers from contraction-induced damage (5). Skeletal muscle fibers are continuously regenerated following exercise and injuries when satellite cells (6) are induced to differentiate into myoblasts that form myotubes and replace the damaged myofibers (7, 8). This muscular regeneration is observed at a much higher frequency in DMD patients (9). Continuous damage to myofibers and constant activation of resident satellite cells due to loss of dystrophin leads to the exhaustion of the satellite cells (10, 11), and the eventual depletion of satellite cells is primarily responsible for the onset of DMD symptoms.

Successful transplantation of normal satellite cells into the skeletal muscle of DMD patients may enable *in situ* production of normal muscle tissue and create a treatment option for this otherwise fatal disease. A recent report has shown that the transplantation of satellite cells collected from mouse muscle tissues can produce muscle fibers with normal dystrophin expression in mdx mice (12-14), a model mouse for DMD (15). This study suggests that stem cell transplantation may be a viable therapeutic approach for the treatment of DMD (16).

Satellite cells are monopotent stem cells that have the ability to self-renew and to differentiate into myoblasts and myotubes to maintain the integrity of skeletal muscle (17). Satellite cells lie dormant beneath the basal lamina and express transcription factors such as Pax3 (13, 18) and Pax7 (19). Pax7, a paired box transcription factor, is particularly important for satellite cell function. A recent study of *Pax7-null* mice revealed that Pax7 is essential for satellite cell formation (19) and that the *Pax7-null* mice exhibit a severe deficiency in muscle fibers at birth and premature mortality with complete depletion of the satellite cells. Surface markers such as M-cadherin and c-met (20) are also expressed by satellite cells. However, these markers are not specific to satellite cells because they are also expressed in the cerebellum (21) and by hepatocytes (22). To specifically identify quiescent satellite cells, a

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doi: 10.1096/fj.08-123661

novel monoclonal antibody, SM/C-2.6, has recently been established (23). Satellite cells purified with this antibody regenerate muscle fibers on implantation into mdx mice (15).

The use of satellite cells for clinical therapies would require the establishment of a reliable source of these cells. Embryonic stem (ES) cells are totipotent stem cells that are able to differentiate into various types of somatic cells *in vitro*. While mouse embryonic stem (mES) cells can be readily induced to differentiate into muscle fibers (24, 25) and the myogenicity of human ES cells was recently validated (26), the induction of mES cells into functional satellite cells has not been reported. Here we have successfully induced mES cells to generate cells expressing Pax7 *in vitro* by forming embryoid bodies (EBs). These ES cell-derived (ES-derived) Pax7-positive cells can be enriched using the SM/C-2.6 antibody (23) and possess a great potential for generating mature skeletal muscle fibers both *in vitro* and *in vivo*. The Pax7-positive cells display a self-renewal ability that can repopulate Pax7-positive cells *in vivo* in the recipient muscles following an injury. Furthermore, these ES-derived Pax7-positive cells could engraft in the recipient muscle for long periods, up to 24 wk, and could also be serially transplanted. These results indicate that ES-derived Pax7-positive cells possess satellite cell characteristics. This is the first report of effective induction of functional satellite cells from mES cells, and these novel findings may provide a new therapeutic approach for treatment of DMD.

MATERIALS AND METHODS

Cell culture

D3 cells, mES cells (27) that ubiquitously express the *EGFP* gene under the *CAG* promoter (28) (a gift from Dr. Masaru Okabe, Osaka University, Osaka, Japan), were used in this study. ES cells were maintained on tissue culture dishes (Falcon) coated with 0.1% gelatin (Sigma, Oakville, CA, USA), in DMEM (Sigma) supplemented with 15% fetal bovine serum (FBS; Thermo Trace, Melbourne, Australia), 0.1 mM 2-mercaptoethanol (Nakalai Tesque, Japan), 0.1 mM nonessential amino acids (Invitrogen, Burlington, CA, USA), 1 mM sodium pyruvate (Sigma), penicillin/streptomycin (50 μg/mL), and 5000 U/ml leukemia inhibitory factor (Dainippon Pharmaceutical Co., Japan).

In vitro differentiation of ES cells into a muscle lineage

To induce EB formation, undifferentiated ES cells were cultured in hanging drops for 3 d at a density of 800 cells/20 μl of differentiation medium, which consisted of DMEM supplemented with penicillin/streptomycin, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptonethanol, 5% horse serum (HS), and 10% FBS. EBs were transferred to suspension cultures for an additional 3 d (d 3+3). Finally, the EBs were plated in differentiation medium in 48-well plates (Falcon) coated with Matrigel (BD Bioscience, Bedford, MA, USA). The medium was changed every 5 d.

Immunofluorescence and immunocytochemical analysis

Immunostaining of cultured cells and recipient mouse tissues were carried out as described previously (29). Briefly, the left tibialis anterior (LTA) muscle of the recipient mouse was fixed with 4% paraformaldehyde and cut into 6 μm cross sections using a cryostat, and samples were fixed for 5 min in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After incubation in 5% skim milk for 10 min at room temperature to block nonspecific antibody binding, cells were incubated for 12 h at 4°C with anti-mouse monoclonal antibodies. Antibodies used in this study were mouse anti-Pax7, which was biotinylated using a DSB-X Biotin Protein Labeling Kit (D20655; Molecular Probes, Eugene, OR, USA), mouse anti-Pax3 (MAB1675, MAB2457; R&D Systems, Minneapolis, MN, USA), rabbit anti-mouse Myf5 (sc-302; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse M-cadherin (205610; Calbiochem, San Diego, CA, USA), mouse anti-myosin heavy chain (MHC; 18-0105; Zymed Laboratories, San Francisco, CA, USA; reacts with human, rabbit, rat, mouse, bovine, and pig skeletal MHC), mouse anti-mouse myogenin and mouse anti-mouse Myo-D1 (M3559, M3512; Dako, Carpinteria, CA, USA), monoclonal rabbit anti-mouse laminin (LB-1013; LSL, Tokyo, Japan), and mouse anti-mouse dystrophin (NCL-DYS2; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Cy3-labeled antibodies to mouse or rabbit IgG, fluorescein isothiocyanate-labeled antibodies to mouse or rabbit IgG (715-005-150, 711-165-152; Jackson ImmunoResearch Laboratory, Bar Harbor, ME, USA), or Alexa 633-labeled goat anti-rabbit IgG (A21070; Invitrogen, Molecular Probes) were applied as secondary antibodies. Hoechst 33324 (H3570; Molecular Probes) was used for nuclear staining. The samples were examined with a fluorescence microscope (Olympus, Tokyo, Japan) or an AS-MDW system (Leica Microsystems, Wetzlar, Germany). Micrographs were obtained using an AxioCam (Carl Zeiss Vision, Hallbergmoos, Germany) or the AS-MDW system (Leica Microsystems). In sections of muscles transplanted with ES-derived satellite cells, the number of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells were counted, per field, at ×100. More than 10 fields in each tissue sample were observed. To prevent nonspecific secondary antibody binding to Fc receptors, all immunostaining of frozen sections used the Vector® M.O.M™ Immunodetection Kit (BMK-2202; Vector Laboratories, Burlingame, CA, USA).

PCR analysis

Total RNA was isolated from cultured cells in 48-well plates, using TRIzol reagent (Invitrogen). The following specific primers were used for PCR:

Pax3, sense, 5′-AACACTGGCCCTCAGTGAGTTCTAT-3′, and antisense, 5′-ACTCAGGATGCCATCGATGCTGTG-3′; Pax7, sense, 5′-CATCCAGTGCTGGTACCCCACAG-3′, and antisense, 5′-CTGTGGATGTCACCTGCTTGAA-3′; Myf5, sense, 5′-GAGCTGCTGAGGGAACAGGTGG-3′, and antisense, 5′-GTTCTTTCGGGACCAGACAGGG-3′; MyoD, sense, 5′-AGGCTCTGCTGCGCGACCAG-3′, and antisense, 5′-TGCAGTCGATCTCTCAAAGC-3′; myogenin, sense, 5′-TGAGGGAGAAGCGCAGGCTCAAG-3′, and antisense, 5′-ATGCTGTCCACGATGGACGTAAGG-3′; M-cadherin, sense, 5′-CCACAAACGCCTCCCCTACCC-3′, and antisense, 5′-GTCGATGCTGAAGAACTCAGGGC-3′; C-met, sense, 5′-GAATGTCGTCCTACACGGCCAT-3′, and antisense, 5′-CACTACACAGTCAGGACACTGC-3′; GAPDH, sense, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and antisense, 5′-TGTTGGGGGCCGAGTTGGGATA-3′. AmpliTaqGold (Applied