研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
金子英雄	色素脱失を伴う免疫不全症	近藤直実、平家俊男	自己炎症性疾患・自然免疫不全症とその近縁疾患	診断と治療社	東京	2012	156 － 158
金子英雄	DNA 修復障害	近藤直実、平家俊男	自己炎症性疾患・自然免疫不全症とその近縁疾患	診断と治療社	東京	2012	248
金子英雄	毛細血管拡張性小脳失調症(ataxia-telangiectasia)	近藤直実、平家俊男	自己炎症性疾患・自然免疫不全症とその近縁疾患	診断と治療社	東京	2012	249-250
金子英雄	Bloom 症候群	近藤直実、平家俊男	自己炎症性疾患・自然免疫不全症とその近縁疾患	診断と治療社	東京	2012	251-252
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Ⅳ. 研究成果の刊行物・別冊

Augmented cell death with Bloom syndrome helicase deficiency

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Abstract. Bloom syndrome (BS) is a rare autosomal genetic disorder characterized by lupus-like erythematous telangiectasias of the face, sun sensitivity, infertility, stunted growth, upper respiratory infection, and gastrointestinal infections commonly associated with decreased immunoglobulin levels. The syndrome is associated with immunodeficiency of a generalized type, ranging from mild and essentially asymptomatic to severe. Chromosomal abnormalities are hallmarks of the disorder, and high frequencies of sister chromatid exchanges and quadriradial configurations in lymphocytes and fibroblasts are diagnostic features. BS is caused by mutations in BLM, a member of the RecQ helicase family. We determined whether BLM deficiency has any effects on cell growth and death in BLM-deficient cells and mice. BLM-deficient EB-virus-transformed cell lines from BS patients and embryonic fibroblasts from $BLM^{-/-}$ mice showed slower growth than wild-type cells. BLM-deficient cells showed abnormal p53 protein expression after irradiation. In $BLM^{-/-}$ mice, small body size, reduced number of fetal liver cells and increased cell death were observed. BLM deficiency causes the up-regulation of p53, double-strand break and apoptosis, which are likely observed in irradiated control cells. Slow cell growth and increased cell death may be one of the causes of the small body size associated with BS patients.

Introduction

Bloom syndrome (BS) is a rare genetic disorder caused by mutations in BLM, a member of the RecQ helicase family (1). There are five human RecQ-like proteins (RECQL1, BLM, WRN, RECQL4 and RECQ5), each having 3' to 5' DNA helicase activity, but little sequence similarity outside the helicase motifs (2,3). Three of these helicases (BLM, WRN and Rothmund-Thomson) show genomic instability and cancer susceptibility; however, each also has distinctive features (4,5). The unique features of BS are severe pre- and post-natal growth retardation and a wide spectrum of cancer types that develop at a young age. Other BS phenotypes include facial sun sensitivity, immunodeficiency and male sterility/female subfertility (6,7). Compared with Werner syndrome, small body size is one of the characteristic features associated with BS patients.

Here, we determined whether BLM deficiency has any effects on the cell growth and death of BLM-deficient cells and mice.

Materials and methods

BS patient. AsOk, who was identified in the BS registry as number 97, weighed 2,250 g at birth. Café-au-lait spots and mandibular hypoplasia were prominent. A 3-bp deletion was detected in the BLM sequence of AsOk DNA (8). This deletion caused the generation of a stop codon at amino acid 186.

Cell culture. EB-virus-transformed cell lines from BS patients and control subjects were developed as previously reported (9). In brief, PBMCs were isolated from the heparinized blood of patients by gradient centrifugation in Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), and suspended at a density of 10^6 ml in culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, l-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml). The PBMCs (10^6 ml) were then cultured in the presence of 10 μg/ml phytohemagglutinin (PHA) for 3 days.

Detection of p53 protein. PBMCs cultured with PHA for 3 days were irradiated (6 Gy). After 1 h, the cells were collected by centrifugation and protein was extracted. Using anti-human p53 antibody (Santacruz, USA), immunoblotting was performed.

BLM-deficient embryonic fibroblasts. Heterozygous BLM-deficient ($BLM^{+/-}$) mice were kindly provided by P. Leder. $BLM^{-/-}$ mice were obtained by mating $BLM^{+/-}$ mice (10). Embryonic fibroblasts from $BLM^{-/-}$ mice were obtained from 12.5-day embryos. None of the $BLM^{-/-}$ embryos survived more than 13 days.

Cell proliferation assay. Cell proliferation and cell viability were determined by the trypan blue or MTT assays. The MTT assay was performed following the manufacturer's protocol.

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Key words: Bloom syndrome, small body size, BLM deficiency, cell death

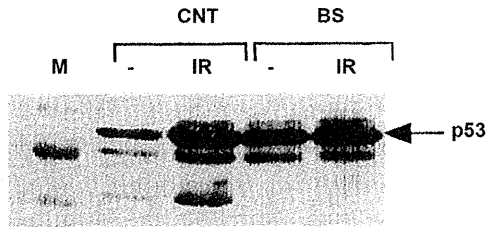

Figure 1. p53 protein expression in PBMCs from a control subject and a BS patient. PBMCs cultured with PHA for 3 days were irradiated (6 Gy). After 1 h, the cells were collected and p53 protein expression was detected.

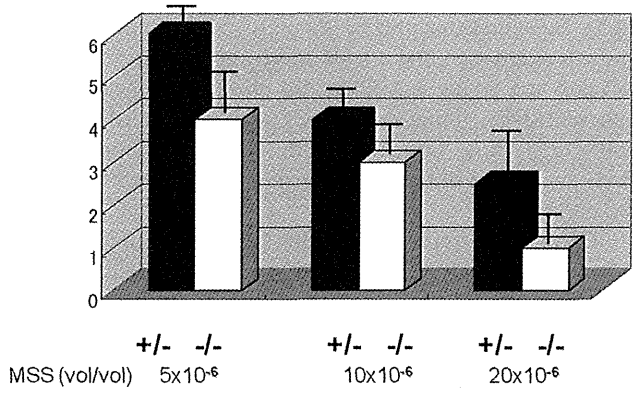

Figure 2. Fig. 2 Cell proliferation and cell viability were determined using trypan blue. Embryonic fibroblasts were established from $BLM^{+/-}$ and $BLM^{-/-}$ mice at 12.5 days post-coitus. Embryonic fibroblasts from $BLM^{-/-}$ mice showed a slow growth rate and a high sensitivity to MMS compared to those from $BLM^{+/-}$ mice.

Embryonic fibroblasts were cultured with methyl methanesulfonate (MMS) (Sigma, Japan) for 24 h (11), then the viable cell number was determined on trypan blue.

Detection of single-strand DNA. Paraffin and cryostat sections were prepared from the brain of $BLM^{+/-}$ or $BLM^{-/-}$ mice at 12.5 days post-coitus. Polyclonal rabbit anti-ssDNA antibody (IgG, 100 μg/ml, Dako Japan, Kyoto, Japan) at a dilution of 1:300 was used to detect the formation of single-stranded DNA (ssDNA) for 1 h at room temperature. Immunoreactivity was detected with peroxidase-labeled goat anti-rabbit immunoglobulins.

Results

Abnormal regulation of p53 protein expression. After the irradiation of PHA-stimulated PBMCs, p53 protein expression was induced in control cells (Fig. 1). In the PBMCs of the BS patient, high p53 protein expression was detected even without irradiation. Irradiation slightly induced p53 protein in BS cells. In the BS EB cell line, p53 phosphorylation by ATM was up-regulated compared with that in the control EB cell line (data not shown). These results suggested that BLM-deficient cells have abnormal regulation of p53 protein expression and an elevated frequency of apoptosis. Next, apoptosis was investigated *in vivo* and *in vitro* using BLM-deficient cells.

Slow growth in BLM-deficient cells. The growth rate of EB cells from BS patients was slower than that of control cells. After irradiation, the growth rate of BS cells was slower than that of control cells. MMS action caused double-stranded DNA breaks. The sensitivity of $BLM^{-/-}$ cells to MMS was higher than that of wild type cells. Embryonic fibroblasts originating from $BLM^{-/-}$ mice also showed a slowed growth rate (Fig. 2).

Augmented cell death in embryonic brain of $BLM^{-/-}$ mice. Anti-single-stranded DNA was detected in the brain of $BLM^{-/-}$ mice, with the number being higher than that detected in the brain of $BLM^{+/-}$ mice (Fig. 3). This result suggested the occurence of augmented cell death in $BLM^{-/-}$ mice.

Discussion

In this study, we showed the abnormal regulation of p53 protein expression and augmented cell death in BLM-deficient cells both *in vitro* and *in vivo*. Stalled replication forks can result in double-strand breaks, thereby triggering the activation of ATM (12). Consistent with a previously reported study, the deficiency of BLM was radiomimetic (13).

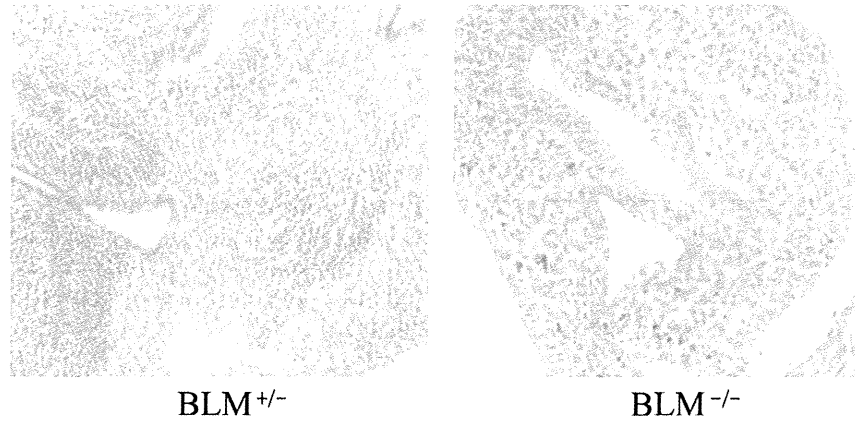

Figure 3. Detection of single-stranded DNA. Immunohistochemical staining of $BLM^{+/-}$ and $BLM^{-/-}$ embryos at 12.5 days post-coitus was performed.

Originally, MMS was considered to directly cause double-stranded DNA breaks, since homologous-recombination-deficient cells are particularly vulnerable to the effects of MMS. However, it is now considered that MMS stalls replication forks, and cells that are homologous-recombination-deficient have difficulty repairing the damaged replication forks.

Studies in yeast and human cells suggest a pivotal role of RECQ-like helicases in maintaining genomic integrity during the S phase (14). BS patients show small body size from birth. This small body size persists throughout their lifetime. At 12.5 days post-coitus, BLM-deficient mice have a smaller body size than wild-type mice (10).

BLM deficiency renders cells highly susceptible to apoptosis, which is a possible explanation for the pre- and post-natal growth retardation observed in BS patients. In the absence of BLM, many cells fail to repair damage rapidly enough, whereupon p53 signals those cells to die. Individuals with BS may continually lose cells, owing to excessive apoptosis, particularly during pre- and post-natal development, when cell proliferation is excessive (15). Excessive apoptosis would leave many tissues with chronic cellular insufficiency, and hence a small size, thereby explaining the pre- and post-natal growth retardation.

p53 is crucial for the apoptosis of BS cells. This apoptosis is not accompanied by an increase in BAX or p21 protein expression. Thus, p53 may induce apoptosis independent of its transactivation activity, consistent with the finding that p53 is transcriptionally inactive during the S phase. p53 may mediate the death of damaged BS cells by directly inducing mitochondria-mediated apoptosis, or by means of its transactivation activity.

In conclusion, BLM deficiency causes the dysregulation of p53 and augmented apoptosis, similar to that observed in irradiated wild-type cells. This slow cell growth and increased cell death may cause the small body size associated with BS patients.

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A rapid screening method to detect autosomal-dominant ectodermal dysplasia with immune deficiency syndrome

To the Editor:

A patient presented to us with autosomal-dominant anhidrotic ectodermal dysplasia with immune deficiency syndrome (EDA-ID). By using a rapid flow cytometric screening system, we detected a novel mutation of the *IKBA* gene in the patient.

Toll-like receptors are one of the major groups of pathogen-associated molecular pattern recognition receptors in the innate

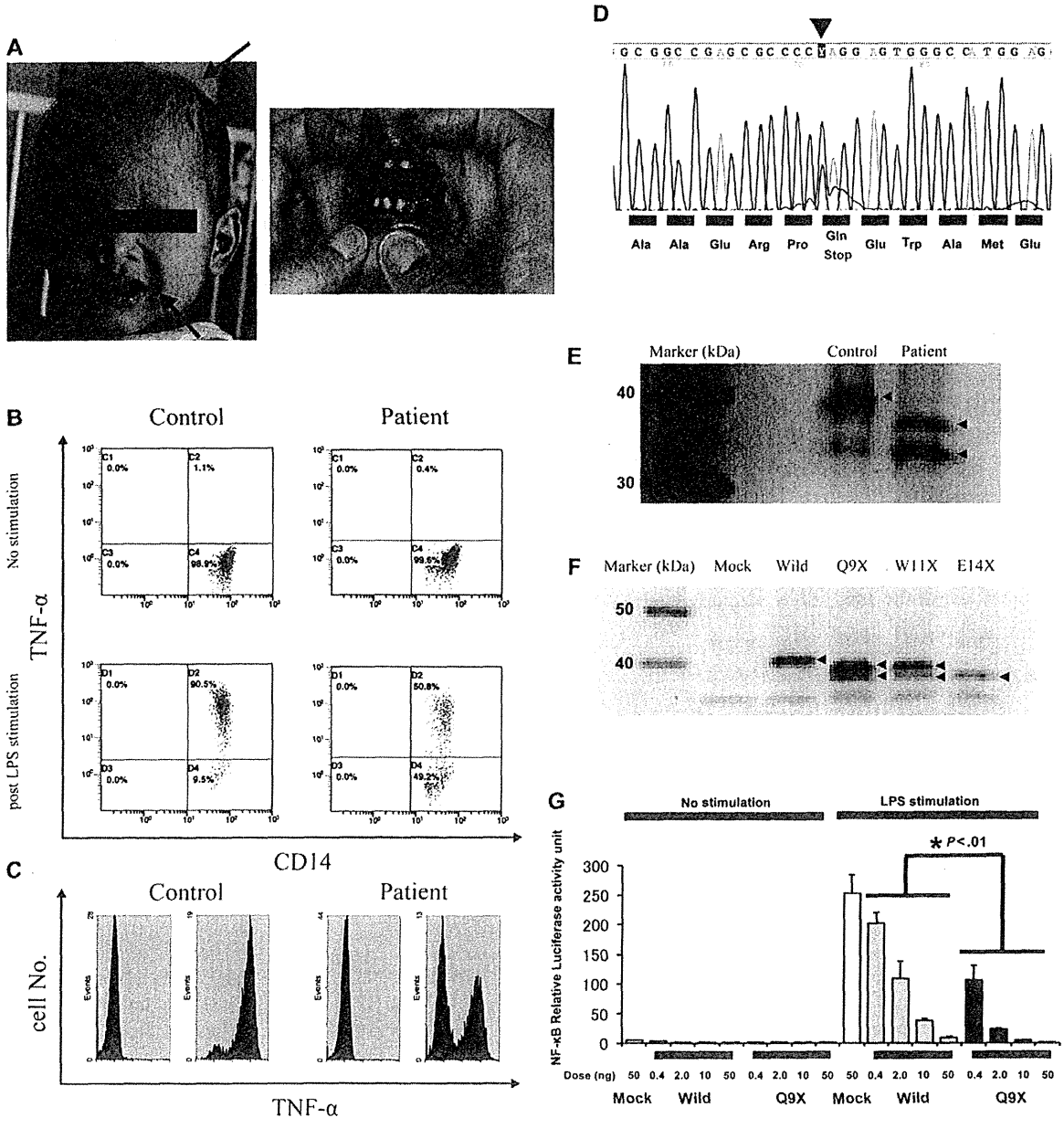

FIG 1. A, External abnormalities of the AD-EDA-ID patient at the age of 16 months. **B** and **C,** Flow cytometric analysis of intracellular TNF-α production in $CD14^+$ cells in response to LPS stimulation. **D,** Genetic analysis of the *IKBA*. **E** and **F,** Western blot analyses of expressions of wild-type and mutant types IκBα proteins. **G,** NF-κB reporter gene activities.

immune system. Following Toll-like receptor activation, intracellular signaling components such as interleukin-1 receptor–associated kinase 4 (IRAK4) and NF-kappa-B essential modulator (NEMO) are sequentially activated. This leads to the degradation of inhibitor of κB (IκB), which causes the activation of nuclear factor-κB (NF-κB) and expression of inflammatory cytokines.[1] Recently, defects in various components of this signaling pathway have been reported; IRAK4 deficiency was seen to cause high susceptibility to bacterial infections such as *Streptococcus pneumoniae*,[2] and NEMO deficiency was observed to lead to X-linked recessive anhidrotic ectodermal dysplasia with immune deficiency syndrome.[3] In 2003, a hypermorphic mutation of the *IκBα* gene was reported as another causative gene defect for EDA-ID.[4] As the hereditary form of this disease is autosomal dominant, it is termed AD-EDA-ID.

The patient we assessed was a 5-month-old male infant with some dysmorphisms (Fig 1, *A*). His umbilical separation date was 18 days after his birth. The patient's body temperature regulation was poor because of his anhidrosis. He suffered recurrent infections from his first month, including hepatitis with *Cytomegalovirus* infection, enteritis with *Rotavirus*, bronchiolitis with respiratory syncytial virus, bacterial pneumonia, urinary tract infection, and acute otitis media. His family has no history of primary immunodeficiency. The results of a blood examination at the age of 5 months are shown in Table I. Serum immunoglobulin values were normal for his age. It should be noted that his serum IgA levels, but not IgM levels, increased with age (IgA, 751 mg/dL, and IgM 125, mg/dL at 9 months; normal ranges of IgA and IgM in Japanese infants are 10-56 and 55-200 mg/dL, respectively).

We used a previously described rapid screening method for IRAK4 deficiency syndrome using the patient's blood cells.[5] Flow cytometric analysis of intracellular TNF-α production in $CD14^+$ cells in response to 4 hours of LPS stimulation (1.0 μg/mL) showed a substantially lower proportion of CD14 and TNF-α double-positive cells in the patient than in age-matched healthy subjects (mean, 95.7%; SD, 2.78; n = 10) (Fig 1, *B*). The histogram of LPS-stimulated TNF-α positive monocytes (blue) of this patient showed a twin peak pattern (mean fluorescence intensity of the peaks, 0.878 and 56.6) compared with the histogram of nonstimulated TNF-α monocytes (red). Monocytes from healthy subjects displayed a single right-shifted peak pattern (mean fluorescence intensity of the peaks, 73.0; SD, 40.2; n = 10) (Fig 1, *C*).

We next analyzed the *IRAK4*, *MyD88*, *NEMO*, and *IKBA* genes and found a novel mutation (c. 25C>T) (p. Q9X) in the *IKBA* gene (Fig 1, *D*). The *IRAK4*, *MyD88*, and *NEMO* genes were normal. Other *IKBA* gene mutations have been previously reported in AD-EDA-ID, namely, S32I, W11X, and E14X.[4,6,7] The serine residues of the N terminus of IκBα, S32 and S36, are functionally important phosphorylation sites. Phosphorylation leads to degradation of this protein and release of active NF-κB. If these residues are substituted or deleted, NF-κB cannot be inactivated by IκBα. Interestingly, a mechanism by which disease onset is caused by gene substitution at the stop codon near the 5′ end of the gene sequence has been reported, and this causes a hypermorphic effect of N terminus–truncated IκBα protein.[6]

We confirmed the functional effect of the Q9X mutation in the *IκBα* gene in our patient by analyzing for endogenous IκBα protein in his lysed blood cells. Western blots using an anti-IκBα antibody (C-21, Santa Cruz) showed 2 shorter bands for the patient than for a control subject (Fig 1, *E*). A subsequent *in vitro* protein expression study on HEK293 cells of C-terminal FLAG-tagged IκBα Q9X and W11X showed 2 shorter bands compared with wild-type IκBα, while IκBα E14X showed a single shorter band. We believe that the 2 bands are likely to be N terminus–truncated IκBα proteins that are translated from M13 (IκBα Δ1-12) or M37 (IκBα Δ1-36) (Fig 1, *F*). If this is correct, the N terminus–truncated IκBα Δ1-36 should have no serine phosphorylation site. An NF-κB reporter gene activity assay showed a significant dose-dependent inhibitory effect of IκBα Q9X compared with wild-type IκBα on LPS-stimulated Toll-like receptor 4-MD2-CD14 coexpressed HEK293 cells (Fig 1, *G*). On the basis of these results, we diagnosed this patient as having AD-EDA-ID.

Because NF-κB is an essential component of immune responses, some EDA-ID patients have combined T-cell dysfunction.[4] There are also reports of EDA-ID patients dying from complications of mycobacterial disease. In addition, NEMO deficiency was recently reported to be one of the candidate deficiencies of Mendelian susceptibility to mycobacterial disease syndrome.[8] We therefore evaluated the patient's T-cell response by using lymphocyte proliferation assays. Lymphocytes stimulated with phytohemagglutinin and concanavalin A proliferated only to low levels (Table I). As an additional feature, inflammatory bowel disease has also often been reported in XL-EDA-ID patients. The mechanism of the onset of inflammatory bowel disease with EDA-ID remains unknown, but our AD-EDA-ID patient also showed symptoms of inflammatory bowel disease.

TABLE I. Immunological findings of the AD-EDA-ID patient at the age of 5 months

	Patient	Normal values
Number of blood cells (/μL)		
Leukocytes	21,190	6,000-17,500
Lymphocytes	11,230	4,000-13,500
Monocytes	1,270	Unknown
Lymphocytes subsets (%)		
CD3	59.0	58-84
CD4	31.9	25-54
CD8	23.4	23-56
CD19	33.9	5-24
CD20	34.1	3-20
Serum immunoglobulin levels (mg/dL)		
IgG	930	290-960
IgA	91	7-44
IgM	101	41-161
IgG subclass (%)		
IgG1	60.4	39.3-89.0
IgG2	30.0	7.4-50.4
IgG3	9.1	1.3-12.6
IgG4	0.5	0.1-7.8
Lymphocyte proliferation assay (cpm)*		
First time		
No stimulus	151	70-700
PHA	8,660	26,000-53,000
Con A	1,260	20,000-48,000
Second time		
No stimulus	123	
PHA	24,600	
Con A	11,200	

Con A, Concanavalin A; *PHA*, phytohemagglutinin.
*Lymphocyte proliferation assay was performed at the age of 10 and 11 months.

This is the first report of an AD-EDA-ID patient with a novel Q9X mutation of the *IKBA* gene. This case also demonstrates that the screening method using LPS-stimulated intracellular TNF-α–producing CD14 cells is an effective method for the rapid diagnosis of innate immune defects, not only in IRAK4-deficient patients but also in EDA-ID patients.

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