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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.

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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.

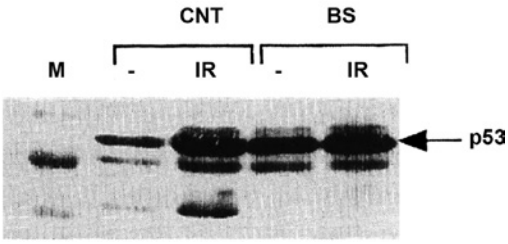

Figure 1. p53 protein expression in PBMCs from a control subject and a BS patient. PBMCs cultured with PHA for 3 days were irradiated (6 Gy). After 1 h, the cells were collected and p53 protein expression was detected.

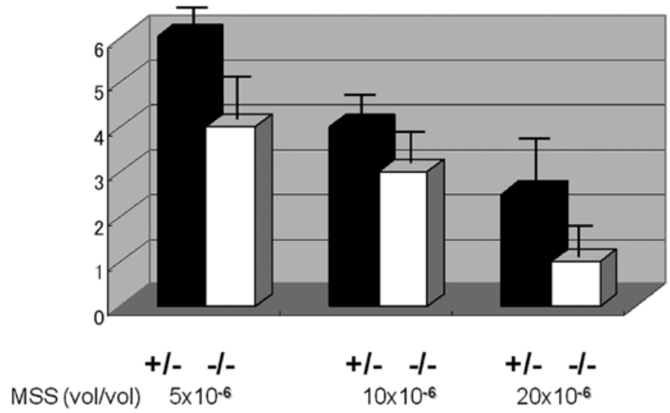

Figure 2. Fig. 2 Cell proliferation and cell viability were determined using trypan blue. Embryonic fibroblasts were established from $BLM^{+/-}$ and $BLM^{-/-}$ mice at 12.5 days post-coitus. Embryonic fibroblasts from $BLM^{-/-}$ mice showed a slow growth rate and a high sensitivity to MMS compared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

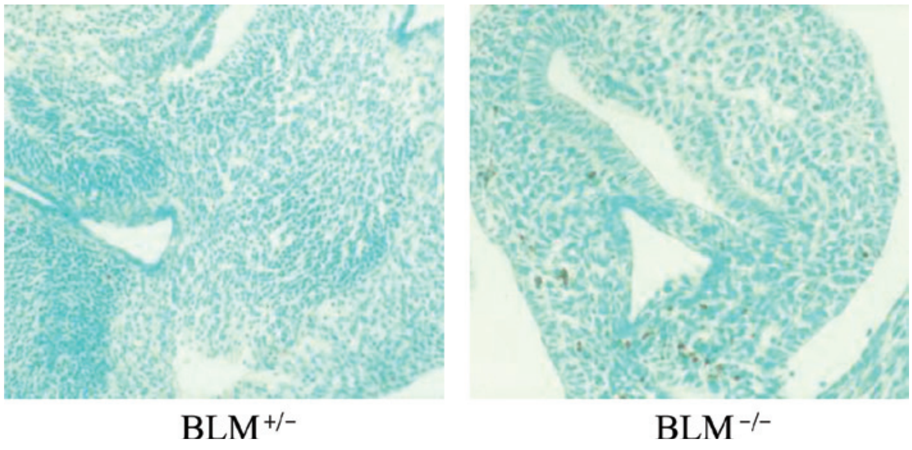

Figure 3. Detection of single-stranded DNA. Immunohistochemical staining of $BLM^{+/-}$ and $BLM^{-/-}$ embryos at 12.5 days post-coitus was performed.

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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