Modulation of the Expression of Bloom Helicase by Estrogenic Agents

Takako ISO, Kazunobu FUTAMI, Teruaki IWAMOTO, and Yasuhiro FURUICHI

*GeneCare Research Institute Co., Ltd.; Kamakura, Kanagawa 247–0063, Japan; b Core Research for Evolution Science and Technology, Japan Science and Technology Agency; Kawaguchi, Saitama 332–0012, Japan; and c Department of Urology, St. Marianna University School of Medicine; Kawasaki, Kanagawa 216–8511, Japan.

Received September 19, 2006; accepted November 22, 2006

We report that the expression of Bloom helicase (BLM) was up-regulated by 17β-estradiol (E2) in estrogen receptor (ER)-positive mammary tumor MCF-7 cells, but was hardly modulated in ER-negative mammary tumor MDA-MB-231 cells. ER antagonist ICI182780 blocked the E2 effect on BLM expression in MCF-7 cells. From these results we conclude that ER participates in up-regulation of BLM expression in MCF-7 cells by means of E2. Similar results were obtained when MCF-7 cells were treated with bisphenol A (BPA), an endocrine-disrupting chemical having a weak estrogenic activity. The ER binding ability of BPA is estimated at 1/1000 of E2 ability, and in this study about 1000-times more BPA was needed for the same levels of estrogenic effect of E2. The expression of cell-cycle associated genes, cdc6, MCM5, MCM2, Myt1, PCNA and AuroraA were up-regulated by E2 and BPA treatment in MCF-7 cells accompanied by up-regulation of BLM. In this BLM promoter study, Sp1 elements in the upper region of BLM modulated transcription, but were not indispensable for E2 response. Our results suggested that up-regulation of BLM expression by E2 and BPA is ER-dependent and may be responsible for repair of DNA damage caused by the genotoxicity of these estrogenic agents.

Key words estrogen; endocrine-disrupting chemical; Bloom helicase; cell cycle

Carcinogenesis by 17β-estradiol (E2) is believed to be associated with its genotoxicity and stimulating activity of cell proliferation.1,2) Studies using laboratory animals3,4) and cultured cells5–8) showed that E2 induces tumors. E2 stimulates antiproliferative and proapototic genes, resulting in cell cycle arrest and cell death.9) We showed previously that E2 causes DNA damage5–8) and cultured cells5–8) showed that E2 induces tumors. E2 suppresses detoxifying enzymes, such as catalase and glutathione-S-transferase, leading to an increase in genotoxicity by E2.10) We showed previously that E2 causes DNA damage in mammary tumor MCF-7 cells assessed by single cell gel electrophoresis, the so-called Comet assay, and that the estrogen receptor (ER) is responsible for DNA damage.10) A similar effect was also observed for bisphenol A (BPA), an endocrine-disrupting chemical,11) widely used as material for polycarbonate plastic and epoxy resin. BPA has a weak estrogenic activity to stimulate cell proliferation of ER-positive mammary tumor cells, and so is attracting considerable attention.11,12) We showed previously the genotoxic effect of BPA as assessed by Comet assay,10) but the BPA effect was much less than the effect of E2.

DNA microarray technology identifies profiles of estrogen responsive genes.13,14) Cell cycle-associated genes whose expression is up-regulated by E2, particularly those having a role in DNA synthesis15) have been studied. E2 down regulates antiproliferative and proapototic genes, resulting in cell proliferation and cell survival in mammary tumor cells. During active proliferation cells need genome-maintaining enzymes, such as RecQ helicases,16) to keep DNA fidelity in replication. In this study, we focused on the expression of Bloom helicase (BLM), a RecQ helicase, in MCF-7 cells stimulated by E2 to study the possible roles of BLM in E2-treated cells.

MATERIALS AND METHODS

Chemicals E2 and BPA were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka). Estrogen receptor antagonist ICI182780 was obtained from TOCRIS (Ellisville, MO, U.S.A.).

*To whom correspondence should be addressed. e-mail: furuichi@geneicare.co.jp

© 2007 Pharmaceutical Society of Japan
cholate. The amounts of protein were determined by using the Bradford protein assay kit (BioRad, Hercules, CA, U.S.A.). Solubilized proteins (50 μg) were electrophoresed on 2—15% SDS-polyacrylamide gels (Daiichi Pure Chemicals, Tokyo) at 200 V for 1 h, and were electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, U.S.A.) for 90 min at 6 V. Goat polyclonal antibodies against human BLM (C-18) was applied (Santa Cruz, CA, U.S.A.), and then by rabbit anti-goat immunoglobulin conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark). The membrane was developed by using increased chemiluminescence using ECL Plus Western Blotting Detection Reagents (Amersham Life Science, Buckinghamshire, U.K.). Anti α-actin (ICN, Costa Mesa, CA, U.S.A.) was used to normalize the sample loading.

**Cap Site Cloning** The transcription start sites of *BLM* mRNA were determined by using an oligonucleotide-capping method according to the manufacturer’s instructions. The cap site of the *BLM* gene was amplified by PCR using 5’-replaced testis cDNA library supplied in Cap Site cDNA kit (NIPPONGENE, Tokyo). Briefly, primary PCR products were generated by the 5’ anchor primer supplied in the kit and human *BLM* specific 3’ primer, 5’-TCTCTGTGTTTCTGTCCTGCTG-3’, and then underwent nested PCR using the 5’ anchor primer supplied in the kit and human *BLM* specific 3’ nested primer 5’-TGTGTTGGGTAGAGGTTC-ACCTGAAGG-3’. The second PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.).

**Plasmid Construction** The upstream 2341 bp fragment of *BLM* containing putative promoter region of *BLM* was amplified by using PCR and was inserted into the BglII site of pGL3-Basic vector (Promega, Madison, WI, U.S.A.) upstream of the firefly luciferase gene, which was used as a reporter gene (pGL3-BLM). A series of 5’ deletion mutants were generated from pGL3-BLM by digesting with Sce I and Xho I, and then treated with exounuclease III and mung bean nuclease. *BLM* promoter regions with various lengths were amplified by using PCR with specific primers and were inserted again into pGL3-3 Basic plasmid. pGL3-rand was made by replacing the *BLM* upstream region (~258 to +18) with a random DNA sequence (103 bp) obtained from multi cloning sites of Bluescript II KS+ (Sac I to Kpn I) (Stratagene, La Jolla, CA, U.S.A.). The directions and sizes of the modified gene inserts were confirmed by sequencing.

**Transient Transfection Assay** The *BLM* promoter activity was assessed by firefly luciferase activity using Lumat LB9507 (Berthold, Bad Wildbad, Germany). Cells were subconfluently grown in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped FBS and were transfected with *BLM* promoter-Luc plasmid DNA mixed with FuGENE (Roche Diagnostics, Indianapolis, IN, U.S.A.) (lipofectin method). After 4 h incubation, the medium was changed and the cells were stimulated with E2 or BPA for 48 h. Other cells were treated with IC1182780 for 1 h, and then by E2 or BPA treatments. Luciferase activity and protein concentrations in lysates were measured according to the manufacturer’s instructions (Promega). Each treatment was done in quadruplicates and the data were shown as a mean with standard deviations.

**Statistical Analysis** Statistical analysis was done by using Dunnett’s test.

**RESULTS**

**Stimulation of *BLM* Expression by E2 in MCF-7 Cells**

E2-responsive MCF-7 cells were grown in a medium containing 10⁻¹²—10⁻¹⁰ M E2 for 48 h. Figure 1A shows that *BLM* mRNA levels increase markedly in a dose dependent manner. A time-course analysis indicated that *BLM* mRNA
levels increased significantly even at 6 h after treatment with $10^{-10}$ M E2 (Fig. 1B). Because physiological concentrations of E2 in the blood are between pg/ml and ng/ml ($10^{-10}$, $10^{-8}$ M), the effective concentrations of Fig. 1A ($10^{-11}$ or $10^{-10}$ M) are within or even less the physiological concentration. BLM mRNA was quantified for cells pre-treated with ER antagonist ICI182780 and then by E2. Pre-treatment with ICI182780 eliminated the increase in BLM mRNA expression by E2 to the control level (Fig. 1C) suggesting participation of ER in the stimulation of BLM mRNA. Basal levels of BLM mRNA expression slightly decreased in the presence of ICI182780, but ICI182780 itself did not affect the cell number (data not shown). To study further the participation of ER in the up-regulation of BLM expression, ER negative MDA-MB-231 cells were treated with $10^{-12}$—$10^{-9}$ M E2. BLM mRNA expression did not significantly increase in estrogen receptor-negative MDA-MB-231 cells at 48 h (Fig. 1D). These results support the idea that E2 up-regulates BLM expression by ER.

BP A has a weak estrogenic activity and binds to ER. If ER participates in the regulation of BLM expression, BP A is also expected to affect BLM expression. Figure 2A shows that $10^{-9}$—$10^{-8}$ M BP A stimulates the expression of BLM dose dependently. A time-course analysis showed that $10^{-5}$ M BP A significantly stimulated BLM mRNA expression at 24 h after treatment (Fig. 1B). BP A also stimulates BLM expression, but needs a higher concentration to attain a similar level achieved by the effect of E2. Studies of ER affinity of BP A showed that its ER binding ability is about 1/1000 of E2 ability. Thus, the difference in the effect on BLM expression between E2 and BP A corresponds to the difference in their affinity with ER. BP A failed to increase BLM mRNA expression in ER-negative MDA-MB-231 cells (data not shown). Figure 2C shows that ICI182780 inhibits the BP A effect on BLM expression in MCF-7 cells similarly to the ICI182780 inhibition of the E2 effect on BLM expression. ICI182780 and BP A did not affect the cell number (data not shown). These results also support the idea that ER participates in the induction of BLM expression by BP A.

Response of Cell Cycle-Associated Genes

E2 and BP A stimulate cell proliferation in MCF-7 cells and E2 up-regulates the expression of cell cycle-associated genes in MCF-7 cells. Stimulation of the expression of most cell cycle-associated genes by E2 was obvious at 24 h or later after E2 treatment in this study. Figure 3 indicates that the expression of cell cycle-associated genes cdc6, MCM5, MCM2, Myt1, PCNA and AuroraA is up-regulated at 24 h after treatment with E2 or BP A. These results are consistent with those of Frasor et al. Generally, when E2 directly regulates genes, the expression of target genes is modulated within a couple of hours. From this fact, together with our results for the time profile of gene expression, we assume that up-regulation of cell cycle-associated genes and BLM genes may be a secondary response, not a primary response, by E2 stimulation.

Increase in BLM Protein Expression

Figures 4A, B
show by Western blotting the expression of BLM protein in MCF-7 cells by 72 h-treatment with \(10^{-12} - 10^{-9}\) M E2 or \(10^{-9} - 10^{-5}\) M BPA. E2 or BPA stimulated the expression of BLM protein at higher doses. A similar result was also observed by a 48 h-treatment (data not shown). The BLM protein was not apparently up-regulated in the ER-negative MDA-MB-231 cells (Fig. 4A). The basal BLM protein level of MDA-MB-231 cells was higher than for MCF-7 cells.

**Determination of Transcription Start Sites**

To study the regulation of BLM expression by E2 treatment by identifying cis-regulatory elements in the BLM promoter region,

![Fig. 4. Expression of BLM Protein in MCF-7 Cells and MDA-MB-231 Cells as Assessed by Immunoblotting](image)

(A) MCF-7 cells and MDA-MB-231 cells were treated with E2 at the indicated concentrations for 72 h. (B) MCF-7 cells were treated with BPA at the indicated concentrations for 72 h.

![Fig. 5. (A) Sequence of the Proximal Upstream Region of BLM](image)

The BLM transcription start sites are indicated by asterisks. The cis-acting Sp1 element binding sites are underlined.

![Fig. 5. (B) Promoter Activities Detected in the Upstream Region of BLM](image)

The structure of the plasmids are shown schematically on the left and the luciferase activities are shown in the central. Estrogen response of the BLM upstream region are shown on the right. Reporter plasmids were transfected into MCF-7 cells with or without \(10^{-5}\) M E2.

![Fig. 5. (C) Effects on Promoter Activity of a Random DNA Sequence by Replacing the BLM Upstream Region](image)

Promoter activities detected in −257→+78 BLM upregion and random DNA sequence are shown in the central. Estrogen response of −257→+78 BLM upregion and random DNA sequence are shown on the right. Each point represents mean±standard deviation of quadruplet samples.
we determined the transcription start sites of BLM by using a Cap site cloning method. The 5′-BLM cap site of cDNA amplified by second PCR was cloned into pCR2.1. The DNA sequence that included the capping site of BLM mRNA was determined to identify the transcription start sites of the BLM gene. From DNA sequencing of 20 independent clones, BLM was judged to be transcribed from multiple positions (Fig. 5A, asterisks); the position (+72) was most frequent. We assume that most upstream start sites are at position (+1) (Fig. 5A).

Deletion Analysis of the BLM Promoter  To define the region responsible for the BLM promoter, serial deletion mutants were constructed as described in Materials and Methods. Three cis-acting Sp1 element binding sites clustered around the 300 bp region (Fig. 5A). Although BLM mRNA expression increased by E2-treatment (Figs. 1A, B), suggesting that ER participates in its regulation (Figs. 1C, D), a consensus estrogen responsive element was not found in the upstream 2 kbp region of BLM. Figure 5B shows the comparative promoter activity of a series of 5′-deletion mutants of pGL3-257—169 and the E2 response to these mutants as assessed by luciferase activity in MCF-7 cells. The activities of pGL3(−257—+78) containing three Sp1 elements and pGL3(−192—+78) containing two Sp1 elements were distinct from other mutants. The activity of pGL3(−332—+78) containing three Sp1 elements was less than for pGL3(−257—+78), indicating that region −332 to −257 induces a negative effect on the promoter activity. Once again, pGL3(−192—+78) showed marked promoter activity and pGL3(−176—+78) and pGL3(−116—+78) containing two Sp1 elements had little promoter activity, suggesting the region at −192 to −177 may be indispensable for BLM transcription. Both pGL3(+20—+78) and pGL(+69—+78), which have no Sp1 element, showed minimal or slight promoter activity. Figure 5B in the right shows the ratio of activity of these 5′-deletion mutants of pGL3-257—169 and without E2. Apparent stimulation of the promoter activity by E2 was observed for pGL3(+20—+78) lacking Sp1, suggesting that Sp1 does not directly participate in E2 response.

When the BLM upstream region at −258 to +18 was replaced with a random DNA sequence (103 bp), the BLM transcription ability decreased to the level of the pGL3-basic vector (Fig. 5C), suggesting the BLM upstream sequence (−257 to +18) is essential for BLM promoter activity.

Participation of ER in Stimulation of BLM Promoter Activity  Figure 6 shows that E2 and BPA stimulates BLM promoter activity in dose dependently. BPA needed about 1000 times higher concentration to attain the same levels of E2 effect. Figure 7 shows that ICI182780 eliminated the effect of E2 as assessed by luciferase activity in MCF-7 cells. Basal levels of BLM expression slightly decreased in the presence of ICI182780, but ICI182780 did not affect the number of viable cells (data not shown). These results of E2 and BPA of the effect on promoter activity by BLM support the idea that they up-regulate the expression of BLM protein by increasing the transcription of BLM.

DISCUSSION

We showed that E2 or BPA stimulated the expression of BLM protein by ER. BLM protein relates to DNA replication and recombination or DNA repair and is expressed during late S and G2 phase.23) The expression of BLM protein is up-regulated during a log-phase and decreases at a stationary

![Fig. 6. Promoter Activities Induced by E2 and BPA](image_url)

![Fig. 7. ER Participation in Inducing Promoter Activity](image_url)
phase for various tumor cells.16) The expression of BLM and cell cycle-associated genes was up-regulated by E2 and BPA that responding to increased cell proliferation. The up-regulation was observed at later stages after their treatment, that is, at 24 h (Figs. 1B, 2B), suggesting that the BLM expression may be secondary after stimulation of cell proliferation by E2. On the other hand, the DNA damaging effect by E2 and BPA was observed much earlier than growth stimulation,10) suggesting that the DNA damaging effect is a direct effect by E2 and BPA.

The 2.3 kb BLM upstream region contains no consensus estrogen responsive element, supporting also the speculation that up-regulation of BLM is not a primary but a secondary, response by E2. We believe this study is the first on BLM transcription regulation by E2 or BPA. Our findings suggest that basal BLM expression is sustained by a Sp1 transcriptional control system similar to other housekeeping genes, and that up-regulation of BLM by E2 is not mediated by Sp1.

Finally, we suggest a possible role of up-regulated BLM proteins by E2 or BPA. We previously showed that E2 and BPA cause DNA damage by ER in MCF-7 cells and that BLM protein forms foci at DNA damage sites.10) Both E2 and BPA accelerated ER positive MCF-7 cells growth, and BPA was observed much earlier than growth stimulation,10) suggesting that DNA damaging effect is a direct effect by E2 and BPA.

Acknowledgements We thank Dr. Masanobu Sugimoto, GeneCare Research Institute, for his excellent discussion and help in reviewing this manuscript. This study was supported by the Japan Science and Technology Agency, Japan.

REFERENCES