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High Induction of Poly(ADP-ribose) Polymerase Activity in Bleomycin-resistant HeLa Cells

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Poly(ADP-ribose) polymerase activity was measured in bleomycin (BLM)-resistant HeLa (HeLa-BLM^r) and the parental HeLa cells after BLM treatment. HeLa-BLM^r cells, which had been subcultured in growth medium containing 1 μg/ml of BLM, showed a 3.75-fold higher enzyme activity than did HeLa cells, but this activity was decreased to the same level as that of HeLa cells after 48 h of BLM-free cultivation. When HeLa and HeLa-BLM^r cells after a 48-h cultivation in BLM-free growth medium were treated with BLM, the enzyme activity was induced at a higher level (2.5–3.8 times) in HeLa-BLM^r than in HeLa cells and was inhibited markedly in HeLa-BLM^r and slightly in HeLa cells by nicotinamide, an inhibitor of this enzyme. The BLM-induced cell killing by nicotinamide was highly potentiated (about 18 times) in HeLa-BLM^r as compared to HeLa cells.

Key words: HeLa cells — Bleomycin resistance — Poly(ADP-ribose)polymerase — Nicotinamide

Poly(ADP-ribose) polymerase is a nuclear enzyme of eukaryotic cells which catalyzes the formation of poly(ADP-ribose) covalently linked to histones and nonhistone chromosomal proteins.[1,2] Since this enzyme activity is stimulated by DNA strand breaks induced by DNA-damaging agents such as γ-ray and UV irradiations,[3,4] chemical carcinogens,[5] and antitumor agents including bleomycin (BLM[2]),[6,7] it has been suggested that poly(ADP-ribose) is involved in DNA repair.[8] In previous studies, we found that BLM-resistant HeLa cells carry an elevated DNA repair activity as one of the main mechanisms for BLM resistance, as compared to HeLa cells.[9,10]

In this study, we investigated the poly(ADP-ribose) polymerase activity levels in BLM-resistant HeLa and the parental HeLa cells after BLM treatment and the potentiation of BLM-induced cell killing by nicotinamide, an inhibitor of this polymerase.

MATERIALS AND METHODS

Cells and cell culture HeLa[11] and HeLa-BLM^r[9] were used in this study. These cells were cultured in Eagle's MEM (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% calf serum and 2 m*M* L-glutamine as growth medium in a 5% CO_2 incubator at 37°C, except that growth medium containing 1 μg/ml of BLM was used for HeLa-BLM^r. Subculture was done routinely by dissociation with 0.14% EDTA-0.08% trypsin dissolved in Ca^{2+}- and Mg^{2+}-free PBS.

Assay for poly(ADP-ribose) polymerase activity The assay was done according to the method of Huet and Laval.[7] The cells were plated at a density of 5×10^5 cells per 16 mm well in a 24-well tissue culture plate (Cell Wells, Corning Glass Works, Corning, N.Y.) and incubated at 37°C for 48 h in a 5% CO_2 incubator. After treatment with various concentrations of BLM for 1 h at 37°C in growth medium, cells were washed 3 times, scraped and suspended (2.5×10^5 cells in 100 μl) in a permeabilizing buffer containing 10 m*M* Tris-HCl (pH 7.8), 1 m*M* EDTA, 4 m*M* $MgCl_2$, and 1 m*M* dithiothreitol. [^{3}H]Nicotinamide adenine dinucleotide (NAD, 5×10^4 dpm/well, sp. act. 3.2 Ci/mmol, Amersham Japan, Tokyo) was added to the cell suspension and incubated at 37°C for 30 min. The reaction was terminated by adding 0.2 ml of 20% TCA and then kept at 4°C for 2 h. The acid-precipitable fraction was collected on a membrane filter (cellulose nitrate, Toyo Roshi Kaisha, Ltd., Tokyo) and counted for radioactivity.

DNA synthesis Cells (10^5/16 mm well) were plated in growth medium and incubated at 37°C for 24 h. The culture medium was discarded, and fresh medium or medium containing 50 μg/ml of BLM in the presence or absence of 1 μg/ml of nicotinamide (NA, Wako Pure Chemical Industries, Ltd., Osaka), which is noncytotoxic,[9] was added. After a 1-h incubation at 37°C, the culture medium was aspirated and cells were incubated in growth medium containing 1 μg/ml of NA for a further 24 h. Then, cells were pulse-labeled for 30 min with 0.5 μCi/ml of [^{3}H]thymidine (sp. act. 2.0 Ci/mmol). The incorporation into the TCA-precipitable fraction was determined.

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[2] Abbreviations used: BLM, bleomycin; MEM, minimum essential medium; NA, nicotinamide; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; IC_{50}, 50% inhibitory concentration.

Cell growth assay Cells (10^5/16 mm well) were plated and incubated at 37°C in growth medium with various concentrations of BLM in the presence or absence of 1 μg/ml of NA. After incubation for 4 days at 37°C, grown cells were harvested with EDTA-trypsin mixture and viable cells were counted on a hemocytometer by using the nigrosin exclusion test.[12]

Alkaline sucrose gradient centrifugation To examine the single-strand scission of cellular DNA by BLM, alkaline sucrose gradient centrifugation was employed as described before.[10] Briefly, cells (10^4/well) were cultured in a 96-well tissue culture plate in growth medium at 37°C for 24 h and were labeled with 2.5 μCi/ml [^{3}H]-thymidine for a further 20 h. After that, the culture medium was discarded, and the cells were washed twice with MEM and treated at 37°C for 60 min with 50 and 100 μg/ml of BLM in the presence or absence of 1 μg/ml of nicotinamide. Then, culture medium was replaced with BLM-free fresh growth medium or growth medium containing 1 μg/ml of nicotinamide and cultured for 30 min at 37°C to permit DNA repair. The DNA sedimentation patterns were analyzed in a 5–20% (w/v) alkaline sucrose gradient (pH 12.5) containing 0.1 *N* NaOH, 0.1 *M* NaCl, 1 m*M* EDTA and 0.01% SDS after lysing cells with lysing buffer (10 m*M* Tris-HCl, pH 7.5, 1% SDS, 10 m*M* EDTA and 150 μg/ml of pronase). The ultracentrifugation was carried out at 36,000 rpm for 90 min at 20°C in a Beckman SW50.1 rotor (152,000*g*).

RESULTS AND DISCUSSION

In order to investigate the mechanism of acquired resistance to antitumor agents, we established the BLM-resistant HeLa (HeLa-BLM^r) cells *in vitro* as a model system. Analysis of the resistance mechanism strongly suggested that elevated DNA repair activity, as well as decreased drug accumulation and retention, is involved in the BLM resistance of HeLa-BLM^r cells.[9,10] Recently, the following findings have implied that poly(ADP-ribose) participates in DNA repair; 1) treatment of the cells with DNA-damaging agents markedly reduces the cellular NAD content, and 2) elicits a pronounced increase of poly(ADP-ribose) polymerase activity with a concomitant increase in the intracellular level of poly-(ADP-ribose),[3-7] 3) potent inhibitors of poly(ADP-ribose) polymerase inhibit the rejoining of DNA strand breaks caused by DNA-damaging agents.[8] Therefore, we measured the poly(ADP-ribose) polymerase activity levels in HeLa and HeLa-BLM^r cells.

As shown in Table I, the enzyme activity of HeLa-BLM^r cells subcultured in growth medium containing 1 μg/ml of BLM was 3.75-fold higher than that of HeLa cells. When HeLa-BLM^r cells were cultured in BLM-free medium, however, their enzyme activity was decreased to the same level as that of HeLa cells after 48 h. This result indicates that the high enzyme activity in HeLa-BLM^r cells is being induced by their continuous exposure to BLM.

To examine whether BLM can induce poly(ADP-ribose) polymerase activity, the cells were treated with BLM. When cells were incubated in growth medium containing 10 μg/ml of BLM, the polymerase activity was increased, reaching a plateau at 30 min in both cells, but it was 3.8-times higher in HeLa-BLM^r than in HeLa cells (Fig. 1). When cells were treated with various concentrations of BLM for 60 min, the enzyme activity increased with BLM concentration up to 5 μg/ml and reached a plateau (Fig. 2). HeLa-BLM^r cells showed 2.5-times higher enzyme activity than did HeLa cells. The cell survival at the time of polymerase assay was 81.3%, 75.9%, 70.3% and 65.6% in HeLa and 95.2%, 93.0%, 87.5%, 82.7% in HeLa-BLM^r, respectively, for 60-min treatment with BLM at concentrations of 5, 10, 50 and 100 μg/ml. The efficient induction of the polymerase activity by BLM was inhibited markedly in HeLa-BLM^r cells and slightly in HeLa cells by simultaneous treatment with 1 μg/ml of NA, an inhibitor of this enzyme (Figs. 1 and 2). This concentration of NA was chosen as a noncytotoxic level giving maximal potentiation of the cytocidal effect of BLM.[9]

Table I. Poly(ADP-ribose) Polymerase Activity Level in HeLa and HeLa-BLM^r Cells

Cells	[^{3}H]NAD incorporated per 2×10^5 cells (dpm±SD)	Ratio
HeLa	400.9±55.2	1
HeLa-BLM^r		
in maintenance medium containing BLM (1 μg/ml)	1,506.7±31.2	3.75
after 48-h cultivation in BLM-free growth medium	400.1±24.5	1

The enzyme activities of HeLa-BLM^r cells subcultured in maintenance medium containing 1 μg/ml of BLM, or cultured in BLM-free medium for 48 h, were measured and compared to that of HaLa cells.

Since we have found that NA inhibits the induction of poly(ADP-ribose) polymerase activity more efficiently in HeLa-BLM^r than in HeLa cells, it is expected that the BLM-induced cell killing is potentiated by NA in HeLa-BLM^r cells. Thus, we examined the combined effect of BLM and NA on DNA synthesis and cell survival of HeLa-BLM^r cells in comparison with those of HeLa cells. As shown in Table II, the inhibition of DNA synthesis in HeLa-BLM^r cells by BLM was greatly stimulated with the combined use of NA. However, the combination of BLM with NA resulted in little stimulation of the inhibition of DNA synthesis in HeLa cells because of the strong inhibitory action of BLM. The former study with alkaline sucrose gradient centrifugation revealed that DNA strand breakage caused by treatment with 50 and 100 μg/ml of BLM for 60 min recovered markedly in HeLa-BLM^r but not in HeLa cells during a 30-min incubation at 37°C in BLM-free medium (Fig. 3 A, B, D, E). This efficient recovery of damaged DNA in HeLa-BLM^r was clearly inhibited by NA and the extent of DNA strand breaks remained similar to that

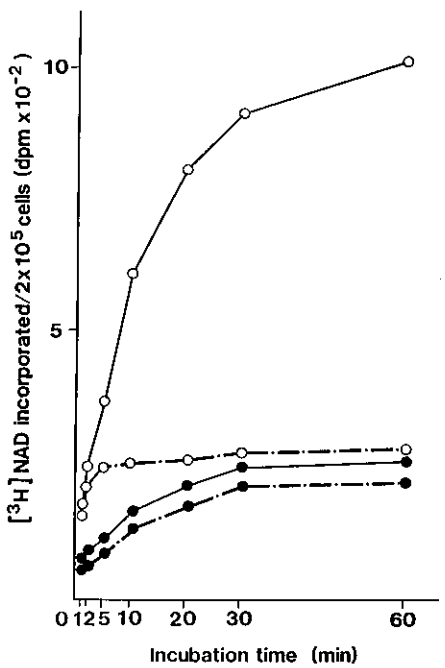

Fig. 1. Time course of poly(ADP-ribose) polymerase activity after treatment with BLM (10 μg/ml) in the presence or absence of nicotinamide (1 μg/ml). ●——●, HeLa treated with BLM; ●—·—●, HeLa treated with BLM and NA; ○——○, HeLa-BLM^r treated with BLM; ○—·—○, HeLa-BLM^r treated with BLM and NA.

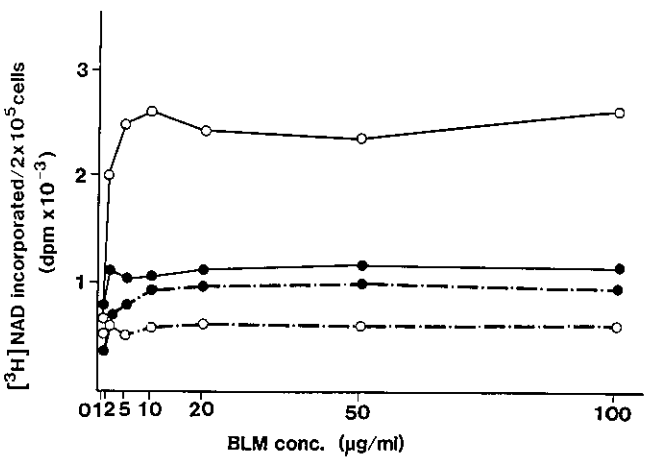

Fig. 2. Poly(ADP-ribose) polymerase activity after treatment with various concentrations of BLM for 1 h in the presence or absence of nicotinamide (1 μg/ml). ●——●, HeLa treated with BLM: ●—·—●, HeLa treated with BLM^r and NA; ○——○, HeLa-BLM^r treated with BLM; ○—·—○, HeLa-BLM^r treated with BLM and NA.

Table II. Inhibition of DNA Synthesis in HeLa and HeLa-BLM^r Cells by BLM in the Presence or Absence of Nicotinamide (NA)

Cells	Drug concentration (μg/ml)	[^{3}H]thymidine incorporation (dpm±SD)	%Inhibition
HeLa	Control	12,659±3,107	—
	BLM (50)	3,920±881	69.0
	BLM (50) + NA (1)	1,607±482	87.3
HeLa-BLM^r	Control	11,955±778	—
	BLM (50)	11,078±1,183	7.3
	BLM (50) + NA (1)	2,495±819	79.1

Cells (10^5/16 mm well) were plated and incubated at 37°C for 24 h, and then treated for 1 h with BLM in the presence or absence of NA at the concentrations indicated. After that, cells were incubated in growth medium containing NA for a further 24 h and DNA synthesis was determined.

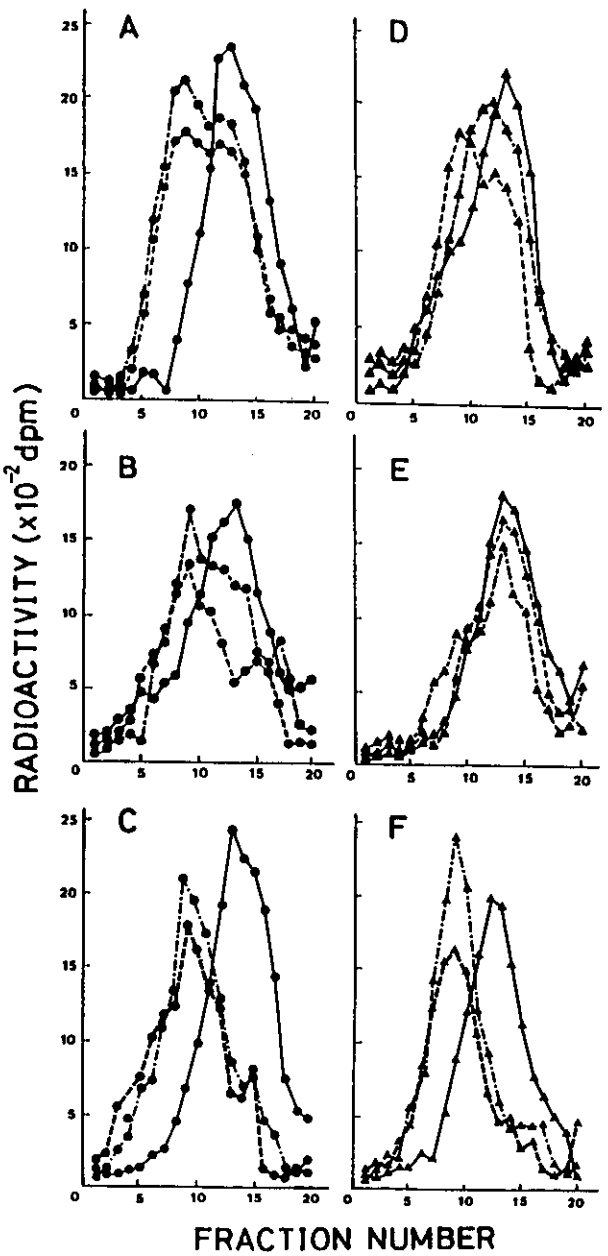

Fig. 3. DNA sedimentation patterns of HeLa and HeLa-BLM^r untreated or treated with BLM in the presence or absence of nicotinamide. (A–C) HeLa: ●——●, untreated; ●—·—●, treated with 50 μg/ml of BLM for 60 min; ●----●, treated with 100 μg/ml of BLM for 60 min. (D–F) HeLa-BLM^r : ▲——▲, untreated; ▲—·—▲, treated with 50 μg/ml of BLM for 60 min; ▲----▲, treated with 100 μg/ml of BLM for 60 min. (A, D) Just after BLM treatment, (B, E) BLM-free incubation for 30 min at 37°C after BLM treatment, (C, F) incubation with 1 μg/ml of nicotinamide for 30 min at 37°C after combined treatment with BLM and nicotinamide. BLM-untreated cells were treated with nicotinamide alone.

of HeLa cells (Fig. 3 C, F). When HeLa and HeLa-BLM^r cells were plated and incubated at 37°C for 4 days in growth medium containing various concentrations of BLM with or without 1 μg/ml of NA, the IC_{50} was 0.18 μg/ml in HeLa and 0.4 μg/ml in HeLa-BLM^r with NA, and 0.25 μg/ml in HeLa and 7 μg/ml in HeLa-BLM^r without NA (Fig. 4). Thus, the cell killing by BLM was potentiated with NA about 18 times in HeLa-BLM^r cells and only 1.4 times in HeLa cells, in terms of IC_{50}.

Similar results concerning the potentiation of BLM-induced cell killing by inhibitors of poly(ADP-ribose) polymerase such as benzamide, 3-aminobenzamide and 3-methoxybenzamide have already been reported in HeLa[13] and CHO cells[7] *in vitro*, and in Ehrlich ascites carcinoma *in vivo*.[13] In the present study, however, we demonstrated that BLM-resistant HeLa cells induce the poly(ADP-ribose) polymerase activity at a higher level

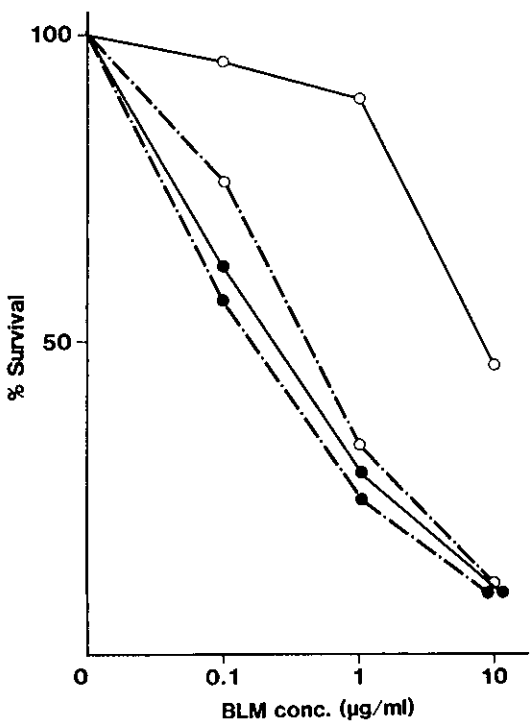

Fig. 4. Combined effect of BLM with nicotinamide on cell killing. Cells (10^5/16 mm well) were plated and incubated at 37°C in growth medium with various concentrations of BLM in the presence or absence of 1 μg/ml of nicotinamide. After incubation for 4 days at 37°C, cell survival was determined. The cell number of HeLa-BLM^r after cultivation for 4 days in growth medium without BLM was almost the same as that of HeLa cells. ●——●, HeLa treated with BLM; ●—·—●, HeLa treated with BLM and NA; ○——○, HeLa-BLM^r treated with BLM; ○—·—○, HeLa-BLM^r treated with BLM and NA.

than do the parental HeLa cells after BLM treatment, and the elevated enzyme activity is markedly inhibited by NA. Furthermore, the BLM-induced cell killing was highly potentiated by NA in HeLa-BLM^r as compared to HeLa cells. This finding was supported by the result of alkaline sedimentation analysis demonstrating that DNA strand-rejoining after BLM and NA treatment in HeLa-BLM^r is clearly inhibited by NA. In addition, we obtained two interesting findings in the previous[9] and present studies. One is that the stability of BLM-resistant phenotype of HeLa-BLM^r was maintained even in cultivation with BLM-free medium for 30 passages. The other is that the high level of poly(ADP-ribose) polymerase activity of HeLa-BLM^r subcultured in maintenance medium containing 1 μg/ml of BLM was decreased to the same level as that of HeLa cells by cultivation with BLM-free medium for 48 h. These findings may suggest that BLM resistance of HeLa-BLM^r is expressed by elevation of DNA repair activity subsequent to rapid and high induction of poly(ADP-ribose) polymerase activity, although decreased drug accumulation and retention are also important mechanisms. Recently, a similar situation has been proposed for peplomycin (a BLM derivative)-supersensitive Chinese hamster lung cells.[14] In these cells, the increase of poly(ADP-ribose) synthesis following peplomycin treatment was significantly reduced as compared with the parental cells, but nevertheless the same degree of peplomycin-induced DNA strand scission was observed in both cell lines. These results also suggest that decreased poly(ADP-ribose) synthesis is related to BLM hypersensitivity and some deficiency in DNA repair.

BLM was previously reported to inhibit the activity of DNA ligase, one of the repair enzymes.[15] Also, it has recently been reported that poly(ADP-ribose) activates DNA ligase activity, especially DNA ligase II, the presumed repair enzyme.[16, 17] Together with these results, our findings may imply that DNA ligase is strongly involved in the DNA repair mechanism or BLM resistance in HeLa-BLM^r cells. Overcoming cellular resistance to antitumor agents is currently an important and attractive topic in cancer chemotherapy. This study provides a useful clue to potentially overcoming acquired resistance to BLM.

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