Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 55-60

BLEOMYCIN-RESISTANT CELLS CONTAIN INCREASED BLEOMYCIN-HYDROLASE ACTIVITIES

Shin-ichi Akiyama[1], Kiyonobu Ikezaki[1], Hiroshi Kuramochi[2], Katsutoshi Takahashi[2] and Michihiko Kuwano[1]

Department of Biochemistry[1], Oita Medical School, Oita 879-56, Japan and Research Laboratories[2], Pharmaceutical Division, Nippon Kayaku Co., Kita-Ku, Tokyo, Japan

Received May 26, 1981

SUMMARY

We have previously isolated mutants relatively resistant to bleomycin, a potent anticancer agent, from mutagenized Chinese hamster ovary (CHO) cells that are more sensitive to the antibiotic (BLM^S). Three bleomycin-resistant mutants (BLM^R-1, -3, -4) showed higher bleomycin-inactivating activity than the parental CHO cell (BLM^S), using an assay for DNA degradation to measure active drug. A hybrid clone, CBH-1, derived from a BLM^R x BLM^S cell fusion, was as resistant to bleomycin-A2 as was BLM^R-1; it also contained increased bleomycin-inactivating activity comparable to that in BLM^R-1 cells.

Bleomycin-hydrolase activity of these cell lines was compared in cell-free extracts by assaying the conversion of bleomycin into its deamidated form. The specific activities of bleomycin hydrolase in the BLM^R clones were shown to be 2-3 fold higher than that in CHO cells.

The bleomycins, potent anticancer agents, were first identified by Umezawa and his colleagues in culture filtrates of Streptomyces verticillus (1). They have been shown to inhibit preferentially the growth of squamous and small cell-type lung cancer, testicular cancer, cervical cancer, hepatoma AH-66, HeLa cells in culture, and sarcoma 180 cells in mice (2, 3). Although DNA strand breaks (4) and chromosomal aberrations (5) are somehow involved, the mechanism of cell death is not fully understood. We have therefore isolated bleomycin-resistant (BLM^R) mutants from Chinese hamster ovary (CHO) cells in order to study the cellular sites which can be modified to affect bleomycin action. We first studied the differential sensitivities of these

0006-291X/81/130055-06$01.00/0

cell lines to bleomycin (6). Our previous study indicated that BLM^R-1 cells contained higher bleomycin-inactivating activities than did parental CHO cells (6). In the present study, we examine the bleomycin-inactivating activity more extensively.

MATERIALS AND METHODS

Cell cultures and cell lines: CHO cells were routinely grown at 37°C in minimal essential medium (MEM)(Nissui Seiyaku Co., Tokyo) containing 10% newborn calf serum (Flow Lab. Stanmore, N. S. W. Australia), 1 mg/ml of bactopeptone, 0.292 mg/ml of glutamine, and 100 units/ml of penicillin-G (Meiji Seiyaku Co., Tokyo).

Cell survival by colony formation: Dose-response curves of CHO cells and sublines were determined by plating 300 cells in replicate 60 mm dishes with varying drug doses. After incubation at 37°C for 7 days, the dishes were stained with Giemsa and colonies were counted.

Bleomycin inactivation in extracts: Labeled DNA (7055 dpm/μg DNA) was isolated from E. coli strain W3110, according to the procedure of Marmur (7). Chain breakage in [^{3}H]thymidine-labeled DNA was assessed by the decrease in acid-insoluble radioactive DNA. A standard reaction mixture contained, in a volume of 500 μl: 7.0 μg [^{3}H]DNA from E. coli, 40 mM Tris HCl (pH 7.8), 10 mM 2-mercaptoethanol, 60 mM KCl, 6 mM $MgCl_2$, and 1 mg bovine serum albumin. One hundred μl of 10 μg/ml bleomycin-A2 and 100 μl of cell extract of CHO or BLM^R cells at indicated concentration were mixed thoroughly and incubated for three hours at 37°C. Then the standard reaction mixture was added and incubated for 30 min more at 37°C. The reaction was stopped with 0.5 ml of cold trichloroacetic acid. The acid-insoluble fraction was collected on a glass fiber filter and counted in a liquid scintillation counter as described previously (8). Protein concentrations of cell extracts were determined by the method of Lowry et al. (9).

Determination of BLM-hydrolase activities: BLM-hydrolase activities were determined by a high-pressure liquid chromatographic method (10). CHO cells were homogenized with 1/15 M phosphate buffer (pH 7.2) in an Potter-Elvejhem homogenizer, and centrifuged at 105,000g for 60 min. The supernatants were collected and dialysed overnight against 1/15 M phosphate buffer (pH 7.2). These dialysates were used as enzyme solutions. For the reaction mixture, 0.4 ml of a 1 gm/l solution of bleomycin-B2 (-Cu) was added to 0.1 ml of the enzyme solution. These were then incubated for 2 hr at 37°C, and 0.4 ml of 10% trichloroacetic acid was then added to terminate the reaction. Mixtures were centrifuged at 3,000 rpm for 10 min and the supernatants were neutralized with 3.2 N KOH. Intact and deamidated-BLM in the solution were changed to the copper chelated form by the addition of 2 - 3 mg of cupric carbonate (basic). Then 5 μl of each sample was injected into the high-pressure liquid chromatography column (HPLC)(Hitachi, type 638) to measure each peak area. Conditions of HPLC analysis were as follows. Column, 4.0 x 250 mm, Lichrosorb SI60, particle size = 5 μm (Merck). Mobile phase, methanol : acetonitrile : 20% ammonium acetate in water : acetic acid = 620 : 380 : 100 : 0.5.

Chemicals: Pure bleomycin-A2 was kindly donated by Nippon Kayaku Co., Tokyo.

Table 1. Bleomycin resistance of parental and hybrid cell lines

Cell lines	Derivation	D_{10} values (μg/ml)[4]
CHO	Chinese hamster ovary cell, blm^S [1] (ATCC)	3.0
BLM^R-1	CHO, blm^R (EMS)[2]	11.0
BLM^R-3	CHO, blm^R (EMS)	10.0
BLM^R-4	CHO, blm^R (SP)	15.0
COT-1	CHO, blm^S, oua^R (EMS), $6tg^R$ (SP)	3.0
CBH-1	COT-1 x BLM^R-1 (hybrid clone)[3]	11.5
CWH-1	COT-1 x CHO (hybrid clone)	4.6

[1]Abbreviation for genotypes - blm^S: bleomycin-sensitive, blm^R: bleomycin-resistance, oua^R: ouabain resistance, $6tg^R$: 6-thioguanine-resistance.

[2]EMS: ethylmethanesulfonate mutagenesis, SP: spontaneous mutagenesis.

[3]Representative hybrid clones CBH-1 and CWH-1 from respective crosses were used.

[4]The D_{10} value represents the concentration of bleomycin which reduces plating efficiency of a cell line to 10% of its original value.

RESULTS AND DISCUSSION

The dose response to bleomycin-A2 was examined for CHO, BLM^R sublines and hybrid clones, CBH-1 (BLM^R x BLM^S) and CWH-1 (BLM^S x BLM^S). D_{10} values are listed in Table 1. The D_{10} value represents the concentration of bleomycin which reduces plating efficiency of a cell line to 10% of its original value. D_{10} values of BLM^R-1, -3, -4 and CBH-1 were 3 - 4 fold higher than those of CHO, COT-1 and CWH-1.

We previously showed that enhanced level of BLM-inactivating activity in a BLM^R clone can be correlated with the drug-resistant phenotype in BLM^R-1 mutant (6). We compared the cellular levels of the inactivating activity of other BLM^R cell lines or hybrid cell lines with that of the parental CHO cells. The cellular level of inactivating activity in cell extracts was tested by assaying for DNA chain breakage by bleomycin-A2 *in vitro*. Table 2 indicates that bleomycin-inactivating activities in BLM^R cell lines (BLM^R-1, -3, -4) and a hybrid cell line, CBH-1 (COT-1 x BLM^R-1), were about two-fold higher than that of CHO cells. The hybrid clone, CWH-1, derived from a hybrid of

Table 2. Bleomycin-inactivating activity and bleomycin-hydrolase activity of CHO, BLM^R, and hybrid cell lines.

Cell lines	[^{3}H]DNA retained on filter[1] (DPM)	Specific activity of BLM-hydrolase[2] (μg deamidated BLM-B2/mg protein/min)
CHO	8714 (46)[3]	1.09
BLM^R-1	12202 (72)	2.58
BLM^R-3	10637 (60)	1.94
BLM^R-4	15087 (93)	3.23
CBH-1	12403 (72)	N. T.[4]
CWH-1	9465 (52)	N. T.

[1]One hundred μl of bleomycin-A2 (10 μg/ml) was preincubated with same volumes of cell extracts (protein concentration: 8 mg/ml) from each cell lines, then incubated with [^{3}H]DNA. The radio activity (dpm/assay) in the absence of bleomycin-A2 and cell extracts was 16549 and the value dropped to 1940 when treated with bleomycin-A2 alone. The data are given as the mean of two experiments.

[2]Bleomycin-B2 was used as the substrate for determining BLM-hydrolase activity. The intact bleomycin and the deamidated bleomycin in the reaction mixture were both measured by a high-pressure liquid chromatographycal method as shown in Fig. 1.

[3]Percent inactivation of bleomycin-A2. From each value, 1940 was subtracted and the value was divided by 14609 (100%).

[4]N. T., not tested.

both bleomycin-sensitive parents contained a slightly higher inactivating activity than that of CHO cells.

Bleomycin-inactivating activity was not dialysable, and cell lysates of CHO or BLM^R cells filtered through PM 10 retaining molecules more than 10,000 daltons (Diaflo ultrafiltration membrane, Amicon Corp., Ireland) had no detectable inactivating activity. On the other hand, the inactivating activities of both parental and BLM^R cells were also heat-labile. Activities were completely inactivated when heated at 100°C for 10 min.

We then assayed for the activity of the bleomycin-inactivating enzyme, BLM-hydrolase (10). The product, deamidated BLM, formed by the BLM-hydrolase, and intact BLM were separated using high-pressure liquid chromatographic method as in Fig. 1. The BLM-hydrolase activities of BLM^R cell lines were 2 - 3 fold higher than that of CHO cells (Table 2).

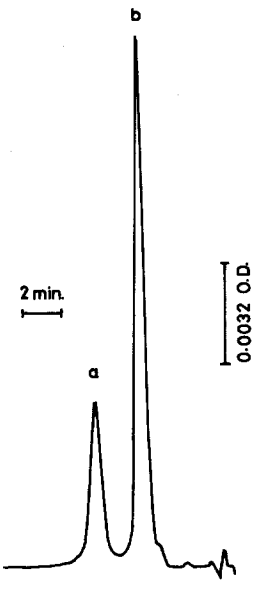

Fig. 1. Separation of intact and deamidated BLM-B2 by HPLC method. The deamidated BLM-B2 (a) formed by BLM-hydrolase in cell extracts from BLM^R-1 cells (0.933 mg/ml) and intact bleomycin-B2 (b) were separated.

Our previous study (11) suggested a membrane barrier to bleomycin in cultured Chinese hamster cells. However, using radioactive bleomycin-A2, we found no decreased permeation of bleomycin in BLM^R sublines compared with CHO cells (data not shown). Instead, higher levels of bleomycin-inactivating activity in cell extracts of BLM^R sublines compared to CHO cells seem to account for the resistant phenotype of BLM^R sublines and CBH-1 hybrid cells.

Yoshioka and colleagues (10) reported that there are two types of inactivation activities of BLM in animal cells, BLM-hydrolase and low molecular components. Lysates filtered through Diaflo membrane (PM 10) had no low molecular components which inactivate bleomycin, but showed the correlation between cell resistance to the drug and BLM-hydrolase content. The same correlation has been reported in squamous cell carcinoma and sarcoma induced by 3-methylcholanthrene (12), as well as for AH66 and AH66F hepatoma cells (13). Our study thus shows that the cellular sensitivity to the drug is closely correlated with the level of BLM-hydrolase.

The mechanism of augmented BLM-hydrolase activity remains unclear. The enzyme structure or translation yield from mRNA could be modified. Alternatively, the number of gene copies could be increased as in the case of Schimke and his colleagues (14, 15). In the latter case, one might expect that continued selection might produce very much higher levels of drug resistance, corresponding to the methotrexate-resistant cell lines with amplified genes coding for the enzyme.

ACKNOWLEDGEMENTS

We greatly thank Dr. D. Schlessinger (Washington University) for critical reading of this manuscript. This work is supported by the Naito Research Fund for 1979 and also by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. Umezawa, H., Maeda, K., Takeuchi, T., and Okami, K. (1966) J. Antibiot. 25, 409-420.
2. Carter, S. K. (1976) J. Natl. Cancer Inst. 57, 235-224.
3. Sartiano, G. L., Coetzee, M. L., Klein, K., and Ove, P. (1977) J. Natl. Cancer Inst. 58, 1357-1364.
4. Suzuki, H., Nagai, K., Akutsu, E., Yamaki, H., Tanaka, N., and Umezawa, H. (1970) J. Antibiot. 23, 473-480.
5. Ohama, K., and Kadotani, T. (1970) Jap. J. Human Genet. 14, 293-297.
6. Akiyama, S., and Kuwano, M. (1981) J. Cell. Physiol. 107, 147-153.
7. Marmur, J. (1961) J. Mol. Biol. 3, 208-218.
8. Akiyama, S., Hidaka, K., Komiyama, S., and Kuwano, M. (1979) Cancer Res. 39, 5150-5154.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
10. Yoshioka, O., Amano, N., Takahashi, K., Matsuda, A., and Umezawa, H. (1978) Bleomycin, pp. 35-56, Academic Press, New York.
11. Akiyama, S., Tabuki, T., Kaneko, M., Komiyama, S., and Kuwano, M. (1980) Antimicrob. Agents Chemother. 18, 226-230.
12. Umezawa, H., Takeuchi, T., and Komi, T. (1972) J. Antibiot. 25, 409-420.
13. Miyaki, M., Ono, T., Hori, S., and Umezawa, H. (1975) Cancer Res. 35, 2015-2019.
14. Kaufman, R. J., Brown, P. C., and Schimke, R. T. (1979) Proc. Natl. Acad. Sci. USA 76, 5669-5673.
15. Dolnick, B. J., Berenson, R. J, Bertino, J. R., Kaufman, R. J., Nunberg, J. H., and Schimke, R. T. (1979) J. Cell. Biol. 83, 394-402.