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Induction of Bleomycin Resistance in a Human Oral Squamous Carcinoma Cell Line and Characterisation of Bleomycin-resistant and -sensitive Clones

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We examined the change of sensitivity to antitumour agents by repeated treatment with bleomycin (BLM) using two oral squamous carcinoma cell lines, SCCTF and SCCKN. SCCTF exhibited minimal sensitivity to BLM and strong heterogeneity in BLM sensitivity, whereas SCCKN was highly sensitive to BLM and showed weak heterogeneity. When SCCTF was treated continuously with low-dose BLM (0.1 μg/ml) but not intermittently with high-dose BLM (1 μg/ml), the BLM sensitivity was rapidly decreased to acquire drug resistance. On the other hand, SCCKN was completely killed by the same treatments. To investigate the mechanism of induction of resistance in SCCTF, BLM-sensitive and -resistant clones, TF-S and TF-R, were isolated and analysed. Consequently, TF-R showed decreased cellular accumulation and retention of BLM, increased BLM hydrolase activity and elevated DNA repair activity concomitant with increased poly(ADP-ribose) polymerase activity as compared with TF-S. Therefore, it was suggested that antitumour drug-resistant clones were selectively grown from heterogeneous tumour cell population.

Key words: oral squamous carcinoma cells, heterogeneity, bleomycin resistance, bleomycin-resistant and -sensitive clones.

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INTRODUCTION

THE VARIETY of combination chemotherapy has been designed to improve the efficiency of antitumour agents. However, recurrent or metastatic tumours occurring after initial treatment with irradiation and chemotherapy are still refractory to combination chemotherapy as secondary treatment. Thus, antitumour drug resistance has become a serious problem in cancer chemotherapy [1, 2].

In order to investigate the mechanism for the acquisition of antitumour drug resistance in oral cancer, we have studied bleomycin (BLM) resistance in cultured human carcinoma cells and the factors involved in resistance [3–7]. As a consequence, especially in the clinial setting, heterogeneity of the tumour cell population was suggested to be one of the important factors by which the tumour becomes resistant to antitumour agents. Among tumour cell populations with a strong heterogeneity, cell clones exist which have advantageous mechanisms that can evade chemotherapeutic pressure.

In the present study, we report the induction of BLM resistance in a human oral squamous carcinoma cell line by repeated treatment with low doses of BLM and discuss the induction mechanism by characterising BLM-sensitive and -resistant clones isolated from the cell line.

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MATERIALS AND METHODS

Cells and cell culture

Human squamous carcinoma cell lines, SCCKN and SCCTF, derived from tongue carcinomas, were used in this study [7]. SCCKN is highly sensitive and SCCTF is minimally sensitive to BLM. Both cell lines demonstrated a similar growth rate and produced moderately differentiated squamous cell carcinoma by heterotransplantation into nude mice. These cells were cultured in Dulbecco's modified Eagle's medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah, U.S.A.) and 4 mM L-glutamine as growth medium in a 5% carbon dioxide incubator at 37°C. They were subcultured routinely by dissociation with

0.14% ethylene diamine tetraacetic acid (EDTA) and 0.08% trypsin dissolved in Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline [PBS(−)].

Drugs

The following antitumour agents were used: bleomycin (BLM), BLM-B_2 for BLM hydrolase assay, peplomycin (PEP) and cisdiammine dichloroplatinum (CDDP) obtained from Nippon Kayaku Co. (Tokyo, Japan); doxorubicin (DOX, Adria Laboratories, Columbus, Ohio, U.S.A.), mitomycin C (MMC) and 5-fluorouracil (5-FU) from Kyowa Hakko Kogyo Co. (Tokyo, Japan); methotrexate (MTX) from Lederle Laboratories (Pearl River, New York, U.S.A.); and vincristine sulfate (VCR) from Shionogi and Company (Osaka, Japan). These agents were dissolved in 0.85% saline before use.

In vitro *drug sensitivity assay*

Drug sensitivity assays were described elsewhere [7]. Briefly, exponentially growing cells (5×10^4/well) were plated in a 24-well tissue culture plate (Cell Wells®, Corning Glass Works, Corning, New York, U.S.A.) and incubated in growth medium at 37°C for 24 h. Culture medium was discarded and replaced with fresh medium containing drugs at various concentrations. Three days after incubation at 37°C, viable cells were counted on a haemocytometer with the nigrosin exclusion test [8], and the percentage of survival was determined.

Cell cloning

A single cell suspension in growth medium was added at a density of 10^3 cells per 90 mm plastic dish. After incubation at 37°C for 3 weeks, growth medium was discarded and cells were cloned from well-isolated colonies formed on a dish surface by covering with tiny filter paper soaked in EDTA and trypsin mixture and dissociating the cells.

Assay for cellular accumulation and retention of [^{3}H]BLM

The cell-associated radioactivity after exposure with CuII[^{3}H]BLM A_2 (Du Pont NEN Research Products, Boston, Massachusetts, U.S.A.; specific activity 63.6 Ci/mmol) was measured for the drug accumulation assay, and the residual radioactivity of the cells prelabelled with [^{3}H]BLM at 37°C for 60 min was determined after incubation in [^{3}H]BLM-free medium for the drug retention assay [7]. At intervals, the culture medium was aspirated and the cells were washed quickly three times with cold PBS(−) and then dissolved in 0.1 ml of 1 N NaOH. After being neutralised with 1 N HCl, the sample was counted for radioactivity.

Assay for BLM hydrolase activity

BLM hydrolase activity was assayed by measuring the deamidated BLM-B_2 formed after incubation of BLM-B_2 with the supernatant of a cell homogenate by high performance liquid chromatography (HPLC, model 655, Hitachi Ltd., Tokyo, Japan) [7]. Briefly, a cell suspension containing $3–5 \times 10^6$ cells/ml in 1/15 M PBS (pH 7.2) was homogenised with a Teflon-glass homogeniser in an ice bath. The sample was ultracentrifuged at 105 000 *g* for 60 min at 4°C. The supernatant was dialysed at 4°C against about 300 volume of PBS overnight to remove the low molecular weight inhibitor [9] and was used as the enzyme sample.

Alkaline elution technique

DNA strand breakage and DNA repair activity were measured with the alkaline elution technique by a modification of the method of Kohn *et al.* [10]. Briefly, cultured cells (10^6) labelled with [^{14}C]thymidine were treated with BLM at 37°C for 60 min and collected on a polycarbonate filter (pore size 1.0 μm, Nucleopore Corporation, Pleasanton, California, U.S.A.). After treatment of the cells with a cell lysis solution (0.2% sodium lauryl sulfate, 2 M NaCl, 0.04 M Na_2EDTA, pH 10.0) and proteinase K (0.5 mg/ml), single-stranded DNA was eluted from the filter by pumping 0.02 M EDTA solution adjusted to pH 12.1 with tetrapropylammonium hydroxide. The extent of DNA single strand breakage was determined by calculating the percentage of the radioactivity remaining on the filter. DNA repair activity was examined by the recovery of DNA strand breakage after incubation with BLM-free medium at 37°C for various times subsequent to BLM treatment.

Assay for poly(ADP-ribose) polymerase activity

The assay was performed according to the method of Huet and Laval [11]. The cells were plated at a density of 5×10^5 cells per 16-mm well in a 24-well tissue culture plate (Corning) and incubated at 37°C for 48 h in a 5% carbon dioxide incubator. After treatment with various concentrations of BLM for 1 h at 37°C in growth medium, cells were washed three times with PBS(−), scraped and suspended at a density of 2.5×10^5 cells in 100 μl of a permeabilising buffer containing 10 mM Tris-HCl (pH 7.8), 1 mM Na_2EDTA, 4 mM $MgCl_2$ and 1 mM dithiothreitol. [^{3}H]Nicotinamide adenine dinucleo-

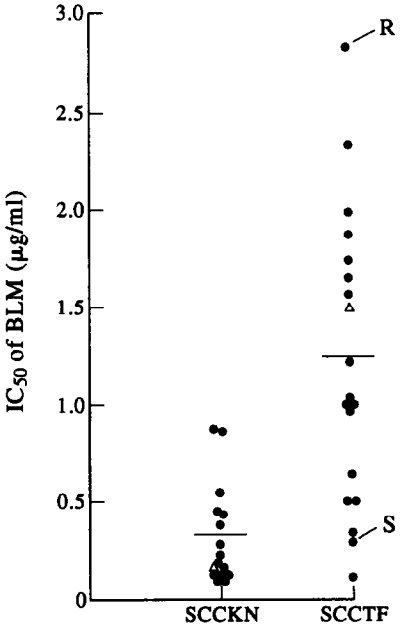

Fig. 1. BLM sensitivity of cell clones isolated from SCCKN and SCCTF. Bar indicates the mean of IC_{50}s. △: IC_{50} of parental cell, S: TF-S isolated as a BLM-sensitive clone, R: TF-R isolated as a BLM-resistant clone.

tide (0.03 μCi/reaction; specific activity 3.2 Ci/mmol; Amersham Japan, Tokyo, Japan) was added to the cell suspension and incubated at 37°C for 60 min. The reaction was terminated by addition of 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, Tokyo, Japan) and counted for radioactivity.

RESULTS

Tumour cell heterogeneity in BLM sensitivity and induction of BLM resistance

Fifteen clones isolated from highly BLM-sensitive SCCKN and 18 clones from minimally BLM-sensitive SCCTF were examined for the sensitivity to BLM. As shown in Fig. 1, IC_{50}s of cell clones in SCCKN were distributed in narrow range (0.08–0.95 μg/ml), whereas those in SCCTF were in wide range (0.15–2.85 μg/ml). Thus, SCCTF was found to have a strong heterogeneity in BLM sensitivity.

For these cell lines, two kinds of protocols in BLM treatment were designed: (a) the cells were treated continuously with low doses of BLM (0.1 μg/ml) for 1 week, followed by incubation without BLM for a further week, and (b) the cells were treated for 2 h once a day with high doses of BLM (1 μg/ml) for 6 successive days, followed by incubation without BLM for a further week. Two courses of method (a) resulted in a significantly decreased sensitivity to BLM in SCCTF (untreated 1.53 ± 0.13 μg/ml, treated 3.54 ± 0.94 μg/ml; $P < 0.01$), whereas no change of BLM sensitivity was detected in method (b). The same treatments caused total cell killing in SCCKN. Furthermore, SCCTF treated with BLM according to method (a) demonstrated decreased sensitivity to peplomycin (3.3-fold), mitomycin C (1.6-fold), cisdiammine dichloroplatinum (3.1-fold) and methotrexate (1.9-fold) (Table 1). These results indicated that continuous treatment with low doses of BLM for the cells with a strong heterogeneity readily induced resistance to antitumour agents.

Isolation of BLM-sensitive and -resistant clones from SCCTF

To investigate the mechanism of induction of BLM resistance in SCCTF, BLM-sensitive and -resistant clones, TF-S and TF-R, were isolated from among 18 clones of SCCTF (Fig. 1). Both cell clones exhibited similar polygonal shapes; the population doubling time was 24.0 h in TF-S and 20.8 h in TF-R. The IC_{50} to BLM of TF-S and TF-R were 0.30 and 2.85 μg/ml, respectively. Thus, TF-R was 9.5-fold more resistant to BLM than TF-S (Fig. 1). In addition, both cell lines produced moderately differentiated squamous cell carcinoma histologically by inoculation into nude mice, but nude mouse tumours by TF-R were significantly less sensitive to BLM than those by TF-S (data not shown).

Analysis of resistance mechanism in TF-R

Cellular accumulation and retention of [^{3}H]BLM, BLM hydrolase activity, DNA repair activity after BLM treatment and poly(ADP-ribose) polymerase activity were examined in comparison with TF-R and TF-S. The cellular accumulation and retention times of [^{3}H]BLM were approximately 50 and 20% lower, respectively, in TF-R than in TF-S (Fig. 2). BLM hydrolase activity in cell extracts measured by HPLC demonstrated a time-dependent increase linearly up to 120 min, and was 2.1-fold greater in TF-R than in TF-S (Fig. 3). DNA single-strand scission caused by treatment with 100 μg/ml of BLM for 60 min occurred about 5-fold less in TF-R than in TF-S (Fig. 4). When incubated at 37°C in BLM-free medium for various times after BLM treatment, the damaged DNA tended to be repaired in a time-dependent manner and more efficiently in TF-R than in TF-S. Since incubation at 4°C in BLM-free medium for 60 min did not repair the damaged DNA, it was suggested that DNA repair enzyme was involved in the process. Therefore, the activity of poly(ADP-ribose) polymerase, a presumed repair enzyme, was measured (Fig. 5). Although it was approximately 1.5-fold higher in TF-R than in TF-S before BLM treatment, it increased rapidly by two to three times in TF-R as compared to TF-S by BLM treatment. The reaction reached a plateau within 10 min of incubation time when the cells were treated with 10 μg/ml of BLM, and at 1 μg/ml of BLM concentration when the cells were treated with various concentrations of BLM for 60 min.

DISCUSSION

In clinical experience, we have often found that the antitumour effect of chemotherapeutic agents was diminished by repeated chemotherapy against oral cancer. This fact

Table 1. Changes of sensitivity to various antitumour agents of SCCTF after BLM treatment with two courses of method (a)

Agents	IC_{50} (μg/ml)		Ratio of IC_{50}
	Pretreatment	Post-treatment	Post/Pre
Bleomycin	1.80	3.80	2.1
Peplomycin	0.55	1.80	3.3
Doxorubicin	0.081	0.074	0.9
Mitomycin C	0.032	0.050	1.6
Cisdiammine dichloroplatinum	0.27	0.85	3.1
5-Fluorouracil	0.050	0.072	1.4
Methotrexate	0.026	0.050	1.9
Vincristine	0.009	0.011	1.2

Method (a): the cells were treated continuously with low dose of BLM (0.1 μg/ml) for 1 week, followed by incubation without BLM for further 1 week. Note: After treatment with various concentrations of antitumour agents at 37°C for 3 days, viable cells were counted in triplicate and the IC_{50} was determined from the cell survival curve.

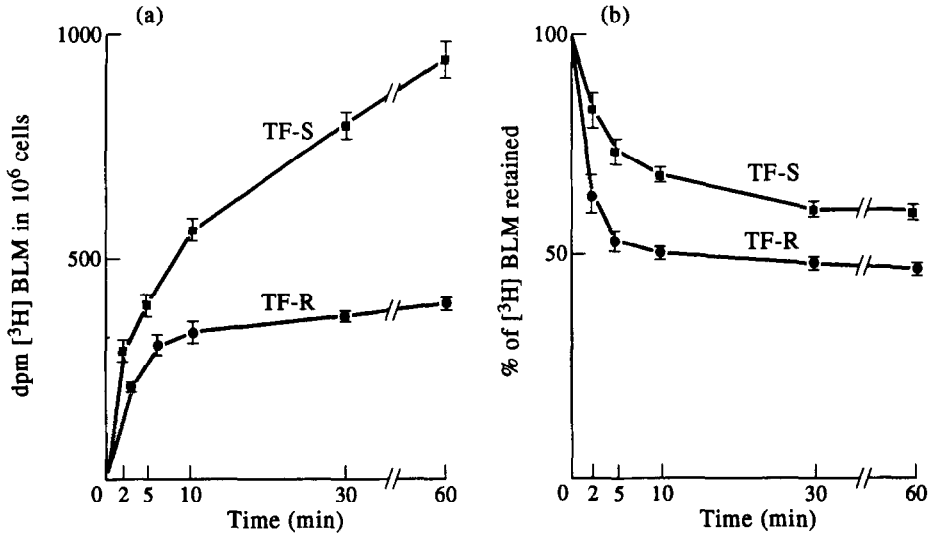

Fig. 2. Cellular accumulation (a) and retention (b) of [^{3}H]BLM in TF-S and TF-R. Cells grown at 37°C for 2 days were exposed to 0.05 μCi (approximately 5×10^4 dpm) of [^{3}H]BLM per dish. The cell-associated radioactivity was counted at intervals after washing cells twice with ice-cold PBS(−) and dissolving in 1 N NaOH. For the retention assay, [^{3}H]BLM-prelabelled cells were incubated in growth medium at 37°C for the times indicated. After the culture medium was aspirated and the cells were washed, the cell-associated radioactivity was counted. Each point represents the mean ± S.D. of three determinations.

suggests that the tumour cell population which was originally sensitive to antitumour agents has changed to the low-sensitive or resistant cell population. Several investigators also suggested that tumour cell heterogeneity is strongly involved in the acquisition of antitumour drug resistance [12, 13].

Cells	Deamidated BLM-B_2 formed (μg/mg protein/min)	Ratio
TF-S	0.53	1
TF-R	1.11	2.1

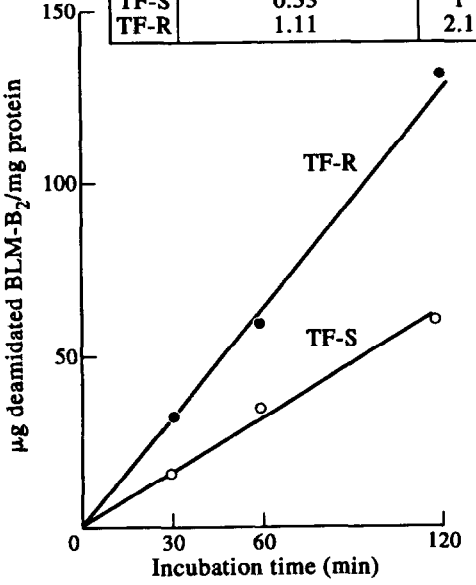

Fig. 3. BLM hydrolase activity in cell extracts of TF-S and TF-R. The method of the enzyme assay by using HPLC is described in Materials and Methods. The deamidated BLM-B_2 was measured in a single sample in each incubation time. Values presented in the table were calculated from the increasing rate of the deamidated BLM-B_2 per minute determined by the regression line for the amount of deamidated BLM-B_2 formed in each incubation time, and protein content of the sample.

In order to investigate this phenomenon, we used BLM and two oral squamous carcinoma cell lines with different BLM sensitivities, and designed the two chemotherapeutic protocols. BLM was chosen as an antitumour agent preferentially effective for squamous cell carcinoma. As a consequence, SCCTF with minimal BLM sensitivity and strong heterogeneity was readily changed to be less sensitive to several antitumour agents including BLM when treated continuously with low doses of BLM as in method (a). The sensitivity of SCCTF to antitumour agents treated with two courses of method (a) decreased 2.1-fold to BLM, 3.3-fold to peplomycin, 1.6-fold to mitomycin C, 3.1-fold to cisdiammine dichloroplatinum and 1.9-fold to methotrexate as compared to those of untreated SCCTF. The extent of decreased sensitivity was not high, but it is expected to be significant clinically and does exhibit resistance to cancer chemotherapy. When SCCTF was treated intermittently with high doses of BLM as in method (b), BLM sensitivity was not changed. On the other hand, SCCKN with high BLM sensitivity and weak heterogeneity was totally killed by the same treatments. It is unclear why the difference between methods (a) and (b) in induction of resistance in SCCTF occurred. However, this finding led us to suggest that the method of chemotherapy has an effect on the induction of resistance. This has clinical importance in cancer chemotherapy.

To examine the induction mechamism of resistance, we isolated two kinds of clones from SCCTF; the BLM-sensitive TF-S and -resistant TF-R. These two clones showed a similar morphology and population doubling time, indicating that the difference of sensitivity to antitumour agents based on the cell cycle can be rule out.

TF-R displays a 9.5-fold degree of resistance to BLM as compared to TF-S *in vitro* and nude mouse tumours produced by TF-R also were significantly less sensitive to BLM than those produced by TF-S. Analysis of the resistance mechanism revealed that TF-R exhibited decreased cellular accumulation and retention of BLM, increased BLM hydrolase activity and elevated DNA repair activity concomitant with

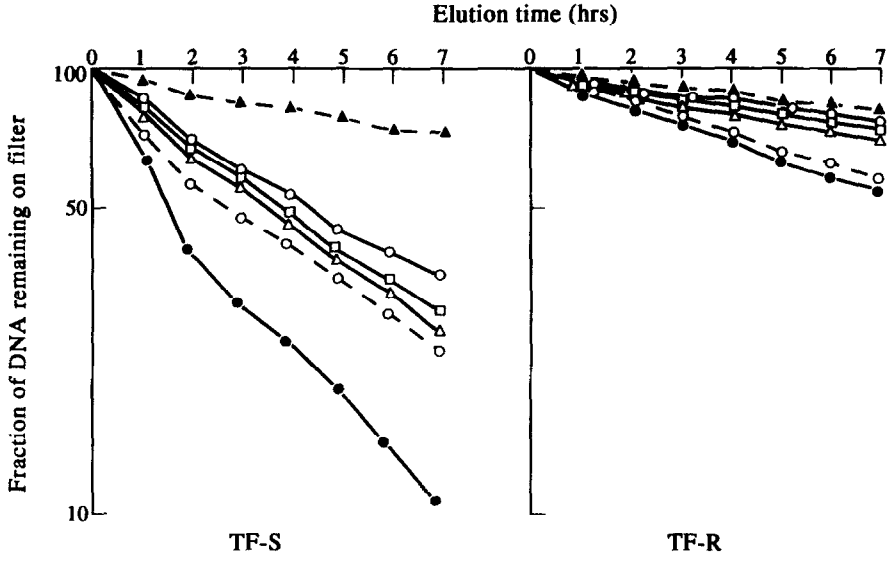

Fig. 4. Alkaline elution profiles of DNA single-strand breakage in TF-S and TF-R with BLM treatment. [^{14}C]Thymidine-labelled cells were incubated with no drug (▲- - -▲) or 100 μg/ml of BLM at 37°C for 60 min (●——●). To permit DNA repair, cells were incubated at 37°C for 15 min (△——△), 30 min (□——□), and 60 min (○——○) or incubated at 4°C for 60 min (○- - -○) in BLM-free medium after BLM treatment. Then, cells were assayed for DNA strand breaks with the use of an alkaline elution technique described in Materials and Methods.

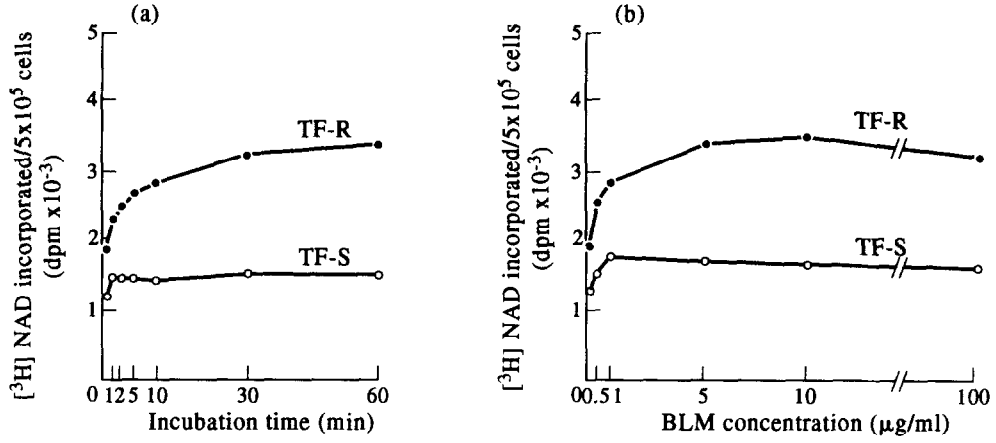

Fig. 5. Poly(ADP-ribose) polymerase activity in TF-S and TF-R after BLM treatment. After BLM treatment, cells were suspended in a permeabilising buffer containing [^{3}H]nicotinamide adenine dinucleotide and incubated at 37°C. The acid-precipitable radioactivity was counted for the enzyme activity. (a) Treatment with 10 μg/ml of BLM for various times indicated. (b) Treatment with various concentrations of BLM for 60 min.

increased poly(ADP-ribose) polymerase activity as compared with TF-S. In the previous studies, we reported that BLM-resistant HeLa cells showed about 40% decreased accumulation and 2–3-fold reduced retention of BLM, elevated DNA repair activity and poly(ADP-ribose) polymerase activity as compared with parental HeLa cells [4, 5]. Also, SCCTF which is minimally sensitive to BLM demonstrated approximately 20% decreased accumulation and retention of BLM, 1.2-fold increase of BLM hydrolase activity, elevated DNA repair activity, and increased poly(ADP-ribose) polymerase activity as compared with SCCKN which is highly sensitive to BLM [7]. Therefore, the findings obtained from TF-R in comparison with TF-S basically were in good agreement with those obtained from BLM-resistant HeLa cells and SCCTF, except that TF-R carried a higher BLM hydrolase activity.

Two types of mechanisms have been postulated for the acquisition of drug resistance in tumours [14]: (i) it is due to the heterogeneity of tumour cell population and antitumour drug-resistant clones selectively survive and grow by chemotherapy and (ii) originally chemosensitive tumour cell induce drug resistance by the expression of a drug resistance gene during the course of chemotherapy. Since BLM is a chemotherapeutic agent with very low mutagenicity [15] and the acquisition of cellular resistance to BLM generally requires long-term exposure to BLM [3], it was speculated that the induction of BLM resistance in SCCTF is due to the selective growth of a BLM-resistant clone existing in the cell population.

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