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Abstract

An adriamycin (ADM)-resistant subline was established by continuous exposure of the SBC-3 cells, a cell line of human small cell lung cancer, to increasing concentrations of ADM, followed by the cloning procedure. The resistant sublines (SBC-3/ADM) thus established were 30-fold more resistant to ADM than the parent SBC-3 cells, in terms of the 70% lethal dose determined by soft agar clonogenic assay. The doubling times of the SBC-3 and SBC-3/ADM cells were 36 h and 22 h, respectively. When transplanted into athymic nude mice, the parent as well as resistant cells formed tumors, and serial passage was successful. Although the transplanted tumors from the two cell lines were very similar in histology, the resistance of the SBC-3/ADM cells to ADM developed in vitro was maintained in serially transplanted tumors. The uptake studies with [3H]daunomycin revealed decreased influx and enhanced active efflux of the drug in the resistant cells, whereas cytogenetic analysis showed that the cell lines had an identical karyotype. These results indicate that ADM resistance may be attributed to alternations in membrane transport, resulting in reduced intracellular accumulation of the drug.

KEYWORDS: human small cell lung cancer, adriamycin-resistant subline, morphological characteristics, uptake studies, chromosome analysis

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Establishment and Characterization of an Adriamycin-resistant Subline of Human Small Cell Lung Cancer Cells

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An adriamycin (ADM)-resistant subline was established by continuous exposure of the SBC-3 cells, a cell line of human small cell lung cancer, to increasing concentrations of ADM, followed by the cloning procedure. The resistant sublines (SBC-3/ADM) thus established were 30-fold more resistant to ADM than the parent SBC-3 cells, in terms of the 70% lethal dose determined by soft agar clonogenic assay. The doubling times of the SBC-3 and SBC-3/ADM cells were 36 h and 22 h, respectively. When transplanted into athymic nude mice, the parent as well as resistant cells formed tumors, and serial passage was successful. Although the transplanted tumors from the two cell lines were very similar in histology, the resistance of the SBC-3/ADM cells to ADM developed *in vitro* was maintained in serially transplanted tumors. The uptake studies with [^{3}H]daunomycin revealed decreased influx and enhanced active efflux of the drug in the resistant cells, whereas cytogenetic analysis showed that the cell lines had an identical karyotype. These results indicate that ADM resistance may be attributed to alternations in membrane transport, resulting in reduced intracellular accumulation of the drug.

Key words : human small cell lung cancer cells, adriamycin-resistant subline, morphological characteristics, uptake studies, chromosome analysis

Small cell lung cancer (SCLC) is a rapidly growing, early and widely metastasizing tumor ; untreated patients have a median survival of only a few months (1). This neoplasm is frequently beyond successful intervention by surgical or radiological means and therefore constitutes the major requirement for chemotherapy (2, 3). Over the past decades, the utilization of combination chemotherapy with or without radiotherapy has led to an improved response rate and significant increases in survival for patients with SCLC (4, 5).

Despite these developments, few patients survive longer than two years. Even those patients achieving a complete response eventually relapse and die of the disease. The mechanism by which tumors become unresponsive remains obscure. Among several factors for treatment failures, a major one is the acquisition of drug resistance by the tumor cells. To date several investigators have selected *in vitro* or *in vivo* mammalian cell lines resistant to anticancer agents (6-9); however, there is little information regarding the isolation of resistant sublines from human tumors (10, 11). No work has been carried out with SCLC cell lines.

The anthracycline antibiotic, adriamycin (ADM), plays an important role in the chemotherapeutic management of SCLC. Ob-

jective response rates of approximately 30% are obtained by single administration of the drug (12, 13). In this paper, we describe the establishment and characterization of SCLC cells with acquired resistance to ADM, for the purpose of elucidating the mechanisms of drug resistance and exploring ways to combat the resitant cells.

Materials and Methods

Chemical agents. ADM formulated for clinical use was obtained from Kyowa Hakko Kogyo Co, Ltd, Tokyo, Japan. The drug was dissolved in 0.9% NaCl solution just before use. [^{3}H]daunomycin (3.8 Ci/m mol) was purchased from New England Nuclear, Boston, MA. The radiochemical purity was higher than 95% by thin layer chromatography.

Tumor cells and culture medium. SCLC cells, SBC-3, were used as parent cell lines. The SBC-3 cells were established in our laboratory from bone marrow aspirates of a 24-year-old male with SCLC (oat cell type) obtained at diagnosis (14). The growth medium for the SBC-3 cells was RPMI 1640 medium (Grand Island Biological Co, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cells were maintained in tissue culture flasks (Falcon 3013) containing the growth medium at 37°C in a humidified atmosphere of 5% CO_2, and subcultured twice weekly.

Isolation of an ADM-resistant subline. An ADM-resistant subline was derived from the parent SBC-3 cells *in vitro* by continuous exposure to increasing concentrations of ADM, followed by the cloning procedure. Initially, the SBC-3 cells were passaged continuously in growth medium containing 10^{-10} M ADM. The concentration of the drug was gradually elevated every two or three weeks, and about 6 months later, cells growing in medium with 5×10^{-8} M ADM were obtained. These cells were then plated in a double-layer soft agar system after exposure to 10^{-6} M ADM for 1 h. The rare colonies surviving this treatment were picked up and distributed to each of 24-Multiwell Tissue Culture Plates (Falcon 3047) containing RPMI 1640 medium supplemented with 20% FBS. After a few weeks, the cells attached to the surface of the plate were shifted to continuous passage in an ADM-free growth medium. For one of these clonal lines, favorable growth was observed ; this clonal line has been used as an ADM-resistant subline, designated SBC-3/ADM, in the further studies reported herein.

Assay of drug sensitivity. Effects of ADM on cell survival of the SBC-3 and SBC-3/ADM cells were determined using a soft agar clonogenic assay similar to that described by Hamburger and Salmon (15). In brief, after exposure to graded concentrations of ADM for 1 h, single cell suspensions were plated in a double-layer soft agar system. Feeder layers of RPMI 1640 medium supplemented with 15% FBS and 0.5% agar were prepared in 35-mm plastic Petri dishes (LUX 5217). Cells to be assayed were suspended in 0.3% agar and RPMI 1640 medium supplemented with 15% FBS, and plated over the feeder layer. Cultures were incubated at 37°C in humidified 5% CO_2-air for 14 days. Dose response curves of the two cell lines were obtained by calculating the ratio of the number of colonies surviving at each drug concentration to the number of colonies growing in control plates.

In vitro growth characteristics. The doubling times of the parent and resistant cells were obtained by daily counting of the viable cell number using the trypan blue dye exclusion method. Morphological features of cells growing in liquid medium were observed under a phase contrast microscope in the late logarithmic phase. The cell size was determined by centrifugation of single cell suspensions in capillary tubes (cytocrit method).

Light and electron microscopy. The cytologic smears derived from the SBC-3 and SBC-3/ADM cells were fixed with 95% ethanol and stained by a modified Papanicolaou method. For ultrastructural analysis, cultured cell pellets were fixed with 2.5% glutaraldehyde, postfixed with 10% osmium tetroxide, and dehydrated in a graded alcohol series. Epok 812-embedded ultrathin sections were stained with uranyl acetate and lead citrate, and then viewed under a H-300 electron microscope (Hitachi Co, Ltd, Tokyo, Japan).

Transplantation in nude mice and treatment of xenografts with ADM. Nude mice (nu/nu) with a BALB/c genetic background were obtained from

Nihon CLEA Co, Ltd, Tokyo, Japan, and were housed with autoclaved food and bedding. All cages were placed in a laminar-air-flow unit. Six- to seven-week old male mice weighing about 25 g were used for the experiments. For primary transplantation, attempts were made to inoculate 10^7 cells of the parent and resistant cells per mouse subcutaneously into the right back of mice. The tumors growing at the injection site were excised, fixed with 10% formalin, and stained with hematoxylin and eosin. The transplanted tumors were maintained *in vivo* by implanting tumor fragments of about 2 mm in diameter subcutaneously with a trocar.

To evaluate *in vivo* response to ADM, treatment was initiated when the tumor mass reached approximately 150-200 mm^3 in volume. A single intravenous dose of 15 mg/kg of ADM was given through the tail vein of mice, while control mice were treated with 0.9% NaCl solution by the same route. The dosage was selected on the basis of the maximum tolerated dose of ADM determined in BALB/c nude mice (16). The tumor size was measured twice a week after the initiation of treatment, and tumor volumes were estimated by the formula $V=(a^2\times b)/2$, where a is the width in mm and b is the length in mm. To standardize the variability in initial tumor volumes among the different test groups, relative tumor volumes at different times were calculated from ratio of the tumor volume of a group at any given time to the initial tumor volume of that group. At least six mice were used for each experimental group.

Uptake studies with [^{3}H]daunomycin. Since [^{3}H]ADM was not available and the SBC-3/ADM cells were completely cross-resistant to daunomycin (DM) as described afterwards, uptake studies were undertaken using [^{3}H]DM. For the cellular drug uptake, the SBC-3 and SBC-3/ADM cells were washed twice and resuspended in Hank's balanced salt solution at a cell concentration of 5×10^6/ml. The cells were incubated with [^{3}H]DM (1 μM) at 37°C to initiate the reaction. At various time intervals (0 to 60 min), triplicate 0.2-ml aliquots were removed and filtered with a Labo Mash cell harvester. The cells on the filter were solubilized by ACS II (Amersham, USA) and were placed in scintillation vials. The radioactivity was counted in an Aloka LSC-700 liquid scintillation counter. The results were expressed as the net uptake as calculated by the total uptake less the background level determined by using cell-free blanks.

Efflux of the drug was measured by incubating tumor cells in the presence of [^{3}H]DM (1 μM) for 30 min at 37°C. Subsequently, the cells were washed at 0°C, and resuspended in drug-free medium at 37°C. At appropriate times, triplicate 0.2-ml aliquots were withdrawn from incubation flasks, and the residual radioactivities in the cells were analyzed in the same manner as mentioned above. For kinetic analysis of the initial uptake of the drug, [^{3}H]DM at graded concentrations (2 to 10 μM) was added to cell suspensions at 37°C. After 2.5 min, the reaction was terminated and the radioactivity was determined.

Cytogenetic studies. For chromosome preparation, logarithmically growing cells in the early phase were exposed to 0.5 μg colcemid per ml for 2-3 h. The cells arrested in metaphase were then treated with 0.075 M KCl hypotonic solution for 13 min at 37°C, fixed in methanol : glacial acetic acid (3 : 1) solution, dropped onto clean glass slides immediately, and air dried. For banding studies, the Q-banding method was used.

Results

Establishment of SBC-3/ADM sublines. Isolation of the SBC-3/ADM cells was initiated *in vitro* by continuous exposure of the SBC-3 cells to increasing concentrations of ADM. The resistant sublines were then established by the cloning procedure from a single colony that developed in soft agar. Cytotoxic effects of ADM on the SBC-3 and SBC-3/ADM cells determined by soft agar clonogenic assay are shown in Fig. 1. The 70% lethal dose (LD_{70}) of ADM was 6.0×10^{-8} M for the parent cells, and 180×10^{-8} M for the resistant cells. Thus, the SBC-3/ADM cells were 30-fold more resistant to ADM than the SBC-3 cells in terms of LD_{70} values. Further continuous cultivation of the resistant sublines in drug-free medium for more than 6 months did not lead to any decrease in the

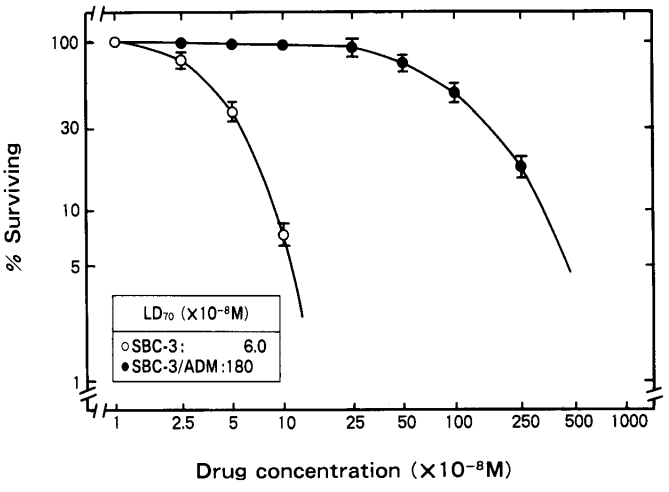

Fig. 1 Dose response curves of the SBC-3 and SBC-3/ADM cells to ADM determined by soft agar clonogenic assay. The parent and resistant cells were treated with graded concentrations of ADM for 1 h, and plated in a double-layer soft agar system as described in Materials and Methods. Dose response curves were obtained by calculating the ratio between the number of colonies in drug-treated dishes and that in control dishes. Each point represents the mean of three estimations ± SD.

degree of resistance.

Doubling times and morphological characteristics. The doubling times of the SBC-3 and SBC-3/ADM cells were 36 h and 22 h, respectively. *In vitro* morphology by phase contrast microscopy demonstrated that the parent cells grew, attaching themselves loosely to the substrate, whereas the resistant cells were attached tightly to the wall of culture flasks. The size of tumor cells was 1100 μ^3 for the SBC-3 cells, and 1600 μ^3 for the SBC-3/ADM cells.

Cytologic findings of the SBC-3 cells were highly characteristic of SCLC cells (oat cell type), with scanty cytoplasm, hyperchromatic nuclei, and inconspicuous nucleoli. In contrast, the SBC-3/ADM cells with relatively abundant cytoplasm showed an increase in cell size, although cellular cytology corresponded to SCLC cells. Ultrastructural analysis also revealed that the resistant cells possessed an increased amount of cytoplasm with better developed organelles including endoplasmic reticulum and Golgi apparatus, as compared with the parent cells, which showed scanty cytoplasm and poorly developed organelles. Dense-cored secretory granules were not present in any of the cell lines.

Heterotransplantation and in vivo response to ADM. The SBC-3 and SBC-3/ADM cells were transplantable to nude mice. Mice given injections of cell suspensions (10^7 cells per mouse) developed progressively growing tumors 10-14 days following inoculation. Light microscopic findings of the excised tumors derived from both cell lines corresponded to SCLC. Although the resistant cells were slightly larger in size than the parent cells, there was no significant difference in histological features between the two cell lines. Serial passage of the initially transplanted tumors *in vivo* was successful, and the efficiency of transplantation was more than 90%. The mass doubling time of both cell lines during the logarithmic growth phase was 3-4 days.

The effect of ADM on the parent and resistant cells growing as xenografts in athymic nude mice is illustrated in Fig. 2. A single intravenous administration of 15 mg/kg of ADM induced a period of tumor regression in the SBC-3 xenograft, although the tumor grew again after 2 weeks. The maximum rate of tumor regression was 28% 10 days after the treatment. In contrast,

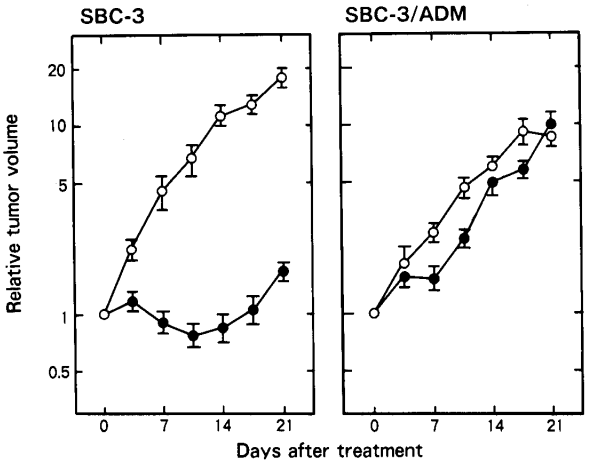

Fig. 2 Activities of ADM against the SBC-3 and SBC-3/ADM cells growing as xenografts in athymic nude mice. Mice were treated with 15 mg/kg of ADM through the tail vein (●), or 0.9% NaCl solution by the same route (○). Relative tumor volumes were calculated as described in Materials and Methods. Each point represents the mean of six estimations ± SD.

tumor growth of the SBC-3/ADM xenograft was only delayed to some degree immediately after the treatment, and the tumor grew progressively thereafter.

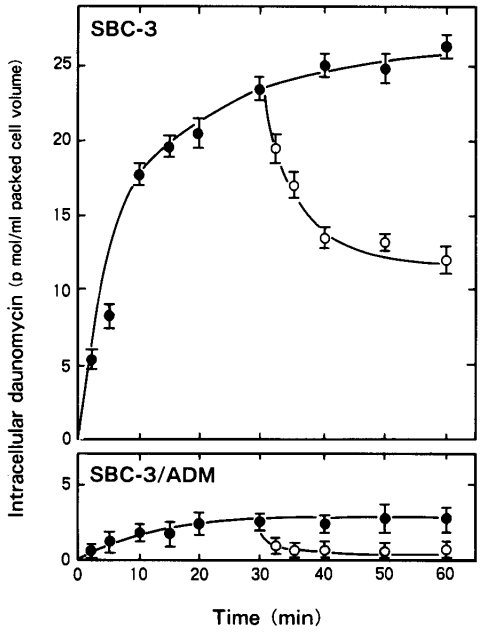

Fig. 3 Time course of the uptake (●) and efflux (○) of [^{3}H]DM in the SBC-3 and SBC-3/ADM cells. [^{3}H]DM at 1 μM was added to cell suspensions of both cell lines at 0 time. Serial samples were withdrawn at the time indicated, and intracellular drug content was determined as described in Materials and Methods. Each point represents the mean of three estimations ± SD.

Cellular influx and efflux of [^{3}H]DM. For both cell lines, the uptake of [^{3}H]DM was characterized by an early linear phase for about 10 min ; thereafter, the rate of net uptake gradually decreased. After incubation with [^{3}H]DM for 60 min, intracellular accumulation of the drug in the SBC-3 and SBC-3/ADM cells was 25.9 and 2.65 p mol/ml packed cell volume, respectively (Fig. 3). Thus, the net uptake of the drug in the resistant cells was approximately 10-fold less than in the parent cells.

The efflux was biphasic with an initial rapid loss of the drug, after which the rate of release became constant. Approximately 50% of [^{3}H]DM taken up at steady state was retained 30 min after resuspension in drug-free medium by the parent cells as compared to only 17% by the resistant cells. Accordingly, the efflux of the drug from the resistant cells was greater than from the parent cells. In Fig. 4, the initial rate of influx of [^{3}H]DM over a range of 2 to 10 μM is compared in both cell lines. At any of the concentrations studied, the initial rate of influx in the SBC-3 cells was significantly higher than in the SBC-3/ADM cells. The Vmax values were 45 and 8.3 n mol/2.5 min/ml packed cell volume for the parent and resistant cells, respectively,

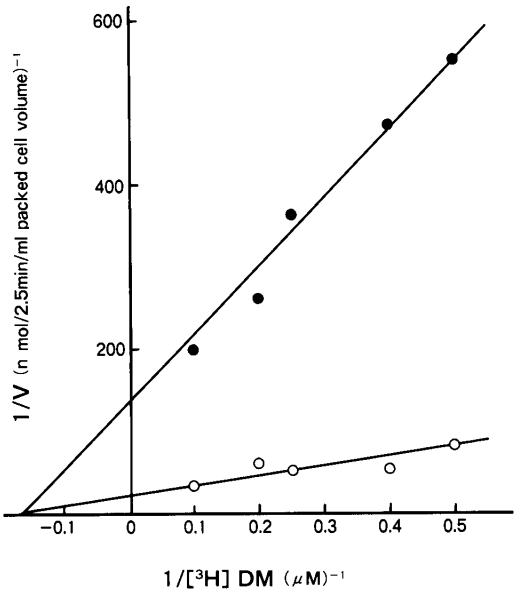

Fig. 4 Lineweaver-Burk plot for the initial rate of influx of [^{3}H]DM in the SBC-3 cells (○) and SBC-3/ADM cells (●). V is expressed as an initial rate of influx of the drug. For determination of Vmax and Km, the line was fitted by the least squares method. Each point represents the mean of three estimations.

whereas the Km values remained the same for the two cell lines (6.9 μM).

Chromosome analysis. For both cell lines, the modal chromosome number was 46. Although chromosomal aberrations were present, the parent and resistant cells demonstrated an identical karyotype of : 46, XY, 1p−q+, 12p+, −15, 19p or q+, +mar. Neither double minute chromosomes nor homogeneously staining regions were found.

Discussion

Although the resistance to anticancer drugs has been extensively studied in non-human systems, few investigations with human tumor cell lines have been performed. To our knowledge, this report is the first one of an ADM-resistant subline of SCLC cells having been established. To isolate the resistant sublines, cells growing in medium containing 5×10^{-8} M ADM were cloned in this report , because noncloned cells are considered to be heterogeneous and unstable as described previously (17). Since the resistance of the SBC-3/ADM cells in ADM-free medium has not changed during the 6-month observation period, the cell line seems to be a stable mutant.

It has been described in some studies that there are no differences in the morphological appearance or cell size between drug-sensitive and drug-resistant cell lines (10, 11). Nevertheless, morphological modifications in drug-resistant sublines have been reported by several investigators (18, 19). The SBC-3/ADM cells revealed an increase in cell size and in cellular cohesiveness to the substrate, in comparison with the SBC-3 cells. The light and electron microscopic findings showed that the resistant cells had an increased amount of cytoplasm with better developed organelles. No complete explanation is available for the relation between the biological or morphological changes and the development of ADM resistance. These observations, however, suggest the possibility of biochemical or physiological alternations in the plasma membrane of the SBC-3/ADM cells.

Although the doubling times of drug-resistant cell lines have been reported to be almost the same (10, 11) or much longer (18, 20) than drug-sensitive cell lines, the *in vitro* growth of the SBC-3/ADM cells was significantly faster than that of the SBC-3 cells. The findings indicate that ADM resistance is not a result of decreased growth fraction due to a prolonged doubling time.

Human tumors serially passaged in nude mice preserve their original chemosensitivities as well as morphological characteris-

tics through many transplant generations. The SBC-3 and SBC-3/ADM cells showed tumorigenicity in athymic nude mice, and the resistance to ADM was also preserved in the SBC-3/ADM xenograft. Therefore, this xenograft system would be a useful model in further studies of ADM resistance in man.

Among several action mechanisms of anthracycline antibiotics such as ADM, intercalative binding to DNA is considered to be primary for cytotoxicity (21). However, defective membrane transport may be an important determinant of ADM resistance, since altered cellular drug content has been described in several drug-resistant sublines (9, 22). Principally, net uptake of the drug in cells is determined by two reciprocally reverse movements across the cell membrane, namely, influx and efflux. It has been demonstrated that anthracycline antibiotics enter into cells by passive diffusion and are actively transported out (23). Evidence has been presented by Inaba and co-workers (24) to suggest enhanced active outward transport of ADM as a possible mechanism of resistance for an ADM-resistant subline of P388 leukemia cells. Tsuruo and co-workers (25, 26) have found that a variety of calcium channel blockers, as well as calmodulin inhibitors, increase cellular accumulation of vincristine and ADM in drug-resistant cells by inhibition of active efflux of the drugs, thereby attaining a reversal of both acquired and inherent drug resistance.

In order to elucidate the mechanism of resistance for the SBC-3/ADM cells, the influx and efflux of [^{3}H]DM across the plasma membrane were examined. Drug uptake profiles showed that the cellular content of [^{3}H]DM in the resistant cells was approximately 10-fold lower than in the parent cells. From the efflux experiments, release of [^{3}H]DM was faster from the resistant cells than from the parent cells. Thus, an enhanced active efflux mechanism may be involved in the SBC-3/ADM cells. Kinetic analysis of the initial uptake of [^{3}H]DM revealed that the SBC-3/ADM cells did not have an altered Km (6.9 μM), but had a decreased Vmax of about 18% of the SBC-3 cells. When applied to the concept of carrier-mediated transport, the modifications of kinetic parameters demonstrate decreased influx in the resistant cells. Based on these data, reduced intracellular accumulation of the drug in the SBC-3/ADM cells would be a result of at least two different mechanisms of decreased influx and enhanced active efflux.

Another possible mechanism of drug resistance may be gene amplification. Overproduction of the methotrexate (MTX) target enzyme, dihydrofolate reductase, is known to be a cause of MTX resistance in murine cells (27, 28). Recently, cytogenetic studies showed that an ADM-resistant Chinese hamster V79 cell line contained increased numbers of small paired extrachromosomal chromatin bodies termed double minute chromosomes, which were considered to be the site of amplified gene sequences (29). However, karyotype analysis of the SBC-3 and SBC-3/ADM cells revealed an identical chromosome profile, and cytogenetic evidences for gene amplification such as homogeneously staining regions (30) or double minute chromosomes were not present. The observations indicate that a membrane mutation resulting in reduced intracellular accumulation of the drug is mainly responsible for the development of resistance in the SBC-3/ADM cells.

Studies are currently under way to evaluate the *in vitro* chemo- and radio-sensitivities of the SBC-3/ADM cells, as well as to search for a means of circumventing the drug resistance. The resistant sublines reported herein may provide important clues as to new drug development, rational selection of combination chemotherapy, and strat-

egies for preventing or overcoming acquired drug resistance.

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