Establishment and Characterization of Resistant Cells to Etoposide (VP16) from a Mouse Breast Cancer Cell Line, FM3A

Eishi Takagi,[*1] Hidetaka Kawatsu,[*1] Kaoru Shimokata,[*1] Yukihiro Nishiyama,[*2] Kiyohide Kojima[*3] and Shonen Yoshida[*3, *4]

[*1]First Department of Internal Medicine, [*2]Laboratory of Virology, Research Institute for Disease Mechanism and Control, and [*3]Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466

We established 4 cell lines resistant to VP16 from a mouse breast cancer cell line, FM3A. The IC_{50} values of all 4 resistant strains were approximately 2 μg/ml as measured by colony formation in soft agar; about 40 times higher than that of parent cell (0.05 μg/ml). These cells showed a cross-resistance to VM26, a compound related to VP16, but not to a variety of other antitumor drugs including adriamycin, mitomycin C, cis-platinum, 5-fluorouracil, bleomycin, vincristine, 4-hydroperoxycyclophosphamide, methotrexate and cytosine arabinoside. Topoisomerase II, the putative target of VP16, was partially purified from cells, and was assayed using knotted P4 phage DNA as a substrate. However, no significant difference was observed between enzymes from resistant cells and from the parent cells in either activity per cell or sensitivity to VP16. On the other hand, the resistance of these cell lines to VP16 was greatly reduced by adding a calcium antagonist, verapamil, to the soft agar at a concentration as low as 5μM, at which the viability of cells was hardly affected. A similar verapamil-induced reduction in the resistance of the cells to VM26 was also observed. These results suggest that the acquired resistance may be largely due to an altered membrane permeability to drugs, which may be overcome by verapamil, rather than to an altered topoisomerase II.

Key words: Etoposide — Topoisomerase II — Drug resistance — FM3A — Verapamil

VP16,[*5] an antitumor drug, exerts its cytotoxic effect by inducing cleavage in DNA mediated by topoisomerase II.[1-5] VP16 has also been shown to interfere with the growth of herpes simplex virus by inhibiting the host cell enzyme.[6] Since VP16 is now available for general clinical use, the common problem of acquisition of resistance in tumor cells may soon arise. Selection for resistance usually results in the development of cross-resistance to other drugs unrelated in their structure and mode of action to the original selective agents, for example vinca alkaloids and anthracyclines.[7-11] Such pleiotropic drug resistance is now thought to be related to the expression of P-glycoprotein in the cell membrane.[11-13]

Interestingly, multidrug resistance can be overcome to some extent by treatment of resistant cells with the calcium antagonist verapamil, which is supposed to block the efflux of drugs from the cell.[14, 15]

In this study, we isolated 4 variant cells resistant to VP 16. They showed cross-resistance to only VM26, a compound related to VP16, but not to other antitumor drugs. No difference was observed between the parent cell and resistant cells in either total activity or VP16-sensitivity of topoisomerase II, the putative target of VP16. The addition of verapamil to the variant cells greatly reduced the resistance to VP16.

MATERIALS AND METHODS

Chemicals and Reagents Etoposide (VP16), teniposide (VM26) and cis-platinum were gifts of Bristol Myers Japan (Tokyo). Adriamycin, mitomycin C, and 5-fluorouracil were from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Methotrexate was from Lederle Japan (Tokyo). Cytosine arabinoside was from Nippon Shinyaku Co., Ltd.

[*4] To whom all correspondence should be sent.
[*5] The abbreviations used are: VP16, etoposide; VM26, teniposide; EGTA, ethyleneglycol bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

(Kyoto). Bleomycin was from Nihon Kayaku Co., Ltd. (Tokyo). Vincristine and 4-hydroperoxy-cyclophosphamide, which is a metabolite of cyclophosphamide, were from Shionogi Co., Ltd. (Osaka). Verapamil was obtained from Eisai Co., Ltd. (Tokyo). Bacteriophage P4 was supplied by Dr. H. Hanaoka of Tokyo University.

Cell Culture Mouse breast cancer cell line, FM3A, which is suitable for establishing mutant strains and shows high plating efficiency in soft agar, was given by Dr. H. Koyama (Cancer Institute, Tokyo), and was maintained in ES mdium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 3% fetal bovine serum (FBS) (Flow Laboratories, North Ryde, Australia).

Establishment of FM3A Cells Resistant to VP16 After exposure to ultraviolet ray irradiation (150 erg/mm^3), a mouse breast cancer cell line, FM3A, was cultured for ten days, and selected in the presence of 1 μg/ml of VP16. Four strains of variant colonies resistant to VP16 were isolated (VPr-1, 2, 3, 4).

Drug Sensitivity by Clonogenic Survival Cells to be tested were suspended in 1 ml of 0.3% soft agar (Bacto-agar, Difco, Laboratories, Detroit, USA) in ES medium, supplemented with 3% FBS to yield a final concentration of 200 cells/ml.[16] Freshly prepared antitumor drug was added at various concentrations immediately before duplicate plating of the cells. One milliliter of the mixture was pipetted into a 35 mm plastic Petri dish. Cultures were incubated at 37° in 5% carbon dioxide in a humidified atmosphere. Colonies (collection of more than 40 cells) appeared in 5 days, and their numbers were counted after 7–8 days. Plating efficiency (%) was calculated according to the formula: number of colonies without drug × 100/200. The IC$_{50}$ value refers to the concentration of a drug which reduces the colonies to half the number of colonies without the drug.

Partial Purification of Topoisomerase II All procedures were performed at 4°. Nuclei were prepared from FM3A parent and resistant cells, according to the method of Pommier et al.[17, 18] Briefly, approximately 5×10^8 cells were transferred to nucleus buffer (150mM NaCl, 1mM KH$_2$PO$_4$, 5mM MgCl$_2$, 1mM EGTA, 0.2mM DTT, 10% (v/v) glycerol, 0.1mM PMSF, pH 6.4) and centrifuged at 1,200 rpm for 10 min. After washing of the cells by centrifugation in nucleus buffer, the cell pellets were resuspended in 5 ml of nucleus buffer and then mixed with an additional 45 ml of nucleus buffer containing 0.3% Triton X-100. The cell suspension was mixed gently by rotation for 10 min and then centrifuged at 1,000g for 10 min. The nuclear pellets were washed once in Triton-free nucleus buffer, and resuspended in 2 ml of nucleus buffer containing 0.35M NaCl (final concentra-

tion). The salt extraction was performed by gentle rotation for 30 min and then sonication, followed by centrifugation at 12,000g for 20 min. The nuclear extracts were diluted with 4 ml of MS buffer (50mM Tris-HCl, pH 7.5, 10% glycerol, 0.25mM DTT), and applied to a Mono S column on an FPLC liquid chromatography system (Pharmacia, Stockholm, Sweden). Elution was performed with a linear gradient of 0 to 500mM potassium chloride in MS buffer, and the peak fraction of topo-isomerase II activity eluted at 0.3M salt was used immediately for the topoisomerase II reaction.

Assay of Topoisomerase II Naturally knotted P4 phage DNA was used to assay for the strand passing activity of topoisomerase II (DNA gyrase: EC 5.99.1.3.).[19-21] P4 knotted DNA (0.3 μg) was incubated in a total volume of 20 μl of reaction mixture (50mM Tris-HCl, pH 7.5, 100mM KCl, 10 mM MgCl$_2$, 0.5mM DTT, 0.5mM EDTA, 30 μg/ml bovine serum albumin, 1mM ATP) with or without VP16 at the concentrations indicated, for 30 min at 37°. The reaction was stopped by the addition of 5 μl of stop solution containing 6.6% SDS, 67mM EDTA, 30% sucrose, and 0.1% bromphenol blue,[22] and the products of the reaction were analyzed by electrophoresis on a 0.7% agarose gel in TAE buffer (40mM Tris-acetate, pH 8.0, 1.6mM EDTA-Na$_4$), and at 10 V/cm for 40 min. After electrophoresis, gels were stained with ethidium bromide and photographed under ultraviolet illumination. One unit of topoisomerase II activity was defined as the greatest dilution of enzyme that could unknot 0.3 μg of knotted P4 phage DNA completely under the standard reaction conditions.

RESULTS

Establishment of Variant Cells Resistant to VP16 VP16 resistant clones were established as described in "Materials and Methods." Plating efficiencies of FM3A parent and variant cells were in the range of 40–60%. Figure 1 shows clonogenic survival curves of parent and 4 variant cells in different concentrations of VP16. The dose-response curve of FM3A parent cells showed a sharp decline in colony formation at drug concentrations above 0.01 μg/ml; the IC$_{50}$ value was 0.05 μg/ml. All 4 variant cells proved to be much more resistant to VP16 as compared to the parent cells; the IC$_{50}$ values were 1.7–2.3 μg/ml. Based upon their IC$_{50}$ values, these variant cell lines were approximately 40-fold more resistant to VP16 than the parent cells.

Cross-resistance of Variant Cells to Other Antitumor Drugs In order to know whether
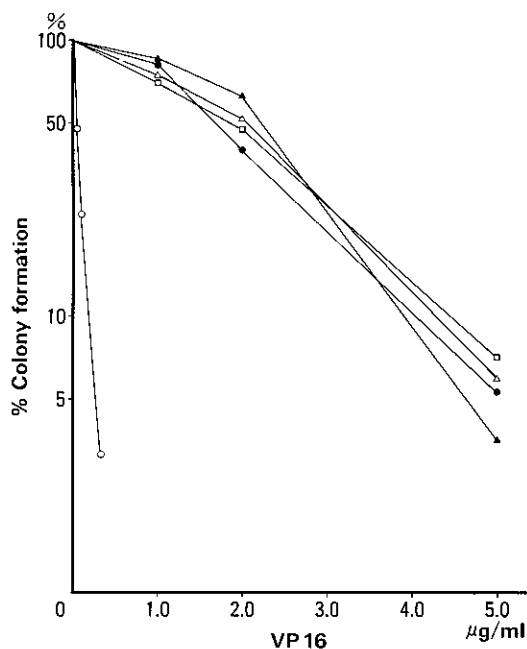
Fig. 1. Effect of VP16 on colony formation of FM3A parent and variant cells. Colony formation in soft agar was measured as described in the text. Parent cells (○), VPr-1 (□), VPr-2 (●), VPr-3 (△), VPr-4 (▲).

Table I. IC_{50} Values (μg/ml) for Various Antitumor Drugs

	Parent cell	VPr-1	VPr-2	VPr-3	VPr-4
VP16	0.05	2.0	1.7	2.0	2.3
VM26	0.004	0.2	0.2	0.1	0.2
4HPCP	0.25	0.25	0.5	0.4	0.6
CDDP	0.007	0.006	0.006	0.009	0.01
BLM	0.02	0.03	0.04	0.03	0.05
VCR	0.001	0.001	0.001	0.001	0.001
MTX	0.015	0.015	0.007	0.015	0.009
ADM	0.009	0.010	0.008	0.009	0.008
MMC	0.007	0.009	0.008	0.007	0.007
Ara-C	0.03	0.015	0.03	0.013	0.025
5-FU	0.02	0.03	0.01	0.025	0.03

The IC_{50} value was estimated from the clonogenic survival curve for each drug. Standard error for each value was within 10%. Other conditions, see the text. Abbreviations: 4HPCP, 4-hydroperoxycyclophosphamide: CDDP, cis-platinum; BLM, bleomycin; VCR, vincristine; MTX, methotrexate; ADM, adriamycin; MMC, mitomycin C; Ara-C, cytosine arabinoside; 5-FU, 5-fluorouracil.

these variant cells had acquired multidrug resistance or not, their sensitivities were tested to a wide variety of antitumor drugs in the same way as described for VP16 (Table I). It was found that all of the variant cell lines also exhibited increased resistance to VM26. The IC_{50} values of 3 out of 4 variant cells were 0.2 μg/ml and that of the other one was 0.1 μg/ml; 50- and 25-fold higher than the IC_{50} exhibited by the parent FM3A (0.004 μg/ml). However, these variant cells did not exhibit any cross-resistance to other antitumor drugs tested (Table I).

Topoisomerase II Activities in Parent and Variant Cells The crude nuclear extracts from parent cells were directly assayed for topoisomerase II activity in order to assess the total activity per cell. There was no significant difference between the parent and variant cells (data not shown). Then the nuclear extracts from the parent and variant cells were partially purified on a Mono S column to separate the desired activity from topoisomerase I, and

topoisomerase II fractions were assayed for sensitivity to VP16 using the same amount of enzyme in each case. The result of a typical experiment is shown in Fig. 2. Final concentrations of VP16 in reaction mixtures were 0, 1, 10, 50, and 100 μg/ml. In the absence of topoisomerase II, the knotted P4 DNA migrated in the gel as linear, circular and various concatemeric forms and the bands appeared smeared (Fig. 2, a and a'). In the presence of topoisomerase II, knotted DNA changed to unknotted form (Fig. 2, b and b'). Addition of 1 μg/ml VP16 had no influence on the activity of topoisomerase II from either parent or variant cells (Fig. 2, c and c'). The inhibition of topoisomerase II by VP16 was observed at the drug concentration of 10 μg/ml or higher, and the extent of inhibition increased in a dose-dependent manner (Fig. 2, d and e, d' and e'). Topoisomerase II activity was completely inhibited by 100 μg/ml of VP16 (Fig. 2, f and f'). As shown in Fig. 2, the extents of inhibition of topoisomerase II from both parent and variant cells were essentially the same.

Restoration of Sensitivity to VP16 in Variant Cells by Verapamil The effect of verapamil on VP16 resistance was then tested. In a preliminary experiment, it has been noted that
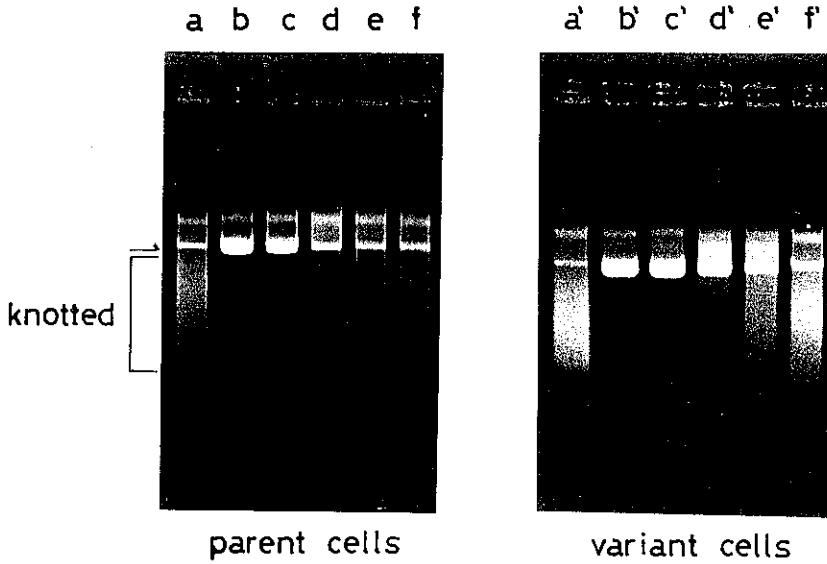
Fig. 2. Sensitivity to VP16 of topoisomerase II from parent and variant cells. Lanes a and a′, P4 knotted DNA only; lanes b–f and b′–f′, 1 unit of topoisomerase II from parent cells and variant cells (VPr-3), respectively. Lanes b and b′, control reactions, without VP16; lanes c and c′, 1 μg/ml of VP16; lanes d and d′, 10 μg/ml of VP16; lanes e and e′, 50 μg/ml of VP16; lanes f and f′, 100 μg/ml of VP16. The arrow indicates unknotted DNA.
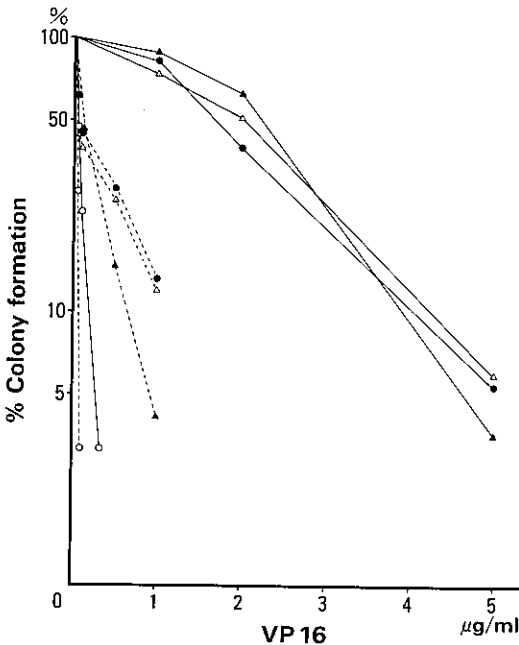
verapamil itself is cytotoxic to FM3A at high concentrations (over $20\mu M$). The relative plating efficiency in the presence of $10\mu M$ verapamil was approximately 80% compared to control, and 95% at $5\mu M$. Therefore, $5\mu M$ verapamil was added to soft agar and sensitivity to VP16 was examined (Fig. 3). Addition of $5\mu M$ verapamil produced only a slight effect on the sensitivity of the parent cells to the drug; the IC_{50} value, 0.03 μg/ml. With variant cells, however, a striking change in the sensitivity to VP16 was observed upon addition of verapamil. The IC_{50} value of each variant cell decreased remarkably (VPr-2, 0.07 μg/ml; VPr-3, 0.05 μg/ml; VPr-4, 0.09 μg/ ml) (Fig. 3). The sensitivity of variant cells to

Fig. 3. Effect of verapamil on the sensitivity to VP16. Sensitivity to VP16 was measured in the presence of $5\mu M$ verapamil (broken lines) with parent cells (○), VPr-2 (●), VPr-3 (△), and VPr-4 (▲). Control experiments in the absence of verapamil are also shown (solid lines). Other conditions were as described in the text.
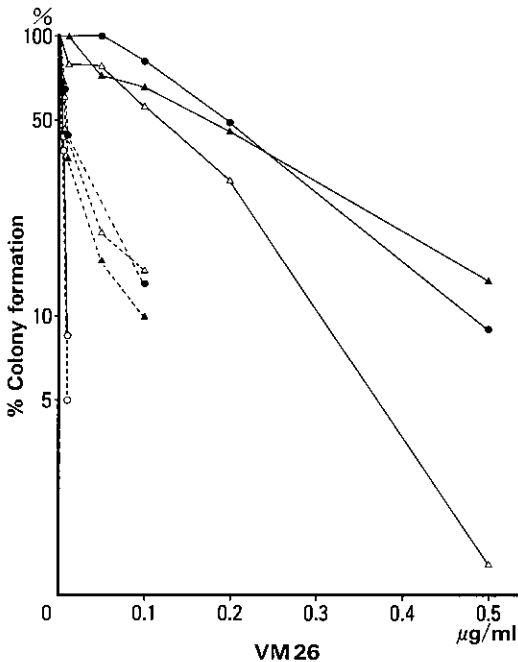
Fig. 4. Effect of verapamil on the sensitivity to VM26. Sensitivity to VM26 was measured in the presence of $5\mu M$ verapamil (broken lines) with parent cells (○), VPr-2 (●), VPr-3 (△), and VPr-4 (▲). Control experiments in the absence of verapamil are also shown (solid lines). Other conditions were as described in the text.

VP16 was thus restored (VPr-2, 24-fold; VPr-3, 40-fold; VPr-4, 25-fold) by the addition of verapamil. Moreover, a similar effect was also seen with respect to VM26 (Fig. 4). In the presence of $5\mu M$ verapamil, the IC_{50} values for VM26 of variant cells were reduced to approximately 0.008 μg/ml, comparable to the value of the parent cells. The sensitivity of the parent cells to VM26 was slightly affected by adding verapamil; the IC_{50} value was 0.003 μg/ml.

DISCUSSION

We isolated FM3A variant cells resistant to VP16 and cross-resistant only to VM26. The mechanism of resistance may well be related to topoisomerase II. However, we failed to detect any difference in topoisomerase II with respect to either total activity per cell or sensitivity to VP16.

Calcium antagonists and calmodulin inhibitors have been found to restore drug sensitivity in resistant cells.[7, 8, 15, 23, 24] One of the mechanisms of resistance is supposed to be an increased efflux of antitumor drugs from cells, which may be controlled by calcium and calmodulin. Calcium antagonists might reduce the formation of calcium-calmodulin complex by lowering the intracellular calcium concentration, or calmodulin inhibitors could directly inhibit the formation of calcium-calmodulin complex.[7, 8]

From this point of view, we examined the effect of a calcium antagonist on the resistant cells. It was found that verapamil markedly increased the VP16-sensitivities of resistant variants, to levels comparable to that of the parent cells. The resistance of the variant cells presented here is thus concluded to be due to an altered cell membrane rather than altered topoisomerase II. In agreement with our results, Yalowich and Ross also reported that verapamil stimulated the effect of VP16.[25] These results strongly suggest that the variant cells have altered permeability to VP16 so as to reduce the intracellular concentration of VP16. Treatment of cells with verapamil might reverse this process, allowing the drug to accumulate in the cells.

Recently, multiple drug resistance has been attributed to 170 kDa P-glycoprotein which is expressed in the cell membrane.[11, 12] A large amount of P-glycoprotein results in the acquisition of resistance to a variety of antitumor drugs. The lack of multi-drug resistance of the variant cells presented here suggests that the cells might have a new mechanism in which the P-glycoprotein is not involved in the acquisition of resistance. The resistance mechanism of our FM3A variant cells may be different from those of previously reported VP16 resistant cells including CHO cells[26, 27] and human cells[28, 29] all of which exhibited multiple drug resistance.

Finally, the striking effect of the calcium antagonist, verapamil, may improve the effectiveness of drugs used in the treatment of cancer in the future.

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