Cancer Chemother Pharmacol (1999) 43: 29–34

ORIGINAL ARTICLE

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Effect of Tween-80 on cell killing by etoposide in human lung adenocarcinoma cells

Received: 22 August 1997 / Accepted: 13 May 1998

Abstract *Purpose*: The non-ionic detergent Tween-80, a surface-active agent, has been shown to modulate the cytocidal effect of certain antitumor agents. In the present study, we sought to determine whether or not Tween-80 could enhance the antitumor effect of etoposide (VP16) in human lung cancer cells in vitro. *Methods*: Survival fractions were measured by growth inhibiton assays of PC14, H69, KB, and PC14/CDDP (the corresponding cisplatin-resistant subline of PC14) cells. An in vitro clonogenic assay of PC14 and PC14/CDDP cells was undertaken after incubation for 10–12 days in RPMI-1640 medium with 20% fetal calf serum and 1.72% methyl cellulose, plus continuous exposure to VP16 with Tween-80. We also investigated the direct toxicity of Tween-80 to PC14 and PC14/CDDP cells using a clonal assay. The intracellular accumulation of VP16 was further analyzed using [^{3}H]VP16 in PC14, PC14/CDDP, A549, KB and H69 cells, and compared with that of daunorubicin (DNR), a hydrophilic anticancer agent, using [^{3}H]DNR in PC14, A549 and KB cells. *Results*: It was found that PC14/CDDP had collateral sensitivity to VP16 and Tween-80 markedly enhanced the killing effect of VP16 not only of PC14 cells but also of PC14/CDDP cells while exerting little cytotoxic effect. Moreover, Tween-80 increased the intracellular accumulation of VP16 in PC14, PC14/CDDP and A549 cells, and not in KB and H69 cells. Tween-80 did not increase the intracellular DNR levels in PC14, A549 and KB cells. *Conclusions*: Tween-80 was shown to potentiate the cytotoxicity of VP16 against several human lung adenocarcinoma cells by increasing the accumulation of VP16 in vitro. Tween-80-mediated sensitization of lung adenocarcinoma cells to VP16 is considered to be related to both the characteristics of the cell membrane in adenocarcinoma cells and the lipotropic properties of VP16. These results suggest that this combination might have the potential to improve the therapeutic index of VP16 in human lung adenocarcinoma.

Key words Tween-80 · Etoposide (VP16) · Human lung adenocarcinoma cells · Drug accumulation

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Introduction

The non-ionic detergent Tween-80, a surface-active agent has wide application as an agent for making lipotropic drugs water soluble. In addition, it has been demonstrated that Tween-80 can modulate the sensitivity of certain antitumor agents in vitro and in vivo [2, 15, 20]. For example, Tween-80 can modulate the sensitivity to anthracyclines, vinca alkaloids and epipodophyllotoxins of multidrug-resistant phenotypes of tumor cells [5, 10, 13] which overexpress P-glycoprotein (P-gp) [1, 7, 8, 18]. Tween-80 also has previously been shown to reverse multidrug resistance in the adriamycin (ADM)-resistant subline of K562 (K562/ADM) [12] by blocking daunorubicin (DNR) and vincristine (VCR) efflux and increasing etoposide (VP16) influx [20]. Furthermore, Tween-80 has been reported to increase the VP16 influx only in K562/ADM cells without increasing that in K562/S cells [20]. The increased influx of VP16 is therefore considered to be related to the characteristics of the cell membrane already altered in K562/ADM cells [16, 19] and to the lipotropic character of VP16 [3, 20].

In the clinical setting, VP16 is one of the most effective anticancer agents currently available for the treatment of malignant tumors such as hematopoietic tumors (for example, acute leukemia and malignant lymphoma), testicular tumors and small-cell carcinoma of the lung. However, for the chemotherapy of non-small-cell lung cancer, VP16 is not a useful drug because of low

sensitivity of the cancer to the drug. On this basis, we were prompted to investigate the potential of Tween-80 to modulate the chemosensitivity of other malignant tumor cells to VP16, especially human non-small-cell lung cancer cells. In the present study, we sought to determine whether or not Tween-80 could enhance the killing effect of VP16 on human lung cancer cells, and also investigated the modulating effect of Tween-80 on the intracellular accumulation of VP16 in several human lung cancer cells.

Materials and methods

Drugs and chemicals

Pure unlabelled VP16 was supplied by Nihon Kayaku Company (Tokyo, Japan). Tween-80 was obtained from Sigma Chemical Company (St. Louis, Mo.). [^{3}H]VP16 (475 mCi/mmol) was obtained from Moravek Biochemical (Brea, Calif.), and [^{3}H]DNR (3.2 mCi/mmol) from New England Nuclear (Boston, Mass.). Protosol and Econofluor were also obtained from New England Nuclear. All other reagents were of reagent grade. Cell culture materials were obtained from GIBCO Laboratories (Grand Island, N.Y.).

Cell lines and culture conditions

The cell lines used in our study included PC14, A549 (human lung adenocarcinoma cell line), PC14/CDDP (The corresponding cisplatin-resistant subline of PC14), KB (human oral epidermoid carcinoma cell line), and H69 (human small cell lung carcinoma cell line). The PC14/CDDP cells were kindly provided by Dr. Nagahiro Saijo (National Cancer Center Research Institute, Tokyo, Japan) [14]. The above cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 100 μg/ml Kanamycin at 37 °C in a humidified incubator under an atmosphere containing 5% CO_2.

Growth inhibition assay

The concentrations of VP16 employed in this study were 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml for PC14 and KB cells, and 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml for PC14/CDDP and H69 cells. The concentrations of Tween-80 were 100 and 250 μg/ml. Cells were suspended at a density of 1×10^5/ml in RPMI-1640 medium supplemented with 10% fetal calf serum. Tween-80 was added from a 100 mg/ml stock solution prepared in deionized distilled water to give final concentrations of 100 and 250 μg/ml. VP16 was also added from a 20 mg/ml stock solution prepared in DMSO to give final concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml. The cells were then incubated for 5 days at 37 °C in a humidified atmosphere of 5% CO_2 in air. Survival fractions were measured after 5 days incubation.

In vitro clonogenic assay

Approximately 400 or 4000 untreated PC14 and PC14/CDDP cells were suspended in RPMI-1640 medium with 20% fetal calf serum and 1.72% methyl cellulose, plus VP16 with or without Tween-80, plated in 35-mm petri dishes (Falcon 3001), and incubated for 10–12 days at 37 °C in a humidified atmosphere of 5% CO_2 in air. The concentrations of VP16 employed in this study were 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml for PC14 cells, and 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml for PC14/CDDP cells. The concentrations of Tween-80 were 100 and 250 μg/ml. Colonies shaped like a balloon, consisting of more than 30 cells, were scored with the aid of an inverted microscope [19]. An additional clonal assay of PC14 and PC14/CDDP cells was undertaken at Tween-80 concentrations of 10, 25, 50, 100, 250 and 375 μg/ml without VP16 under the same conditions of incubation as described above.

VP16 accumulation study

The concentrations of Tween-80 employed in this study were 100 and 250 μg/ml. Untreated PC14, PC14/CDDP, A549, KB and H69 cells were suspended in Falcon 2006 tubes at a density of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum. Tween-80 was added from a 100 mg/ml stock solution prepared in deionized distilled water to give final concentrations of 100 and 250 μg/ml. [^{3}H]VP16 was also added from a 50 nmol/ml stock solution prepared in 99.9% ethanol to give a final concentration of 2 nmol/ml. The cells were then incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After the incubation, 1×10^6 cells were distributed into 15-ml Assist tubes, and washed twice with 10 ml ice-cold RPMI-1640 supplemented with 5% fetal calf serum and once with 10 ml ice-cold PBS by centrifugation and resuspension. Following the washing, 1 ml Protosol was added to dissolve the cells. After 24 h, the cell dissolution products were transferred to scintillation vials with 8 ml Econofluor, and the radioactivities of the cells were measured using an Aloka LSC 1000 liquid scintillation counter [4, 20].

Daunorubicin accumulation study

The concentration of Tween-80 employed in this study was 250 μg/ml and the cell lines were PC14, A549 and KB. The same methods, other than the final concentration of [^{3}H]DNR, were used as for the VP16 accumulation study. [^{3}H]DNR was added from a 10 nmol/ml stock solution prepared in RPMI-1640 medium to give a final concentration of 40 pmol/ml [4, 20]. Each experiment was repeated at least three times.

Results

Growth inhibition assay

Coincubation with Tween-80 had an enhancing effect on the decrease in survival fractions not only of PC14 cells but also of PC14/CDDP cells exposed to VP16. In contrast, in H69 and KB cells exposed to VP16, Tween-80 exerted no enhancing effect on the survival fractions (Fig. 1A,B).

In vitro clonogenic assay of PC14 and PC14/CDDP cells

PC14/CDDP cells showed collateral sensitivity to VP16. Tween-80 enhanced the cytotoxic effect of VP16 not only in PC14 cells but also in PC14/CDDP cells (Figs. 2 and 3). For example, coincubation with Tween-80 reduced the number of colonies by 0.8 log in PC14 cells and 1.6 log in PC14/CDDP cells exposed to 0.5 μg/ml of VP16 at a Tween-80 concentration of 100 μg/ml, and by 0.9 log in PC14 cells and 2.4 log in PC14/CDDP cells exposed to 0.1 μg/ml of VP16 at a Tween-80 concentration of 250 μg/ml. The direct cytotoxicity of Tween-80 on PC14 and PC14/CDDP cells was also evaluated. Cell proliferation was little affected by Tween-80 at

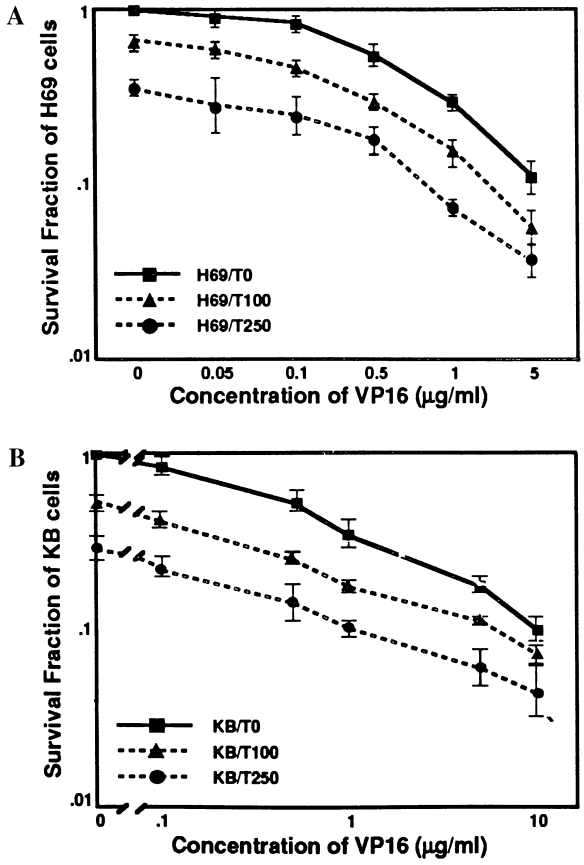

Fig. 1A,B Effect of Tween-80 on cell growth of H69 (**A**) and KB (**B**) cells exposed to VP16. The cells were continuously exposed to VP16 with (▲ 100 μg/ml and ● 250 μg/ml) and without (■) Tween-80 for 5 days. Tween-80 exerted no enhancing effect on the survival fractions. The data shown are the means ± SD of average values obtained from duplicate experiments

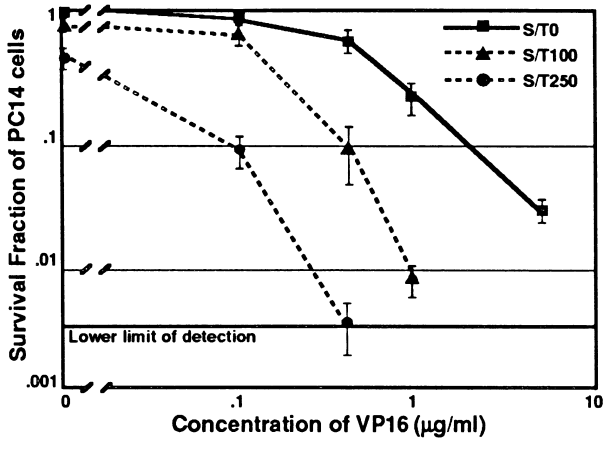

Fig. 2 Effect of Tween-80 on cell killing by VP16 of PC14 cells in a clonogenic assay. The survival fractions of PC14 cells was measured after incubation for 10–12 days. S/T, S/T100 and S/T250 indicate the survival fractions of PC14 cells exposed to VP16 at a Tween-80 concentration of 0 μg/ml (■), 100 μg/ml (▲) and 250 μg/ml (●), respectively. Tween-80 markedly enhanced the cytocidal effect of VP16 on PC14 cells. Tween-80 itself showed little cytotoxicity. The data are the means ± SD of average values obtained from three separate experiments performed on different days

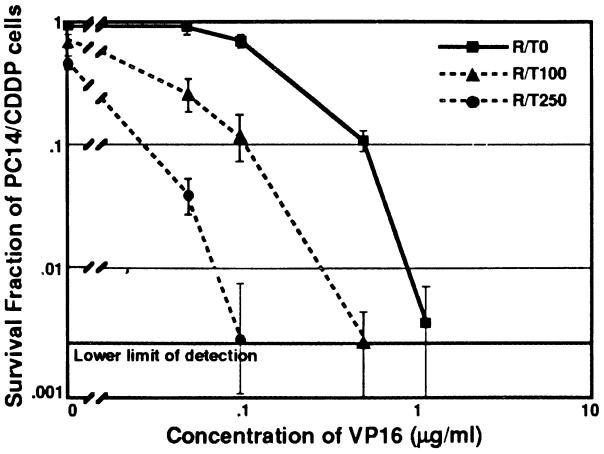

Fig. 3 Effect of Tween-80 on cell killing by VP16 of PC14/CDDP cells in a clonogenic assay. The survival fractions of PC14/CDDP cells was measured after incubation for 10–12 days. R/T0, R/T100 and R/T250 indicate the survival fractions of PC14/CDDP cells exposed to VP16 at a Tween-80 concentration of 0 μg/ml (■), 100 μg/ml (▲) and 250 μg/ml (●), respectively. The PC14/CDDP subline was collaterally sensitive to VP16, and Tween-80 markedly enhanced the cytotoxic effect of VP16 on PC14/CDDP cells. The data are the means ± SD of average values obtained from three separate experiments performed on different days

concentrations up to 100 μg/ml, but the cell growth of both cell lines was moderately inhibited at a Tween-80 concentration of 250 μg/ml (Fig. 4).

Effect of Tween-80 on intracellular VP16 accumulation

Figures 5 and 6 illustrate the VP16 accumulation after exposure to 2 nmol/ml [^{3}H]VP16 with and without

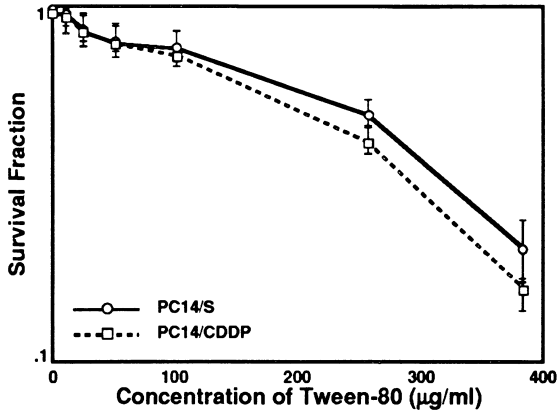

Fig. 4 The direct effect of Tween-80 on PC14 and PC14/CDDP cells in a clonal assay. The survival fractions of PC14 (○) and PC14/CDDP (□) cells were measured after incubation for 10–12 days. Cell proliferation was little affected by Tween-80 at concentrations up to 100 μg/ml, but the cell growth of both cell lines was moderately inhibited at a Tween-80 concentration of 250 μg/ml. The data are the means ± SD of average values obtained from three separate experiments performed on different days

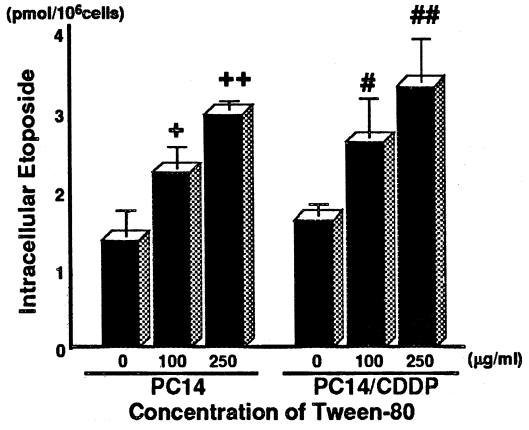

Fig. 5 Modulation of VP16 accumulation by Tween-80 in PC14 and PC14/CDDP cells. The cells were exposed to [^{3}H]VP16 (2 nmol/ml) with and without Tween-80 (100 μg/ml and 250 μg/ml) for 3 h. Tween-80 led to a progressive increase in the cellular level of VP16 in PC14 cells and also in PC14/CDDP cells, as the concentration of Tween-80 increased. The data are the means ± SD of average values obtained from three separate experiments performed on different days. Significant differences between the cellular contents of VP16 with and without Tween-80 were detected by the *t*-test: $^{+}P = 0.009$, $^{++}P = 0.003$ for PC14; $^{\#}P = 0.033$, $^{\#\#}P = 0.031$ for PC14/CDDP

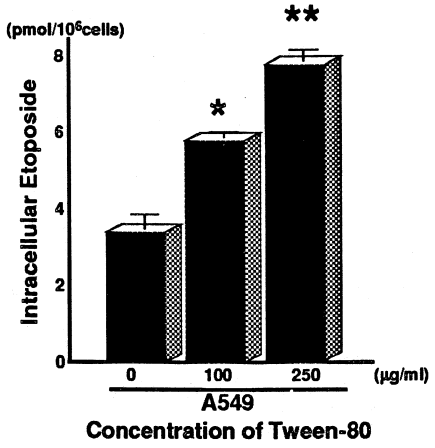

Fig. 7 Modulating effect of Tween-80 on the accumulation of VP16 in A549 cells. The cells were exposed to [^{3}H]VP16 (2 nmol/ml) with and without Tween-80 (100 μg/ml and 250 μg/ml) for 3 h. Tween-80 increased the cellular contents of VP16 in A549 cells in a similar manner to the effects in PC14 and PC14/CDDP cells. The data are the means ± SD of average values obtained from three separate experiments performed on different days. Significant differences between the cellular contents of VP16 with and without Tween-80 were detected by the *t*-test: $^{*}P = 0.014$, $^{**}P = 0.011$

Tween-80 (at 100 and 250 μg/ml) in PC14, PC14/CDDP and KB cells. In H69 cells, only 250 μg/ml of Tween-80 was examined. Tween-80 increased the levels of VP16 in PC14 and PC14/CDDP cells, which were adenocarcinoma cell lines, in a dose-dependent manner. For example, VP16 accumulation was increased 1.62-fold by Tween-80 at 100 μg/ml and 2.17-fold at 250 μg/ml in PC14 cells, and 1.62-fold by Tween-80 at 100 μg/ml and 2.08-fold at 250 μg/ml in PC14/CDDP cells. In contrast, Tween-80 exerted no effect on VP16 accumulation in KB and H69 cells, which are non-adenocarcinoma cell lines.

To determine whether or not Tween-80 could increase the intracellular VP16 level in other human adenocarcinoma cells, we also measured VP16 accumulation in A549 cells coincubated with Tween-80. Figure 7 shows the VP16 accumulation after exposure to 2 nmol/ml [^{3}H]VP16 with and without Tween-80 (at 100 and 250 μg/ml). Tween-80 increased the cellular level of VP16 1.70-fold at 100 μg/ml and 2.30-fold at 250 μg/ml in the A549 cells.

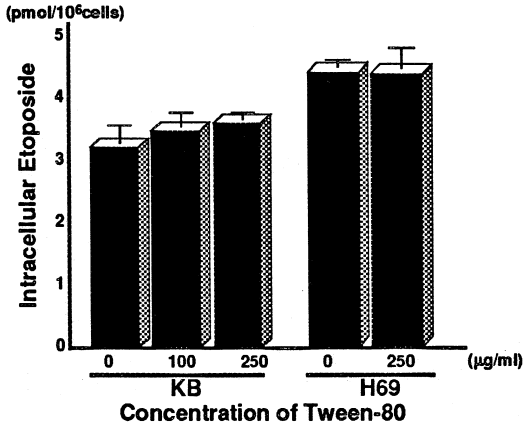

Fig. 6 Effect of Tween-80 on the accumulation of VP16 in KB and H69 cells. Tween-80 had no effect on VP16 accumulation in both KB and H69 cells which are non-adenocarcinoma cell lines. The data are the means ± SD of average values obtained from three separate experiments performed on different days. There were no significant differences between the cellular accumulation of VP16 with and without Tween-80

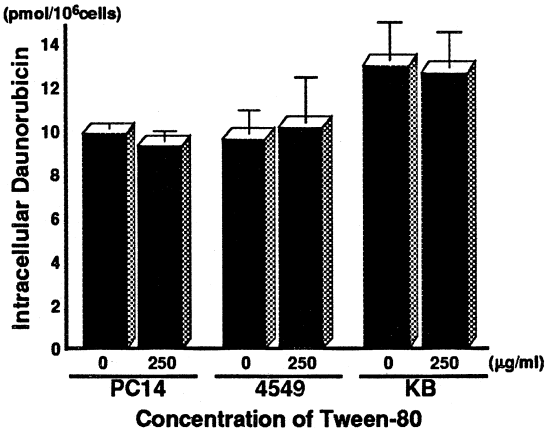

Fig. 8 Effect of Tween-80 on the accumulation of DNR in PC14, A549 and KB cells. Tween-80 had no effect on the DNR accumulation in PC14, A549, and KB cells. The data are the means ± SD of average values obtained from three separate experiments performed on different days. There were no significant differences between the cellular accumulation of VP16 with and without Tween-80 in the three cell lines

Effect of Tween-80 on intracellular DNR accumulation

Figure 8 illustrates the DNR accumulation after exposure to 40 pmol/ml [^{3}H]DNR with and without Tween-80 at 250 μg/ml in PC14, A549 and KB cells which were all adherent cells in in vitro cultures. Tween-80 had no effect on DNR accumulation in the PC14, A549 and KB cells.

Discussion

In the present study, we sought to determine whether or not Tween-80 could enhance the killing effect of VP16 in human lung cancer cells, and also in cisplatin-resistant human lung cancer cells [6, 14], since this could become a very important problem in the chemotherapy of lung cancer. We therefore needed to use cells of the cisplatin-resistant subline PC14 (PC14/CDDP) in our study. PC14/CDDP cells were found to be collaterally sensitive to VP16, and Tween-80 was found to enhance the cytotoxicity of VP16 not only in PC14 cells but also in PC14/CDDP cells. In contrast, the killing effect of VP16 was not enhanced by Tween-80 in H69 (small-cell cancer) and KB (epidermoid cancer) cells. The enhancing effect of Tween-80 on the cytocidal effect of VP16 observed in the growth inhibition assay was confirmed by the clonogenic assay in PC14 and PC14/CDDP cells.

As mentioned in the Introduction, in K562/ADM cells, Tween-80 increases the cytotoxicity of VP16 as a result of an increase in intracellular accumulation (through an increased influx) [20]. We therefore assumed that in PC14 and PC14/CDDP cells the primary cause of the effect of Tween-80 in increasing the cytotoxicity of VP16 was also an increase in the intracellular levels. Indeed, consistent with this assumption in the accumulation study, the intracellular levels of VP16 increased in PC14 and PC14/CDDP cells exposed to VP16 with Tween-80, but did not increase in H69 and KB cells.

To determine whether or not similar results could be obtained in other human lung adenocarcinoma cells, an identical accumulation study to that using PC14 and PC14/CDDP cells was repeated using A549 cells. The outcome was the same as for PC14 and PC14/CDDP cells: the intracellular VP16 level was increased in the A549 cells by Tween-80. Accordingly, this phenomenon might be specific to adenocarcinoma cells, although it remains unknown whether this phenomenon is a characteristic only of lung adenocarcinoma cells or also occurs in adenocarcinoma cells of other organs.

To investigate the underlying mechanism of the increased accumulation of VP16 elicited by Tween-80, we evaluated the intracellular levels of DNR, a hydrophilic anticancer agent. DNR accumulation was not increased by Tween-80 in PC14 cells, A549 cells or KB cells (these three cell lines are adherent cell lines). Based on the above results, therefore, although we did not clarify the detailed processes of VP16 influx and efflux, the following hypothesis can be put forward: owing to its lipotropic character, VP16 might become more readily transported through the cell membrane by Tween-80, a surface-active agent [3, 9]. On the other hand, Tween-80 has been shown not to enhance VP16 accumulation in K562/S cells, in contrast to its effect in K562/ADM cells [20], because the effect of VP16 arises only at cell membranes already altered [16]. On this basis, the membrane of lung adenocarcinoma cells is considered to have undergone modification beforehand [17, 21] (although the precise kind of change still remains unknown).

Furthermore, Tween-80 has been found to activate Na^+/K^+ ATPase and Mg^{2+} ATPase at low concentrations [11]. Further investigations on the relationship between the activating effects of Tween-80 on Na^+/K^+ ATPase and Mg^{2+} ATPase and the specific phenomenon of the already altered cell membrane in adenocarcinoma cells are thus required. As mentioned in the Introduction, VP16 is not at present a key drug for the chemotherapy of human lung adenocarcinoma. The low sensitivity to its action is thought to be caused mainly by a decline in its accumulation. Although not yet tested in vivo, it is possible that the biochemical modulation of VP16 by Tween-80 could make VP16 a key drug in the chemotherapy of human lung adenocarcinoma. In particular, in cases of human lung adenocarcinoma that have acquired cisplatin resistance [6, 14], treatment based on the increased sensitivity to VP16 elicited by Tween-80 is considered to have good clinical potential.

Further studies on the effects of Tween-80 on adenocarcinoma cells (including those of the lung and other organs) should help to clarify the unique characteristics of the cell membrane in cancers that display little sensitivity to certain antitumor agents.

In conclusion, Tween-80 potentiated the cytotoxicity of VP16 against not only lung adenocarcinoma cells but also its corresponding cisplatin-resistant lung adenocarcinoma cells by increasing accumulation of VP16 in vitro. Tween-80-mediated sensitization of lung adenocarcinoma cells to VP16 is considered to be associated with the characteristics of the cell membrane in lung adenocarcinoma cells and the lipotropic properties of VP16. If the increased sensitivity to VP16 elicited by Tween-80 in vitro correlates with in vivo activity, it is considered to have a good potential for the practical treatment of human lung adenocarcinoma.

Acknowledgement This work was supported by aid kindly provided by Dr. Nagahiro Saijo, National Cancer Center Research Institute, Tokyo, Japan.

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