Cross-resistance to tumour promoters in human cancer cell lines resistant to adriamycin or cisplatin

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Summary The growth inhibitory effect of tumour promoters on human leukaemia and lung cancer cell lines was examined using the [3-(4,5 dimethylthiazol)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. The four cell lines used were the K562 human leukaemia cell line, its adriamycin (ADM)-resistant subline (K562/ADM), which shows the *mdr* phenotype, PC-9 (a human lung adenocarcinoma cell line) and its cisplatin (CDDP)-resistant subline (PC-9/CDDP), which does not show the *mdr* phenotype. Phorbol 12-tetradecanoate-13-acetate (TPA) and the TPA-type tumour promoters, aplysiatoxin and debromoaplysiatoxin, inhibited the growth of the two parental cell lines, K562 and PC-9. The non-TPA-type tumour promoter, okadaic acid, also inhibited the growth of the two parental cell lines in a dose-dependent manner. TPA-type and okadaic acid inhibited the growth of K562/ADM more weakly than that of K562, and showed no growth inhibition in PC-9/CDDP. Anhydrodebromoaplysiatoxin, an inactive derivative of the TPA-type tumour promoter, could suppress the growth of K562 and K562/ADM only at high concentration (more than 50 μM) and it showed similar growth inhibitory effects on the two cell lines. Okadaic acid tetramethyl ether, the inactive form of the non-TPA-type tumour promoter did not inhibit the growth of any of the cell lines. The growth inhibitory effect of these compounds was well correlated with their tumour-promoting activity. A study of the accumulation of okadaic acid revealed that the amount of ³H-okadaic acid in K562/ADM and PC-9/CDDP was similar to that in their parental cells indicating that cross-resistance to this tumour promoter in the drug-resistant cell lines is not due to a difference in the amount of drug accumulated in sensitive and resistant cells. These results suggest the presence of another common mechanism for resistance to ADM and CDDP as well as to TPA- or non-TPA-type tumour promoters.

Phorbol esters such as TPA have several biological activities. TPA activates protein kinase C (PKC) which has been considered to be a key enzyme involved in promotion, cell differentiation and proliferation. On the other hand, there are several reports that activation of protein kinase C by phorbol ester modulates resistance to anticancer drugs such as vincristine and etoposide in breast cancer or small cell carcinoma cell lines (Ferguson & Cheng, 1987). Modulation of drug resistance by TPA is possibly due to phosphorylation of P-glycoprotein, although the modulatory effects are transient (Hamada *et al.*, 1987). To investigate this, we examined the sensitizing effects of TPA to anticancer drugs using the K562/ADM cell line which possesses the *mdr* phenotype and its parental cell line K562. Modulatory effects of TPA were not however observed in this assay system (unpublished data). However, in the course of these studies, we observed that TPA showed a growth inhibitory effect on K562 and K562/ADM cells but did not induce differentiation in these cells. Because of this result, we considered that these cells may be useful to study the growth inhibitory effects of tumour promoters in the absence of cell differentiation. We also found TPA to have different inhibitory effects on the growth of parental and drug-resistant cell lines. K562/ADM and PC-9/CDDP revealed cross-resistance to TPA. These data suggested that tumour promoters may be useful compounds in the elucidation of new mechanisms of drug resistance.

On the other hand, recent reports suggest that some compounds such as bryostatin II, which have activity resembling that of phorbol ester, show cytotoxic effects towards some tumour cells. The mechanisms of antitumour activity of these compounds are considered to be activation of a specific isozyme of PKC, or down regulation of PKC. We considered that the elucidation of the relationship between the growth inhibitory effects and the tumour promoting activity of tumour promoters would be useful not only for the develop-

ment of new anticancer drugs, but also for the elucidation of unknown mechanisms of resistance to drugs. We have, therefore, examined the growth inhibitory effect of several tumour promoters on drug sensitive and resistant cell lines.

Materials and methods

Cell lines The human leukaemia cell line, K562, and the human lung cancer cell line, PC-9 together with their drug-resistant sublines were used for the study. K562 was established from a patient with chronic myelocytic leukaemia. Its adriamycin-resistant subline, K562/ADM, was kindly donated by Professor T. Tsuruo, Tokyo University. PC-9, derived from a previously untreated patient with adenocarcinoma of the lung, was provided by Professor Y. Hayata, Tokyo Medical College. Its cisplatin-resistant subline, PC-9/CDDP was established by continuous exposure to a stepwise-increasing concentration of cisplatin (to 0.1 $\mu\text{g ml}^{-1}$) and selection by limiting dilution technique. This cell line showed 4.2-fold resistance to cisplatin compared to the respective parental cell line, PC-9, based on the 50% inhibitory concentrations (IC_{50}) in a tetrazolium dye (MTT) assay with continuous drug exposure (Hong *et al.*, 1988). All cell lines grew in RPMI 1640 medium (Gibco Laboratories, Tokyo Japan) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U ml^{-1}) and streptomycin (100 $\mu\text{g ml}^{-1}$) (RPMI-FBS) in a humidified 5% CO_2 atmosphere at 37°C. All cell lines grew mainly as floating aggregates, although PC-9 was also partially attached to the surface of the culture flask. The lines were subcultured once or twice per week.

Tumour promoters TPA and the TPA-type tumour promoters, aplysiatoxin and debromoaplysiatoxin, and a non-TPA-type tumour promoter, okadaic acid, were used in these experiments. Anhydrodebromoaplysiatoxin and okadaic acid tetramethyl ether were used as inactive, control compounds. TPA was purchased from Sigma Chemical Co. St. Louis MO. Other tumour promoters and their derivatives, aplysiatoxin and debromoaplysiatoxin have an acetogenic, phenolic bislactone structure (Figure 1). Okadaic acid is a

Figure 1 Structures of tumour promoters.

toxic polyether compound of a C38 fatty acid. Structures of the tumour promoters are shown in Figure 1. [27-³H]okadaic acid was synthesised as reported previously (Suganuma *et al.*, 1989). Tumour promoters were dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and stored at -70°C until use.

Growth inhibition assay The MTT assay used was essentially the same as that previously reported by Mosmann (1983). This assay is dependent on the reduction of MTT (3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) mitochondrial dehydrogenase in viable cells to a blue formazan product that can be measured spectrophotometrically. Single cell

suspensions were obtained by mechanical disaggregation of the cell lines. After viability had been confirmed, the suspensions were diluted with RPMI-FBS to the seeding concentrations, and plated in 96-well microculture plates (Falcon 3072). The cells were plated 10³ cells per well in 200 µl of medium. Anticancer agents and tumour promoters were dissolved in DMSO and diluted with RPMI + FBS to give a final concentration of DMSO of 0.1%. Tumour promoters at various concentrations were added in 20 µl culture medium, the control culture receiving 20 µl of RPMI containing 0.1% DMSO. The plates were then incubated at 37°C in a CO₂ incubator. After 4 days of drug treatment, 20 µl of MTT solution (5 mg ml⁻¹ in PBS, Sigma) was added into each well

of the culture and the plates incubated at 37°C for a further 4 h. After centrifugation of the plates at 1,500 r.p.m. for 5 min, the medium was aspirated from the wells as completely as possible without disturbing the formazan crystals and cells on the plastic surface. A volume of 200 µl of DMSO was then added to each well and the plate was agitated on a plate shaker for 5 min resulting in good solubilization of the formazan crystals. The optical density (O.D.) was measured at 560 and 690 nm using a Titertek Multiskan MCC spectrophotometer (Flow Laboratories Japan Inc., Tokyo, Japan). As a background, a well containing only RPMI-FBS plus MTT containing 0.1% DMSO was used. Each experiment was performed in triplicate. The IC₅₀ was defined as the concentration needed for 50% reduction of optical density in each test and fractional absorbance was calculated as (mean absorbance in three test wells – absorbance in a background well)/(mean absorbance in three control wells – absorbance in a background well) × 100. Relative resistance was defined as IC₅₀ for resistant subline/IC₅₀ for parental cell line.

Intracellular uptake of [27-³H] okadaic acid K562, K562/ADM, PC-9 or PC-9/CDDP cells (1 × 10⁶) in 0.5 ml of RPMI-FBS were incubated at 37°C for 30 min in a shaking water bath, then at 37°C in the presence of various concentrations of [³H]okadaic acid solution. The specific activity of labelled okadaic acid was 14 Ci nm⁻¹. At 2 h intervals, the suspensions were well mixed and 0.5 ml quantities were transferred to Eppendorf tubes containing 0.5 ml of a mixture of silicon oil (HIVAC-F-4, Shin-Etsu Chemical Co. Ltd., Tokyo, Japan) and paraffin oil (Wako Pure Chemical Co. Ltd., Osaka, Japan) (50:15), and centrifuged at 15,000 r.p.m. for 3 min. After removal of the supernatant the pelleted cells were lysed overnight with 1 ml of 1% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, Calif., USA) and transferred to scintillation vials and 10 ml of scintillator cocktail (ACS II, Amersham Japan Ltd., Tokyo, Japan) was added. The radioactivity was counted in a Beckman LS 3801 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif., USA). The experiment was repeated in order to confirm the reproducibility.

Assay of tumour promoting activity Carcinogenesis was initiated by a single application of 100 µg of DMBA dissolved in 0.1 ml of acetone to the skin of the backs of 8-week-old female CD-1 mice as reported previously (Fujiki *et al.*, 1989a). Starting one week after initiation, 2.5 µg of a tumour promoter, dissolved in 0.1 ml of acetone, was applied to the same area on the mice, twice weekly. Control groups were treated with DMBA alone. Each group consisted of 15 mice. Tumour promoting activity was evaluated macroscopically in terms of percentage of tumour-bearing mice and average number of tumours per mouse. Histological evaluation was not performed in this experiment. The percentages of tumour-bearing mice and average number of tumours per mouse were recorded 30 weeks after the beginning of tumour promotion as reported previously (Fujiki 1989b).

Results

Drug resistance of cell lines The adriamycin-resistant cell line K562/ADM, was 8.4 times more resistant to adriamycin than K562 but did not show cross-resistance to cisplatin. On the other hand, the cisplatin resistant line PC-9/CDDP was 4.2-fold resistant to cisplatin compared with PC-9, but showed no cross-resistance to adriamycin. This is consistent with the evidence that the *mdr-1* gene is not involved in cisplatin resistance. Doubling times of K562 and K562/ADM were 16.8 h and 12.0 h, respectively, while doubling times of PC-9 and PC-9/CDDP were 62.4 h and 52.8 h respectively. (Table I).

Growth inhibitory effects of tumour promoters The growth inhibitory effects of TPA and okadaic acid, as well as their derivatives without tumour-promoting activity in these cell lines, were determined by MTT assay. The growth of these cell lines was inhibited by TPA and okadaic acid in a dose-dependent manner. However, K562/ADM was relatively resistant to both tumour promoters and the cross-resistance indices were 15.0 and 3.6 for TPA and okadaic acid, respectively. On the other hand, both cell lines were quite resistant to the inactive derivatives, anhydrodebromoaplysiatoxin and okadaic acid tetramethyl ether. There was no difference in the growth inhibitory effects of these compounds on K562 and K562/ADM cells. PC-9 cells were relatively resistant to TPA and okadaic acid compared with K562, and PC-9/CDDP cells were completely resistant to all the compounds (Table II).

The growth inhibitory activities of the other two TPA-type tumour promoters such as aplysiatoxin and debromoaplysiatoxin with moderate tumour promoting activity were tested against the four cell lines (Table II). Both compounds showed lower growth inhibitory effect on K562, K562/ADM and PC-9 cells than those produced by TPA. From these results, there seems to be some correlation between the growth inhibitory and tumour promoting activity of the tumour promoters.

Correlation between tumour promoting activity and growth inhibition by tumour promoter in K562 and K562/ADM The correlation between tumour promoting activity and growth

Table I Characteristics of resistant cell lines

Cell line	ADM ^a IC ₅₀ ^c (nM ml ⁻¹)	CDDP ^b IC ₅₀ ^c (nM ml ⁻¹)	Doubling time (h)
K562	13.3		16.8
K562/ADM	112.1 (8.4 ^d)		12.0
PC-9		11.5	62.4
PC-9/CDDP		48.0 (4.2) ^d	52.8

^a ADM: adriamycin, ^b CDDP: cisplatin, ^c IC₅₀: the drug concentration reducing the fractional absorbance in the MTT assay to 50%, ^d Relative resistance value: IC₅₀ of resistant cell lines/IC₅₀ of parent cell lines.

Table II Growth inhibitory effect and tumour promoting activity of tumour promoters

Tumour promoter	IC ₅₀ ^a (pM)				Tumour promoting activity (Number of tumours/ mouse in 30 weeks)
	K562	K562/ADM	PC-9	PC-9/CDDP	
TPA ^b	0.3	4.5 (15.0) ^c	15.6	> 1620 (> 104)	11.0
Aplysiatoxin	19.3	24.1 (1.7)	19.8	> 760 (> 38.5)	3.4
Debromoaplysiatoxin	> 3460	> 3460	1505	> 8650 (> 5.7)	2.9
Anhydrodebromoaplysiatoxin	69.6	69.6 (1.0)	> 5220	> 5220	0.4
Okadaic acid	2.2	7.9 (3.6)	24.0	> 372 (> 19.4)	4.6
Okadaic acid tetramethyl ether	> 25200	> 25200	> 25200	> 25200	0.0

^a IC₅₀: the drug concentration inhibiting the growth of tumour cells by 50%; ^b TPA: Phorbol 12-tetradecanoate-13-acetate; ^c Relative resistance value: IC₅₀ of resistant cell line/IC₅₀ of parent cell line.

inhibition by these compounds was further examined in K562 and K562/ADM cells (Figure 2). The tumour-promoting activity was expressed as the average numbers of tumours per mouse at 30 weeks of tumour promotion as described in 'Material and methods' and was found to be well correlated with the IC_{50} s in K562 and K562/ADM cells. The correlation coefficients between these two parameters were $r = 0.915$ and 0.822 . There was a similar trend in PC-9/CDDP cells. However, it was impossible to calculate the correlation coefficient because the IC_{50} value in PC-9/CDDP could not be obtained for the majority of compounds.

Accumulation of 3H -okadaic acid In order to answer the question of whether the sensitivity to a tumour promoter is associated with a difference in drug accumulation, a study of [3H]okadaic acid accumulation was carried out in these four cell lines. Labelled TPA and other TPA-type tumour promoters were not used in this experiment, because the majority of TPA-type tumour promoters bind to membrane located receptors for phorbol ester and thus are not suitable for such a direct accumulation study. There were no differences in the accumulation of 3H -okadaic acid between K562 and K562/ADM (Figure 3, Table III) or between PC-9 and PC-9/CDDP (Table III). The efflux of 3H -okadaic acid

Figure 2 Correlation between tumour promoting activity and growth inhibition by tumour promoter K562 (○), K562/ADM (●).

Figure 3 Kinetics of intracellular net uptake and efflux of 3H -okadaic acid in K562 (○) and K562 ADM (●).

Table III Accumulation of 3H -okadaic acid

3H -OA (nM)	Intracellular 3H -okadaic acid (dpm/ 2×10^6 cells)			
	K562	K562/ADM	PC-9	PC-9/CDDP
2 ^a	569.2	408.2	358.0	290.8
10	1089.5	2775.2	2049.0	1739.8
50	4718.2	5080.2	7334.2	9575.7

^a Concentration of 3H -okadaic acid.

was also evaluated in K562 and K562/ADM. There was no difference in the efflux of 3H -okadaic acid between these cell types. The slow efflux of 3H -okadaic acid in both cells suggests that okadaic acid may bind with an intracellular substrate (Figure 3). These results suggest that the cross-resistance to tumour promoters in drug-resistant cells is not caused by changes in drug uptake and efflux.

Discussion

Inherent and acquired resistance have been considered to be the main cause of failure in cancer chemotherapy. Several factors have been demonstrated to be involved in such resistance. Two resistant cell lines K562/ADM and PC-9/CDDP, were used in this study. K562/ADM has a typical (multidrug-resistance) phenotype and its main mechanism of resistance to adriamycin is thought to be the increased efflux of the anticancer drug caused by overexpression of P-170 glycoprotein coded by the *mdr-1* gene (Tsuruo *et al.*, 1986; Fojo *et al.*, 1985; Katner *et al.*, 1983; Ueda *et al.*, 1986). We confirmed the overexpression of the *mdr-1* gene in K562/ADM (Minato *et al.*, 1990) and we demonstrated that efflux of adriamycin was more than 2-fold greater in K562/ADM than in K562. Although K562/ADM was only 8.4-fold resistant to adriamycin by the MTT assay, it showed about 130-fold resistance in a growth inhibition assay. (Horichi *et al.*, 1990). These results are consistent with the characteristics of K562/ADM reported by Tsuruo *et al.* (1986). On the other hand cisplatin resistance has usually been considered to be multifactorial. We have recently demonstrated that an increase of glutathione (Fujiwara *et al.*, 1990) and a decrease in the formation of DNA interstrand crosslinking due to a decreased uptake of cisplatin (Bungo *et al.*, 1990) are both involved in cisplatin resistance. It has however been demonstrated that expression of the *mdr-1* gene is not related to cisplatin resistance (Nakagawa *et al.*, 1988).

In the present study, K562/ADM and PC-9/CDDP cells showed cross resistance to TPA-type and non-TPA-type tumour promoters. The inactive derivatives did not, however, produce any effects on the growth of parental and resistant cells except for the high concentration of anhydrotetrabromoaplysiatoxin in K562 and K562/ADM. We have also demonstrated that growth inhibition by tumour promoters correlates well with their tumour-promoting activity. This result suggests the possibility of a common mechanism for tumour-promoting activity and growth inhibition by tumour promoters. The same phenomenon was observed for both TPA- and non-TPA-type tumour promoters. We therefore consider that TPA- and non-TPA-type tumour promoters may be useful in the elucidation of the common mechanisms of anticancer drug resistance. TPA-type tumour promoters, such as TPA, aplysiatoxin, and debromoaplysiatoxin, activate protein kinase C (PKC) by binding to its phorbol ester binding site (Fine *et al.*, 1988). Unlike TPA and aplysiatoxin, okadaic acid does not inhibit the specific binding of 3H -TPA to PKC, and does not activate PKC *in vitro* (Fujiki & Sugimura, 1987). This is why okadaic acid is classified as a non-TPA-type tumour promoter. PKC is believed to be related to drug resistance because: of the association of a 20-kD phosphoprotein with the *mdr* phenotype in human breast and small cell lung cancer cell lines (Fine *et al.*, 1985, 1986); of reversibility of the *mdr* phenotype by calmodulin antagonists which also inhibit protein kinases (Tsuruo *et al.*, 1983); of high baseline PKC activity in an adriamycin-resistant

subline of a human nasopharyngeal carcinoma cell line compared with the parental cell line (Cowan *et al.*, 1982); and increased vincristine efflux and resistance by the activation of PKC in drug-sensitive MCF-7 cells (Fine *et al.*, 1986). Indeed, the baseline PKC activity in K562/ADM cells is about 2-fold higher than that in K562 (unpublished data), and a phosphoprotein of about 20 kDa is observed only in K562/ADM cells labelled with ^{32}P -ATP (unpublished data). These findings suggest that decreased drug accumulation in K562/ADM may be associated with a change in phosphorylation of P-glycoprotein and/or 20 kDa phosphoprotein which is reversible by TPA-type tumour promoters. However, it is questionable whether a similar phenomenon occurs in PC-9/CDDP because increased drug efflux is not involved in the resistance of this line. K562/ADM and PC-9/CDDP

also showed cross-resistance to okadaic acid which does not activate PKC. There was no difference in the accumulation of ^3H -okadaic acid in the sensitive and the resistant cell lines. These results therefore suggest that the cross-resistance to okadaic acid in drug-resistant cells is not caused by changes in drug uptake and efflux. Recently, it has been demonstrated that okadaic acid can act on cells through inhibition of protein phosphatases which induced 'apparent' activation of protein kinases (Sassa *et al.*, 1989). Therefore, we consider that 'apparent' phosphorylation of cellular proteins might influence the drug sensitivity of cells. This is the first report demonstrating that adriamycin and cisplatin resistant cell lines each show cross-resistance to TPA- and non-TPA-type tumour promoters.

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