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Characteristic Cross Resistance to Anti-Cancer Drugs and Different Responses for Various Growth Factors in Cisplatin Resistant-Human Ovarian Cancer Cell Line (TYK-R10)

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Summary. **Cis-diamminedichloroplatinum (II) (CDDP) resistant-human ovarian cancer cell line (TYK-R10) was examined for its drug resistance to other anti-cancer drugs, and growth response to various growth factors, compared with the parental cell line (TYK-nu). TYK-R10 was established by a gradual increase in the concentrations of CDDP compared with the parental line. TYK-R10 showed about a ten-fold resistance, with 1×10^{-5} M CDDP of IC_{50}, which represents the concentration of an anti-cancer drug to produce a 50% inhibition of cell growth. TYK-R10 revealed a cross resistance to various anti-cancer drugs including carboplatin (CBDCA), vincristin (VCR) and adriamycin (ADR). However, no significant alternations in topoisomerase II, glutathione-S-transferase levels or glutathione concentrations were detected. In addition, P-glycoprotein (P-gp) was not expressed on the cell surface of TYK-R10 and verapamil had no reverse effect on resistance to CDDP, CBDCA, VCR and ADR. Surface marker analysis using epidermal growth factor receptor antibody disclosed a high positivity in TYK-R10, compared with TYK-nu, and consequently epidermal growth factor activated the growth of TYK-R10. Transforming growth factor α had no effect on the growth of TYK-R10, whereas it promoted the growth of TYK-nu. In contrast, transforming growth factor β promoted the growth of TYK-nu, whereas it inhibited the growth of TYK-R10. These results indicate that the mechanism of multidrug resistance of TYK-R10 is not mediated through P-gp, and that altered responsibility for some growth factor in TYK-R10 is closely associated with the acquisition of CDDP resistance.**

Key words—cisplatin, multidrug resistance, growth factors, ovarian cancer cell line.

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INTRODUCTION

Cis-diamminedichloroplatinum (II) (CDDP), an effective therapeutic agent, is widely used for various solid malignant tumors, including ovarian cancer. There is now considerable evidence that DNA is a principal intracellular target of CDDP action, and that the formation of CDDP-DNA cross-links can inhibit DNA replication.[1] There appear to be various mechanisms of CDDP resistance. Some studies have shown a decrease in DNA intrastrand cross-links in proportion to the degree of tumor cell CDDP resistance,[2] whereas others have claimed that this is not always the case. Cross resistance to the CDDP-analogues that are different platinum compounds is generated by a common mechanism.[3] However, the mechanism involved in the induction of CDDP-resistance is not well understood. There are four possible mechanisms for the induction of resistance: 1) decreased cellular accumulation of CDDP; 2) increased in glutathione-S-transferase (GST) activity or glutathione (GSH) levels; 3) increased levels of intracellular metallothioneins (MTs); and 4) enhanced ability of DNA repair. On the other hand, drug resistant cells show different responses for cytotoxic cytokines or cell mediated cytokines. We and some other authors demonstrated the lack of any effect of tumor necrosis factor α or interferon γ in drug resistant leukemia cells.[4] These findings indicate limitations for the therapeutic application of growth factors. The design of effective clinical strategies for overcoming CDDP resistance would be greatly facilitated by a more complete understanding of the various mechanisms that may operate along different cell lines.

In this study, we have compared additional fea-

tures of parental cell line (TYK-nu) and resistant-human ovarian cancer cell line (TYK-R10) in drug resistance and cell proliferation involved with various growth factors to assess the correlation between the acquisition of drug resistance and response patterns for growth factors.

MATERIALS AND METHODS

Cells

An ovarian cancer cell line, TYK-nu, was derived from a patient with ascites of a disseminated state of undifferentiated ovarian carcinoma.[5] We treated it with a gradual increase in the concentrations of CDDP, finally maintaining it at 1×10^{-5} M in a concentration of CDDP, designated TYK-R10. These cell lines were maintained in α-MEM (Irvine, CA, USA) containing 10% fetal bovine serum (IBL, Gunma, Japan), 100 μg/ml streptomycin and 100 IU/ml penicillin G. These cultures were incubated under conditions of absolutely humidified 5% carbon dioxide in air at 37°C. Cell culture was performed on plastic dishes (Corning, Co. New York, USA) and exchanged with a freshly prepared medium every three days.

Drugs

CDDP was purchased from Nihon Kagaku, Ltd., and diammine (1,1-cyclobutanedicarboxylato) platinum (II) (CBDCA) was from the Bristol-Myers Squibb Company. Mitoxantrone (MX) was purchased from Takeda Yakuhin Kogyou, Ltd. 1-β-D arabinofuranosylcytosine (AraC) from Nippon Shinyaku Ltd., (Tokyo, Japan). Etoposide (VP-16) was also from the Bristol-Myers Squibb Company. Adriamycin (ADR) was purchased from Sigma (St. Louise, USA). Vincristin (VCR) was provided by Shionogi pharmaceutics, Ltd., (Osaka, Japan).

MTT assay

The degrees of drug resistance and cross resistance for various drugs were assayed using a MTT assay kit (Funakoshi, Ltd. Tokyo, Japan) as described by Green et al.. Cells of TYK-nu and TYK-R10 were placed in 96 well culture plates (Corning, Co. St. Louis, USA) at a concentration of 2×10^4 cells/100 μl in each well with various concentrations of various drugs, together with or without verapamil (VER), and incubated for 72 h under the same conditions as described above. After incubation, a reagent was added to the plate, mixed thoroughly and incubated for three hours. The absorption rates were obtained using a Biorad Model 210 fluorometer (Biorad, Ltd. Tokyo, Japan) in 577 nm. Dose-response curves of TYK-nu and TYK-R10 were determined and the IC_{50} was defined as concentrations of 50% cell growth inhibition by cell growth curves in each drug.

Monoclonal antibodies

Analysis of surface antigens was performed using the following monoclonal antibodies: epithelial membrane antigen (EMA) (Nichirei, Co. Tokyo, Japan) and carcinoembryonic antigen (CEA) (DAKO, Ltd. Tokyo, Japan) as epithelial markers; JSB-1 (Novocastra Laboratories Ltd., London, UK)[6] and MRK16 (Kyowa Hakko Co., Tokyo, Japan)[6] as surface marker for P-glycoprotein (P-gp); TO73 and TO77 as monoclonal antibodies for VCR resistant leukemic cells[9]; anti-pan-cadherin (pan-Cad) (Sigma, Co. St. Louis, USA) and ICAM-1 (CD58) (IBL) as markers for a cell-to-cell adhesion molecule; c-met (IBL) as a receptor protein for the hepatocyte growth factor (HGF); and an epidermal growth factor receptor antibody (EGF-R-Ab) (Chemicon International, California, USA).

Measurement of GST activity

GST activity was assayed using 1 mM 1 chrolo-2, 4-dinitrobenzene and 1 nM GSH according to the method by Habig et al..[7]

Measurement of GSH concentrations

Cells were tripsynized and centrifuged. The medium was poured off and the packed cells were frozen on dry ice. GSH was measured by the glyoxalase method. The frozen cells were promptly treated with cold $HClO_4$ in ethylenediaminetetraacetic aid (EDTA), and the supernatants obtained by centrifugation were neutralized at 0°C and assayed for their GSH concentrations. An equal volume of medium, concurrently subjected to the same procedure as a negative control, was used as a blank. The assay was validated by using a freshly prepared solution of GSH.

Immunoblotting

Immunoblotting of membranes was carried out as described by Kakihara et al..[8] Membranes and nuclear proteins were transferred to a nitrocellulose membrane using standard procedures. For immunostaining of the blots, the nitrocellulose membrane was incubated overnight with the diluted anti-topoisomerase II antibody (Funakoshi, Tokyo, Japan) and

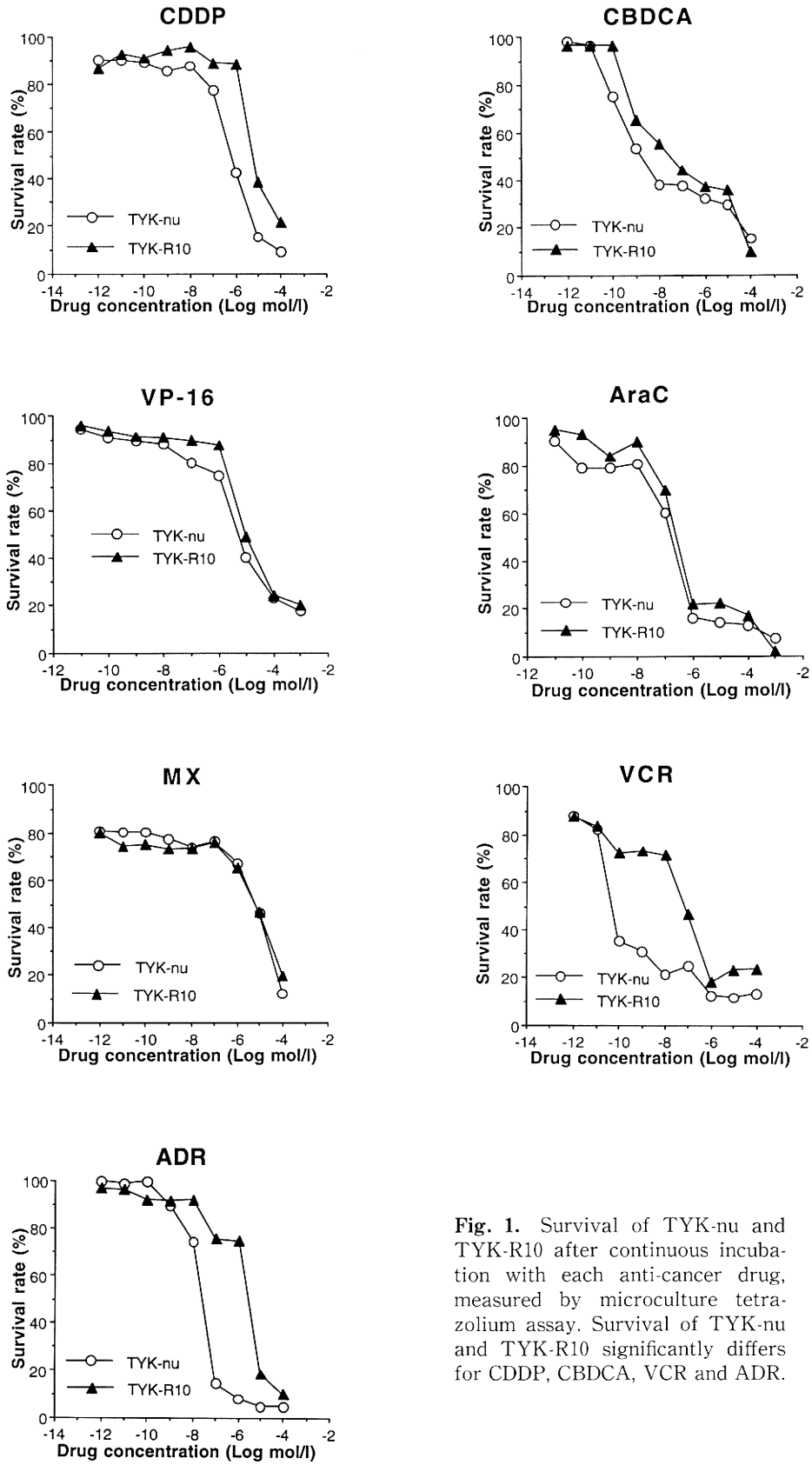

Fig. 1. Survival of TYK-nu and TYK-R10 after continuous incubation with each anti-cancer drug, measured by microculture tetrazolium assay. Survival of TYK-nu and TYK-R10 significantly differs for CDDP, CBDCA, VCR and ADR.

treated by the avidin-biotin complex method using the Nichirei ABC kit (Nichirei, Tokyo, Japan). 3, 3-diaminobenzidine (Dojin, Ltd. Kyoto, Japan) was used as the enzyme substrate.

Thymidine incorporation

^{3}H-thymidine was purchased from Amersham (Tokyo, Japan). 2×10^4 cells of each cell line were suspended in 100 μl medium and incubated for 72 h in 96 hole well plates (Corning, St. Louis, USA) with or without various cytokines including transforming growth factor α (TGF α) (Wakunaga Pharmaceutical Co. Ltd., Tokyo, Japan), transforming growth factor β (TGF β) (Kurashiki Bohseki, Ltd. Kurashiki, Japan), epidermal growth factor (EGF) (Mallinckrodt Speciality Chemicals, Paris, France), insulin-like growth factor I (IGF-I) (Genzyme, Cambridge, USA), and hepatocyte growth factor (HGF) (Chemicon International, California, USA). 0.5 μCi ^{3}H-thymidine was added to each hole and the plates were incubated under similar culture conditions as described above. After incubation for four hours, cells were harvested on glass filters and radioactivity was counted by a liquid scintillation counter (Aloka, Ltd., Tokyo, Japan).

Table 1. Sensitivity measured by microculture tetrazolium assay after continuous drug incubation of TYK-nu and TYK-R10 for various chemotherapeutic drugs

Drug	IC_{50} of TYK-nu	IC_{50} of TYK-R10	RF
CDDP	9×10^{-7}	1×10^{-5}	11.1
CBDCA	2×10^{-9}	6×10^{-8}	30
MX	8×10^{-6}	8×10^{-6}	1.0
AraC	4×10^{-7}	3.8×10^{-7}	1.0
VP-16	4×10^{-6}	1×10^{-5}	2.5
VCR	7×10^{-11}	1×10^{-7}	1430
ADR	4×10^{-8}	5×10^{-6}	125

RF (Resistance factors), defined as IC_{50} of the TYK-R10/IC_{50} of TYK-nu.

Table 2. GST concentration in TYK-nu and TYK-R10

Cell line	GST concentration (nmol/min/mg protein)
TYK-nu	248±41 (n=3)
TYK-R10	294±27 (n=3)

No significant difference between the cell lines is seen (mean±S.E.).

Statistical analysis

Statistical analysis was carried out using paired Students's *t*-test.

RESULTS

Drug resistance of TYK-nu and TYK-R10

The surviving curves shown in Fig. 1 indicate the resistant levels of TYK-nu and TYK-R10 and the IC_{50} values of drugs evaluated (Table 1). TYK-R10 exhibited approximately 11.1-fold resistance to CDDP, compared to TYK-nu. To determine the cross-resistance patterns in both TYK-nu and TYK-R10, several agents were examined. TYK-R10 expressed a cross resistance to platina drugs (CBDCA). Unexpectedly, TYK-R10 showed a cross resistance to ADR, a marked one to VCR, and to VP-16 to a lesser degree. TYK-R10 had no resistance to MX or AraC. The resistance factors, defined as IC_{50} of TYK-R10/IC_{50} of TYK-nu, were 30 in CBDCA, 2.5 in VP-16, 125 in ADR and 1430 in VCR.

Topo II activity

The density of the band in the TYK-R10 extract was compared with that of TYK-nu. There was no significant change in band density in either cell line (data not shown).

GST activity

GST activities (nmol/min/mg protein, mean±S.E.) of each cell line were 248±41, 294±27 for TYK-nu and TYK-R10, respectively (Table 2). There was no statistically significant difference in GST activity.

GSH concentrations

BSO inhibited GSH in both TYK-nu and TYK-R10. TYK-R10 showed slightly higher levels of GSH than TYK-nu ($P<0.05$) (Table 3).

Analysis of surface markers

The results of surface marker analysis are summarized in Table 4. MRK16 and JSB-1, which could detect P-gp, failed to react with either TYK-nu or TYK-R10. In addition, both cell lines showed no positive immunostaining for antibodies of VCR-resistant leukemic cells (TO73 and TO77). Positive

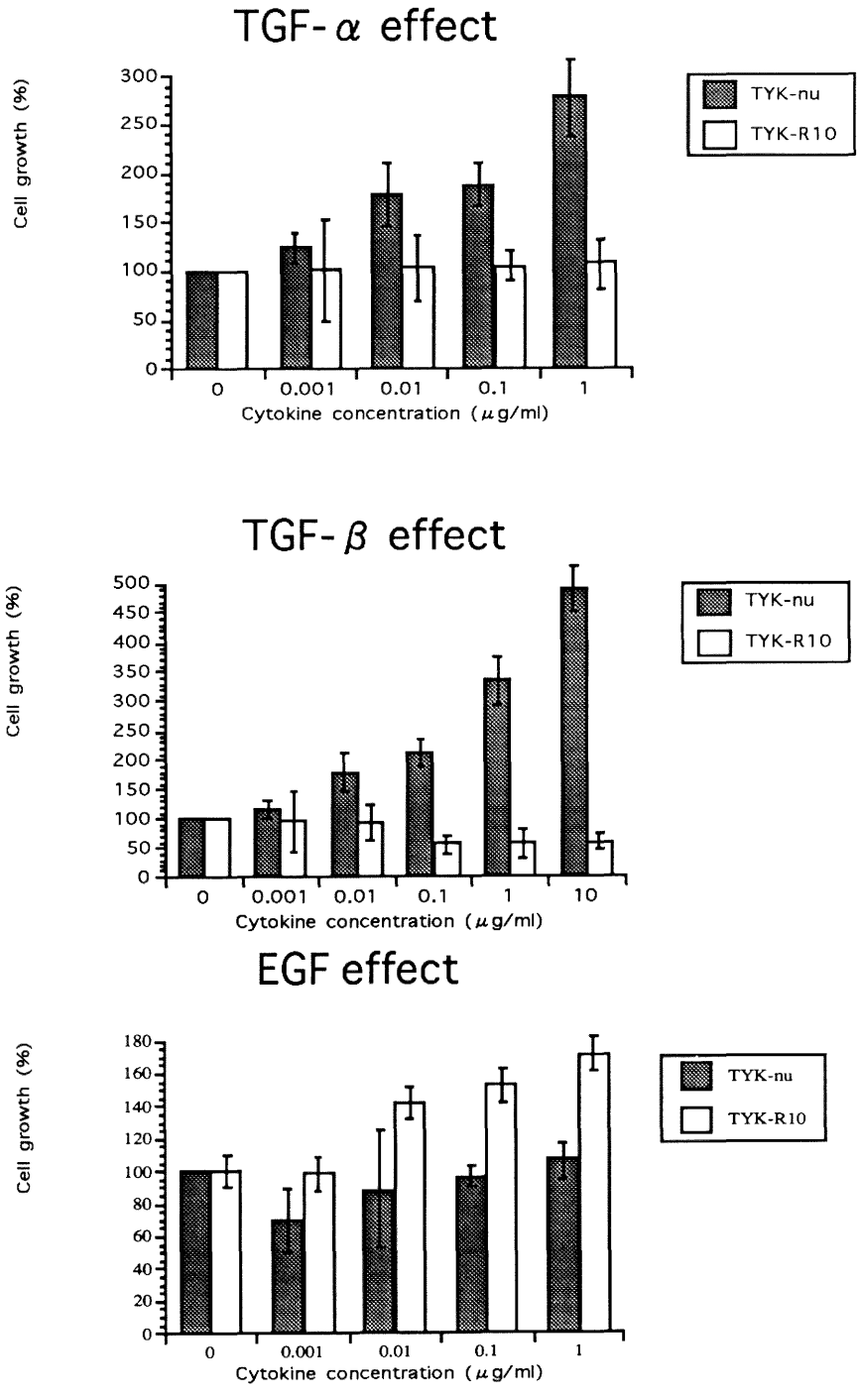

Fig. 2. The effects of various growth factors on TYK-nu and TYK-R10.

Table 3. Effect of BSO-induced GSH depletion on the cytotoxicity of the drugs for TYK-nu and TYK-R10 (μg/mg/protein)

Cell line	BSO (−)	BSO (+)
TYK-nu	15.0±0.4	3.2±0.4
TYK-R10	18.0±0.7*	4.1±0.5

BSO inhibited GSH in both TYK-nu and TYK-R10. TYK-R10 showed a slightly higher level of GSH than TYK-nu (mean ± S.D., n=4, *; $P<0.05$)

Table 4. Results of FACScan analysis

Antibodies	TYK-nu	TYK-R10
EMA	0.7±0.1	3.7±0.6*
CEA	0.9±0.2	0.8±0.2
JSB-1	0.5±0.3	0.4±0.2
MRK16	7.5±1.5	6.4±0.3
Pan-Cadherin	3.0±0.1	7.0±1.7
ICAM-1	9.2±1.3	21.7±1.9**
c-met	1.2±0.1	1.6±0.2
EGF-R-Ab	0.6±0.1	42.8±5.5**
TO73	6.9±1.5	6.2±0.3
TO77	4.9±0.2	7.9±2.1

Percentages of positive cells for each antibody are shown in the Table. (mean±S.D.; n=3) Immunopositivity for EGF-R-Ab of TYK-R10 is significantly different from TYK-nu. (*; $P<0.05$, **; $P<0.01$)

Table 5. Results of proliferation response with various growth factors

Cytokines	TYK-nu	TYK-R10
TGFα	↑	base
TGFβ	↑	↓
EGF	base	↑
HGF	base	base
IGF-I	base	base

cells for EMA were slightly increased in TYK-R10, compared with TYK-nu. Other epithelial markers such as carcinoembryonic antigen (CEA) and adhesion molecule as pan-Cad revealed no enhancement. Positivities of EGF-R and ICAM-1 in TYK-R10 was higher, at 42.8±5.5% and 21.7±1.9%, respectively, than these of TYK-nu.

Growth response for various growth factors

The effect of several growth factors on tritiated thymidine incorporation in TYK-nu and TYK-R10 are shown in Fig. 2. TGF α and TGF β stimulated thymidine incorporation in TYK-nu in a dose dependent manner. TYK-nu increased 2.8-fold in culture with 1 μg/ml TGF α and 4.9-fold with 10 μg/ml TGF β. In contrast, thymidine incorporation of TYK-R10 was slightly inhibited by TGF β but not significantly changed by TGF α. EGF promoted the growth of TYK-R10. No other growth factors used in this study showed any effect on the growth of TYK-R10 or TYK-nu (Table 5). No promotional or inhibitory effect of thymidine incorporation was seen in TYK-nu when the cells were treated with EGF.

DISCUSSION

Resistance to multiple chemotherapeutic drugs is one of the main reasons for treatment failure in cancer chemotherapy. Shen et al. have demonstrated a cross resistance with some anti-cancer drugs in the CDDP resistant cell line,[9] although the exact mechanism of this cross resistance in the CDDP resistant cell line has not been explained. Kuppen et al. reported the cross resistance to CDDP in a mitomycin C resistant cancer cell line which showed an elevated GSH level.[3] Slight elevations of the GSH level may be partly responsible for CDDP resistance in TYK-R10. However, cross resistance to VCR, ADR and VP-16 may be mediated by other mechanisms. This resistance pattern of TYK-R10 suggests a multidrug resistant phenotype. The most extensively studied type of multidrug resistance (MDR) is associated with the overproduction of P-gp,[10] which is encoded by the MDR-1 gene and acts as an ATP dependent pump protein excluding different types of anti-cancer drugs. In addition, there are some other resistant mechanisms contributing to the anti-cancer drug resistance in non-P-gp multidrug resistant cells, including the overexpression of the 190-kDa ATP binding membrane protein, which is encoded by the MDR-associated protein (MRP) gene,[11] alternations in the level of topo-II activity,[12,13] GST activity[14] and an increased GSH level.[15] Although TYK-R10 showed cross resistance for not only platinum analogues but also ADR and VCR, it expressed no P-gp. There were no significant differences in topo-II, the cellular activity of GST or GSH concentrations co-cultured with buthionine sulfoximine(BSO) between TYK-nu and TYK-R10. Unfortunately, since we have not examined the expression of MDR-associated protein

(MRP) in this study, we cannot evaluate the correlation between CDDP resistance and MRP expression. Calcium channel blockers such as VER modulate the function of P-gp by involving the competitive inhibition of P-gp.[16] It can lead to increase intracellular anti-cancer drug accumulation in MDR cell lines.[17] However VER showed no significant effect for reversal in this study. These findings indicate the presence of some MDR mechanisms other than P-gp in TYK-R10.

We and other authors have reported a different response for some growth factors in drug resistant cell lines, when compared with those of parental lines.[4] Meyers et al. observed that Chinese hamster and mouse tumor cells selected for resistance to VCR and actinomycin D had increased numbers of EGF-R, compared with those of their parental cells.[18] They reported that cells expressing high numbers of EGF-R were more drug sensitive than control cells.[19] However, other authors showed that the drug concentration of 90% cell kill was not altered by the responsiveness of the tumor to EGF in cell cycle-independent drugs such as ADR and CDDP.[19] Then the effects of some kinds of growth factors was evaluated on the *in vitro* growth of TYK series. In this study, TYK-R10 expressed greater numbers of EGF-R, compared with TYK-nu, and the consequent addition of EGF promoted the growth of TYK-R10. The cause of the different expression of the EGF receptor remains unknown. One possibility is that the clone expressing EGF-R is selected by a step-wise increase of CDDP, and another is that the non-EGF-R expressing clone expresses EGF-R in association with the acquisition of CDDP resistance.

Examination of responses for other growth factors also showed interesting results. TGF α shares structural and functional properties with EGF.[20,21] Both peptides bind to the EGF-R with high affinity.[20] In the present study, there was a converse relationship on the effect of TGF α and EGF for TYK-nu and TYK-R10. That is, EGF enhanced the growth of TYK-R10 in a dose dependent manner, although TGF α had no proliferation effect. Conversely, the growth of TYK-nu was enhanced by TGF α with no proliferation effect by EGF. These findings indicate that TGF α cannot bind to EGF-R expressed on TYK-R10, and that TYK-nu expresses the original receptor of TGF α on its cell surface. To determine whether a structural abnormality is present in EGF-R on TYK-R10 is a future issue to be resolved. Regarding the biological effect of TGF β, Fynan reported that most immortalized cancer cell lines were usually resistant to the growth inhibitory effect of TGF β.[22] However, Havrilesky et al. found that TGF β inhibited the proliferation of ovarian cancers through apoptosis.[23] In this study, the growth of TYK-R10 was inhibited by TGF β, whereas TGF β promoted the proliferation of TYK-nu, in a dose dependent manner. Thus, our finding indicates that TGF β has a dual effect of progression and inhibition in tumor cell growth. Differing effects of growth factors in drug sensitive and resistant cells have been reported previously.[4] Such different effects were not induced by alternation of receptor numbers but rather would be mediated through alternation of the intracellular transduction.[4] These different response patterns for TGF α and β in the TYK series suggest an alternation of the intracellular transduction, although the numbers of TGF α and β receptor have not been examined in this study.

In conclusion, our results suggest that cross-resistance to ADR and VCR in the CDDP resistant TYK-R10 cell line may be mediated through a non-P-gp mechanism and the acquisition of CDDP resistance alters the growth response for some growth factors, probably through abnormal intracellular transduction.

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