Jpn. J. Cancer Res. (Gann), 79, 1238–1246; November, 1988

Vincristine-resistant Human Cancer KB Cell Line and Increased Expression of Multidrug-resistance Gene

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A multidrug-resistant clone of human cancer KB cells was isolated by stepwise selection on exposure to increasing doses of vincristine. The final clone, VJ-300, obtained after ethylmethane sulfonate mutagenesis showed 400-fold higher resistance to vincristine than did KB cells. Cellular accumulation of vincristine in VJ-300 was decreased to less than one-tenth of that in KB. The cells were also cross-resistant to daunomycin, adriamycin, actinomycin D, colchicine and VP-16. During continuous culturing in the absence of any drug for several months, a different colchicine-resistant and multidrug-resistant clone, KB-C1, reverted almost completely to drug sensitivity, whereas drug resistance in VJ-300 was stably maintained. Amplification of the multidrug-resistance-1 (*mdr-1*) gene was more than 20-fold in KB-C1, but less than 2-fold in VJ-300. *mdr-1* mRNA was, however, expressed in VJ-300 at a rate comparable to KB-C1. Acquisition of high multidrug resistance in VJ-300 might be correlated with both activated transcription of *mdr-1* gene and amplification.

Key words: KB cells — Drug-resistant cell variant — *mdr-1* gene

Simultaneous resistance of tumor cells to multiple anticancer agents (multidrug resistance) is a major problem in cancer chemotherapy. Multidrug resistance occurs frequently in tissue culture cells selected for resistance to a single agent, and the study of multidrug-resistant (MDR) cell lines has been very useful for understanding the molecular mechanism of multidrug resistance.

Since Biedler and Riehm[1] reported the first study to select MDR clones after stepwise selection of variants increasingly resistant to either actinomycin D or daunomycin, many other MDR variants have been selected in a similar manner. These MDR clones include Chinese hamster ovary cells resistant to colchicine,[2,3] human cancer KB cells resistant to colchicine,[4] human lymphoid cells resistant to vincristine,[5] and Chinese hamster lung cells resistant to adriamycin[6] or vincristine.[7] As a biochemical mechanism for MDR, a reduction in net drug accumulation, possibly through enhanced efflux, has been reported.[8–11] Sirotnak *et al.*[12] showed low intracellular binding of vincristine and altered membrane transport in MDR cells.

In many MDR cell lines, overexpression of a 170,000 molecular weight membrane glycoprotein (P-glycoprotein) is correlated with multidrug resistance.[5,13–16] Relevant studies have also shown amplification of a multidrug-resistance gene, *mdr-1* gene, in MDR cell lines.[17–19] These and other data[18,20,21] have suggested that *mdr-1* gene encodes P-glycoprotein, which is supposed to function as an energy-dependent efflux pump. In these MDR cell lines, the *mdr* gene is often amplified, leading to increased efflux. In this report we describe the isolation of a vincristine-resistant clone, VJ-300, from KB cell line after selection in increasing doses of vincristine. This clone acquired levels of drug resistance similar to the colchicine-resistant MDR clone, KB-C1, from KB,[4] but the increased expression of the *mdr-1* gene in VJ-300 was proved to result from a different mechanism.

Materials and Methods

Cell Lines and Cell Culture KB-3-1-4 (KB) was derived from a single clone of human KB epidermoid carcinoma cells after four subclonings. A multidrug-resistant mutant, KB-8-5-11-24 (KB-C1), was selected with increasing concentrations of colchicine.[4] Cells were grown in mono-

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layer in MEM (Nissui Seiyaku Co., Tokyo) containing 10% newborn calf serum (Microbiological Associates, Bethesda, MD), 1 mg/ml Bactopeptone (Difco Laboratories, Detroit, MI), 0.292 mg/ml glutamine, 100 μg/ml kanamycin, and 100 units/ml penicillin.[22,23]

Drugs and Chemicals Adriamycin, vincristine, actinomycin D, and daunomycin were obtained from Sigma Chemial Co. (St. Louis, MO). [^{3}H]-Daunomycin (3.8 Ci/mmol) and [^{3}H]vincristine (4.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

Isolation of Vincristine-resistance Clone Vincristine-resistant clones were isolated after multiple steps of selection in the presence of increasing doses of vincristine. Exponentially growing KB cells at 1×10^6 per 100-mm dish in 10 ml of MEM containing 10% NCS were treated for 24 hr once with 200 μg/ml ethylmethane sulfonate: this treatment decreased the survival fraction to 60–70% of the initial viable cells. The cells were then incubated in mutagen-free medium for 5 days for expression of the resistant phenotype. Vincristine was then added to the medium for 10-day periods at increasing concentrations from 1 ng/ml to 300 ng/ml: 1, 2, 4, 5, 10, 20, 50, 100, 200, and 300 ng/ml. Colonies selected at 5, 50 and 300 ng/ml of vincristine were repurified, and the purified clones were named VJ-5, VJ-50 and VJ-300.

Cell Survival Assay by Colony Formation Cell survival was determined by plating 300 cells in 60-mm dishes in the absence of any drug.[22,23] Various drugs were added 16 hr later. After incubation for 10 days at 37°, the colonies were stained with 0.5% methylene blue in 50% ethanol and counted. Solutions of all the drugs were freshly prepared before use in dimethyl sulfoxide. Relative resistance was determined by dividing the D_{10} of various anticancer agents against MDR clones by that against KB cells.

Drug Accumulation Cells (4×0^5/60-mm dish) were plated and incubated overnight at 37°. Then the medium was replaced with serum-free MEM, and the cells were incubated with 0.25 μCi/ml [^{3}H]-daunomycin or [^{3}H]vincristine for 60 min.[22,23] Cells were washed once with cold PBS (g/liter: NaCl, 8.0; $Na_2HPO_4\cdot12\,H_2O$, 2.9; KCl, 0.2; KH_2PO_4, 0.2) and harvested. The cells were washed 3 times with cold PBS, and the cell pellets were suspended in 0.7 ml of H_2O and mixed thoroughly with 7 ml of Scientisol EX-H (Wako Chemical Co., Osaka). The radioactivities were determined.

Membrane Vesicle Preparation and Photoaffinity Labeling Membrane vesicles from KB, KB-C1, VJ-300 and KB-C1-R cells were prepared as described.[24,25] Membrane vesicles were incubated with N-(*p*-azido)-[3-^{125}I](salicyl)-N′-(β-aminoethyl)vindesine (^{125}I-NASV) (10^5 dpm) for 15 min at room temperature: ^{125}I-NASV was synthesized by Drs. Y. Inoue and K. Suzuki (Omiya Research Laboratory, Nikken Chemical Co., Saitama). After continuous UV irradiation at 25°, samples were solubilized in SDS sample buffer as described.[26,27] Samples labeled with ^{125}I-NASV were fractionated by electrophoresis on an SDS-polyacrylamide-urea-gel as described by Debenham *et al.*[17] on 5% polyacrylamide-4.5*M* urea, pH 7.6, without a stacking gel.

DNA and RNA Isolation High-molecular DNA was isolated from each cell line essentially by the method of Maniatis *et al.*[28] RNA was isolated using a buffer containing 8*M* guanidine HCl as described.[29]

DNA and RNA Analysis DNA was digested with the appropriate restriction endonuclease, electrophoresed on 0.7% agarose, and transferred to Nylon filters by the method of Southern.[30] Dot blot filters were prepared with DNA from KB cell lines by using a BRL blot apparatus. Total cellular RNA was separated on 1% agarose gels containing 2.2*M* formaldehyde, then transferred to a Nylon filter. DNA and RNA were cross-linked to the filter by UV irradiation. A ^{32}P-labeled DNA probe (0.8 kb *Eco*RI-*Hin*dIII fragment containing the *mdr-1* coding regions from pMDR105) was prepared by the random priming method of Feinberg and Vegelstein[31]; pMDR105 was provided by Dr. M. M. Gottesman (National Cancer Institute, Bethesda, MD). Hybridization and washing conditions were as previously described.[32]

Results

Drug Sensitivity of a Series of Vincristine-resistant Cell Lines Dose-response curves of VJ-5, VJ-50 and VJ-300 to vincristine showed respectively about 5-fold, 50-fold and 400-fold higher resistance than that of the parental KB cell line (Fig. 1).

Stability of the drug resistance of VJ-300 was examined in the absence of selected drugs. Multidrug resistance is usually unstable, and revertants can be easily isolated, for example from KB-C1 by culturing in the absence of colchicine.[4] We purified clones of VJ-300 and KB-C1 after each cell line had been continuously cultured in the absence of any drug for two months. As can be seen in Fig. 2, a clone, KB-C1-R, derived from KB-C1 showed almost the same dose response to vincristine as KB cells, whereas a clone, VJ-300-R, from VJ-300 showed the same sensitivity to vincristine as VJ-300. Drug resistance of

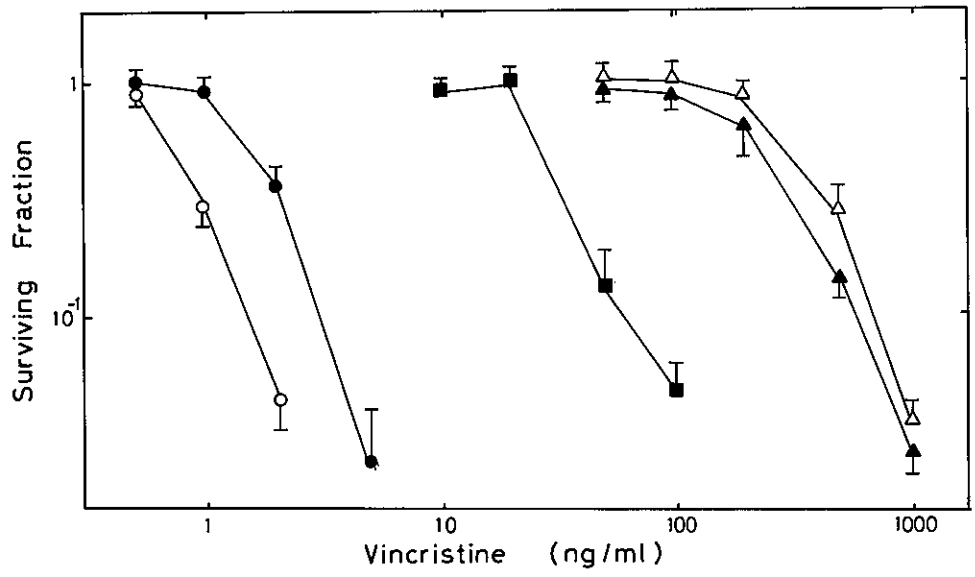

Fig. 1. Dose-response curve of KB cell lines. Drug resistance of KB cell lines in the presence of vincristine was assayed in terms of colony-forming ability as described in "Materials and Methods." The surviving fraction was obtained by normalizing colonies of parental KB cells. Cell lines are shown as follows: KB (○), KB-C1 (△), VJ-5 (●), VJ-50 (■) and VJ-300 (▲)

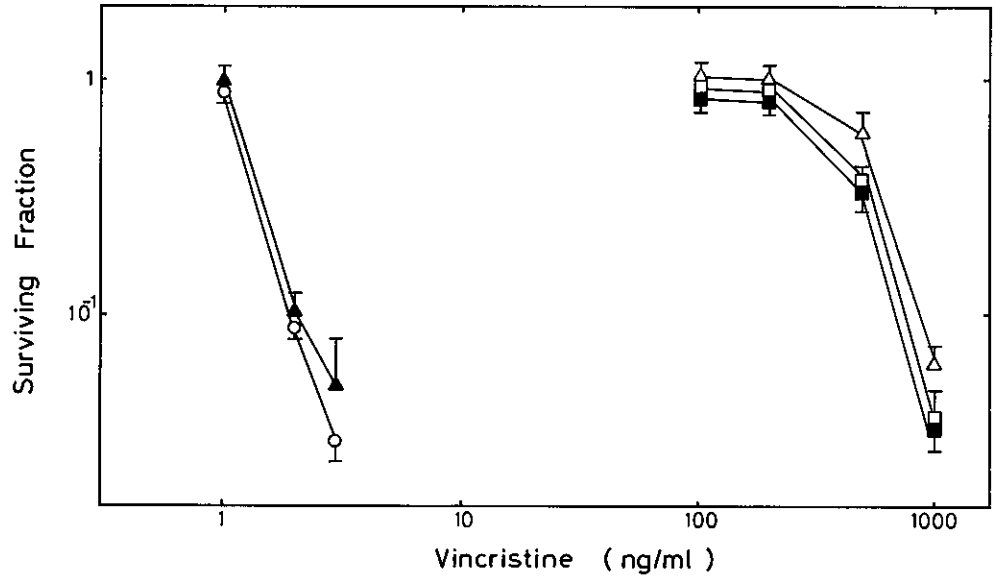

Fig. 2. Effect of drug-free cultivation on resistance to vincristine. Drug resistance of KB cell lines is shown by the dose-response curve. Cell lines are shown as follows; KB (○), KB-C1 (△), KB-C1-R (▲), VJ-300 (□) and VJ-300-R (■).

Table. I. Comparison of Durg Resistance in KB, KB-C1, KB-C1-R and VJ-300

Anticancer agent	Relative resistance[a]			
	KB	KB-C1	KB-C1-R	VJ-300
Vincristine	1	450	1.1	394
Daunomycin	1	106	1.0	18
Adriamycin	1	21	1.1	10
Actinomycin D	1	22	0.9	92
5-Fluorouracil	1	1.4	1.0	1.3
Colchicine	1	323	0.9	21
VM-26	1	18	0.6	3.8
VP-16	1	13	0.5	4.8
Camptothecin	1	24	1.4	4.0

Relative resistance of KB-C1, KB-C1-R and VJ-300 is presented as D_{10} of each cell line when D_{10} of KB was normalized as 1.0: D_{10} is the dose required to reduce the initial survival by 10%.
a) Average of duplicate trials as seen in Fig. 1.

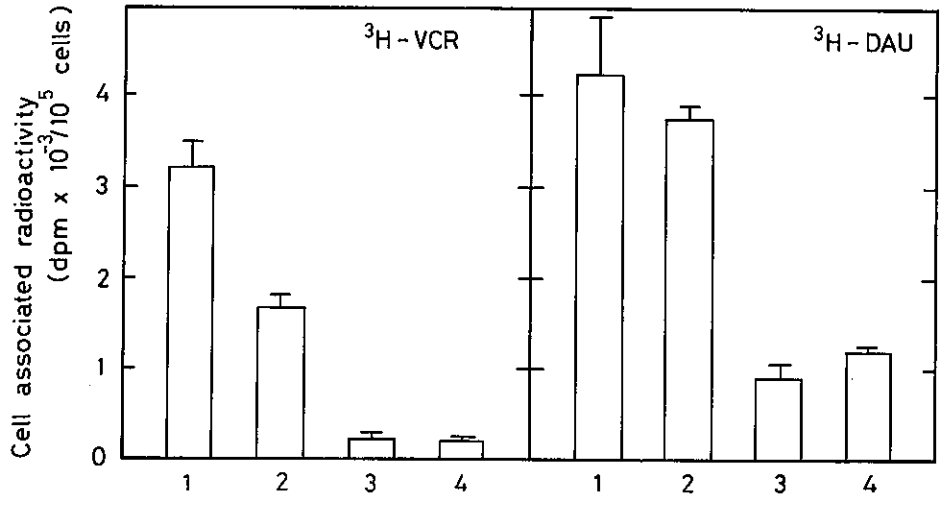

Fig. 3. Drug accumulation in KB and its MDR cell lines. KB, KB-C1-R, KB-C1 and VJ-300 cell lines were seeded and then incubated with [^{3}H]vincristine (^{3}H-VCR) or with [^{3}H]daunomycin (^{3}H-DAU) for 60 min. Cell-associated radioactivity was counted and each value is the average of duplicate trials. Bar ± SD. Lane 1, KB; lane 2, KB-C1-R; lane 3, KB-C1; lane 4, VJ-300.

KB-C1 appears to be unstable, but that of VJ-300 appears to be stably maintained.

Relative resistance of KB-C1 and JV-300 with respect to KB and KB-C1-R was examined by colony formation assay. KB-C1, originally selected in 1000 ng/ml colchicine,[4] was 323-fold more resistant to colchicine than KB and was cross-resistant to vincristine, daunomycin, adriamycin, actinomycin D and VP-16. Its revertant, KB-C1-R, showed almost the same sensitivity to these agents as KB cell line (Table I). VJ-300, selected in 300 ng/ml vincristine, was 394-fold more resistant to vincristine than KB, and was cross-resistant to daunomycin, actinomycin D, colchicine and VP-16. Although the cellular levels of cross-resistance were different between VJ-300 and KB-C1, VJ-300 was found to be a MDR cell variant.

Drug Accumulation and Photoaffinity Labeling of 170-kDa Protein Cellular accumulation of vincristine or daunomycin is

decreased in KB-C1 cells in comparison with KB.[2, 22, 23, 33] In agreement with the previous reports, KB-C1 cells showed reduced accumulation of [^{3}H]vincristine and [^{3}H]daunomycin (Fig. 3), and the accumulation of both agents was restored in a revertant of KB-C1, KB-C1-R. VJ-300 cells showed reduced accumulation of both agents, like KB-C1 cells (Fig. 3).

MDR cells express a 170-kDa P-glycoprotein that can be photoaffinity labeled with a vinblastine analog, ^{125}I-vindesine. This 170-kDa protein of membrane vesicles from KB-C1 is also specifically labeled with ^{125}I-NASV. As can be seen in Fig. 4, 170-kDa protein (P-glycoprotein) was overexpressed in KB-C1 and we could observe photolabeling of this protein, but no similar labeling of 170-kDa protein was seen in KB or KB-C1-R cells. We could also observe photolabeling of the 150–170 kDa molecular weight band when membrane vesicles were extracted from VJ-300 cells (Fig. 4).

Comparison of *mdr-1* Gene Expression between VJ-300 and KB-C1 VJ-300 cells express a P-glycoprotein which is encoded by the *mdr-1* gene, as shown in Fig. 4. To determine the presence of *mdr-1* mRNA in VJ-300, Northern analysis was performed using RNA from KB cell lines. Equal amounts of RNA (10 μg of total cellular RNA) were run on a 1% formaldehyde gel. The *mdr* specific probe hybridized to a 4.5 kb RNA species, which is highly expressed in both VJ-300 and KB-C1 (Fig. 5). VJ-300 had almost the same level of *mdr-1* mRNA as KB-C1, but no *mdr-1* mRNA was detected in similar experiments with KB or KB-C1-R cells.

To test the possibility that *mdr-1* mRNA expression in VJ-300 cells might result from *mdr-1* gene amplification or rearrangement, genomic DNAs were isolated from KB cell lines. These DNAs were digested to completion with two restriction endonuclease (*Eco*RI and *Hin*dIII), electrophoresed on a 0.7% agarose gel and transferred to Nylon mem-

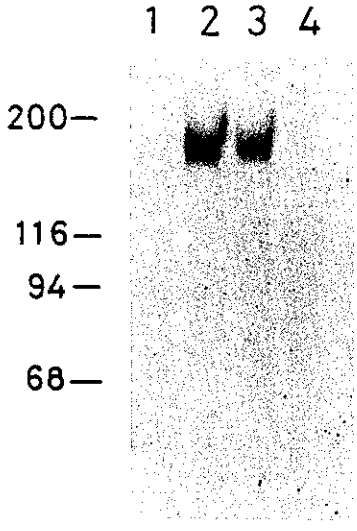

Fig. 4. Photoaffinity labeling by ^{125}I-NASV of membrane vesicles of KB, KB-C1, VJ-300 and KB-C1-R cells. Membrane vesicles of various cell lines were incubated with ^{125}I-NASV. Autoradiograms were developed after exposure for 1 day. Molecular size markers on the left are in kilodaltons. Lane 1, KB; lane 2, VJ-300; lane 3, KB-C1; lane 4, KB-C1-R.

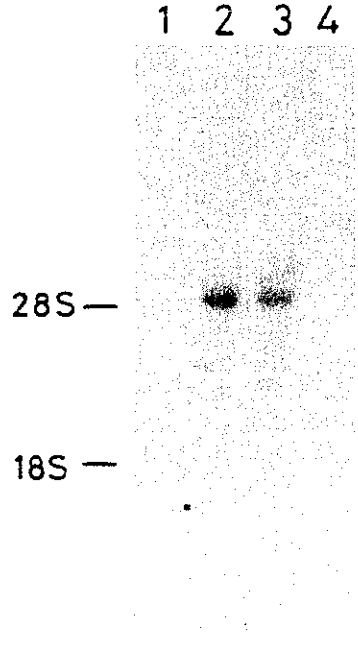

Fig. 5. Northern hybridization. Total cellular RNAs (10 μg) from each cell line were separated on a formaldehyde agarose gel and transferred to Nylon membrane. Preparation of *mdr-1* specific probe and hybridization were carried out as described in "Materials and Methods." Lane 1, KB; lane 2, VJ-300; lane 3, KB-C1; lane 4, KB-C1-R.

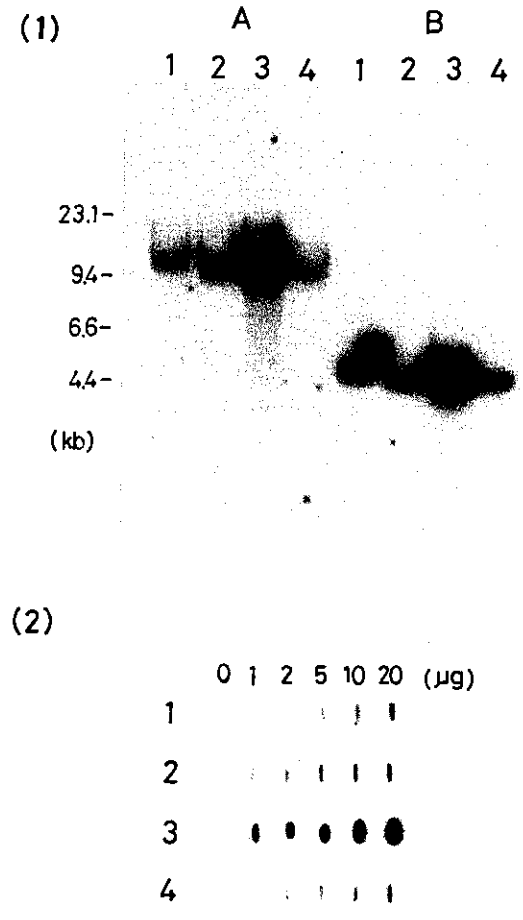

Fig. 6. (1) Southern hybridization of *mdr-1* gene. DNAs (20 μg) extracted from KB, VJ-300, KB-C1 and KB-C1-R cell lines were digested with *Eco*RI (panel A) or *Hin*dIII (panel B) and electrophoresed on a 0.7% agarose gel, transferred to Nylon membrane and autoradiographed. Lane 1, KB; lane 2, VJ-300; lane 3, KB-C1; lane 4, KB-C1-R. Phage DNA *Hin*dIII-generated fragments were used as molecular weight markers in kilobases. (2) Slot blot hybridization. DNAs from KB, KB-C1, VJ-300 and KB-C1-R cell lines were suspended in 0.2*N* NaOH and 6×SSC, heated at 80° for 10 min, and then chilled in ice. Samples were neutralized by addition of 1*M* Tris-HCl (pH 7.4) and then applied to the BRL slot blot apparatus. Lane 1, KB; lane 2, VJ-300; lane 3, KB-C1; lane 4, KB-C1-R.

brane. Hybridization of the Southern blot with a ^{32}P-labeled *mdr-1* specific probe revealed no rearrangements in the 3′ end of *mdr-1* gene as assessed by a comparison of fragment size with that of control KB cell DNA (Fig. 6(1)). Three other genes (actin, epidermal growth factor receptor and c-*myc*) also showed no rearrangements (data not shown). DNA dot blot analysis shows more than 20-fold amplification of *mdr-1* gene in KB-C1 cells over KB cells (Fig. 6(2)), in good agreement with the previous report by Shen *et al.*[16] In contrast, there was no apparent amplification of *mdr-1* gene in VJ-300 cells (Fig. 6(2)).

Discussion

Akiyama *et al.*[4] isolated an MDR clone, KB-C1, by selecting a colchicine-resistant clone from KB cell line. We have now isolated another MDR clone, VJ-300, from KB by selecting a vincristine-resistant clone. Cellular accumulation of vincristine or daunomycin in both KB-C1 and VJ-300 is very low. Decreased accumulation of these anticancer agents in KB-C1 is partly caused by enhanced efflux .[2,22,23,33] Efflux of vincristine or daunomycin is also found to be enhanced in VJ-300 (J. Kikuchi, unpublished data). We have recently established five cell lines from human head and neck tumor which exhibit different sensitivities to adriamycin or vincristine,[34] and in one of the most resistant tumor cell lines, there appears to be no active expression of *mdr-1* gene. The drug-resistance level in naturally occurring drug-resistant tumor cells might not be high enough to allow detection of the mRNA.[34] Alternatively as discussed by Skovsgaard[11] or by Siegfried *et al.*,[35] there might exist more than one mechanism besides that through the *mdr-1* gene product. Known mechanisms of acquired drug resistance such as MDR or methotrexate resistance should provide a basis to understand underlying mechanisms of drug resistance in human tumors.

Photoaffinity labeling of P-170 protein with a vinblastine analog showed similar amounts of the protein in both KB-C1 and VJ-300. (1) Both KB-C1 and VJ-300 show about 400-fold higher resistance to vincristine than their parental cell line, but VJ-300 is 20-fold more resistant to colchicine and KB-C1 is 300-fold more resistant to colchicine than KB. (2) Multidrug resistance of KB-C1 is very unstable in the absence of drug (see also ref. 4), whereas that of VJ-300 is very stably maintained. (3) Overexpression of *mdr-1* gene is

mediated through gene amplification in KB-C1 but it is mainly mediated through elevated expression of *mdr-1* mRNA in VJ-300.

Shen *et al.*[16] reported that a slightly increased expression of *mdr-1* gene precedes amplification of the gene. During selection of colchicine-resistant clones for increased levels of resistance,[2] *mdr-1* mRNA expression is elevated, but no gene amplification is observed, in KB-8 and KB-8-5 isolated at earlier steps in the selection. KB-8 and KB-8-5 are about 2–4 fold more resistant to colchicine or adriamycin than KB.[16, 19] However, in KB-8-5-11, selected as a clone resistant to a 40-fold higher dose of colchicine and a 23-fold higher dose of adriamycin than KB, expression of *mdr-1* mRNA was increased simultaneously with amplification of *mdr-1* DNA. It has recently been reported that amplification of *mdr-1* gene still remains in the drug-sensitive revertant of a human MDR clone. Expression of the *mdr-1* gene might be turned off in the revertant through an unknown mechanism.[36]

Fuque *et al.*[37] have isolated an adriamycin-resistant clone from MDA-231 human breast cancer cell line which is about 40-fold more resistant to adriamycin than its parent. A 5.0 kb mRNA of *mdr-1* gene was found to be expressed in this adriamycin-resistant clone, but Southern blot analysis showed no amplification.[37] Scott *et al.* have also isolated several MDR-variants which show increased expression of *mdr-1* mRNA due to amplification of *mdr-1* gene.[38] But they suggest no direct correlation between the level of *mdr-1* gene expression and the copy number of the gene. These data suggest that amplification of *mdr-1* gene may not be obligatorily required for acquisition of multidrug resistance in human cancer cells. In VJ-300 cells, either the *cis* regulatory region of the *mdr-1* gene or transacting factor(s) might be responsible for increased expression of *mdr-1* mRNA. Increased levels of *mdr-1* mRNA in human tissue or human tumor specimens are clearly correlated with enhanced transcription of mRNA rather than with gene amplification.[16] Since the MDR-phenotype of VJ-300 is very stably maintained, direct comparison of its gene transcription rates and gene sequences with those in the wild-type material should reveal the critical mechanism for elevated *mdr-1* gene expression in human tumors.

Acknowledgments

We thank Dr. D. Schlessinger (Washington Univ., St. Louis) for a critical reading of the manuscript. This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. We thank Mariko Ando for her editorial help.
(Received July 25, 1988/Accepted October 7, 1988)

References

1) Biedler, J. L. and Riehm, H. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*, cross-resistance, radioautographic, and cytogenic studies. *Cancer Res.*, **30**, 1174–1183 (1970).
2) Akiyama, S., Shiraishi, N., Kuratomi, Y., Nakagawa, M. and Kuwano, M. Circumvention of multiple-drug resistance in human cancer cells by thioridazine, trifluoperazine, and chlorpromazine. *J. Natl. Cancer Inst.*, **76**, 839–844 (1985).
3) Ling, V. and Thompson, L. H. Reduced permeability in Chinese hamster ovary cells as a mechanism of resistance to colchicine. *J. Cell. Physiol.*, **83**, 103–106 (1975).
4) Akiyama, S., Fojo, A., Hanover, J. A., Pastan, I. and Gottesman, M. M. Isolation and genetic characterization of human KB cell line resistant to multiple drugs. *Somatic Cell Mol. Genet.*, **11**, 117–126 (1985).
5) Beck, W. T., Mueller, T. T. and Tanger, L. R. Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. *Cancer Res.*, **39**, 2070–2076 (1979).
6) Garman, D. and Center, M. S. Alteration in cell surface membranes in Chinese hamster lung cells resistant to adriamycin. *Biochem. Biophys. Res. Commun.*, **105**, 157–163 (1982).
7) Biedler, J. C. and Peterson, R. H. F. "Chemotherapeutic Agents: Molecular Actions and Targets for Cancer," pp. 453–482 (1981). Academic Press, New York.
8) Danø, K. Development of resistance to adriamycin (NSC-123127) in Ehrlich ascites tumor *in vitro*. *Cancer Chemother. Rep.*, **56**, 321–326 (1972).

9) Inaba, M., Fujikura, R., Tsukagoshi, S. and Sakurai, Y. Active efflux common to vincristine and daunorubicin in vincristine-resistant P388 leukemia. *Biochem. Pharmacol.*, **30**, 2191–2194 (1981).
10) Peterson, C. and Trouet, A. Transport and storage of daunorubicin and doxorubicin in cultured fibroblasts. *Cancer Res.*, **38**, 4645–4649 (1978).
11) Skovsgaard, T. Mechanism of resistance to daunorubicin in Ehrlich ascites tumor cells. *Cancer Res.*, **38**, 1785–1791 (1978).
12) Sirotnak, F. M., Yang, C. H., Mines, L. S., Oribe, E. and Biedler, J. L. Markedly altered membrane transport and intracellular binding of vincristine in multidrug resistant Chinese hamster cells selected for resistance to vinca alkaloids. *J. Cell. Physiol.*, **126**, 266–274 (1986).
13) Juliano, R. L. and Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta*, **455**, 900–912 (1976).
14) Kartner, N., Riordan, J. and Ling, V. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science*, **221**, 1285–1288 (1983).
15) Kartner, H., Evemden-Porelle, D., Bradley, G. and Ling, V. Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature*, **316**, 820–823 (1985).
16) Shen, D. W., Fojo, A., Chin, J. E., Roninson, I. B., Richert, N., Pastan, I. and Gottesman, M. M. Human multidrug-resistant cell lines: increased mdrl expression can precede gene amplification. *Science*, **232**, 643–645 (1986).
17) Debenham, P. G., Kartner, K., Siminoritch, L., Riordan, J. R. and Ling, V. DNA mediated transfer of multidrug-resistance and plasma membrane glycoprotein expression. *Mol. Cell. Biol.*, **2**, 881–884 (1982).
18) Gros, P., Croop, J. and Housman, D. Mammalian multidrug-resistance gene: complete cDNA sequence indicates strong homology to bacteria transport proteins. *Cell*, **47**, 371–380 (1986).
19) Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D-W., Gottesman, M. M. and Pastan, I. Isolation of the human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **83**, 4538–4542 (1986).
20) Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M. and Roninson, I. B. Internal duplication and homology with bacterial transport proteins in the *mdr1* (P-glycoprotein) gene from multidrug-resistant human cells. *Cell*, **47**, 381–389 (1986).
21) Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. Expression of a full-length cDNA for the human "*MDR1*" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA*, **84**, 3004–3008 (1987).
22) Nakagawa, M., Akiyama, S., Yamaguchi, T., Shiraishi, N., Ogata, J. and Kuwano, M. Reversal of multidrug resistance by synthetic isoprenoids in the KB human cancer cell line. *Cancer Res.*, **46**, 4453–4457 (1986).
23) Shiraishi, N., Akiyama, S., Nakagawa, M., Kobayashi, M. and Kuwano, M. Effect of bisbenzylisoquinoline (biscoclaurine) alkaloids on multidrug-resistance in the human cancer cells. *Cancer Res.*, **47**, 2413–2416 (1987).
24) Cornwell, M. M., Gottesman, M. M. and Pastan, I. Increased vinblastine binding to membrane vesicles from multidrug resistant KB cells. *J. Biol. Chem.*, **261**, 7921–7928 (1986).
25) Cornwell, M. M., Safa, A. R., Felsted, R. L., Gottesman, M. M. and Pastan, I. Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA*, **83**, 3847–3850 (1986).
26) Akiyama, S., Cornwell, M. M., Kuwano, M., Pastan, I. and Gottesman, M. M. Most drugs that reverse multidrug-resistance also inhibit photoaffinity labeling of P-glycoprotein by a vinblastine analog. *Mol. Pharmacol.*, **33**, 144–147 (1988).
27) Nogae, I., Kohno, K., Kikuchi, J., Kuwano, M., Akiyama, S., Kiue, A., Suzuki, K., Yoshida, Y., Cornwell, M. M., Pastan, I. and Gottesman, M. M. Analysis of structural features of dihydropyridine analogs needed to reverse multidrug-resistance and inhibit photoaffinity labeling of P-glycoprotein. *Biochem. Pharmacol.* (1988), in press.
28) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning: A Laboratory Manual," pp. 191–193 (1982). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29) Adams, S. L., Sobel, M. E., Howard, B. H., Olden, K., Yamada, K. M., Crombrugghe, B. and Pastan, I. Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA*, **74**, 3399–3403 (1977).

30) Southern, E. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503–517 (1975).
31) Feinberg, A. P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132**, 6–13 (1983).
32) Church, G. M. and Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995 (1984).
33) Fojo, A., Akiyama, S., Gottesman, M. M. and Pastan, I. Reduced drug accumulation in multiple drug-resistant human KB carcinoma cell lines. *Cancer Res.*, **45**, 3002–3007 (1985).
34) Komiyama, S., Matsui, K., Kudoh, S., Nogae, I., Kuratomi, Y., Saburi, Y., Asoh, K., Kohno, K. and Kuwano, M. Establishment of tumor cell lines from patient with head and neck cancer and their different sensitivities to anticancer agents. *Cancer* (1989), in press.
35) Siegfried, J. M., Burke, T. G. and Tritton, T. R. Cellular transport of anthracyclines by passive diffusion: implication for drug resistance. *Biochem. Pharmacol.*, **34**, 593–598 (1985).
36) Sugimoto, Y., Roninson, B. I. and Tsuruo, T. Decreased expression of the amplified *mdr-1* gene in revertants of multidrug-resistant human myelogenous leukemia K562 occurs without loss of amplified DNA. *Mol. Cell. Biol.*, **7**, 4549–4552 (1987).
37) Fuque, S. A. W., Moretti-Rojas, I. M., Schneider, S. L. and McGuire, W. L. P-Glycoprotein expression in human breast cancer cells. *Cancer Res.*, **47**, 2103–2106 (1987).
38) Scott, W. K., Biedler, L. J. and Melera, W. P. Amplification and expression of genes associated with multidrug-resistance in mammalian cells. *Science*, **232**, 751–755 (1986).