In vivo Acquired Drug Resistance and Multidrug Resistance Gene (MDR1) Expression in the KB Carcinoma Cell Line Xenotransplanted in Nude Mice

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We studied the correlation between in vivo responsiveness of KB xenografts to anticancer drugs and the expression level of the human multidrug resistance gene (MDR1) encoding P-Glycoprotein (P-Gp). We established KB xenografts (xeKB3-1 and xeKB8-5) by inoculating these in vitro lines into nude mice. The responsiveness was evaluated by an in vivo chemosensitivity assay (T/C; sensitive, <50%). Xenograft xeKB3-1 was sensitive to vincristine (VCR) (T/C, 48%), and xeKB8-5 was resistant to VCR (T/C, 72%). We selected a VCR-resistant variant (xeKB3-1-R, T/C, 76%) by treating xeKB3-1 with VCR (1.2 mg/kg, x3) in vivo. The MDR1 expression was evaluated by a semi-quantitative assay using reverse transcription-polymerase chain reaction. A MDR1 expression pattern in xeKB3-1 and xeKB8-5 in vivo was the same as that to KB3-1 and KB8-5 in vitro. The xenograft xeKB3-1-R expressed definitive but significantly lower levels of MDR1 than xeKB8-5. These results suggest that acquired drug resistance is related to minimally enhanced expression of the P-Gp protein/MDR1 gene in KB xenografts in vivo.

(Key Words: acquired drug resistance, P-glycoprotein, in vivo chemotherapy)

INTRODUCTION

The development of drug resistance in tumors that are initially responsive to chemotherapy often presents a major problem in the treatment of solid tumors. Recently many studies of multidrug resistance (MDR) in vitro have revealed the mechanisms induced by P-Glycoprotein (P-Gp), a drug efflux pump (4, 5, 8, 10, 23, 30). P-Gp is encoded by the human multidrug resistance gene (MDR1), in chromosome 7q. Although MDR mechanisms in vivo remained unclear, tumors that originated in organs which expressed levels of P-Gp, also expressed high levels of MDR1, followed by intrinsic drug resistance. Our previous reports showed that intrinsic MDR in some solid tumor xenografts in vivo was related to MDR1 gene expression (1). In this study, we established human KB squamous cell carcinoma xenografts from in vitro KB cell lines, and then selected vincristine (VCR) resistant xenografts (xeKB3-1-R) from those that were drug sensitive (xeKB3-1). We confirmed that the resistant xenografts (xeKB3-1-R) expressed MDR1, and that the parent xeKB3-1 did not, by means of the reverse transcription-polymerase chain reaction (RT-PCR) (19, 21).

MATERIALS AND METHODS

Human Tumor Cell Lines and Xenografts:

A drug-sensitive epidermoid carcinoma cell
line KB3-1 and its resistant derivative KB8-5, which was clonally selected by inoculation of colchicine (15 ng/10^6 cells, for 28 days) in vitro (provided by Dr. Y. Kakehi, Kyoto University), were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 5% fetal bovine serum at 37 °C in a fully humidified 5% CO₂ atmosphere. We transplanted these KB lines into nude mice (BALB/c-nu/nu mice, Clea Japan, Inc., Tokyo) subcutaneously, and established human carcinoma xenografts (xeKB3-1 and xeKB8-5). The xenografts, maintained by serial subcutaneous transplantation into nude mice, were removed after sacrificing the animals under deep anesthesia and rapidly frozen at −80°C until used. Total RNA was prepared from the frozen specimens by standard procedures (24). Tumor xenografts were also prepared for routine histopathological analysis.

**Antitumor Agents:** Vincristine (VCR) was purchased from Shionogi Co., Osaka, and Doxorubicin (DOX) was supplied by Kyowa Hakko Kogyo Co., Tokyo, as pure crystals for experimental use.

**In vivo selection of VCR-resistant derivatives:** We established in vivo VCR-resistant derivatives (xeKB3-1-R) by in vivo selection. Three doses of VCR (1.2 mg/kg) were administered intravenously to nude mice bearing xeKB3-1. No significant morphological differences were noted between parent xenografts and the VCR-resistant derivatives (Fig. 1).

**In Vivo Chemosensitivity Test:** The mice received in vivo chemotherapy according to the previously published protocol (12, 13, 14). Four female nude mice, 6–15 weeks old, bearing xenografts of the same size (100–300 mm³), were given CEDs of VCR, 0.4 mg/kg and DOX, 12 mg/kg. The CED values were determined by pharmacokinetic measurements on the concentration of these drugs in human and mouse plasma (12). Tumor volumes were calculated by a previously described formula on day 14 of the anticancer drug treatment (13). Growth of the tumor xenografts was shown by relative tumor volume (RV%), expressed as RV=V₁₄/V₀, where V₁₄ is the tumor volume on day 14 and V₀ is the initial tumor volume on the day that treatment was started (day 0). The effects of the drugs were represented by RV of the xenografts and the T/C% values defined as the ratio of the RV of the treated tumor xenografts to controls after 14 days of drug administration (18, 29).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR):** Recently, MDR1 gene expression has been detected by means of the reverse transcription of RNA followed by the polymerase chain reaction (RT-PCR) (19, 21). Here, we used the same reported procedure with minor modifications (21). Complementary DNA (cDNA) was synthesized with 5 to 1,000 ng of total cellular RNA and 20 pmoles of a

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**Fig. 1** Xenograft histopathology: Standard sections stained with hematoxylin and eosin (x300). (A) xeKB3-1, (B) xeKB8-5, (C) xeKB3-1-R

No significant histopathological differences are apparent between the in vivo selected VCR resistant xeKB3-1-R and the parent xeKB3-1.
downstream antisense primer in 10 μl of a reaction mixture (50 mM Tris-Cl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 500 M dNTPs), containing 10 units of Molony murine leukemia virus (MMLV RT) reverse transcriptase (SuperScript, Bethesda Research Laboratories), at 24°C for 10 minutes and at 43°C for 60 minutes.

PCR proceeded with cDNA in a final volume of 100 μl of reaction mixture (10 mM Tris-Cl pH 8.2, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin) containing 2.5 units of thermostable DNA polymerase (AmpliTaq, PerkinElmer/Cetus). Each PCR cycle consisted of 2 minutes of denaturation at 94°C, 2 minutes of primer annealing at 55°C, and 2 minutes of extension/synthesis at 72°C. PCR primers (ampprimers) were synthesized using a DNA synthesizer (Applied Biosystems, model 391).

We prepared an original set of ampprimers (#B, sense: AAGCTTAGTACAAAGAGGCTCTG, residues 2041-2064, antisense: GGCTAGAAACAATAGTGAACCAA, residues 2260-2283) to evaluate the expression level specific to the human MDR1 gene. The ampprimer sequences (#B) were derived from various exons separated by introns to prevent amplification of genomic DNA. RT-PCR with ampprimers #B amplified a 243 bp segment of MDR1 cDNA. The sense and antisense ampprimers (#B) revealed 96 and 75% homology with the murine MDR1 gene, respectively, with no amplification of the MDR1 gene from normal murine tissues (brain, heart, lung, kidney, liver, spleen). We avoided the amplification of murine MDR1 gene transcripts contaminating the tumor xenografts specimens (data not shown). We also used ampprimers for β2 microglobulin (β2m-#D), as published, previously to qualify the RNA by evaluating housekeeping gene expression (21).

PCR products, separated by electrophoresis through a 3% agarose gel, were blotted onto membranes (Zeta Probe, BioRad). Specific PCR products were detected by hybridization with synthetic oligonucleotide probes (AGGATTATGAAGCTAATTSA, residues 2094-2115, for MDR1-#B, GTGTTGAACCATGTGACTTTGTCACA, residues 1574-1598, for β2m-#D) labeled with ³²P (16). The relative expression levels of the MDR1 genes were evaluated by densitometry using the Interactive Build Analysis System (Carl Zeiss.) (15). MDR1 gene expression levels were calculated by multiplying the mean density by the densitometric area.

RESULTS

In Vivo Multidrug Resistance

The growth rates of tumor xenografts in the in vivo chemosensitivity test are presented in Figs. 2 and 3. Each group included 4 nude mice bearing tumor xenografts. Evaluated as "sensitive" was strictly defined, based both on statistical significance determined by the Mann-Whitney U-test (P<0.01, one-sided), and on T/C values of 50% or less, according to previous reports (12-14, 18, 29). Xenograft xKB3-1 was sensitive to both VCR and DOX (T/C, 48%, 14%, respectively), whereas xKB8-5 was resistant to both VCR and DOX (T/C, 72%, 51%, respectively). The xKB3-1-R selected by VCR administration was drug resistance to VCR (T/C, 76%), whereas xKB3-1-R was not cross resistant to DOX (T/C, 6%) (Table 1).

MDR1 Gene Expression

The amount of the PCR product is proportional to the initial amount of the template cDNA reverse-transcribed from a specific mRNA under conditions in which PCR proceeds exponentially at a constant efficiency. We kinetically analyzed the RT-PCR of MDR1 mRNA in the KB3-1 and KB8-5 cell. The amounts of specific MDR1 products were amplified exponentially by 22 to 28 cycles of PCR. The yield of the MDR1-specific RT-PCR product, using over 500ng of RNA template from the KB8-5 cell line, approached a plateau at 28 cycles of amplification, whereas the slope of the RT-PCR products, using a KB8-5 RNA template (25 ng to 1000 ng), showed a linear correlation with 26 amplification cycles. These results suggested that the initial amount of the RNA template (25 to 1000 ng) could be evaluated in different samples before the amplification procedure became saturated, that is, after 26 PCR cycles of MDR1 cDNA (Fig. 4). We used β2m mRNA as an internal control for MDR1 expression, according to Noonan et al. (21). The ratio of the MDR1/β2m specific PCR product in KB8-5 RNA samples was stable. These results suggest that MDR1 gene expression can be semi-quantified by 26 cycles.
of RT-PCR with 500 ng of sample RNA. The amounts of \textit{MDR1} mRNA in the samples were evaluated in relation to a standard set of RNAs (500 ng), isolated from KB3-1 and KB8-5 and processed simultaneously with the test samples. RT-PCR under the same conditions revealed no \textit{MDR1} expression in KB3-1. RT-PCR of \textit{\beta}2m revealed a homogeneous level of expression, suggesting that the RNA was reliable as the initial template. We also examined the \textit{MDR1} gene expression level in several tumor xenografts that were serially transplanted, including passage numbers below 10. Xenograft xelKB3-1 did not express \textit{MDR1} and xelKB8-5 showed apparent \textit{MDR1} expression in vivo, reflecting their behavior in vitro. No significant changes in \textit{MDR1} expression levels were evident throughout the serial passages of KB xenografts (Fig. 5-A). Xenograft xelKB3-1-R expressed a definitive, but lower level of \textit{MDR1} than xelKB8-5 (Fig. 5-B).

The graphs in Fig. 2 and Fig. 3 illustrate the growth rate of tumor xenografts in the in vivo chemosensitivity test to vincristine (VCR) and doxorubicin (DOX), respectively. Each group included 4 nude mice bearing tumor xenografts. Solid line, untreated control; Broken line, treated group. (A) xelKB3-1, (B) xelKB8-5, (C) xelKB3-1-R.
DISCUSSION

KB3-1 is sensitive to many anticancer drugs, whereas KB8-5 selected by the administration of two courses of colchicine (5 ng/ml, 10 ng/ml), has shown multidrug resistance (MDR) to colchicine, VCR, DOX, and etoposide (3, 20, 23). Several studies using these human KB cell lines have revealed that in vitro MDR mechanisms are correlated with P-Gp/MDR1 overexpression (3, 9, 25-28, 31, 32). However, little is known about the apparent relationship between acquired drug resistance and P-Gp/MDR1 overexpression in human carcinoma xenografts in vivo, such as lung cancer and rhabdomyosarcoma (6, 11, 28). We established KB carcinoma xenografts from KB cell lines to examine the discrepancy between in vitro and in vivo behavior.

After establishing the human epidermoid cell carcinoma KB xenografts (xeKB3-1 and xeKB8-5), a VCR-resistant variant (xeKB3-1-R) was selected by treating the drug sensitive xeKB3-1 with VCR in vivo. RT-PCR has increased the sensitivity of detection of low levels of MDR1 expression (19, 21). By means of this assay, we demonstrated apparent MDR1 expression in xeKB3-1-R but not in xeKB3-1. The xenograft xeKB3-1-R expressed less MDR1 than xeKB8-5. These results suggest that the inducible drug resistance in vivo is related to the low level enhancement of P-Gp protein/MDR1 expression in KB xenografts. We previously reported that the levels of MDR1 gene expression were low in the various intrinsic MDR-xenografts in vivo (1).

The sensitivity of these KB xenografts was examined in nude mice treated with the CED of anticancer drugs (VCR and DOX). The sensitivities of the tumor xenografts in vivo were determined both by the Mann-Whitney U-test and by T/C values of 50% or less (12-14, 18, 29). Xenograft xeKB3-1 was sensitive to the CEDs of VCR and DOX, whereas xeKB8-5 was resistant to the drugs. VCR-resistant xeKB3-1-R was not cross resistant to DOX in the chemosensitivity test in vivo, whereas xeKB3-1-R was resistant to DOX in the adhesion tumor cell culture system (ATCCS) assay in vitro (data not shown) (2). These results suggest that DOX is more effective in vivo than in vitro. In addition, the expression of topoisomerase II may represent an alternative mechanisms in the resistance to DOX (17).

The chemotherapeutic experimental system presented herein, with nude mice bearing human tumor xenografts, is useful for investigating the MDR phenomenon in vivo and to develop new anticancer agents. Further analysis of acquired MDR in vivo is in progress using KB xenografts.

| Table 1 | In vivo sensitivity of tumor xenografts to VCR or DOX |
|---------------------------------|---------------------------------|---|---|---|
| Xenograft /Drugs | Relative Tumor Volume (%) | | | | |
| | Control | Treated | U-Test | T/C | Sensitivity |
| /VCR | | | | | |
| xeKB3-1 | 13.14 ± 1.44 | 6.30 ± 0.92 | + | 48 | S |
| xeKB8-5 | 16.02 ± 4.50 | 11.52 ± 3.10 | - | 72 | R |
| xeKB3-1-R | 13.98 ± 2.51 | 10.67 ± 1.18 | - | 76 | R |
| /DOX | | | | | |
| xeKB3-1 | 13.14 ± 1.44 | 1.84 ± 0.20 | + | 14 | S |
| xeKB8-5 | 8.50 ± 2.35 | 4.33 ± 0.41 | - | 51 | R |
| xeKB3-1-R | 13.98 ± 2.51 | 0.90 ± 0.41 | + | 6 | S |

Relative Tumor Volume (RV), RV=Vt/V0 where Vt is the tumor volume at day 14 and V0 is the initial tumor volume on the day treatment was started (day 0).

U-Test, statistical differences were estimated by the Mann-Whitney U-Test (p<0.01, one-sided). (+, significant; -, insignificant)

T/C, growth ratio of the relative volume of the treated xenografts to controls (untreated) on day 14 of treatment. S, sensitive; R, resistant.

*Data expressed as mean values ± SD
Fig. 4  The signal level of MDR1 specific products at 26 cycles of PCR increases in a dose-dependent manner with the initial amount of the KB8-5 RNA template. MDR1 expression was detected in over 25 ng of KB8-5 mRNA under these conditions. DNA size markers (puc19 DNA digested with Hae III) are also shown. The co-amplified \(\beta\)2m gene PCR products (120bp) were rehybridized with the oligonucleotide probe.

Fig. 5  Gene expression of MDR1 in the tumor xenografts. (A) Lane 1, \textit{in vitro} KB8-5 cell line; Lane 2, \textit{in vitro} KB3-1 line; Lane 3, \textit{ex-KB}8-5 xenograft (3 passages); Lane 4, \textit{ex-KB}3-1 xenograft (3 passages); Lane 5, \textit{ex-KB}8-5 xenograft (8 passages); Lane 6, \textit{ex-KB}3-1 xenograft (8 passages). Each KB xenograft expressed similar levels of MDR1 compared with KB cell lines; significant changes were not seen in MDR1 expression throughout 10 serial passages. (B) Lane 1, \textit{ex-KB}8-5 xenograft; Lane 2, \textit{ex-KB}3-1 xenograft; Lane 3, \textit{ex-KB}3-1 xenografts from mice given 1 dose of 1.2 mg/kg VCR intravenously; Lane 4, \textit{ex-KB}3-1-R (resistant tumor xenografts selected in mice by administering 3 doses of VCR from \textit{ex-KB}3-1 xenografts).
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REFERENCES


