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Establishment and characterization of renal cell carcinoma cell lines with multidrug resistance

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Abstract Many of the discoveries of multidrug resistance (MDR) have resulted from studies using drug-resistant cultured tumor cell lines as experimental models. To date, there has been no report on the detailed characterization of such a cell line from renal cell carcinoma (RCC). By long-term exposure of an established RCC (RCC8701) to increasing concentrations of adriamycin, we established a series of subcultures that were considerably more resistant to the cytotoxic effect of this drug. Biological morphology and cell cycles were analyzed by morphometry and flow cytometry. The chemoresistance index of cells were measured by methyl tetrazolium assay. For evaluation of the expression of MDR-related protein (MRP), mdr-1, glutathione transferase (GST-π), and topoisomerase II mRNAs, the reverse transcription-polymerase chain reaction was used. Membranous expression of mdr-1-related p-glycoprotein was analyzed by immunofluorescence cytometry. The intracellular content of both glutathione (GSH) and glucose-6-phosphate dehydrogenase (G-6-PDH) were measured using a capillary electrophoresis method. Compared with parent cells, the resistant sublines had a slower growth rate and lower confluent density. They were smaller and mixed with giant cells in different sizes and with different numbers of nucleoli. Flow cytometric analyses showed that resistant cells had a greater percentage of cells in the G2/M phase. The resistant cells, RCC8701/ADR800, were 122 times more resistant to adriamycin and 238 times more resistant to epirubicin than the parent cells. The resistant cells also demonstrated cross-resistance to cisplatin and 5-fluorouracil. In addition to MRP, the contents of mRNA coding for mdr-1, GST-π, and topoisomerase II in the MDR sublines were higher than in the native cell line. A higher content of cytoplasmic GSH and G-6-PDH were found in the resistant cells; however, the expression of the MDR-related membranous glycoprotein, p-glycoprotein, was not raised. The adriamycin-induced MDR sublines may be used as an experimental system for the search of a means to overcome drug resistance and elucidate possible mechanisms of acquired MDR involved in human renal cancer.

Key words Renal cell carcinoma · Multidrug resistance · p-Glycoprotein · MDR-related protein · Glutathione transferase · Topoisomerase II

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Introduction

Chemotherapy has proven effective in the cure or palliation of many human tumors such as testicular cancer and leukemia; however, drug resistance remains a major obstacle in the treatment of other carcinomas. Human renal cell carcinoma (RCC) displays a characteristically high degree of chemoresistance toward a broad spectrum of natural cytotoxic compounds that do not possess obvious functional or structural similarities [28]. This phenomenon is termed multidrug resistance (MDR) [26]. Since reliable therapeutic alternatives to chemotherapy are still lacking, this resistance contributes considerably to the poor prognosis of patients with disseminated RCCs.

At least three distinct mechanisms that instigate or are associated with MDR have been identified. The first one is gp-170 p-glycoprotein [7, 23], an active, energy-dependent multidrug transporter in the membrane, which impedes intracellular cytotoxic drug accumulation by rapid extrusion. MDR-related protein (MRP), which was recently discovered [9], is very similar to gp-170 in function. The second one is the cytoplasmic glutathione redox cycle, which is involved in the intracellular binding and detoxification of chemotherapeutic agents [13, 16]. The existence of both mechanisms has been

documented in human RCCs and a relationship to adriamycin (doxorubicin) resistance has been clearly demonstrated [6]. The third is the nuclear enzyme topoisomerase II, which can inhibit drug-DNA adducts in repair [10]. Mickish et al. have explored the multiple drug resistance of RCCs. They used an in vitro model system of primary cultures of renal tumor to examine the relative contribution of the p-glycoprotein drug efflux (classic MDR-vinca alkaloids) and the glutathione redox detoxification system to doxorubicin resistance (atypical MDR) [16].

The aim of this study is to establish MDR cell lines of human RCC against the clinically important chemotherapeutic compound adriamycin, to trace the underlying resistance mechanisms of acquired MDR. Glucose-6-phosphate dehydrogenase (G-6-PDH) is included in this study because it is an energy and metabolism-related cytoplasmic enzyme and we presumed it may have a certain relationship to the MDR of RCC cancer cells.

Materials and methods

Establishment of RCC MDR cell lines

A human RCC cell line, RCC8701, was used in this study. The cell line was established in our laboratory from a localized RCC specimen. It was maintained in a RPMI-1640 culture medium containing 5% heat-inactivated fetal calf serum in an atmosphere of 5% CO_2 in a humidified incubator. Drug resistance sublines were cultured long-term in gradually increasing concentrations of adriamycin over a period of 16 months from 30 to 800 ng/ml.

Growth morphometry and DNA flow cytometry

The growth rate, saturation density, size, nucleus to cytoplasm (N/C) ratio, and DNA content were analyzed individually. Cultured parent and MDR subline cells were fixed, HE stained, and measured for size and N/C ratio under image analyzer (L2 system; Yen-Hau, Taiwan). DNA content and cell cycle were analyzed by flow cytometer (FACScan; B-D) using propidium iodide (50 μg/ml in citrate buffer; Sigma) as the staining dye [29].

Cytotoxicity assay of RCC MDR cell lines

The cytotoxicity assay and ID_{50} determination of anticancer drugs on RCC cells were analyzed by the microculture methyl tetrazolium (MTT) method as previously described [4]. Briefly, equal numbers of cells were inoculated into each well in 180 μl of RPMI-1640 medium, to which 20 μl of 10× concentration of anticancer drug or phosphate-buffered saline (PBS) was added. For each drug 5–10 concentrations were used, ranging from 0.001 mg/ml to 10 μg/ml, covering a 3- to 5-log concentration range chosen to span the 50% inhibitory concentration determined by preliminary assays. After 2 days of culture, with or without drug, 0.1 mg (50 μl of 2 mg/ml) of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical) was added to each well and then incubated at 37 °C for an additional 4 h. Plates were centrifuged at 450 ×*g* for 5 min in a plate holder and then the media was aspirated from plates leaving about 30 μl of media in each well. One hundred and fifty microliters of dimethyl sulfoxide (DMSO; Merck) was added to each well and the plates were placed in a shaker for 10 min to solubilize the formazan crystals. The plates were then read immediately at 540 nm on a scanning multiwell spectrophotometer (Titetek Instruments). All data points represent the mean of a minimum of six wells. Absorbance levels from drug-tested cells were corrected against untreated control absorbance values. The ID_{50} value is defined as the dosage of drugs in which 50% of cellular death (50% reduction of absorbance at 540 nm) occurred after 48 h of treatment.

Reverse transcription-polymerase chain reaction study of MRP, mdr-1, GST-π, and topoisomerase II mRNAs

The mRNA was prepared by a Fast Track 2.0 mRNA isolation kit (Invitrogen, USA). Cells plated in two 150T flasks, about 4×10^7 cells, were lysed in 15 ml Fast Track 2.0 lysis buffer. The NaCl of the lysate was adjusted to a 0.5 M final concentration. Seventy-five milligrams Oligo(dT) cellulose was added to the lysate and rotated gently for 60 min. RNA was eluted with elution buffer (10 mM TRIS-HCl, pH 7.5) and precipitated with 0.15 volume of 2 M sodium acetate and 2.5 volume of 100% ethanol. The RNA pellet after centrifuge was resuspended in 35 μl of elution buffer. The RNA yield was determined using a spectrometer at 260 nm to read the absorbance by the following formula: RNA = A260 × 0.04 μg × dilution factor. For evaluation of the expression of MRP, mdr-1, GST-π, and topoisomerase II mRNAs, the reverse transcription-polymerase chain reaction (RT-PCR) was used [24]. In brief, 1 μl of purified cellular mRNA (0.1–0.5 μg) was converted to single-strand cDNA by mixing it with 1 μl of $Oligo(dT)^{12-18}$ (0.5 μg/μl; BRL, Gaithersburg, Md.), 2 μl rTth DNA polymerase (250 units/ml; Perkin-Elmer, N.J.), 2 μl of dNTP (10 mM), 2 μl of $MnCl_2$ (10 mM), 2 μl of 10× reverse transcriptase buffer, and 10 μl of DEPC-treated deionized water. The cDNAs homologous with the MRP, mdr-1, GST-π, topoisomerase II-positive-strand RNA, 2 μl in each, were subjected to 35 cycles of treatment in a DNA thermal cycler 480 (Perkin-Elmer, N.J.) for amplification after adding 1 μl of 20 μM primer, 1 μl of Taq DNA polymerase (2–5 units/μl), 2 μl of 10 mM dNTP, and 6 μl of 25 mM $MgCl_2$ solution. These primers used were: MDR-1 (243 bp) sequence with upstream AAGCTTAGTACCAA-AGAGGCTCTG and downstream GGCTAGAAACAATAGTGAAAACAA; MRP (181 bp) sequence with upstream ATCAAGACCGCTGTCATTGG and downstream GAGCAAGGATGATGACTTGCAGG; GST-π (227 bp) sequence with upstream CATGCTGCTGGCAGATCAGG and downstream CATTCATCATGTCCACCAGG; and topoisomerase II (200 bp) sequence with upstream GCTGTGGATGACAACCTCCT and downstream GCCATCTAGCATTCGTCTGA. The cycle conditions included a 94 °C denaturation step for 5 min, a 50 °C annealing step for 30 s, and a 72 °C extension step for 30 s. β2-microglobulin-specific primers were used in each study as an internal control. Following PCR reaction, approximately 20% of the reaction mixture was run on a 1% agarose/ethidium bromide gel, and the presence of specific base pair fragments detected by UV fluorescence. The intensity of band was read by densitometer and calculated as the ratio to β2-microglobulin.

Immunofluorescence measurement of cell membrane p-glycoprotein expression

Trypsinized cells were suspended in PBS with a concentration of $2–4 \times 10^6$ cells/ml [2, 30]. An aliquot of 1 ml was incubated with 60 μl of rabbit polyclonal antibody MRK16 against p-glycoprotein (1:10 dilution; Oncogene). The suspension was incubated for 40 min at 37 °C. In parallel, a second identical aliquot was incubated with 60 μl of control antibody to allow for quantification of nonspecific binding. After washing with PBS three times, 60 μl of FITC-conjugated swine-anti-rabbit immunoglobulin antibody (1:50 dilution; DAKO) was added to the suspension, which was then incubated for 30 min at 4 °C. After the incubation was complete, the specimens were centrifuged and washed twice in PBS. The cell pellets were then suspended in 1 ml of PBS and made ready for flow cytometry analysis. Ten thousand cells were measured in each tube using the FACScan flow cytometer (Becton-Dickinson,

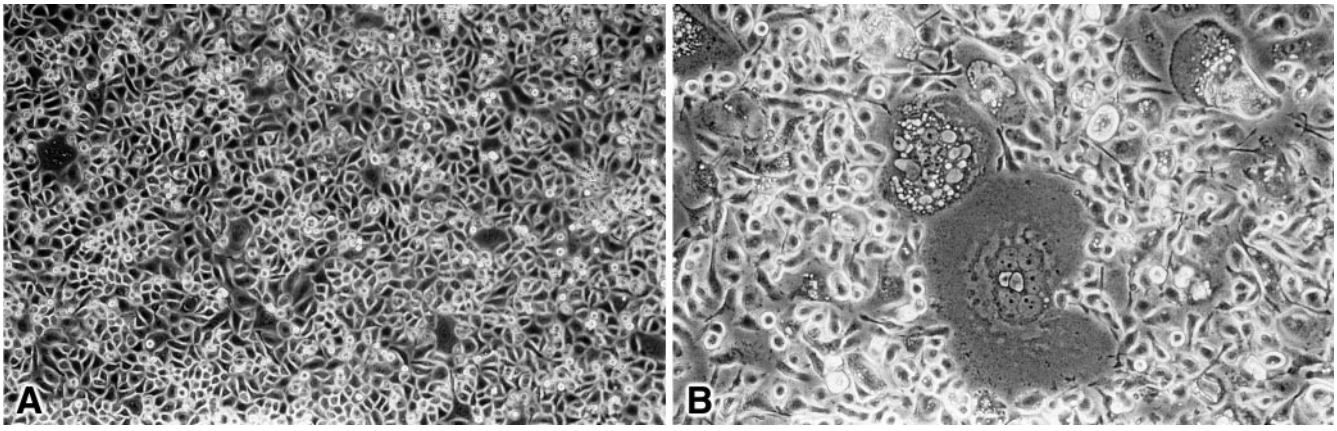

Fig. 1A, B Inverted microscopic examination of **A** RCC8701 (×100) and **B** RCC8701/A800 (×200) cultured tumor cells

Mountain View, California), and the results analyzed using the LYSIS software package (Becton-Dickinson) [11]. The intensity of fluorescence emitted by each of the 10,000 cells per sample was measured on a logarithmic scale of 10^4 channels, and the data displayed as a single parameter histogram and mean fluorescence value. All FACS detection of p-glycoprotein included positive and negative control cell lines. The mean fluorescence is a "net fluorescence" obtained from an experimental group subtracted from a negative control group and is measured on a logarithmic scale.

Capillary electrophoresis analysis of cytoplasmic GSH and G-6-PDH

Exponentially growing cells were harvested by a rubber scratcher after washing twice with cold PBS on the 3rd day after subculture. Cells were homogenized by ultrasound (Ultraturrax) and centrifuged at 42,000 rpm for 60 min. The cold supernatant was used for measurement of the GSH and G-6-PDH levels. The protein content was determined from aliquots of the solution using folin-phenol reagent (Sigma). The extinction was measured at 595 nm in a spectrophotometer (GBC UVIVIS 911A, UK) and bovine serum albumin served as a standard. The supernatant (adjusted to 1 mg protein/ml) was run in the capillary electrophoresis using a microsampler capillary cartridge (14 cm × 25 m) (HPE100; Bio-Rad) and the GSH and G-6-PDH contents were determined by comparison with a standard using a chromatocorder (α-Tech CC-12) and expressed as micrograms of GSH or G-6-PDH/mg protein [25]. Experiments were run in duplicate.

Statistical analysis

The values of mRNAs ratio, p-glycoprotein expression, GSH levels, and G-6-PDH activities were expressed as mean ± standard deviation and tested by Student's *t*-test. A difference was considered statistically significant if the *P*-value was less than 0.05.

Results

Changes of cellular morphology, kinetics, and cycle

A series of MDR sublines, from RCC8701/A50 to RCC8701/A800, were established after more than 1 year of culture, with adriamycin concentration in the medium increased from 50 to 800 ng/ml. Cells adapted to clonal growth, smaller in size and with a lobulated giant nuclei after the drug challenge (Fig. 1). They were slow in growth with increased doubling time after long-term culture. Saturation density decreased and nucleus-to-cell ratio increased (Table 1). Flow-cytometric DNA analysis showed a decreased synthesis phase fraction cell population with unchanged DNA stemline (Table 1).

Drug-resistant strength of MDR sublines

The drug resistance of MDR subline RCC8701/A800 to adriamycin was 80-fold stronger than the native line at the ID_{50} level (Table 2). There was a 238-fold increased drug resistance against epirubicin. The resistance index remained stable even after adriamycin was withdrawn from the culture medium for several weeks. Cross-resistance to cisplatin, vinblastine, 5-fluorouracil (5-FU), 6-thioguanine, and hydroxyurea was also observed in RCC MDR sublines (Table 3).

Table 1 The biological characteristics of the RCC8701 cell line and adriamycin-induced multidrug resistance sublines

Cell line	RCC8701	RCC8701/A50	RCC8701/A500	RCC8701/A800
Doubling time (h)	30	30	22	44*
Saturation density ($\times 10^5$ cells/cm^2)	3.25	1.32	1.54	1.50*
Cell area (μ^2)	419	336	369	247
Cell perimeter (μ)	109	90	109	79
N/C ratio	0.48 ± 0.10	0.67 ± 0.11	0.65 ± 0.17	0.69 ± 0.18*
FCM DNA index	1.18	1.23	1.21	1.20
S-phase fraction (%)	46.5	36.3	29.6	25.4*

*$P < 0.05$ when compared with native cell line by Student's *t*-test

Table 2 The ID_{50} drug resistance index of multidrug resistant renal cancer cell sublines to adriamycin and epirubicin when compared to native cell line

Cell line	Adriamycin		Epirubicin	
	ID_{50} (μg/ml)	Resistance index	ID_{50} (μg/ml)	Resistance index
RCC8701	0.13		0.10	
RCC8701/A30	0.44	3.4	0.29	2.9
RCC8701/A50	0.60	4.6	0.54	5.4
RCC8701/A300	0.82	6.3	1.80	18
RCC8701/A500	3.82	29.4	16.21	162.1
RCC8701/A800	10.38	79.9	23.84	238.4

Table 3 The ID_{50} drug resistance index of RCC8701/A800 subline to various nonrelated anticancer drugs when compared with native cell line RCC8701

Anticancer drugs	ID_{50}	Resistance index
Cisplatin	1.41 μg/ml	329.2
Vinblastine	8.29 μg/ml	2.9
5-FU	1.83 μg/ml	9.8
6-thioguanine	25.71 μM	1.3
Hydroxyurea	76.00 μM	3.3

Changes of mdr-1, MRP, GST-π, and topoisomerase II mRNA

The expression of mdr-1, MRP, GST-π, and topoisomerase II mRNA that was detected by RT-PCR is shown in Fig. 2. In addition to MRP, which was slightly lower than native cells, the contents of mRNA coding for mdr-1, GST-π, and topoisomerase II in RCC8701/A800 subline were higher than for the native cell line RCC8701 (Table 4).

Change of membranous p-glycoprotein

As shown in Table 5, the amount of p-glycoprotein in the tumor cell membrane of RCC MDR sublines decreased after adriamycin challenge, as measured by the immunofluorescent method.

Fig. 2 RT-PCR amplification of mRNAs of mdr-1, MDR-related protein (*MRP*), glutathione transferase, (*GST*), and topoisomerase (*Topo-II*) in RCC8701 (*lane B*) and RCC8701/A800 subline (*lane C*). *Lane A* is standard for reference

Changes of cytoplasmic GSH and G-6-PDH

The MDR subline RCC8701/ADR800 had significantly higher GSH and G-6-PDH content than their parent cell lines (Fig. 3, Table 6).

Discussion

The putative underlying resistance mechanisms, including expression of mdr-1-related p-glycoprotein, drug reflux-related MRP, GST-related glutathione, energy-related G-6-PDH, and nuclear enzyme topoisomerase II were measured and correlated with the chemosensitivity of the cultured RCC cell line and its acquired MDR sublines.

Mickisch et al. have extensively elucidated the MDR mechanisms involved in fresh renal tumor tissues [17, 19]. They noted that, in clinical patients, the intrinsic resistance of RCC tumor cells to anticancer drugs, including carboplatin, doxorubicin, and vinblastine, is related to the expression of membranous p-glycoprotein and the cytoplasmic content of GSH. Subsequently, they also evaluated the circumventional efficacy of various modulators on these intrinsic MDR of RCC specimens, such as calcium channel blocker (verapamil) [20, 21] and GSH inhibitor (buthionine sulfoximine, BSO) [22], and found a dramatic effect.

Our study is the first report on the characterization of acquired MDR in RCC tumor cells. These tumor cells adapted MDR phenotype without changing their DNA stemline, which indicates that the production of MDR in tumor cells does not necessarily refer to the stemline changing. Significant changes of cellular morphology, growth rate, and increased N/C ratio during MDR adaptation were seen, although the stemline did not indicate change. No such similar data were available in other reports for a comparison between RCC cell lines.

In transitional cell carcinoma (TCC), McGovern et al. reported that an adriamycin-resistant TCC cell line, MGH-UIR, had a stemline change which was similar to our previous report on another cultured TCC MDR subline, TCC8803/A200 [15, 32]. The stability of the stemline in cancer cells appears to be different between inter- and intracellular levels and is not absolutely related to MDR generation. Nevertheless, decrease in growth rate, decreased saturation density, and increased cellular size and N/C ratio are the common rule for cancer cells acquiring MDR.

The adriamycin-induced RCC MDR sublines demonstrated cross-resistance to various anticancer drugs, including cisplatin, vinblastine, 5-FU, 6-thioguanine,

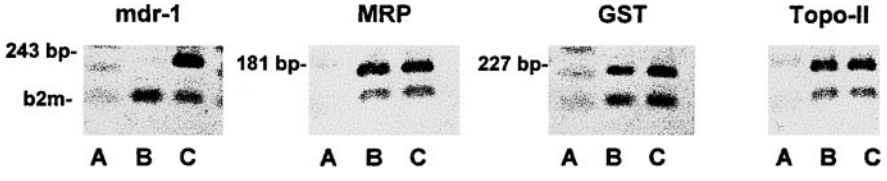

Table 4 The content ratio of mRNAs coding for mdr-1, multidrug resistant-related protein (*MRP*), glutathione transferase (*GST-π*), and topoisomerase II to β2-microglobulin in RCC8701 and RCC8701/A800 sublines

Cell line	mdr-1	MRP	GST-π	Topoisomerase II
RCC8701	0.07 ± 0.01	1.55 ± 0.32	0.94 ± 0.12	1.23 ± 0.22
RCC8701/A800	1.94 ± 0.24*	1.43 ± 0.08	1.26 ± 0.16*	1.40 ± 0.32

*$P < 0.05$ when compared with native cell line

Table 5 The mean fluorescence of p-glycoprotein in native RCC cell line and adriamycin-induced multidrug resistant sublines

Cell line	RCC8701	RCC8701/A50	RCC8701/A500	RCC8701/A800
p-Glycoprotein (mean fluorescence)	5.59 ± 1.02	1.26 ± 0.24*	0.35 ± 0.06*	1.82 ± 0.82*

*$P < 0.05$ when compared with native cell line

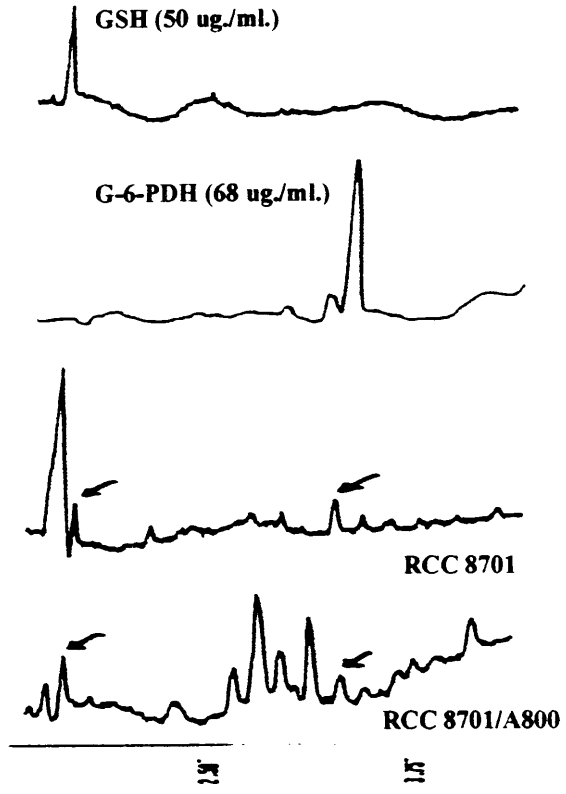

Fig. 3 The capillary electrophoresis of cytoplasmic proteins in RCC8701 and RCC8701/A800 tumor cells using pure cytoplasmic GST-π (*GSH*) and glucose-6-phosphate dehydrogenase (*G-6-PDH*) reagents as standard

Table 6 Concentration of cytoplasmic glutathione (*GSH*) and glucose-6-phosphate dehydrogenase (*G-6-PDH*) in native RCC8701 and multidrug resistant subline RCC8701/ADR800

	GSH (μg/mg protein)	G-6-PDH (μg/mg protein)
RCC8701	1.78 ± 0.34	9.72 ± 1.62
RCC8701/ADR800	6.85 ± 1.56*	28.00 ± 4.78*

*$P < 0.05$ when compared with native cell line

and hydroxyurea, in addition to adriamycin and epirubicin which are in the same drug group. The degree of resistance of RCC8701/A800 to cisplatin and epirubicin is much higher than in the original MDR-inducing drug, adriamycin. The real mechanisms are not yet defined. These anticancer drugs have differences in structure and anticancer mechanisms. This means some common pathways may participate in the antidrug function of acquired MDR RCC cells at the same time.

The generation of MDR should be started from the genetic changes in the nucleus of tumor cell (MDR-related genes) and their final products distributed from the nucleus to cytoplasm and membrane locations. The mRNAs from various common factors, including nuclear topoisomerase II, cytoplasmic GST-π (GSH), membranous mdr-1 (p-glycoprotein), and MRP have been well evaluated in RCC8701/A800 cells. A marked increase in mRNAs coding for mdr-1 and GST-π had been noted in the same way as for topoisomerase II, which showed a mild increase when compared with native cells.

Theoretically, an increased expression of p-glycoprotein membranous protein should be seen, reflecting the increased mdr-1 mRNA. On the contrary, a stepwise decrease in p-glycoprotein in MDR RCC cells acquired from long-term culture was observed in repeated tests and is different to the increased p-glycoprotein expression in the intrinsic MDR of RCC cells. Unlike the high expression rate (30–89%) of p-glycoprotein in original renal carcinoma cells [17, 18], the expression of p-glycoprotein in MDR RCC cells is very low. This phenomenon stresses the totipotential adaptation of cancer cells to chemotherapy. This discrepancy in mRNA content and p-glycoprotein expression may be due to the misreading of mRNA or unknown factors which interfere with precise steps of p-glycoprotein protein synthesis during the translation process. Interestingly, we had observed that in the early phase of drug challenge the membranous p-glycoprotein increased initially then it decreased in the late phase.

Amplification of MRP gene with overexpression of a 190-kDa membrane-bound glycoprotein, which is

thought to be related to MDR, has been reported in doxorubicin-resistant lung cancer cell line [3, 8], etoposide-resistant breast carcinoma [27], and leukemias [1]. The downregulation of MRP mRNA in RCC8701/A800 MDR cells indicates that the non-p-glycoprotein does not play an important role in mediating the MDR of RCC cells.

Increased cytoplasmic GSH is parallel to increased mRNA content for GST-π in the MRD RCC cells. The GSH also plays an important role in intrinsic MDR of RCC cells. The increased GSH should be related to the production of acquired MDR in RCC8701 cells. The relationship can be confirmed in a further study by MDR modulators or inhibitors on GSH activity such as BSO.

Although G-6-PDH also increased more in RCC MDR sublines than in the native line, the exact function of G-6-PDH in these cells or its relationship to MDR generation still needs further confirmation. In our previous study on TCC, increased cytoplasmic G-6-PDH was also observed in over 90% of TCC cell lines and all the adriamycin induced MDR sublines [31, 32], but no correlation with drug resistance was seen.

Close association of topoisomerase II and MRP has been reported in other cancers [13, 27]. However, there was no relationship between topoisomerase II and MRP expression in RCC observed in this study. Increased expression of topoisomerase I in RCC8701/A800 has also been demonstrated in northern blot in our laboratory (data not shown). We did not study further the differential expression of topoisomerase I and II. The proposed possible relationship of elevated topoisomerase-II mRNA and mdr in RCC cells needs further confirmation since usually it is reduced during development of drug resistance. The underlying mechanisms and function of topoisomerase II in the acquired MDR of RCC cells need further elucidation.

MDR-related oncogenes have been reported in various cancers such as c-*myc*, c-*jun*, c-*bax*, c-*ras* and *p-53* [5, 12]. The relationship between these oncogenes and aforementioned MDR-related gene products still needs further evaluation.

Conclusion

Morphological adaptation and intracellular changes could be evoked by drug challenge on RCC cancer cells with acquired high drug resistance. In addition to MRP, the contents of mRNA coding for mdr-1, GST-π, and topoisomerase II in the MDR sublines were higher than in the native cell line. The well-characterized MDR sublines may be used as an experimental system for the search of a means to overcome drug resistance and elucidate possible mechanisms of acquired MDR involved in human renal cancer.

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