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Expression of the mdr1 gene in human colorectal carcinomas: relationship with multidrug resistance inferred from analysis of human colorectal carcinoma cell lines*

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Summary. To investigate whether mdr1 gene products are involved in conferring the chemoresistant phenotype to human colorectal carcinomas (HCCs), we determined the mdr1 mRNA expression level (mdr1 EL) in surgical specimens from 29 pharmacologically untreated patients and analyzed the relationship between mdr1 EL and drug resistance in an in vitro experimental model. This consisted of 7 HCC cell lines chosen to cover the range of mdr1 ELs detected in the neoplastic specimens. No relationship was observed between the mdr1 EL of the HCC cell lines and the degree of chemosensitivity found for each drug tested, regardless of whether mdr1 gene products may [doxorubicin (DOX), vincristine (VCR), and actinomycin-D (ACT-D)] or may not affect [*cis*-diamminedichloroplatinum (CDDP)] drug-transmembrane equilibria. Conversely, a direct relationship was found between the mdr1 EL of HCC cell lines and the number of drug-resistant (DR) colonies arising from each parent cell line treated in continuous culture with high DOX concentrations. In addition, the chemoresistance index and mdr1 EL of the DR cell variants were roughly proportional to the mdr1 EL of the parent cell line. Our findings suggest that primary HCCs derive multidrug resistance from biochemical mechanism(s) other than mdr1 gene products. However, the mdr1 EL might be indicative of a predisposition to develop DR cell variants after chemotherapeutic treatment.

Introduction

Drug resistance, whether intrinsic or consequent to pharmacological treatment, is a major obstacle to cancer chemotherapy [9]. Drug resistance is often not limited to a single chemotherapeutic agent but may extend to structurally and functionally unrelated drugs [8]. Multidrug-resistant (MDR) cells generally express varying degrees of cross-resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, and actinomycin-D (ACT-D) but not to alkylating agents, antimetabolites, and *cis*-diamminedichloroplatinum (CDDP) [8, 12]. In vitro studies have shown that the MDR phenotype is frequently consequent to the expression of cell-surface high-molecular-weight glycoproteins (P-glycoproteins) that determine an active, energy-dependent drug extrusion from the cells [6]. In human cells, a glycoprotein of about 170 kDa (gp170) that is encoded by the mdr1 gene seems to play a major role in this phenomenon [3, 20].

It has been hypothesized that mdr1 gene expression could be responsible for the MDR phenotype in primary human tumors. Intrinsic mdr1 gene expression has been detected mainly in poorly chemoresponsive human neoplasms such as colon carcinomas, renal-cell carcinomas, and hepatomas [10]. Moreover, increased expression of mdr1 mRNA frequently occurs in many tumors at relapse after chemotherapy [22]. However, this relationship is not complete since tumors that do not express mdr1 gene products can exhibit the MDR phenotype, which suggests that such a property is not exclusively related to gp170 activity [17, 18].

The purpose of the present study was to investigate whether mdr1 gene products are involved in conferring the MDR phenotype on human colorectal carcinomas (HCCs). The relationship between the mdr1 expression level (EL) and drug resistance was studied in an in vitro experimental model so as to obviate both the many factors that affect the response to in vivo chemotherapeutic treatment [21, 26] and the possible heterogeneous gp170 expression within the tumor cell population [23]. The experimental model consisted of HCC cell lines chosen on the grounds of their constitutive mdr1 EL so as to cover the range of mdr1 ELs observed in primary HCCs. Within this experimental model, we also investigated the pharmacological and biochemical properties of drug-resistant (DR) cell variants arising

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after exposure of the HCC cell lines to high DOX concentrations.

Materials and methods

Chemicals. DOX (Adriamicin; Farmitalia-Carlo Erba, Milan, Italy), ACT-D (Cosmegen; Merck, Sharp, Rome, Italy), VCR (Oncovin; Lilly, Florence, Italy), and CDDP (Platinex; Bristol, Latina, Italy) were sterilely dissolved in saline just before their use.

Cell lines. DLD-1, LoVo, HT-29, SW480, SW948, SW1116, and SW1417 HCC cell lines were obtained from the American Type Culture Collection (Rockville, Md., USA). Cultures were propagated in RPMI 1640, F12 (LoVo), or Dulbecco's modified Eagle's medium (DMEM, HT-29) supplemented with 10% heat-inactivated fetal calf serum (FCS; Seralab, Sussex, UK), 1 mM Na-pyruvate, 50 μg streptomycin/ml, and 50 units penicillin G/ml. Cell lines were cultured at 37° C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Tissue samples. Colon carcinoma samples were obtained at surgery from patients who had never been treated with anticancer drugs. The corresponding normal mucosa specimens were obtained from the surgical margins. Fragments of the tissue specimens were pathologically examined and samples consisting entirely of neoplastic or normal tissue were used. Samples were quick-frozen in liquid nitrogen and stored at –80° C. The anatomic location, Duke's stage, and histological differentiation of the HCCs analyzed are reported in Table 1.

Cytotoxicity tests. Pharmacological treatments were carried out for 24 h at 37° C, then cell viability was tested by clonogenic assay. Clonogenic assays were performed in liquid medium by seeding $0.5-5 \times 10^5$ cells/6-cm petri dish as previously described [13]. Plates were incubated for 10–14 days at 37° C, then colonies were counted. Cytotoxicity was calculated as the drug concentration inhibiting the colony number by 50% (IC_{50}). The IC_{50} value was extrapolated by linear regression of experimental data.

Induction and selection of DR cell variants. DR cell clones were obtained by exposing in continuous, exponentially growing cell cultures to DOX concentrations as specified in Results. Medium containing DOX was substituted twice a week. After about 8 weeks, single colonies arising in culture containing the highest drug concentration compatible with cell survival were picked up and stabilized by growing them in the presence of DOX for at least 3 months at the same concentration used during selection. The incidence of DR clones was determined by counting the colonies arising in culture containing the same DOX concentration used for their isolation. DOX-resistant cell variants were termed with the monogram of the parent cell line followed by R, the level of DOX concentration used for selection, and the number of the clone (i.e. SW948-R-100-1 represents clone 1, derived from the SW948 cell line at 100 ng DOX/ml).

Southern blot analysis. High-molecular-weight DNA was extracted from bioptic specimens and cultured cells in accordance with standard methods [14]. DNA was digested with the appropriate restriction endonucleases, electrophoresed in agarose gel, and blotted onto a Gene Screen Plus membrane (New England Nuclear, Florence, Italy) by the Southern method [24]. Filters were prehybridized, hybridized with probes labelled with ^{32}P, and washed as described elsewhere [25]. Membranes were exposed to X-ray films using an intensifying screen at –80° C.

Northern blot and dot-blot analysis. Total cellular RNA was extracted by the guanidine chloride method from frozen or fresh samples as previously described [5]. For Northern blots, mRNAs were separated by electrophoresis in a denaturing 1% agarose/6% formaldehyde gel and transferred to Gene Screen Plus membrane by electroblotting in 0.025 M phosphate buffer pH 6.5 (10 V overnight and 40 V for 1 h). For dot-blot analysis, membranes were presoaked in distilled water and then 1-, 3-, and 10-μg aliquots of denatured RNA were loaded using a Hybri-Dot Manifold apparatus (BRL, Gaithersburg, Md., USA). Membranes were baked in a vacuum oven at 80° C for 2 h and then prehybridized, hybridized, and washed as for Southern analysis. Values for the mdr1 EL were determined by densitometry of the autoradiograms.

Table 1. Clinical profile and mdr1 mRNA expression in HCCs

Case	Tumor location	Stage[a]	Differentiation[b]	mdr1 EU[c]	
				T	N
1	Ascending colon	C2	P	9	7
2	Sigmoid-rectum	C2	M	ND	ND
3	Ascending colon	C2	M	ND	ND
4	Transverse colon	C2	M	18	15
5	Transverse colon	C2	M	26	11
6	Transverse colon	B2	M	2	3
7	Descending colon	B2	M	12	8
8	Descending colon	C2	M	1	2
9	Rectum	C2	M	ND	ND
10	Descending colon	B2	M	15	13
11	Rectum	C2	M	5,10,7	6
12	Ascending colon	C2	M	20	16
13	Sigmoid-rectum	B1	M	11	7
14	Sigmoid-rectum	B2	M	17	19
15	Rectum	C1	NA	3	3
16	Sigmoid	C2	M	18	20
17	Sigmoid-rectum	B1	M	13	9
18	Sigmoid-rectum	C1	M	14	12
19	Transverse colon	NA	NA	23	19
20	Sigmoid	C2	M	15	14
21	Sigmoid-rectum	C2	M	5,5,5	7
22	Sigmoid-rectum	C2	M	21	NT
23	Descending colon	C1	M	34	9
24	Rectum	B1	M	20	16
25	Rectum	C1	M	7,9,12	7
26	Ascending colon	C2	W	5,10,7	12
27	Rectum	C2	M	22	17
28	Rectum	C1	M	14	10
29	Sigmoid-rectum	C2	M	19,12,17	18

[a] According to Duke's classification (Astler-Coller modification)
[b] Degree of differentiation: W, well; M, moderate; P, poor
[c] T, Neoplastic tissue; N, normal tissue. Cases 11, 21, 25, 26 and 29 were subjected to 3 independent determinations
NA, Not available; ND, not detectable; NT, not tested

Probes. Southern, Northern, and dot blots were hybridized with a human mdr1 cDNA probe (1.2 kb) derived from EcoRI/digested plasmid pHuP170 1 [15]. The amounts of RNA and DNA loaded on the blots were estimated using a human β-actin probe (0.7 kb) derived from BamHI-EcoRI-digested plasmid pHFβA-3'UT [19] and a probe specific for the human c-*myc* third exon (1.6 kb) that was derived from ClaI-EcoRI-digested plasmid pHSR-1 [1], respectively. Probes were labelled with ^{32}P using a multiprime labelling system (Amersham, Buckinghamshire, UK) at a specific activity of $>10^9$ cpm/μg DNA.

Results

Transcriptional and structural mdr1 gene analyses in HCC specimens and HCC cell lines

Values for the mdr1 EL were estimated by dot-blot analysis using total RNAs. The specificity of the hybridization signal was confirmed by Northern blot analysis. mdr1 ELs

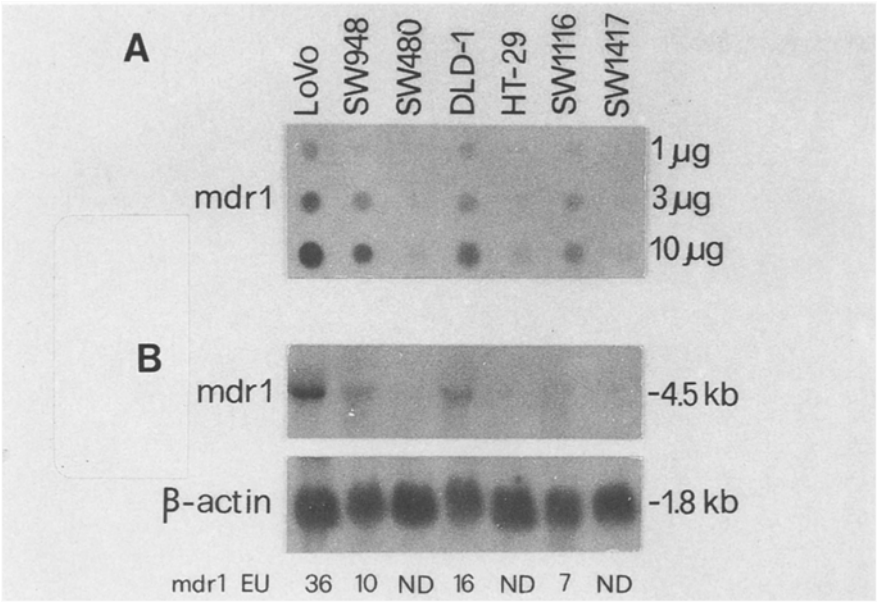

Fig. 1 A, B. Observation of mdr1 mRNA expression in HCC cell lines. **A** Dot-blot analysis. Total amounts of cytoplasmic RNA loaded are indicated at the *right*. **B** Northern blot analysis. In all, 10 µg total RNA was loaded in each lane. A β-actin probe was used as the internal standard. *EU*, Expression units; *ND*, not detectable

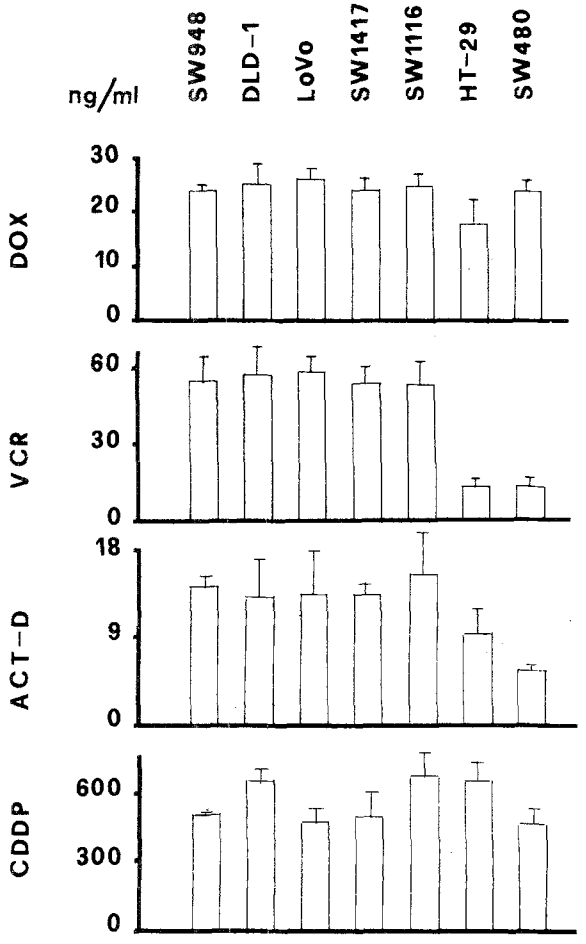

Fig. 2. Sensitivity of HCC cell lines to DOX, VCR, ACT-D and CDDP. Data (mean of at least 3 independent experiments) are reported as IC_{50} values; bars indicate SDs

were evaluated in arbitrary expression units (EU) by assigning a 10-EU value to the hybridization signal displayed by 10 µg total RNA obtained from the SW948 cell line. Primary HCC samples expressed mdr1 mRNA levels ranging from undetectable to 34 EU (Table 1). No substantial difference in the mdr1 EL was observed between the neoplastic and the corresponding normal tissues, the only exceptions being cases 23 and 26, which presented higher mdr1 ELs in neoplastic specimens than in normal tissues.

The variability in mdr1 EL within each tumor was also determined. Five randomly selected tumors were subjected to multiple independent determinations. Three fragments were obtained from different locations of the same specimen and were separately processed and analyzed. The intratumoral variability in mdr1 EL was narrow and lower than that detected in the overall series of HCC samples (Table 1).

HCC cell lines also differed in their mdr1 EL: HT-29, SW480, and SW1417 did not express detectable (ND) mdr1 mRNA, whereas SW1116, SW948, DLD-1, and LoVo expressed 7, 10, 16, and 36 EU, respectively (Fig. 1). Structural characteristics of the mdr1 gene were analyzed by Southern blot analysis after DNA digestion with PvuII, EcoRI, and BamHI restriction enzymes. Neither gene amplification nor gene rearrangements were detected in HCC specimens or cell lines (data not shown).

Chemosensitivity of HCC cell lines

The chemosensitivity of HCC cell lines was estimated by clonogenic assay in liquid medium. Five cell lines (DLD-1, LoVo, SW948, SW1116, and SW1417) were almost equally sensitive to DOX, VCR, ACT-D, and CDDP (Fig. 2). In contrast, HT-29 and SW480 displayed a different pattern of chemosensitivity as compared with the former cell lines. SW480 was characterized mainly by higher sensitivity to VCR and ACT-D, whereas HT-29 exhibited higher sensitivity to VCR only. The sensitivity of

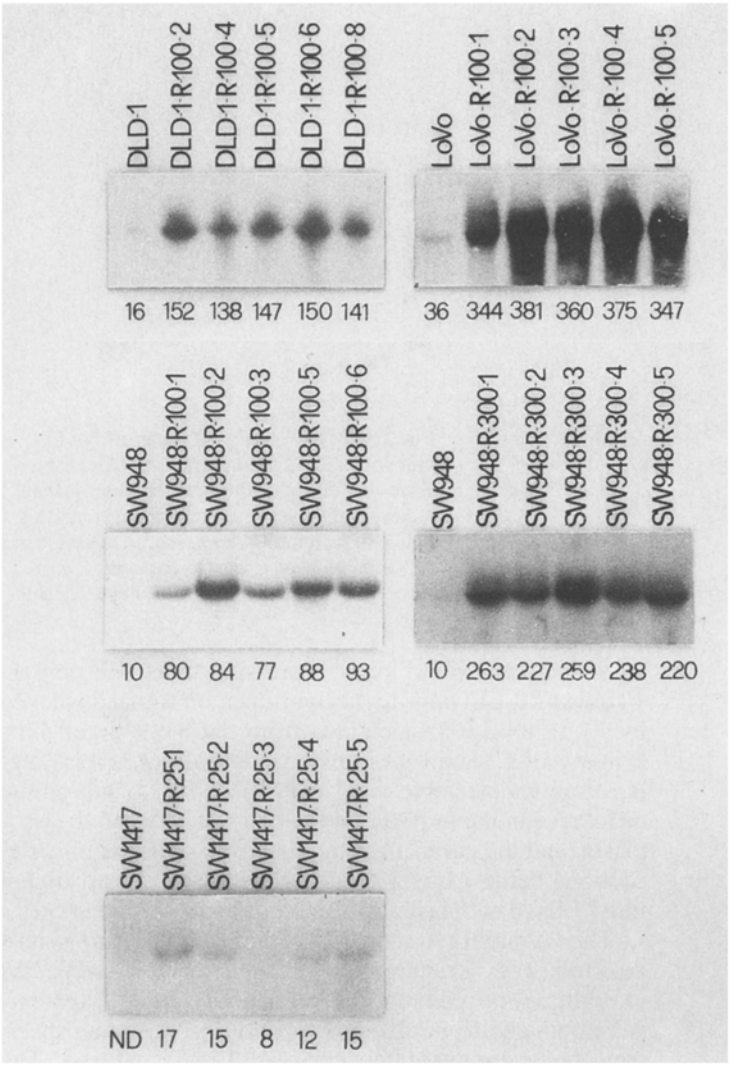

Fig. 3. Observation of mdr1 mRNA expression in DLD-1, LoVo, SW948 and SW1417 cell lines and DR variants. Each lane contained 10 μg total RNA. Values in mdr1 EU (reported under each lane) were calculated as the area under the densitometric peak for the mdr1 signal normalized by the corresponding β-actin level (data not shown). Exposure times for the five autoradiograms were different. *ND*, Not detectable

Table 2. Observation of mdr1 mRNA expression in HCC cell lines and incidence of DR cell clones

Cell lines	mdr1 EL (EU)	DOX[a] (ng/ml)	Number of colonies[b]
LoVo	36	100	17 ±9.2
DLD-1	16	100	5.3±2.5
SW948	10	100	0.9±0.4
SW1417	ND	25	13.7±6

[a] DOX concentration at which DR clones were derived
[b] Number of colonies for 1×10^6 cells seeded (mean value ± SD for 3 independent experiments)
ND, Not detectable

HT-29 and SW480 to DOX and CDDP was roughly similar to that of the other cell lines (Fig. 2).

Selection of DOX concentrations and incidence of DOX-resistant clones

Exponentially growing SW1417, SW948, DLD-1, and LoVo HCC cells ($0.5-5 \times 10^5$ cells/plate in 10 ml medium) were exposed to 2× serial DOX concentrations ranging from 6.25 to 100 ng/ml for about 8 weeks. LoVo, DLD-1, and SW948 gave rise to DR colonies at all drug concentrations tested, whereas SW1417 yielded DR colonies in plates containing up to 25 ng DOX/ml. Higher DOX concentrations resulted in the complete killing of the latter cells. The incidence of DR colonies arising from each parent cell line at the respective peak selective DOX concentration is reported in Table 2.

SW948-R-100-1 cells were additionally subjected to selection with 300 ng DOX/ml under the same conditions described above. Five independent clones were recovered. The incidence of SW948-R-300 clones was not determined.

DOX-resistant clones: mdr1 mRNA expression and DR pattern

Five independently derived DOX-resistant clones from each parent cell line were analyzed for both mdr1 mRNA expression and drug-resistance pattern. The clones derived from each cell line showed roughly identical mdr1 ELs

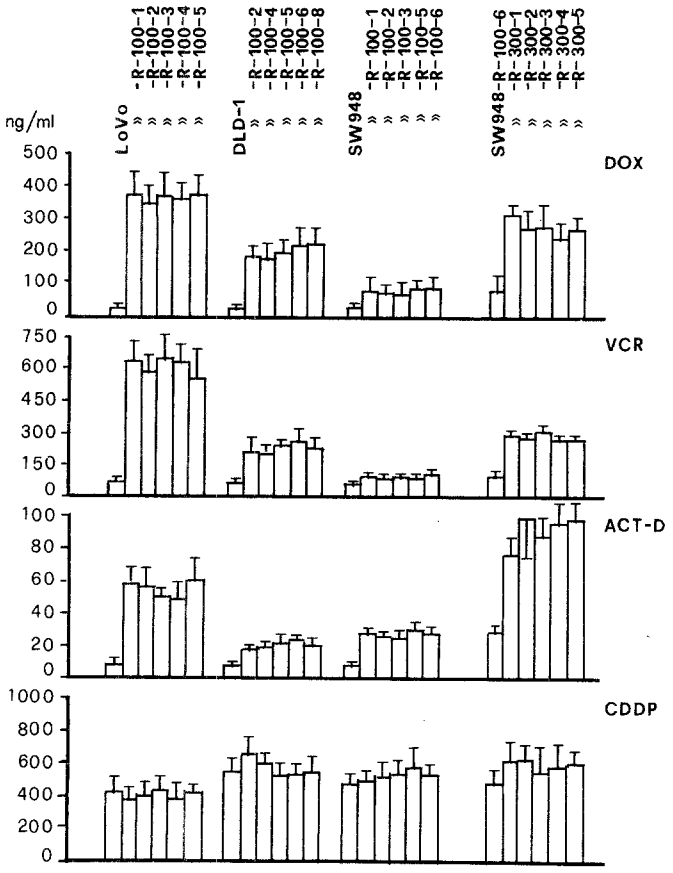

Fig. 4. Sensitivity of HCC cell lines and DR clones to DOX, VCR, ACT-D and CDDP. Data (reported as IC_{50} values) were obtained from at least 3 independent experiments; bars indicate SDs

(Fig. 3). As compared with the mdr1 ELs of the parent cell line, the LoVo-R-100, DLD-1-R-100, and SW948-R-100 cell variants displayed an almost identical (10-fold) increase in mdr1 gene expression. Since SW1417-R-25 clones were derived from a cell line in which the mdr1 EL was undetectable, the entity of such an increase could not be established. In all 5 LoVo-R-100 clones, the increase in mdr1 mRNA expression was associated with mdr1 gene amplification (about 8–10 gene copies for the diploid genome; data not shown). DOX-resistant variants derived from the other parent cell lines showed neither gene amplification nor structural gene rearrangements (data not shown).

DOX-resistant clones derived from LoVo, DLD-1, and SW948 displayed the typical MDR phenotype. In relation to the parent cell lines, they showed increased resistance to DOX, ACT-D, and VCR but not to CDDP (Fig. 4). The pattern of cross-resistance was very similar among the five clones derived from each parent cell line, but it differed among subsets of resistant clones. LoVo-R-100 variants showed the greatest degree of resistance, whereas SW948-R-100 variants displayed a moderate increase in resistance to DOX and VCR and a marked increase in resistance to ACT-D. DLD-1-R-100 variants exhibited intermediate increases in drug resistance. Therefore, in spite of the almost identical drug sensitivity of the LoVo, DLD-1, and SW948 parent cell lines, their DR variants showed varying degrees of increases in drug resistance. Only slight, statistically nonsignificant increases in chemoresistance to ACT-D, VCR, and CDDP were detected in SW1417-R-25 DOX-resistant clones (data not shown).

The five SW948-R-300 clones were analogously characterized for mdr1 EL and chemoresistance. Both parameters were higher than those displayed by the parent SW948-R-100-1 cell line (Figs. 3, 4). As previously observed in DR clones derived from the first selection with DOX, essentially no clonal heterogeneity was induced by the second selective treatment.

Discussion

HCCs show a generalized chemoresistance to many different chemotherapeutic agents and this phenotypic property is the major obstacle to the clinical management of such tumors [4]. An understanding of the molecular bases of the MDR phenotype displayed by HCCs may therefore be crucial to the development of more effective chemotherapeutic regimens. At present, a major hypothesis ascribes the MDR phenotype of HCCs to mdr1 gene products [10, 11]. This hypothesis, however, is mainly based on the generalized constitutive expression of mdr1 gene products in HCCs, but conclusive experimental demonstrations of such a relationship are lacking. Moreover, the findings that even HCCs that do not express mdr1 gene products have a MDR phenotype and that gp170 inhibitory agents such as verapamil are ineffective in reverting the drug resistance of HCCs seem to weaken such a hypothesis [7].

Chemosensitivity data on HCC biopsy samples are very difficult to organize and interpret, mainly as a consequence of both the extremely low survival of HCC cells in primary cultures and the possible selection of cell clone(s) that are not truly representative of the neoplastic cell population. Because of these methodological problems, the relationship between the mdr1 EL and the drug resistance of HCC cells was analyzed in a selected group of HCC cell lines chosen on the basis of mdr1 ELs detected in our series of primary HCCs.

Low variability in mdr1 was observed among the different tissue fragments derived from each tumor. This finding indicates that primary HCCs that have not been subjected to selective pressure from antineoplastic drugs do not undergo a heterogeneous evolution of the MDR phenotype such as that observed in chemotherapeutically treated tumors [2]. In contrast, a fairly wide range of mdr1 ELs was observed in the overall series of primary HCCs. On the basis of histological analysis of tissue samples contiguous to those used for mdr1 EL determination, we can reasonably exclude that the observed variability could be attributable to a considerable contamination of the neoplastic specimens by normal cells. More probably, as previously observed [16], such a variability depends on the genetic characteristics of individual patients and is not a consequence of neoplastic transformation. In fact, no significant difference in mdr1 EL was detected between the neoplastic and the corresponding normal tissues in the large majority (27/29) of cases analyzed. Finally, the level of mdr1 gene expression in HCC did not depend on the ana-

tomic location, the Duke's stage, or the histological differentiation of the tumor.

In spite of the variability observed, the values of 0 and 34 EU were the lowest and highest limits, respectively, of mdr1 gene expression in our series of primary HCCs. Seven HCC cell lines were chosen so as to cover such a range of mdr1 ELs. Chemosensitivity tests performed on the HCC cell lines did not reveal any relationship between drug resistance and mdr1 EL. Therefore, biochemical factors other than mdr1 gene products must have determined the chemoresistant properties of the HCC cells following exposure to 24-h drug treatments.

In vivo chemotherapeutic regimens involve sequential drug treatments that may induce or select DR cell variants, which in turn are ultimately responsible for the failure of the pharmacological treatment. To ascertain whether a relationship exists between the mdr1 EL and the cellular response to a protracted drug treatment, we subjected SW1417, SW948, DLD-1, and LoVo HCC cell lines to DOX selection. These cell lines were chosen on the basis of a similar pattern of chemosensitivity but differential mdr1 ELs (ND, 10, 16, and 34 mdr1 EU, respectively). SW948, DLD-1, and LoVo developed DOX-resistant colonies even at the highest DOX concentration tested (100 ng/ml), whereas SW1417 gave rise to DOX-resistant variants only at lower selective pressures (25 ng/ml). Moreover, HCC parent cell lines expressing detectable levels of mdr1 gene products developed DOX-resistant clones at a frequency roughly proportional to their mdr1 ELs.

Five DOX-resistant clones derived from each cell line (at 100 ng DOX/ml for SW948, DLD-1, and LoVo and at 25 ng/ml for SW1417) were characterized for chemoresistance pattern and mdr1 EL. No clonal heterogeneity was observed among the clones derived from each parent cell line, but each subset displayed a specific level of chemoresistance and mdr1 mRNA expression. Such levels were roughly proportional to the mdr1 EL of the parent cell line. These findings indicate that both chemoresistance as detected by clonogenic assay and adaptation to a protracted selective pharmacological pressure are dependent on different biochemical mechanisms. The latter phenomenon seems to be genetically determined since the DR variants arising from each HCC cell line displayed identical pharmacological and biochemical properties. In addition, the level of drug-selective pressure seems to affect the adaptative evolution of HCC cells. In fact, the five SW948-R-300 variants arising from the SW948-R-100-1 cell line at 300 ng DOX/ml displayed greater drug resistance and more elevated mdr1 ELs than did the parent cell line.

In conclusion, although the inference of in vivo properties from data obtained from experimental models involves approximate assumptions, our findings suggest that the chemoresistant phenotype displayed by primary HCCs is not related to their constitutive expression of mdr1 gene products. However, a direct relationship exists between the constitutive mdr1 EL exhibited by HCC cells and the adaptative response to selective pharmacological stimuli. This finding might be useful in enhancing our understanding of the molecular bases of the development of DR neoplastic populations from primary HCCs as a consequence of chemotherapeutic treatment.

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