Human hepatocellular carcinoma cell lines exhibit multidrug resistance unrelated to \textit{MDR}1 gene expression

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Summary

Multidrug resistance of human cancer cells may result from expression of P-glycoprotein, the product of the \textit{MDR}1 gene, acting as an energy-dependent drug efflux pump. However, direct evidence that expression of the \textit{MDR}1 gene contributes to the multidrug resistance of human liver carcinomas has not been established. In this study, we tested five cell lines derived from human hepatocellular carcinomas for sensitivity to a variety of drugs used widely as anticancer agents: these included vinblastine, doxorubicin, actinomycin D, mitomycin C, 5-fluorouracil, 6-mercaptopurine, melphalan, methotrexate, \textit{cis}-platinum and etoposide (VP-16). All five hepatoma cell lines were resistant at different levels to these chemicals compared to human KB cells. Although it has been demonstrated that resistance to vinblastine, colchicine, doxorubicin and actinomycin D in human multidrug-resistant cells is associated with overexpression of P-glycoprotein, very little expression of P-glycoprotein was found in these human hepatoma cells. Neither verapamil nor quinidine, inhibitors of the drug efflux pump, were able to overcome multidrug resistance in hepatoma cells. These results indicate that the multidrug resistance phenotype in human hepatocellular carcinoma cells cannot be attributed to expression of the \textit{MDR}1 gene, but that novel mechanisms may account for the resistance of these cancer cells.

Key words: P-glycoprotein, hepatoma cells, doxorubicin, \textit{cis}-platinum.

Introduction

The phenotype of multidrug resistance (MDR) has been studied extensively in tissue culture cells as well as in human cancers (Pastan and Gottesman, 1987; Endicott and Ling, 1989; Shen \textit{et al.} 1986a; Goldstein \textit{et al.} 1989), where it is commonly associated with expression of the \textit{MDR}1 gene, which encodes the 170 000 M\textsubscript{r} membrane glycoprotein, acting as an ATP-dependent efflux pump to prevent accumulation of drugs in resistant cells (Gottesman and Pastan, 1988). It has been reported recently that \textit{MDR}1 RNA is expressed at substantial levels in human colon, kidney, small intestine and liver (Fojo \textit{et al.} 1987b; Gottesman \textit{et al.} 1989), and at increased levels in rat liver induced by carcinogens, hepatocytome and malignant transformation (Thorgeirsson \textit{et al.} 1987; Fairchild \textit{et al.} 1987; Burt \textit{et al.} 1988). In the case of renal adenocarcinomas, correlative evidence and data based on the use of inhibitors of the multidrug transporter suggest that the \textit{MDR}1 gene contributes directly to the intrinsic multidrug resistance of this cancer (Fojo \textit{et al.} 1987a; Kakehi \textit{et al.} 1988; Kanamaru \textit{et al.} 1989).

Despite the large number of patients throughout the world who die of primary liver cancer, the number of patients with this tumor who have entered carefully planned clinical chemotherapy regimens from which valid conclusions can be drawn remains limited. However, most existing studies indicate that the disease is resistant to most chemotherapeutic regimens tested. The intrinsic mechanisms by which liver carcinomas resist chemotherapy or acquire drug resistance after treatment remain to be determined.

In the present study, five cell lines derived from human hepatocellular carcinomas (Chen \textit{et al.} 1980; Shen and Chen, 1985) were used to explore the association between multidrug resistance and expression of the \textit{MDR}1 gene in human liver carcinoma cells.

Materials and methods

Cell lines and cell culture

Five human hepatoma cell lines, BEL-7402, BEL-7404, BEL-7405, QGY-7703 and SMMC-7721, were derived from different specimens of primary liver cell carcinomas not subjected to chemotherapy prior to surgery. Their biological characteristics have been previously described in detail (Shen and Chen, 1985; Chen \textit{et al.} 1986). These hepatoma cell lines were all grown as monolayer cultures at 37°C in 5 % CO\textsubscript{2}, using Dulbecco's modified Eagle's medium with 4.5 g l\textsuperscript{-1} of glucose (Gibco), supplemented with 15 % fetal bovine serum (Whittaker, M. A. Bioproducts), L-glutamine, penicillin and streptomycin.

KB-3-1, a human KB epidermoid carcinoma cell line, and its colchicine-selected derivative, KB-8-5, which was maintained in colchicine, 10 ng ml\textsuperscript{-1}, were used for comparison (Akizawa \textit{et al.}...
The dose–response curves of the hepatoma cell lines and the KB-3-1 cell line that served as a drug-sensitive control were determined by seeding $5 \times 10^4$ cells in 1 ml medium in each well of 24-well dishes. At the time of seeding, the chemicals at desired concentrations were introduced into the cell cultures. After incubation for 3 days, the cells were counted with a Coulter Counter. The $IC_{50}$ value was determined as the concentration of drug inhibiting cell growth to 50% of that in control (drug-free) medium. The relative resistance factor was determined by dividing the mean $IC_{50}$ value of the drug for the hepatoma cell lines by that for the KB-3-1 cell line that served as their control. The values are means of triplicate determinations.

Most of the chemicals tested in this study were purchased from Sigma, except mitomycin C (Calbiochem) and cis-platinum (platinum; Bristol Myers Laboratories).

Nucleic acid hybridization and protein gel
High molecular weight genomic DNAs and total RNAs were prepared as described (Shen et al. 1986b). RNA slot blots and Southern blots were hybridized with a 1383 base-pair insert of the cDNA probe pHDR 5A (Ueda et al. 1985; Shen et al. 1986a). The culture conditions for the KB cell lines were the same as for the human hepatoma cell lines described above.

Drug sensitivity assay
The dose–response curves of the hepatoma cell lines and the KB-3-1 cell line that served as a drug-sensitive control, were determined by seeding $5 \times 10^4$ cells in 1 ml medium in each well of 24-well dishes. At the time of seeding, the chemicals at desired concentrations were introduced into the cell cultures. After incubation for 3 days, the cells were counted with a Coulter Counter. The $IC_{50}$ value was determined as the concentration of drug inhibiting cell growth to 50% of that in control (drug-free) medium. The relative resistance factor was determined by dividing the mean $IC_{50}$ value of the drug for the hepatoma cell lines by that for the KB-3-1 cell line that served as their control. The values are means of triplicate determinations.

Results and discussion

Multidrug resistance phenotype
Human liver carcinoma is one of the most common cancers in males and females in the world (Yu, 1985; Munoz and Busch, 1987). However, no chemotherapeutic agents have been found to provide a clinically effective treatment of this disease. In this study we screened a variety of anticancer drugs or cytotoxic agents, including natural products affected by the multidrug resistance phenotype, using five human hepatocellular carcinoma-derived cell lines as an in vitro model.

Colchicine, vinblastine, actinomycin D, doxorubicin and etoposide (VP-16), drugs known to be transported by the P-glycoprotein efflux pump (Pastan and Gottesman 1987; Endicott and Ling 1989; Gottesman and Pastan, 1988), were tested for their toxicity against five human hepatoma cell lines as compared to KB-3-1 cells. Table 1 shows that the BEL-7404 cell line was 9.6 times more resistant to colchicine than KB-3-1 cells, while the other four cell lines exhibited only slightly higher resistance to this drug. All five liver cell lines showed a similar level of resistance to vinblastine, actinomycin D and doxorubicin, which was 2- to 5-fold greater than for KB-3-1 cells. All the hepatoma cell lines were even more resistant to VP-16, except for the SMMC-7721 cells.

We also examined the toxicity of some anti-cancer agents that are poor substrates for P-glycoprotein, such as cis-platinum, mitomycin C, melphalan, methothrexate, 5-fluorouracil (5FU) and 6-mercaptopurine (6MP). The results are shown in Table 2. All five hepatoma cell lines were quite resistant to mitomycin C, with 11- to 15-fold more resistance than KB-3-1 cells. Most of the cell lines also showed substantial resistance to all of the other agents tested. Thus, the five hepatoma cell lines displayed a broad multidrug resistance phenotype as evidenced by resistance to all of the chemicals tested here including drugs known to be affected by the multidrug transporter, or unrelated compounds. These results confirm the clinical impression that hepatocarcinomas are resistant to the commonly used anticancer agents (Falkson and Coetzer, 1987; Kamiyama and Tobe, 1987). The broad spectrum of resistance suggest that the resistance cannot be explained by expression of the MDR1 gene, which encodes part of a multidrug resistance transporter.
The effect of reversing agents
We reported that quinidine, at a clinically achievable concentration, enhanced sensitivity to vinblastine in cells from several renal cell lines and primary renal cell cultures that are naturally multidrug resistant (Fojo et al. 1987a; Kakehi et al. 1988; Kanamaru et al. 1989). Several calcium-channel blockers (i.e. verapamil), and many other agents (i.e. reserpine, phenothiazines, cyclosporin A) are also known to reverse the multidrug resistance phenotype, due to expression of the MDR1 gene in vitro (Tsuruo, 1988). To determine whether the MDR phenotype in hepatoma cells could be overcome by reversing agents, verapamil, quinidine, reserpine and thiadiazine were tested. The results are shown in Figs 1 and 2. Verapamil was effective at reducing resistance of renal cell lines as well as resistance of KB colchicine-resistant cells at a concentration of 10 μg ml⁻¹ (Fojo et al. 1987a). However, verapamil failed to overcome resistance in the hepatoma cell line BEL-7404 to the P-glycoprotein substrate colchicine, or to cis-platinum or mitomycin C when the same concentration was used (Fig. 1). Fig. 2A and B shows that the resistance of QGY-7703 cells to colchicine or mitomycin C was not overcome by quinidine at 7.5 μg ml⁻¹, a concentration known to reverse drug resistance in many cell lines. Neither reserpine nor thiadiazine at concentrations indicated in Fig. 2C and D enhanced the sensitivity of the hepatoma cell line BEL-7404 to colchicine. These results indicated that the mechanism(s) involved in the multidrug resistance phenotype in hepatoma cells was probably not associated with overexpression of P-glycoprotein.

Expression and amplification of the MDR1 gene
The most common form of resistance to multiple chemotherapeutic agents results from expression of a 170 000 Mr, membrane protein (P-glycoprotein, P170), encoded by the MDR1 gene. We determined the level of P-glycoprotein in each of the hepatocarcinoma cell lines by immunoprecipitation. As shown in Fig. 3, although a very light band of unknown origin is seen with a molecular weight of 170 000 Mr, this band is much lighter than the P170 band found in KB-8-5 cells which are 3- to 6-fold drug-resistant compared to KB-3-1. These data, taken together with the evidence presented above that inhibitors of P-glycoprotein do not reverse the MDR phenotype in hepatoma cells, indicate that the drug resistance of these cells cannot be attributed to expression of P-glycoprotein.

To determine with greater specificity whether the hepatomas express the MDR1 gene, MDR1 mRNA levels were measured by slot blot hybridization with a 32P-labeled MDR1 cDNA probe. Only one of the five hepatoma cell lines, QGY-7703, showed slightly higher MDR1 RNA levels than that of the other hepatoma cell lines or the KB-3-1 cells, but still less than the low-level multidrug-resistant cell line KB-8-5 (Fig. 4). No evidence of MDR1 gene rearrangement or amplification was detected in any of the hepatoma cell lines (data not shown).

The broad range of multidrug resistance of these hepatoma cells, and their failure to express significant levels of MDR1 RNA, or P-glycoprotein, or for their resistance to be overcome by verapamil, indicate that novel mechanisms of multidrug resistance are responsible for their phenotype. The general resistance of the hepatomas may be derived in some way from mechanisms related to the important role that the liver plays in

**Fig. 1.** Effect of verapamil on *in vitro* sensitivity of BEL-7404 cells to colchicine, cis-platinum and mitomycin C. The concentration of verapamil used was 10 μg ml⁻¹. (□) -Verapamil; (●) +verapamil.
detoxification of xenobiotics and chemical toxins in vivo. Recently, an increasing body of evidence has shown atypical or multiple patterns of drug resistance in human leukemia cell lines that fail to overexpress P-glycoprotein (Norris et al. 1989; Finalay et al. 1990), but none of these resistance patterns corresponds to those observed in the hepatoma cells. Increased levels of glutathione-S transferase and decreased topoisomerases I and II are thought to be associated with drug resistance in human breast cancer cells (Batist et al. 1986; Cazenave et al. 1989) and in some

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Continued analysis of these hepatoma cell lines should yield valuable information about these and other novel mechanisms of drug resistance.

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References


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