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Prediction of Doxorubicin Resistance in Gastrointestinal Cancer by P-Glycoprotein Staining

Masaji Yamauchi, Heiji Kumazawa, Tetsuya Satta, Isamu Sugawara, Kenichi Isobe, Yasuliro Kodera, Katsuki Ito, Tadashi Watanabe and Hiroshi Takagi

Feasibility of immunohistochemical staining of P-glycoprotein for the prediction of doxorubicin resistance in gastrointestinal cancers was examined. Among 10 cancer cell lines which consist of two gastric cancer cell lines and eight colon cancer cell lines, seven cell lines were stained positively by the monoclonal antibody to P-glycoprotein, C219. In consequence of the evaluation on the effect of doxorubicin on these tumour cells by means of succinic dehydrogenase inhibition test (SDI test), zero out of seven cell lines stained positively by C219 was sensitive to doxorubicin, but two out of three cell lines stained negatively were sensitive. Among 23 fresh surgical specimens of gastrointestinal cancers which consisted of 15 gastric cancers and eight colon cancers, seven tumour tissues were stained positively by C219. All P-glycoprotein positive tumours were resistant to doxorubicin. On the other hand, four of 16 P-glycoprotein negative tumours were sensitive to doxorubicin. These data indicate that positively stained cancer cells by C219 are resistant to doxorubicin.
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INTRODUCTION

FOR THE CHEMOTHERAPY of gastrointestinal cancers, doxorubicin is one of the most effective drugs. Combination chemotherapy regimens including doxorubicin have been used on patients with gastrointestinal cancers, although the response rates are not satisfactory and complete responses are generally uncommon. Recently, it has become clear that a 170 kD membrane glycoprotein (P-glycoprotein) encoded by the MDR1 gene functions as an energy-dependent drug efflux pump of specified anticancer drugs such as doxorubicin and vincristine [1]. When various molecular and immunologic techniques were used, P-glycoprotein expression was detected in human gastric and colorectal carcinoma cell lines and surgical specimens from patients with gastrointestinal cancers [2–5]. From these basic findings, P-glycoprotein is thought to play an important role in the doxorubicin resistance of patients with gastrointestinal cancers who have not been treated by doxorubicin before the surgery. In this study, we compared the *in vitro* sensitivity patterns of cytotoxic drugs and P-glycoprotein expression by immunohistochemical staining in cell lines and fresh specimens of gastrointestinal carcinomas.

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MATERIALS AND METHODS

Cell lines and clinical samples

Human gastric cancer cell lines that are now well established in serial subculture were used. MKN-28 and AZ521 were provided by Japanese Cancer Research Resources Bank. Human colorectal carcinoma cell lines, SW1083, SW1116 and SW1222 were presented by Sloan Kettering Cancer Research Institute and LoVo, Colo201, DLD-1, CaR-1 and SW-837 were provided by Japanese Cancer Research Resources Bank. Human epidermoid carcinoma cell line, KB and its colchicine resistant cell line KB^{chr} were donated by Dr Kuwano, Ohita Medical University. Clinical samples were obtained by surgical specimen in our department of surgery. Specimens were resected from the patients with stomach and colon cancer.

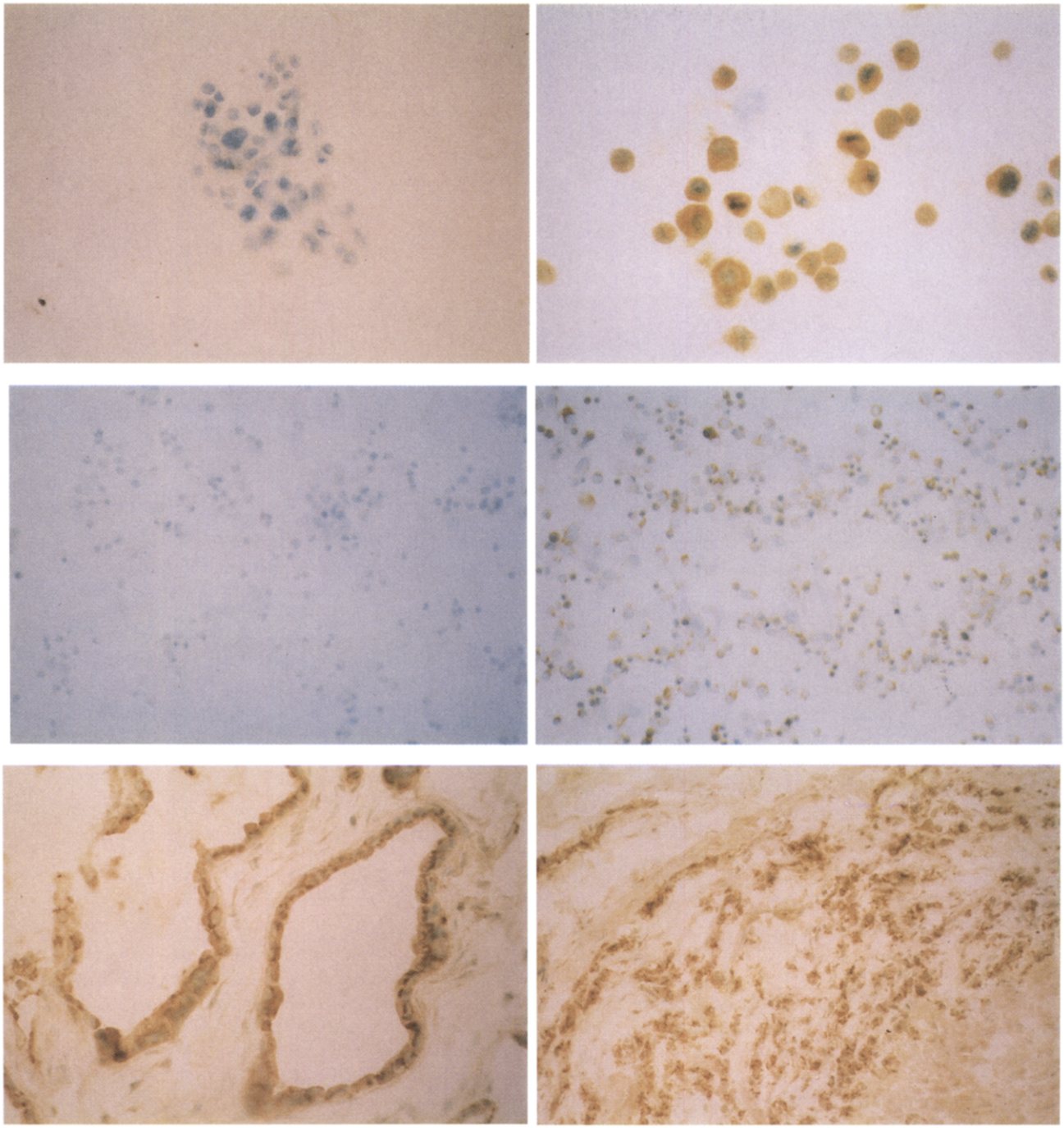

Fig. 1. Immunohistochemical C219 staining of cancer cell lines KB (left upper), AZ521 (left middle) and SW837 (left lower)—cells were negatively stained. KB^{chr} (right upper) MKN28 (right middle) and SW1222 (right lower) cells were positively stained.

Immunohistochemical staining

Expression of P-glycoprotein in the cell lines was assessed on cytospin slides and that in surgical specimens was assessed on the slides of frozen sections. Specific murine monoclonal antibody, C219, was obtained from the Centocor in Malvern, PA [6, 7]. C219 recognises a single 170 kD glycoprotein (determined by immunoprecipitation analysis), which has been identified as the P-glycoprotein associated with the multidrug resistant phenotype or mdr1. Immunohistochemistry was performed as follows. Each slide was incubated for 30 min at room temperature with normal horse serum. Primary antibody was incubated on the slides overnight at 4°C. Endogenous peroxidase activity was blocked in C219 preparations by incubation of slides in 0.3% H_2O_2 in absolute methanol for 30 min at room temperature. Slides were rinsed in phosphate buffered saline (PBS) and incubated for 30 min at room temperature in secondary antibody. Slides were rinsed in PBS and incubated for 30 min at room temperature in the avidin biotin horseradish peroxidase macromolecular complex. After rinsing in PBS, slides were incubated for 3 min in 0.05% 3.3′-idiaminobenzidine tetrahydrochloride dehydrate in PBS with 5 ml of 0.03% H_2O_2. Slides were rinsed in deionised water and then counterstained with methylgreen. KB^{chr} expressing known levels of the P-glycoprotein served as positive controls (Fig. 1). The cases in

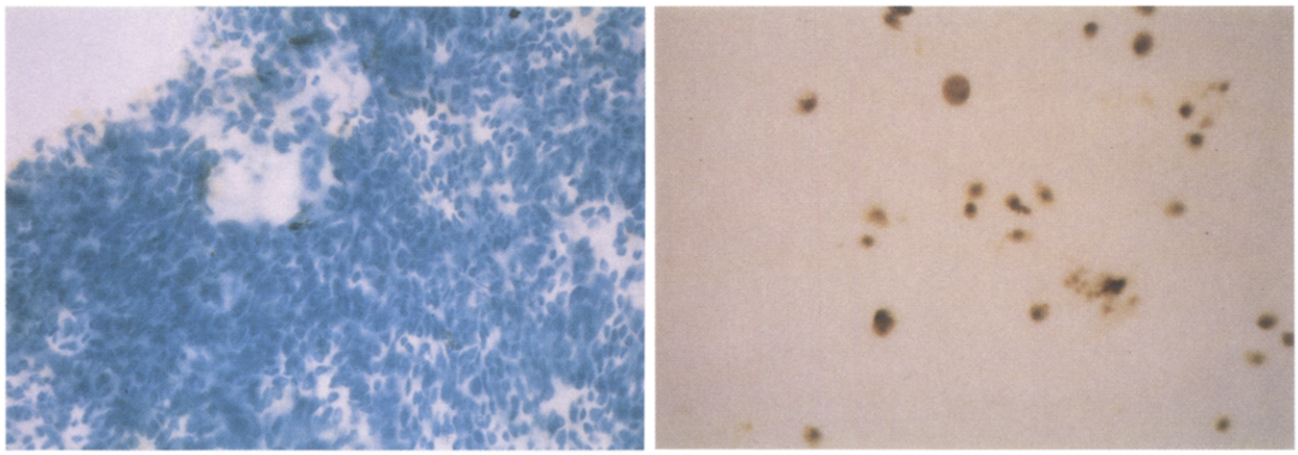

Fig. 2. Immunohistochemical C219 staining of fresh surgical specimen. Left figure from moderately differentiated adenocarcinoma of stomach is positively stained. Right figure from poorly differentiated adenocarcinoma of stomach is also positively stained.

which only cell surface membrane was stained clearly by this method are judged positive, but the cases in which both cell surface and cytoplasm were stained weakly or neither were stained are judged negative.

Chemosensitivity assay (SDI test)

Doxorubicin and mitomycin were obtained from Kyowa Hakko, Tokyo. Cisplatin was provided by Nihon Kayaku Pharmaceutical Industries, Tokyo. The succinic dehydrogenase inhibition test (SDI test) was developed in our laboratory in 1963 [8]. At first, we used tetrazolium triphenyl tetrazolium chloride (TTC) as the hydrogen receptor, but now we are using 3-(4,5-dimethylthiazol-2-yl)-2,5-d:phenylformazan bromide (MTT), which is also a tetrazolium salt but which is more sensitive than TTC. This method was first developed by Carmichael *et al.* [9]. MTT is a straw-yellow colour, but when a hydrogen donor is present, it is reduced and forms purple formazan. This reaction is extremely sensitive, and when cancer cells are destroyed by anticancer drugs, succinic dehydrogenase activity is lost, and the development of the purple colour no longer occurs. By measuring the differences in the colour of formazan, comparison of the effectiveness of various drugs is possible [10]. In the case of solid tumours, cell suspensions are prepared by mincing, and the cell number is adjusted 3×10^5 cells per ml to 5×10^5 cells per ml, divided into 100 μl lots, and poured into 96-well microplates, which contain 100 μl dilutions of each type of anticancer drugs in the RPMI 1640 medium containing 20% fetal calf serum. Then the cancer cells are incubated for 3 days at 5% CO_2. After washing the cells with PBS, the cells were spun down, and 10 μl 0.1 mol/l sodium succinate and 10 μl 0.4% MTT were added, after that the mixture was left to react for 3 h at 37°C. After completion of the reaction, formazan was dissolved in 150 μl dimethylsulphoxide (DMSO), and the optical density (OD) was measured with an ELISA reader at 540 nm. The OD was found to be accurately reflected the number of living cells when the cell count was over 10^4 cells per ml in the previous experiments and this test is evaluable when the OD is over 0.1 and the coefficient of variation (CV) from the OD of six wells without the addition of the anticancer drug is under 20%.

The inhibition index (I.I.) is calculated from $(a\text{-}p)/(a\text{-}m)$ 100 where p is the OD when the tumour cells are exposed to the anticancer drugs, a is the OD determined by the same procedure but without the addition of the anticancer drug, and m is the OD when neither the anticancer drug nor MTT is added. The drug was judged to be effective when the I.I. was 50% or more and ineffective when under 50%. In the SDI test, adequate concentrations necessary to evaluate the effect of doxorubicin and mitomycin are 1 μg/ml for the cancer cell lines and 10 μg/ml for the fresh specimen. In the case of solid tumours, the admixture of normal fibroblasts is an unavoidable drawback. In addition, the viability of cancer cells themselves in culture medium is low, and so higher drug concentrations for evaluation are necessary rather than those used in the cell lines [11].

RESULTS

P-glycoprotein staining

The criteria of positivity in staining are shown in Fig. 2. A small portion of KB cells were stained by C219 but almost all KB^{chr} cells were stained positively. In the previous study, Ding-Wu Shen *et al.* reported that KB^{chr} has amplified mdr1 which was detected by immunoprecipitation [5]. In human gastric cancer cell lines, AZ521 was negatively stained and MKN28 was positively stained. In human colon cancer cell lines, SW1083, SW1116, LoVo, Colo201, SW1222 and DLD-1 were positively stained, and CaR-1, SW-837 were negatively stained. In clinical samples, the specimens of four gastric cancers among 15 cases and three colon cancers among eight cases were stained positively. Positive rate of P-glycoprotein staining of these 23 cases was 30.4%.

Evaluation of the effect of anticancer drugs

In the study on the Selection of "Optimum AntiCancer Drugs Using Nude Mice" organised by the Ministry of Health and Welfare, we investigated in 1981 the administered doses of anticancer drugs used in *in vivo* sensitivity tests and established standard doses for these tests.

The maximum anticancer drug serum concentrations in nude mice, administered the standard doses of drugs, are applied to the critical drug concentration for the evaluation of the effectiveness of drugs in the SDI test. Although the doses in

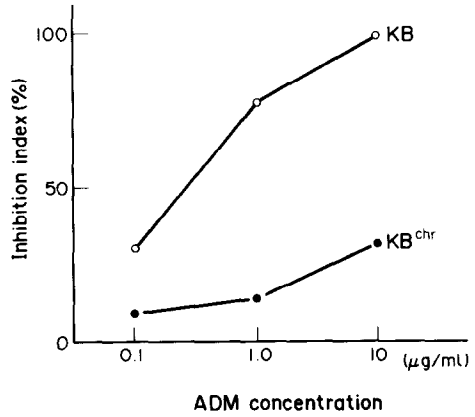

Fig. 3. Effect of doxorubicin on KB and KB^{chr} cells assessed by SDI test. ○KB = P-glycoprotein negative; ●KB^{chr} = P-glycoprotein positive.

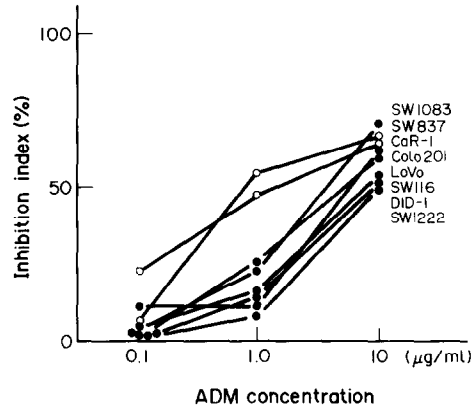

Fig. 5. Effect of doxorubicin on colon cancer cell lines assessed by SDI test. ○SW837, CaR-1 = P-glycoprotein negative; ●SW1083, Colo201, LoVo, SW1116, DLD-1, SW1222 = P-glycoprotein positive.

this test were much longer than those used clinically, they were thought suitable for the screening of drugs for gastrointestinal cancers, which composed with leukaemia and malignant lymphoma, are markedly drug resistant. Figure 3 showed the effect of doxorubicin on KB and KB^{chr}. Inhibition index of KB cells, which were stained negatively by C219, at the concentration of 1 μg/ml of doxorubicin was 78.2% and that of KB^{chr} stained positively was 13.1%. Figure 4 showed the effect of doxorubicin on gastric cancer cell lines. Inhibition index of AZ521 cells which were stained negatively by C219 was 95.5%. On the other hand, that of MKN28 cells which were stained positively was 15.4%. Figure 5 showed the effect of doxorubicin on colon cancer cell lines. Inhibition indexes of CaR-1 and SW-837 cells which were stained negatively were 46.8 and 54.7%, respectively. The other colon cancer cell lines were stained positively by C219 and the inhibition indexes of these cells were all under 50%. Mitomycin was effective to AZ521, but ineffective to the other cell lines. Mitomycin was more ineffective in KB than in KB^{chr}, so this ineffectiveness was caused by the mechanism excepting P-glycoprotein. Cisplatin was effective to KB^{chr}, AZ521, SW1083, LoVo, CaR-1 and SW-837. P-glycoprotein positivity was related to the effectiveness of doxorubicin but not cisplatin (Table 1). In these cell lines, four cell lines were stained negatively by C219 and three of them were sensitive to doxorubicin. Eight cell lines were stained positively by C219 and all of them were resistant to doxorubicin. The chi square (χ^2) test or Fisher's exact test were used for the evaluation of the significance of differences of the C219 staining between the resistant cell line group to doxorubicin and the sensitive group. There is a significant association of C219 positive stained cell lines and the resistance to ADM (Fisher's exact test: $P = 0.0182$, $\chi^2 : P = 0.03390$. In the clinical samples stained positively by C219, all cases were resistant to doxorubicin, but one gastric cancer case was sensitive to mitomycin and four cases were sensitive to cisplatin. On the other hand, in the 16 clinical samples stained negatively, four cases were sensitive to doxorubicin (Table 2). Sensitivities of mitomycin are similar to those of doxorubicin in both cell lines and surgical specimens except KB and SW-837 in Table 1 and cases four and seven of gastric cancer case VII of colon cancer in Table 2. The causes of similarity of sensitivities between mitomycin and doxorubicin

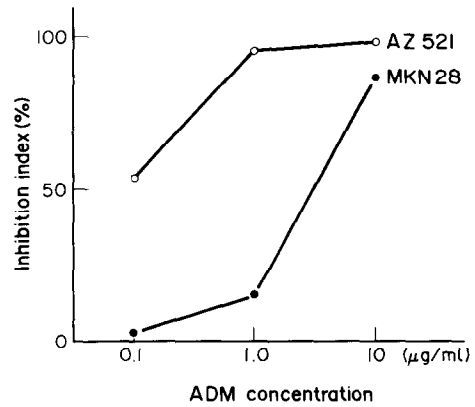

Fig. 4. Effect of doxorubicin on gastric cancer cell lines assessed by SDI test. ○AZ 521 = P-glycoprotein negative; ●MKN28 = P-glycoprotein positive.

Table 1. Effect of anticancer drugs on cancer cell lines and C219 immunohistochemical staining

Cell line	C219 staining	Inhibition index on SDI test (%) ADM (1 μg/ml)	MMC (1 μg/ml)	CDDP (10 μg/ml)
Renal cancer				
KB^{chr}	+	13.1	25.1	53.1
KB	−	78.2	7.5	32.0
Gastric cancer				
MKN28	+	15.4	18.3	32.4
AZ521	−	95.5	71.9	67.1
Colon cancer				
SW1083	+	23.1	22.3	53.9
SW1116	+	16.6	15.6	38.9
LoVo	+	25.1	17.7	63.9
Colo201	+	11.9	30.5	14.1
SW1222	+	7.2	33.8	40.9
DLD-1	+	13.6	11.4	7.6
CaR-1	−	46.8	31.7	61.7
SW-837	−	54.7	43.5	57.3

Effective: inhibition index is over 50% and figure is enclosed.

Table 2. Effect of anticancer drugs on surgical specimen from gastrointestinal cancers and C219 immunohistochemical staining

Case	C219 staining	Inhibition index on SDI test (%) Doxorubicin (10 μg/ml)	Mitomycin (10 μg/ml)	Cisplatin (15 μg/ml)
1	+	31.5	37.6	50.4
2	+	8.8	14.1	40.7
3	+	36.1	37.6	51.7
4	+	20.0	60.6	42.9
Gastric cancer				
5	−	33.4	30.3	32.6
6	−	10.8	24.7	47.6
7	−	26.2	61.3	25.0
8	−	3.7	32.5	49.7
9	−	19.2	33.6	43.2
10	−	9.3	21.1	36.3
11	−	51.6	56.9	—
12	−	57.3	57.5	66.9
13	−	4.04	49.1	43.0
14	−	15.4	15.9	32.9
15	−	50.8	69.3	65.6
I	+	9.5	37.6	51.7
II	+	39.8	34.5	39.0
III	+	31.1	33.1	51.8
Colon cancer				
IV	−	5.6	3.3	34.9
V	−	32.0	43.0	34.9
VI	−	47.2	47.2	60.6
VII	−	49.5	52.7	66.8
VIII	−	57.8	63.1	61.4

Effective: inhibition index is over 50% and figure is enclosed.

are unknown, but 12 P-glycoprotein-negative cases were resistant to doxorubicin. These results suggest that the other factor is concerned with the resistance of the anticancer drug.

DISCUSSION

Decreased drug accumulation with enhanced drug efflux occurring by P-glycoprotein in tumour cell lines has been well described. P-glycoprotein has recently been detected in tumour cells from patients with gastric cancer and colon cancer. Specific murine monoclonal antibody to P-glycoprotein, C219, was originally developed by Ling and colleagues as described in [12]. We used this antibody in this study. Among the cell lines used in this study, KB^{chr} and SW1222 have been revealed to have mdr1 in RNA by northern blot analysis and these cells were stained positively by C219. Sugawara *et al.* demonstrated that fresh human cancer tissues obtained from patients were stained successfully by using the other monoclonal antibody to P-glycoprotein, MRK-16 [7]; C219 stained successfully both in cell lines and fresh specimen. Weinstein *et al.* [13, 14] reported the non-specific reactivities of the antibody C219 to the normal human colon and ureter. They found high levels of putative Golgi P-glycoprotein in normal columnar and transitional epithelium in subpopulations of patients with specific blood types. In blood type A patients, Golgi staining by C219 was present in 46% of normal colon samples. On the other hand, in blood type 0 patients, Golgi staining was present in only 6% of normal colon samples [13, 14].

In spite of these non-specific cross-reactivities, we found inappropriate hyperexpression of P-glycoprotein in gastrointestinal cancer cell lines and cancer tissues of surgical specimens, and also confirmed by means of immunoelectron microscopy that in the cases of the positive staining, the cell membranes were apparently stained by C219.

The SDI test is a method of evaluating the influence of anticancer drugs on the survival and proliferation of tumour cells by measuring with a spectrophotometer the red formazan produced by mitochondrial succinate dehydrogenase in tumour cells, with the tetrazolium salt MTT. The study revealed a correlation between positive immunohistochemical staining for the P-glycoprotein and *in vitro* tumour cell resistance to doxorubicin in gastric cancers and colon cancers.

The chemosensitivity tests such as the SDI test, an isotope incorporation assay and subrenal capsule assay have a common weak point in that the normal fibroblasts are admixed to a greater or lesser degree when fresh surgical specimens are used [15–17]. Human tumour cell clonogenic assay (HTCA) is the only method without this drawback containing normal cells but the evaluable rate for the fresh gastrointestinal cancer tissues of HTCA is very low compared with the other assays. Salmon *et al.* reported that immunohistochemical staining is useful to determine the intrinsic cellular resistance of mutliple myeloma, malignant lymphoma or breast cancer to doxorubicin and the good correlation between positive immunohistochemical staining and *in vitro* tumour resistance to doxorubicin examined by HTCA [18].

Immunohistochemical staining method has advantages—the resistance of tumours to anticancer drugs can be determined by using small pieces of tumours without the influence of contamination by normal cells [19–21].

Recently, topoisomerase II has also been seen to take part in the resistance of tumour cells to doxorubicin and the mechanism of this drug resistance is revealed to be extremely complicated [22]. Hereafter, further studies are necessary to know the factors of resistance to individual anticancer drugs.

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Relationship of Cell Cycle Parameters to *in vitro* and *in vivo* Chemosensitivity for a Series of Lewis Lung Carcinoma Lines

Karen M. Holdaway, Graeme J. Finlay and Bruce C. Baguley

The sensitivity of three Lewis lung carcinoma sublines, which grow in culture and *in vivo*, and vary in *in vivo* drug sensitivity, have been compared using topoisomerase II poisons amsacrine, amsacrine analogue CI-921, doxorubicin and etoposide. D_{10} (drug concentration for 10% clonogenic survival) values were determined *in vitro* for low and high density cultures, and *ex vivo* for cells from subcutaneous tumours. The cytokinetic parameters of these populations were obtained by flow cytometric analysis of bromodeoxyuridine-labelled cells. Regression analysis showed that logarithmic D_{10} values were significantly correlated ($r > 0.95$) with G_1- and S-phase proportions and highly correlated ($r = 0.99$) with calculated G_1 transit times. The slopes of the regression lines were similar for all topoisomerase II poisons tested and it is suggested that this slope reflects the disappearance of topoisomerase II during G_1 phase.
Eur J Cancer, Vol. 28A, No. 8/9, pp. 1427–1431, 1992.

INTRODUCTION

ONE OF the goals of present day cancer chemotherapy is to determine, from a tumour biopsy, the spectrum of drug sensitivity of the patient's cancer cells. Three principal methods have been developed with this intent: drug sensitivity testing of cultured primary cancer cells in culture, inhibition of growth of tumour cell xenografts in immune deficient mice, and the analysis of biochemical, cytological or molecular characteristics of tumour cells which may predispose it towards responding to a given drug. One particular example of the third method which has found widespread clinical use is the technique of flow cytometric analysis of cellular DNA following labelling with 5-bromodeoxyuridine (BUdR) [1] and for some tumours there is an inverse correlation between the proportion of S-phase cells in the tumour and the response to chemotherapy with a variety of agents [2,3].

The use of model systems in which the cytokinetic properties of tumour cells can be related to chemosensitivity both *in vivo* and *in vitro* can provide valuable information relevant to the interpretation of clinical studies. Few studies have been carried out using variants of the same tumour cell line with varying cytokinetics and chemosensitivity. In this report we describe such a system using the murine Lewis lung adenocarcinoma, a tumour which initially arose spontaneously in $C_{57}B1$ mice, and which has a number of features which make it a good model for clinical carcinomas. It grows easily both *in vitro* and *in vivo* and is aneuploid, heterogeneous, metastatic and resistant to many, but not all, clinical antitumour agents. It has been used extensively as one of the main tumour models in the *in vivo* drug

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