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Correlations between natural resistance to doxorubicin, proliferative activity, and expression of P-glycoprotein 170 in human kidney tumor cell lines

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Summary. The natural resistance to doxorubicin of 15 human renal carcinoma cell lines was analyzed and compared to proliferative activity and expression of P-glycoprotein. We found a significant negative correlation between proliferative activity and natural resistance to doxorubicin, as well as between proliferative activity and the expression of P-glycoprotein. A positive correlation between resistance and expression of P-glycoprotein was found.

Key words: Doxorubicin resistance – P-glycoprotein – Proliferation activity – Monoclonal antibodies

The natural resistance of kidney tumors to chemotherapy remains a serious problem in clinical oncology. In previous investigations with sensitive and resistant tumors we showed that the tumor's response to treatment with cytostatics depends on the growth rate [17]. On the other hand, the phenomenon of drug resistance is correlated with molecular biological changes in the tumor, e. g., overexpression of membrane proteins. Frequently drug resistance is combined with cross-resistance. The cross-resistance between hydrophobic compounds of high molecular weight without apparent structural or functional similarities, e. g., anthracyclines, antibiotics, and alkaloids is termed "multidrug resistance" [14, 19]. The most frequently reported biochemical alteration in multidrug-resistant cells is the overexpression of a surface glycoprotein with a M_r 170 kDa (P-glycoprotein 170) [9, 12]. P-glycoprotein 170 (P-gp170) is thought to act as an energy-dependent efflux pump for anti-tumor agents. This transport protein is detected in multidrug-resistant animal tumors [20, 21], as well as in surgical human tumor specimens [7, 8, 22]. For example, kidney tumors are known to express high levels of P-gp170 [1, 4, 10].

The aim of this study was to analyze whether the proliferative activity of tumors and the overexpression of P-glycoprotein represent two independent factors in the development of drug resistance or whether there exists an association between these factors. For this reason, we investigated a panel of 15 human kidney tumor cell lines. For immunofluorescent detection of P-glycoprotein we used the monoclonal antibodies C219 and 265/F4 [12, 13], and for detection of the proliferative activity the monoclonal antibody Ki-67 [5]. The resistance of tumors was determined by the nucleotide incorporation assay [17].

Materials and methods

Human kidney tumor cell lines

The tumor cell lines were derived from specimens of kidney tumors and were obtained from Dr. S. Pomer (Urological Clinic, University of Heidelberg, FRG). Patients had not been exposed to chemotherapy before surgical removal of the tumors. After enzymatic treatment with collagenase, hyaluronidase and deoxyribonuclease, the resulting cell suspensions were centrifuged, resuspended and cultured in DMEM medium (Seromed, Berlin, FRG) supplemented with 10% fetal calf serum and 1% L-glutamine. The characterization of the cell lines by different tumor markers has recently been described [3]. All the investigations were carried out using subconfluent cell cultures, so the proliferative activity of the various cell lines are comparable.

Detection of doxorubicin resistance by the nucleotide incorporation assay

The nucleotide incorporation assay was carried out by the method described earlier [17, 18]. Briefly, tumor cells were incubated with doxorubicin at different concentrations for 3 h at 37°C. Radioactive nucleic acid precursors were added during the last hour of incubation. The incorporation activity of aliquots of the cell suspension was measured by scintillation counting. A correlation between incorporation of ^{3}H-uridine and ^{3}H-thymidine and the growth rate of tumors has been reported previously [16]. In a recent investigation we showed that the use of doxorubicin in the nucleotide incorporation assay is sufficient for detection of the multidrug-resistance phenotype [2].

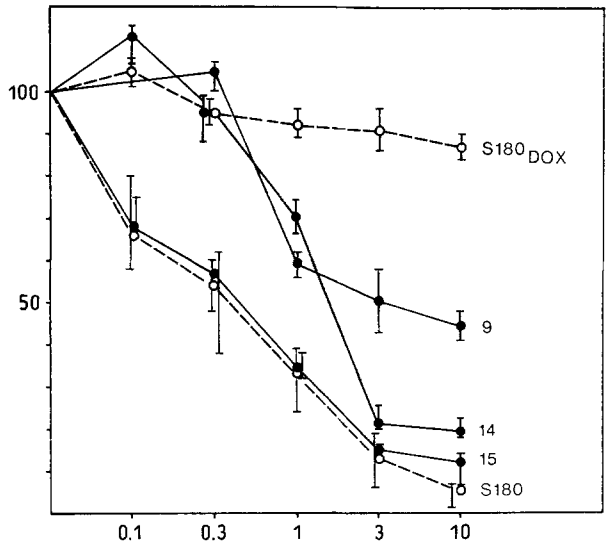

Fig. 1. Dose-response curves for the effect of doxorubicin on three kidney tumor cell lines (nos. 9, 14, 15) in the nucleotide incorporation assay. Sensitive (S180) and doxorubicin-resistant sarcoma 180 cells ($S180_{DOX}$) grown in tissue culture served as controls. The degree of resistance of $S180_{DOX}$ was 100-fold [19]. Mean values and range of each set of four measurements are shown. *Abscissa:* concentration of doxorubicin (μg/ml); *ordinate:* incorporation of ^{3}H-uridine (% of control)

Table 1. Resistance to doxorubicin, P-glycoprotein 170 expression, and proliferative activity of human kidney cancer cell lines[a]

Cell lines	Resistance to doxorubicin	P-glycoprotein		Proliferative activity Mab Ki-67
		Mab C219	Mab 265/F4	
1	94	75	55	16
2	77	48	36	19
3	75	52	44	15
4	71	42	46	16
5	70	54	51	23
6	69	59	61	15
7	69	53	45	12
8	48	33	13	16
9	45	52	46	31
10	44	39	42	33
11	36	11	3	41
12	30	9	9	45
13	22	9	5	46
14	20	8	–	39
15	12	5	4	49

[a] Incorporation of ^{3}H-uridine: % of control; mean values of each set of four measurements: immunopositive cells (%), 800–1,000 counted cells

Immunofluorescence

The immunofluorescent streptavidin-biotinylated phycoerythrin complex method was used as described previously [22]. After preincubation with non-immune sheep serum (dilution 1:10, 10 min; Dianova, Hamburg, FRG), the cell smears were incubated with primary antibody for 20 h at 4°C. The primary antibodies were monoclonal antibodies C219, 265/F4 and Ki-67. Mab C219 and Mab 265/F4 are specific for P-glycoprotein [12, 13]. Mab C219 was obtained from Centocor, Malvern, Pennsylvania; Mab 265/F4 was the kind of gift of Dr. B. Lathan (Medical Clinic, University of Cologne, FRG). The antibodies against P-glycoprotein were used at a concentration of 5 μg/ml. Mab Ki-67 reacts with a nuclear antigen in proliferating cells [5, 6]; this antibody was obtained from Dianova and used at a dilution of 1:10. After washing three times with PBS, cell smears were incubated with biotinylated sheep anti-mouse Ig previously diluted with 5% non-immune human serum (Amersham, Braunschweig, FRG; diluted 1:100) for 30 min. The streptavidin-biotinylated phycoerythrin complex (Amersham) was then applied at a dilution of 1:50 for 40 min in a darkened humidity chamber. Endogeneous biotin may lead to problems with streptavidin-based detection systems. Therefore, endogeneous biotin was suppressed in accordance with Wood and Warnke [23]. As negative control, the staining procedure was performed omitting the primary antibody. Monitoring expression of endogeneous biotin, control smears were incubated with streptavidin-biotinylated phycoerythrin complex alone. Doxorubicin-resistant sarcoma 180 cells served as positive controls for P-glycoprotein 170 expression [19]. The percentages of immunopositive cells were determined by counting 800–1,000 cells per cell line.

Results

For detection of the doxorubicin-resistance of kidney tumor cell lines, the incorporation of nucleic acid precursors was determined after preincubation of these cell lines with different doses of doxorubicin. Examples of dose-response curves of three different kidney tumor cell lines (Nos. 9, 14, 15) are depicted in Fig. 1. Doxorubicin-sensitive (S180) and doxorubicin-resistant murine S180 cells ($S180_{DOX}$) served as controls [19]. The dose-response curves demonstrate that it is sufficient to use one concentration of doxorubicin (10 μg/ml) to determine resistance. Therefore, further measurements of the other tumor cell lines were carried out using the concentration of 10 μg/ml doxorubicin. As expected, the tumor cell lines responded differently to doxorubicin (Table 1). The incorporation of nucleic acid precursors after addition of 10 μg/ml doxorubicin varied between 94% and 12% of control.

The expression of P-glycoprotein was determined by immunofluorescence using two monoclonal antibodies (Mab C219 and Mab 265/F4). Positive immunostaining of P-glycoprotein (P-gp170) in a kidney tumor cell line is demonstrated in Fig. 2a. The percentages of P-gp170 positive cells in the cell lines investigated varied considerably (Table 1; range with Mab C219: 5–75%, range with Mab 265/F4: 4–61%). The proliferative activity was determined by means of Mab Ki-67. A representative example of the Ki-67 staining in the nucleoplasm of kidney tumor cells is given in Fig. 2b. The fraction of Ki-67 positive cells varied between 12% and 49%.

In order to examine whether correlations exist between proliferative activity, doxorubicin resistance, and P-gp170 expression we analyzed the data by linear regression (Fig. 3a–c). As can be seen in Fig. 3a, there is a significant correlation between resistance to doxorubicin and P-glycoprotein expression measured using Mab C219 ($r = 0.92$, $p = 0.05$). This was confirmed using Mab 265/F4 ($r = 0.83$; $p = 0.05$, data not shown). Furthermore, we

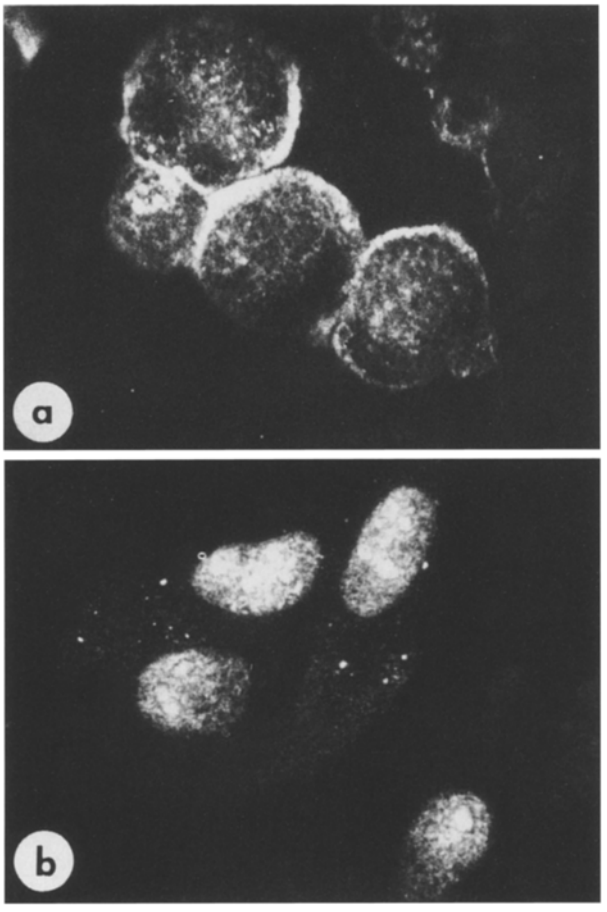

Fig. 2. a Detection of P-glycoprotein using Mab 265/F4 and b detection of proliferative activity using Mab Ki-67 in cultured kidney tumor cells. ×500

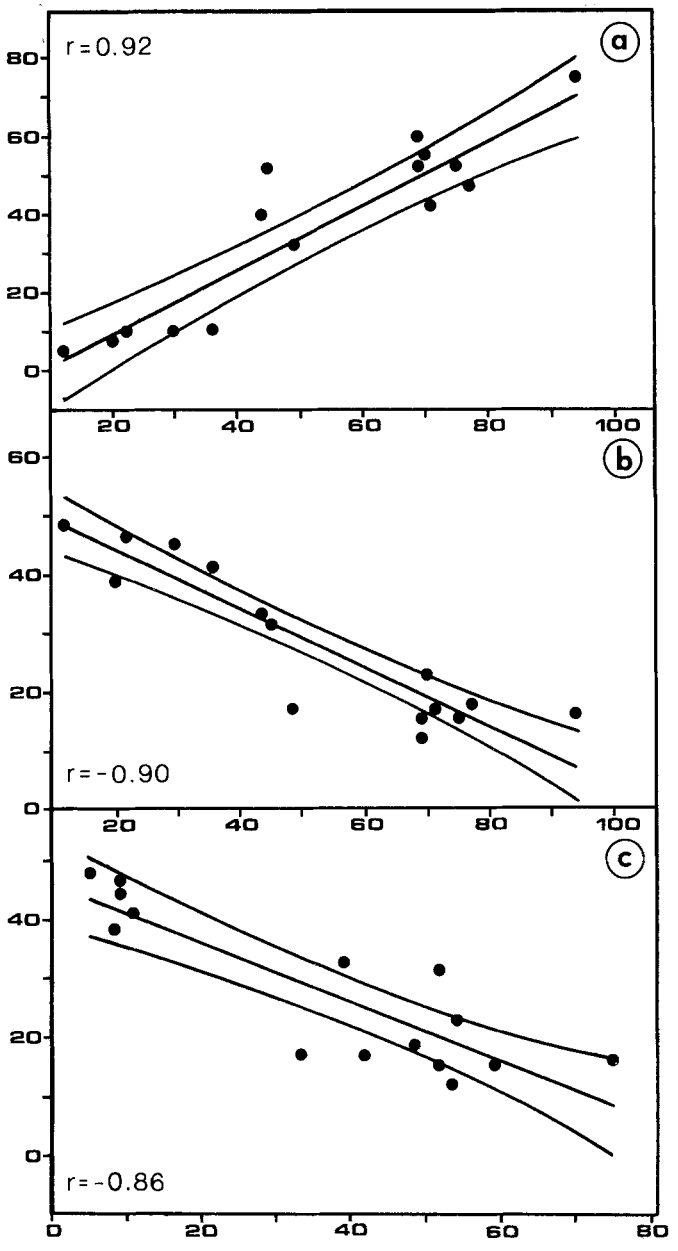

Fig. 3a–c. Relationships [linear regression ($p = 0.05$) with confidence interval (>95%)] between **a** incorporation of ^{3}H-uridine after treatment with 10 μg/ml doxorubicin (% of control) (*abscissa*) and P-glycoprotein expression (% immunopositive cells using Mab C219) (*ordinate*); **b** incorporation of ^{3}H-uridine after treatment with 10 μg/ml doxorubicin (% of control) (*abscissa*) and proliferative activity (% immunopositive cells using Mab Ki-67) (*ordinate*); **c** P-glycoprotein expression (% immunopositive cells using Mab C219) (*abscissa*) and proliferative activity (% immunopositive cells using Mab Ki-67) (*ordinate*)

correlated the level of resistance and the proliferative activity and found a strong correlation ($r = -0.90$; $p = 0.05$; Fig. 3b). Moreover, we correlated the immunoreactivity of Mab C219 (P-glycoprotein) and Mab Ki-67 (proliferative activity) and again found a negative correlation ($r = -0.86$; $p = 0.05$; Fig. 3c). This finding was confirmed using Mab 265/F4 (P-glycoprotein) ($r = -0.76$; $p = 0.05$; data not shown). The correlation between immunoreactivity of both antibodies against P-glycoprotein (Mab C219 and Mab 265/F4) was highly significant ($r = 0.94$; $p = 0.05$; data not shown).

Discussion

In order to prove whether proliferative activity plays a role in the development of doxorubicin resistance in kidney tumors, we investigated the proliferative activity by means of monoclonal antibody Ki-67 in 15 kidney cancer cell lines. Indeed, the correlation between resistance to doxorubicin and proliferative activity was significant. Nevertheless, whether the Ki-67 antigen can serve as a prognostic tool for drug resistance requires further investigation because of the small number of cases.

In the past few years, considerable clarification has been obtained on molecular mechanisms of drug resistance. In this context, P-glycoprotein 170 (P-gp170) has been intensively investigated in animal and human tumors in order to establish its possible role as marker for drug resistance. We and others have shown that renal carcinomas express high levels of P-gp170 [1, 4, 10], but there is

also high level of expression of P-gp170 in normal kidney [15]. In accordance with these data, we also found a high expression of P-gp170 in cultured kidney tumor cells.

In order to obtain comparable values, the determination of proliferative rate and P-glycoprotein expression was carried out in subconfluent cell cultures at identical time points. Although the number of investigated cases is small, the data reveal a negative correlation between proliferative activity and the expression of P-glycoprotein. Thus, the proliferative activity and the expression of P-glycoprotein do not seem to be independent factors in the development of drug resistance. There are also other hints that P-gp170 may be associated with cellular proliferation. In a recent study, Kanamaru et al. [11] suggested that there is a correlation between the state of differentiation and levels of MDR1 RNA expression in human renal cell carcinomas. We also found a tendency for cell lines of less differentiated kidney tumors to be more sensitive to doxorubicin and to show lower reactivity with Mab C219 and Mab 265/F4, and higher reactivity with Mab Ki-67 than cell lines of more differentiated kidney tumors. These correlations, however, were not statistically significant (data not shown). Whether the proliferative activity is dependent on the state of differentiation of tumors and whether the state of differentiation is a factor responsible for failure of drug response requires further study.

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