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Antioxidant defenses in a B16 melanoma line resistant to doxorubicin: an *in vivo* study

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A B16 melanoma line was repeatedly transplanted subcutaneously in C57BL/6 mice. On day 4 after every transplant, the animals were treated with doxorubicin (DXR), 10 mg/kg i.p. The aim of the work was to develop an in-vivo model of resistance to the antiblastic in order to analyze some possible mechanistic aspects of the process in the course of time. After 16 transplants and treatments the melanoma completely lost its sensitivity to the antiproliferative effects of maximal tolerated doses of DXR and showed over-expression of P-glycoprotein. Compared to the parental line, the *in vitro* resistance index was 4.6. After 27 transplants and treatments the melanoma did not increase its *in vitro* resistance to DXR further, and this resistance was completely reversed by verapamil. The behavior of the antioxidant defenses (superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, glutathione reductase and glutathione) was evaluated after 4, 16 and 27 transplants and treatments with DXR. At no stage did the treated melanoma show any variation in the antioxidant enzymes. Compared to the parental counterpart its glutathione levels were elevated after four treatments (+80%), when, however, the line was still sensitive to the *in vivo* effects of DXR, and after 16 treatments (+30%). Instead, no variation of the glutathione content was seen after 27 treatments with DXR. These results seem to exclude the possibility that the antioxidant defenses play a major role in the resistance of this B16 melanoma line to DXR. On the other hand, the low but, however, 'clinically' significant resistance of the tumor to the antiblastic seems mainly related to the mechanisms linked to the P-glycoprotein over-expression.

Key words: Antioxidant defenses, B16 melanoma, doxorubicin, resistance.

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Introduction

Doxorubicin (DXR) is an anthracycline agent with good clinical activity on a wide range of solid and liquid tumors. However, its effectiveness is limited by the problem of resistance, which is frequently of the multiple type (multidrug resistance). Certain characteristics have been associated with this phenomenon and they include reduced intracellular accumulation of the drug due to its rapid efflux,[1,2] over-expression of P-glycoprotein,[3] altered drug-topoisomerase interactions,[4] and others (for review see for example Refs. 5–7). The possibility that DXR may exert cytotoxic effects by inducing free radical generation has also been considered, and it has been suggested that the elevation of some antioxidant activities may contribute to the lack of cell sensitivity to the drug.[8–15] However, on the whole, the mechanistic basis of resistance to DXR has not been fully pinpointed; the experimental models that have been used to study it have also often been criticized because they were frequently represented by *in vitro* cells with very high levels of resistance.[6] *In vivo*, tumors which are refractory to DXR may have a resistance of only a few folds.[6]

The aim of this work was to develop an experimental model of resistance to DXR *in vivo* in order to study some biochemical aspects which might be responsible for the phenomenon at the onset of the process, such as the behavior of the antioxidant defenses. The murine B16 melanoma was used for this purpose.

Materials and methods

Mice and tumors

Female C57BL/6 mice were obtained from Nossan s.r.l., Correzzana, Italy. B16 melanoma was kindly provided as an *in vivo* line by Dr G. Pratesi, Istituto Nazionale Tumori, Milan, Italy. The tumor was homogenized in volumes of saline (100 mg/ml) and 0.5 ml of the cellular suspension was injected s.c. in the mice on day 0. The mice were divided into two groups: those bearing B16 melanoma (B16) and those bearing B16 melanoma treated with DXR (B16/DXR). On day 4 after each transplant the B16/DXR group was treated i.p. with DXR (Adriblastina, supplied by Farmitalia, Milan, Italy) at the dose of 10 mg/kg. About every 15 days the tumors were transplanted in new mice.

In vivo studies

After 4 and 16 transplants (and, in the case of the B16/DXR bearing mice, 4 or 16 treatments with DXR) mice received s.c. 0.5 ml of a cellular suspension (100 mg/ml) of B16 or B16/DXR melanomas. The mice (eight per group) were treated i.p. with saline or DXR at the dose of 4 mg/kg i.p. on days 1, 4, 8 and 12 after injection of the tumors or vincristin (VCR) (Vincristina, Lilly, Sesto Fiorentino, Italy) at the dose of 1.7 mg/kg on days 1, 5 and 9 (the VCR treatment was done only after 16 transplants). The number of deaths was checked daily at the same hour.

P-glycoprotein

P-glycoprotein expression was evaluated on cytological imprints of B16 or B16/DXR melanomas which had been inoculated in new mice after the completion of 16 and 27 transplants and treatments. The evaluation was performed by immunocytochemistry using the commercially available kit Glycochek C-219 TM (CIS diagnostici SpA, Santhiā, Italy).

In vitro resistance to DXR

The B16 and B16/DXR cells were obtained from tumors which had been inoculated in mice after 16 and 27 transplants. After mechanical disaggregation the cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 1 mM sodium pyruvate and 1% penicillin and streptomycin. In order to assay the cytotoxicity, after two subcultures the cells were seeded at 1×10^5 per ml in the presence of various concentrations of the drugs. After 72 h, the cells were harvested by trypsin–EDTA and counted by Trypan blue exclusion. The survival curves were derived as previously described.[16]

Biochemical determinations

The levels of the antioxidant enzyme activities and the content of total glutathione were tested on tumors which had been inoculated in mice after the completion of 4, 16 and 27 transplants and treatments with DXR. The B16 and the B16/DXR tumors were obtained from the animals, homogenized, sonicated and centrifuged at $100{,}000 \times g$ for 60 min. On the supernatants, the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase and glutathione reductase were tested by standard spectrophotometric techniques, as previously described.[16] Total glutathione was determined according to Griffith.[17]

Results

The activity of DXR was tested in mice bearing B16 or B16/DXR melanomas after the completion of 4 and 16 cycles of transplants (and, in the case of B16/DXR, treatments). The activity of VCR was assessed only after 16 transplants. After four transplants (Table 1) DXR increased the mean survival by 28.1% in mice bearing B16 melanoma and by 37.9% in those with B16/DXR melanoma. Thus, after four transplants the two lines were shown to be still equally sensitive to the antiblastic. At the 16th transplant, DXR and VCR increased the survival of mice with B16 melanoma by 42.1 and 60.9%, respectively, but only marginally modified (+7.3 and +9.6%, respectively) that of the B16/DXR mice.

The two tumors were cultured *in vitro* after the 16th and the 27th transplant (Table 2). The concentration of DXR inhibiting the growth by 50% in the cell lines derived from the 16th transplant was 9.6 ng/ml for the sensitive cells and 44.0 ng/ml for the resistant cells. Thus, the resistance index was equal to 4.6. On the lines derived from the 27th transplant the activity of DXR and also that of VCR was tested. At that time

Table 1. Activity of doxorubicin (DXR) and vincristin (VCR) in mice bearing melanoma B16 or B16/DXR

Tumor line	No. of transplants	Treatment	Mean survival time in days ± SD	Increase (%)	*p*
B16	4	Saline	23.8 ± 4.2		
B16	4	DXR	30.5 ± 5.7	28.1	0.05*
B16/DXR	4	Saline	21.1 ± 3.9		
B16/DXR	4	DXR	29.1 ± 4.9	37.9	0.01**
B16	16	Saline	22.8 ± 2.6		
B16	16	DXR	32.4 ± 7.1	42.1	0.01*
B16	16	VCR	36.7 ± 7.0	60.9	0.01*
B16/DXR	16	Saline	24.4 ± 3.4		
B16/DXR	16	DXR	26.2 ± 4.8	7.3	NS
B16/DXR	16	VCR	25.0 ± 4.2	9.6	NS

C57BL/6 mice (eight per group) treated s.c. with 0.5 ml of 10% tumor brei received i.p. on days 1,4,8 and 12 after injection of the tumor DXR at the dose of 4 mg/kg or on days 1,5 and 9 VCR at the dose of 1.7 mg/kg.
* *p* versus untreated B16; ** *p* versus B16/DXR; NS not significant.

Table 2. Drug sensitivity profiles of B16 and B16/DXR melanoma cell lines

Drug	No. of transplants	ID_{50} (ng/ml)[b]		RI[c]
		B16	B16/DXR	
DXR	16	9.6	44.0	4.6
DXR	27	10.8	39.5	3.6
DXR + VRP[a] 1 μM	27	10.5	12.5	1.1
DXR + VRP[a] 10 μM	27	10.6	12.2	1.1
VCR	27	18.6	50.5	2.7

The data are the mean of at least four separate experiments. The standard deviations are less than 10%.
[a] VPR 1 and 10 μM alone did not affect the cell growth of B16 or B16/DXR.
[b] ID_{50} is the dose inhibiting the cellular growth by 50%.
[c] RI is the ratio between the ID_{50} of B16/DXR and the ID_{50} of B16.

(Table 2) the concentration of DXR inhibiting the growth by 50% was 10.8 ng/ml for the sensitive line and 39.5 ng/ml for the B16/DXR. The resistance index was 3.6. Verapamil (VRP) at 1 or 10 μM completely restored the sensitivity to DXR of the B16/DXR line (Table 2). The concentration of VCR inhibiting the growth by 50% was 18.6 ng/ml for the B16 cells and 50.5 ng/ml for the B16/DXR cells. The resistance index was 2.7 (Table 2). After 16 (Figure 1) and 27 transplants the B16/DXR melanoma clearly showed expression of P-glycoprotein. At the same stages a positivity for P-glycoprotein was not detected in the B16 melanoma (Figure 2).

The activities of the antioxidant enzymes and the content of glutathione in the tumors are shown in

Table 3. Antioxidant enzyme activities and total glutathione in B16 and B16/DXR melanoma

Tumor	No. of transplants	Superoxide dismutase (U/mg protein)	Catalase (k/mg protein)	Glutathione peroxidase[a,b]	Glutathione transferase[c]	Glutathione reductase (mU/mg protein)	Total glutathione (nmol/mg protein)
B16	4	5.8 ± 04	0.28 ± 0.01	54.5 ± 3.8	108.9 ± 9.2	51.5 ± 2.1	13.8 ± 1.0
B16/DXR	4	5.6 ± 0.5	0.29 ± 0.04	49.4 ± 6.5	121.4 ± 7.5	45.4 ± 3.7	24.9 ± 3.8
B16	16	5.4 ± 0.2	0.27 ± 0.02	55.9 ± 0.7	103.2 ± 3.4	46.3 ± 0.7	14.5 ± 0.8
B16/DXR	16	4.8 ± 0.1	0.32 ± 0.02	54.1 ± 3.0	113.7 ± 2.8	45.1 ± 0.4	19.0 ± 1.4
B16	27	5.5 ± 0.4	0.34 ± 0.02	55.1 ± 4.9	105.3 ± 8.2	47.7 ± 1.3	14.1 ± 1.2
B16/DXR	27	6.1 ± 0.3	0.28 ± 0.01	55.8 ± 2.3	114.1 ± 9.5	48.7 ± 2.6	11.4 ± 0.9

The data are the mean of at least three observations ±SD.
[a] The activity of glutathione peroxidase was similar using H_2O_2 or cumene hydroperoxide as substrate.
[b] Substrate used was H_2O_2 (expressed as nmol NADPH oxidized/min/mg protein).
[c] Substrate used was 1-chloro-2,4-dinitrobenzene (expressed as nmol/mg protein).

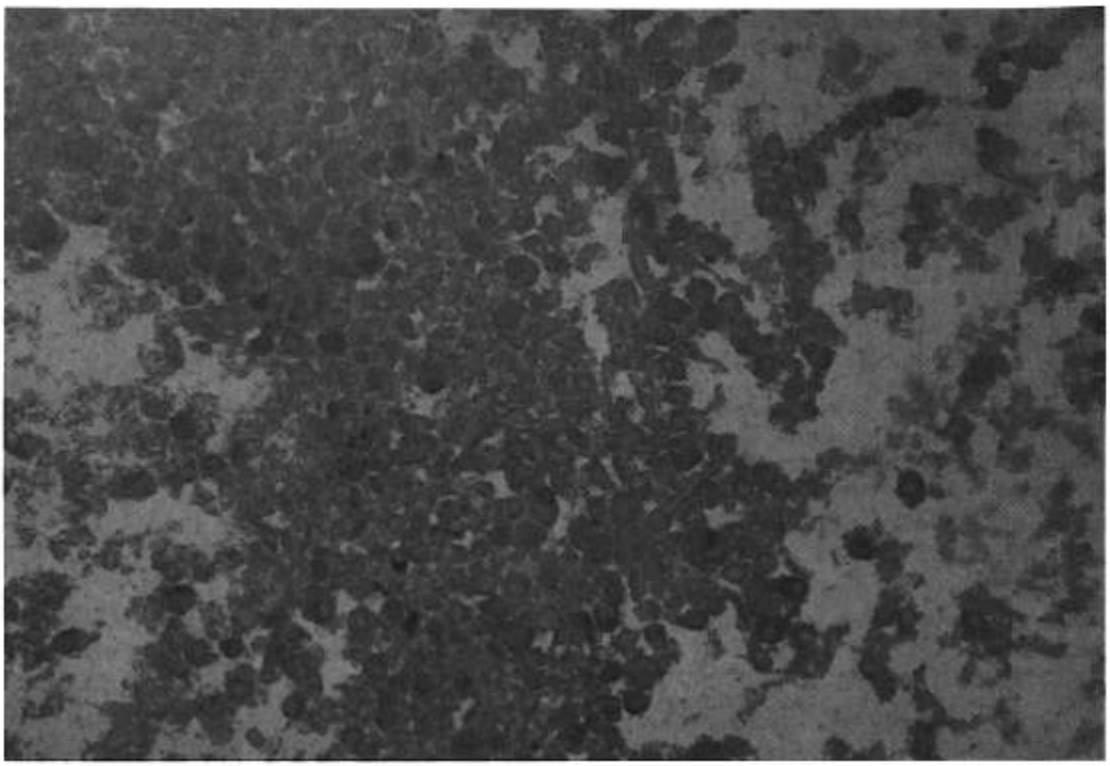

Figure 1. Immunocytochemical search of P-glycoprotein expression on cytological imprints of B16/DXR melanoma after 16 transplants and treatments with DXR. The red staining (avidin–biotinilated peroxidase complex) denotes positivity for P-glycoprotein. For further details see Materials and Methods.

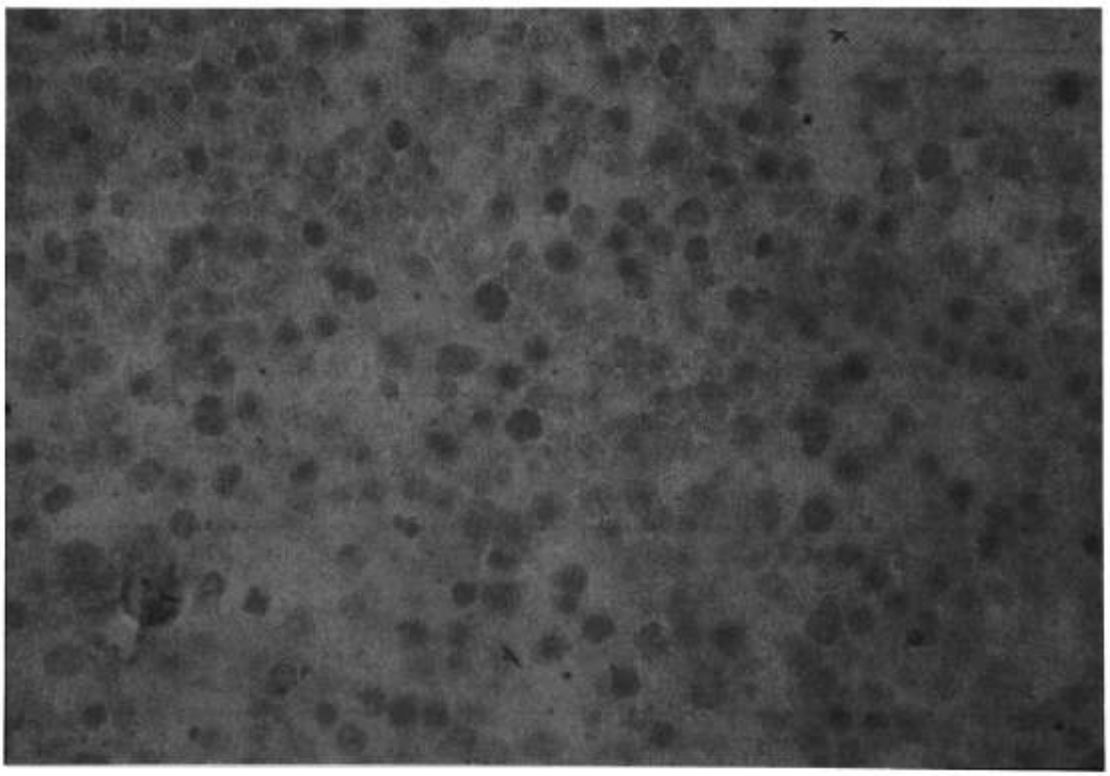

Figure 2. Immunocytochemical search of P-glycoprotein expression on cytological imprints of B16 melanoma after 16 transplants. For further details see Materials and Methods.

Table 3. B16 and B16/DXR melanoma did not show any variation in glutathione peroxidase activity using hydrogen peroxide or cumene hydroperoxide as a substrate and thus they seemed to lack the selenium-independent form of glutathione peroxidase. No significant variation in the antioxidant enzymes was found in B16/DXR melanoma after 4, 16 and 27 transplants. However, glutathione increased in B16/DXR melanoma after 4 (+80%) and 16 (+31%) transplants and it returned to the B16 value after 27 transplants.

Discussion

We have tried to characterize some biochemical aspects of the resistance to DXR during its *in vivo* development in a line of B16 melanoma. The tumor showed complete resistance to the treatment with roughly maximal tolerated doses of DXR and also of VCR after 16 cycles of transplants and treatments with the anthracycline. At that time the treated melanoma had become 4.7-fold more resistant *in vitro* to DXR than its original counterpart and clearly expressed P-glycoprotein. These data seem to indicate that the selected clone had acquired the multidrug-resistant phenotype and confirm that moderate levels of *in vitro* resistance to DXR are sufficient to confer 'clinical' resistance to the drug. We think it worthwhile mentioning that, from a kinetic point of view, the treated melanoma did not differ from its parental line. The *in vivo* growth rates, the mitotic indexes and the [^{3}H]thymidine labeling indexes were almost identical in the two tumors. We did not encounter any major difference morphologically if we excluded a steadily greater content of melanin (about 30% more) in the resistant line. This last observation is in close agreement with the findings of other authors who have developed similar models of resistance to DXR in the B16 melanoma,[18] and would indicate a more differentiated attitude of the resistant line.

Some characteristics of the B16 melanoma were studied after 27 cycles of transplants and treatments with DXR. At that stage, the *in vitro* resistance to the anthracycline had not increased, indeed it had slightly regressed: with respect to the parental line the resistance index was 3.6. This resistance was completely abolished by co-treatment with VRP. Other authors have already shown that the latter drug can completely reverse the resistance to DXR of human tumor cells when their level of resistance is moderate, in the order of 3- to 6-fold.[19] Finally, the *in vitro* index of resistance to VCR was equal to 2.7 and the treated melanoma continued to express P-glycoprotein.

It has been suggested that an elevated generation of free radicals may contribute to the cytotoxic effects of the anthracyclines.[20] On the other hand, increases in the defenses against these toxic species could be involved in the mechanistic aspects of tumor cell resistance to these drugs. Thus, resistance to the anthracyclines has frequently been proposed to be associated with increased levels of selenium-dependent glutathione peroxidase,[8–11] but not universally.[14,21] Some resistant tumors show an elevation in their content of glutathione[9,10,14,15] and others do not.[9,11,21] Over-expression of glutathione-S-transferase (of the anionic type, π) was present in some DXR-resistant tumors,[10,12,13] but not in others.[9,21,22] In previous studies on Friend erythroleukemia cells with increasing levels of resistance to DXR, we did not find any clear-cut evidence of a better equipment against free radicals; however, the appearance of a unique alpha subunit of glutathione-S-transferase was seen. This isoform was not present in the parental clone and presumably accounted for an increased selenium-independent glutathione peroxidase activity which was expressed by the resistant lines.[16] From the overall data from the literature, which were often obtained from *in vitro* models with high resistance indexes, one could conclude that resistance to the anthracyclines may be associated with an elevation of some antioxidant activities, but not in an obligatory and/or stereotyped way. It is not entirely clear if these changes contribute to an increased tolerance of a cell to these drugs. For example, it has been reported that Chinese hamster ovary cells with acquired high levels of superoxide dismutase, catalase, glutathione peroxidase and glutathione are equally sensitive to DXR as other clones not endowed with the same elevated activities.[23] However, depletion of cell glutathione with buthionine sulfoximine seems to be a way to increase or restore sensitivity to DXR.[9,10]

In this paper we have analysed the behavior of the various antioxidant defenses during the in-vivo development of resistance to DXR by B16 melanoma. When compared with other tumors and normal tissues,[23] this melanoma does not possess especially high levels of antioxidant defenses which could account for a lack of sensitivity to the hypothetical free radicals generated by DXR. However, at no stage of the study did it show an increase in its enzymatic antioxidant activities. Only a transitory elevation of glutathione was found;

however, it was present after four transplants and treatments (when the melanoma was still sensitive to the *in vivo* effects of DXR) and after 16 treatments, but not after 27 treatments with the drug.

Conclusions

In conclusion, our data seem to exclude the possibility that, in this *in vivo* model of B16 melanoma with moderate but indeed significant resistance to DXR, the behavior of the antioxidant defenses plays a role in the cell refractoriness to the antiblastic. They cast further doubts that elevated defenses against free radicals may be a constant mechanism in tumor cell resistance to the anthracyclines.

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