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Expression of resistance to etoposide and vincristine *in vitro* and *in vivo* after X-irradiation of ovarian tumor cells

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Two human overian carcinoma cell lines (JA-T/P and SK-OV-3/P) were exposed to 10 fractions of 5 Gy X-irradiation *in vitro*. Surviving populations generated sublines designated DXR-10 which expressed significant resistance to etoposide (VP-16) and vincristine (VCR), but not to adriamycin (ADR) or acute X-irradiation, as judged by clonogenic assays. JA-T/P and JA-T/DXR-10 tumor cells were xenografted into nude mice and treated with a single dose of VCR (1.8 mg/kg), VP-16 (24.5 mg/kg) or ADR (10.0 mg/kg) and 48 h later the surviving clonogenic cells in each tumor were quantitated. Significantly fewer colonies grew from the JA-T/P xenografts treated with either VCR or VP-16, as opposed to the JA-T/DXR-10 tumors, whilst comparable colony numbers were recorded after ADR treatment. These data suggest that the resistant phenotype following exposure to fractionated X-irradiation *in vitro* is also expressed *in vivo*.

Key words: Drug resistance, fractionated X-irradiation, ovarian cancer.

Introduction

Clinical drug resistance has been documented in patients after treatment not only with antitumor drugs, but also with radiotherapy. Although it has been argued that hypoxic tumor cell populations, decreased host immunity and/or radiation-induced vascular fibrosis may contribute to the observed drug resistance after radiation, Hill *et al.* have emphasized that certain cellular and biological phenomena may also be involved.[1,2]

In a general *in vitro* study of interactions between a series of clinically-useful antitumor drugs and radiation it has been shown that prior exposure of rodent and human tumor cells to fractionated X-irradiation can result in the expression of subsequent resistance to etoposide (VP-16) and the Vinca alkaloids, but *not* to the anthracylines or to acute X-irradiation.[3]

Since these earlier experiments had all been performed *in vitro*, the main objective of this present study was to investigate whether this drug resistance is also expressed *in vivo*. As an initial approach, rather than irradiating tumor-bearing animals, we aimed to establish whether tumor cells expressing resistance following fractionated X-irradiation *in vitro* retained their resistance phenotype if xenografted into nude mice.

Materials and Methods

Cell lines

The JA-T/P cell line, derived from JA-1 cells, was established from a human ovarian cystadenocarcinoma obtained from a previously untreated patient.[4] The SK-OV-3/P cell line, derived from malignant ascites of a patient treated with thiotepa,[5] was obtained from the American Type Culture Collection (Rockville, MD, USA). Monolayer cultures were maintained in Ham's F12 or Eagle's medium, supplemented with 10% fetal calf serum (Gibco-Biocult, Glasgow, UK), for JA-T and SK-OV-3 cell lines, respectively.

The DXR-10 sublines were derived by exposing

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logarithmically-growing cultures of JA-T/P and SK-OV-3/P cells to 10 fractions of 5 Gy as described elsewhere.[6,7] The 50 Gy total radiation dose administered was within the range used in clinical radiation therapy protocols for ovarian cancers.[8] Population doubling times, colony-forming efficiencies in soft agar, cell volumes, DNA and protein contents of each parental cell and its DXR-10 subline were determined, and no significant differences in any of these parameters were noted initially or during a 6 months period in culture.

Cytotoxicity assays

The drugs used were kindly donated for these studies: VP-16 by Bristol-Myers Squibb (Evansville, IN, USA), vincristine (VCR) by Lederle Laboratories (Gosport, Hampshire, UK) and adriamycin (ADR) by Farmitalia Carlo Erba (Milan, Italy). Radiation sensitivity assays were performed under identical conditions to those used for the derivation of the subline, as described earlier.[3] Survival curves after X-irradiation were fitted using a linear least-squares computer programme, and X-ray doses reducing the surviving fraction of cells to 0.37 on the exponential region of the survival curve (D_0) and the extrapolation number (n) were determined. The Courtenay soft agar clonogenic assay[9] was used and IC_{50} drug concentrations (i.e. those required to reduce survival by 50% of control levels) were calculated from full dose–response curves.

Animal studies

Exponentially-growing cells were trypsinized, washed twice with phosphate buffered saline (PBS) and approximately 5×10^6 cells per line in 0.5 ml PBS were injected subcutaneously into the flank of an outbred nude mouse [female *nu/nu* of mixed genetic background maintained by the ICRF Biological Services Unit under specified pathogen-free (SPF) conditions]. For each cell line at least six mice aged 6–8 weeks were used. Animals were kept under sterile conditions in autoclaved cages with filter bonnets in a laminar flow unit, examined twice a week for gross evidence of tumor development and killed by cervical dislocation when tumors reached 1.0 cm in diameter. For routine propagation tumors were removed under sterile conditions, minced into small fragments (2–3 mm in diameter) and pooled. Fragments were then transplanted with a trocar subcutaneously. Tumors reaching 1 cm^3 were histologically processed and paraffin sections were stained with hematoxylin–eosin.

Chemotherapy studies were performed according to the method described by Mirabelli *et al.*[10] with minor modifications. Xenografts were used between the 10th and 14th passages in nude mice. The growth of each tumor was measured three times a week, with volumes being calculated from two perpendicular caliper measurements. On days 8–10 after transplantation, animals with an tumor diameter of approximately 0.8 cm were selected for examination and divided into groups of six animals. One group served as a control and the others were treated with the LD_{10} (10% lethal dosage) concentration (single dose) of each drug tested, i.e. VP-16 24.5 mg/kg i.p. (R Osieka, personnel communication); VCR 1.8 mg/kg i.p.;[11] and ADR 10.0 mg/kg i.v.[12] Exactly 48 h after treatment all tumor-bearing mice were killed, individual tumors were excised, minced and then trypsinized to obtain a single cell suspension. Cell counting was done using a hemocytometer and cell viability was measured by the trypan blue dye exclusion test before the colony-forming assays were performed using three different cell concentrations from each tumor suspension.

Results

In vivo cytotoxicity assays

Dose–response curves for the four tumor cell lines following a 24 h exposure to VP-16 or to VCR are shown in Figure 1. IC_{50} values were interpolated and a comparison (see Table 1) shows that both irradiated sublines were significantly resistant to VP-16 (2-fold, $p < 0.05$) and VCR (2- to 3-fold, $p < 0.01$), but *not* to ADR or to acute X-irradiation.

Animal studies

The four human ovarian carcinoma cell lines were next tested for their ability to form xenografts in nude mice. The results are summarized in Table 2. Histological examination revealed no significant differences between the transplanted parental tumors and the xenografts formed by the DXR-10 sublines. A comparison with the original tumor histologies from the patient was not possible since paraffin sections were not available. Since JA-T/P and JA-T/DXR-10 cells most readily formed tumors, within 10–14 days after transplantation,

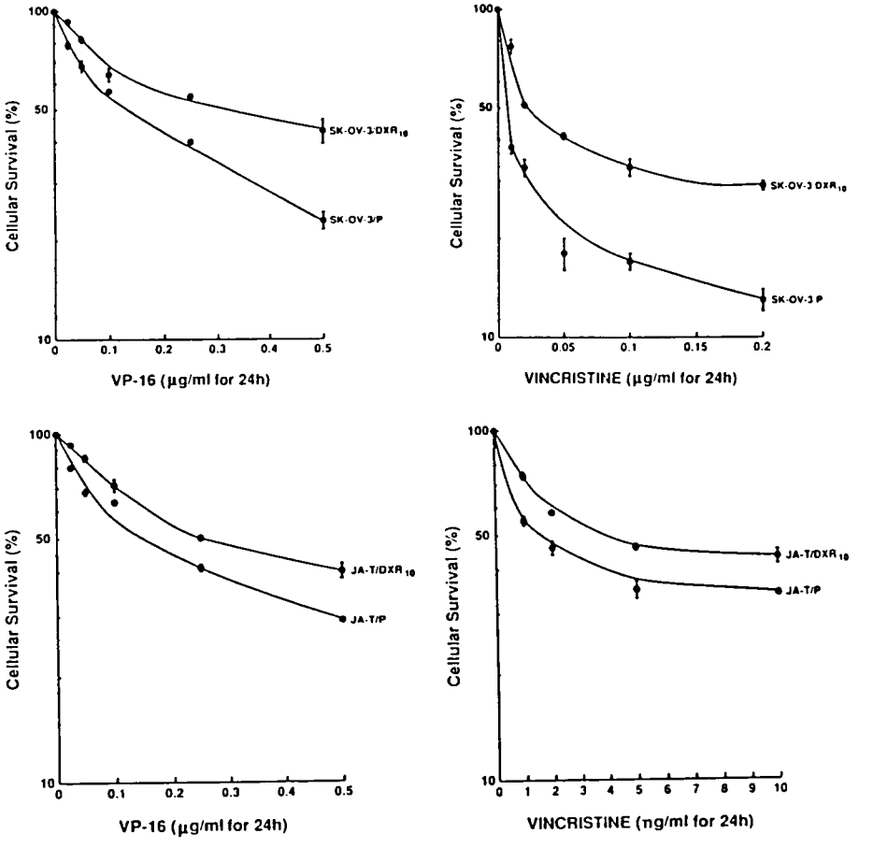

Figure 1. Survival of SK-OV-3/P, SK-OV-3/DXR-10, JA-T/P and JA-T/DXR-10 cells after a 24 h exposure to VP-16 (left panel) or VCR (right panel). Values are the mean ±SE (bars) of two or three experiments in which triplicate cultures were treated.

these xenograft lines were selected to perform the drug sensitivity studies.

We first attempted to monitor tumor growth rates *in vivo* after a single dose of VCR (0.9 or 1.8 mg/kg). However, by measuring tumor size no significant differences was detectable between JA-T/P and JA-T/DXR-10 xenografts, and since even using groups of six animals a large variation in tumor size was noted, we considered that modest levels of drug resistance were unlikely to be identified by this procedure. However, when the number of surviving clonogenic cells in each tumour was

Table 1. Cytotoxicity data on SK-OV-3/P and JA-T/P cells and their DXR-10 sublines

Cell line	D_0[a] (Gy)	n[b]	IC_{50}VCR[c] (ng/ml)	IC_{50}VP-16[c] (μg/ml)	IC_{50}ADR[c] (μg/ml)
SK-OV-3/P	2.89 ± 0.19	1.25 ± 0.09	12.0 ± 0.5	0.16 ± 0.01	0.020 ± 0.004
SK-OV-3/DXR-10	3.12 ± 0.05	1.19 ± 0.06	27.0 ± 1.4	0.33 ± 0.04	0.023 ± 0.003
p values	>0.1	>0.1	<0.01	<0.05	>0.1
JA-T/P	1.46 ± 0.19	1.67 ± 0.29	3.8 ± 0.2	0.14 ± 0.02	0.016 ± 0.002
JA-T/DXR-10	1.25 ± 0.15	1.99 ± 0.36	11.0 ± 0.6	0.28 ± 0.03	0.016 ± 0.004
p values	>0.1	>0.1	<0.01	<0.05	>0.1

[a] X-ray dose reducing the surviving fraction of cells to 0.37 in the exponential region of the survival curve.
[b] Extrapolation number
[c] Cells were exposed to drug for 24 h and mean values ± SE (assays of two or three separate experiments) derived from full dose–response curves are listed.

Table 2. Subcutaneous growth of four human ovarian carcinoma xenografts in nude mice

Tumor line[a]	Tumor take rate (>3 passages)	Mean doubling time[b]	Days to reach a diameter of 1 cm (range)
JA-T/P	6/6	5.3 ± 1.4	14–19
JA-T/DXR-10	6/6	5.1 ± 1.6	13–21
SK-OV-3/P	4/6	13.5 ± 2.8	42–54
SK-OV-3/DXR-10	5/6	12.3 ± 3.0	40–50

[a] Results from two experiments with tumors obtained between passages 3 and 5 in nude mice.
[b] Days ± SE calculated as the number of days to grow from 200 to 400 mm^3.

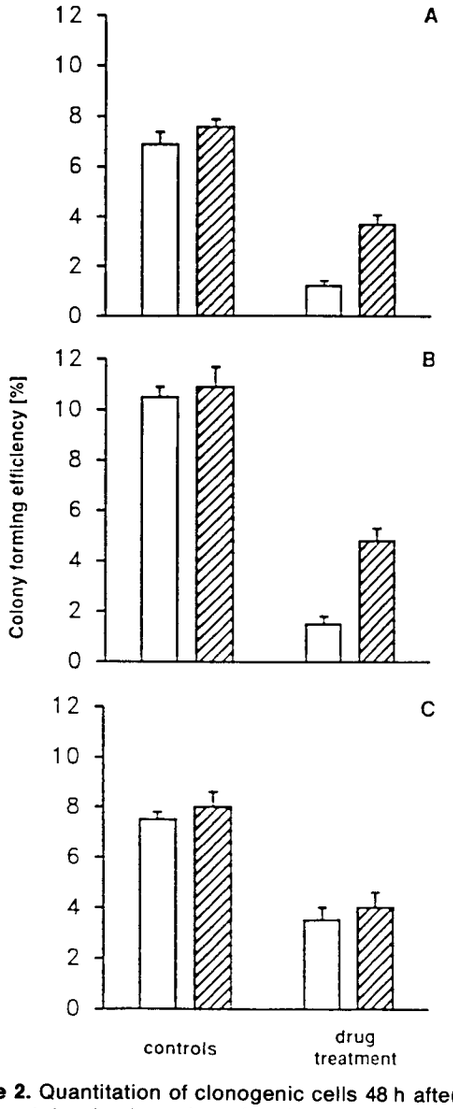

Figure 2. Quantitation of clonogenic cells 48 h after drug treatment *in vivo* by colony forming assay. Mice were treated with a single dose of VP-16 (24.5 mg/kg, panel A), VCR (1.8 mg/kg, panel B) or ADR (10.0 mg/kg, panel C). After 48 h tumors were removed and colony forming assays were performed as outlined in Materials and methods. Open boxes: JA-T/P; hatched boxes: JA-T/DXR-10.

quantitated by colony forming assay 48 h after drug treatment, significantly fewer colonies grew from the JA-T/P, as opposed to the JA-T/DXR-10 VCR-treated xenografts ($p < 0.01$). Similar differential colony numbers were obtained after treatment with a single dose of VP-16 ($p < 0.01$), whilst comparable colony numbers were recorded after ADR treatment ($p > 0.1$) (Figure 2).

Discussion

In attempting to characterize interactions between radiation and antitumor drugs we have exposed human ovarian carcinoma cells (JA-T and SK-OV-3) to fractionated X-irradiation *in vitro*. The derived DXR-10 sublines expressed significant resistance to VP-16 and VCR, but *not* to acute X-irradiation or to ADR. This confirms earlier reports[1-3] that stable resistance to VP-16 or to the Vinca alkaloids can be expressed not only after exposure of tumor cells to the drug itself, but also after *in vitro* exposure to radiation.

The lack of significant cross-resistance to ADR distinguishes our irradiated sublines from drug-selected multidrug-resistant cells, although there are reports of more highly resistant sublines showing more resistance to the Vinca alkaloids than to the anthracyclines.[13,14] Hill *et al.*[2] have provided the first evidence that resistance to VP-16 and VCR in Chinese hamster ovary following fractionated X-irradiation *in vitro* was associated with increased levels of the membrane protein P-glycoprotein (P-170). The possibility that P-170 overexpression may also be implicated in the resistance phenotype of our similarily-derived human ovarian carcinoma sublines is currently being investigated.

Since none of our previously derived X-irradiated human tumor sublines[3] successfully xenografted into nude mice, we selected these two human ovarian carcinoma cell lines for this study since they readily formed xenografts.[4] The JA-T

xenografts proved particularly suitable for these types of drug sensitivity assays since they were readily disaggregated mechanically yielding a single cell suspension with $\geq 90\%$ viability. Our observation that the resistance phenotype of these tumor cells is still expressed after xenografting and subsequent drug treatment of the tumor-bearing mice suggests that this observed drug resistance is stable not only *in vitro*, but also *in vivo*. Further support for this finding comes from a study by Mattern *et al.*[15] who have pre-exposed human lung cancer xenografts to fractionated X-irradiation *in vivo* and also found that the irradiated xenografts proved resistant to the Vinca alkaloids. Consistent with our earlier *in vitro* data[2] these irradiated xenografts also overexpressed P-glycoprotein.

In conclusion, these data add weight to the proposal that a biochemical basis is implicated in the clinical problem of drug resistance in some previously irradiated tumors. These established human tumor xenografts provide a useful *in vivo* model for further investigating this phenomenon.

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