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Effect of etoposide, carmustine, vincristine, 5-fluorouracil, or methotrexate on radiobiologically oxic and hypoxic cells in a C3H mouse mammary carcinoma in situ*

Cai Grau and Jens Overgaard

Danish Cancer Society, Department of Experimental Clinical Oncology, Radiumstationen, Nörrebrogade 44, DK-8000 Aarhus C., Denmark

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Summary. The effect of etoposide (VP16), carmustine (BCNU), vincristine (VCR), methotrexate (MTX), and 5-fluorouracil (5-FU) on the oxic and hypoxic cells in a C3H mammary carcinoma in CDF_1 mice was investigated using an in situ local-tumor-control (TCD_{50}) assay. The surviving fraction (SF) was calculated from the size of the radiation dose needed to inactivate the surviving tumor cells in drug-treated tumors relative to untreated controls. Preferential drug cytotoxicity towards oxic and hypoxic cells was evaluated from the difference in the response to irradiation under ambient and clamped conditions, respectively. Three drugs caused a significant ($P < 0.05$) reduction in the survival of hypoxic cells, the SFs being 0.31 (VP-16), 0.13 (BCNU) and 0.16 (VCR). VCR was also toxic towards oxic cells (SF, 0.17), whereas VP16 and BCNU had no significant effect on these cells (SF, 0.5 and 0.76, respectively). Two drugs produced significant killing of cells in the oxic compartment: 5-FU (SF, 0.10) and MTX (SF, 0.22); these two drugs had no effect on hypoxic cells (SF, 0.78 and 1.11, respectively).

Introduction

Hypoxia in solid tumors is known to result in a decreased effectiveness of ionising irradiation [5]. Hypoxic cells distant from blood vessels may also be resistant to drug therapy because of insufficient drug delivery, reduced drug activation under hypoxic/acidic conditions, or low activity against non-cycling hypoxic cells [4, 19, 22]. On the other hand, some drugs may be selectively toxic to hypoxic cells and might be used to improve the treatment outcome in radiotherapy [1, 2, 4, 6, 8, 19, 22]. Information about the specific effect of cytotoxic drugs on well-oxygenated or hypoxic tumor cells is necessary for the development of rational chemotherapeutic regimens designed to attack each of the physiological tumor subpopulations.

We have previously reported a method for the evaluation of the preferential cytotoxicity of antineoplastic agents towards oxic and hypoxic cells in situ [6]. Three drugs (Adriamycin, cyclophosphamide and mitomycin C) were toxic towards both oxic and hypoxic cells, whereas bleomycin and cisplatin were toxic towards oxic cells only. In the present study, we extended these experiments to test five other drugs that are frequently used both in chemotherapeutic regimens and in conjunction with radiotherapy: etoposide (VP16), carmustine (BCNU), vincristine (VCR), 5-fluorouracil (5-FU) and methotrexate (MTX).

Materials and methods

Animal tumor system. The C3H/Tif mammary carcinoma was grown in the right rear foot of 10- to 14-week-old male $C_3D_2F_1$ mice. Non-anaesthetised mice were treated when tumors had reached an average volume of 200 mm^3 (range, 150–257 mm^3) as determined by the formula $\pi/6 \times D_1 \times D_2 \times D_3$, where D_1, D_2 and D_3 represent the three orthogonal diameters.

Irradiation. Radiation was given as single doses of 250-kV X-rays (10 mA; half-value layer, 3.1 mm Cu; dose rate, 2.3 Gy/min). The mice were placed in a lucite jig with the tumor-bearing leg exposed, loosely taped to the jig and immersed in a water bath to ensure a homogeneous dose distribution in the tumor. In animals receiving X-rays under hypoxic conditions, the tumor-bearing leg was clamped at 5 min prior to and during the period of irradiation. Clamping was achieved by constriction of the blood flow using a rubber tube tightened around the leg; the validity of this procedure for the generation of complete radiobiological hypoxia has previously been documented [6, 10].

Drugs. For each drug, the maximum tolerated dose (MTD), defined as the dose that killed approximately 1% of the mice within 150 days, was used in all experiments. The drugs were injected intraperitoneally at a constant volume of 0.02 ml/g body weight. BCNU was dissolved in alcohol and diluted with isotonic saline. All other drugs were dissolved in sterile water and diluted with isotonic saline at room temperature. A 4-h interval between radiation and chemotherapy was used. Previous studies have shown that this interval is sufficiently long to allow full repair of sublethal radiation damage but short enough to prevent significant reoxygenation in the surviving tumor cells [6, 7].

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Offprint requests to: C. Grau

Table 1. Effect of VP16, BCNU, VCR, 5-FU or MTX on the growth of a C3H mammary carcinoma

Treatment	Volume-doubling time (days)[a]	Time to 3 × treatment volume (days)
Untreated control	2.7±0.1	3.6±0.1
VP16 (15 mg/kg)	2.9±0.1	5.0±0.1*
BCNU (30 mg/kg)	2.6±0.3	7.6±0.5*
VCR (2 mg/kg)	2.6±0.1	9.7±0.3*
5-FU (150 mg/kg)	4.1±0.9	6.6±0.2*
MTX (150 mg/kg)	2.6±0.1	4.9±0.2*

Data represent mean values ±1 SE
[a] Volume-doubling time in the exponential regrowth phase
* Significantly different from untreated control values (P <0.05)

Tumor response. The response to chemotherapy alone was evaluated in terms of tumor-growth time (TGT), defined as the time required for a tumor to reach 3 times the treatment volume. The exponential regrowth phase was used to calculate the volume-doubling time (DT). All calculations were based on individual growth data. Specific drug cytotoxicity was quantified by the radiation dose required to inactivate the surviving cells after drug therapy. The effect of graded doses of radiation alone or in combination with drugs was evaluated as the radiation dose required to produce local tumor control in 50% of the treated animals (TCD_{50}). A total of 6–18 mice/dose point and 5–8 dose points/treatment were used in all TCD_{50} experiments, which were repeated at least once. The mice were followed every 2–3 weeks for a total of 90 days, and tumor control was defined as persistent disappearance of the tumor within that period. Data were assessed using computerised logit analysis [16].

Derived parameters. The surviving fraction (SF) was calculated from the size of the radiation dose (TCD_{50}) required to inactivate surviving tumor cells after drug therapy relative to untreated controls as described in detail elsewhere [6, 9]. A two-compartment model was assumed, one being fully oxic and the other fully hypoxic, and both containing cells giving exponential survival curves. Naturally and artificially hypoxic cell-survival curves were assumed to have the same slope and intercept. The same level of cell killing was also assumed to be required to control clamped and normally aerated tumors, illustrating that clamping of the tumor produced no cell killing or persistent changes in the tumor bed by itself and that naturally occurring hypoxic cells had the same probability of causing recurrence as did artificially hypoxic cells. The steps used in the calculation of the various parameters are outlined below.

The hypoxic fraction (HF) was calculated from local-tumor-control data as:

$$HF = \exp-(TCD_{50,\,clamp} - TCD_{50,\,air})/D_{o,\,hypoxic},$$

where $TCD_{50,\,air}$ and $TCD_{50,\,clamp}$ represent the doses in normal and clamped tumors, respectively, and $D_{o,\,hypoxic}$ represents a dose of 3.2 Gy for hypoxic cells [16]. The total number of tumor cells (N_{total}) was determined as:

$$N_{total} = \exp(TCD_{50,\,clamp}/D_{o,\,hypoxic})(\ln(2)/n),$$

where n is the extrapolation number of 3 [16]. The total number of hypoxic cells ($N_{hypoxic}$) was estimated using the formula:

$$N_{hypoxic} = \exp(TCD_{50,\,air}/D_{o,\,hypoxic})(\ln(2)/n),$$

and the number of oxic cells, by the equation:

$$N_{oxic} = N_{total} - N_{hypoxic}.$$

The relative proportion of hypoxic tumor cells surviving a given treatment ($SF_{hypoxic}$) was defined as:

$$SF_{hypoxic} = N_{hypoxic,\,radiation+treatment}/N_{hypoxic,\,radiation},$$

and, finally, the relative number of surviving aerobic tumor cells was calculated as:

$$SF_{oxic} = N_{oxic,\,radiation+treatment}/N_{oxic,\,radiation}.$$

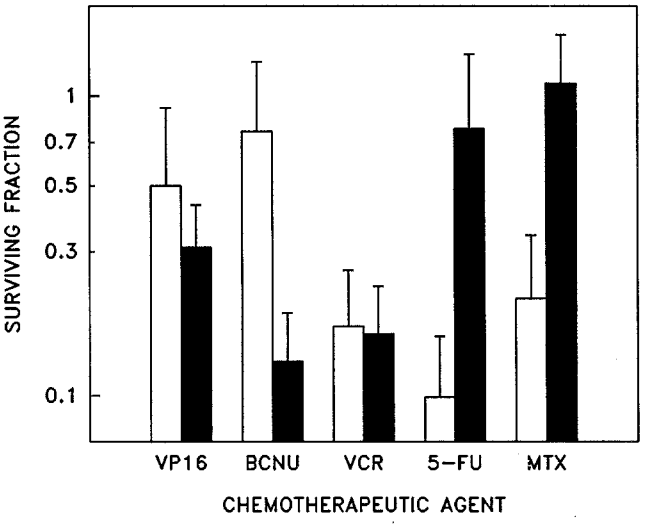

Fig. 1. Effect of VP16, BCNU, VCR, 5-FU or MTX on the surviving fraction of aerobic (□) and hypoxic (■) cells in the C3H mouse mammary carcinoma. Data represent mean values ±1 SE

Statistical analysis was done using the propagation-of-error technique as described in detail elsewhere [9]. A significance level of P <0.05 was used in all statistical analyses.

Results

Table 1 shows the effect of drug treatment on the growth of the C3H mammary carcinoma. After drug-dependent initial lag phases, all tumors regrew with volume-doubling times similar to those of untreated controls (2.6–2.9 days). For 5-FU, the doubling time increased from 2.7 to 4.1 days, but due to a considerable spread in the data, this increase was not statistically significant. Since the volume-doubling time in the regrowth phase remained unchanged, the time required to reach a specific volume could be assumed to reflect drug cytotoxicity. The TGT was most prominently increased by VCR (from 3.6 to almost 10 days), whereas the effect of other drugs was weaker, although statistically significant (TGT, 4.9–7.6 days). A series of control experiments showed that clamping of the tumors for 30 min (as used in the irradiation experiments) 4 h prior to drug injection did not change the TGT or DT relative to non-clamped-tumor values (data not shown).

The TCD_{50} values for radiation delivered alone and in combination with drugs are listed in Table 2 together with the derived values for HF and SF. Untreated tumors were found to have a HF of 0.6%. BCNU caused a significant reduction in the HF (0.1%, P <0.05), whereas the other drugs resulted in either unaltered HFs (VP16 and VCR) or increased values (5-FU and MTX). The preferential drug cytotoxicity towards oxic and hypoxic cells is illustrated in Fig. 1. Three drugs caused a significant reduction in the cells in the hypoxic compartment, the SFs being 0.31 (VP16), 0.13 (BCNU) and 0.16 (VCR). The other drugs, 5-FU and MTX, had no effect on the hypoxic cells. The SF of oxic cells was significantly reduced by VCR (0.17), 5-FU (0.10) and MTX (0.22). Thus, three drugs (VP16,

Table 2. Effect of VP16, BCNU, VCR, 5-FU or MTX on the TCD_{50} values the hypoxic fraction, and the surviving fraction of aerobic and hypoxic cells

Adjuvant treatment	$TCD_{50, air}$ (Gy)[a]	$TCD_{50, clamp}$ (Gy)[b]	Hypoxic fraction (%)[c]	Surviving fraction[d]	
				Aerobic	Hypoxic
None (radiation only)	54.4±0.9	68.6±1.7	0.6±0.2	(1.00)	(1.00)
VP-16 (15 mg/kg)	50.1±1.1*	68.4±2.5	0.4±0.3	0.50±0.42	0.31±0.14*
BCNU (30 mg/kg)	47.9±1.1*	69.8±2.2	0.1±0.1*	0.76±0.55	0.13±0.06*
VCR (2 mg/kg)	48.5±1.2*	65.0±1.5*	0.6±0.3	0.17±0.09*	0.16±0.07*
5-FU (150 mg/kg)	53.6±2.3	63.4±1.6*	4.7±4.1	0.10±0.06*	0.78±0.60
MTX (150 mg/kg)	54.8±1.0*	65.9±1.7	3.1±1.8	0.22±0.13*	1.11±0.47

Data represent mean values ± 1 SE. All drugs were applied 4 h following irradiation

[a] Irradiation under aerobic (unclamped) conditions

[b] Irradiation under 100% hypoxic (clamped) conditions

[c] Hypoxic fraction = exp $[(TCD_{50, air}-TCD_{50, clamp})/D_{0, hypoxic}]$

[d] Surviving fraction = surviving cells after (radiation+drug)/surviving cells after radiation only

* Significantly different from values obtained following radiation only ($P<0.05$)

BCNU and VCR) were toxic to hypoxic cells and three compounds (VCR, 5-FU and MTX) were toxic to oxic cells.

Discussion

The aim of the present study was to evaluate the specific effect of five major chemotherapeutic drugs towards oxic and hypoxic cells. Only one of the five drugs tested (VCR) had a significant effect on both aerobic and hypoxic cells. Two compounds (VP16 and BCNU) caused a marked reduction in the survival of hypoxic cells only, and two drugs (5-FU and MTX) produced major cell killing in the oxic compartment without showing any activity against hypoxic cells. Such preferential effects have been dealt with in only a limited number of in vivo studies, and with the exception of our earlier studies [6, 8, 9, 14, 15], in situ clonogenic assays have not previously been reported. A classification of drugs on the basis of their selective toxicity towards tumor subpopulations has recently been proposed [22]. Using a FSaIIC sarcoma treated in vivo and cell sorting based on the Hoechst 33342 dye content, the ratio of cell survival in the 10% brightest (putatively aerobic) and 20% dimmest (putatively hypoxic) cells was taken as a measure of selective toxicity. In contrast to our experience, these results suggest that except for mitomycin C, the most commonly used antineoplastic drugs are considerably more toxic to aerobic cells than to hypoxic cells.

In our tumor system, the epipodophyllotoxin VP16 had no effect on oxic cells and only a modest effect on cells that were hypoxic. Other investigators have found a greater effect on oxic cells than on hypoxic cells both in vitro [20] and in vivo [22]. An increased effect has also been observed when VP16 is combined with carbogen and fluosol-Da [21]. These results also suggest aerobic cytotoxicity for VP16. Similarly, the cytotoxic effect of the nitrosurea BCNU was found to be mostly directed towards hypoxic cells. This finding is in good agreement with the results recently reported by Durand [3], who studied the effect of BCNU and/or cisplatin on different cell layers in V79 spheroids. This experimental setup simulates in vivo microenvironmental conditions, as hypoxic/necrotic regions are known to develop with increasing depth within the spheroid. BCNU was found to kill internal cells more efficiently than it killed external (well-oxygenated) cells. Cisplatin has been demonstrated to be preferentially cytotoxic to aerobic cells (also in agreement with results obtained in our laboratory [6, 15]), and the combination of cisplatin and CCNU/BCNU resulted in interesting synergistic effects. It should be noted that other investigators have found either a lack of preferential cell killing by BCNU in vitro [18, 19] or a direct sparing of hypoxic cells in B16 melanoma in vivo [11]. In a recent study, Teicher et al. [22] classified this drug as being preferentially toxic to aerobic cells, yielding a bright/dim ratio of 3.2. In our tumor system, a considerable effect was noted for the vinca alkaloid VCR. The regrowth delay was >6 days, and the cytotoxicity was found to be directed towards both aerobic and hypoxic cells. In the FSaIIc tumor, survival fractions of 0.47 (bright) and 1.00 (dim) have been found [22]. In EMT6 cells in vitro, the effect was also mostly limited to aerobic cells [19]. The antimetabolite 5-FU showed prominent activity against aerobic cells, whereas its effect on hypoxic cells was insignificant. This result is in accordance with the in vivo results of Teicher et al. [22], who reported a bright/dim ratio of 2.3. Other studies have shown equal sensitivity for aerobic and hypoxic cells in EMT6 cells [19] or KHT and 16/C tumors [17]. The other antimetabolite, MTX, showed a similar but somewhat weaker effect; its slight aerobic cytotoxicity was marginally significant, and it had no effect on hypoxic cells. This observation is in agreement with a study conducted by Tannock [17], who found a weak effect for MTX on aerobic cells in the KHT tumor. In vitro, no preferential cytotoxicity was found in EMT6 cells [19], and Tannock found no significant effect of MTX on either cell type in the Lewis lung tumor [17].

The evident inconsistency between the results of different studies may well be related to the use of different tumors and endpoints. Most other experiments reported have been based on cell-survival curves for tumor cells either treated in vitro under aerobic and anaerobic conditions [4, 17–19] or treated in vivo with subsequent excision and plating [22]. Although such methods may provide important 'mechanistic' information, the removal of cells from their natural environment introduces methodological problems since the excision procedure may rescue subpopulations (e.g. hypoxic cells) that would have been

doomed to die if left in situ [12]. Furthermore, the survival assay follows the responses of perhaps 10^4 of 10^7-10^8 cells, whereas the TCD_{50} endpoint provides information about the response of *all* clonogenic cells. This also contrasts with regrowth-delay assays, which reflect the killing of both clonogenic and non-clonogenic cells. In the present study, we included regrowth-delay data to show the apparent inconsistency between the results obtained from the two endpoints. All five drugs significantly increased the TGT, whereas only three of them improved the treatment outcome when they were combined with irradiation in the local-tumor-control assay. A similar lack of correlation has been shown for a range of other drugs in our laboratory [13]. We therefore believe that local tumor control is the most valid and clinically relevant endpoint to use.

In conclusion, the present study showed that two drugs (VP16 and BCNU) were toxic to hypoxic cells but not to oxic cells and two others (5-FU and MTX) were toxic to aerobic cells but not to hypoxic cells. Finally, VCR was found to be cytotoxic towards both tumor subpopulations. Effective treatments for solid tumors should take into consideration the physiological status of all clonogenic tumor cells, and rational chemotherapeutic regimens should be designed to attack each of the physiological tumor subpopulations, i.e. with a combination of agents directed against cycling and non-cycling cells as well as oxic and hypoxic cells.

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