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Short communication

Severe depletion of cellular thiols and glutathione-related enzymes of a carmustine-resistant L1210 strain associates with collateral sensitivity to cyclophosphamide

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Abstract. Cyclophosphamide (CPA) increased the life span of both carmustine (BCNU)-resistant (L1210/BCNU) and BCNU-sensitive L1210 (L1210/0) leukaemic mice; their sensitivity to CPA, however, was extremely different. The BCNU-resistant strain was much more sensitive (collaterally) to CPA than was its sensitive counterpart. The collateral sensitivity was accompanied by a severe reduction in the activity of glutathione-related enzymes and in protein thiol (SH) and non-protein SH levels in BCNU-resistant cells. The activity of glutathione reductase (GSSG-R) was 2 times higher in the L1210/0 cells than in the L1210/BCNU cells. Glutathione-S-transferase (GST) was also almost 2 times more active in the sensitive cells than in the resistant strain. To develop resistance against CPA with a single treatment (60 mg/kg) per passage, the L1210/BCNU strain needed 26 passages, whereas the L1210/0 strain required significantly fewer. The resistance developed against CPA was associated with a moderate elevation of thiols in the L1210/CPA cells, whereas this elevation was approximately 3 times more pronounced in the L1210/BCNU/CPA cells. The severely reduced activity of GST in the L1210/BCNU strain was markedly increased when these cells were made resistant to CPA; the GSSG-R activity, however, remained low, suggesting an irreversible injury of this enzyme by BCNU.

Introduction

According to the study of Schabel and co-workers [18], the L1210 leukaemia resistant to carmustine (BCNU) shows approximately the same degree of sensitivity to cyclophosphamide (CPA) as does its sensitive parent strain. In the present work, however, we found that the BCNU-resistant L1210 leukaemia (L1210/BCNU) was more sensitive (collaterally) to CPA than was its sensitive counterpart. The collateral sensitivity between the two drugs may be explained by the different resistance mechanism(s) developed by tumour cells against them. A major mechanism of resistance to BCNU has been shown to be the capacity of cells to repair the O^6-chloroethyl guanine adduct [8]. An additional mechanism may be the drug detoxification involving glutathione (GSH) and its related enzymes. Elevated glutathione-S-transferase (GST) activity has been found in BCNU-resistant melanoma [24] and astrocytoma cells [1], the latter also contained increased level of GSH, whereas a rat brain tumour resistant to BCNU (9L-2) has shown GSH depletion and a decrease in the activity of GSH-related enzymes [9]. The inhibition of GSSG-R by 2-chloroethyl nitrosourea [6, 10] has been attributed to the carbamoylation of the enzyme [2, 22]. The nature of the resistance developed against CPA is only partially known, although CPA is a widely used antitumour agent. An increased level of aldehyde dehydrogenase has been observed in L1210 cells resistant to CPA [13]. Some evidence suggests that GSH exerts a protective effect against the toxicity of CPA and, thus, depletion of cellular GSH levels can potentiate the cytotoxicity of CPA and its metabolites [5, 7, 17]. Other investigators have found an increase in the activity of GST and an elevation of GSH levels in some cell lines resistant to CPA [15, 24].

The experiments reported herein were performed to get some information on the role of cellular thiols and GSH-related enzymes in the collateral sensitivity observed between BCNU and CPA in L1210 leukaemic cells.

Materials and methods

CPA (Endoxan, Asta) was purchased from Asta-Werke Degussa Pharma Gruppe. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine) was obtained from Bristol Laboratories (Paris). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Sigma Chemical Co. Reduced Glutathione (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine

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Table 1. Effect of CPA on BCNU-sensitive and -resistant L1210 leukaemia

Doses (mg/kg)	L1210/0 (T/C%)	L1210/BCNU (T/C%)
200	212	>1,200
130	189	469
60	125	202

Life span of the treated group (days) T/C% $\times 100$
Life span of the control group (days)

dinucleotide phosphate (NADPH) and sodium nitrite were purchased from Reanal (Hungary). 5-5′-Dithio-bis(2-nitrobenzoic acid) (DTNB) was obtained from Calbiochem (California, USA). Ammonium sulphamate was purchased from Ferak Laboratories (Berlin, Germany).

Animal experiments. The BCNU-resistant strain of L1210 leukaemia was originally obtained from Dr. A. E. Bogden, NCI, E. G. & G. Mason Research Institute (Worcester, Mass.), and the standard L1210 leukaemia cell line was supplied by Dr. D. Gericke, Hoechst AG (Frankfurt/Main). Groups of BDF_1 mice (6 animals each) were inoculated i.p. with 10^6 L1210/0 or L1210/BCNU cells. In the survival studies, the tumour-bearing mice were injected i.p. with single doses of BCNU or CPA on the 2nd day after transplantation. Sensitivity to the drugs was evaluated on the basis of survival in days (T/C $\times$ 100%). For development of resistance against CPA, BCNU-sensitive and -resistant L1210 leukaemic mice were treated i.p. with single sub-effective doses of CPA (60 mg/kg) on the day following transplantation in each passage. The strain was considered to be resistant when 260 mg/kg CPA had not caused any prolongation in the life-span of animals. The cell number and cell volume were measured with a Laborscale (PSL-1)-Analisator (PSA-1; Medicor, Budapest.

Sulphydryl measurements. Mice bearing sensitive or resistant L1210 leukaemic cells were killed 6 days after implantation. Ascitic cells were collected by centrifugation, washed with saline and resuspended in cold distilled water for 1 min to lyse erythrocytes. After centrifugation the cells were homogenized in 0.02 *M* ethylene diaminetetraacetic acid (EDTA, pH 4.7). The total and non-protein sulphydryl contents were determined according to the procedure of Sedlak and Lindsay [19].

GST and GSSG-R assays. Ascitic cells harvested from mice bearing sensitive or resistant leukaemia were freed of erythrocytes and suspended in 50 m*M* potassium phosphate buffer (pH 6.5), disrupted by sonication and centrifuged at 100,000 *g* at 4°C for 1 h. The GST activity in the supernatant was measured by the method of Habig et al. [12] using 1 m*M* CDNB as an electrophilic substrate. GST activity is expressed as the amount (in nanomoles) of GSH-CDNB conjugate formed per minute per milligram of protein. The GSSG-R activity in the supernatant was determined by measuring the decrease in absorbance at 340 nm caused by utilization of NADPH by the enzyme [16]. Enzyme activity is given as the amount (in nanomoles) of NADPH oxidized per minute per milligram of protein.

Results

CPA increased the life span of both BCNU-resistant and BCNU-sensitive L1210 leukaemic mice; their sensitivity to CPA, however, was extremely different (Table 1). The BCNU-resistant strain was much more sensitive (collaterally) to CPA than was its sensitive counterpart. The maximal dose (200 mg/kg) used in this experiment practically cured the mice bearing BCNU-resistant L1210 leukaemia, whereas this dose resulted in a merely 2-fold increase in the life span of the sensitive tumour-bearing animals. The lowest dose (60 mg/kg) that had no effect on the sensitive strain caused an approximately 2-fold prolongation of the life span of BCNU-resistant leukaemic mice. In an attempt to understand the mechanism(s) of collateral sensitivity between BCNU and CPA, the level of protein thiol (PSH) and non-protein thiol (NPSH) and the activity of two GSH-related enzymes were determined in L1210/0 and L1210/BCNU cells. The L1210/0 cells contained approximately twice as much NPSH and PSH as did the L1210/BCNU cells ($P < 0.001$, Table 2). Among the GSH-related enzymes, the activity of GSSG-R and GST was also severely reduced in the resistant strain. The activity of GSSG-R was almost 2 times higher in the L1210/0 cells than in the L1210/BCNU cells ($P < 0.001$). The GST activity measured by reactivity with CDNB was approximately 2 times higher in the sensitive cells than in the resistant cells ($P < 0.001$).

Development of resistance against CPA in BCNU-resistant and -sensitive leukaemia was carried out by treatment with single sub-effective doses of CPA (60 mg/kg) in every passage. Repeated treatment initiated a decrease in sensitivity to CPA for both strains, but whereas the L1210/BCNU strain became resistant to 200 mg/kg CPA after the 26th passage, the sensitive strain did so relatively earlier, after the 11th passage. When both strains had become solidly resistant against CPA, their sulphydryl contents and the activities of their GSH-related enzymes were

Table 2. Changes in the sulphydryl content and enzyme activity related to GSH in L1210/0 and L1210/BCNU strains made resistant to CPA

	NPSH		PSH		GSSG-R		GST		Cell volume[b],*** (μm^3)
	nmol	nmol	nmol	nmol	mU	mU	mU	mU	
	mg protein	10^7 cells[d]	mg protein	10^7 cells[d]	mg protein[c]	10^7 cells[d]	mg protein[c]	10^7 cells[d]	
L1210/0	30.0 ± 7[a]	39.0	116.5 ± 18[a]	144.4	190 ± 21	107.6	321.1 ± 61	181.4	998 ± 140
L1210/BCNU	15.1 ± 5[a], *	19.6	59.5 ± 11[a], *	72.0	90 ± 9*	52.6	175.1 ± 49	102.0	1,044 ± 180
L1210/BCNU/CPA	45.7 ± 3[c], *	57.6	209.6 ± 12[c], *	272.5	97 ± 8*	54.9	296.0 ± 30	166.9	986 ± 91
L1210/CPA	41.6 ± 6[c], **	52.8	176.3 ± 17[c], *	218.6	185 ± 19	105.8	301.0 ± 50	172.1	1,112 ± 210

[a] Mean values ± SD for 5 determinations
[b] Mean values ± SD for 6 determinations
[c] Mean values ± SD for 7 determinations
[d] Mean values for 3 determinations

* $P < 0.001$ (statistically significant difference from L1210/0); ** $P < 0.01$ (statistically significant difference from L1210/0; *** $P > 0.2$ (no significant difference)

compared with those of the original L1210/0 and L1210/BCNU strains (Table 2). The development of resistance to CPA resulted in elevated levels of PSH and NPSH in both the sensitive and the resistant cells. The increase in NPSH and PSH levels was especially high, approximately 3 times greater, when the L1210/BCNU strain was made resistant to CPA ($P < 0.001$). The elevated NPSH content measured in both strains was not associated with an increase in the activity of GSSG-R. The GST activity, which was strongly suppressed in the BCNU-resistant cells, increased almost 2 times ($P < 0.01$) during the development of resistance to CPA, but this activity was no higher than that of the GST in the L1210/0 cells. There was no significant difference in the cell volume of the four strains studied in this work ($P > 0.2$, Table 2).

Discussion

The role of GSH as a detoxifier of electrophilic compounds was first postulated by Boyland and Chasseaud [3], who suggested that GST may catalyse such detoxification in cells. Since then, increasing amounts of data have shown a significant association between increases in GSH and GST activity and the resistance of animal and human tumour cells against various anticancer agents, mainly bifunctional alkylators [4, 11, 23]. The resistance to chloroethyl nitrosoureas, however, was not frequently accompanied by elevated GSH contents and GST activity, but rather GSH depletion and decreases in GST and GSSG-R activity were observed in some cell lines [9, 24]. The collateral sensitivity found between nitrogen mustard and BCNU has been explained by a reduction in the protective effect of GSH and the down-regulation of GST enzymes [21, 22, 24]. In our experiment the L1210/BCNU cells showed a severe reduction in the sulphydryl content and in the activity of GSH-related enzymes as compared with the L1210/0 cells. This reduction could not be attributed to different cell sizes, since their cell volumes proved to be approximately the same (Table 2). Thus, the collateral sensitivity we observed against CPA may be due to the decreased detoxifiying capacity of the BCNU-resistant cells. The increase in NPSH and PSH levels was more pronounced in the L1210/BCNU cells than in the L1210/0 strain when they were rendered resistant to CPA. Interestingly, the elevated NPSH levels observed in the L1210/BCNU/CPA cells were not accompanied by a stimulation of GSSG-R, but rather the activity of the latter remained as low as that measured before the onset of resistance to CPA, indicating an irreversible deficiency in the enzyme function caused by BCNU. An explanation for the survival of L1210/BCNU cells with reduced NPSH levels and lowered GSSG-R activity may be that these cells have a greater detoxification capacity (i.e., greater sulphydryl content and higher levels of GSH-related enzymes) than that required to sustain their normal cellular function. These phenomena, however, require further detailed studies on the effect of these drugs on the GSH cycle and PSH content of various tumour cells.

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