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Glutathione antagonized cyclophosphamide- and acrolein-induced cytotoxicity of PC_3 cells and immunosuppressive actions in mice[1]

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KEY WORDS glutathione; cyclophosphamide; acrolein; cytoprotection; immunosuppression

ABSTRACT

AIM: To study the antagonistic effect of glutathione (GSH) on toxicity of PC_3 cell induced by cyclophosphamide (Cyc) and acrolein (Acr) and on immunosuppressive actions caused by Cyc. **METHODS**: Splenocyte, PC_3 cell proliferation and cell protein content were measured by tetrazolium (MTT) assay and Coomassie brilliant blue assay. Serum anti-SRBC hemolysin, agglutinin, and splenocyte proliferation were measured in normal and S-180-bearing mice. Tumors were weighed. **RESULTS**: Pretreatment with GSH 2 mmol·L^{-1} reduced splenocyte proliferation inhibition from 18.64 %, 49.72 % to 6.78 %, 18.36 % (induced by Cyc 1, and 5 mmol·L^{-1}), and PC_3 cell proliferation inhibition from 27.7 %, 45.3 %, and 74.6 % to 14.6 %, 18.8 %, and 49.1 % (induced by Cyc 1, 3, and 5 mmol·L^{-1}), and from 62.6 %, 85.4 %, and 90.6 % to 41.9 %, 57.7 %, and 86.4 % (induced by Acr 10, 25, and 50 μmol·L^{-1}), respectively. In normal mice, sc GSH 75 or 150 mg·kg^{-1} bid × 5 d after ip Cyc 40 mg·kg^{-1}, the hemolysin and the splenocyte proliferation were higher than those in normal mice ip Cyc 40 mg·kg^{-1} alone. Hemolysin, serum agglutinin, and splenocyte proliferation in S-180-bearing mice given sc GSH 150 mg·kg^{-1} bid × 10 d after ip Cyc 40 mg·kg^{-1} were also markedly higher than those in S-180-bearing mice given ip Cyc alone. But, according to tumor weight, GSH did not interfere the antitumor activity of Cyc in S-180-bearing mice. **CONCLUSION**: GSH exhibited protective effects against Cyc and Acr, but had no effect on the antitumor action of Cyc.

INTRODUCTION

Cyclophosphamide (Cyc), has been widely used for the treatment of many tumors and for immunosuppression prior to organ transplantation as well as for the treatment of some autoimmune diseases. However, it is cytotoxic, especially to the hemopoietic and immunologic systems. Acrolein (Acr), a very toxic compound, is believed to be the primary toxic metabolite of Cyc[1]. Acr exhibited toxicity at 10 μmol·L^{-1} concentrations[1]. Glutathione (GSH) protected against Cyc and Acr cytotoxicity and did not interfere the antitumor activity of Cyc[2]. The subject of this paper was to observe the effects of GSH on PC_3 cell cytotoxicity induced by Cyc and Acr, and on immunosuppressive actions caused by Cyc.

MATERIALS AND METHODS

Chemicals GSH was purchased from Biomedica Foscama, Roma, Italy. Cyc was from Shanghai Hualian Pharmaceutical Co. Acr was from Niansha Chemical Factory, Jiangsu Province, China. MTT, concanavalin A (Con A) and Coomassie brilliant blue G250 were from Sigma. Hams' F12 and RPMI-1640 medium were from Gibco/BRL. Fetal Bovine Serum (FBS) and SRBC were provided by Hangzhou Sijiqing Corp. All other chemicals were AR.

Cell culture PC_3 cell, a human prostate adenocarcinoma cell line, was obtained from ATCC (American Tissue Culture Collection), USA, and cultured in Hams' F_{12} medium containing 10 % FBS in

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5 % CO_2 atmosphere at 37 ℃. Splenocytes of mice were cultured in RPMI-1640 medium containing 10 % FBS in 5 % CO_2 atmosphere at 37 ℃.

Mice ICR mice, ♀, weighing 18-22 g were supplied by Zhejiang Experimental Animal Center. Mice were caged in groups of 6 or 8 and received commercial chow and water *ad lib*.

Cytotoxicity *in vitro* Cytotoxicity of Cyc and Acr and the protective action of GSH were measured by modified MTT assay[3] and Coomassie brilliant blue assay[4].

1) MTT assay was used to measure cell proliferation (PC_3 and splenocyte). Briefly, cell suspension was prepared in corresponding medium containing 10 % FBS. The cell concentration was: PC_3 5×10^7 cells·L^{-1}, splenocyte 1×10^{10} cells·L^{-1}. Cells were seeded on Nunclon 96-well plate, 100 μL for each well. In splenocyte experiment, after adding splenocyte (4 wells for one mouse splenocyte), Con A 5 mg·L^{-1} (in RPMI-1640) 100 μL was added. In splenocyte experiment of normal mice, after adding splenocyte and Con A, Cyc or Acr was added (10 μL for each well, 4 wells for one concentration). In PC_3 cell experiment, after 24-h incubation in 5 % CO_2 atmosphere at 37 ℃, indicated chemicals (dissolved in PBS) were added, 100 μL for each well, 8 wells as a group. Serum-free medium 100 μL was added as control. In GSH protective experiment, splenocyte or PC_3 cell was pretreated with GSH 2 mmol·L^{-1} (in incubation) for 15 min. Then GSH was removed and Cyc or Acr was added. After incubation for 94 h (PC_3 cell) or 70 h (splenocyte), MTT (1 g·L^{-1} in serum-free medium) 100 μL was added, and the plate was incubated for 2 h. Then all chemicals and medium in each well were discarded, acid-isopropyl alcohol 150 μL was added. The plate was kept in dark at 22 – 25 ℃ for 30 min. Using a DG 3022 A type ELISA-READER, absorbance (A) was read at 570 nm. The result was expressed as $\bar{x} \pm s$. The cell proliferation inhibitory rate (%) = ($A_{\text{control group}} - A_{\text{tested group}}$)/$A_{\text{control group}} \times 100$.

2) Coomassie brilliant blue assay was used to measure cell protein content. Briefly, PC_3 cell (5×10^7 cells·L^{-1}) was seeded on Costar 24-well plate, 1 mL for each well. After 24-h incubation, GSH, Cyc or Acr was added, 1 mL in each well, 4 wells as a group (After adding GSH 15 min, removed GSH, then added Cyc or Acr). Plate was incubated for 96 h. Cell protein content was measured by Coomassie brillient blue assay[4]. Using UV-754 spectrophotometer, A was measured at 595 nm. The results were expressed as reduced protein content rate. Reduced protein content rate (%) = ($A_{\text{control group}} - A_{\text{tested group}}$)/$A_{\text{control group}} \times 100$. The cytotoxicity assays were performed for 4 independent times.

Antitumor activity *in vivo* On the day after inoculating S-180 (1×10^6 cells/mouse), mice were ip Cyc 40 mg·kg^{-1} alone or at the same time, sc GSH 75 or 150 mg·kg^{-1}·d^{-1} bid × 10 d. For control group, mice were sc 0.9 % NaCl solution bid × 10 d. On d 11, the tumor was weighed.

Immunologic assay Normal mice or S-180-bearing mice were treated with Cyc or Cyc + GSH, or 0.9 % NaCl. Each mouse was injected ip 20 % SRBC 0.2 mL and was killed after 4 d. The blood taken from femoral artery was used for determining immunologic effects. Splenocytes proliferation was measured by MTT assay. Hemolysin, serum agglutinin, and splenocyte proliferation were measured[3,5,6].

Statistical analysis Results were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Protective effects of GSH against PC_3 cell cytotoxicity induced by Cyc and Acr MTT assay indicated that both Cyc and Acr inhibited PC_3 cell proliferation in a concentration-dependent manner. Pretreatment with GSH 2 mmol·L^{-1} reduced the inhibitory rate of Cyc or Acr, except Acr 50 μmol·L^{-1} (Tab 1).

Coomassie brilliant blue assay indicated that Cyc reduced the protein content of PC_3 cell in a concentration-dependent manner. After pretreatment with GSH 2 mmol·L^{-1}, the protein content was less reduced than that without GSH pretreatment (Tab 2).

Protective effects of GSH against immunosuppresive actions caused by Cyc *in vitro* and *in vivo* *In vitro* splenocyte proliferation of normal mice was inhibited by Cyc in a concentration-dependent manner. GSH 2 mmol·L^{-1} pretreatment reduced the inhibitory rate of Cyc (Tab 1).

Tab 1. Effects of GSH on splenocyte and PC_3 cell proliferation inhibited by Cyc and Acr. $n = 4$ tests. $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Cyc. $^fP < 0.01$ *vs* Acr.

Cell	Drug/mmol·L^{-1}	Absorbance	Inhibition rate/%
Splenocyte	Control	0.73 ± 0.07	0
	Cyc 1	0.65 ± 0.06	18.64
	5	0.51 ± 0.05	49.72
	GSH 2 + Cyc 1	0.70 ± 0.05	6.78
	GSH 2 + Cyc 5	0.65 ± 0.08^b	18.36
PC_3	Control	0.86 ± 0.10	0
	Cyc 1	0.62 ± 0.03	27.7
	3	0.47 ± 0.02	45.3
	5	0.22 ± 0.03	74.6
	GSH 2 + Cyc 1	0.73 ± 0.07^b	14.6
	GSH 2 + Cyc 3	0.69 ± 0.06^c	18.8
	GSH 2 + Cyc 5	0.44 ± 0.11^c	49.1
	Acr 0.010	0.32 ± 0.03	62.6
	Acr 0.025	0.13 ± 0.02	85.4
	Acr 0.050	0.08 ± 0.02	90.6
	GSH 2 + Acr 0.010	0.50 ± 0.09^f	41.9
	GSH 2 + Acr 0.025	0.36 ± 0.08^f	57.7
	GSH 2 + Acr 0.050	0.12 ± 0.03	86.4

Tab 2. Effects of GSH on PC_3 cell protein content reduced by Cyc. $n = 4$ tests. $\bar{x} \pm s$. $^bP < 0.05$ *vs* Cyc 1 mmol·L^{-1}. $^fP < 0.01$ *vs* Cyc 5 mmol·L^{-1}.

Drug/mmol·L^{-1}	Absorbance	Reduced protein/%
Control	0.664 ± 0.051	0
Cyc 1	0.472 ± 0.060	28.9
Cyc 3	0.444 ± 0.064	33.1
Cyc 5	0.333 ± 0.089	49.8
GSH 2 + Cyc 1	0.663 ± 0.023^b	1.5
GSH 2 + Cyc 5	0.624 ± 0.112^f	6.0

In vivo hemolysin, serum agglutinin, and splenocyte proliferation were markedly reduced in normal and S-180-bearing mice after ip Cyc 40 mg·kg^{-1}. However, in normal mice ip Cyc 40 mg·kg^{-1}, at the same time sc GSH 75 or 150 mg·kg^{-1} bid × 5 d, hemolysin and splenocyte proliferation were higher than those in normal mice ip Cyc alone ($P < 0.05$). In S-180-bearing mice ip Cyc 40 mg·kg^{-1}, at the same time sc GSH 150 mg·kg^{-1} bid × 10 d, hemolysin, serum agglutinin, and splenocyte proliferation were higher than those in S-180-bearing mice ip Cyc alone ($P < 0.05$) (Tab 3).

Effect of GSH on antitumor activity of Cyc

Tab 3. Effects of GSH on immunosuppresion induced by Cyc in normal and S-180-bearing mice. $n = 6$ mice. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* normal mice ip Cyc 40 mg·kg^{-1}. $^eP < 0.05$, $^fP < 0.01$ *vs* S-180-bearing mice ip Cyc 40 mg·kg^{-1}.

Mice	Drug/ mg·kg^{-1}	Hemolysin /HC_{50}	Serum agglutinin/antibody score	Splenocyte proliferation /A
Normal	Control	692 ± 120	164 ± 0	0.55 ± 0.02
	Cyc 40	247 ± 99	121 ± 10	0.46 ± 0.02
	GSH + Cyc			
	75 bid × 5 + 40	485 ± 14^b	133 ± 21	0.53 ± 0.03^c
	150 bid × 5 + 40	407 ± 93^b	130 ± 10	0.60 ± 0.02^c
S-180	Control	448 ± 138	131 ± 26	0.54 ± 0.15
	Cyc 40	370 ± 163	111 ± 15	0.40 ± 0.14
	GSH + Cyc			
	75 bid × 10 + 40	399 ± 101	116 ± 23	0.59 ± 0.16^e
	150 bid × 10 + 40	533 ± 79^e	132 ± 17^e	0.73 ± 0.17^f

According to the weight of tumor, the antitumor activity was not interfered by GSH. Body weight (subtracting tumor weight) of S-180-bearing mice between groups GSH 75, 150 mg·kg^{-1} bid × 10 d + Cyc and group Cyc showed little difference ($P > 0.05$). But body weight (subtracting tumor weight) of mice in group GSH 150 mg·kg^{-1} bid × 10 d + Cyc was heavier than that in control group ($P < 0.05$) (Tab 4).

Tab 4. Effect of GSH on antitumor activity of Cyc and body weight of S-180-bearing mice. $n = 8$ mice. $\bar{x} \pm s$. $^aP > 0.05$ *vs* Cyc 40 mg·kg^{-1}. $^eP < 0.05$, $^fP < 0.01$ *vs* control.

Drug/ mg·kg^{-1}	Tumor wt/ g	Tumor inhibition/ %	Body wt/g before	Body wt/g after	Reduced body wt/%
Control	5.99 ± 1.55	0	21.5 ± 0.9	19.7 ± 1.1	8.3
Cyc 40 × 1	3.04 ± 0.51^f	49.2	21.4 ± 1.1	20.4 ± 2.3	4.6
GSH 75 bid × 10 + Cyc 40 × 1	2.86 ± 0.57^{af}	52.3	21.6 ± 1.2	20.9 ± 2.3^a	3.2
GSH 150 bid × 10 + Cyc 40 × 1	2.68 ± 0.61^{af}	55.2	21.4 ± 0.5	21.1 ± 1.4^{ae}	1.4

DISCUSSION

Our study indicated that *in vitro* GSH exhibited protective effect against the cytotoxicity of Cyc and Acr

643-646

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in PC_3 cell and it did not interfere with the antitumor activity in S-180-bearing mice. This is consistent with previous studies, though they used A_{549} cells and Walker 256 carcinoma in rats[1,2]. In our experiments, GSH 2 mmol·L^{-1} did not reduce PC_3 cell proliferation inhibition induced by Acr 50 μmol·L^{-1}, it was most likely due to the high dose of Acr, GSH could only partly antagonize Acr-induced cytotoxicity of PC_3 and showed no difference. The mechanism of GSH protective action has not been very clear yet. However, it is useful for those patients using Cyc as a chemotherapeutic agent to use GSH to get the same therapeutic effects and to have less side effect. On the other side, it has not been reported that GSH exhibited actions against immunosuppression of Cyc *in vivo* and *in vitro*. As our experiments showed that *in vitro* pretreatment with GSH reduced splenocyte proliferation inhibition (induced by Cyc) and in normal and S-180-bearing mice sc GSH 5 – 10 d after ip Cyc 40 mg·kg^{-1}, the hemolysin, serum agglutinin, and splenocyte proliferation were higher than those in mice given ip Cyc alone. And this is important for cases using Cyc to treat tumor or using Cyc as an immunosuppressive agent.

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谷胱甘肽拮抗环磷酰胺和丙烯醛所致 PC_3 细胞毒性及小鼠免疫抑制[1]

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关键词 谷胱甘肽; 环磷酰胺; 丙烯醛; 细胞保护; 免疫抑制

目的: 观察谷胱甘肽(GSH)在体外对环磷酰胺(Cyc)及其代谢产物丙烯醛(Acr)所致正常小鼠脾细胞增殖抑制和人前列腺癌 PC_3 细胞体外生长抑制的对抗作用, 同时观察 GSH 在体内对 Cyc 所致正常小鼠及荷 S-180 小鼠免疫抑制及抑瘤的影响. **方法**: 用 MTT 法和考马斯亮蓝法测定正常小鼠脾细胞及人前列腺癌 PC_3 细胞体外增殖抑制率, 并测定小鼠抗 SRBC 血清溶血素, 凝集素含量及脾细胞增殖反应. **结果**: 预先用 GSH 2 mmol·L^{-1}处理使 Cyc 1 – 5 mmol·L^{-1}对小鼠脾细胞的增殖抑制率由 18.64 % – 49.72 %降为 6.78 % – 18.36 %. 对 PC_3 细胞的生长抑制率由 27.7 % – 74.6 %降到 14.6 % – 49.1 %. Acr 10 – 50 μmol·L^{-1}对 PC_3 细胞的生长抑制率为 62.6 % – 90.6 %, 预先用 GSH 处理亦可使抑制率降低. GSH 对 Cyc 所致正常小鼠血清溶血素减少与脾细胞增殖抑制有明显的对抗作用, GSH 处理并不影响 Cyc 对荷 S-180 小鼠的抑瘤作用, 但使血清溶血素和凝集素水平及脾细胞增殖能力均显著高于单用 Cyc 组. **结论**: GSH 减少 Cyc, Acr 对小鼠脾细胞及 PC_3 细胞的细胞毒性, GSH 与 Cyc 合用减少 Cyc 的免疫抑制作用, 但不影响 Cyc 的抗肿瘤作用.

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