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一、中文摘要

Glutathione (GSH)麩胱甘太是含tripetide的半胱胺酸(cysteine)，而OTZ是cysteine的先驅物，其會增加GSH的合成；而BSO為*r*-glutamyl cysteine合成的抑制劑，其會抑制GSH的合成；本試驗探討抗癌藥物平陽黴素作用後對KB細胞內GSH的影響、改變細胞內GSH的濃度後對平陽黴素細胞毒性的影響、細胞內GSH的濃度對平陽黴素引發KB細胞凋亡的影響。研究結果發現，平陽黴素會造成KB細胞內GSH值下降，GSH的消耗增加會促進細胞死亡且細胞死亡模式會由細胞壞死轉變為細胞凋亡。
關鍵詞：麩胱甘太、平陽黴素、細胞凋亡

Abstract

The effects of intracellular glutathione (GSH) concentration on the toxicity of pingyangmycin in human squamous cell carcinoma cell line were evaluated. By using the GSH synthesis inhibitor D,L-buthionine-S,R-sulfoximine (BSO) and the precursor of cysteine 2-oxothiazolidine-4-carboxylate (OTZ), intracellular glutathione levels were artificially changed. After exposed to different GSH concentrations cultured tumor cells were treated with pingyangmycin and the resultant mode of cell death was analyzed using morphological and biochemical criteria. It was found that the toxicity of pingyangmycin was obviously increased to cultured tumor cells on lowering GSH levels, with the mode of cell death switching from necrosis to apoptosis. In contract, treatment with OTZ increased GSH level compared with that of control cells, inhibited cell death induced by pingyangmycin via a necrotic rather than apoptotic process. These observations suggest that modulation of GSH levels effects the toxicity of pingyangmycin and that GSH influences the mode of cell death induced by pingyangmycin.
KeyWords: glutathione; pingyangmycin; apoptosis

二、緣由與目的

The anti-tumour antibiotic pingyangmycin (bleomycin A_5) is fairly extensively used in chemotherapy for treatment of neoplasms in head and neck region [1,2]. Our previous study has shown that pingyangmycin can induce two distinct modes of cell death [3]. Cells became enlarged, multinucleated,

and displayed an arrest in G_2-M phase of the cell cycle in the presence of low concentrations of pingyangmycin (5μg/ml, 50μg/ml). In contrast, cells exhibited the morphological and biochemical changes associated with apoptosis when treated with high concentrations of pingyangmycin (500μg/ml, 5mg/ml). The mechanism of the mode of cell death changes is closely related to the number of pingyangmycin molecules accumulated in the target cells. At high concentrations, pingyangmycin can be considered as an apoptosis mimetic drug.

It is well-known that D,L-buthionine-S,R-sulfoximine (BSO), a specific inhibitor of γ-glutamyl cysteine synthetase, inhibits GSH synthesis, is relatively non-toxic, and is quite efficient in decreasing intracellular GSH levels. 2-oxothiazolidine- 4-carboxylate (OTZ), a precursor of cysteine, metabolically promotes GSH synthesis, increasing intracellular GSH levels by as much as 2- to 3- times the control level [4, 5].

In this study, we artificially changed the intracellular GSH levels and analyzed the mode of pingyangmycin induced cell death affected by intracellular GSH levels. We found that the toxicity of pingyangmycin was obviously increased to cultured tumor cells on lowering GSH levels, with the mode of cell death switching from necrosis to apoptosis. These observations suggest that modulation of GSH levels effect the toxicity of pingyangmycin and that GSH influences the mode of cell death induced by pingyangmycin.

三、結果與討論

The effect of BSO and OTZ exposed on the GSH concentrations of KB cells was examined. As shown in Figure 1, dose-dependent depletion of GSH levels was observed by the treatment of KB cells with BSO. KB cells treated with 50μM BSO for 24 hours reduced the GSH levels to 28% of that of the control. In contrast, the treatment with OTZ resulted in a significant increase of GSH levels in KB cells. The KB cells treated with 5mM OTZ for 24 hours had 160% level of the GSH, compared to the control cells. The effect of pingyangmycin on the intracellular total GSH content of KB cells was analyzed. Although the total GSH consists of both oxidized and reduced GSH forms, oxidized GSH accounts for less than 5% of total GSH [6]. The total GSH level was used as a parameter in the present study. The amount of GSH in the cell affected by pingyangmycin is shown in Figure 2. Pingyangmycin depleted intracellular GSH in KB cells in a dose-dependent manner ($p < 0.05$). KB cells treated with 5μg/ml pingyangmycin for 24 hours reduced the GSH levels to 77% of that of the control ($p < 0.05$). When treated with 5mg/ml pingyangmycin for 24 hours the GSH levels reduced to 47% of that of the

control. KB cells survival was determined by MTT assays practically reflected the viable cell number as previously described [7].

As can be seen in Figure 3, incubation of KB cells with pingyangmycin higher than 5μg/ml elicited a cytotoxicity response which was concentration-dependent. In the cell pretreated with BSO (5, 50μM), this cytotoxic effect of pingyangmycin was heavily potentiated, as also shown in Figure 3. When treated with 0.5μg/ml pingyangmycin in the presence of 50μM BSO KB cell viability was obviously inhibited as compared to that of control cultures ($p < 0.05$). In contrast, the cells pretreated with OTZ (0.5, 5mM) showed resistance to the cytotoxicity of pingyamgmycin.

Morphological changes in KB cells having altered levels of GSH treated with pingygngmycin were observed by the May-Grünwald Giemsa staining (Figure 4). The percentage of apoptotic and necrotic cells in the cultures were assessed using morphological criteria. The cytotoxicity of pingyangmycin in KB cells was enhanced when the intracellular GSH levels were lowered; however, under this condition pingyangmycin induced cell death by apoptosis rather than necrosis (Figure 5). In contrast, when intracellular GSH levels were raised, the cytotoxicity of pingyangmycin in KB cells was reduced, and pingyangmycin induced cell death by necrosis rather than apoptosis (Figure 5). These results indicated that GSH levels affected the mode of cell death.

Chromosomal DNA fragmentation in KB cells was evaluated after treatment with various doses of pingyangmycin. A definite characteristic patter of oligonucleosomal sized DNA fragments consisting of multimers of approximately 180 bps was observed by modified agarose gel electrophoresis. DNA ladder bands were weakly detectable by the treatment of 500μg/ml and were clearly observed by the treatment of 5mg/ml. No ladder bands were detected when pingyangmycin concentrations were below 50μg/ml. Next, we examined the effects of 50μM BSO and 5mM OTZ on the formation of DNA ladders induced by pingyangmycin. In cells pretreated with BSO for 24 hours, DNA ladders were observed within 24 hours of treatment with 50μg/ml pingyangmycin. However, in cells pretreated with OTZ, DNA ladders were weakly detected within 24 hours of treatment with 5mg/ml pingyangmycin (Figure 6).

From a clinical viewpoint, our present experiments indicate that decreasing intracellular GSH level may enhance the toxicity of pingyangmycin in chemotherapy for treatment of malignant tumors. GSH and its associated enzyme activities might have important implications in the reversion of MDR in tumor clinical work [8]. On the other hand, it might be very beneficial to induce tumor cell apoptosis in cancer chemotherapy. Necrotic cell

death would release intracellular constituents into tissue upon cell destruction. Therefore, if we could induce tumor cell apoptosis in the treatment of malignant tumor, it would prevent secondary tissue damage due to inflammatory reaction caused by necrotic cell death [9,10]. In our study, decreasing GSH levels could promote pingyangmycin responsiveness to induce tumor cells apoptosis *in vitro*. Our earlier result indicated that in order to induce cell apoptosis, the concentration of pingyangmycin present in the culture medium is too high for clinical use [1]. Therefore, GSH and its associated enzyme activities could affect the transport function of pingyangmycin through the plasma membrane, resulting in inducing apoptosis of tumor cells at low concentration of pingyangmycin might resolve this problem.

五、參考文獻

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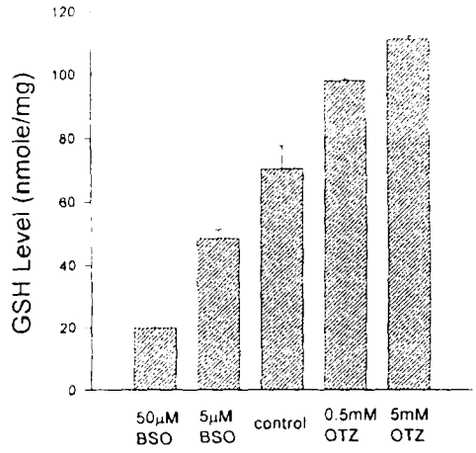

Fig. 1. Intracellular glutathione (GSH) levels in KB cells treated with various concentrations of BSO (5 and 50 μM) or OTZ (0.5 and 5 mM) for 24 h.

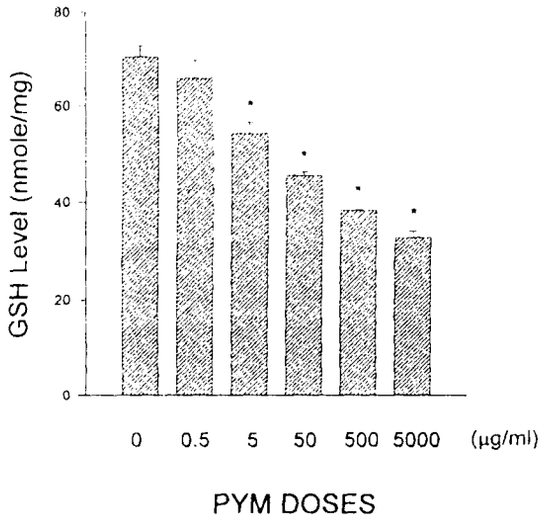

Fig. 2. Intracellular glutathione (GSH) levels in KB cells treated with different doses of pingyangmycin (PYM; 0.5, 5, 50, 500 μg/ml, and 5 mg/ml) for 24 h. PYM depleted intracellular GSH in KB cells in a dose-dependent manner ($P<0.05$).

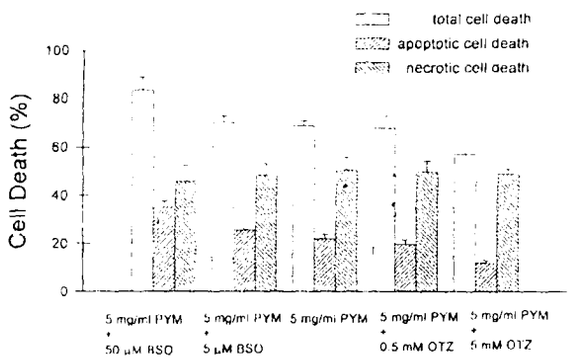

Fig. 5. The percentage of apoptotic and necrotic cells in the cultures were assessed using morphological criteria. When the intracellular glutathione (GSH) levels were lowered; pingyangmycin (PYM)-induced cell death by apoptosis rather than necrosis. In contrast, when GSH levels were raised, PYM-induced cell death by necrosis rather than apoptosis.

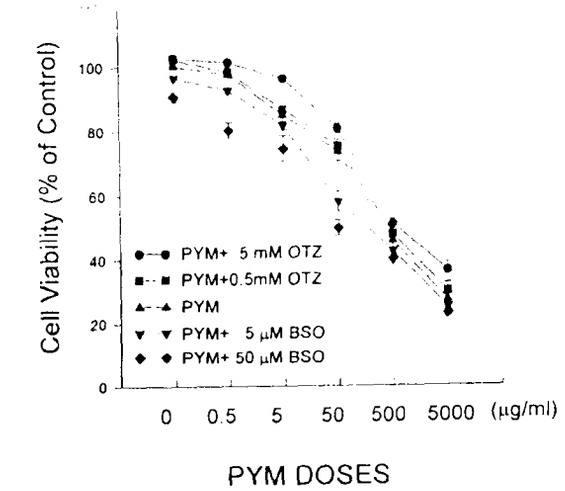

Fig. 3. Cytotoxicity assay with MTT staining of viable cells. Incubation of KB cells with pingyangmycin (PYM) higher than 5 μg ml represented a cytotoxicity response which was concentration-dependent. In the cell pretreated with BSO, this cytotoxic effect of PYM was heavily potentiated ($P<0.05$). In contrast, the cells pretreated with OTZ showed resistance to the cytotoxicity of PYM ($P<0.05$).

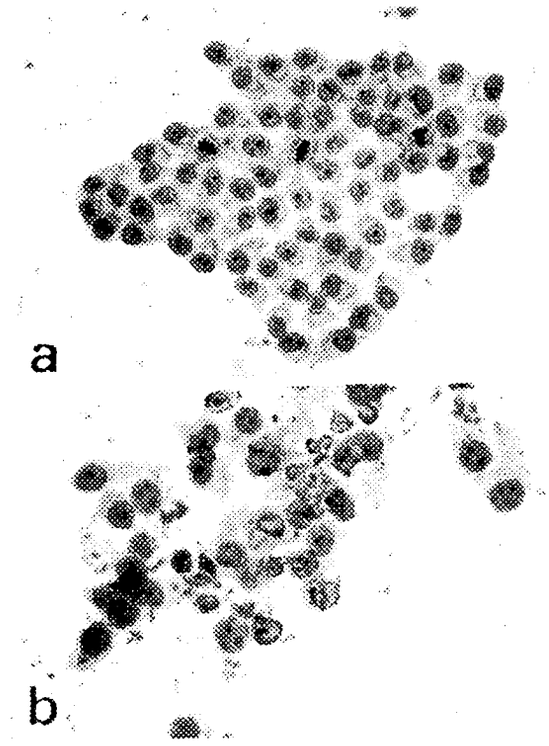

Fig. 4. Dependence of the mode of cell death induced by pingyangmycin on intracellular glutathione (GSH) levels (×400). (a) Untreated cells; (b) cells pretreated with 50 μM BSO for 24 h. apoptotic cells were observed within 24 h of treatment with 50 μg ml pingyangmycin.

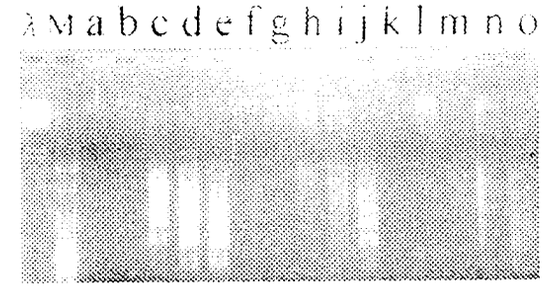

Fig. 6. Chromosomal DNA fragmentation in KB cells was evaluated after treatment with various doses of pingyangmycin (5, 50, 500 μg ml, and 5 mg ml). DNA ladder bands were weakly detectable by the treatment of 500 μg/ml and were clearly observed by the treatment of 5 mg ml (land f to land j). In cells pretreated with 50 μM BSO for 24 h, DNA ladders were observed within 24 h of treatment with 50 μg ml pingyangmycin (land a to land e). However, in cells pretreated with 5 mM OTZ, DNA ladders were weakly detected within 24 h of treatment with 5 mg/ml pingyangmycin (land k to land o). λ, λ-HindIII; M, 100 bp ladder.