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行政院國家科學委員會補助專題研究計畫成果報告

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一、中文摘要

經過嚴格的篩選過程，我們首度證實部分抗黴菌藥物具有使抑制癌細胞週期停滯的作用，根據其作用位置可分成三大類：(A). 誘發 G0/G1 phase arres：包括 ketoconazole，fluconazole，及 flucytosine。(B). 誘發 S phase arrest：Ketoconazole 在低劑量(10 μg/mL)時有大部分細胞停滯於 S phase。(C). 誘發 G2/M phase arrest：如 griseofulvin；我們已經初步證實 griseofulvin 造成人類癌細胞內α-tubulin 異常糾結(Figure 9)、Cyclin B 蛋白量表現增加、cdc-2 kinase 活性增加及 Wee-1，Myt-1 活性下降，是導致 G2/M phase 停滯的重要因素。由於參與細胞週期的基因調控目前已經相當清楚，因此本計劃以有系統的分析方式，逐步探討藥物處理後細胞週期之基因變化情形。

關鍵詞：抗黴菌藥、細胞週期、細胞凋亡

Abstract

We first demonstrated that the several oral antifungal agents could induce cell cycles arrest. These agents were divided into 3 class according to their effects: (A). Agents that induce Go/G1 phase arrest: Such as ketoconazole, fluconazole, and flucytosine. (B). Agent that induces S phase arrest: As shown in Figure 2, low dose of ketoconazole (10 μg/mL) induce cell cycle arrest in S phase. (C). Agent that induce G2/M phase arrest: Flow cytometry analysis demonstrated that griseofulvin may cause G2/M cell cycle arrest in human hepatoma cell lines (Hep G2 and Hep 3B). Our preliminary data have demonstrated that the mechanisms were: (1), induce abnormal intracellular α-tubulin stabilization (2), enhance the expression of cyclin B and cdc2 kinase activity that regulate G2/M cell cycle arrest (3), inhibit the expression of the Wee-1, and Myt-1 protein . (4), induce Bcl-2 protein hyper-phosphorylation in drug-induced G2/M arrest cells.

Keywords: Oral antifungal agents, apoptosis, cell cycle

二、緣由與目的

According to recent reports, microtubule-stabilizing agents such as paclitaxel and docetaxel (Srivastava et al., 1998) and microtubule-disrupting drugs such as vincristine, vinblastine, and colchicine have anti-mitotic and apoptosis-inducing activity (Donaldson et al., 1994). In these studies, human leukemic, breast cancer, and prostate cancer cells exposed to paclitaxel expressed a phosphorylated form of Bcl-2 and underwent apoptosis, suggesting that phosphorylation of Bcl-2 may inhibit Bcl-2 function (Srivastava et al., 1998). In addition, phosphorylation of Bcl-2 appears to inhibit its binding to Bax, since less Bax was observed in an immunocomplex with Bcl-2 in taxol-treated cancer cells (Halder et al., 1996). Other studies have indicated that Bcl-2 phosphorylation is tightly associated with mitotic arrest, but is not a determinant of progression into apoptosis after mitotic arrest induced by antitubulin agents such as taxol and nocodazole (ND) (Halder et al., 1997; Ling et al., 1998; Roth et al.,1998). The correlation of G2/M arrest and apoptosis was investigated in reports which demonstrate that activation of protein kinase A (or raf-1) due to microtubule damage is an important event in Bcl-2 (or Bcl-x_L) phosphorylation (Poruchynsky et al., 1998; Srivastava et al., 1998). How disruption of the microtubules can lead to cell death is only now being

explored.

The ability of cancer chemotherapeutic agents to initiate apoptosis plays an important determinant of their therapeutic response. Our previous results showed that the oral antifungal agent, ketoconazole, caused apoptosis in human cancer cell lines (Ho et al., 1998). The present work extends the previous study, and the data indicate that another antifungal agent, griseofulvin (GF), at micromolar concentrations, rapidly initiates apoptosis. The goal of this study was not only to increase our understanding of GF-induced apoptosis and G2/M cell cycle arrest but also to elucidate general mechanisms through which intracellular signals can mediate these responses.

三、結果與討論

Griseofulvin Induces Apoptosis and G2/M Cell Cycle Arrest in Human Cancer Cells

In this study, human cancer cells including colon adenocarcinoma cells (COLO 205 and HT 29), hepatoma cells (Hep G2 and Hep 3B), and leukemia cells (HL 60), and normal keratinocytes (#76 KhGH), were treated with various concentrations (0 to 50 μM) of GF. The minimal dose of GF which induced cytotoxicity in human cancer cells was 1 μM (Figure 1). As described previously (Enari et al., 1998), apoptosis is characterized by specific changes including DNA fragmentation and the presence of a subdiploid peak following flow cytometry analysis. Figure 2 showed that GF induced DNA laddering in various cancer cells in a dose-dependent manner (0 to 50 μM). Our results from figures 1 and 2 indicate that human cancer cell lines were more sensitive to GF as compared to normal human keratinocytes. The doses of GF that induce DNA fragmentation were found to be consistent with effective cytotoxic concentrations in various cell lines. Such results imply that the cytotoxic action of GF is due to its ability to induce apoptosis.

Molecular Mechanisms of GF-Induced G2/M Arrest

As shown in figure 3A, a higher dose of GF (>20 μM) could induce G2/M arrest in human HT 29 cells. To further investigate the molecular events in GF-induced G2/M arrest, HT 29 cells were synchronized at the G0/G1 phase by 0.04% serum starvation for 24 h. After synchronization, the medium was replaced with the complete medium containing 10% FCS. The subpopulation during cell cycle in cells treated with either mock- (Figure 6A) or GF (40 μM, Figure 6B) were then measured by flow cytometry analysis. As shown in figure 6B, G2/M arrest was observed initially at 6 h and reached the maximal level (>90%), at 24 h after GF treatment. Similar results were also seen in COLO 205 cells (data not shown).

We then examined changes of G2/M regulatory proteins in HT 29 cells after GF treatment. Expression of cyclin B1/cdc2, a key regulator of cell entry into mitosis, was first monitored by immunoblotting and assaying the cyclin B1-immunoprecipitated cdc2 kinase activity. The time points according to figure 6 were selected as 0 h (representing the G0/G1 phase), 15 h (representing the S phase), 18 h (representing the G2/M phase) and 24 h (representing the 2nd G0/G1 phase). Immunoblotting analysis showed that cyclin B1 in mock-treated cells was elevated at 18 h and then decreased at 24 h (Figure 7A). In the GF-treated group, the cyclin B1 levels accumulated initially at 15 h and reached the maximal level at 24 h. In addition, cdc2 kinase activity was elevated significantly in the GF-treated group although the change of protein level was not observed. The other G2/M regulatory protein, myt-1, which inhibits cdc2 kinase, was down-regulated in GF-treated cells.

It has been suggested that Bcl-2 phosphorylation is associated with G2/M cell cycle arrest (Halder et al., 1996; Scatena et al., 1998; Srivastava et al., 1998; Stewart et al., 1999). Figure 7A showed that the slower migration form of phosphorylated-Bcl-2 was present initially at 15 h in cells treated with GF. The GF-induced phosphorylation changes of Bcl-2 were consistent with cells accumulating in the G2/M phase with an active cyclin B1/cdc2 complex. In this study, GF-mediated Bcl-2 phosphorylation coincides with cdc-2 kinase activation (Figure 7A, lanes 6, 7, and 8). Others (Stewart et al., 1999) and

our results indicate that cdc-2 kinase might be involved in Bcl-2 phosphorylation.

It has been shown that Bcl-2 may protect cancer cells from apoptosis (Halder et al., 1997). Human cancer cells exposed to paclitaxel expressed a phosphorylated form of Bcl-2 and underwent apoptosis, suggesting that phosphorylation of Bcl-2 may inhibit Bcl-2 function. In this study, we demonstrate that Bcl-2 phosphorylation appears to inhibit its binding to Bax, since less Bax was observed in an immunocomplex with Bcl-2 in GF-treated cancer cells (Figure 7B). Our results demonstrate that Bcl-2 phosphorylation affects the binding ability to Bax and promotes abnormal microtubule polymerization, although the intracellular level of α-tubulin is not affected (Figure 7B).

Treatment with GF and ND Combination Enhances Cancer Chemotherapeutic Efficacy in vivo.

We further examined the therapeutic efficacy of GF and ND in vivo by treating athymic mice bearing COLO 205 tumor xenografts with GF (50 mg/Kg), ND (5 mg/Kg), or GF and ND combination. After establishment of palpable tumors (mean tumor volume, 200 mm^3), animal received intraperitoneal injections of GF, ND, or both agents together 3 times per week, as well as DMSO and saline for a negative control. After 6 weeks, tumor volumes in mice treated with either GF or ND was significant reduced in comparison with DMSO or saline-treated controls (Figure 9). Treatment with GF and ND together significantly enhanced the efficacy of ND, leading to cessation of tumor growth (Figure 9 A and B). In mice receiving these treatment regimens, no gross signs of toxicity were observed (body weight, visible inspection of general appearance and microscopic examination of individual organs). Our results provide further evidence that GF may have significance of application for cancer chemotherapeutic purposes.

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