行政院國家科學委員會專題研究計畫　成果報告

探索在人類急性髓性白血病骨髓微環境中對三氧化二砷產生抗藥性之重要因子

計畫類別： 個別型計畫
計畫編號： NSC94-2314-B-038-041-
執行期間： 94 年 08 月 01 日至 95 年 07 月 31 日
執行單位： 臺北醫學大學醫學研究所

計畫主持人： 劉興璟

計畫參與人員： 周志銘,李進成,林書帆

報告類型： 精簡報告

處理方式： 本計畫可公開查詢

中　華　民　國 95 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫

■成果
報　告

探索在人體急性髓性白血病骨髓微環境中對三氧化二砷產生抗藥之重要因子

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC94－2314－B－038－041
執行期間：94 年 08 月 01 日至 95 年 07 月 31 日

計畫主持人：劉興璟
共同主持人：
計畫參與人員：　周志銘,李進成,林書帆

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

(二)中、英文摘要及關鍵詞(keywords)

本研究在於探索骨髓微環境中用以調控三氧化二砷的抗藥機轉.我們發現微環境中之 fibronectin 及 V-CAM-1 對經三氧化二砷處理之急性髓性白血病細胞之分裂,及死亡有明顯影響, fibronectin 可以使不同的白血病細胞產生抗藥性,這些抗藥性是透過 fibronectin 的 ligand,VLA-4 來進行訊息傳遞,同時以 VLA-4 抗體可以阻斷 fibronectin 產生之抗藥性,同時 VLA4 的活化使細胞內 AKT 及 MAPK 訊息路徑的磷酸化增加,更進一步以 AKT 之抑制劑 LY294002 及 MAPK 抑制劑 SB202190 處理後發現可以阻斷 fibronectin 誘導產生之抗藥性進而使加強三氧化二砷,防止三氧化二砷的抗藥性發生.因此本研究初步發現骨髓微環境中用以煤介三氧化二砷抗藥性發生的分子, 藉此可以做爲未來新藥開發之理論依據.

關鍵詞：急性髓性白血病, 骨髓微環境, 三氧化二砷, AKT, VLA-4

In this study, we intended to study the mechanism underlying the stromal cells-mediated resistance to arsenic trioxide in acute myeloid leukemia. We found that fibronectin is the most critical factor that mediated the resistance to ATO in vitro, and possibly in vivo. When AML cells grown in fibronectin coated plates, they exhibited less apoptosis, increased survival and proliferation. Furthermore we demonstrate that interaction with fibronectin downregulates the levels of p21 and p27 in NB4 cells, increases the expression of cyclin D1, antiapoptotic Bcl-xl and Bcl-2. Furthermore, the fibronectin mediated effects can be partially abrogated by anti-VLA-4 but not anti-VLA-5 antibody. Interaction with fibronectin increases the phosphorylation of AKT and p38 MAPK. To circumvent the fibornectin-mediated effect, we show that AKT inhibitor LY294002 can block the fibronectin-mediated effects. We also tested the effect of MAPK inhibitor SB 202190, but the activity of MAPK inhibitor was less pronounced than LY294002. Therefore, this study indicates that fibronectin in the stromal cells play a critical role in ATO resistance and blocking with novel AKT inhibitor might represent a potential strategy for improving anti-leukemic therapy in vitro.

Keyworkds: acute myeloid leukemia, bone marrow microenvironment, arsenic trioxide, fibronectin, VLA-4

(三)報告內容

1. 前言與研究目的

Arsenic trioxide (ATO) is a novel agent for treating acute promyelocytic leukemia (APL). However, studies on the use of arsenic trioxide on different types of cancer such as multiple myeloma, hepatocellular carcinoma, etc has shown that these cancer cells are sensitive to ATO in vitro but become resistant in vivo. Our previous study on the use of ATO on APL has shown that APL cells are quite sensitive to ATO in vitro but gradually develop resistance in vivo. Our study further shows that ATO can upregulate glutathione in vivo, inducing resistance. Furthermore, using in vitro system, we previously demonstrated that stromal cells are crucial for developing resistance in vivo. Some of the resistance mechanisms appear to be the upregulation of antiapoptotic protein and changes of cell cycle proteins. To further elucidate the mechanisms, we intended to use in vitro system to dissect the molecular mechanisms underlying the resistance in vivo. Several stromal proteins have been shown to cause drug resistance to conventional chemotherapeutic agents, such as fibronectin. Therefore, in this study, we hypothesized that stromal factors such as fibronectin play a crucial role in mediating the ATO resistance in vivo. We used an in vitro system to emulate in vivo situation, hoping that through understanding of the molecular mechanisms, we can identify the causes of resistance and help develop anti-resistance mechanisms in future cancer therapy.

2.研究方法

In this study, we used the following assays to answer various questions.

1. All cell lines were purchased from 食品研究所, AKT and MAPK inhibitors from Calbiochem. Fibronectin, V-CAM-1 and type I collagen were from Sigma-Aldrich.
2. **MTT assays**: we used this assay to study the growth of acute leukemic cells. Briefly, cells were grown in indicated conditions and then treated with MTT for 4 hours and viable cells were converted to yellowish MTT to bluish formazan and measured by spectrophotometry. For detailed methods please refer to reference 6.
3. **Cell cycle analysis:** cells were lyzed with propidium iodide-containing hypotonic citrate solution and analyzed by flow cytometry within two hours. For detailed methods please refer to reference 6.
4. **Apoptosis assays:** cells were stained with annexin-V-FITC and propidium iodide for 30 minutes and the unused propidium iodide and annexin-V-FITC were washed away and Cells were analyzed by flow cytometry. For detailed methods please refer to reference 6
5. **Western blotting:** cells were lysed with NETN lysis buffer and total cell lysates were resolved on polyacrylamide gel and probed with different antibodies. Antibodies used in this study included p21WAF1, p27cip1, cyclin D1, cyclin B, cyclin D, Bcl-xL, Bcl-2, BAD, BAX, actin, phospho-AKT, AKT, and p38MAPK. For detailed methods please refer to reference 6.
6. Statistical analysis: the data are presented as mean±SEM. Statistical analysis was done by Student's t tests, and $p<0.05$ was regarded as statistically significant.

5.結果與討論

1. **Extracellular matrix protein (ECM) fibronectin (FN) significantly improved the survival of AML cells when grown in the presence of 1 microM ATO.** We tested the effects of 3 different major ECM that have been shown to play a role in stromal cell microenvironment on 4 different types of acute myeloid leukemia (HL-60, NB4, U937 and THP-1). We found that FN significantly improved the survival of cells in all four kinds of AML cells, but the effects were most evident in NB4 cells (Figure 1).

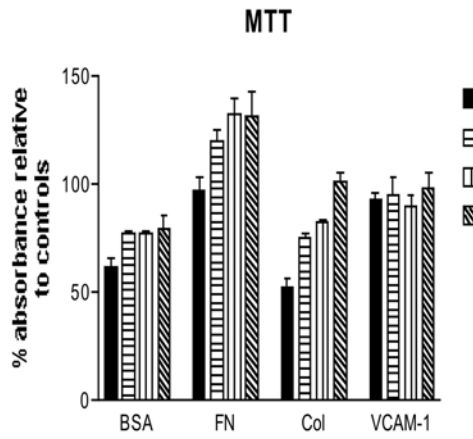

Figure 1. Effects of ECM on MTT assays. Four types of AML cells were treated with 1 mcM ATO for 24 hours before MTT assays. Cells grown in fibronectin (FN)-coated cultures grow significantly better in the presence of ATO. The differences are most evident in NB4 cells. The other types of ECM were not significantly different from BSA controls

2. **FN prevented the apoptosis of ATO-treated cells.** We then tested whether ECM can prevent the apoptosis of AML cells in vitro. Cells were treated with 1 microM ATO on the ECM for 24 hours before assaying apoptosis. We found that cells grown on FN and VCAM-1 had significantly less apoptosis then on other ECM (Figure 2)

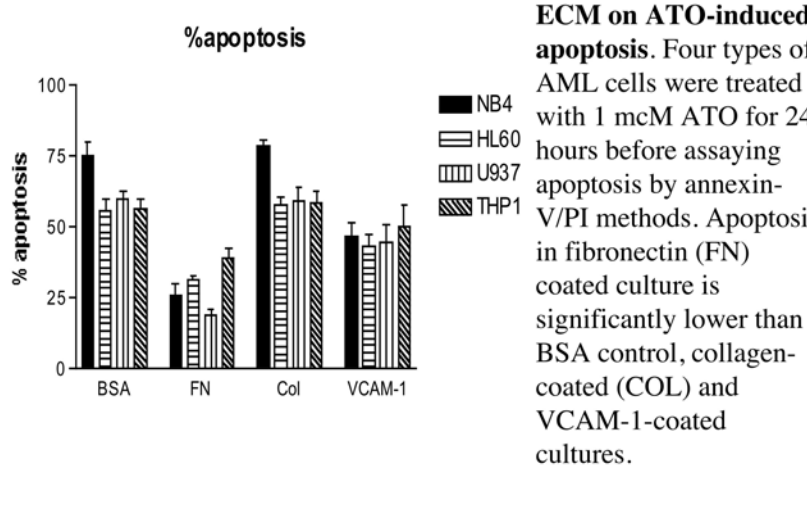

Figure 2.Effects of ECM on ATO-induced apoptosis. Four types of AML cells were treated with 1 mcM ATO for 24 hours before assaying apoptosis by annexin-V/PI methods. Apoptosis in fibronectin (FN) coated culture is significantly lower than BSA control, collagen-coated (COL) and VCAM-1-coated cultures.

3. **FN aboraged the ATO-induced cell cycle arrest. To further evaluate the effect of ECM on ATO resistance,** we measured the cell cycle changes on 4 different AML cells. Then cells were treated with 1 microM ATO on ECM for 24 hours. Still cells on FN exhibited the most evident cell cycle arrest, followed by V-CAM1 and type I collagen (Figure 3).

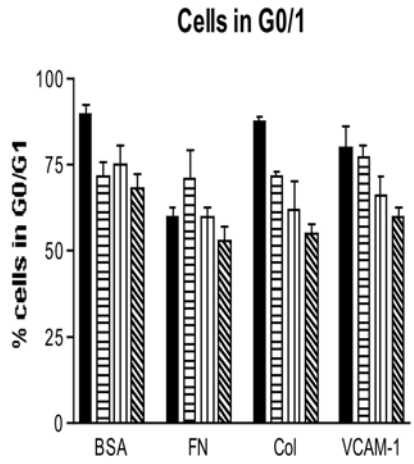

Figure 3. The effects of FN on cell cycle analysis. Four different types of AML cells treated with 1mcM ATO for 24 hours before cell cycle analysis. Adhesion to FN overcomes the cycle-arrest induced by ATO, especially in NB4 cells.

4. Effect of FN on cell-cycle-related protein, apoptosis-related protein. We then characterized the changes of proteins in cells treated with ATO. We found that when cells were grown on V-CAM1 and FN, the levels of p21 and p27 were significantly reduced. But the cyclin D1 levels were increased. We did not detected any changes in cyclin B or E on either of the treated. Cells. In contrast, V-CAM-1 and FN increased the antiapoptotic protein Bcl-xL and Bcl-2 (Figure 4). These changes support the prior findings that FN coating enhanced the proliferation and inhibited the apoptosis of ATO-treated cells.

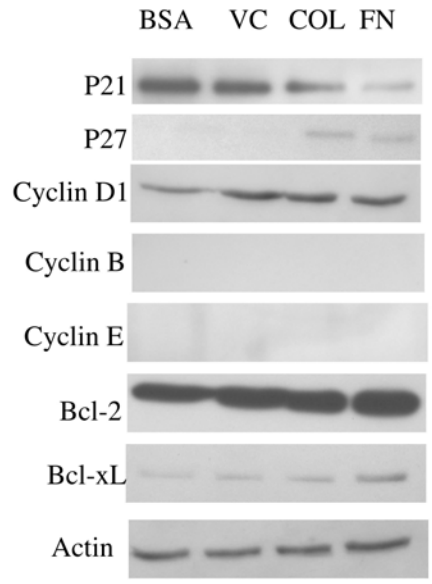

Figure 4. Effect of FN on cell-cycle-related protein, apoptosis-related protein. NB4 cells cultured with 1 mcM ATO for 24 hours before evaluation. Tissue culture plates were precoated with BSA, VCAM-1, type I collagen and fibronectin.

5. Effects of anti-VLA-4 and VLA-5 on the AML cells. Since both VLA-4 and VLA-5 on AML cells can interact with FN, we then tried to identify which ligand is responsible the increased proliferation and reduced apoptosis. Cells were cotreated with either 1 mcrogram/mL VLA-4 or VLA-5 blocking antibodies for 24 hours before MTT, cell-cycle and

apoptosis assays. We found that the effects of anti-VLA-4 were more prominent, indicating that activation through VLA-4 is responsible for the observed effects.

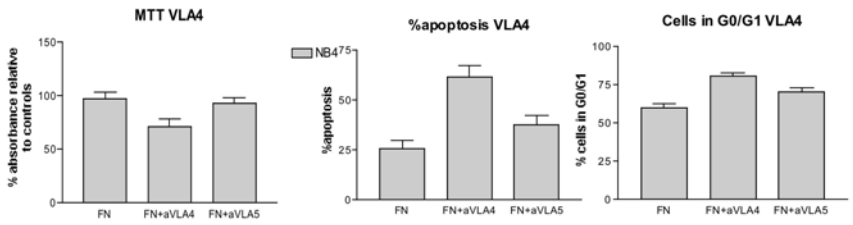

Figure 5. Effects of anti-VLA-4 and VLA-5 on the AML cells. Cells grown on FN-coated plates were treated 1 mcM ATO for 24 hours at the same time with anti-VLA4 or VLA-5 antibodies. Survival, apoptosis and cell cycle status were assayed as previously.

6. **FN mediated AKT phosphorylation is responsible for the increased survival of AML cells.** To further characterized the molecular mechanisms underlying the FN-mediated activity. We measured the phosphorylation of AKT and MAPK pathway. FN coating increased the phosphorylation of AKT phosphorylation and total AKT were not affected (Figure 6A). At the same time, FN also increased the levels of MAPK, but JNK was not affected (data not shown). The activation of AKT can be blocked by AKT inhibitor LY294002 and LY294002-treated cells exhibited increased apoptosis (Figure 6B), indicating that AKT activation is the major pathway responsible for the ATO resistance. We also tested the role

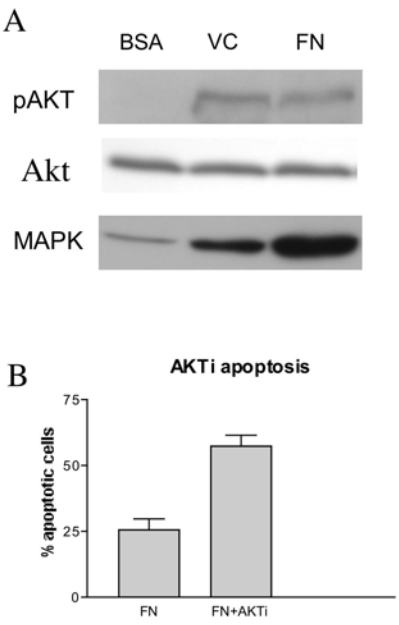

Figure 6. FN mediated phosphorylation. (A) NB4 cells treated with 1 mcM ATO for 24 hours were grown on BSA, VCAM-1, and FN. Phospho-AKT, total AKT and p38 MAPK were evaluated. (B) FN-mediated anti-apoptosis can be blocked with AKT inhibitor LY294002

of MAPK on ATO resistance using MAPK inhibitor SB202190, but the effect of MAPK inhibitor was less pronounced.

參考文獻

1. Wang X et al. XIAP is upregulated in HL-60 cells cocultured with stromal cells by direct cell contact. Leukemia Research Oct 2006 (ahead of print)
2. Hazelehurst LA et al. Cell adhesion to fibronectin influences acquired mitoxantrone resistance in U937 cells. Cancer Research 66:2338-45, 2006.
3. Karst AM et al. Role of p53 upregulated modulator of apoptosis and phosphorylated Akt in melanoma cell growth, apoptosis and patient survival. Cancer Research 66, 9221-26, 2006.
4. Yamamandra N et al. Tipifarnib and bortezomib are synergistic and overcome cell adhesion-mediated drug resistance in myeloma and acute myeloid leukemia. Clinical Cancer Research 12:59199, 2006.
5. Lee C et al. Increased cellular glutathione and protection by bone marrow stromal cells account for the resistance of non-acute promyelocytic leukemia acute myeloid leukemia cells to arsenic trioxide in vivo. Leukemia and Lymphoma 47:521-29, 2006.
6. Huang MJ. A small molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis and myeloid differentiation of human acute myeloid leukemia. Experimental Hematology, 34, 1480-89, 2006.

(三)計畫成果自評部份

We have identified the bone marrow stromal factor, i.e. fibronectin that plays an important role in the ATO resistance. Inhibition of the signaling pathways initiated by fibronectin will be of critical importance for future therapy of patients with acute promyelocytic leukemia and may also benefit patients with different types of leukemia especially for myeloma. For patient with acute promyelocytic leukemia, in the future, a combination of arsenic trioxide, all-trans retinoic acid, chemotherapy and AKT inhibitor might be able to eradicate the leukemic cells and prevent the development of drug resistance. For myeloma, which is characterized by cell-adhesion molecule-mediated drug resistance, our findings provide a new approach, since the use of ATO in myeloma is effective in vitro but ineffective in vivo. In the future the inclusion of AKT inhibitor should greatly benefit these patients. Part of this work has recently been published in 2006 March issue of Leukemia and Lymphoma (Reference 5).

We are currently pursuing using this finding to see if the inhibition of AKT will also improve the treatment of acute myeloid leukemia in vivo.