行政院國家科學委員會專題研究計畫成果報告

Glutathione 及去氧核糖核酸甲機化對癌細胞氧化砷抗藥性的影響

Effect of Glutathione and DNA Methylation on Arsenic Resistance in Cancer Cells

計畫編號：NSC 89-2314-B002-113
執行期限：88 年 8 月 1 日至 89 年 7 月 31 日
主持人：陳耀昌教授　楊志新醫師
執行機構及單位名稱:台大醫院檢驗醫學部,台大醫院腫瘤醫學部

一、中文摘要

砷元素廣泛的散佈在自然界中。長期的暴露在過量的砷會增加得到皮膚癌、肺癌、膀胱癌等癌症的機會。但自古以來，砷就被西方醫學及傳統中醫用來治療多種疾病，最近發現，氧化砷能治療對化學治療、全反式維生素甲酸完全沒有效用的急性前骨髓型白血病病人。砷也被應用在其他癌症的臨床試驗中。

細胞排除砷的能力似乎和 glutathione 系統有關，此外砷對細胞的毒性似乎和去氧核糖核酸甲機化的程度有關。我們初期研究顯示對氧化砷有抗藥性的癌細胞，glutathione 的量較高，使用抗藥逆轉劑 buthionine sulfoximine (BSO) 降低 glutathione 可增加氧化砷的敏感度。

本計畫的目的在探討癌細胞抗氧化砷和 glutathione，及去氧核糖核酸甲機化的關係。我們發現在一系列癌細胞中 glutathione 的量和氧化砷的毒性很有相關。對氧化砷敏感的 NTU-B1 細胞(低 glutathione)去氧核糖核酸甲機化的程度，比對氧化砷有抗藥性的 NTU-B1/P14(高 glutathione)為低。用 BSO 處理 NTU-B1/P14 細胞可增強氧化砷對癌細胞的毒殺能力，但去氧核糖核酸甲機化的程度並無改變。全細胞去氧核糖核酸甲機化的程度應和氧化砷有抗藥性無關，也許只有和特定基因的去氧核糖核酸甲機化有關。細胞內 glutathione 的改變也無法改變全細胞去氧核糖核酸甲機化的程度。

關鍵字: 氧化砷，癌症治療，抗藥性

Abstract

Arsenic is a ubiquitous element that presents in environment. Chronic exposure of arsenic compound is associated with increased risk of skin cancer, lung cancer, bladder cancer, etc. Arsenic is an ancient medication and was used widely in both western and Chinese medicine. It has been used to treat acute promyelocytic leukemia in the past few years. Arsenic has been used in clinical trials involving other cancer types.

Cellular glutathione system and DNA hypomethylation has been linked to arsenic toxicity to normal cells. Our preliminary study on a panel of cancer cell lines suggested that high cellular glutathione was associated with arsenic resistance in cancer cells. The objective of this project is to correlate cellular glutathione level, DNA methylation level with arsenic resistance in cancer cells. In a panel of cell lines, cytotoxicity to arsenic correlate well to glutathione content in cancer cells. NTU-B1 cells that contain low level of glutathione has lower level of global DNA methylation than high glutathione containing arsenic resistant NTU-B1/P14 cells. However, there was no further DNA hypomethylation when BSO were added to either NTU-B1 or NTU-

B1/P14 cells. There is no correlation between arsenic sensitivity to global DNA methylation. Arsenic sensitivity may be related to only some specific gene methylation. Depletion of glutathione by BSO was not able to change global DNA methylation in cancer cells.

二、緣由與目的

Arsenic

Arsenic is widely distributed in water, mineral, soils, air, plants and animals. Chronic exposure to arsenic was also associated with increase risk of cancer in skin, lung, liver, kidney, urinary bladder and hematopoietic system[1].

Arsenic compounds have been used widely for a long time in both western and Chinese medicine. Fowler's Solution is made of arsenic trioxide dissolved in potassium hydroxide, hydrochloric acid and chloroform water Arsenic trioxide (As_2O_3) is known for a long time as ingredient of a poison called **砒霜**. Local application of arsenic was used in the past in the treatment of cancer of skin, oral cavity, cervix etc. Doctors have been using arsenic in the treatment of chronic myelogenous leukemia, lymphoma, stomach cancer and esophageal cancer in Mainland China. Arsenic trioxide has revived recently with the revelation that arsenic trioxide infusion is one of the most effective treatment for acute promyelocytic leukemia (APL).[2]

Arsenic trioxide is cytotoxic to several cancer cell lines

We have tested arsenic trioxide in several cancer cell lines. Cancer cells from various origins were killed at concentrations that can be reached in the plasma of patients who have been treated with arsenic trioxide (peak level 4-6μM). Of note, NTU-B1, BFTC905 cells, both bladder cancer cell lines, are extremely sensitive to arsenic trioxide. Our results suggest that arsenic trioxide may be useful in the treatment of patients with solid cancer.[3] There are currently several clinical trials worldwide to explore the role of arsenic in cancers other than APL.

Arsenic detoxification system in cells

Cells contain several detoxification systems in response to environmental hazardous toxins. These include phase I and phase II enzymes that metabolize toxins, heat shock proteins, ATP-cassette binding proteins (mdr-1) and a variety of other stress-responsive proteins. Glutathione and/ or glutathione-s-transferase pi (GST-π) overexpression were linked to arsenic resistance in Chinese hamster ovarian cells[4]. Recently, MRP (multidrug-associated related protein) was found to carry many characteristics of a putative GS-X pump that help cells to expel glutathione conjugated toxins out of the cells[5]. A combination of overexpression of GST-π and MRP or high cellular reduced glutathione content therefore, may confer high resistance to arsenic in cancer cells[6]. Our prior data showed that glutathione may be the most important factor correlated to arsenic resistance. [3]

Arsenic exposure and DNA hypomethylation in cells.

Arsenic-induced malignant transformation was associated with DNA hypomethylation and aberrant gene expression.[7] Thus, it is possible that DNA methylation levels are correlated with arsenic toxicity in cancer cells. Glutathoine depletion may resulted in global hypomethylation in cells because glutathione turnover at the expense of methione, which is an important source of DNA methylation.[8] Since depleted glutathione in cancer cells were associated with arsenic sensitivity, we plan to investigate whether global DNA hypomethylation is present in arsenic sensitive cancer cells.

Specific aim

I. Correlation of reduced glutathione level and arsenic cytotoxicity in cancer cells.

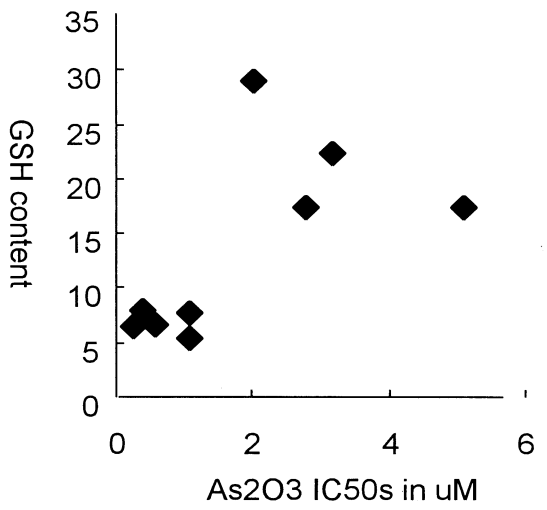
II. Correlation of global DNA methylation with arsenic cytotoxicity (and glutathione contents) in NTU-B1 and NTU-B1/P14 cells.

III. Study the effect of glutathione depletion by BSO on arsenic cytotoxicity and global DNA methylation in NTU-B1 and NTU-B1/P14 cells.

三、結果與討論

1. IC50s vs. glutathione content in cancer cells.

Glutathione contents were measured by colorimetric assay. IC50s were measured by SRB method. Spearman's rho correlative coefficient was 0.661 (P=0.026, one-tail). Five cell lines that were intrinsically sensitive to arsenic (IC50s<1.5μM) all contained a low level of GSH (GSH<10μg mg^{-1} protein), whereas four cell lines that were intrinsically resistant to arsenic (IC50s >1.5μM) all contained a high level of GSH (GSH>10μg m^{-1}



2. BSO deplete glutathione in NTUB1 and NTUB1/P14 cells

BSO is known to deplete cellular GSH via inhibition of gamma-glutamylcysteine synthetase which is required for GSH biosynthesis. NTU-B1, NTU-B1/P14, were incubated with various concentrations of As_2O_3 and 10 , 30 and 50μM of BSO for 4 days. (IC_{10}s of BSO in NTU-B1, and NTU-B1/P14 were 37μM, >50μM, respectively). IC_{50}s of As_2O_3 and GSH contents in BSO-treated GSH depleted cells (drug-sensitive and –resistant NTU-B1) are shown below. Cells became very sensitive to arsenic (IC_{50}s 0.1μM to 0.4μM) when glutathione was depleted by BSO. IC50s were not performed in 50 uM BSO since in that concentrations, half of the cells were dead.

Cell lines	As_2O_3*	GSH content#
NTU-B1	0.47±0.08	8.3±2.2
NTU-B1/P14	2.59±0.41	50.9±15.4
Coincubation 10uM BSO		
NTU-B1	0.19±0.04	2.27±1.11
NTU-B1/P14	0.14±0.01	14.20±2.2
Coincubation 30uM BSO		
NTU-B1	0.088±0.008	NA
NTU-B1/P14	0.095±0.009	NA
Coincubation 50uM BSO		
NTU-B1	NA	1±0.75
NTU-B1/P14	NA	8.7±0.61

3. DNA Global methylation in BSO treated NTU-B1 and NTU-B1/P14 cells.

Since BSO treated arsenic sensitive and resistant NTU-B1 cells serve as a good model for correlation of arsenic cytotoxicity to GSH content of the cells, we measure DNA global methylation in BSO treated NTU-B1 and NTU-B1/P14 cells. If the hypothesis by Lertratanangkoon[8] was correct, depletion of glutathione in cells will result in global hypomethylation in cells because glutathione turnover at the expense of methionine, which is an important source of DNA methylation, then, we should observe hypomethylation in BSO treated NTU-B1 and NTU-B1/P14 cells. DNA of cancer cells were isolated and treated with HpaII or MspI restriction enzyme. The resulting DNA fragments are end-labelled with P32 dCTP. After incubation at 72°C for 1-2 hours,. labeled reaction products are transferred to wet treated paper disc. After washing, disc is air-dried and counted in scintillation counter.

%methylation=

1-(HpaII-Uncut)/(MspI-Uncut)

Results are shown in table below.

NTUB1	BSO 0	BSO 10uM	BSO 50uM
Exp 1	37%	43%	55%

Exp 2	27%	50%	34%
Exp 3	45%	48%	36%
Exp 4	48%	33%	43%
Ave	**39%**	**40%**	**42%**
SD	**9%**	**8%**	**9%**
NTUB1 /P14	BSO 0	BSO 10uM	BSO 50uM
Exp 1	53%	57%	54%
Exp 2	65%	55%	61%
Exp 3	48%	40%	45%
Exp 4	58%	62%	60%
Exp 5	55%	69%	49%
Exp 6	47%	48%	52%
Ave	**54%**	**55%**	**52%**
S.D.	**6%**	**10%**	**8%**

Global DNA methylation in NTU-B1 were lower than that in NTU-B1/P14 cells. There was marginal statistical differences in global DNA methylation between arsenic sensitive NTU-B1 cells and resistant NTU-B1/P14 cells. There were no differences in global DNA methylation when glutathione contents were depleted by BSO in both NTU-B1 and NTU-B1/P14 cells.

四、計劃成果自評

Our study suggest that GSH contents in cancer cells correlate well with arsenic cytotoxicity. DNA global methylation seems to be lower in arsenic sensitive NTU-B1 cells that also contain low level of glutathione. However, there was no further hypomethylation in cancer cells when glutathione was depleted by BSO in both NTU-B1 and NTU-B1/P14 cells. In our previous study, depletion of BSO were associated with increased sensitivity to arsenic. Therefore, DNA global methylation is not associated with changes in resistance to arsenic in cancer cells. Glutathione contents in cancer cells may not directly correlate to percentage of global DNA methylation. Other factors may be more important to affect methylation. Specific gene methylation may be involved in arsenic resistance (such as LINE methylation) rather than global methylation as tested in this study.

五、參考文獻

[1] Chiou HY, Hsueh YM, Liaw KF, Horng SF, Chiang MH, Pu YS, Lin JS, Huang CH, Chen CJ: Incidence of internal cancers and ingested inorganic arsenic: a seven-year follow-up study in Taiwan. Cancer Research 55:1296, 1995

[2] Soignet SL, Maslak P, Wang ZG et. al. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 1998;339:1341-8.

[3] Yang CH, Kuo ML, Chen JC and Chen YC. Arsenic trioxide sensitivity is associated with lowe level of glutathione in cancer cells. Br J Cancer 81(5),796-799,1999

[4] Lee TC, Ho IC. Differential cytotoxic effects of arsenic on human and animal cells. Environmental Health Perspectives. 1994;102(Suppl 3):101-5.

[5] Ishikawa T, Akimaru K, Kuo MT, Priebe W, Suzuki M. How does the MRP/GS-X pump export doxorubicin? [letter]. Journal of the National Cancer Institute. 1995;87(21):1639-40.

[6] Ishikawa T, Bao JJ, Yamane Y, Akimaru K, Frindrich K, Wright CD, Kuo MT. Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. Journal of Biological Chemistry. 1996;271(25):14981-8.

[7] 1. Zhao, C. Q., Young, M. R., Diwan, B. A., Coogan, T. P., and Waalkes, M. P. Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression, Proceedings of the National Academy of Sciences of the United States of America. *94:* 10907-12, 1997.

[8] 1. Lertratanangkoon, K., Orkiszewski, R. S., and Scimeca, J. M. Methyl-donor deficiency due to chemically induced glutathione depletion, Cancer Research. *56:* 995-1005, 1996.