行政院國家科學委員會專題研究計畫　成果報告

三氧化二砷激發氧化自由基之分子機轉

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-002-129-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：周文堅
共同主持人：鄭安理，田蕙芬
計畫參與人員：徐悅淳

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 6 月 15 日

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 ■期中進度報告

（計畫名稱）

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計畫類別：■ 個別型計畫　　□ 整合型計畫

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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

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執行單位：國立臺灣大學醫學院內科

中　華　民　國　94　年　5　月　31　日

中文摘要

在吞噬性白血球中，三氧化二砷可以刺激 NADPH 氧化酶進而激發 superoxide 之形成，這是免疫反應中很重要的一環．但是三氧化二砷刺激 NADPH 氧化酶的分子機轉卻不甚明瞭．在此研究中，我們證實三氧化二砷在各種白血病細胞中均有刺激 NADPH 氧化酶並激發 superoxide 之能力．而大量的 superoxide 似乎有增加 NADPH 氧化酶活化之現象．分化的吞噬性白血球有很強的 NADPH 氧化酶，但三氧化二砷並未促使細胞分化，因此，細胞分化不是三氧化二砷刺激 NADPH 氧化酶的原因．我們並製備了可以產生 superoxide 的 XCGD 細胞 (XCGD-gp91)，在細胞分化時，此細胞可以產生大量之 superoxide，而 XCGD 細胞仍無任何可偵測的 superoxide．我們相信這是進一步研究氧化自由基生物學的一個重要的工具．我們進一步運用 luciferase assay 來嘗試探討三氧化二砷刺激 NADPH 氧化酶的原因．我們把 $p47^{PHOX}$ (此乃 NADPH 氧化酶之一個成分) 之 promoter 接到 Renilla luciferase reporter 之上游，並在三氧化二砷給予前後來測試 luciferase 之活性．結果顯示雖然三氧化二砷可以增加 $p47^{PHOX}$ 之表達，但是並未明顯提升 luciferase 之活性．這個矛盾可以以下列三種可能性來解釋: (1) 雖然三氧化二砷可以增加 $p47^{PHOX}$ 之表達，但是 $p47^{PHOX}$ 之 transcription 並未增加，而是 $p47^{PHOX}$ 之半衰期被三氧化二砷所延長．我們將運用 actinomycin D 來阻斷新的 mRNA 之合成，並測量 $p47^{PHOX}$ 之半衰期，來驗證此假說． (2) 三氧化二砷增加 $p47^{PHOX}$ 之表達之幅度可能不夠高，使得 $p47^{PHOX}$ promoter-luciferase 之增加 (if any) 不夠明顯．為了證實這假設，我們將用藥物促使細胞分化，此時之 NADPH 氧化酶 (包括 $p47^{PHOX}$) 之表達已大幅提升，再運用這個系統去測試 $p47^{PHOX}$ promoter-luciferase 是否有上升． (3) $p47^{PHOX}$ promoter-luciferase 之 reporter assay 太過於人工化，無法運用於解答我們的問題． 我們相信下一年度是關鍵的一年．在過去的一年中，很多技術上的問題已經克服．只要再給予我們支持，我們相信很快會有令人振奮的結果．

關鍵詞: 三氧化二砷, NADPH 氧化酶, superoxide, $p47^{PHOX}$, luciferase

英文摘要

Arsenic trioxide (ATO) can induce superoxide production by up-regulation of NADPH oxidase, an enzyme complex in phagocytes for bactericidal effects via “respiratory burst”. However, the mechanisms of NADPH oxidase activation remain to be defined. In this report, we showed that arsenic trioxide can induce superoxide in various kinds of leukemia cells via activation of NADPH oxidase. Reciprocally, large amount of superoxide produced by cell differentiation can further augment the activation of NADPH oxidase. Thus, there seems to be a positive feedback loop of NADPH oxidase and superoxide production. Furthermore, we show that ATO did not induce leukemia cell differentiation, thus excluding the possibility of cellular differentiation as a mechanism of NADPH oxidase activation and superoxide formation. We also generate a cell line, XCGD-gp91, which replenishes NADPH oxidase function in XCGD cells. Upon differentiation, this cell line exhibits huge amount of superoxide production while the parental cells still do not have detectable superoxide. We believe this pair of cell lines can be an excellent tool for research of biological effects of superoxide. Finally, we encounter a problem in matching the induction

of *in vivo* NADPH oxidase and *in vitro* luciferase reporter activity. Several possibilities exist, but we have solutions to solve this problem. We have solved the technical problems in the past year, and we are ready to collect the data by use of these techniques. We believe there will soon be exciting data coming up by your further support next year.

Keywords: arsenic trioxide, NADPH oxidase, superoxide, $p47^{PHOX}$, luciferase

Introduction

Reactive oxygen species (ROS) remains an enigmatic topic in biology. ROS is important in tumor initiation, progression, and maintenance. But it also can lead to apoptosis when cancer cells are under stress (Gupta et al., 2003) (Jackson and Loeb, 2001). ROS can promote cancer cell death by activation of pro-apoptotic signaling such as ASK1, JNK and p38 (Benhar et al., 2001) (Davis, 2000) (Morita et al., 2001). Overall, ROS is very pleiotropic and the real physiology awaits further investigation.

We and others have shown that arsenic can induce ROS production (Jing et al., 1999) (Chou et al., 2005; Liu et al., 2003) (Chou and Dang, 2005). We further pointed out unambiguously that arsenic-induced ROS in myeloid leukemia cells is exclusively through activation of NADPH oxidase, which is an enzyme complex present in phagocytes, and is important for innate immunity (Chou et al., 2004).

It is plausible that arsenic might exert a significant proportion of its biological effects via ROS production. Based on this hypothesis, elucidation of the molecular mechanisms by which arsenic induces ROS is a critical step to understanding arsenic physiology. Our previous observation that arsenic-induced ROS came exclusively from NADPH oxidase helped us focus on the molecular mechanisms of arsenic activation on NADPH oxidase.

In this report, we have tried various methodologies to explore the molecular mechanisms of arsenic activation of NADPH oxidase.

Methods and Materials

1. Cell lines: PLB985 is a human acute myeloid leukemia cell line. XCGD cells are derived from PLB985, but the CYBB gene, a critical component of NADPH oxidase, has been knocked out by recombination. Since NADPH oxidase is responsible for production of superoxide from these cells, XCGD cells are not able to emit superoxide upon encountering lipopolysaccharide (LPS) or arsenic trioxide (Zhen et al., 1993). These cells are maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin in 37°C humidified incubator.
2. Treatment with arsenic trioxide: Cells in logarithmic phase of growth are seeded in 6-well plates with 10^5/ml for 5 ml/well, and treated with arsenic trioxide of different concentrations and durations before harvesting for subsequent experiments.
3. Detection of superoxide: Cells after treatment are collected and washed with 1x PBS, and then 50 μM lucigenin and 50 mM phrobol ester were added to 10^6 cells in 2 ml of aerated complete PBS (PBS with 0.5 mM $MgCl_2$/0.7 mM $CaCl_2$/0.1% glucose). The chemiluminescence was measured continuously in a Berthold Sirius luminometer (Pforzheim, Germany) single-tube luminometer at 37°C for 30 min.
4. Extraction of total RNA and preparation of cDNA: Cellular total RNA is extracted by kits purchased from Sigma. cDNA was generated by heating a mixture of 15 μl containing 15 μg total RNA and 1 μg random primers (Promega) to 70°C for 5 minutes. After immediate chilling on ice, 5 μl of 5x reaction buffer, 5 μl of dNTP (2.5 mM each), 40 units of RNase inhibitor, and 200 units of MMLV reverse transcriptase were added and the mixture was

incubated at 37°C for 1 hours.

5. Measurement of *p47^{PHOX}* expression by real time PCR: Taqman real time PCR is used to measure the mRNA of *p47^{PHOX}* with a ribosomal protein, *huPo*, as a loading control. The primers and probe sequences for *p47^{PHOX}* and huPo are:
   NCF1-taqman545F: 5'-TGGTGGAGGTCGTGGAGAA-3'
   NCF1-taqman612R:5'-CCAGCCTCGCTTTGCTTTC-3'
   PROBE:5'FAM-AGCGGTTGGTGGTTCTGTCAGATG-3'TAMRA. In a well of 20 μL of reaction mixture, 10 μL of universal PCR mix (Applied Biosystems), 10 ng of cDNA, 500 nM of each primer, 0.2 μM probe, 1 μL of 20x *hupo* mix (Applied Biosystems) and distilled water are run for 40 cycles in ABI 7700 sequence detection system (Applied Biosystems).
6. Generation of XCGD-gp91 cell line: gp91 coding sequence is amplified by PCR with primers containing *XhoI* site. The PCR product is then digested with *XhoI* and cloned into pMSCV-puro (Clonetec) which has been cut with *XhoI.* The vector is confirmed by sequencing. The vector is then transfected into retrovirus-producing cells, phoenix, and the viral supernatant is harvest to transducer XCGD cells. Stable cells are selected by puromycin 2 μg/ml.
7. Induction of cell differentiation by dimethylformamide (DMF): DMF of 0.5% final concentration for 6 days is used to induce cell differentiation.
8. Generation of promoter-containing luciferase vectors: Gateway technology (Invitrogen) is adopted. Briefly, we first generate a phRL DEST vector by inserting a attR fragment into phRL-TK vector (Promega). Each promoter is amplified with attB fragment-containing primers, followed by recombination with pDNTR vector (Invitrogen) containing attp fragment by BP clonase (Invitrogen) to get attL-containing entry vectors. Finally, the promoter-containing vectors are obtained by recombination with the entry vector and phRL DEST vector by LR clonase (Invitrogen).
9. Transfection by electroporation: Cells are transfected with either empty plasmids or promoter-containing reporter plasmids by electroporation. Three million cells growing in log phase were washed twice and re-suspended in 500 μl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4 and 6 mM glucose, pH=7.15) containing either 10 μg of experimental or control pGL3 plasmids. The mixture was then electroporated with 1500 μF and 200 volts using Gene Pulser II (Biorad, Hercules, California). After 48 hours, the cells were treated with 2 μM arsenic for another 48-72 hours. The cells were harvested for luciferase reporter determination.
10. Luciferase reporter determination: Renilla luciferase activities are determined using Berthold Sirius luminometer (Pforzheim, Germany) according to the instruction (Promega). phRL-TK and phRL-null are used as an internal control and negative control, respectively.

Results

1. Arsenic trioxide induces superoxide production in leukemia cells:

We have shown definitive evidence of arsenic-induced superoxide by activation of NADPH oxidase. To test if induction of NADPH oxidase is reciprocally related to

superoxide formation, we use PLB985 and its NADPH oxidase-deficient (gp91 knock-out) derivative XCGD cells as starting materials. We see baseline and arsenic-inducible superoxide in PLB985 cells but not in XCGD cells (Fig. 1a)

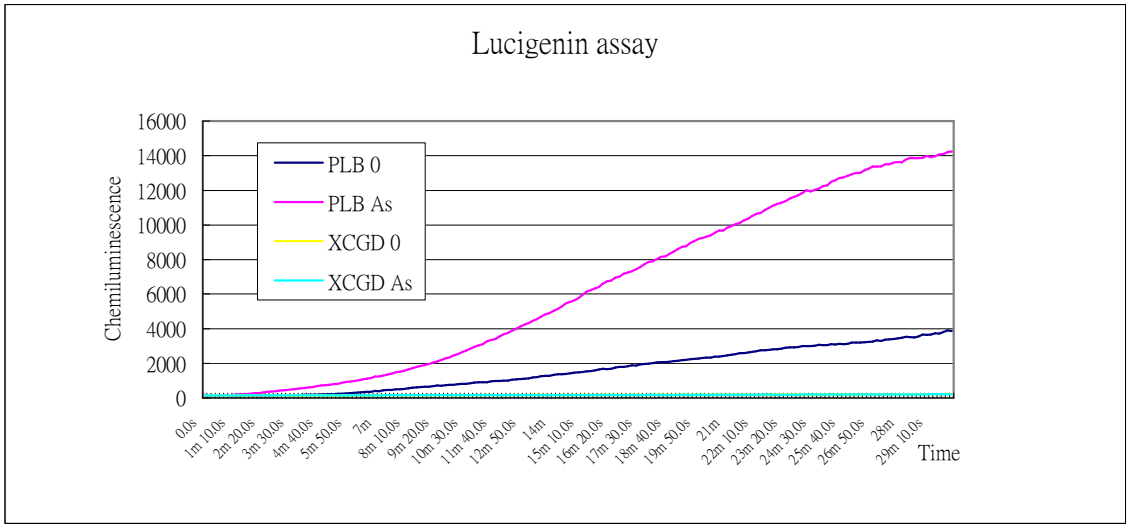


Fig. 1a

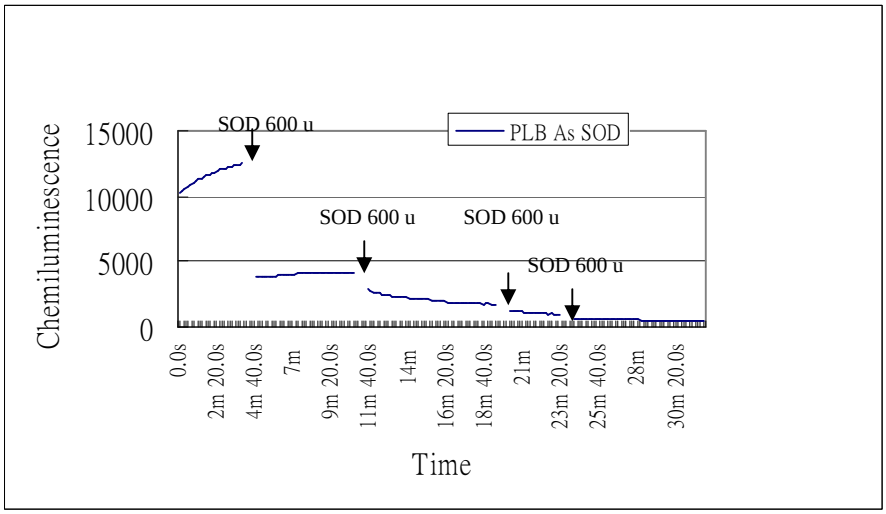


Fig. 1b

Legend: A: Arsenic induces ROS formation in PLB985 but not XCGD cells. The former exhibit both baseline and inducible ROS as measured by lucigenin chemiluminescence. B: The signal can be blunted by superoxide dismutase in a dose-dependent fashion, thus confirming the identity of ROS as superoxide.

The identity of the reactive oxygen species was confirmed to be superoxide because the chemiluminescence was nearly completely quenchable by superoxide dismutase (SOD) (Fig. 1b)

2. *$p47^{PHOX}$* expression can be further augmented by large amount of superoxide

Subsequently we test the *$p47^{PHOX}$* induction by arsenic in these two cell lines before and after

arsenic treatment. The *p47^{PHOX}* induction seems to be stronger in XCGD cells compared with PLB985. But this does not imply an inverse correlation of induction of *p47^{PHOX}* and superoxide production, since NB4 cells, which also can produce significant amount of superoxide (data not shown), have even higher induction of *p47^{PHOX}* (Fig. 2a-c). Based on this observation, it seems that the amount of superoxide production in cells does not influence the induction of *p47^{PHOX}*. To explore the possible hypothesis that **LARGE AMOUNT** of superoxide production might affect *p47^{PHOX}* expression, we generate an XCGD cell lines replenished with gp91 component of NADPH oxidase to restore the ability of superoxide generation upon differentiation. When these cells go through differentiation by DMF, the difference of superoxide formation is very dramatic (Fig. 3). We then test if induction of *p47^{PHOX}* expression is different in XCGD cells with empty vector (XCGD-puro) and in XCGD cells with gp91 vector (XCGD-gp91). We found *p47^{PHOX}* induction is very dramatic in DMF-induced differentiation about 40 and 80 fold higher after differentiation, in XCGD-puro and XCGD-gp91 cells, respectively. We conclude that *p47^{PHOX}* induction is not affected by arsenic-induced superoxide, which is relatively small. But there is positive effect of *p47^{PHOX}* induction by large amount of superoxide produced by cell differentiation (Fig. 4).

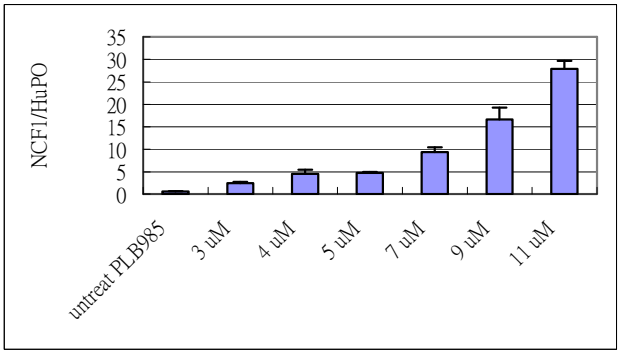


Fig. 2a

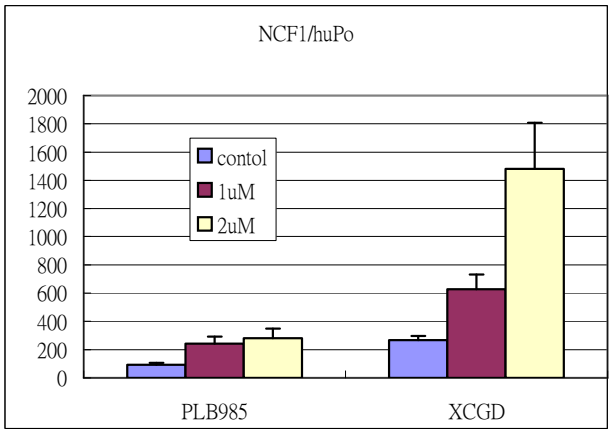


Fig. 2b

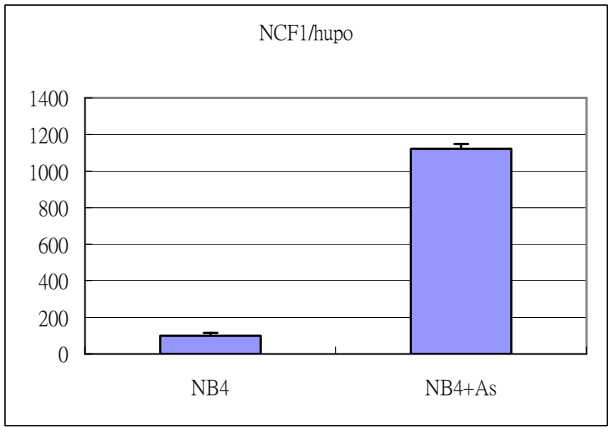


Fig. 2c

Legend: *p47^{PHOX} (NCF1)* induction by arsenic trioxide in various cell lines. A: a dose-responsive induction of *p47^{PHOX} (NCF1)* is observed in PLB985 cells. B: Both PLB985 and XCGD can express higher *p47^{PHOX} (NCF1)* upon arsenic treatment. C: NB4 cells exhibit higher induction of *p47^{PHOX} (NCF1)* compared with other cells.

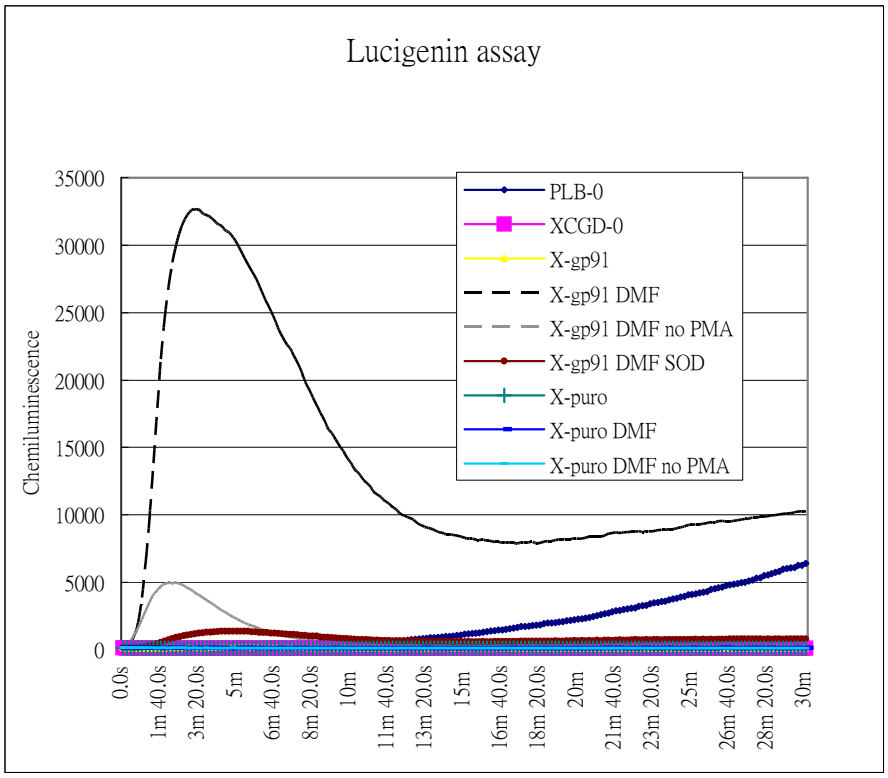


Fig. 3

Legend: When XCGD expressing gp91, the missing NADPH oxidase component, its ability to produce superoxide is restored upon cell differentiation by DMF. The signals are so strong that considerable amount of superoxide can be detected even without addition of phorbol ester (PMA). Again, the signal can be blunted upon addition of superoxide dismutase. On the other hand, XCGD with empty vector (XCGD-puro) did not exhibit any detectable superoxide.

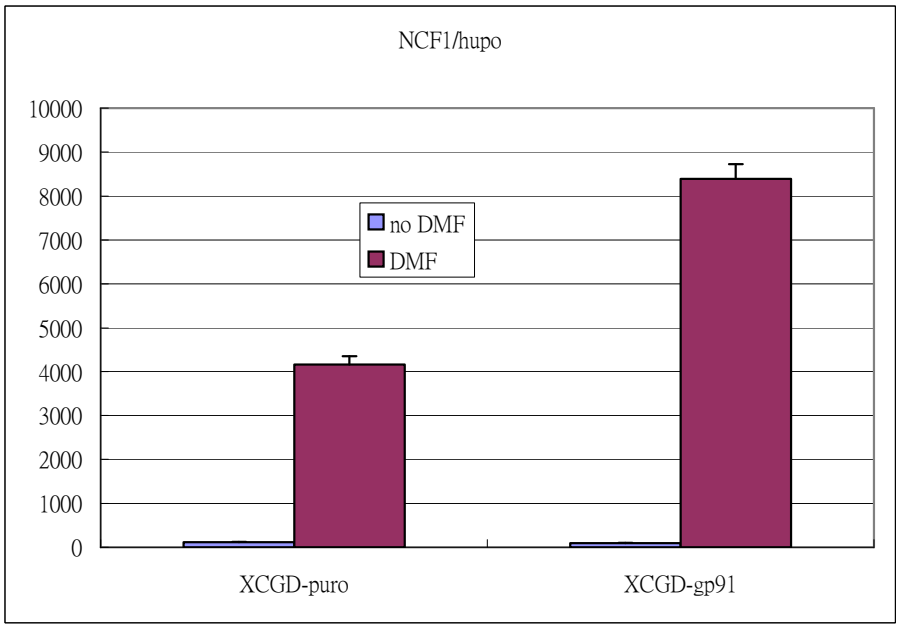


Fig. 4

Legend: Dramatic induction of $p47^{PHOX}$ (NCF1) after cell differentiation in both XCGD-puro and XCGD-gp91, regardless the ability to produce superoxide, although the latter exhibit one fold higher induction than the former (40 vs 90 fold induction)

3. Induction of superoxide by arsenic trioxide is independent of cellular differentiation:

Since leukocyte differentiation is accompanied by up-regulation of superoxide production and some studies have implied a role of differentiation induction by arsenic trioxide, it is very plausible that arsenic induces superoxide formation via induction of cell differentiation. To test this hypothesis, we compare the cell morphology and immunophenotype on the PLB985 and XCGD cells before and after arsenic treatment. We found that while dimethylformamide induces cell differentiation as revealed by morphology (Fig. 5a-d) and immunophenotyping (Fig. 6a-d), arsenic trioxide treatment does not induce differentiation at all.

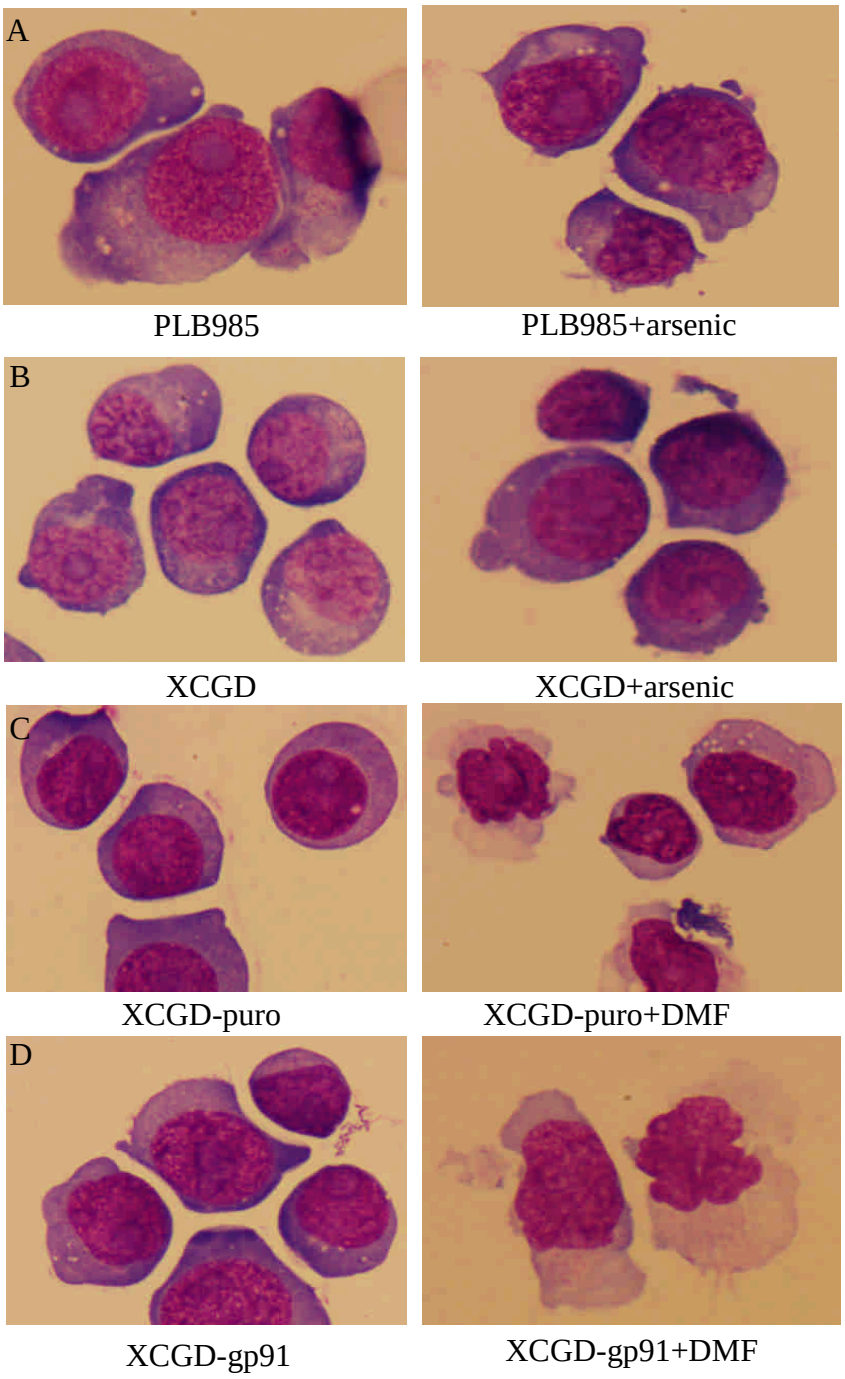
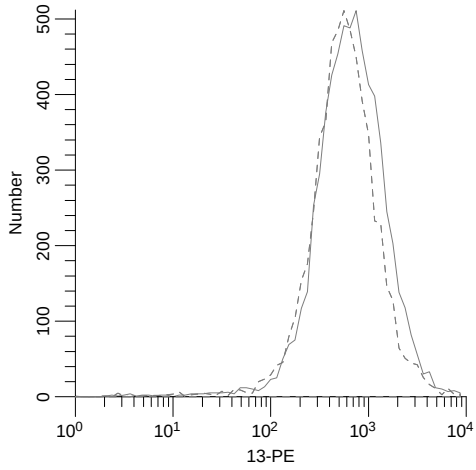


Fig. 5

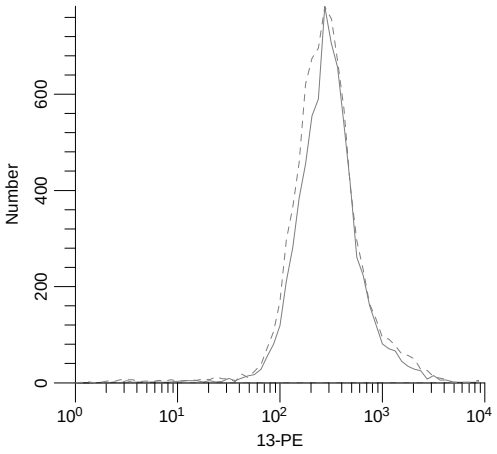
Legend: The differentiation effect of arsenic and DMF in leukemia cells. In A and B, the arsenic-treated cells reveal blue cytoplasm and round nuclei with nucleoli. The morphology is not different from that of untreated cells. In contrast to C and D, DMF induced differentiation as the cytoplasm becomes pink, and the nuclei become lobulated and disappearance of nucleoli.



A

XCGD with and without Arsenic 0.75 uM for 7 days

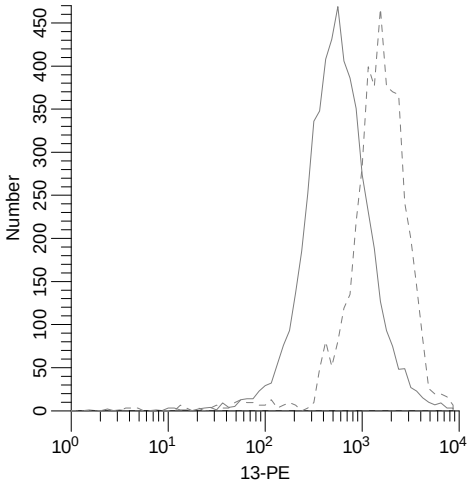
Solid line: without arsenic; dot line: with arsenic



B

PLB985 with and without Arsenic 0.75 uM for 7 days

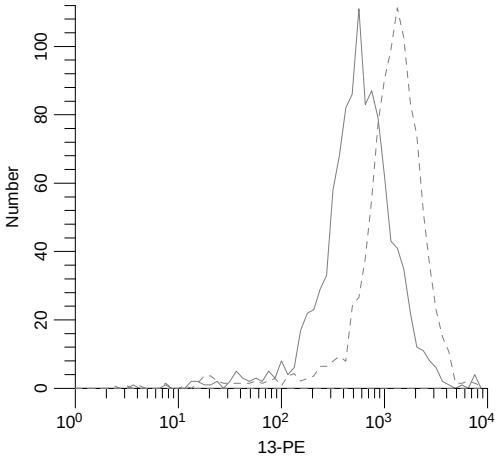
Solid line: without arsenic; dot line: with arsenic



C

XCGD-puro with and without 0.5% DMF for 6d

Solid line: without DMF; dot line: with DMF



D

PLB985 with and without 0.5% DMF for 6d

Solid line: without DMF; dot line: with DMF

Fig 6

Legend: The differentiation of leukemia cells as revealed by change of immunophenotyping. CD13 expression will elevated upon myeloid cell differentiation into mature myeloid cells.

4. Arsenic trioxide does not induce *p47^{PHOX}* promoter in luciferase assay:

Because XCGD cells have the highest efficiency of electroporation (data not shown), we choose this cell line as a material for luciferase assay. We construct a plasmid p47^{PHOX}-1200-Renilla reporter containing the 1200 bp promoter region upstream *p47^{PHOX}* transcription start site fused with Renilla open reading frame, and transfect the plasmid into XCGD cells by electroporation. After 24 hours, the cells are treated with arsenic trioxide 2 μM for another 48 hours. The cell lysate is collected for Renilla luciferase assay. As shown in Fig. 7, the luciferase activity is not induced after arsenic treatment.

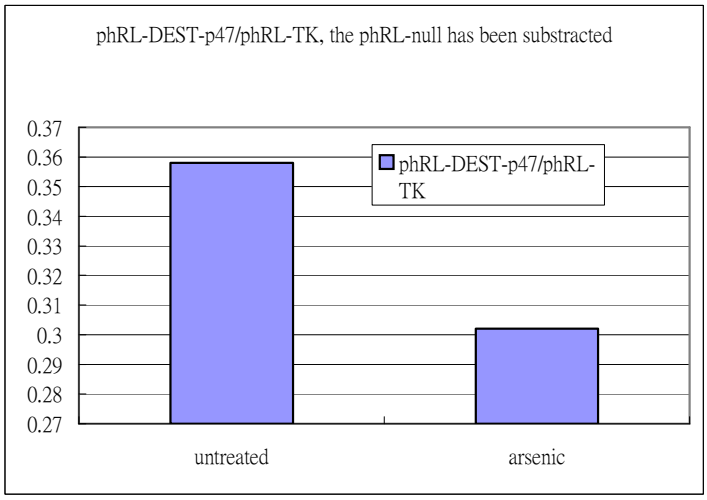


Fig. 7

Legend: The luciferase activity driven by *p47^{PHOX}* promoter is not increased after arsenic treatment. This does not match the induction of the endogenous *p47^{PHOX}* expression.

Although arsenic can induce endogenous *p47^{PHOX}* expression, but it can not induce luciferase activity driven by *p47^{PHOX}* promoter. Several possibilities exist for this discrepancy:
(1). The induction of *p47^{PHOX}* expression by arsenic exposure for 48 hours is not strong enough (about 6 fold, see Fig. 2b) for a clear increase of luciferase activity, if any. One way to test this possibility is to use NB4 cells as a material, since the *p47^{PHOX}* induction is more than 10 fold (see Fig. 2c). Another way to test this hypothesis to measure the luciferase activity after induction of differentiation by DMF, because the treatment augments *p47^{PHOX}* induction to as high as 80 fold (Fig. 4). We expect to see clear increase of luciferase activity in cells induced to differentiate by DMF.
(2). Another hypothesis is that arsenic increases *p47^{PHOX}* expression not by increasing the rate of transcription but by prolongation of its half life. To test this hypothesis, we plan to use actinomycin D, which can block production of nascent mRNA. Subsequently, the half life of *p47^{PHOX}* mRNA before and after arsenic treatment can be measured.
(3). Another possibility is that this artificial, *in vitro* luciferase assay is not able to represent the *in vivo p47^{PHOX}* mRNA induction.

Concluding remark:
In this preliminary report, we showed that arsenic trioxide can induce superoxide in various kinds of leukemia cells via activation of NADPH oxidase. Reciprocally, large amount of superoxide produced by cell differentiation can further augment the activation of NADPH oxidase. Thus, there seems to be a positive feedback loop of NADPH oxidase and superoxide production:

NADPH oxidase ⟶ Superoxide
⟵

Also, we showed that arsenic induction of superoxide and NADPH oxidase activation is not

through cell differentiation.

We also generate a cell line, XCGD-gp91, which replenishes NADPH oxidase function in XCGD cells. Upon differentiation, this cell line exhibits huge amount of superoxide production while the parental cells still do not have detectable superoxide. We believe this pair of cell lines can be an excellent tool for research of biological effects of superoxide.

Finally, we encounter a problem in matching the induction of in vivo NADPH oxidase and in vitro luciferase reporter activity. Several possibilities exist, but we have solutions to this problem.

我們相信下一年度是關鍵的一年. 在過去的一年中, 很多技術上的問題已經克服. 只要再給予我們支持, 我們相信很快會有令人振奮的結果

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