# The Effect of Auranofin on Thrombomodulin Expression in Acute Promyelocytic Leukemia Cell

Il-Ha Lee, Ph.D.[1,*], Jong Youl Jin, M.D.[2,*], Myung Shin Kim, M.D.[3] and In-Sook Kim, Ph.D.[1]

Departments of [1]Natural Sciences Chemistry Section, [2]Internal Medicine and [3]Clinical Pathology, College of Medicine, The Catholic University of Korea, Seoul, Korea

**Background:** Acute promyelocytic leukemia (APL) is distinguished from other forms of leukemia by its association with bleeding diatheses. All-*trans* retinoic acid (ATRA) and arsenic trioxide ($As_2O_3$), which have been effectively used in the treatment of the APL, promptly improve coagulation/bleeding syndromes by regulating the expressions of tissue factor (TF) and thrombomodulin (TM). We have previously shown a novel activity of auranofin (AF), in that it induced apoptosis and differentiation of NB4 cells. To study whether AF also possesses similar anticoagulant effects to those of ATRA and $As_2O_3$, its effects on the expressions of TM and TF were investigated.

**Methods:** NB4 cells derived from APL were incubated with 1 $\mu$M of AF. After incubation for 12, 24 and 48 hours, the AF-regulated expressions of TM and TF were analyzed by RT-PCR, Northern blot and Western blot. The assay for the TM antigen on the cell surface was performed using a flow cytometry.

**Results:** The expression of the TM gene was increased for upto 12 hours after the AF treatment, but no change was observed in the expression of the TF gene. Western blot analysis also demonstrated that AF increased the level of TM proteinin a time-dependent manner. FACS data showed the TM antigen on the cell surface to gradually increase for upto 48 hours in AF-treated cells.

**Conclusion:** The results of this study indicate that AF can have an antithrombotic function via the up-regulation of the expression of TM, which suggests it may partially contribute to the improvement of coagulopathies in APL. ***(Korean J Hematol 2005;40:135-141.)***

**Key Words:** Auranofin, Thrombomodulin, Tissue factor, Coagulopathy, Acute promyelocytic leukemia

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교신저자 : 김인숙, 서울시 서초구 반포동 505번지
㉾137-701, 가톨릭대학교 의과대학 화학과
Tel: 02-590-1269, Fax: 02-592-7068
E-mail: ikim@catholic.ac.kr

Correspondence to : In-Sook Kim, Ph.D.
Department of Natural Sciences, College of Medicine, The Catholic University of Korea
505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea
Tel: +82-2-590-1269, Fax: +82-2-592-7068
E-mail: ikim@catholic.ac.kr
*These authors contributed equally to this study.

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## INTRODUCTION

Acute promyelocytic leukemia (APL) presents the abnormal accumulation of promyelocytic cells caused by a failure in cell differentiation. The character of APL is a reciprocal chromosomal translocation [t(15; 17)], which leads to the fusion of the genes encoding promyelocytic leukemia (PML) and retinoic acid receptor alpha (RAR$\alpha$). The resultant PML-RAR$\alpha$ fusion protein plays an important role in the pathogenesis of APL.[1] The APL is also characterized by bleeding diatheses which usually become more serious during chemotherapy. Bleeding diatheses in APL are generally attributed to disseminated intravascular coagulation (DIC) and hyperfibrinolysis. In fact, APL patients have showed elevated levels in thrombin-antithrombin complex, prothrombin fragment 1+2, fibrinopeptide A and elastase, and decreased levels in protein C and coagulation inhibitors.[2,3]

Thrombomodulin (TM) is the specific cell surface receptor that forms a complex with thrombin. The thrombin-TM complex activates protein C that forms a complex with its cofactor protein S. The protein C-protein S complex proteolytically degrades factor Va and VIIIa, and thereby suppresses thrombin generation. In contrast, tissue factor (TF), an essential cofactor for the activation of the extrinsic coagulation pathway, combines with coagulation factor VII or VIIa and activates factor X which contributes to the thrombin generation.

Because the pharmacologic high dose of all-*trans* retinoic acid (ATRA) induces neutrophilic differentiation of the APL cells both *in vitro* and *in vivo*, the differentiation-inducing therapy with ATRA has been effectively used in APL patients. Recently, $As_2O_3$ has also been used as an effective therapeutic agent in APL patients including relapsed cases that are resistant to ATRA treatment.[4,5] It has been reported that the ATRA or $As_2O_3$ treatment in APL patients improve bleeding symptoms through significantly decreasing procoagulant activity and TF expression.[6,7]

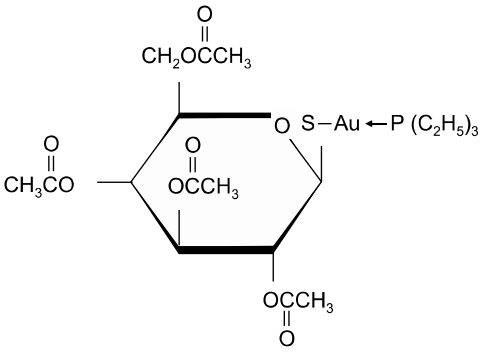


**Fig. 1.** The structure of AF.

Auranofin (AF; 2,3,4,6-tetra-*o*-acetyl-1-thio-$\beta$-D-glucopyranosato-*S*-(triethyl-phosphine) gold) is a thiol- reactive gold (I) compound (Fig. 1). It has been widely used in the treatment of rheumatoid arthritis. Recent studies have reported the anti-inflammatory mechanism of the AF, and demonstrated that it inhibits NF-$\kappa$B activation by blocking I$\kappa$B kinase activity and thereby reduces the gene expression of proinflammatory cytokines such as IL-1$\beta$ and TNF-$\alpha$.[8,9] Our previous study showed that the AF possesses the novel activity to induce apoptotic cell death and to synergistically enhance differentiation of APL cells in a combined treatment with ATRA.[10,11] These findings suggested that the AF had the potential as a new drug for APL therapy.

To study whether AF has an anticoagulant property, we investigated the effect of the AF on expressions of TM and TF in APL-derived NB4 cell line in this paper.

## MATERIALS AND METHODS

### 1. Cell culture

NB4 human acute promyelocytic leukemia cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). To study the regulation of TM and TF expression, the NB4 cells were plated at a density of $2\times10^5$/mL and

stimulated with 1 $\mu$M AF for various durations.

### 2. Total RNA preparation and RT-PCR

Total cellular RNA was extracted by using RNAzol B solution (Tel-test Inc., Friendswood, TX), according to the procedure recommended by the manufacturer. The RT-PCR was carried out as the same procedure described in the previous report.[12] In brief, 2 $\mu$M total RNA was reverse-transcribed for 30 minutes at 42°C in 1×PCR buffer containing 5mM $MgCl_2$, 1mM dNTPs, RNase inhibitor, random hexamer, and 0.1U/$\mu$L AMV reverse transcriptase in a total volume of 50 $\mu$L. A 2 $\mu$L aliquot of the RT product was amplified in a total volume of 50 $\mu$L. The amplification was performed 30 cycles under the following reaction conditions: 5mM $MgCl_2$, 1mM dNTPs, 10pmol TM or TF primers,[13] 0.1U/$\mu$L *Taq* DNA polymerase (denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute), and then a 10 $\mu$L aliquot of the PCR product was separated electrophoretically on a 1.5% agarose gel and stained with ethidium bromide.

### 3. Northern blot analysis

Prepared RNA samples (10 $\mu$g) were separated electrophoretically on a 1.2% agarose-formaldehyde gel, and transferred to a nylon membrane (Boehringer Mannheim, Mannheim, Germany). The hybridization was performed at 45°C in ExpressHyb hybridization solution (Clontech, palo Alto, CA) for 2 hours with the probe for TM into which DIG-11-dUTP nucleotides were incorporated. The transcripts were analyzed by using DIG chemiluminescent detection kit (Boehringer Mannheim, Germany).

### 4. Western blot analysis

The cells were washed twice with phosphate-buffered saline (PBS), and then lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM phenylmethylsulfonyl fluoride, 100 $\mu$M/mL trypsin inhibitor, 50 $\mu$M leupeptin, and 100 $\mu$M antipain, pH 8.0). After the protein concentration of each lysate was determined, equal amounts of the samples (50 $\mu$g) were separated on a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane in an XCell$^{TM}$ blot module (Novex, San Diego, CA). The proteins of interest were detected with rabbit polyclonal anti-TM antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

### 5. FACS analysis

The NB4 cells were incubated with or without 1 $\mu$M of AF for 12, 24 and 48 hours and washed twice with buffer A (PBS, 0.1% sodium azide, 1% heat-inactivated FBS). After being resuspended in buffer A, 50 $\mu$L of aliquot ($1\times10^6$ cells) was incubated with antibody against TM or isotype control rabbit IgG. Following incubation on ice for 30 minutes, the cells were washed three times and further incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG for 30 minutes on ice. The incubated cells were washed again, resuspended in 500 $\mu$L of buffer A containing propidium iodide, and then analyzed on a FACScan flow cytometer (BD Biosciences, San Diego, CA).

## RESULTS

### 1. The effect of AF on TM and TF gene expressions

To examine the regulation of procoagulant factors by AF, NB4 cells were incubated with 1 $\mu$M of AF for 12 and 24 hours, and the expressions of TM and TF were investigated. According to RT-PCR data, the level of TM mRNA was increased by AF within 12 hours, while TF mRNA level was not changed until 24 hours (Fig. 2A). Up-regulation of TM gene expression was also confirmed by Northern blot analysis in which AF enhanced a specific 3.7kb band correspondent to TM transcript (Fig. 2B).

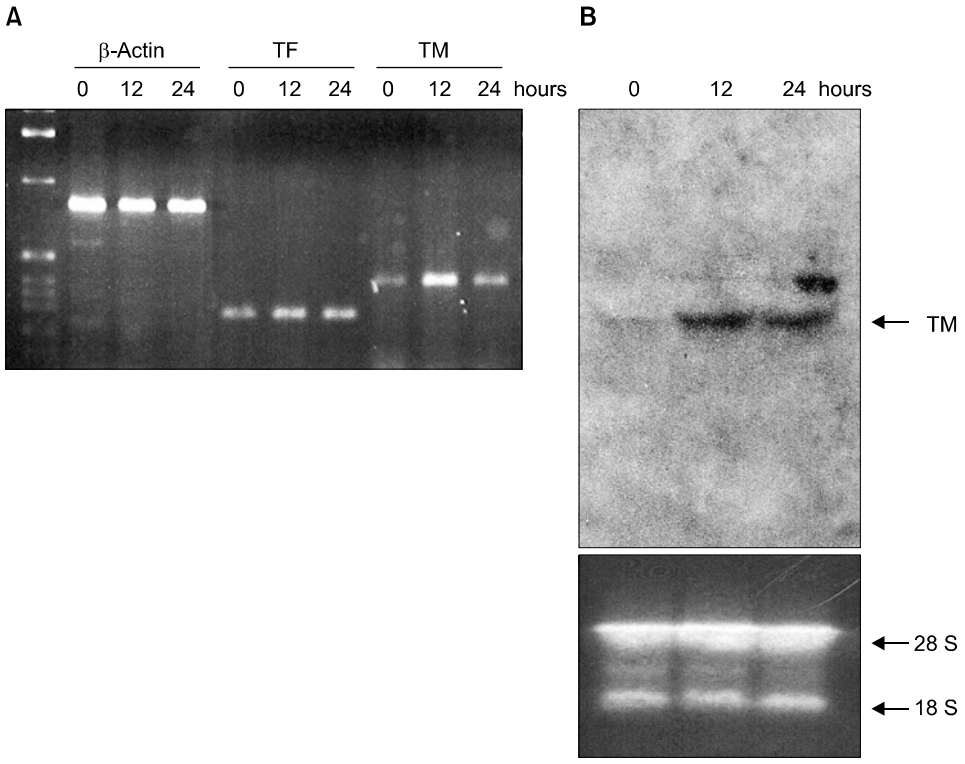


**Fig. 2.** The effect of AF on TM and TF gene expressions. NB4 cells were treated with 1 $\mu$M of AF for 12 and 24 hours, and then total cellular RNA was isolated using RNAzol B solution. (A) To analyze TM and TF gene expressions, RT-PCR was performed as described in materials and methods. Equal amounts of RNA used in each sample were evaluated by detecting $\beta$-actin expression. (B) Northern blot analysis. 20 $\mu$g of total RNA per lane was loaded and electrophoresed on a 1.2% agarose/formaldehyde gel. DIG-labeled PCR product of human TM gene was used as a probe. 28 S and 18 S ribosomal RNA shown in the EtBr-stained gel indicate the equal amount of loaded RNA samples. The results shown (A and B) are representative of at least two independent experiments.

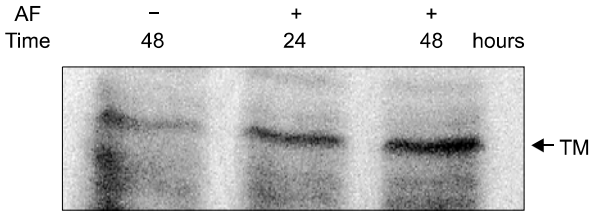


**Fig. 3.** The enhancement of TM antigen level by AF. After treatment with 1 $\mu$M of AF for the indicated time, 50 $\mu$g of cellular proteins were subjected to SDS-polyacrylamide gel electrophoresis and the TM protein was analyzed by Western blot by using anti-TM antibody. The blot shown is representative of three independent experiments.

### 2. The enhancement of TM antigen level by AF

To investigate whether the increased TM mRNA level was consistent with TM protein level, TM antigens were detected by Western blot analysis. When the cells were treated with AF, a 75kD band correspondent to TM protein was gradually increased until 48 hours (Fig. 3).

TM, high affinity thrombin receptor, is an integral membrane protein. Therefore, we further verified the increased TM antigen on cell surface by using a flow cytometry method. As shown in Fig. 4, the treatment of NB4 cells with 1 $\mu$M of AF enhanced cell surface TM antigen level in a time-dependent manner. These findings indicate that the AF can diminish a coagulant reaction.

## DISCUSSION

In this paper, we demonstrated that AF upre-

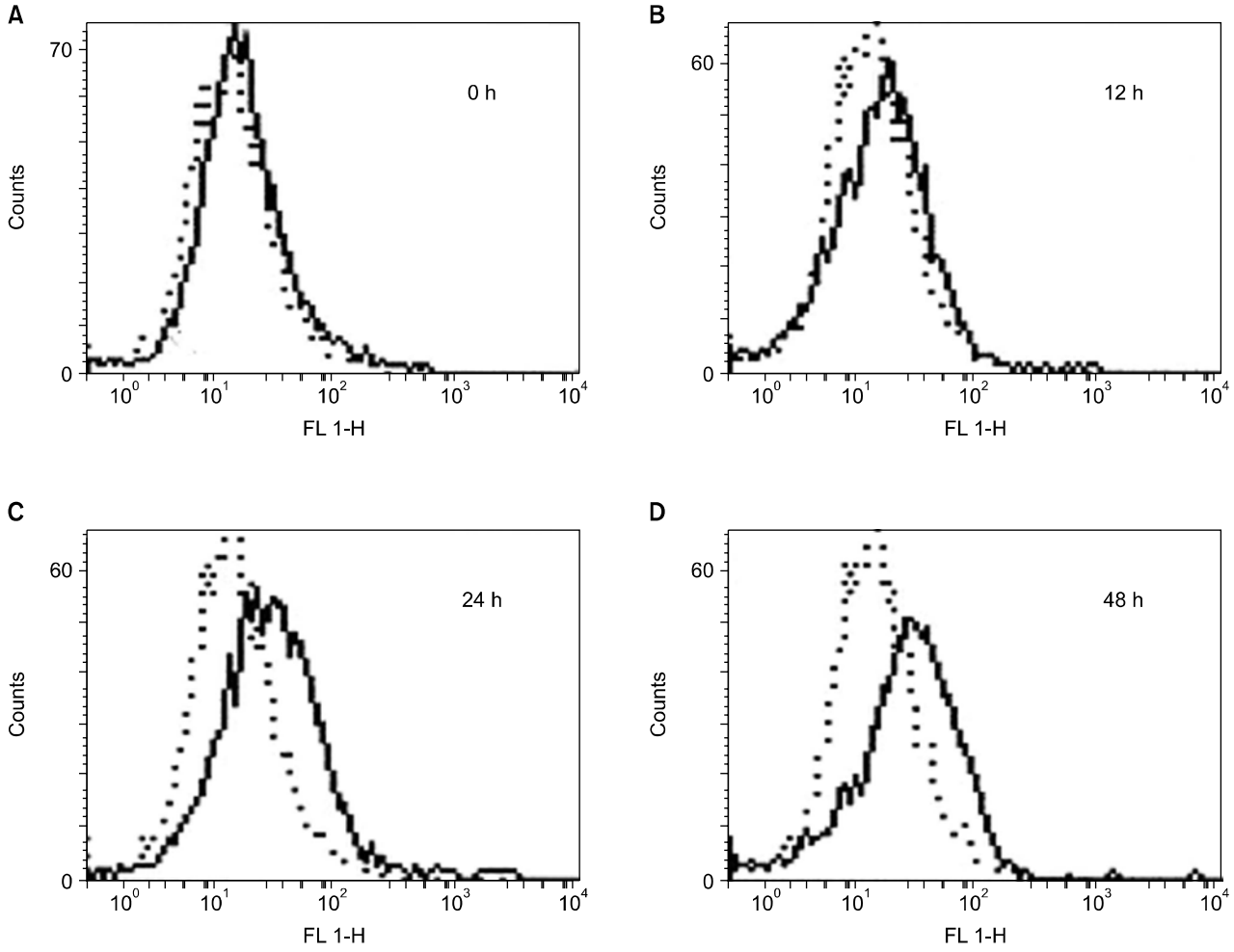


**Fig. 4.** The flow cytometric analysis of surface TM antigen. The NB4 cells were treated with 1 $\mu$M of AF for 0, 12, 24 and 48 hours. After that, the cells were sequentially incubated with anti-TM antibody and FITC-conjugated anti-rabbit IgG, and analyzed by a flow cytometer. Note that the population of cells with FITC-fluorescence was elevated in a time-dependent manner. Dashed line is for isotype control by using rabbit IgG instead of anti-TM antibody. The data shown represent a typical result obtained from two independent experiments.

gulated TM expression in NB4 cells. The induction of TM expression occurred within 12 hours and was accompanied by the increase of transcript level. We also found the enhancement of cell surface TM antigen, which forms a complex with thrombin. These results indicate that the AF exerts antithrombotic activity.

The AF has been widely used in the treatment of rheumatoid arthritis, which is based on its anti-inflammatory and immunosuppressive properties.[14] Because it reacts with thiol and/or selenol groups, the AF also potently inhibits mitochondrial thioredoxin reductase (TrxR), which is a selenocysteine-containing enzyme.[15] Recent reports have showed that the AF induces cytochrome c release into cytoplasm by inhibiting mitochondrial TrxR[16,17] and ultimately leads to apoptotic cell death.[18] In addition, we have found that the AF exhibits dual effects on apoptosis and differentiation of APL cells. This drug not only induced apoptotic cell death via ROS generation and p38 MAP kinase activations but also synergistically enhanced the differentiation of APL cells in a combined treatment with ATRA.[10,11]

On the other hand, $As_2O_3$ has been effectively treated for management of APL patients because

it has a proapoptotic activity and a partial differentiation-inducing activity.[4] The $As_2O_3$ also improves the coagulant problems in APL patients by regulating TM and TF expression.[7] Since both AF and $As_2O_3$ are thiol-reactive organometallic compounds and act in a similar manner on apoptosis and differentiation of APL cells, we thought that AF might have similar functions to that of $As_2O_3$ in blood coagulation, and investigated its effect on TM and TF expressions.

For the first time, a novel antithrombotic property of AF is identified in the present study, which suggests that the AF might improve thrombotic complications of APL patients. However, further studies showing the effects of AF on other procoagulant/anticoagulant factors will be required.


## 요 약

**배경**: 급성전골수성백혈병(acute promyelocytic leukemia, APL)에서는 혈액응고장애가 특징적으로 나타난다. 효과적인 APL 치료제로 사용되고 있는 all-*trans* retinoic acid (ATRA)와 arsenic trioxide ($As_2O_3$)는 혈액응고와 관련된 인자인 thrombomodulin (TM)과 tissue factor (TF)의 발현을 조절함으로써 혈액응고장애를 신속하게 개선시킨다고 알려져 있다. 최근에 저자들은 auranofin (AF)이 NB4 세포의 사멸과 분화유도에 관여함을 보고한 바 있으며 AF의 작용이 $As_2O_3$의 작용과 유사하였기 때문에 AF도 항응고기능이 있는지를 알아보고자 본 연구에서는 TM과 TF의 발현조절에 대한 AF의 효과를 조사하였다.

**방법**: APL 세포주인 NB4 세포에 AF을 1 $\mu$M 농도로 가하고 12, 24 및 48시간 동안 배양하였다. 배양 후, AF에 의해 조절되는 TM 및 TF 발현양상은 RT-PCR, Northern blot과 Western blot 방법으로 확인하였다. 또한 유동세포분석법을 사용하여 세포표면에 존재하는 TM의 변화도 동시에 측정하였다.

**결과**: TM mRNA 발현량은 AF 처치 후 12시간부터 증가하였으나 TF mRNA 발현량은 변화를 보이지 않았다. 또한 AF에 의해 TM 단백질량도 함께 증가함을 알 수 있었으며 세포표면에서도 TM이 48시간까지 지속적으로 증가되어 있음을 확인하였다.

**결론**: NB4 세포의 TM 발현을 상승조절하는 AF의 새로운 기능을 처음으로 규명하였다. 이러한 결과로 AF가 APL의 혈액응고장애를 개선시킬 수 있을 것으로 기대된다.


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