# Declined Asparagine Synthetase mRNA Expression and Enhanced Sensitivity to Asparaginase in HL-60 Cells Committed to Monocytic Differentiation

KEN HASHIMOTO, FUMIKA SUZUKI and HIROSHI SAKAGAMI

*Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences,
Meikai University School of Dentistry, Saitama, Japan*

**Abstract.** *During 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of human promyelocytic leukemia HL-60 cells toward maturing monocytes/macrophages, asparagine synthetase (ASNS) mRNA expression declined time and dose-dependently. The effect of TPA was inhibited by inhibitors for PKC and MEK 1/2, but not by those for JNK and p38 MAPK. Combination treatment with TPA and asparaginase synergistically enhanced the growth retardation accompanied by apoptotic cell death characterized by internucleosomal DNA fragmentation. These data suggest the possible involvement of MEK1/2 MAPK in the inhibitory effect of TPA on ASNS mRNA expression and that the induction of the down-regulation of ASNS (via MEK1/2 activation) may be a new strategy for the treatment of leukemia blast cells.*

Asparaginase catalyzes the hydrolysis of asparagine to aspartic acid. It is widely used for acute lymphoblastic leukemia therapy, since certain types of leukemia require an external supply of asparagine for their growth due to the diminished production of asparagine synthetase (ASNS). On the contrary, normal cells are relatively resistant to asparginase due to their higher capability to synthesize asparagines (1-3). Thus, asparaginase is effective against childhood acute lymphoblastic leukemia (ALL) during the induction of remission or the intensification phases of treatment (4-7). Asparagine in the blood, cerebrospinal fluid and bone marrow can be depleted by asparaginase. A reduction of asparagine may lead to cell death since it has been observed that exposure to asparaginase *in vitro* induced the fragmentation of DNA and morphological changes typical of apoptosis in a mouse lymphoma cell line (8). It has also been seen that asparaginase activated ASNS expression possibly by augmenting the asparagine insufficiency, and, furthermore, the overexpression of ASNS protein induced asparaginase-resistance in human T-cell leukemia (9). The correlation between the expression of ASNS and the resistance to asparaginase has been reported not only in leukemic but also in ovarian cancer cells (10). These findings suggest the importance of monitoring ASNS activity in clinical trials of asparaginase therapy (11).

It has recently been reported that asparagine production (12) and ASNS expression at protein (13) and mRNA (14) levels declined during the activation of mouse macrophage-like RAW264.7 cells induced by lipopolysaccharide (LPS) or lignin. However, it is not clear whether the enhanced production of asparagine is related to the differentiation process toward macrophages or the activation process of macrophages. To distinguish these possibilities, the changes in ASNS expression and the sensitivity against asparaginase during the differentiation of human promyelocytic leukemia cell line HL-60 toward maturing macrophage-like cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter (15, 16), were investigated. Since TPA is an activator of protein kinase C (PKC) (17), the effect of PKC inhibitor and other popular inhibitors for various kinases was also studied.

## Materials and Methods

*Materials.* The following chemicals and reagents were obtained from the indicated companies: RPMI-1640 medium, fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), asparaginase (Sigma-Aldrich Inc., St. Louis, MO, USA); dimethyl sulfoxide (DMSO), ISOGEN (RNA isolation reagent) (Wako Pure Chem. Ind., Osaka, Japan); GF-109203X (PKC inhibitor), U0126 (MEK 1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) (EMD Chemicals, Inc., Gibbstown, NJ, USA), Rever Tra Ace (reverse transcriptase), KOD *plus* (DNA polymerase) (Toyobo Co., Ltd, Japan).

*Correspondence to:* Ken Hashimoto, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 492792758/2759, Fax: +81 492855171 e-mail: kenhashi@dent.meikai.ac.jp/sakagami@dent.meikai.ac.jp

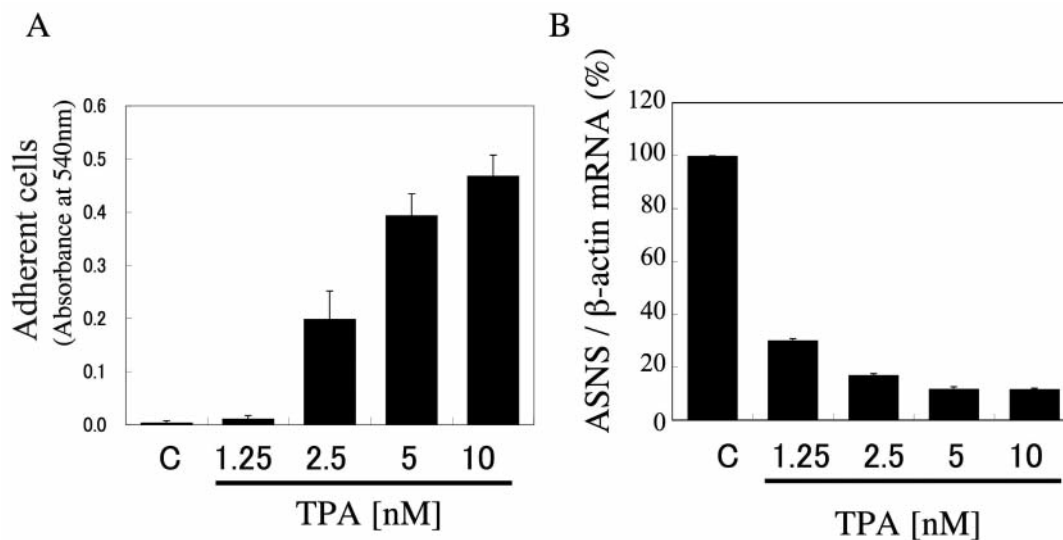*Key Words:* HL-60, asparagine synthetase, TPA, differentiation, asparaginase, apoptosis.

Figure 1. *Down-regulation of ASNS mRNA expression during TPA-induced differentiation of HL-60 cells. Cells were incubated for 24 hours with the indicated concentrations of TPA. A, The cells were washed twice with PBS(–), and the number of adherent cells was determined by the MTT assay. B, The cells were harvested, total RNA was isolated and RT-PCR analysis for ASNS mRNA was performed. C: Incubated without TPA. Each value represents the mean±S.D. from four independent experiments.*

*Cell culture.* Human promyelocytic leukemia cell line (HL-60) (Riken Cell Bank, Tsukuba, Japan) was cultured at 37°C in RPMI-1640 medium supplemented with 10% heat-inactivated FBS under a 5% $CO_2$ humidified atmosphere.

*Assay for cytotoxic activity.* Near confluent cells were incubated for 48 hours in RPMI-1640 medium supplemented with 10% FBS containing various concentrations of the asparaginase in 96-microwell plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The relative viable cell number was determined by MTT method, as described previously (18).

*Assay for differentiation induction.* The percentage of adherent macrophage-like cells were used as markers of monocytic differentiation as described previously (19, 20).

*Assay for mRNA expression.* Total RNA was isolated according to the ISOGEN protocol. HL-60 cells were lysed in 500 μL ISOGEN, and mixed with 100 μL chloroform. After centrifugation at 12,000 × g for 15 minutes, the supernatant was collected and mixed with 250 μL isopropanol. After centrifugation at 12,000 × g for 10 minutes, the pellet was washed in 500 μL 75% ethanol, air dried and dissolved in DEPC-treated $H_2O$. A reverse-transcriptase reaction (RT) was performed with 1.0 μg of total RNA, using the Rever Tra Ace and oligo $(dT)_{20}$ primer. Single-strand cDNA obtained by RT reaction was amplified, using the KOD *plus*, using ASNS specific primers (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3') and β-actin specific primers (5'-GAGGCCCAGAGCAAGAGAGG-3', 5'-TACATGGCTGGGGTGTTGAA-3'). The RT-PCR products were applied to 2% agarose gel electrophoresis. After staining with ethidium bromide, the DNA was visualized by UV irradiation and photographed by a charge coupled device (CCD) camera (Bio Doc-It, UVP, Inc., Upland, CA, USA).

*Assay for DNA fragmentation.* The cells were washed once with PBS (–) and lysed with 50 μL lysate buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution). The solution was incubated for 2 hours at 50°C with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K and then mixed with 50 μL NaI solution (7.6 M NaI, 20 mM EDTA-2Na, pH 8.0, 40 mM Tris-HCl, pH 8.0) and then 250 μL of ethanol. After centrifugation for 20 minutes at 20,000 × g, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-2Na, pH 8.0). Each sample (10 μL) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized as described above.

## Results

*Down-regulation of ASNS expression.* When HL-60 cells were incubated for 24 hours with increasing concentrations (1.25-10 nM) of TPA, the number of adherent cells with typical macrophage-like morphology dose-dependently increased (Figure 1A). This was accompanied by a corresponding decrease in the expression of *ASNS* mRNA with a maximum inhibition observed at 10 nM TPA (Figure 1B). The appearance of macrophage-like cells and the decline of *ASNS* mRNA expression were observed progressively with incubation time until 24 hours (Figure 2A, 2B).

*PKC inhibitor partially blocked the TPA-induced decrease of ASNS mRNA expression.* HL-60 cells were pretreated with 1-1,000 nM GF-109203X (protein kinase C inhibitor) for 2 hours and then treated with 2.5 nM TPA for an
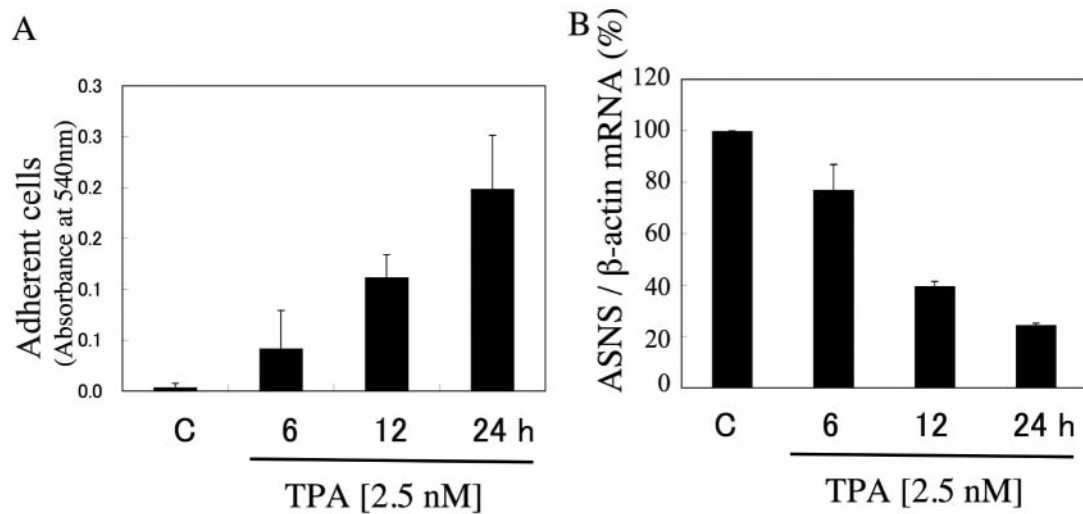
Figure 2. *Time-dependent decrease in ASNS mRNA expression during TPA-induced differentiation of HL-60 cells. HL-60 cells were exposed to TPA (2.5 nM) for 6-24 hours. A, The cells were washed twice with PBS(−) and the number of adherent cells was determined by the MTT assay. B, Cells were harvested, total RNA was isolated and RT-PCR analysis for ASNS mRNA was performed. C: Incubated without TPA. Each value represents the mean±S.D. from four independent experiments.*

additional 24 hours. The treated and untreated (control) cells were harvested and RT-PCR analysis was performed for *ASNS* mRNA (Figure 3). GF-109203X partially blocked the TPA-induced decrease of *ASNS* mRNA levels at high concentrations. This suggests that the decrease in *ASNS* mRNA expression is mediated at least in part by the activation of PKC.

*MEK inhibitor blocked the TPA-induced decrease of ASNS expression.* To further evaluate the signaling pathways triggered by TPA in the HL-60 cells, the effects of the inhibitors of MAPK family were examined. HL-60 cells were pretreated with 1-2 μM U0126 (MEK 1/2 inhibitor), SP600125 (JNK inhibitor) or 10-20 μM SB203580 (p38 MAPK inhibitor) for 1 hour and then treated with 2.5 nM TPA for an additional 24 hours. SP600125 and SB203580 did not affect the *ASNA* mRNA levels. In contrast, U-0126 blocked the inhibitory effect of TPA on *ASNS* mRNA levels (Figure 4). These results indicate the possible involvement of MEK1/2 MAPK in inhibition of the TPA-induced reduction of *ASNS* mRNA expression.

*TPA enhanced the cytotoxicity and the DNA fragmentation by asparaginase.* There was a possibility that the decline of *ASNS* mRNA expression by TPA may enhance the susceptibility of the cells to asparaginase. To test this possibility, HL-60 cells were treated with 0.13-1 units/mL of asparaginase for 48 hours in the presence or absence of TPA (2.5 nM) and the viable cell number was determined. TPA alone was essentially non-cytotoxic, but asparaginase was
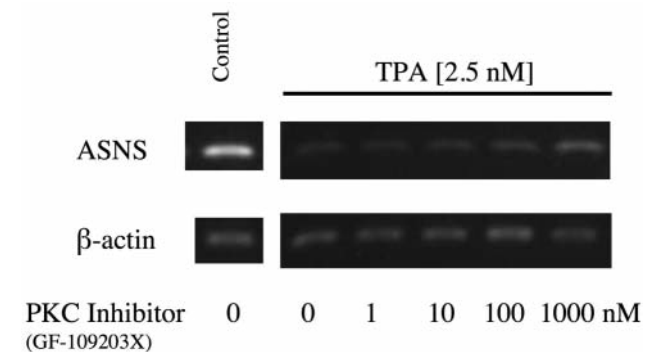


Figure 3. *Inhibition of TPA-induced decrease of ASNS expression by a PKC inhibitor. HL-60 cells were pretreated with 1-1,000 nM GF-109203X for 2 hours and then treated with 2.5 nM TPA for an additional 24 hours. The treated and untreated (control) cells were harvested and RT-PCR analysis was performed for ASNS mRNA.*

cytotoxic at higher concentrations (Figure 5). Combination of TPA and asparaginase induced internucleosomal DNA fragmentation to a greater extent than did TPA or asparaginase alone (Figure 6).

## Discussion

The present study demonstrated that ASNS mRNA expression consistently declined during TPA-induced differentiatin of HL-60 cells toward maturing macrophage-like cells. Furthermore, the combined treatment with TPA and asparaginase synergistically induced apoptotic cell
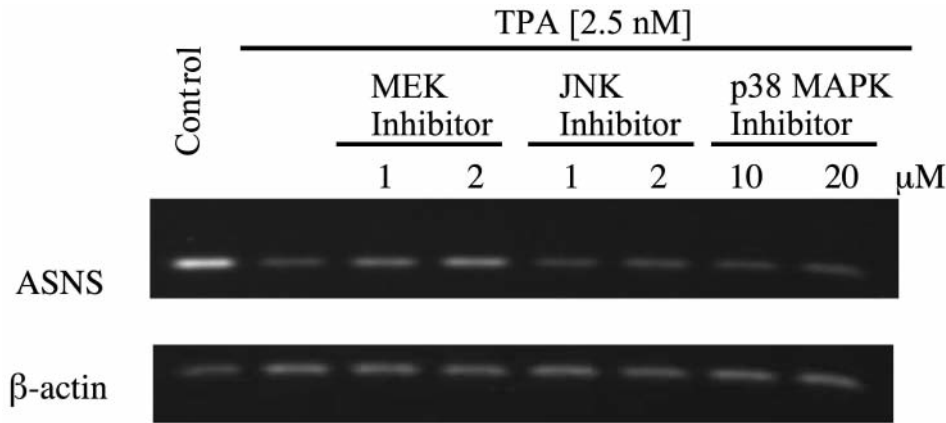
*Figure 4. Inhibition of TPA-induced decrease of ASNS expression by a MEK inhibitor. HL-60 cells were pretreated without (control) or with U0126 (MEK inhibitor) (1-2 μM), SP600125 (JNK inhibitor) (1-2 μM) or SB203580 (p38 MAPK inhibitor) (10-20 μM) for 1 hour and then treated with 2.5 nM TPA for an additional 24 hours. RT-PCR analysis was performed for ASNS mRNA.*

death characterized by growth retardation and internucleosomal DNA fragmentation. It has been previously reported that ASNS protein and mRNA expressions were elevated during the activation of RAW264.7 macrophages (13, 14). These data suggest that the declined ASNS expression in HL-60 cells is due to the induction of growth retardation or cell death, supporting the previously reported negative correlation between the expression of ASNS and the cell death linked to the enhanced susceptibility to asparaginase (10). It was found that RAW264.7 cells can grow in asparagine-free DMEM medium (12, 13), whereas HL-60 cells consume asparagine at about one third of the rate that of serine (21).

Irino *et al*. demonstrated that the *ASNS* mRNA expression level is an index of resistance to L-asparaginase in the cell lines (11). In HL-60 cells, treatment with TPA, which inhibits ASNS expression, and asparaginase, which degrades asparagine, enhanced the cytotoxicity in asparagine-requiring HL-60 cells. The absence of sufficient ASNS activity in leukemia blast cells is thought to be the basis of the antileukemic effect of asparaginase.

Since TPA is a well-known activator for PKC, the role of PKC activation in the inhibition of *ASNS* mRNA levels by TPA was investigated using PKC inhibitor, GF-109203X, which blocks a wide spectrum of PKCs, including conventional and novel PKC isoforms (22, 23). As shown in Figure 3, GF-109203X partially blocked the TPA inhibition of ASNS at high concentrations. U-0126, an inhibitor of MAPK kinase (MEK) 1/2, blocked the TPA inhibition of ASNS in a dose-dependent manner at concentrations of 1-2 μM (Figure 4). The present study suggests that TPA probably inhibits *ASNS* gene expression *via* PKC-MEK1/2 pathway, by an as yet not identified mechanism. Thus, the
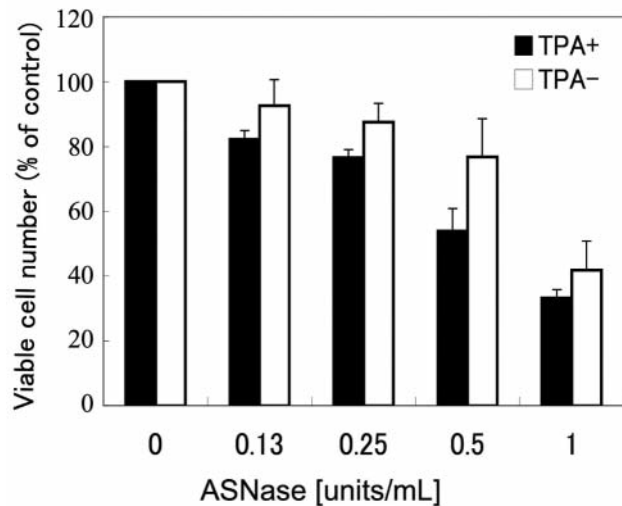


Figure 5. *Enhancement of cytotoxicity of ASNase by TPA. HL-60 cells were treated with the indicated concentrations of ASNase for 48 hours in the presence or absence of TPA (2.5 nM). Viable cell number was determined by modified MTT assay. Each value represents the mean±S.D. from three independent experiments.*

induction of the down-regulation of ASNS by MEK1/2 activation may be a new strategy for the treatment of leukemia blast cells.
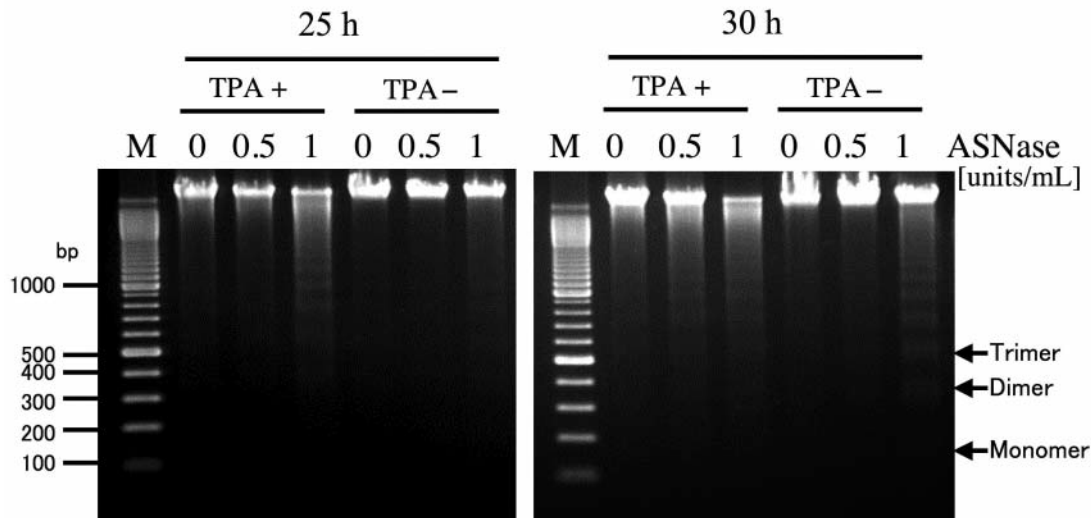
## Acknowledgements

Figure 6. *Enhancement of ASNase-induced DNA fragmentation by TPA. HL-60 cells were treated with 0, 0.5 or 1 units/mL ASNase for 25 or 30 hours in the presence or absence of TPA (2.5 nM). Genomic DNA was isolated from the cells and separated on an agarose gel. M: DNA size marker.*

## References

1 Kiriyama Y, Kubota M, Takimoto T, Kitoh T, Tanizawa A, Akiyama Y and Mikawa H: Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia *3*: 294-297, 1989.

2 Dubbers A, Wurthwein G, Muller HJ, Schulze-Westhoff P, Winkelhorst M, Kurzknabe E, Lanvers C, Pieters R, Kaspers GJ, Creutzig U, Ritter J and Boos J: Asparagine synthetase activity in paediatric acute leukaemias: AML-M5 subtype shows lowest activity. Br J Haematol *109*: 427-429, 2000.

3 Fu CH, Sakamoto KM; PEG-asparaginase. Expert Opin Pharmacother *8*: 1977-1984, 2007.

4 Keuting MJ, Holmens R, Lerner S and Ho DH: L-asparaginase and PEG asparaginase: past, present, and future. Leuk Lymphoma *10*: 153-157, 1993.

5 Koizumi S and Fujimoto T: Improvement in treatment of childhood acute lymphoblastic leukemia: a 10-year study by the Children's Cancer and Leukemia Study Group. Int J Hematol *59*: 99–112, 1994.

6 Hawkins DS, Park JR, Thomson BG, Felgenhauer JL, Holcenberg JS, Panosyan EH and Avramis VI: Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. Clin Cancer Res *15*: 5335-5341, 2004.

7 Avramis VI and Tiwari PN: Asparaginase (native ASNase or pegylated ASNase) in the treatment of acute lymphoblastic leukemia. Int J Nanomedicine *1*: 241-254, 2006.

8 Story MD, Voehringer DW, Stephens LC and Meyn RE: L-asparaginase kills lymphoma cells by apoptosis. Cancer Chemother Pharmacol *32*: 129-133, 1993.

9 Aslanian AM, Fletcher BS and Kilberg MS: Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J *357*: 321-328, 2001.

10 Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, Scudiero DA, Eisen MB, Sausville EA, Pommier Y, Botstein D, Brown PO and Weinstein JN: A gene expression database for the molecular pharmacology of cancer. Nat Genet *24*: 236-244, 2000.

11 Irino T, Kitoh T, Koami K, Kashima T, Mukai K, Takeuchi E, Hongo T, Nakahata T, Schuster SM and Osaka M: Establishment of real-time polymerase chain reaction method for quantitative analysis of asparagine synthetase expression. J Mol Diagn *6*: 217-224, 2004.

12 Suzuki F, Tashiro M, Hashimoto K, Yokote Y, Akahane K and Sakagami H: Stimulation of arginine consumption and asparagine production in LPS-activated macrophages. Anticancer Res *22*: 203-209, 2002.

13 Suzuki F, Okayasu H, Tashiro M, Hashimoto K, Yokote Y, Akahane K, Hongo S and Sakagami H: Effect of lignins and their precursors on nitric oxide, citrulline and asparagine production by mouse macrophage-like Raw264.7 cells. Anticancer Res *22*: 2719-2724, 2002.

14 Hashimoto K, Suzuki F, Tonosaki K and Sakagami H: Changing of asparagine synthetase according to activation of macrophage-like cells. J Oral Biosciences *46(Suppl)*: 181P, 2004 (in Japanese).

15 Wang X and Studzinski GP: Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells. J Cell Biochem *82*: 68-77, 2001.

16 Herblot S, Vekris A, Rouzaut A, Najeme F, de Miguel C, Bezian JH and Bonnet J: Selection of down-regulated sequences along the monocytic differentiation of leukemic HL60 cells. FEBS Lett *414*: 146-152, 1997.

17 Rovera G, Santoli D and Damsky C: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci *76*: 2779-2783, 1979.

18  Sekine T, Takahashi J, Nishishiro M, Arai A, Wakabayashi H, Kurihara T, Kobayashi M, Hashimoto K, Kikuchi H, Katayama T, Kanda Y, Kunii S, Motohashi N and Sakagami H: Tumor specificity and type of cell death induced by trihaloacetylazulenes in human tumor cell lines. Anticancer Res *27*: 133-144, 2007.

19  Sakagami H, Hromchak R and Bloch A: Prevention of phorbol ester receptor down modulation in human myeloblastic leukemia ML-1 cells by differentiation-stimulating serum components. Cancer Res *44*: 3330-3335, 1984.

20  Collins SJ: The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood *70*: 1233-1244, 1987.

21  Sakagami H, Yokote Y, Kochi M, Hara E and Akahane K: Amino acid utilization during apoptosis in HL-60 cells. Anticancer Res *19*: 329-332, 1999.

22  Jun CD, Pae HO, Kwak HJ, Yoo JC, Choi BM, Oh CD, Chun JS, Paik SG, Park YH and Chung HT: Modulation of nitric oxide-induced apoptotic death of HL-60 cells by protein kinase C and protein kinase A through mitogen-activated protein kinases and CPP32-like protease pathways. Cell Immunol *194*: 36-46, 1999.

23  Kazanietz MG: Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumor promoters. Mol Carcinog *28*: 5-11, 2000.