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# Induction of Differentiation of the Human Histocytic Lymphoma Cell Line U-937 by Hypericin

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Hypericin, a photosensitizing plant pigment, was found to be a potent inducer of differentiation of human myeloid leukemia U-937 cells. At a concentration of 0.2 μM, hypericin exhibited 50% growth inhibition. An effect on cell differentiation by hypericin was assessed by its ability to induce phagocytosis of latex particles, and to reduce nitroblue tetrazolium (NBT). Approximately 51% of 0.2 μM hypericin-treated cells were stained with NBT and 63% showed phagocytic activity. In order to establish whether hypericin induces differentiation of U-937 cells to macrophage or granulocyte, esterase activities and cell sizes were measured. When U-937 cells were treated with 0.2 μM and 0.15 μM of hypericin, the α-naphthyl acetate esterase activity was increased by 38.4% and 48.1%, respectively, but naphthol AS-D chloroacetate esterase activity was not influenced. The size of hypericin-treated cells in terms of cell mass was larger than that observed in untreated cells as determined by flow cytometry. Protein kinase C (PKC) inhibitor, NA-382, decreased the NBT reducing activity of hypericin, whereas a cAMP-dependent protein kinase A (PKA) inhibitor, H-89, did not show any influence on the differentiation. These results indicate that hypericin triggers differentiation toward monocyte/macrophage lineage by PKC stimulation.

**Key words** : Hypericin, Differentiation, NBT, Esterase, Flow cytometry

## INTRODUCTION

The development of a malignant phenotype may be viewed as a defect in the control of differentiation process in which the neoplastic cells exhibit a change in the tightly regulated homeostatic balance between proliferation and maturation that occur in normal cells. The cellular mechanisms which control these events are at the center of an understanding of the genesis of malignancy and may be important in the development of novel approaches to combating cancer.

Cell proliferation and differentiation can be accomplished through either cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC) activities. When cells were treated with phorbol ester which induced activation of PKC, the U-937 cells started proliferating and differentiating to a monocyte/macrophage-like cell (Way *et al.*, 1992). Many other studies also suggest that PKA is primarily involved in differentiation and proliferation processes (Cho-chung and Clair, 1993).

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Myeloid leukemic cell lines, which proliferate continuously *in vitro*, are useful models for the study of mechanisms for induction of differentiation in leukemia (Sandrostrom and Vilsson, 1976). Among the human hemopoetic cell lines, U-937 represents a histiocytic lymphoma line with monoblast-like characteristics. It can be induced to morphologically matured macrophage-like cells after treatment with such agents as TPA, cytokines, vitamin analogues, and anti-leukemia drugs. Dimethyl sulfoxide (Collins *et al.*, 1978), retinoic acid (Bretiman *et al.*, 1980), and other substances have been shown to induce HL-60 cells to differentiate along the myeloid pathway, whereas phorbol diesters (Roversa *et al.*, 1979) and $1\alpha,25(OH)_2$-$D_3$ (Miyara *et al.*, 1981) were able to induce HL-60 cells to differentiate toward monocyte/macrophage. IFN-γ (Harris *et al.*, 1985) induces differentiation of ML1 and U-937 cells and was shown to synergize with $1\alpha,25(OH)_2D^3$.

Differentiation therapy for leukemia, using differentiation-inducing compounds and combinations of various inducers, has been shown to be a new approach in the treatment of some human leukemia, since the therapeutic use of retinoic acid led to a complete remission of acute promyelocytic leukemia (Chomienne

*et al.*, 1996).

Hypericin (MW 504.5) is a photoactive polycyclic anthron isolated from *Hypericum triquetrifolium* Turra. This pigment has been found *in vitro* to oxidize lipids, amino acids, and proteins and to disrupt the normal function of cellular membranes. Hypericin exhibits antiviral (Meruelo *et al.*, 1988) and antineoplastic properties (Zhang *et al.*, 1996) in some cell types. These effects have been positively correlated with protein tyrosine kinase (PTK) activity (Agonist *et al.*, 1995; Kil *et al.*, 1996) and PKC activity (Takahashi and Nakanishi, 1989), suggesting that inhibition of PKC is involved in these processes. The present study was undertaken to address whether hypericin has differentiation inducing effects in U-939 cells with comparison to $1\alpha,25(OH)_2D_3$ and retinoic acid, well-known differentiation agents.

## MATERIALS AND METHODS

### Materials

U-937 human histocytic lymphoma cell lines were obtained from Korean Cell Line Bank. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco laboratories (Grand Island, NY), nitrobluetetrazolium (NBT), $1\alpha,25(OH)_2D_3$, all trans-retinoic acid, phorbol 12-myristate 13-acetate (TPA), hypericin and naphthyl AS-D chloroacetic esterase kit were purchased from Sigma Chemical Co. (St. Louis, MO). H-89 and NA-382 were kindly supplied from Dr. Miyamoto (Kanazawa Univ., Japan).

### Cell culture

U-937 cells were grown at 37°C in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml). The cells were grown in a humidified atmosphere of 95% air/5% $CO_2$ and the cells were seeded in plates after three passages.

### Determination of growth inhibition

Cells were seeded at a density of $2\sim3\times10^6$ cells/ml, maintained for logarithmic growth by passaging them every 2~3 days, and incubated for 2~4 days with hypericin at various concentrations. Hypericin dissolved in acetone added in serial dilution. The final acetone concentration in all assays did not exceed 0.1%. All experiments were performed after exposure to light under a standard fluorescent lamp for 20 min. The total light dose was 1.6 J/cm$^2$ as measured with a CC-20930070 radiation indicator (Kippnzomen, Holland). Cell viability was determined by the trypan blue exclusion method (Zhang *et al.*, 1992). The percentage of inhibition was calculated as; $100-\frac{I}{C}\times100$

### Differentiation assay

**NBT reduction test:** The percentage of U-937 cells capable of reducing NBT was determined by counting the number of cells which contained precipitated formazan particles after cells were incubated with NBT (1.0 mg/ml) at 37°C for 30 min. TPA was used as stimulator for the formation of formazan.

**Phagocytosis test:** U-937 cells ($1\times10^6$ cells/ml) were suspended in serum- free RPMI 1640 medium containing 0.2% latex particles (average diameter, 0.81 μM; Sigma Chemical Co.) and incubated at 37°C for 4 hr. After incubation, the cells were washed once with phosphate-buffered saline (PBS). The cells containing more than ten latex particles were scored as phagocytic cells.

**Esterase activity test:** A smear preparation was chemically stained for α-naphthyl acetate esterase and AS-D chloroacetate esterase by the standard techniques.

### Measurement of changes in cell size

U-937 cells were incubated with hypericin for 4 days. After incubation, $5\times10^5$ cells were washed two times with PBS and fixed in 70% cold ethanol at 4°C for 60 minutes. Before analysis, the cells were spun down and resuspended in PBS. The cells were analyzed using FACS Caliber Cellquest program (Becton Dickinson, Germany).

## RESULTS

Since hypericin has been shown to exhibit cytotoxic and PTK inhibitory activity (Agonist *et al.*, 1995; Kil *et al.*, 1996), we examined its antiproliferative and differentiation inducing activity in U-937 cells. Total growth inhibition of these malignant cells by hypericin could be mediated through suppression of cell viability or by induction of cell differentiation. To further study the effect of hypericin on cell differentiation, it is necessary to determine an optimal concentration of hypericin which inhibits cell growth without any significant effect on cell viability. Thus, U-937 cells were treated with various concentrations of hypericin for different incubation periods. The dose-dependent inhibition of cell growth is presented in Fig. 1. In the experiment to determine the effect of hypericin on cell growth and differentiation we chose the concentration of 0.2 μM of hypericin, a concentration that was cytostatic but not cytocidal to U-937 cells. At 0.2 μM concentration, inhibition of cell growth became apparent 3 days after exposure of cells to hypericin and reached around 50% after 4 days treatment (Fig. 1).

The partial growth inhibition of U-937 cells by hypericin was supposed to be associated with terminal differentiation. Differentiation was verified by the NBT test for the detection of granulocytes and monocytes

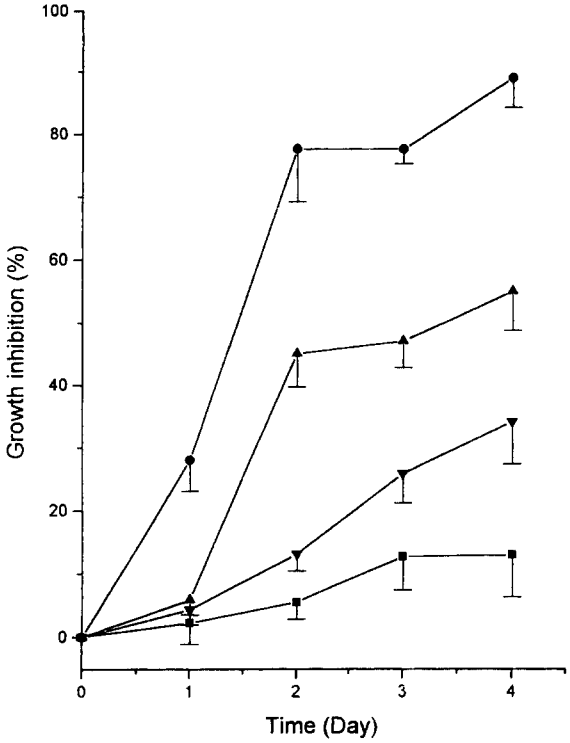


**Fig. 1.** The effect of hypericin on growth of U-937 cells. Cells were treated with increasing concentration of hypericin (0.15~0.25 μM) or were left untreated as control for 4 days. The bars represent the mean and SD of three independent experiments. ■: control, ●: 0.25 μM, ▲: 0.2 μM, ▼: 0.15 μM.

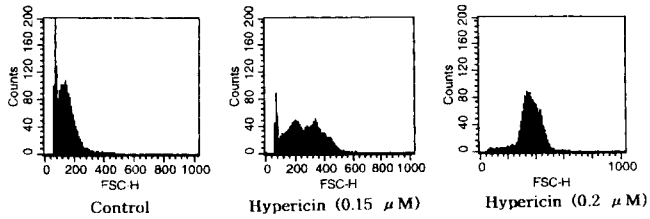


**Fig. 2.** Changes in size of U-937 cells treated with hypericin for 4 days.

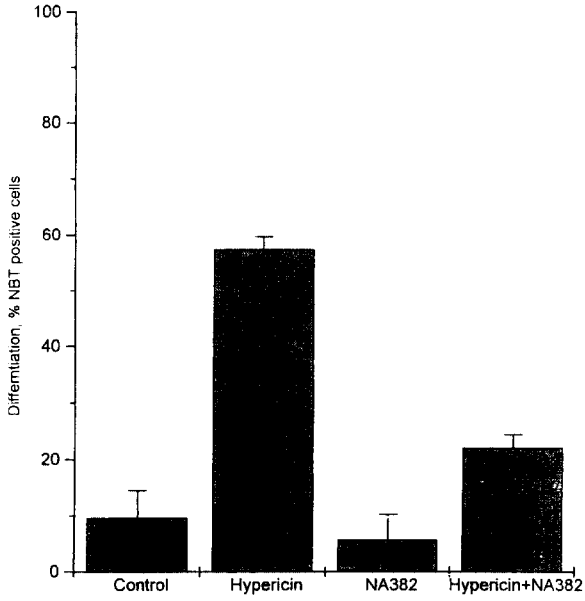


**Fig. 3.** Effect of NA-382 on the hypericin induced differentiation of U-937 cells. U-937 cells were cultured for 4 days in the presence of hypericin (0.2 μM) and/or NA-382 (10 nM). Each value represents the mean±S.D. of three separate experiments.

and also by measuring the phagocytic activity for the presence of macrophage cells. When U-937 cells were incubated with hypericin at concentrations of 0.15 and 0.2 μM for 4 days, approximately 48.5% and 51.2 % of U-937 cells became stainable with NBT, respectively, whereas only 5.8% of the untreated cells were positive (Table I). When U-937 cells were treated with retinoic acid and $1\alpha,25(OH)_2D_3$, 37.8% and 71.5% of the cells, respectively, reduced NBT.

In order to confirm whether hypericin induces U-937 cells to differentiate toward macrophage, esterase activities and phagocytotic activity were measured. When U-937 cells were treated with 0.2 and 0.15 μM of hypericin, α-naphthyl acetate esterase activity was increased to 38.4% and 48.1%, respectively, but naphthol AS-D chloroacetate esterase activity remained unchanged (Table I). The results from the two tests demonstrated that hypericin induced differentiation of U-937 cells to monocyte/macrophage-like cells. Moreover, 40.7% and 47.9% of $1\alpha,25(OH)_2D_3$, and retinoic acid treated U-937 cells were positive for α-naphthyl acetate esterase activity.

**Table I.** Induction of differentiation markers in U-937 cells after treatment with hypericin for 4 days

| Compound | Concentration (μM) | NBT reduction (%) | Naphthol AS-D chloroacetate esterase activity (%) | α-Naphthylacetate esterase activity (%) | Phagocytosis (%) |
|---|---|---|---|---|---|
| Hypericin | 0.15 | 48.5±2.4[a] | 8.4±0.8 | 38.4±1.8 | 30.8±1.7 |
| | 0.2 | 51.3±3.0 | 12.5±0.7 | 48.1±2.3 | 63±4.1 |
| | -[b] | 13.3±0.8 | 12.2±0.3 | 6.9±0.2 | 13.2±1.1 |
| Retinoic acid | 5 | 37.8±2.0 | 9.0±1.2 | 47.9±3.0 | 28.2±2.0 |
| Vitamin $D_3$ | 0.01 | 71.5±4.9 | 12.2±3.0 | 40.7±3.1 | 40.4±1.7 |

[a]Mean±S.D (n=3).
[b]Untreated cells.

Morphological changes were analyzed by a fascan analyzer. As shown in Fig. 2, hypericin-treated U-937 cells became larger than did untreated cells.

In order to further examine the mechanism of cell differentiation induced by hypericin, we analyzed relationship between the differentiation of U-937 cells and the activities of PKA or PKC after exposure to 0.2 and 0.15 μg/ml of hypericin. When U-937 cells were treated with hypericin together with H-89 (PKA inhibitor) or NA-382 (PKA inhibiter) for 4 days, NA-382 reduced differentiation by hypericin (Fig. 3), whereas H-89 did not show any influence (no data shown). Therefore, the specific activity of hypericin to induce differentiation of leukemia cells seems to be associated with its stimulatory effect on PKC activity.

## DISCUSSION

Leukemia cells are blocked at some steps of the maturation processes and display a highly proliferative capacity. The potential value of differentiation inducers as therapeutic agents resides in their ability to overcome the maturation blockade.

We have recently demonstrated that hypericin produced a selective cytotoxicity on leukemia cells and PTK inhibitory activity (Kil *et al.*, 1996). The present study demonstrates that hypericin has a potent differentiation-inducing activity in the myelogenous leukemia-derived U-937 cells. Hypericin produced the expression of differentiation markers such as NBT reducing ability, phagocytic activity, cell size and the appearance of AS-D esterase activity in U-937 cells. In addition, we observed that NA-382, an inhibitor of PKC, reduced the differentiation induced by hypericin whereas, H-89 an inhibitor of PKA, did not. Although the mechanism of differentiation induction by hypericin in U-937 cells is not clear, it appears that PKC rather than PKA plays an important role in modulation of differentiation. These results suggest that differentiation induced by hypericin depends on a chain of events including protein kinase C activity. Results from other studies suggest that tyrosine phosphorylation induced by EGF is significantly inhibited by hypericin in a does-dependant manner (Agonist *et al.*, 1995; Kil *et al.*, 1996). If the target of the action by hypericin is limited to tyrosine kinase or closely associated reaction, the hypothetical common step in differentiation is likely to be related to tyrosine residues in cellular proteins. In this respect, it is noterworthy that genistein and herbimycin A, inhibitor of protein phosphorylation activity of protein tyrosine kinase *in vitro,* also induce differentiation of several cells under certain condition (Kondo *et al.*, 1989). Taken together, it is likely that specific inhibition of protein phosphorylation at tyrosine residues make the cells, directly de indirectly, less proliferative and more susceptible to physiological conditions favoring differentiation.

Tyrosine phosphorylation of PKC δ has been reported to occur in the promyeloid cell line 32D and NIH-3T3 fibroblast treated with the tumor promotor TPA and PDGF. To date, most experiments in which tyrosine phosphorylation of PKC δ is isolated from intact cells support a role of tyrosine phosphorylation in the inhibition of PKC δ. PKC δ regulates myeloid differentiation, cell cycle progression in CHO cells and growth arrest in fibroblasts (Denning *et al.*, 1996).

The present study shows that hypericin-induced differentiation of U-937 cells is inhibited by the PKC inhibitor, NA-382. Therefore, we suggest that differentiation by hypericin is related to PKC activity and is stimulated by inhibition of tyrosine phosphorylation in response to hypericin-mediated signaling.

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