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Humic Acid Induces the Expression of ox-LDL Receptor in HL-60 Cells Through Activation of PPARγ

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Humic acid (HA) is a fluorescent, deep brown organic acid, that is easily found in various water (such as well, lakes, and oceans), humus and sediments (Flaig 1966) Epidemiological studies have shown that there is a high association of HA with Blackfoot disease (BFD), which prevails the southwest coast of Taiwan (Lu et al.1991). Humic acid causes various alterations in the process of vascular thrombosis, such as enhancing the expression of tissue factors and other pro-coagulation factors of cultured endothelium cells (Yang et al. 1994) and induces blackening of tail and feet of mice (Lu 1990).

Recently, HA has been reported as being involved in gene regulation, such as inhibiting lipopolysaccharide-induced NF-κB activation in human umbilical vein endothelium cells (Gau et al, 2000), and inducing peroxisome proliferator-activated receptor γ (PPARγ) activation in preadipocyte (Lee et al, 1999). PPARγ is reported to be expressed in myeloid leukemia cell line HL-60 and is known to induce monocytic differentiation (Tontonoz et al, 1998). Uptake of oxidized low-density lipoprotein (ox-LDL) by macrophage is the initial process of formation of the atherosclerostic plaque (Nagy et al. 1998), which is seen in the arterial endothelium of BFD. We report in this paper the induction of expression of PPARγ and ox-LDL receptor in HL-60 cells by HA, which may partly explain the pathogenesis of BFD.

MATERIALS AND METHODS

HA was obtained from Aldrich chemical company (Milwaukee, Wisconsin, USA). Fetal calf serum, RPMI1640 medium, penicillin, and streptomycin were obtained from Gibco BRL/Life technologies (Rockville, Maryland, USA). Anti-PPARγ (rabbit) polyclonal antibody was obtained from Affinity Bioreagents, Inc (Golden, California, USA). Goat anti-rabbit IgG:HRP (horseradish peroxidase) was obtained from Transduction Laboratories (Lexington, Kentucky, USA). Anti-CD36 (mouse) monoclonal antibody and goat anti-mouse IgG: HRP wass obtained from Neo Marker (Fremont, California, USA). XTT (Sodium 3'-1 [1 - (phenylamino-carbonyl) - 3, 4 - tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) was obtained from Boehringer Mannhein, Germany). ECL (enhanced chemiluminescence) system was obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey, USA)

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HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin and maintained at a density of approximately 5×10^5 cells /ml at the initiation of subculture. HA and phorbol ester (PMA) were added to the medium one day after the passage. In order to examine the cytotoxic effect of HA and PMA on HL-60 cells, XTT assay was based on Jost (1992). HL-60 cells, treated with various concentrations of HA (control, 20, 50, 100, 200, 400 μg/ml) and PMA (0, 2, 5, 10, 20, 40 ng/ml) were seeded in the 96 well-ELISA plates with the density of 1×10^5 cells /0.1 ml, and 0.05 ml XTT (1mg/ml). The reaction mixtures were incubated at 37°C for 4 hours. Absorption values at 450 nm were determined spectrophotometrically. The final absorption values were calculated by subtracting the reading at time zero.

After 24 hrs treatment with HA or PMA, HL-60 cells were harvested and the total nuclear proteins extracted according to Gronowiski et al (1994). Nuclear proteins were then separated by molecular weight on 10% SDS polyacrylamide gel and transferred to PVDF paper by electroblotting. The membrane was hybridized with anti-PPARγ antibody for 60 minutes followed by anti-IgG second antibody. Specific complex was visualized with the ECL system according to the supplier's manual.

We also examined the surface protein in HA and/or PMA treated-HL-60 cells. Cells were washed with ice -cold PBS (phosphate buffer saline) containing 0.5% BSA and incubated in the presence or absence of anti-CD36 monoclonal antibody at 4 °C for 10 minutes. Then, a second incubation with FITC (fluorescein isothiocyanate)-conjugated anti-immunoglobin monoclonal antibody was performed. Cells were analyzed on a Becton -Dickinson FACScan using CellQuest software. A population of at least 10,000 cells was analyzed in each experiment.

RESULTS AND DISCUSSION

The cytotoxic effects of HA and PMA were shown in Fig 1A and 1B, respectively. The HL-60 cells were not affected by HA or PMA up to 48 hrs of culture. HA dose-dependently reduced the number of HL-60 cell on the third day of culture (Fig 1A). PMA also inhibited HL-60 cell proliferation at the third day of culture but reached a steady state, which suggests PMA induces HL-60 cell differentiation This result is consistent with previous reports (Collins 1987, Chang et al, 1981). Figure 2 shows the combination effect of PMA and HA on the HL-60 cells. The curve exhibited that HA enhanced the proliferation cease (differentiation) activity of PMA.

To understand the mechanism of HA inhibitory effect, the effect of HA on the expression of transcription factor PPARγ was examined after one-day treatment. Total nuclear protein was extracted and a 92 KD band was detected by Western blotting with anti-PPARγ polyclonal antibody. Fig 3 shows that HA alone enhances PPARγ expression in HL-60 cells dose dependently. This phenomenon is also found in the lower concentration of HA (data not shown).

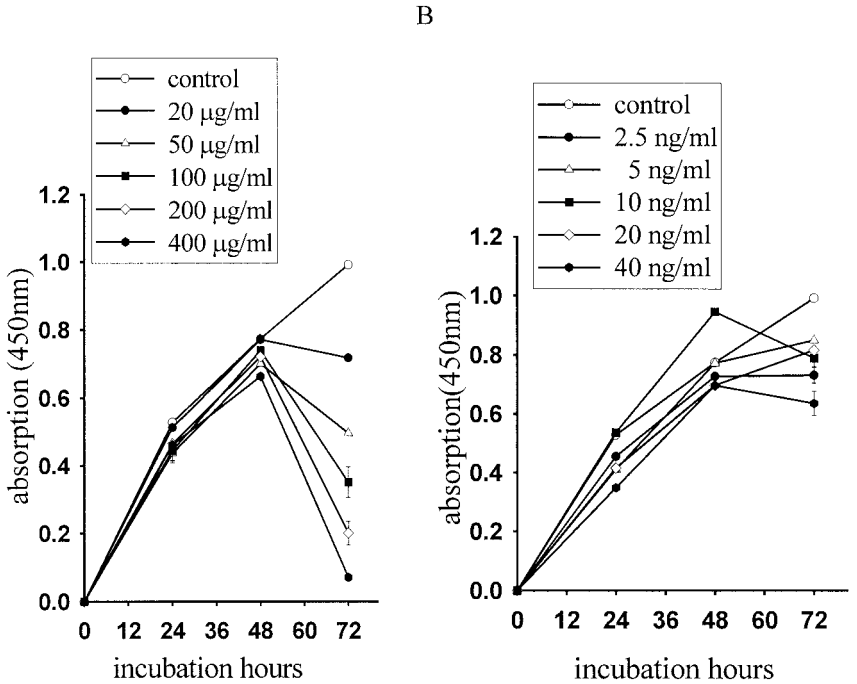

Figure 1. Cytotoxic effect of HA (A) and proliferation inhibitory effect of PMA (B) on HL-60 cells. The absorption values of individual point originated from a representative experiments (n=3). Each experiment was repeated at least three times.

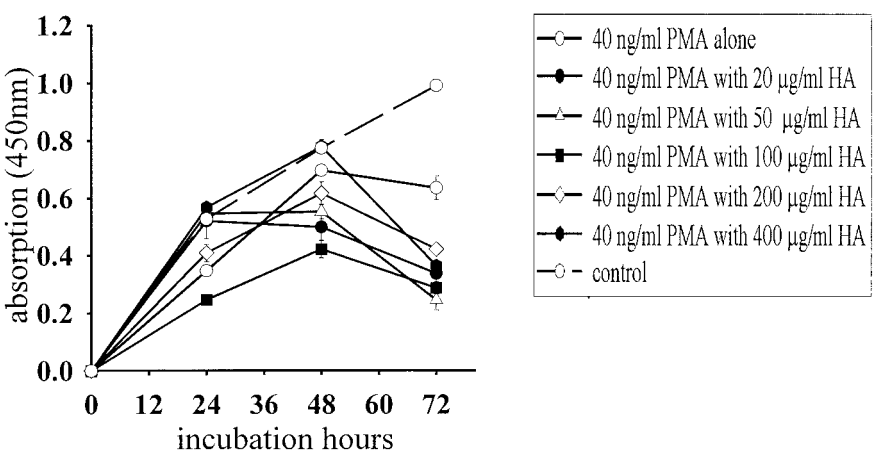

Figure 2. Cytotoxic effect of HA on HL-60 cells in the presence of PMA.

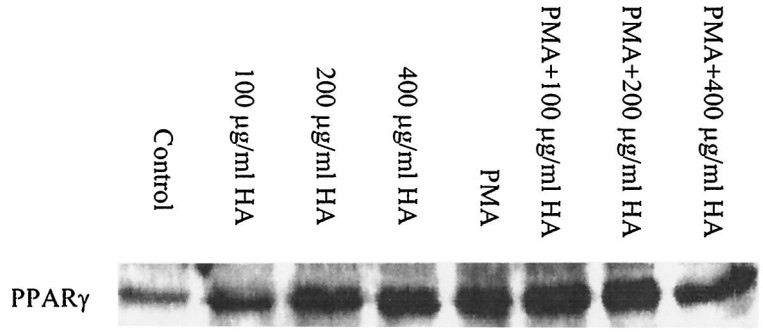

Figure 3. Induction of PPARγ by HA and PMA in the HL-60 cells. HL-60 cells were cultured for one day in the presence of one or more of the following agents: 100 ; 200 ; 400 μg/ml HA or 40 ng/ml PMA. Total nuclear protein (20 μg per lane) was analyzed by Western blotting analysis using anti-human PPARγ polyclonal antibody.

HA enhanced the expression of PPARγ synergistically with PMA. Since HL-60 cell in one-day culture was still in the proliferative phase, HA probably induced PPARγ expression in precursor cells.

In order to know whether PPAR γ, induced by HA, could activate the downstream gene and express CD36 (ox-LDL receptor) in HL-60 cells, the amount of CD36 glycoprotein on the HL-60 cells were detected by cytometer analysis. Fig 4 showed the expression of CD36 after PMA-induced cell differentiation. After PMA treatment for one day, the HL-60 cells have prominent morphological change. Some cells become smaller than original ones, and with increased granularity (Fig 4A), these cells have already differentiated to macrophages. However, there is no significant change in CD36 expression (Fig 4B). The CD36 glycoprotein expression may not accompany early monocyte differentiation. However, there were 15-fold increases of CD36 expression after two days treatment with HA (value from 10.34 to 161.9). This increase of fluorescence intensity is not due to the inherent fluorescence of HA, because when FITC-conjugated IgG second antibody to CD36 is added, the fluorescence of both the control and HA treated cells remain equivalent (Fig 5A).

The cells that express CD36 split into two groups after PMA treatment for two days (Fig 5C). According to the dot plot analysis, the cells that have stronger fluorescent intensity belong to the differentiated ones and are not present in the HA treated HL-60 cells (Fig 5B). Furthermore, if HA is added to the HL-60 previously treated with PMA, the population of this group increases (Fig5B). From the result of flow cytometric analysis, it can be concluded that HA can only induce HL-60 cell to express CD36, but not differentiation. But, in the differentiated cells induced by PMA, the CD36 expression is highly variable. Cotreatment with HA and PMA in HL-60 cells induced better differentiation into monocyte and enhance CD36 expression in theses cells.

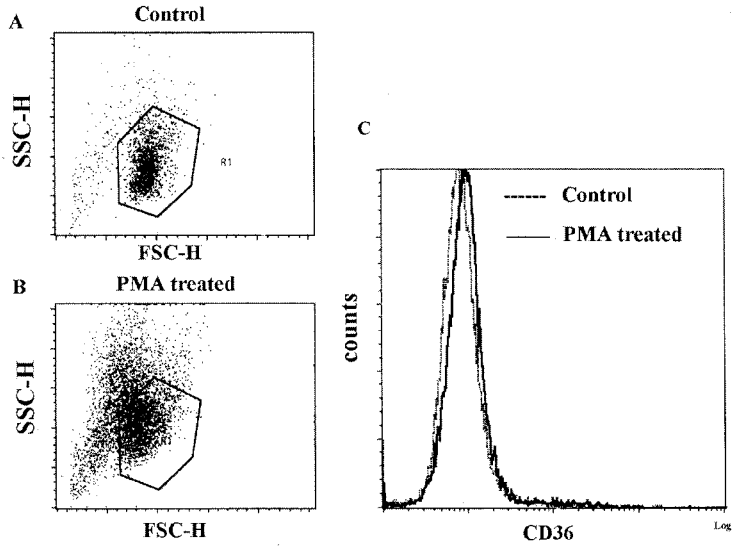

Figure 4. CD36 expression in HL-60 cells before (A) and after (B) treatment with PMA for one day. PMA treated HL-60 cells became smaller (with decreased forward scatter counts, FSC) and abundant granularity (increased side scatter counts, SSC). There is no significant difference of CD36 expression in HL-60 cells after PMA treatment. (C)

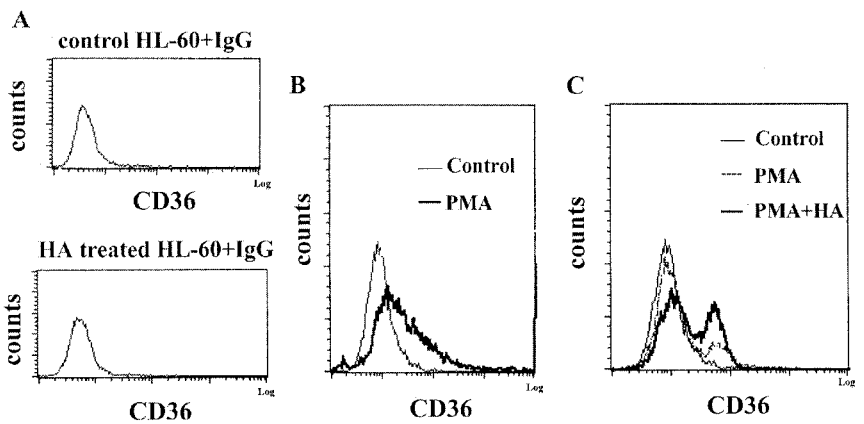

Figure 5. HA induced the expression of scavenger receptor CD36 in HL-60 cells. HL-60 cells were treated with 20 μg/ml HA (B) or 40 ng/ml PMA (C) for two days. Expression of CD36 was determined by flow cytometry using an anti-human CD36 antibody. The fluorescence distribution of the counted cells in this figure is originated from a representative experiment. Each experiment was repeated at least three times with similar results. Addition of HA does not affect the fluorescent intensity detected by CD36 antibody (A)

From the change in cell morphology we can further verify the effects of HA and PMA. HL-60 cells treated with HA alone show no sign of differentiation. (Fig 6B, 6C, and magnifying power: 10x20). HL-60 cells treated with PMA for one day, there are some attached cells showed up (Fig 6D). If cotreated with HA, the population of attached cells increased (Fig 6E, F). HL-60 cells treated with PMA for two days showed some aggregation (Fig 6G). In conjunction with HA (20 μg/ml), almost all cells become aggregated. The cells become sticker and compressed, which may result from bountiful CD36 surface protein expression and the close interaction between cells.

Co-treatment with HA (50 μg/ml) and PMA results in cell death due to unknown toxic effect. This is consistent with the result of the cell viabilities test (Fig 2).

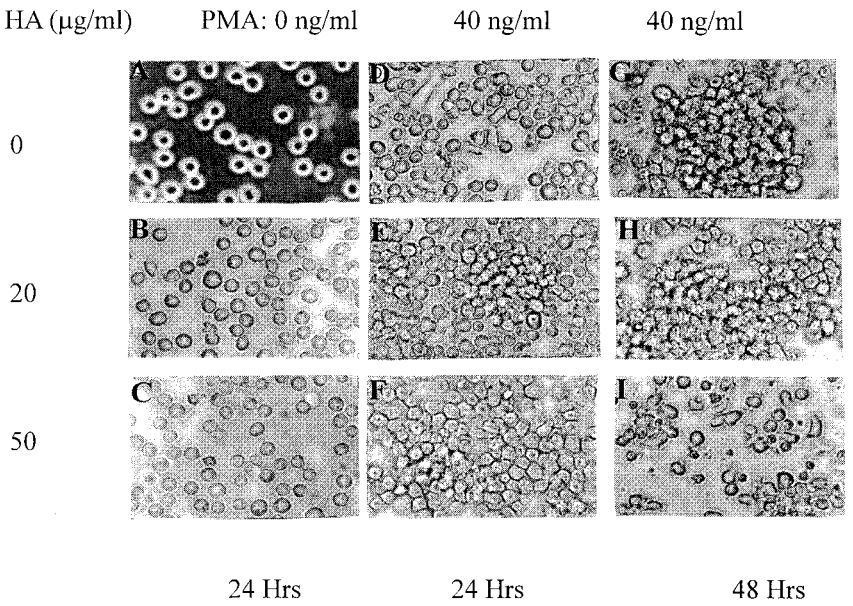

Figure 6. Effect of HA and PMA on the morphologies of HL-60 cells. HL-60 cells were incubated with various concentrations of HA and PMA for 24 hours (A-F) or 48 hours (G-I). Upper panel shows PMA combination, left axis shoes HA concentration and lower panel shows the incubation period. Photograph was taken with inverse microscope.

BFD is an endemic disease in Taiwan with a long history. Epidemiological studies have shown that there is a high correlation between this disease and HA. Clinical studies and microscopic examination revealed that signs and symptoms of BFD are similar to that of atherosclerosis (Yeh et al. 1963). Uptake of ox-LDL by macrophage is thought to play a central role in the foam cell formation and the pathogenesis of atherosclerosis. PPARγ is a nuclear receptor, which forms a heterodimer DNA binding complex with the retinoic acid receptor α(RXR-α), and is expressed in several myeloid leukemia cell lines (Tontonoz et al, 1998). Ligand activation of PPARγ: RXRα heterodimer in myelocytic cell line induces change in

characteristic of monocytic differentiation and promotes the uptake of ox-LDL by regulating the expression of the scavenger receptor, CD36 (Nagy et al. 1998).

Our previous data indicated that HA could induce PPARγ expression in preadipocyte. This finding led us to study whether HA can induce PPARγ expression in the HL-60 cells. The amount of immunoreactive CD36 on HL-60 cells significantly increased when cells were treated with only 20 μg/ml HA. Furthermore, a synergistic effect on induction of surface CD36 expression was observed following the cotreatment with HA and PMA, which is a strong inducer of HL-60 monocytic differentiation. The above data strongly suggests that HA may induce formation of foam cells, and play an important role in the pathogenesis of BFD.

The HA concentration of well water is around 220 μg/ml (220 ppm) (Lu et al. 1986). Each person may ingest about 0.44 mg HA per day by drinking two litter of water. In the rat model, 1.69% of I^{125} HA remained in the blood 24 hours after injection and it accumulated in the liver, kidney, bone and thyroid gland for more than 72 hrs (Huang et al. 1995). Similar distribution pattern is noted through oral route (our unpublished data). According to the animal model by computing the HA concentration in the well water, the amount of water ingested, and the metabolic clearance of HA, the HA concentration in body is estimated to be around 1-100 ppm. Therefore, the concentration of HA used in the experiments is relevant to the physiological condition. Furthermore, the half-life of circulated monocyte is 20-40 hours. Also relevant is that the experiment design is completed within 2 days.

We found that the effect of HA and PMA on expression of CD36 is quite different. Treatment with HA led to a differentiation independent of the induction of CD36, while PMA-treatment resulted in a delayed induction of CD36 after the differentiation of monocyte. Treating the HL-60 cells with HA caused the heamopiotic precursor cell to express low level of PPARγ, which might be insufficient to trigger differentiation. We postulated that small amount of PPARγ may bind with an alternative transcription factor other than RXR α to turn on CD36 expression.

Measurement of DNA by flow cytomer (PI stain) has shown a G1 arrest phenomena in the HA treated HL-60 cells (data not shown). The induction of PPARγ by HA may sufficient to induce growth arrest but not adequate to initiate cell differentiation (There is no detectable expression of CD14-monocytic marker by flow cytometry analysis in the HA-treated HL-60 cells). In summary, we can hypothesize that other essential hibernated proteins, which are not activated by HA, are involved in cell differentiation.

The present study demonstrates the potential of HA in the induction of PPARγ and it's down stream gene for CD36 expression, a major scavenger receptor of ox-LDL, in HL-60 cells. Our finding suggests that HA may act as a proatherosclerotic factor in the pathogenesis of BFD.

REFERENCE

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

Chang LJ, McCulloch EA (1981) Dose-dependent effects of a tumor promotor on blast cell progenitors in human myeloblastic leukemia. Blood 57: 361-7

Flaig W (1966) Chemistry of humic substance in the use of isotopes in soil organic matter studies. Report of FAOIAEA Technical Meeting, Pergamon, and Elmsford, NY. Pp.103-127

Gau RJ, Yang HL, Chow SN, Suen JL, Lu FJ (2000) Humic acid suppresses the LPS-induced expression of cell-surface adhesion proteins through the inhibition of NF-kappaB activation. Toxicol Appl Pharmacol 166: 59-67

Gronowski AM, Rotwein P (1994) Rapid changes in nuclear protein tyrosine phosphorylation after growth hormone treatment in vivo. Identification of phosphorylated mitogen-activated protein kinase and STAT91. J Biol Chem 269: 7874-7878

Huang TS, Lu FJ, Tsai CW (1995) Tissue distribution of absorbed humic acid. Environ Geochem Health 17:1-4

Jost LM, Kirkwood JM, Whiteside TL (1992) Improved short- and long-term XTT-based colorimetric cellular cytotoxicity assay for melanoma and other tumor cells. J Immunol Methods 147: 153-165

Lee Y, Huang TS, Yang ML, Huang LR, Chen CH, Lu FJ (1999) Peroxisome proliferation, adipocyte determination and differentiation of C3H10T1/2 fibroblast cells induced by humic acid: induction of PPAR in diverse cells. J Cell Physiol 179: 218-225

Lu FJ, Liu TM (1986) [Fluorescent compounds in drinking water of blackfoot disease endemic areas: animal experimental model]. [Chinese] Taiwan I Hsueh Hui Tsa Chih - J Formosan Med Assoc 85: 352-358

Lu FJ (1990) Fluorescent humic substance and Blackfoot disease in Taiwan. Appl Organomet Chem 4: 191-195

Lu FJ, Hsieh HP, Yamauchi H, Yamaura T (1991) Fluorescent humic substance-arsenic complex in well water in area where Blackfoot disease in endemic in Taiwan. Appl Organomet Chem 5:507-512

Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR gamma. Cell 93: 229-40

Yeh S, How SW (1963) A pathological study on the blackfoot disease in Taiwan, Reports, Institute of Pathology, National of Taiwan University, 14:25-73

Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM (1998) PPAR gamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93: 241-52

Yang HL, Lu FJ, Wung SL, Chiu HC (1994) Humic acid induces expression of tissue factor by cultured endothelial cells: regulation by cytosolic calcium and protein kinase C. Thromb Haemostasis 71: 325-330