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Colchicine Inhibits Integrin $\alpha_5\beta_1$ Gene Expression during PMA-induced Differentiation of U937 Cells

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Monocyte adhesion involves specific cell surface receptors, integrins and results in cell differentiation. We have stiudied expression and regulation of integrin $\alpha_5\beta_1$ during differentiation along the monocytic pathway in human monocytic cell line, U937 as *in vitro* model. To determine expression of integrin $\alpha_5\beta_1$ during differentiation of U937 cells, cells were cultured for 24 h in RPMI 1640 medium with PMA (2 to 200 ng/ml). We determined expression of integrin $\alpha_5\beta_1$ genes by RT-PCR (reverse transcription and polymerase chain reaction) method. We found that expression of integrin α5β1 was greatly increased during PMA-induced differentiation of U 937 cells and also found that PMA-induced expression of integrin $\alpha_5\beta_1$ was inhibited by colchicine, microtubule depolymerizing agent. These results indicate that microtubular integrity is associated with expression of integrin $\alpha_5\beta_1$ during PMA-induced differentiation of U937 cells.

Key words : Colchicine, Microtubule, Integrin $\alpha_5\beta_1$, RT-PCR

INTRODUCTION

Differentiation along monocytic pathway involves several stages including mononuclear phagocyte, monoblast, promonocyte, monocyte, and tissue macrophage. The expression of genes which are related differentiation of monocyte has been studied *in vitro* system. As an *in vitro* model, U937 cultures of human monocytic cell line has been extensively studied for differentiation into macrophage-like cells in presence of a variety of agents including phorbol esters (Amento *et al.*, 1984; Harris and Ralph, 1985; Bhalla *et al.*, 1989; Hass *et al.*, 1990; Wager and Assoian, 1990).

Adhesion is of fundmental importance to a cell, because it provides anchorage, cues for migration, and signals for growth and differentiation (Ruoslahti, 1991; Sporn *et al.*, 1990; Springer *et al.*, 1990). Integrins are a family of cell surface proteins involved in cell adhesion. Integrins are transmembrane heterodimers (Hynes, 1987; Hynes, 1992). The integrin $\alpha_5\beta_1$ is the predominant fibronectin receptor (Akiyama *et al.*, 1990). The integrin $\alpha_5\beta_1$ is expressed in a variety of cell types and may be involved in their differentiation (Dedhar, 1989; Yamada, 1989).

Microtubules, intermediate filaments, and microfilaments are the main components of the cytoskeleton. Microtubules are composed of two protein subunits, α-tubulin and β-tubulin. By binding to tubulin at a certain site, various agents including colchicine prevent further assembly of microtubules but not further disassembly. As a result, the microtubules are completely disrupted (Alberts *et al.*, 1989). Leung and Sartorelli (1992) reported that microtubule is involved in modulating signal transduction during the initiation of HL-60 cell differentiation. The involvement of cytoskeleton in the process of cellular differentiation has been shown by changes in components of the cytoskeleton during initiation of myeloid leukemia maturation (Bernal and Chen, 1982). Since Chen *et al.* (1992) found that treatment of cytochalacin B, actin depolymerzing agent, increased mRNA levels of the integrin 2 subunit in MG-63 cells, it is highly likely that cytoskeleton is involved in the regulation of integrin gene expression. The effect of microtubule disruption, however, integrin $\alpha_5\beta_1$ gene expression by PMA-differentiated U937 still remains to be examined.

The purpose of this study is to investigate the expression of integrin gene expression during monocyte/macrophage differentiation and also to know if the expression of these genes is related with microtubular integrity. We used RT-PCR method for expression of these genes during differentiation of U937 cells. Here we report that the mRNA levels of integrin $\alpha_5\beta_1$ are related with microtubule disruption during PMA-induced differentiation of U937 cells.

MATERIALS AND METHODS

Cell culture

The human histiocytic lymphoma cell line, U937,

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was received from the American Tissue Type Culture Collection (ATCC, Rockville, MD, USA). U937 cells were cultivated in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin and were maintained in an humidified 5% CO_2 atmosphere at 37°C. Cultures were used after 3 days.

Cells were plated at an initial density of 2×10^5 cells/ml in 100 mm dishes or 24 well plates (Corning, USA) and differentiation of U937 cells was achieved by exposure to 0-200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 3-24 h. Untreated control cells were left in medium containing 1% bovine serum albumin (BSA, Sigma, USA).

Synthesis of DNA primers

The specific primers for integrin $\alpha_5\beta_1$ (Argraves *et al.*, 1987) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Tso *et al.*, 1985) were synthesized according to previously published sequences. DNA primers (Table 1) used in polymerase chain reaction were prepared by a DNA synthesizer.

Reverse transcription and Polymerase chain reaction amplification

Total cellular RNA was isolated by the guanidinium isothiocyanate procedure using RNAzol B modified from Chomczynski and Sacchi (1987).

Reverse transcription was performed by using GeneAmp RNA PCR kit. In brief, 300 ng of total cellular RNA was reverse transcribed in the presence of 2.5 μM oligo d(T)16 as primers, 5 mM dithiothreitol, 1 mM each of dNTP, and 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 20 U of placental RNase inhibitor, in a total volume of 20 μl. Samples were incubated at 42°C for 30 min. This reaction mixture was used immediately in a polymerase chain reaction or was stored at -20°C until use.

The polymerase chain reaction was carried out as follows. cDNA was amplified in a 50 μl reaction mixture. Reaction mixture was composed of 2 μl of cDNA (equivalent to 300 ng RNA), 2.5 μl of dNTP (Sigma, St. Louis, MO, USA, 5 mM each dATP, dCTP, dGTP and dTTP), 1.5 μl each of "sense" and "antisense" primers (100 ng/μl each) and 5 μl of 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) and brought with water to a final of 50 μl. cDNA was then heat denatured at 94°C for 5 min. Then the mixture was cooled down to 60°C and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) were pipetted into each tube. PCR was performed in the Ericomp thermal cycler (Ericomp Co., San Diego, CA) for 35 cycles. A cycle profile consisted of 60 s at 94°C for denaturation, 60 s at 60°C for annealing, and 90 s at 72°C for extension. As negative controls H_2O only and total RNA were amplified under the same conditions.

Electrophoresis of 15 μl reaction mixture was performed on a 3% agarose gel containing ethidium bromide approximately 30 min at 200 V. Gels were photographed using Polaroid 55 film and then PCR products were detested with the naked eye. As size marker 1 μg of *Hind III*-digested $\phi\times174$ DNA was used. GAPDH was assayed on all samples as external control to verify intactness of RNA and efficient cDNA synthesis.

Table 1. Sequences of sense and antisense primers of integrin $\alpha_5\beta_1$ genes

Target mRNA		Primers of sense and antisense of target genes (5'→3')	Expected PCR product (bp)
GAPDH	sense	ATCTA CCGCA TTGAC CACCT G	470
	antisense	CCCAC AGAAG ACATC CAGGA TGAG	
α_5	sense	AGACC CTGCT CATCC AGAAT G	1190
	antisense	GCAGA CTTTG GCTCT CTTGT TG	
β_1	sense	CACAA GTGAA CAGAA CTGCA CC	1300
	antisense	CGTGT CCCAT TTGGC ATTCA T	

RESULTS

Expression of integrin α_5 and β_1 mRNA during PMA-induced differentiation of U937 cells

In order to determine whether PMA induced integrin α_5 and β_1 gene expression, total cellular RNAs were extracted from U937 cells treated with or without PMA (2 to 200 ng/ml) for 24 h and analyzed by RT-PCR. According to Fig. 1, the mRNA level of GAPDH was not changed but that of integrin α_5 and β_1 subunit was increased during differentiation of U 937 cells. The extent of induction was dose dependent and maximal induction was at 200 ng/ml PMA.

Effect of colchicine on integrin α_5 and β_1 gene expression during PMA-induced differentiation of U937 cells

The PMA-induced integrin α_5 and β_1 expression in U937 cells was marked that analysis of the mechanisms could be initiated. To understand the effects of colchicine on PMA-differentiated U937, the amount

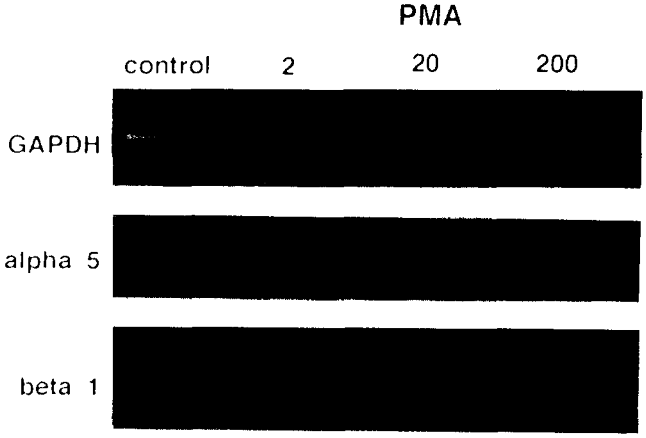

Fig. 1. Expression of integrin α_5 and β_1 subunit during PMA-induced differentiation of U937 cells. U937 cells were differentiated with PMA 2, 20, and 200 ng/ml for 24 h. Total cellular RNA extraction and RT-PCR analysis was done as described in Materials and Methods.

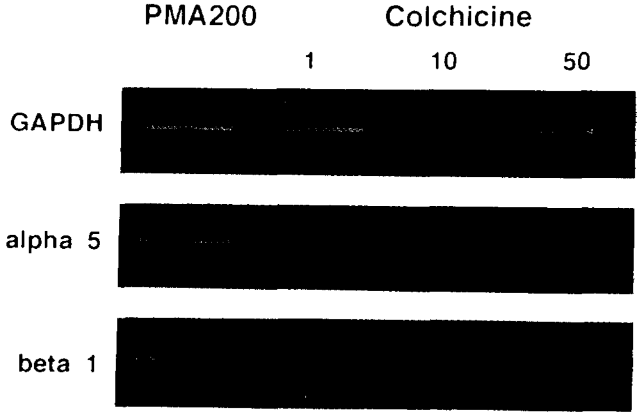

Fig 2. Effect of colchicine on the expression of integrin α_5 and β_1 subunit during PMA-induced differentiation of U937 cells. U937 cells were treated with colchicine 1, 10, and 50 μM for 24 h during PMA-induced differentiation of U937 cells. Total cellular RNA extraction and RT-PCR analysis were done as described in Materials and Methods.

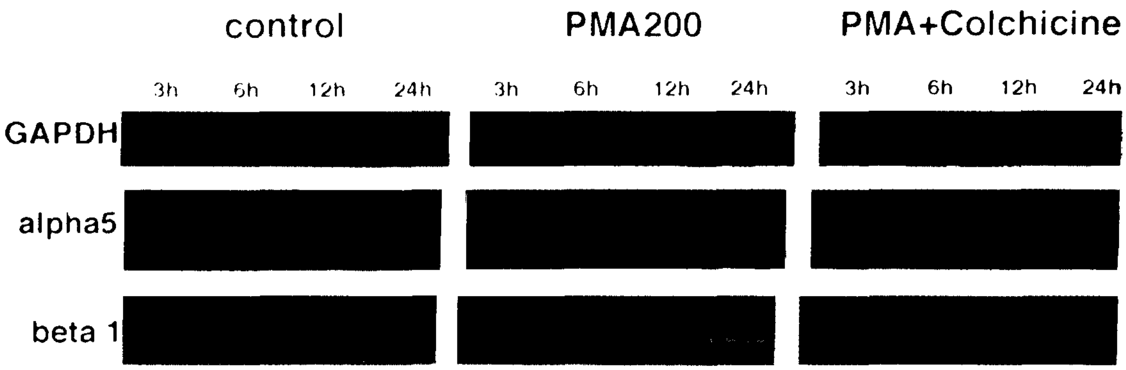

Fig. 3. Time-response relation of integrin α_5 and β_1 subunit mRNA levels on U937 cells. U937 cells (left) were differentiated with 200 ng/ml PMA (middle) or were treated with 200 ng/ml PMA and 10 μM colchicine together (right) for 3, 6, 12, and 24 h. Total cellular RNA extraction and RT-PCR analysis was done as described in Materials and Methods.

of integrin α_5 and β_1 mRNA induced by colchicine (1 to 50 μM) for 24 h was measured. As shown in Fig. 2, the GAPDH mRNA was detected in all samples and there was no significant difference in amount of GAPDH message between different conditions. Inhibition of PMA-induced integrin α_5 and β_1 expression begins at 1 μM colchicine and is maximal at 50 μM colchicine. In this case, the extent of α_5 mRNA inhibition by colchicine was dose-dependent.

Time-response relation of integrin α_5 and β_1 subunit mRNA levels on U937 cells

To determine the dynamics of expression, the kinetics of mRNA levels for integrin α_5 and β_1 subunit were examined. For this experiment, U937 cells were incubated for 3, 6, 12 and 24 h with 200 ng/ml PMA or with 200 ng/ml PMA and 10 μM colchicine together. The time course of integrin α_5 and β_1 subunit mRNA on U937 cells was shown in Fig. 3. The mRNA levels of GAPDH were detected as a control and they remain the same with time in all three cases. Integrin α_5 subunit mRNA was detectable only after 12 h in all three cases and only a low level of integrin α_5 subunit mRNA induction occurred in untreated controls. The level of integrin α_5 subunit mRNA in the presence of PMA after 12 h increased and declined by adding colchicine.

Interestingly, the expression of integrin β_1 subunit mRNA was induced rapidly whin the first 3 h and 3 h value for integrin β_1 subunit mRNA was maintained through 24 h of culture in all three cases. Incubation with PMA for only 24 h resulted in integrin β_1 mRNA expression even greater than that seen as a result of combined treatment with PMA and colchicine.

DISCUSSION

The data obtained in this study indicate that the expressions of integrin $\alpha_5\beta_1$ are changed during PMA-induced differentiation of U937 cells and their expressions are altered when microtubules are disrupted by colchicine.

The differentiation of U937 cells was induced by PMA. PMA is well known as a tumor promoter and differentiation inducing factor (Gidlund *et al.*, 1981). One of the evidences of PMA-induced differentiation in U937 cells was the morphological change which caused cell aggregation (data not shown). This change is in accordance with several other studies (Hass *et al.*, 1990; Hass *et al.*, 1991). Second evidence that PMA-induced U937 cell differentiation was the expression of a human H3 histone gene which associated with cell proliferation. We found that the H3 mRNA level was downregulated during PMA-induced differentiation of U937 cells (data not shown). This result is well in accordance with that of HL-60 promyelotic leukemia cells. Stein *et al.* (1989) show that the downregulation of H4 and H3 histone gene transcription accompanies the shutdown of proliferation and onset of differentiation. Third evidence to support the differentiation of U937 cells was the expression of integrin $\alpha_5\beta_1$ which associated with cell adhesion. The expression of integrin $\alpha_5\beta_1$ and β_1 subunit is gradually increased during PMA-induced differentiation of U937 cells. The up-regulation of integrin $\alpha_5\beta_1$ shows to be concomitant with cell attachment (Dalton *et al.*, 1992) and cell differentiation (Ferreira *et al.*, 1991).

The evidence to support the relation with microtubular integrity during the differentiation of U937 cells was the change of integrin $\alpha_5\beta_1$ expression by colchicine, microtubule disrupting agent. PMA-induced upregulation of integrin genes was suppressed by colchicine with a dose dependent manner. This result indicates that microtubule may be involved in integrin $\alpha_5\beta_1$ gene expression during the differentiation of U937 cells. This finding is in accordance with the result of study in MG-63 cells (Chen *et al.*, 1992) and chick embryo fibroblast (Otey *et al.*, 1990).

Taken together, these evidences support that PMA induces U937 cell differentiation and microtubule may be an essential factor in signal transduction of integrin $\alpha_5\beta_1$ gene expression during the differentiation of U937 cells.

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