The Induction of Differentiation for THP-1 Cells by Activation of CD44-Associated Signal Transduction Pathway; The Elucidation of Its Mechanism

June-Won Cheong

Department of Medicine
The Graduate School, Yonsei University

The Induction of Differentiation for THP-1 Cells by Activation of CD44-Associated Signal Transduction Pathway; The Elucidation of Its Mechanism

Directed by Professor **Yoo Hong Min**

The Doctoral Dissertation
submitted to the Department of Medicine
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

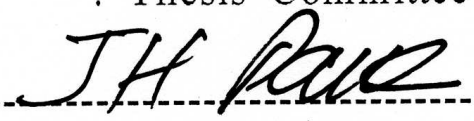
June-Won Cheong

June 2006

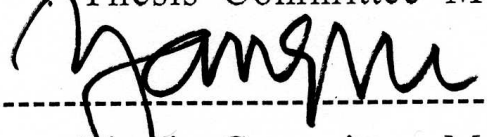
This certifies that the Doctoral Dissertation of June-Won Cheong is approved.

Thesis Supervisor : Yoo Hong Min

[: Thesis Committee Member#1)



[: Thesis Committee Member#2)



[: Thesis Committee Member#3)

[: Thesis Committee Member#4;

The Graduate School
Yonsei University

June 2006

ACKNOWLEDGEMENTS

Foremost, I would like to thank LORD for allowing me to write this dissertation.

I would like to express my gratitude to my supervisor Prof. Yoo Hong Min for his patience, encouragement, support and hearty supervision of my paper. I must also extend my gratitude to all professors of thesis committee for their valuable discussions and suggestions. They are willing to provide me all supports to undergo this study.

Many words are talked to thank all members of laboratory of hematology in clinical research center (especially Mrs. Ji Yeon Kim) for helping me to overcome many scientific problems.

I also appreciate the help of Prof. Jin Seok Kim for giving me a sufficient time to study by sharing a lot of duties.

It is impossible to convey the flavor of my grateful feelings toward my great-hearted wife Hye Kyung, my sister In Hee, and my parents.

I would like to dedicate this paper to my mother, Mrs. Seo, who has struggled against cancer and will finally prevail in the long run.

TABLE OF CONTENTS

ABSTRACT ····· 1
I. INTRODUCTION ····· 3
II. MATERIALS AND METHODS ····· 5
1. Chemicals and antibodies ····· 5
2. Cell culture and induction of differentiation by CD44 ligation ····· 5
3. Measurement of cell proliferation, viability, and apoptosis ····· 6
4. Analysis of cell cycle distribution ····· 6
5. Assessment of cell differentiation ····· 6
6. Western blot analysis ····· 7
7. Statistical analysis ····· 7
III. Results ····· 8
1. CD44 ligation with A3D8 inhibited proliferation and induced the apoptosis of THP-1 cells ····· 8
2. CD44 ligation with A3D8 induced terminal differentiation of THP-1 cells ····· 9
3. CD44 ligation with A3D8 induced a G_0/G_1 arrest in THP-1 cells ····· 10
4. Activation of Raf-1/MEK/ERK kinase signaling pathway during A3D8-induced terminal differentiation of THP-1 cells ····· 11
5. Activation of PI3-K/Akt signaling pathway during A3D8-induced terminal differentiation ····· 13
6. Inhibition of the MEK/ERK signaling pathway by PD98059 and U0126 ····· 14
7. PI3-K/Akt signaling is required for A3D8-induced Raf-1/MEK/ERK activation in THP-1 cells ····· 15
8. MEK/ERK signaling is essential for A3D8-induced terminal differentiation in THP-1 cells ····· 15
9. PI3-K/Akt signaling is essential for A3D8-induced terminal differentiation in THP-1 cells ····· 17
IV. DISCUSSION ····· 18
V. CONCLUSION ····· 22
REFERENCES ····· 23
ABSTRACT(IN KOREAN) ····· 27

LIST OF FIGURES

Figure 1. Time-dependent proliferation in THP-1 cells by A3D8 treatment ····· 8

Figure 2. Time-dependent apoptosis in THP-1 cells by A3D8 treatment ····· 9

Figure 3. Time-dependent differentiation in THP-1 cells by A3D8 treatment ····· 10

Figure 4. Time-dependent phosphorylation of Raf-1, MEK1/2, and ERK1/2 in THP-1 cells by A3D8 treatment ····· 11

Figure 5. The changes of p38 MAP kinase and its phosphorylation in THP-1 cells by A3D3 treatment ····· 12

Figure 6. The changes of PI3-K/Akt pathway in THP-1 cells by A3D3 treatment ··· 13

Figure 7. The changes of ERK and Akt phosphorylation in THP-1 cells by pre-treatment of PD98059 or U0126 before A3D8 treatment ····· 14

Figure 8. The changes of Akt, Raf-1, and MEK1/2 phosphorylation in THP-1 cells by pre-treatment of LY294002 before A3D8 treatment ····· 15

Figure 9. The changes in expression of CD11b and CD14 by treatment with A3D8 and various inhibitors ····· 16

LIST OF TABLES

Table 1. Time- and dose-dependent expression of CD11b and CD14 by A3D8 treatment ····· 10

Table 2. The changes of cell cycle in THP-1 cells by A3D8 treatment ····· 11

ABSTRACT

The Induction of Differentiation for THP-1 Cells by Activation of CD44-Associated Signal Transduction Pathway; The Elucidation of Its Mechanism

June-Won Cheong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor **Yoo Hong Min**)

Ligation of CD44 with anti-CD44 monoclonal antibody (mAb), A3D8, induces monocytic differentiation of human leukemia cell line THP-1. However, the underlying molecular mechanisms remain largely unknown. Herein, we examined the importance of extracellular signal-regulated kinases, p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways in the A3D8-induced terminally differentiated THP-1 cells. THP-1 cells showed cytologic changes typical of mature monocytes and an increased expression of monocyte-specific antigen CD14 (from 1.8± 0.1% to 38.2±3.2%) and of myeloid-specific antigen CD11b (from 6.6±0.6% to 66.6 ±1.6%) 72 hours after A3D8 treatment. The increase in the expression of these differentiation antigens was dose- and time-dependent. CD44 ligation with A3D8 mAb led to rapid and sustained activation of the essential kinases in the extracellular signal-regulated kinase pathway such as phospho-Raf-1, phospho-MEK1/2, and phospho-ERK1/2. In addition, Ser473 Akt phosphorylation was also observed shortly after the cells were treated with A3D8 mAb and was sustained thereafter. In contrast, the phosphorylation of p38 MAPK was dramatically decreased by CD44 ligation. Pre-treatment of cells with MEK1 inhibitors, PD98059 and U0126, potently inhibited THP-1 differentiation. Pre-treatment of cells with PI3-K inhibitor LY294002 also resulted in nearcomplete

inhibition of A3D8 mAb-induced differentiation and also blocked the A3D8-induced MEK/ERK activation, but not the activation of Raf-1.

Taken together, these findings demonstrated that the cross-talk between the activation of A3D8-inducible PI3-K/Akt pathway and the Raf/MEK/ERK pathway in THP-1 cell exists, and plays a critical role during CD44 ligation-induced THP-1 differentiation.

Key Words: CD44, differentiation, THP-1 cells, Raf-1/MEK/ERK, PI3-K/Akt

The Induction of Differentiation for THP-1 cells by Activation of CD44-Associated Signal Transduction Pathway; The Elucidation of Its Mechanism

June-Won Cheong

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor **Yoo Hong Min**)

I. INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the blockage of myeloid differentiation at various stages. The differentiation block can be overcome after treatment with either chemical agents[1] such as all-trans retinoic acid or monoclonal antibodies (mAbs) that target antigens such as on leukemia blasts[2,3]. The CD44 antigen is expressed on the surface of leukemic blasts in the majority of AML[4,5]. Because CD44 is a functional receptor that plays an important role in transmitting signals that can modulate cell proliferation, survival and differentiation4[6-7], it can be used as a target molecule to induce terminal differentiation of AML blasts. It has been reported that the ligation of CD44 with two mAbs, A3D8 and H90, triggers terminal differentiation of AML blasts on the basis of antigenic, functional, and cytologic criteria[8,9]. However, the molecular mechanisms involved in the CD44-induced differentiation of leukemia cells have not been elucidated.

Emerging evidence implicates that the mitogen-activated protein kinase (MEK)/ extracellular signal-regulated kinase (ERK) and p38 MAP kinase (MAPK) pathways are involved in the differentiation of certain cell types[10,11]. In the case of hematopoietic cell differentiation, antisense oligonucleotides and dominant-negative mutants targeting MEK-1 have been shown to inhibit macrophage-like differentiation of TF-1a cells[12]. Sustained activation of the ERK/MAP kinase pathway has been shown to be required

for the megakaryocytic differentiation of K562 cells[13]. Differentiation of U937 cells to monocytes/macrophages induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) was accompanied by activation of ERK1 and ERK2[14] like in HL-60 cells[15] and K562 cells[16] undergoing mononuclear and megakaryocytic differentiation, respectively, induced by this agent. In contrast, inhibition of ERK activation by PD98059 blocked differentiation of ML-1[17] and U937 cells[18]. While these observations indicate an involvement of MEK/ERK/MAP kinases in hematopoietic differentiation, the detailed role of the MEK/ERK/MAP kinase pathway in myeloid differentiation remains poorly understood.

Phosphatidylinositol 3-kinase (PI3-K) and the serine/threonine kinase Akt/PKB pathway regulate widely divergent cellular processes including cell growth, survival, apoptosis, and metabolism[19,20]. PI3-K/Akt pathway also has been demonstrated to be crucial in the regulation of cellular differentiation including chondrocytes[21], myoblasts[22], Th cells[23], neuronal cells[24], and adipocytes[25]. The Raf/MEK/ERK and PI3-K/Akt signaling pathways are often simultaneously activated in response to growth factors and hormones. In some systems, the small guanine nucleotide binding protein, Ras acts as an upstream positive effector of both the Raf/MEK/ERK pathway and the PI3-K/Akt pathway[26]. Cross-talk between the Raf/MEK/ERK and the PI3-K/Akt pathways may switch the biologic response and modulate differentiation, as shown for myoblast differentiation, by cell type-specific mechanisms[27]. However, the roles of these two pathways in the process of CD44 ligation-induced monocytic differentiation of THP-1 cells have not been previously been elucidated. This prompted us to study the roles of Raf/MEK/ERK and PI3-K/Akt in A3D8-induced terminal differentiation of THP-1 cells.

In this study, we demonstrated that CD44 ligation with A3D8 mAb induces the terminal differentiation of THP-1 cells by promoting cross-talk between Raf/MEK/ERK and Akt/PKB pathway in THP-1-leukemia cells. This result may provide a new experimental basis for a differentiation therapy in AML.

II. MATERIALS AND METHODS

1. Chemicals and antibodies

The anti-human CD44 mAb, A3D8 (IgG1), was obtained from Sigma Immunochemicals (St Louis, MO). Isotype control mAb (immunoglobulin G1; IgG1), was from Coulter- Immunotech (Miami, FL). MEK-1 specific inhibitors PD98059 and U0126, PI3-K inhibitor LY294002 and Raf-1 inhibitor ZM336372 were purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal antibodies against total Akt/PKB, Raf-1, MEK1/2, ERK1/2, and p38 MAPK were obtained from Cell Signaling Technology (Beverly, MA). Phospho-specific antibodies against Akt/PKB, Raf-1, MEK1/2 (Ser217/Ser221), ERK1/2 (Thr202/Tyr204), and p38 MAPK (Thr180/Tyr182) were also purchased from Cell Signaling Technology. Probing with anti-tubulin monoclonal antibody from Upstate (Charlottesville, VA) was used to demonstrate equal protein loading. Phycoerythrin (PE)-conjugated anti-CD11b antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody and the Annexin-V apoptosis kit were obtained from BD Phar Mingen (San Diego, CA). HRP-conjugated secondary antibodies for Western blotting were obtained from Promega (Madison, WI).

2. Cell culture and induction of differentiation by CD44 ligation

The human monoblastic leukemia cell line, THP-1, was obtained from the ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; Gibco BRL). Cells were subcultured 2～3 times weekly to maintain a log phase growth. To induce differentiation of THP-1, cells in logarithmic growth were seeded in triplicate at 3×10^5/ml and treated with anti-CD44 mAb, A3D8, dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO in culture medium was 0.1%) as indicated in the text and incubated at 37℃ with 5% CO_2 for various durations. As a negative control, cells were incubated with 10 μg/ml of IgG_1. To test the roles of Akt/PKB, MEK/ERK, and p38 MAPK kinase activation in the A3D8-induced terminal differentiation, THP-1 cells were pre-incubated with variable concentration of PD98059, U0126, LY294002, or SB203580 2 h before

treatment with A3D8 mAb, respectively. They were then processed for proliferation, differentiation, and apoptosis studies as described below.

3. Measurement of cell proliferation, viability, and apoptosis

Cells were seeded at 1.5×10^5/ml in 24-well plates and were allowed to grow for 6 days in normal growth medium or medium containing various amounts of A3D8 as indicated in the text. Viable cells were counted every day using trypan blue dye exclusion. All of the experiments were repeated at least 3 times. The values are reported as the mean±SD of triplicate wells. Apoptosis was quantified by flow cytometry on cells labeled with annexin V-FITC and propidium iodide.

4. Analysis of cell cycle distribution

After treatment with A3D8 mAb, the cells were pelleted and fixed in 70% ethanol on ice for 1 h and resuspended in 1 ml of a cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide) at a concentration of 1×10^6 cells/ml. Cell cycle analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest software (Becton Dickinson).

5. Assessment of cell differentiation

Cell differentiation was determined by cell morphological changes and cell surface markers (CD11b and CD14) expression. For detection of cell surface markers, 1×10^6 cells were harvested at indicated time, washed twice with ice-cold phosphate buffer solution (PBS) before 20 μl of phycoerythrin (PE)-conjugated anti-CD11b and FITC-conjugated anti-CD14 antibodies were added and incubated for 45 min at room temperature. After incubation, the cells were washed twice again with cold PBS and re-suspended in 0.5 ml of PBS. Two-parameter analysis was performed by FACS Calibur flow cytometry with the aid of Cell Quest software (Becton Dickinson). All of the differentiation-induction experiments were independently performed at least 3 times. For morphological assessment of differentiation, 100 μl of cell suspension were cytospun on to slides and examined microscopically using the Wright-Giemsa stain according to

the manufacturer's instruction. Percent differentiation was calculated as the number of cells that were differentiated divided by the total number of cells counted.

6. Western blot analysis

3×10^6 cells were dissolved in 100 μl of a SDS-PAGE sample buffer containing-mercaptoethanol. The lysates were sonicated for 15 sec with a Vibra Cell Sonicator, boiled for 10 min, and further analyzed by Western blotting. The protein yields were quantified using the Bio-Rad Dc protein assay kit (Hercules, CA) and equivalent amounts of the protein (40 μg) were applied to the 15% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked at room temperature with 3% bovine serum albumin (BSA) in TBST (1X TBS, 0.1% Tween 20) for 16 h. After washing twice in TBST, the membranes were incubated with the primary antibodies for 2 h at room temperature. The membranes were then washed four times in TBST, and incubated with the relevant HRP-conjugated secondary antibodies (1:3000 dilution with 3% BSA in TBST) for 1h. After washing four times in TBST, the reactive proteins were visualized by an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Densitometry was performed using the Molecular Dynamics Imaging system and ImageQuant 3.3 software (Amersham Biosciences) to quantify the relative amounts of the protein detected on Western blots.

7. Statistical analysis

Statistical analysis was done with paired-samples t-test using SPSS software, version 12.0 (SPSS Inc, Chicago, IL). Differences were considered to be statistically significant at $p<0.05$.

III. RESULTS

1. CD44 ligation with A3D8 inhibited proliferation and induced the apoptosis of THP-1 cells

Measurement of viable cell numbers over a 4-day period showed that treatment of 2.5 μg/mL A3D8 anti-CD44 mAb strongly inhibited the growth of the THP cell line (Fig. 1). The antibody controled THP-1 cells consistently increased in their numbers, but the number of A3D8-treated THP-1 cells eventually decreased after only slight increment until initial 48 hours.

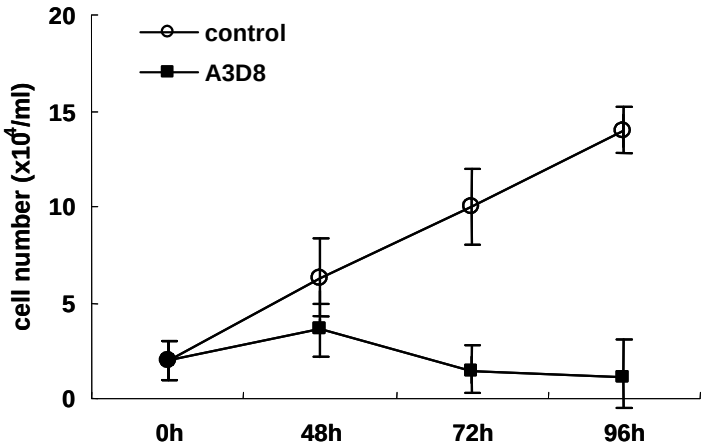


Figure 1. Time-dependent proliferation in THP-1 cells by A3D8 treatment.
Cell suspensions (1×10^5cells/ml) were incubated in triplicate in the presence of 2.5 μg/ ml of A3D8. Controls were cells incubated with isotype matched IgG1 (20 μg/ ml) as an antibody-control. At the indicated times, the viable cell number was determined using trypan blue dye exclusion. The statistical difference between A3D8-treated cells and controls is reached from day 3($p<0.05$).

In addition, A3D8 induced apoptotic death of THP-1 cells. When the THP-1 cells were incubated with A3D8 for 72 hour, an obvious apoptosis of THP-1 cells was observed. The mean percentage of apoptotic cells was significantly increased compared to the control THP-1 cells (13.7±1.2% versus 3.0±0.6%, $p<0.01$) (Fig. 2).

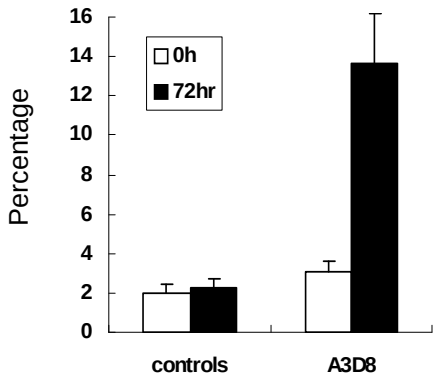


Figure 2. Time-dependent apoptosis in THP-1 cells by A3D8 treatment.
THP-1 cells were incubated in the presence of A3D8 (2.5 μg/ml) for 72 hours. At the indicated times, apoptosis was quantified by measuring the percentage of annexin V-FITC-positive, propidium iodide-negative cells, by flow cytometry.

2. CD44 ligation with A3D8 induced terminal differentiation of THP-1 cells

It was observed that THP-1 cells terminally differentiated into monocytic lineage after CD44 ligation. The THP-1 cells treated with 2.5 μg/ml of A3D8 mAb for 3 days showed an increased expression of the monocytic-specific antigen CD14 (from 1.8± 0.1% to 38.2±3.2%) and of the myeloid-specific antigen CD11b (from 6.6±0.6% to 66.6±1.6%) (Fig. 3A, 3B). It implies that A3D8 induced THP-1 cells to differentiate mainly, but not exclusively, towards the monocytic lineage.

The increase in the expression of these differentiation antigens was time- and dose-dependent(Table 1). Besides induction of cell surface marker expression, A3D8 induce changes in cell morphology. A3D8-treated THP-1 cells showed morphologic features typical of mature monocytes such as decreased nucleus-cytoplasmic ratio and nucleoli numbers, chromatin condensation, and irregular cytoplasmic contours (data not shown).

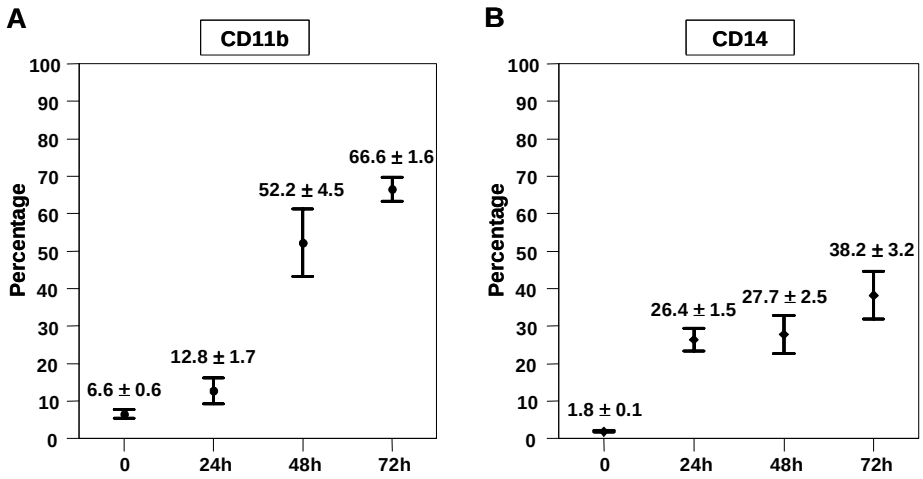


Figure 3. Time-dependent differentiation in THP-1 cells by A3D8 treatment. Increased expression of lineage differentiation antigens. The expression of CD11b (A) and CD14 (B) was measured as described in "Material and methods".

Table 1. Time- and dose-dependent expression of CD11b and CD14 by A3D8 treatment

	A3D8					
	0.63 μg/ml		1.25 μg/ml		2.5 μg/ml	
	CD11b	CD14	CD11b	CD14	CD11b	A3D8
0 hr	6.3±0.3	1.3±0.2	5.1±1.1	2.0±0.4	6.6±0.6	1.8±0.1
24 hr	7.3±1.2	2.1±0.4	8.9±2.1	11.7±2.5	12.8±1.7	26.4±1.5
48 hr	11.1±3.1	2.4±2.0	17.4±2.9	14.2±2.9	52.2±4.5	27.7±2.5
72 hr	12.4±3.3	4.1±1.2	28.7±1.8	16.3±2.4	66.6±1.6	38.2±3.2

3. CD44 ligation with A3D8 induced a G_0/G_1 arrest in THP-1 cells

Cell cycle arrest is a common feature of cells undergoing terminal differentiation and defective proliferation. Based on the growth inhibitory and differentiation-inducing effects of A3D8 on THP-1 cells, cell cycle analysis in response to A3D8 was performed. The DNA content analysis showed that the THP-1 cells underwent a G_0/G_1 arrest (Table 2). The proportion of cells in G_0/G_1 phase increased from 29.5% (controls) to 56.2% in A3D8-treated cells after 48 hours, and from 35.4% to 99.5% after 96 hours. This was mirrored by a decreased in the proportion of cells in S and G/M phase.

Table 2. The changes of cell cycle in THP-1 cells by A3D8 treatment

	48 hour		72 hour		96 hour	
	control	A3D8	control	A3D8	control	A3D8
G0/G1	29.5±2.1	56.2±5.4	33.4±1.5	68.3±3.3	34.4±4.3	99.5±4.3
S	48.9±3.3	24.8±3.1	40.5±2.2	22.8±2.9	43.1±2.4	0.1±0.8
G2/M	21.6±2.7	19.0±2.2	26.1±3.5	8.9±1.4	22.5±4.2	0.4±0.6

4. Activation of Raf-1/MEK/ERK kinase signaling pathway during A3D8-induced terminal differentiation of THP-1 cells

To understand the early molecular mechanism involved in the A3D8-induced terminal differentiation of THP-1 cells, we examined the activation of the Raf-1/MEK/ERK and p38 MAPK pathway by analyzing the phosphorylation status of these molecules using phospho-specific antibodies. A3D8 treatment of THP-1 cells induced the activation of MEK1/2 kinase (Ser217/221), which was coincident with the phosphorylation of upstream kinase Raf-1(Fig. 4). Time-course analysis revealed that the phosphorylation of MEK1/2 (Ser217/221) was detected within 30 min after A3D8 treatment and lasted for more than 48 h. Since differentiation began only 12 h after the A3D8 treatment, and became more prominent after 48 h, these results indicate that the ERK pathway is activated by CD44 ligation with A3D8 before the appearance of the differentiation phenotype.

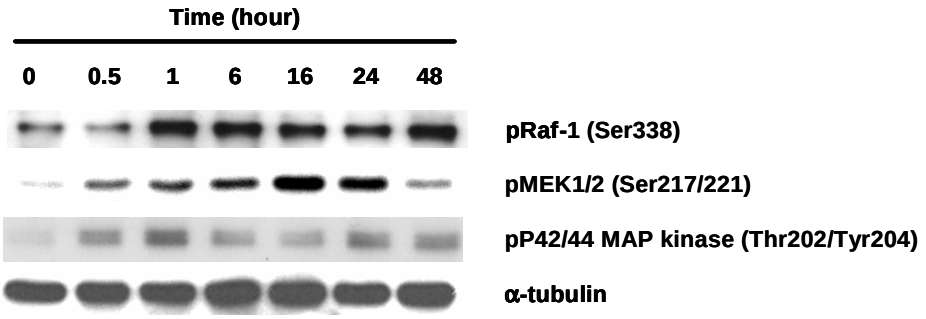


Figure 4. Time-dependent phosphorylation of Raf-1, MEK1/2, and ERK1/2 in THP-1 cells by A3D8 treatment.
After CD44 ligation by 2.5 μg/ml of A3D8, THP-1 cells were harvested at the indicated time and equivalent amounts of the protein (40 μg) were electrophoresed on a 15% acrylamide gels. The protein levels of phosphorylated Raf-1 (pRaf-1), phosphorylated

MEK1/2 (pMEK1/2), and phosphorylated ERK1/2 (p42/44 MAP kinase) were analyzed by immunoblotting. The phosphorylation of Raf-1, MEK1/2 and ERK1/2 were all increased at half hour and sustained up to 48 hour after A3D8 treatment to THP-1 cells.

These levels of phosphorylation were sustained even after 6 days of A3D8 treatment. By contrast, Western blot analysis using the antibody that detects total MEK1/2 protein revealed that A3D8 treatment did not affect the cellular levels of these proteins. The activation of ERK1/2 (p42/44 MAP kinase) was also observed(Fig. 4). At 30 min after 2.5 g/ml of A3D8 was added to the THP-1 cells, phospho-ERK1/2 proteins were elevated and this increase lasted at least for 48 h. Taken together, these data showed that CD44 ligation by A3D8 treatment activates the MEK/ERK signaling, possibly initiated through the activation of Raf-1.

Whether the A3D8-induced terminal differentiation of THP-1 cells was mediated by p38 mitogen-activated protein kinase (MAPK) signaling pathway was examined. We firstly determined the protein levels of phospho-p38 MAPK, an activated form, in A3D8-treated THP-1 cells. As shown in Figure 5, the level of phospho-p38 MAPK was highly elevated in untreated THP-1, and this increased phosphorylation of p38 MAPK was dramatically decreased after 48～72 h of treatment of A3D8. However, total p38 MAPK level remained unchanged through the entire time course.

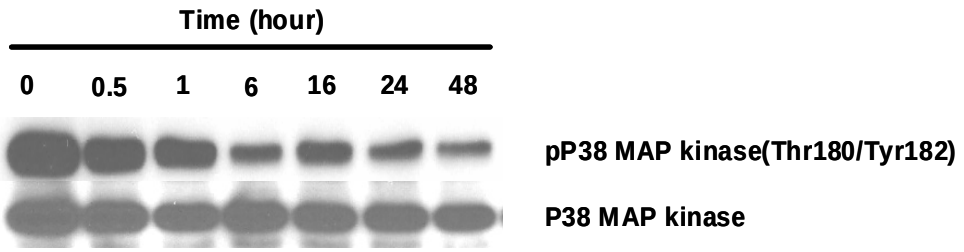


Figure 5. The changes in p38 MAP kinase and its phosphorylation in THP-1 cells by A3D3 treatment.
After CD44 ligation by 2.5 μg/ml of A3D8, THP-1 cells were harvested at the indicated time and equivalent amounts of the protein (40 μg) were electrophoresed on a 15% acrylamide gels. The protein levels of p38 MAPK (P38 MAP kinase), and phosphorylated p38 MAPK (pP38 MAP kinase) were analyzed by immunoblotting. The phosphorylated p38 MAP kinase was decreased and sustained to 48 hour without any changes in the level of p38 MAP kinase in THP-1 cells by A3D8 treatment.

5. Activation of PI3-K/Akt signaling pathway during A3D8-induced terminal differentiation

We examined whether the PI3-K/Akt pathway is activated during the A3D8-induced terminal differentiation process of THP-1 cells. Like the phosphorylation of Raf-1, PI3-kinase was concomitantly increased by A3D8 treatment. However, the time of PDK phosphorylation was delayed until 6 hour after A3D8 treatment(Fig. 6A). In addition, the rapid phosphorylation(Ser473) of Akt was also observed within 30 min of A3D8 treatment(Fig. 6B). These levels of phosphorylation were sustained by day 4 of A3D8 treatment before declining. However, A3D8 treatment did not affect the total levels of Akt proteins in THP-1 cells.

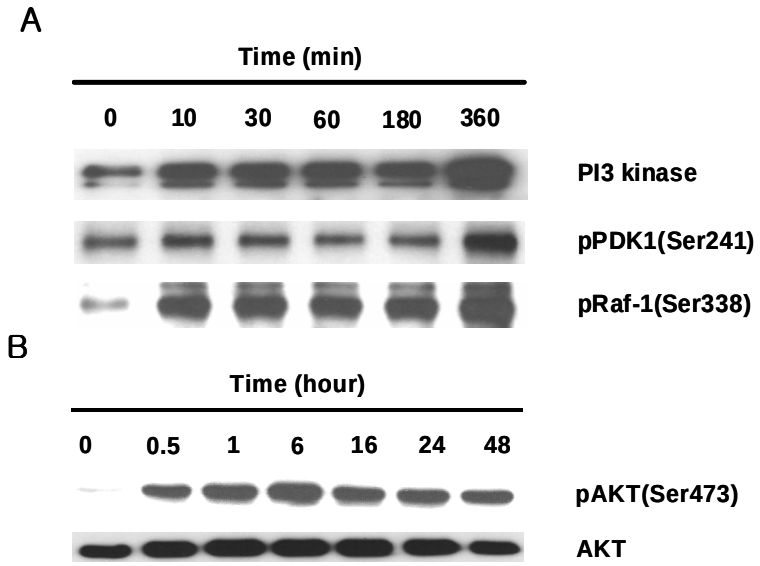


Figure 6. The changes in PI3-K/Akt pathway in THP-1 cells by A3D3 treatment.
After CD44 ligation by 2.5 μg/ml of A3D8, THP-1 cells were harvested at the indicated time and equivalent amounts of the protein (40 μg) were electrophoresed on a 15% acrylamide gels. The protein levels of PI3 kinase, phosphorylated PDK1 (Ser241) (pPDK1 (Ser241)), phosphorylated Raf-1 (Ser338) (pRaf-1(Ser338)), Akt, and phosphorylated Akt (Ser473) (pAkt(Ser473)) were analyzed by immunoblotting. (**A**) Both the level of PI3-K and phosphorylated Raf-1 were concomitantly increased at 10 minutes after A3D8 treatment, but phosphorylation of PDK1 was delayed until 6 hour. (**B**) The phosphorylation of Akt was also increased after A3D8 treatment without changes in total level of Akt.

Since differentiation began only 12 h after the A3D8 treatment and became more prominent after 48 h, these results indicate that the Akt pathway was activated by CD44 ligation before the appearance of the differentiation phenotype. Taken together, these data show that CD44 ligation by A3D8 treatment activates the Akt pathway, and it is possibly initiated through the activation of PI3-K.

6. Inhibition of the MEK/ERK signaling pathway by PD98059 and U0126

To determine whether the activation of ERK1/2 was due to the activity of MEK, a specific MEK inhibitors, PD98059 and U0126 were used. PD98059 potently inhibits MEK-1 enzymes, and U0126 inhibits both MEK-1 and MEK-2 enzymes. THP-1 cells were pre-incubated with varying doses of PD98059 (0-20 μM) or U0126 (0-10 μM) for 30 minutes followed by CD44 ligation by A3D8 treatment for 30 minutes.

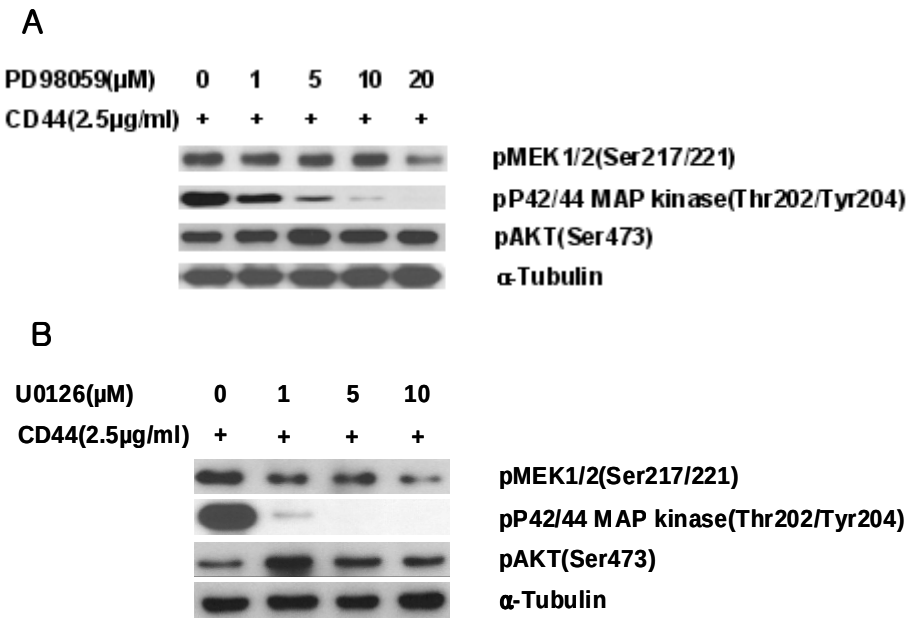


Figure 7. The changes of ERK and Akt phosphorylation in THP-1 cells by pre-treatment of PD98059 or U0126 before A3D8 treatment.
THP-1 cells were pre-incubated with varying doses of (**A**) PD98059 and (**B**) U0126 for 30 minutes followed by CD44 treatment for 30 minutes. Both PD98059 and U0126 inhibited the phosphorylation of ERK1/2.

As shown in Figure 7, there was a dose-dependent inhibition of ERK1/2 phosphorylation by PD98059 and U0126 indicating this activation by CD44 ligation by A3D8 was MEK-dependent.

7. PI3-K/Akt signaling is required for A3D8-induced Raf-1/MEK/ERK activation in THP-1 cells

We next investigated whether the signaling crosstalk between the Raf-1/MEK/ERK and PI3-K/Akt pathway is present during the A3D8-induced THP-1 differentiation. As shown in Figure 8, the pre-treatment of LY294002 diminished the A3D8-induced activation of Raf/MEK pathway. Conversely, the MEK-1 inhibitor PD98059 did not affect the A3D8-induced PI3-K/Akt activation in THP-1 cell(Fig. 7A).

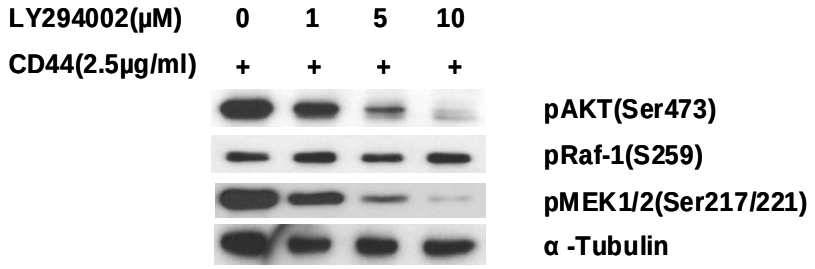


Figure 8. The changes of Akt, Raf-1, and MEK1/2 phosphorylation in THP-1 cells by pre-treatment of LY294002 before A3D8 treatment.
THP-1 cells were pre-incubated with varying doses of LY294002 for 30 minutes followed by CD44 treatment for 30 minutes. The phosphorylation of MEK1/2 by CD 44 ligation was inhibited by LY294002 treatment.

8. MEK/ERK signaling is essential for A3D8-induced terminal differentiation in THP-1 cells

To further determine whether the activation of MEK/ERK signaling was critically responsible for THP-1 cell differentiation induced by CD44 ligation, we examined the effect of MEK1 inhibitor PD98059 on the expression of cell surface differentiation markers. When THP-1 cells were pre-treated with 20 μM of PD98059 for 2 h before the addition of A3D8 mAb, PD98059 abolished the A3D8-induced phosphorylation of

MEK/ERK. Likewise, the A3D8-induced expression of the monocytic-specific antigen CD14 and of the myeloid-specific antigen CD11b were potentially decreased with PD98059 pretreatment (CD14 and CD11b expression at day 6 were reduced from 74.2 % and 81.3% in A3D8-treated cultures to 4.5% and 32.7% in CD44+PD98059 cultures, respectively) (Fig. 9). When THP-1 cells were pre-treated with 10 μM of U0126, another MEK inhibitor, for 2 h before the addition of A3D8 mAb, the A3D8-induced expression of the monocytic-specific antigen CD14 and of the myeloid-specific antigen CD11b were also decreased (CD14 and CD11b expression at day 6 were reduced from 74.2% and 81.3% in A3D8-treated cultures to 10.8% and 11.0% in CD44+U0126 cultures, respectively).

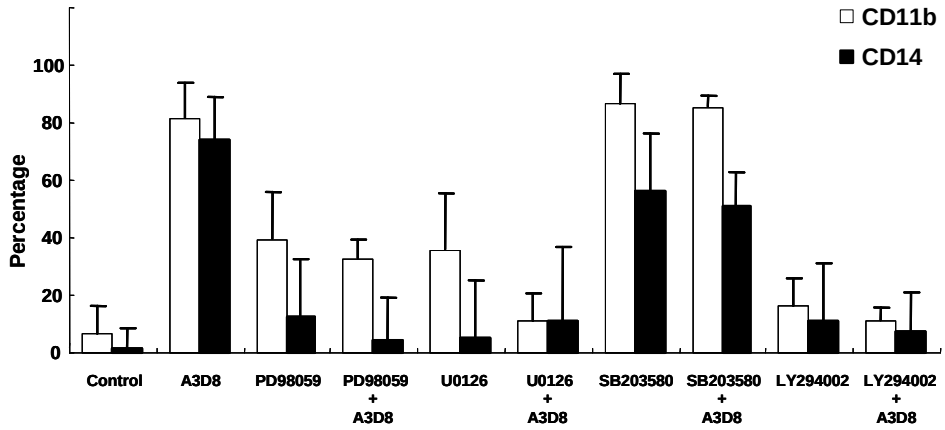


Figure 9. The changes in expression of CD11b and CD14 by treatment with A3D8 and various inhibitors

THP-1 cells were harvested after incubation with A3D8 (lane 2), PD98059 (lane 3), U0126 (lane 5), SB203580 (lane 7), or LY294002 (lane 9) alone. THP-1 cells were also harvested after pre-incubation with PD98059 (lane 4), U0126 (lane 6), SB203580 (lane 8), or LY294002 (lane 10) before A3D8 treatment. The expression of CD11b and CD14 was measured as described in "Material and methods".

MEK inhibitors, PD98059 and U0126, and Akt inhibitors, LY294002, reduced the increased expression of CD14 and CD11b in THP-1 cells by CD44 ligation. However, p38 MAP kinase inhibitor, SB203580, did not reduce the increased expression of CD14 and CD11b. This suggests that ERK activation is responsible for A3D8-induced

terminal differentiation in THP-1 cells. However, when THP-1 was treated with A3D8 and SB203580, the p38 MAP kinase inhibitor, these reduction of CD14 and CD11b by MEK inhibitor was not shown (CD14 and CD11b expression at day 6 were reduced from 74.2% and 81.3% in A3D8-treated cultures to 51.0% and 85.4% in CD44+SB203580 cultures, respectively).

9. PI3-K/Akt signaling is essential for A3D8-induced terminal differentiation in THP-1 cells

We examined the effect of PI3-K inhibitor LY294002 on the A3D8-induced terminal differentiation of THP-1 cells. Pretreatment of cells with 20 μM of LY294002 for 2 h before the addition of A3D8 mAb abrogated the A3D8-induced phosphorylation of Akt as well as acquisition of differentiation surface markers. A3D8-induced expression of CD14 and CD11b was potentially inhibited with LY294002 pretreatment (CD14 and CD11b expression at day 6 were reduced from 74.2% and 81.3% in A3D8-treated cultures to 7.7% and 10.8% in CD44+LY294002 cultures, respectively) (Fig. 9). This suggests that PI3-K/Akt activation is responsible for A3D8-induced terminal differentiation in THP-1 cells.

IV. DISCUSSION

In this study, I have demonstrated that the activation of Raf-1/MEK/ERK and PI3-K/Akt pathway is critically involved in the anti-CD44 mAb A3D8-induced terminal differentiation of THP-1 leukemia cells. Furthermore, the inhibition of PI3-K/Akt pathway blocked the A3D8-induced activation of Raf-1/MEK/ERK pathway, suggesting the existence of cross-talk between the PI3-K/Akt and Raf-1/MEK/ERK pathway in the A3D8-induced terminal differentiation of THP-1 cells.

It was previously reported that the ligation of CD44 with two activating mAbs, A3D8[8] or H90[9], triggers terminal differentiation of leukemia cells with variable extent according to the cell types[8,28]. Since the CD44 antigen is expressed on the surface of leukemic blasts from most AML, these findings suggest new possibilities for the development of CD44-targeted differentiation therapy in AML. However, the molecular mechanisms involved in the CD44 ligation-induced terminal differentiation of leukemic cells have not been fully elucidated.

The evidence indicating the role of MEK/ERK kinase pathway in the hematopoietic differentiation has been accumulated. Sustained activation of the ERK kinase pathway is required for the megakaryocytic differentiation of K562 cells[16], monocytic/granulocytic differentiation of HL-60 cells[15,29,30], and monocytic differentiation of U937 leukemia cells[14], which was induced by TPA treatment. It was also shown that inhibition of ERK activation by PD98059 blocked differentiation of ML-1[17] and U937[18] cells.

Present study clearly demonstrates that A3D8-induced terminal differentiation of THP-1 cells was mediated by activation of the MEK/ERK pathway, as indicated by rapid and sustained increase in the levels of activated forms of kinases such as phospho-Raf-1, phospho-MEK1/2, and phospho-ERK1/2 prior to the monocytic differentiation of THP-1 cells. The finding that PD98059 pretreatment abolished the A3D8-induced terminal differentiation as well as phosphorylation of MEK/ERK supports the notion that Raf-1/MEK/ERK activation is required for the A3D8-induced differentiation of THP-1 cells.

The p38 MAPK pathway was reported to be involved in erythropoietin-induced

erythroid differentiation of mouse erythroleukemia SKT6 cells[31], and butyrate-induced erythroid differentiation of K562 cells[32,33]. Activation of p38 MAPK is also crucial for STI571-induced multi-lineage differentiation in K562 cells[34]. However, p38 MAPK pathway is not activated during the A3D8-induced THP-1 differentiation. Instead, the baseline level of phospho-p38 MAPK was increased in THP-1 cells and this phosphorylation was down-regulated by A3D8 treatment. Pre-incubation of THP-1 cells with SB203589 before A3D8 treatment resulted in a further increase in the level of CD11b and CD14 expression. The finding that MEK inhibitor PD98059 treatment abrogated the A3D8-induced terminal differentiation and down-regulation of phospho-p38 MAPK suggests that the involvement of p38 MAPK pathway in the A3D8-induced terminal differentiation of THP-1 cells is mediated by MEK/ERK pathway. Of relevance to this study, it was recently reported that p38 MAP kinase inhibitor SB203580 could induce HL-60 cells to differentiate along the granulocytic lineage, which was mediated by activation of Raf/MEK/ERK signaling[35]. Since the expression of cell surface differentiation markers was observed well before the subsequent decrease in the phosphor-p38 MAPK level at 48 h, we conclude that the differentiation induction in THP-1 cells cannot be attributable to inhibition of p38 MAPK.

The PI3-K/Akt pathway also has been demonstrated to be crucial in the regulation of cellular differentiation including the chondrocytes[21], myoblasts[22], Th cells[23], neuronal cells[24], enterocytes[36] and adipocytes[25]. However, the roles of PI3-K/Akt pathway in the differentiation of leukemic cells have not been fully elucidated. A previous study by Lin[37] showed that treatment of THP-1 cells with bryostatin-1 induced a rapid and enduring activation of MEK but not Akt kinase during the differentiation process. In contrast, we demonstrated that both of PI3-K/Akt and Raf-1/MEK/ERK pathways were activated during the A3D8-induced THP-1differentiation. Pre-incubation of cells with LY294002 potentially blocked the A3D8-induced terminal differentiation of THP-1 cells, suggesting that PI3-K/Akt activities are critically involved in the A3D8-induced differentiation.

While the current understanding of the regulatory role of PI3-K in Raf/MEK/ERK pathways is not quite clear in the hematopoietic cells, a few reports have documented that ERK kinase activation was inhibited by PI3-K inhibitors[38]. It was recently demonstrated that both of ERK and Akt/PKB are activated by Steel factor in the

hematopoietic stem cell-like cell lines[39]. In addition, PI3-K inhibitors block not only Akt/PKB activation but also activation of Raf and ERK. However, in mast cells and other committed hematopoietic precursors, the activation of ERK by Steel factor is not PI3-K-dependent. Duckworth and Cantley[40] found that PI3-K can regulate MAPKs depending on the cell type, the ligand and also the number of the specific growth factor receptors on the cell surface. Some cross-talk between the IGF-1-inducible PI3-K pathway and the MEK/ERK pathway in myeloma cells exists, as evidenced by the inhibition of MEK1/2 and ERK phosphorylation by LY294002[41]. In contrast, such cross-talk was not seen in other studies using cell lines in which constitutive activation of PI3-K and MAPK pathway was not found[42], suggesting that this cross-talk selectively occurs during IGF-1-dependent activation of these pathways. These results suggest that the type of intercommunication between these pathways depends on the differentiation stage of specific cell types and ligand-receptor specificity. However, the roles of these two pathways in the process of leukemia cell differentiation have not previously been evaluated.

This study showed that p-MEK1/2 was sensitive to LY294002 when the THP-1 cells were stimulated by A3D8, suggesting that cross-talk between PI3-K and Raf-1/MEK/ERK pathway is in place. Furthermore, the finding that the MEK-1 inhibitor, PD98059, did not affect the A3D8-induced PI3-K/Akt activation in THP-1 cells indicates that the PI3-K/Akt-induced activation of Raf-1/MEK/ERK pathway is mediated at the level of (or upstream of) Raf in the A3D8-induced THP-1 differentiation. The findings that the association between the Raf-1/MEK/ERK and PI3-K/Akt pathway was not observed in the A3D8-treated HL-60 and U937 leukemia cells in our study (data not shown) or bryostatin-1-induced differentiation of THP-1 cells[37] indicate that the signaling pathway activated during the leukemia cell differentiation might be different according to the cell type and differentiation inducing agent. Our results suggest that the crosstalk between the Raf-1/MEK/ERK and PI3-K/Akt pathways exists during the process of CD44-induced terminal differentiation of THP-1 cells and not in the same undifferentiated THP-1 cells. These varying effects could be mediated by other as yet unidentified protein partners, which are only capable of bridging Raf-1 and PI3-K/Akt/PKB in certain cell types, under certain conditions. Although the mechanisms by which the Raf-1/MEK/ERK cascade become PI3-K-dependent during A3D8-induced

THP-1 differentiation is not fully understood, it is possible that the cross-regulation between PI3-K and Raf-1/MEK/ERK pathways may be important in other leukemia cell lineages, in which such cross-talk may similarly depend on differentiation stage. Understanding the mechanisms by which the Raf-1/MEK/ERK and PI3-K/Akt pathways regulate CD44-induced terminal differentiation may thus contribute to the development of differentiating agents in leukemic disorders.

V. CONCLUSION

This study has demonstrated that the activation of Raf-1/MEK/ERK and PI3-K/Akt pathway is critically involved in the anti-CD44 mAb A3D8-induced terminal differentiation of THP-1 leukemia cells. Furthermore, the inhibition of PI3-K/Akt pathway blocked the A3D8-induced activation of Raf-1/MEK/ERK pathway, suggesting the existence of cross-talk between the PI3-K/Akt and Raf-1/MEK/ERK pathway in the A3D8-induced terminal differentiation of THP-1 cells.

The results can be summarized as follows.

1. CD44 ligation with A3D8 inhibited proliferation and induced the apoptotic cell death of THP-1 cells.
2. CD44 ligation with A3D8 induced terminal differentiation of THP-1 cells. Both the expression of monocytic-specific antigen CD14 and myeloid-specific antigen CD11b were increased in the A3D8-treated THP-1 cells.
3. CD44 ligation with A3D8 induced a G0/G1 arrest in THP-1 cells. The proportion of cells in G0/G1 phase increased in A3D8-treated THP-1 cells.
4. During the terminal differentiation of THP-1 cells by A3D8 treatment, the Raf-1/MEK/ERK signaling pathway was activated. The phosphorylated form of Raf-1, MEK1/2, and ERK1/2 were increased at half hour and sustained up to 48 hour after A3D8 treatment to THP-1 cells, and decreased by specific MEK inhibitors, PD98059 and U0126. These inhibitors also decreased the expression of CD11b and CD14 of A3D8-treated THP-1 cells. Taken together, these results suggest that Raf-1/MEK/ERK signaling pathway is essential for A3D8-induced terminal differentiation in THP-1 cells.
5. A3D8 treatment also activated the PI3-K/Akt signaling pathway. The phosphorylated Akt (Ser473) was increased at half hour and sustained up to 48 hour after A3D8 treatment to THP-1 cells. Interestingly, the Akt inhibitor, LY294002, inhibited the phosphorylation of MEK1/2 as well as Akt, and the differentiation of THP-1 cells by A3D8. The PI3-K/Akt signaling is another essential pathway for A3D8-induced terminal differentiation in THP-1 cells.

This result may provide a new experimental basis for a differentiation therapy in AML.

REFERENCES

1. Degos L. Differentiating agents in the treatment of leukemia. Leuk Res 1990;14: 717-719
2. Sievers EL. Targeted therapy of acute myeloid leukemia with monoclonal antibodies and immunoconjugates. Cancer Chemother Pharmacol 2000;46(Suppl):S18-S22
3. Waxman S. Differentiation therapy in acute myelogenous leukemia (non-APL). Leukemia 2000;14:491-496.
4. Reuss-Borst MA, Buhring HJ, Klein G, Muller CA. Adhesion molecules on CD34+ hematopoietic cells in normal human bone marrow and leukemia. Ann Hematol 1992;65:169-174.
5. Kortlepe K, Bendall LJ, Gottlieb DJ. Human acute myeloid leukaemia cells express adhesion proteins and bind to bone marrow fibroblast monolayers and extracellular matrix proteins. Leukemia 1993;7:1174-1179
6. Liesveld JL, Frediani KE, Harbol AW, DiPersio JF, Abboud CN. Characterization of the adherence of normal and leukemic CD34+ cells to endothelial monolayers. Leukemia 1994;8:2111-2117.
7. Verfaillie CM, Benis A, Iida J, McGlave PB, McCarthy JB. Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-terminal heparin-binding domain of fibronectin: Cooperation between the integrin alpha 4 beta 1 and the CD44 adhesion receptor. Blood 1994;84:1802-1811.
8. Charrad RS, Gadhoum Z, Qi J, Glachant A, Allouche M, Jasmin C, et al. Effects of anti-CD44 monoclonal antibodies on differentiation and apoptosis of human myeloid leukemia cell lines. Blood 2002;99:290-299.
9. Gadhoum Z, Delaunay J, Maquarre E, Durand L, Lancereaux V, Qi J, et al. The effect of anti-CD44 monoclonal antibodies on differentiation and proliferation of human acute myeloid leukemia cells. Leuk Lymphoma 2004;45:1501-1510.
10. Herrera R, Hubbell S, Decker S, Petruzzelli L. A role of the MEK/MAPK pathway in PMA-induced cell cycle arrest: Modulation of megakaryocytic differentiation of K562 cells. Exp Cell Res 1998;238:407-414
11. Lessor T, Yoo JY, Davis M, Hamburger AW. Regulation of heregulin beta1-

induced differentiation in a human breast carcinoma cell line by the extracellular-regulated kinase (ERK) pathway. J Cell Biochem 1998;70:587-595.

12. Hu X, Moscinski LC, Valkov NI, Fisher AB, Hill BJ, Zuckerman KS. Prolonged activation of the mitogen-activated kinase pathway is required for macrophage-like differentiation of a human myeloid leukemic cell line. Cell Growth Diifer 2000; 11:191-200.
13. Racke FK, Lewandowska K, Goueli S, Goldfarb AN. Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. J Biol Chem 1997;272: 23366-23370.
14. Pulvere BJ, Hughes K, Franklin CC, Kraft AS, Leevers SJ, Woodgett JR. Co-purification of mitogen-activated protein kinase with phorbol ester-induced c-Jun kinase activity in U937 leukemia cells. Oncogene 1993;21:3334-3558.
15. Kharbanda S, Saleem A, Emoto Y, Stone R, Rappa U, Kufe D. Activation of Raf-1 and mitogen-activated protein kinases during monocytic differentiation of human myeloid leukemia cells. J Biol Chem 1994;269:872-878.
16. Whalen AM, Galasinski SC, Sharpiro PS, Nahreini TS, Ahn NG. Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase. Mol Cell Biol 1997;17:1947-1958.
17. He H, Wang X, Gorospe M, Holbrook NJ, Trush MA. Phorbol ester-induced mononuclear cell differentiation is blocked by the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059. Cell Growth Differ 1999;10:307-315.
18. Okuma E, Inazawa Y, Saeki K, You A. Potential roles of extracellular signal-regulated kinase but not p38 during myeloid differentiation of U937 cells stimulated by cytokines. Augmentation of differentiation via prolonged activation of extra-cellular signal-regulated kinase. Exp Hematol 2002;30:571-581.
19. Datta K, Bellacosa A, Chan TO, Tsichlis PN. Akt is a direct target of the phos-phatidylinositol 3-kinase. Activation by growth factors, v-src and v-Ha-ras, in Sf9 and mammalian cells. J Biol Chem 1996;271:30835-30839.
20. Scheid MP, Woodgett JR. PKB/AKT: Functional insights from genetic models. Nat Rev Mol Cell Biol 2001;2:760-768.
21. Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, et al. Runx2

induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J Cell Biol 2004;166:85-95.
22. Sarker KP, Lee KY. L6 myoblast differentiation is modulated by CDK5 via the PI3K-AKT-p70S6K signaling pathway. Oncogene 2004;23:6064-6070.
23. Arimura Y, Shiroki F, Kuwahara S, Kato H, Dianzani U, Uchiyama T, et al. Akt is a neutral amplifier for Th cell differentiation. J Biol Chem 2004;279:11408-11416.
24. Ditlevsen DK, Kohler LB, Pedersen MV, Risell M, Kolkova K, Meyer M, et al. The role of phosphatidylinositol 3-kinase in neural cell adhesion molecule-mediated neuronal differentiation and survival. J Neurochem 2003;84:546-556.
25. Nakae J, Kitamura T, Kitamura Y, Biggs WH 3rd. Arden KC, Accili D. The forkhead transcription factor Foxo 1 regulates adipocyte differentiation. Dev Cell 2003;4: 119-129.
26. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002;296:1655-1657.
27. Rommel C, Clarke BA, Zimmermann S, Nunez L, Rossman R, Reid K, et al. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. Science 1999;286:1738-1741
28. Charrad RS, Li Y, Delpech B, Balitrand N, Clay D, Jasmin C, et al. Ligation of the CD44 adhesion molecule reverses blockage of differentiation in human acute myeloid leukemia. Nat Med 1999;5:669-676.
29. Yen A, Roberson MS, Varvayanis S, Lee AT. Retinoic acid induced mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase-dependent MAP kinase activation needed to elicit HL-60 cell differentiation and growth arrest. Cancer Res 1998;58:3163-3172.
30. Miranda MB, McGuire TF, Johnson DE. Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia 2002; 16:683-692.
31. Nagata Y, Todokoro K. Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. Blood 1999;94:853-863.
32. Witt O, Sand K, Pekrun A. Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathway. Blood 2000;95:2391-2396.

33. Park JI. Choi HS, Jeong JS, Han JY, Kim IH. Involvement of p38 kinase in hydroxyurea-induced differentiation of K562 cells. Cell Growth Differ 2001;12: 481-486.
34. Kohmura K, Miyakawa Y, Kawai Y, Ikeda Y, Kizaki M. Different roles of p38 MAPK and ERK in STI571-induced multi-lineage differentiation of K562 cells. Cell Physiol 2004;198:370-376.
35. Zhang JX, Zhuang WJ, Poon KH, Yang M, Fong WF. Induction of HL-60 cell differentiation by the p38 mitogen-activated protein kinase inhibitor SB203580 is mediated through the extracellular signal-regulated kinase signaling pathway. Anti-cancer Drugs 2003;14:31-38.
36. Gentili C, Morelli S, Russo De Boland A. Involvement of PI3-Kinase and its association with c-Src in PTH-stimulated rat enterocytes. J Cell Biochem 2002;86: 773-783.
37. Lin H, Chen C, Li X, Chen BD. Activation of the MEK/MAPK pathway is involved in bryostatin1-induced monocytic differentiation and up-regulation of X-linked inhibitor of apoptosis protein. Exp Cell Res 2002;272:192-198.
38. Rommel C, Hafen E. Ras - A versatile cellular switch. Curr Opin Genet Dev 1998; 8:412-418.
39. Wandzioch E, Eding CE, Palmer RH, Carlsson L, Hallberg B. Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/ stem cell lines. Blood 2004;104:51-57.
40. Duckworth BC, Cantley LC. Conditional inhibition of the mitogen-activated protein kinase cascade by wortmannin. J Biol Chem 1997;272:27665-27670.
41. Qiang YW, Kopantzev E, Rudicoff S. Insulinlike growth factor-1 signaling in multiple myeloma: Downstream elements, functional correlates, and pathway cross-talk. Blood 2002;99:4138-4146.
42. Liu P, Leong T, Quam L, Billadeau D, Kay NE, Greipp P, et al. Activating mutations of N- and K-ras oncogenes in multiple myeloma show different clinical associations; analysis of the Eastern Cooperative Oncology Group phase III trial. Blood 1996;88:2699-2706.

ABSTRACT(IN KOREAN)

CD44-연관 신호전달체계 활성화에 의한 THP-1 세포 분화유도 및 그 기전 규명

<지도교수 민 유 홍>

연세대학교 대학원 의학과

정 준 원

항 CD44 단클론 항체(A3D8)를 이용한 CD44와의 결합은 사람 백혈병 세포주인 THP-1의 단세포성 분화를 유도한다. 그러나 이러한 세포분화의 정확한 분자적 기전은 정확히 알려져 있지 않다. 본 연구에서 A3D8에 의한 THP-1의 분화에 있어 세포외 신호전달 활성화효소인 p38 mitogen-activated protein kinase (MAPK)와 phosphatidylinositol 3-kinase (PI3-K)/Akt 경로의 중요성에 대해 연구하였다. A3D8 처리 72시간 후에 THP-1 세포는 성숙 단세포의 전형적인 세포 특징을 보였으며, 단세포-특이 세포표면항원인 CD14 (1.8±0.1%에서 38.2±3.2%)와 골수계 특이 항원인 CD11b (6.6±0.6%에서 66.6±1.6%) 표현이 증가하였다. 이러한 분화관련 항원의 증가는 A3D8의 처리량 및 시간에 의존적이었다. A3D8 단클론 항체에 의한 CD44 결합은 인산화 Raf-1, 인산화 MEK1/2 및 인산화 ERK1/2와 같은 세포외 신호조절 활성화효소 경로의 주요 활성화효소들의 빠르고 지속적인 활성화를 유도하였다. 또한, A3D8 단클론 항체에 노출된 직후 THP-1 세포에서 Ser473 Akt 인산화가 관찰되었으며, 이후 인산화가 지속되었다. 반면에 p38 MAPK 인산화는 CD44 결합에 의해 감소되었다. MEK1 억제제인 PD98059 및 U0126을 THP-1세포에 전처지하였을 때, A3D8에 의한 MEK1/2 및 ERK1/2 활성화는 물론 THP-1의 분화가 억제되었다. PI3-K 억제제인 LY294002 전처치 시에도 A3D8 단클론 항체에 의한

세포분화가 거의 완전히 억제되며, A3D8에 의한 MEK/ERK 활성화가 억제 되었고 Raf-1 활성화는 억제되지 않았다. 이상의 결과를 종합하여 볼 때, CD44 결합에 의한 THP-1 세포의 분화유도과정에서 PI3-K/Akt 경로와 Raf/MEK/ERK 경로가 중요한 역할을 하며, 이 두 신호전달체계가 서로 연결되어 있음을 확인할 수 있었다.

핵심되는 말 : CD44, 분화, THP-1 세포, Raf-1/MEK/ERK, PI3-K/Akt