Part Ⅳ Analysis of mechanisms of differentiation induced by cytokinins

In this part, I examined changes in signal transduction pathways, differentiation-associated gene expressions and transcriptional factors during differentiation induced by cytokinins, in comparison with those by ATRA or VD_3, typical inducers of terminal differentiation of the leukemia cells.

Effects of some inhibitors of signal transduction on the IPA-induced differentiation

To assess the involvement of signal transduction pathways which are thought to be related to differentiation in the mechanism of action of IPA, I examined the effects of several inhibitors on the IPA-induced differentiation of HL-60 cells. First, I examined the effect of IPA on activities of various protein kinases including Src, EGF receptor, Flt-1, Ca/calmodulin-dependent and protein kinase C, phosphoinositide 3-kinase, protein kinase A, cAMP-activated protein kinase, and MAPK, using each specific inhibitors. Of the inhibitors tested, PD98059, a specific inhibitor of extracellular signal-regulated kinases 1/2 (ERK1/2) pathway significantly suppressed the differentiation induced by IPA. On the other hand, I found SB203580, a specific inhibitor of p38 MAPK pathway induced the differentiation of human leukemia cells, but this differentiation was not associated with the inhibition of p38 MAPK activity (50). Other inhibitors of signal transduction did not essentially affect the cytokinin-induced differentiation of the cells (49). Next, I examined the effects of Ado receptor A_1, A_2 and A_3 antagonists on the cytokinin-induced differentiation and found that they did not have any significant effects (49). Thus, these results indicated that the effects of

cytokinin on HL-60 cells are unlikely to be mediated by extracellular Ado receptors. Among the inhibitor 9 tested, only PD98059 effectively inhibited IPA-induced differentiation.

Involvement of ERK1/2 activation in the cytokinin-induced differentiation

MAPKs form a large family of serine-threonine protein kinases which are conserved through evolution (81). In manmmalian cells, four distinct MAPK cascades have been identificated: ERK1/2, c-jun amino-terminal kinases (JNK) or stress-activated protein kinases (SAPK), p38 MAPK, and Erk5/BMK. The classical MAP kinases, ERK1/2 are activated by a variety of stimuli for cell growth and differentiation, and play central roles in mitogenic signaling. Activation of ERK1/2 is coordinated with the induction of differentiation of myeloid leukemia cells (71, 110). It was reported that ATRA, a potent inducer of granulocytic differentiation activated ERK1/2 in HL-60 cells before inducing granulocytic differentiation and growth arrest (124). To determine whether IPA induces the differentiation of HL-60 cells by a similar mechanism to that of ATRA, I examined the effects of IPA on ERK1/2 activity in HL-60 cells. The activity was measured as the phosphorylation of Elk-1, a substrate of ERK1/2 by western blotting. IPA significantly increased ERK1/2 activity in a dose dependent manner by 2 hours, and this enhancement persisted for 24 hours (Fig.Ⅳ -1A,C and data not shown). The enhancement of ERK1/2 activity by IPA was more prominent than that by ATRA (Fig.Ⅳ-1C). The increased ERK1/2 activity was reduced by pretreatment with PD98059 which specifically inhibit upstream of ERK1/2 called mitogen-activated protein kinase kinase (MEK) (Fig.Ⅳ-1B). To determine whether the MEK/ERK1/2 pathway plays a role in the process of

IPA-induced differentiation, HL-60 cells were treated with PD98059 prior to induction of differentiation. PD98059 also suppressed granulocytic differentiation, as assessed by NBT reducing activity, and slightly counteracted growth inhibition induced by IPA (Fig.Ⅳ-2). These results suggest that MEK/ERK1/2 signaling is essential for the cytokinin-induced differentiation.

Effect of cytokinins on expression of the transcription factors related to differentiation of myeloid leukemia cells

Development of hematopoietic cells is controlled by several lineage-specific and -nonspecific transcription factors (21, 105 and 125). Then, I examined the effect of IPA on the expression of several transcription factors that are associated with differentiation into granulocytes and monocytes. Although expression of c-myc, c-myb, c-jun, c-fos, and PU.1 was changed by treatment with IPA in a similar pattern of those by well-known inducers such as ATRA and VD_3 (data not shown), the expression of CCAAT enhancer binding protein (C/EBP) family proteins were differently regulated by IPA. C/EBPs form a subgroup of the basic region/leucine zipper super family of transcription factors that function as regulators of cell growth and differentiation in numerous cell types (44, 63 and 68). The six C/EBP members (α, β, δ, ε, γ, and CHOP; the C/EBP homologous protein) have been identified in mammalian cells, and they have their own roles on the functions of the cells (93).

Expression of main members of this family including C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, CHOP in the HL-60 cells treated with IPA, ATRA, or VD_3 for 24 hours was examined by western

blotting. In this family, C/EBPα is the most crucial for granulocytic differentiation (54, 92). It has been reported that C/EBP α-null mice lack the entire granulocyte lineage (107, 127), and dominant-negative mutations of C/EBP α were found in leukemia patients (88). Although ATRA and VD_3 enhanced the expression of C/EBPα, IPA did not affect it (Fig.Ⅳ-3). On the other hand, IPA greatly enhanced the expression of C/EBPδ protein, but ATRA and VD_3 did not. C/EBPβ was constitutively expressed with or without inducers, and the expression of C/EBPε was slightly enhanced with all the inducers I tested. The expression of C/EBPγ was not detected in the cells, and that of CHOP was not changed by either treatment (data not shown). Taken together, IPA specifically enhanced the expression of C/EBPδ (Fig.Ⅳ-3).

In order to understand whether the expression of C/EBPδ is directly correlated with the differentiation of HL-60 cells induced by IPA, the cells were treated with antisense oligonucleotides for C/EBP family to inhibit their expression. HL-60 cells were treated with IPA in the presence of antisense oligomucleotides for C/EBP α, β, δ, or missense-δ for 3days, and the effects of antisense oligonucleotides on the growth inhibition and differentiation were observed. The growth-inhibitory effects and NBT reduction caused by IPA were significantly prevented by pre-incubation with antisense oligonucleotide for C/EBPδ, whereas treatment with antisense oligonucleotides for C/EBPα, C/EBPβ, and missense C/EBPδ did not (Fig.Ⅳ-4AB). Similar results were obtained by examination of morphological changes in HL-60 cells treated with IPA in the presence or absence of antisense oligonucleotides for C/EBP δ. Morphologic differentiation of HL-60 cells treated with IPA into granulocytes was completely prevented by pre-incubation with antisense oligomers for C/EBP δ (Fig.Ⅳ-4C). These results

suggest that C/EBPδ plays a crucial role on the IPA-induced differentiation of myeloid leukemia cells.

Microarray analysis of IPA-induced gene expression

In order to exhaustively investigate the gene expression-profiles, DNA microarray analysis using TaKaRa-Human 1k Chip were conducted, and compared the gene expression patterns of IPA with those of ATRA or VD_3. Total RNA was isolated from HL-60 cells treated with or without IPA, ATRA or VD_3 for 24 hours using Isogen, and commissioned continuous analysis to TaKaRa Bio Custom Service Center. I defined a highly expressed gene in HL-60 cells treated with IPA when the fluorescent ration was three times higher compared to untreated control (tableⅣ). Of these 13 genes, *tumor necrosis factor, alpha-induced protein 2* was only one which also expressed over three fold by treatment with ATRA and VD_3. TableⅣshows that several genes were preferentially up-regulated in HL-60 cells treated with IPA in comparison with ATRA and VD_3.

IPA greatly increased S100P mRNA expression, whereas ATRA or VD_3 hardly affected. S100P is a member of S100 calcium-binding protein family, mainly present in human placenta (6, 25). S100 family comprises 19 members that are differentially expressed in a large number of cell types and implicate in the regulation of variety of intracellular activities (23, 24). Previous studies have showed that some of the S100 family members such as S100A8, A9 and A12 are up-regulated in neutrophils and monocytes during inflammatory responses (22, 89 and 116). S100A8 and A9 exhibit potent chemoatractic activity for leukocytes, and have been suggested to affect

alteration of the cytoskeleton and cell shape (55, 67 and 98). However, the function of S100P is poorly understood.

To confirm the results from the microarray analysis, the expression of S100P mRNA was examined by RT-PCR analysis. As shown in Fig. Ⅳ-5A, the accumulation of the S100P transcript was greatly induced within 4 hours after treatment with IPA. Next, I investigated the expression of another S100 family members including S100A8, S100A9 and S100A12 mRNA in HL-60 cells treated with IPA, ATRA and VD_3 by RT-PCR. IPA hardly affected the mRNA expression of S100A8, S100A9 and S100A12 as well as ATRA and VD_3 for 24 hours, but gene expression of S100A8 and S100A9 was up-regulated within 3 days by these inducers (Fig. Ⅳ-5B and data not shown). These results suggest that the expression patterns of S100A8, S100A9 and S100A12 are not so different among the inducers. On the other hand, expression of S100P mRNA was greatly increased by IPA up to 24 hours, and it was sustained up to 6 days, whereas ATRA and VD_3 did not affect it even by 6-day-treatment (Fig. Ⅳ-6A). S100P was specifically up-regulated by IPA in HL-60 cells. To confirm the preferential up-regulation of S100P mRNA by IPA on HL-60 cells, I investigated the effects of other differentiation inducers and anticancer drugs on S100P mRNA expression by RT-PCR. Besides ATRA and VD_3, other compounds including dimethyl sulfoxide, actinomycin D, hydroxy urea and aphidicolin did not affect the expression of S100P mRNA, despite they greatly inhibited the growth of HL-60 cells (Fig. Ⅳ-6B). On the other hands, methyl jasmonate (MJ) and cotylenin A also significantly increased the expression of S100P mRNA (Fig. Ⅳ-6B). Jasmonate is one of the plant hormones, that play pivotal roles in their biological activities (114, 118), and cotylenin A is a plant-growth regulators. Furthermore, these two compounds have been found to

induce differentiation of the leukemia cells (2, 37, 38, 48 and 123). These results suggest that the expression of S100P mRNA was preferentially increased by agents which affect the growth and differentiation of both human leukemia and plant cells. Next, I investigated whether up-regulation of S100P mRNA is associated with the differentiation of HL-60 cells. HL-60 cells were treated with IPA and/or PD98059, and then, part of which was used for analysis of NBT-reducing activity (Fig.Ⅳ-7A) and the rest of which was used for isolation of mRNA(Fig. Ⅳ-7B). As shown in Fig.Ⅳ-2, PD98059 is an inhibitor of MEK, which was revealed to be necessary for the differentiation by IPA. When PD98059 prevented the NBT-reducing activity caused by IPA (Fig.Ⅳ-7A), the expression of S100P mRNA was examined by RT-PCR, and relative levels are depicted after normalizing to GAPDH mRNA levels. The expression of S100P was reduced in accompany with the inhibition of differentiation (Fig. Ⅳ-7B), suggesting that this gene expression is associated with the cytokinin-induced differentiation.

Figure Ⅳ-1. Induction of MAPK activity in HL-60 cells by IPA or ATRA.

(A) Cells were treated with various concentrations of IPA for 3 hours. ERK1/2 activity was analyzed as Elk-1 phosphorylation, one of the in vivo targets of ERK1/2 using phospho-antibodies by western blotting and chemiluminescent detection. (B) Inhibition of ERK1/2 activity by PD98059. Cells were treated with 0.15 mM IPA in the absence or presence (+) of 0.5 μM PD98059 for 24 hours. (C) Comparison of ERK1/2 activity between IPA and ATRA. Cells were treated with 0.15 mM IPA or 400 nM ATRA for 2 and 12 hours.

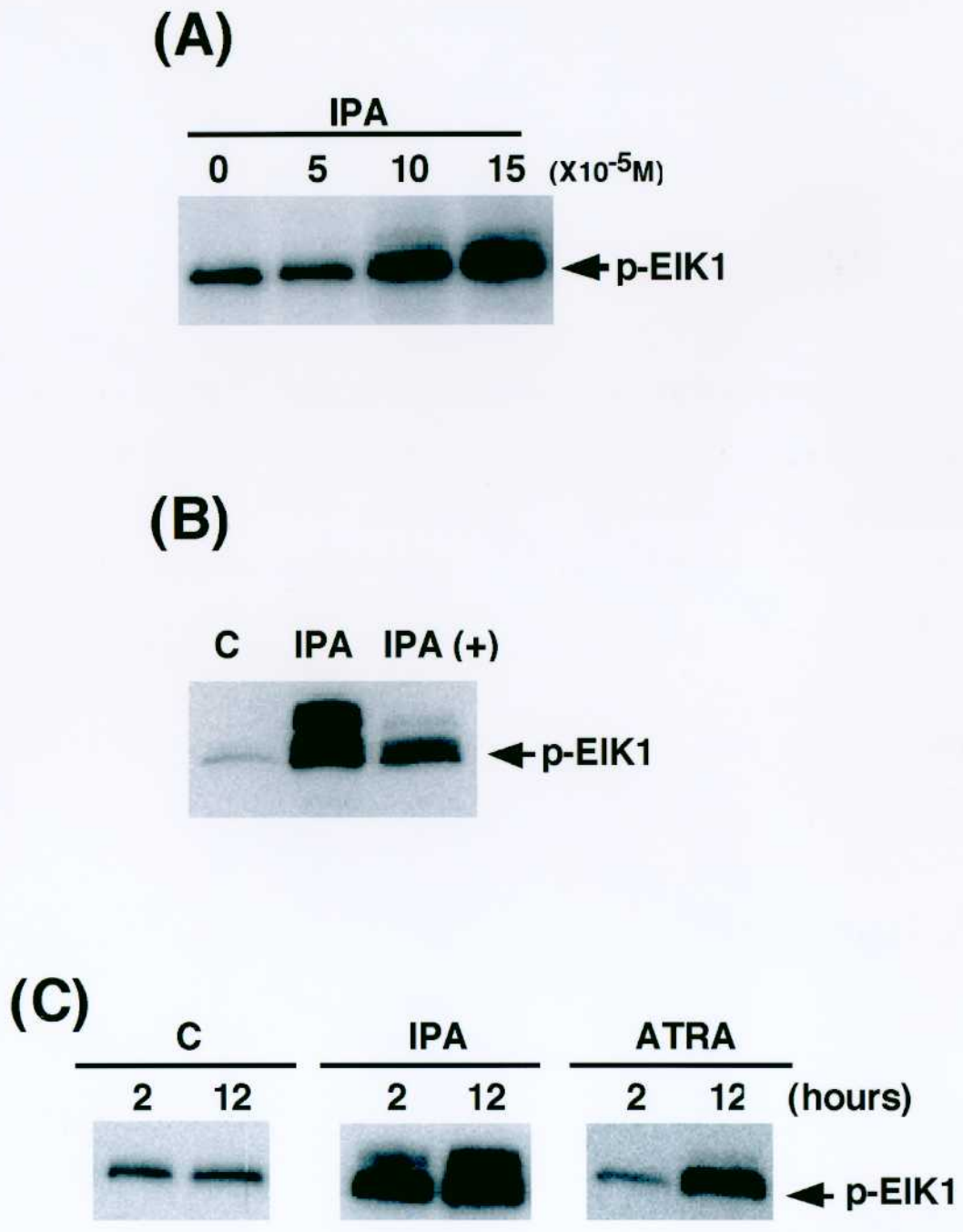
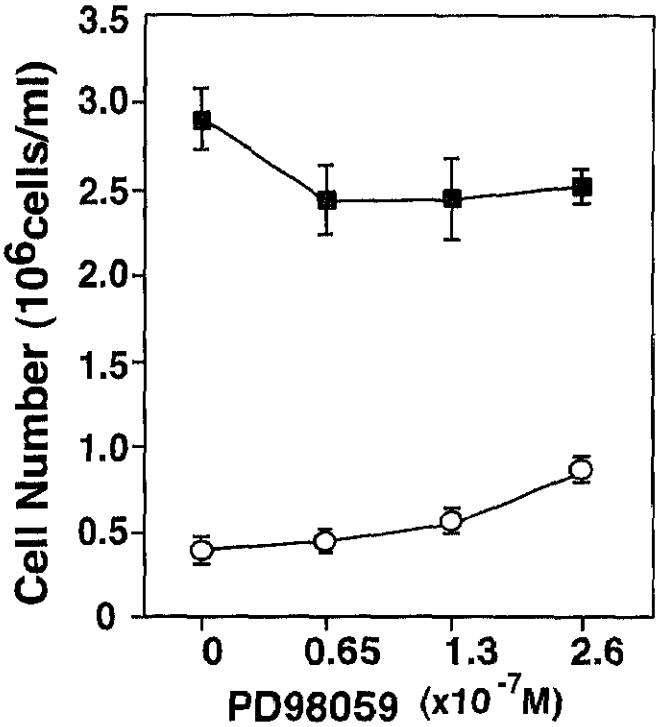

(A)
IPA
0 5 10 15 (X10-5M)
p-EIK1
(B)
C IPA IPA (+)
p-EIK1
(C)
C
IPA
ATRA
2 12 2 12 2 12 (hours)
p-EIK1

Figure Ⅳ-2. Effect of PD98059 on the IPA-induced growth inhibition (A) and NBT reduction (B) in HL-60 cells.

Cells were cultured with various concentrations of PD98059 in the absence (■), or presence of 0.1 mM IPA (○) for 4 days. Values are means of ± SD four separate experiments.

(A)


Cell Number (10^6cells/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.65
1.3
2.6
PD98059 (x10^{-7}M)

(B)

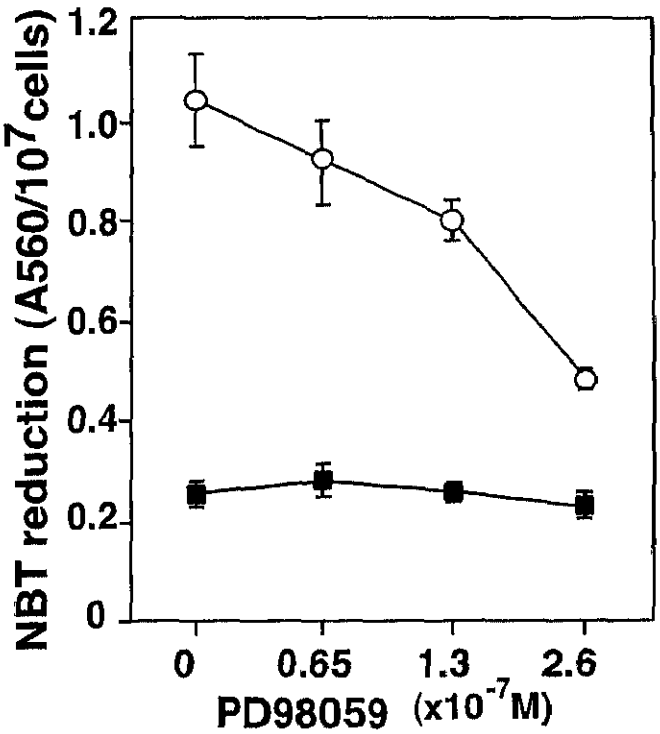

NBT reduction (A560/10^7cells)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.65
1.3
2.6
PD98059 (x10^{-7}M)

Figure Ⅳ-3. Expressions of C/EBP family proteins by differentiation inducers in HL-60 cells.

HL-60 cells were untreated or treated with 0.1 mM or 0.15mM IPA, 40 nM or 400 nM ATRA and 2.4 nM or 24 nM VD_3 for 1 day. C/EBP α, β, δ, ε proteins were detected by western blotting.

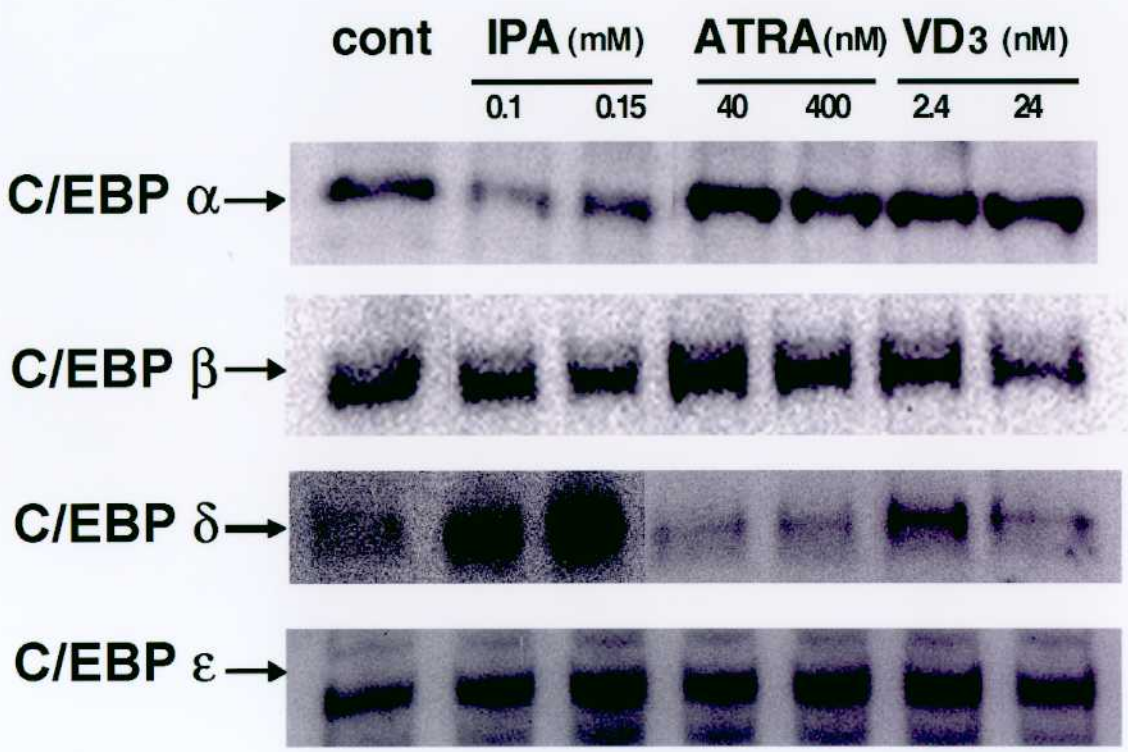
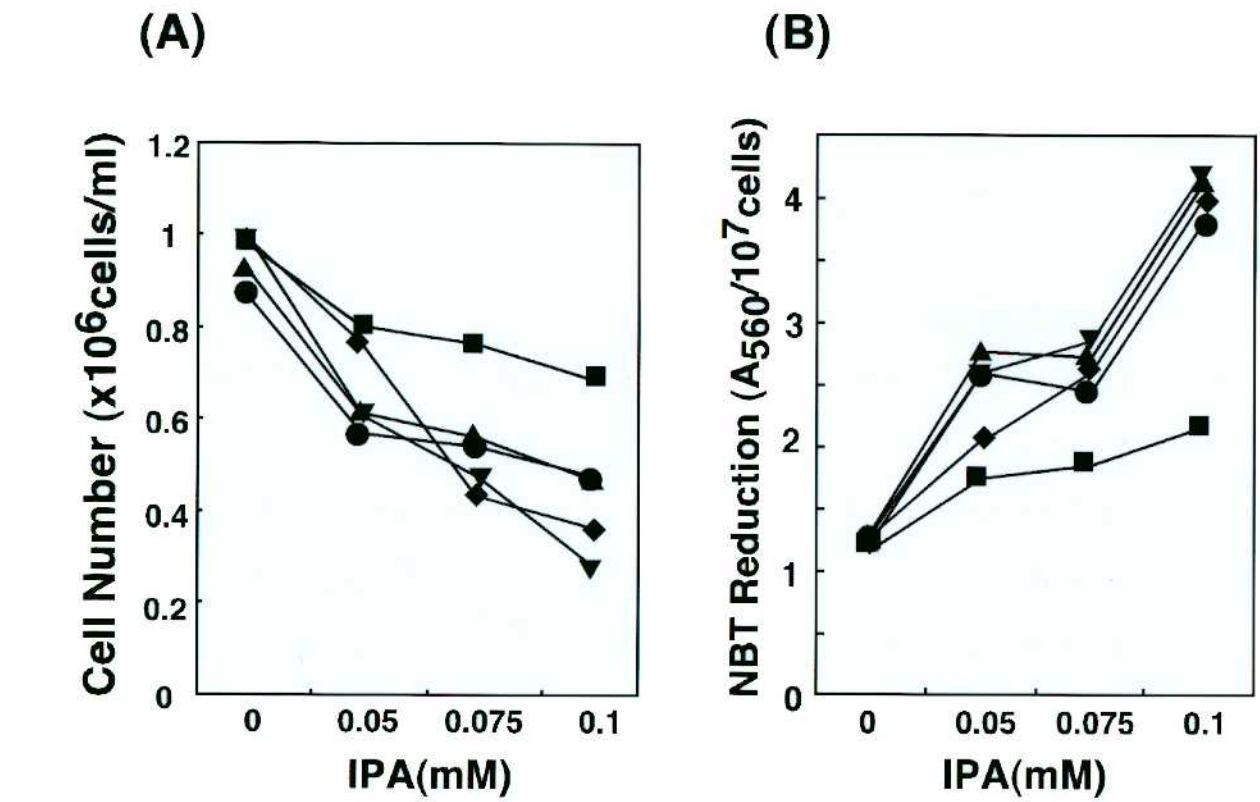

cont
IPA (mM)
0.1
0.15
ATRA (nM)
40
400
VD3 (nM)
2.4
24
C/EBP α
C/EBP β
C/EBP δ
C/EBP ε

Figure Ⅳ-4. Effects of antisense oligonucleotides on the growth inhibition and differentiation by IPA.

Cells were cultured with various concentrations of IPA in the absence (◆) or presence of 12 μM of antisense oligonucleotides for C/EBP α(●), β(▼), δ (■) and missense-C/EBPδ(▲) for 4days, and cell number (A) and NBT reduction (B) were examined. (C) Morphologic changes in HL-60 cells in the presence or absence of IPA and antisense oligonucleotides for C/EBP δ for 4 days.


(A)
Cell Number (x10^6cells/ml)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.05
0.075
0.1
IPA(mM)
(B)
NBT Reduction (A560/10^7cells)
4
3
2
1
0
0
0.05
0.075
0.1
IPA(mM)

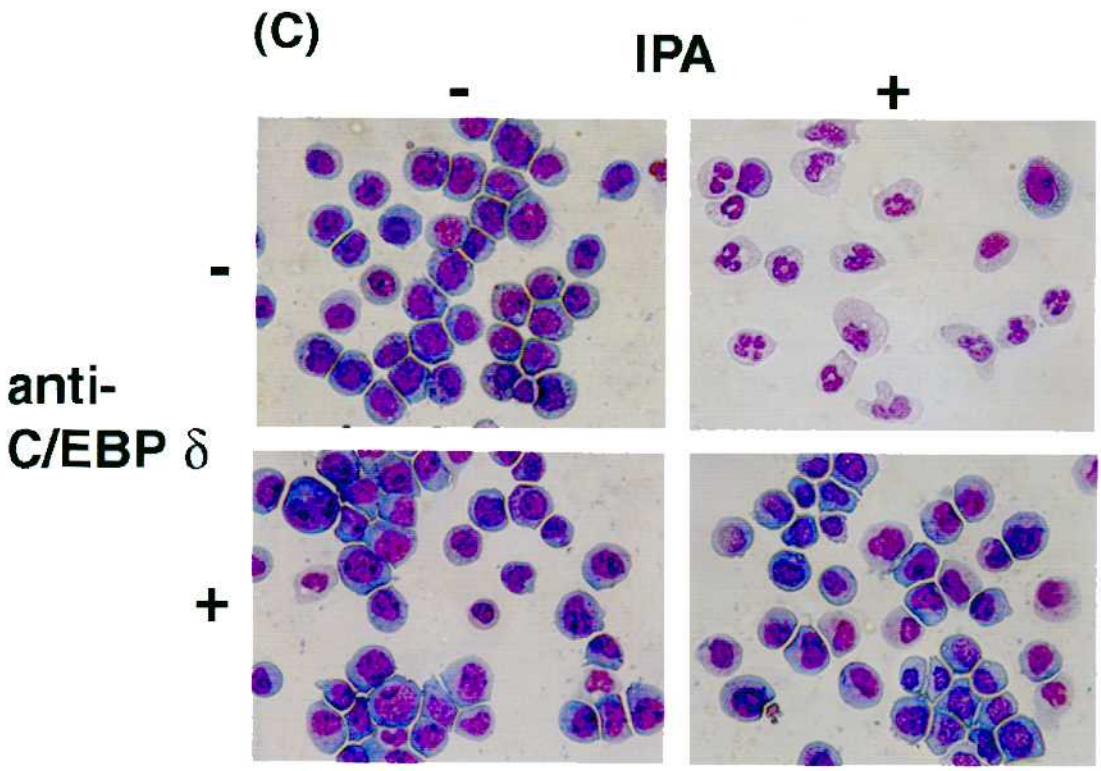

(C)
IPA
-
+
anti-
C/EBP δ
-
+

Table IV.

Highly expressed genes in HL-60 cells treated with IPA by microarray analysis.

Gene name	Fold increase
S100P calcium-binding protein P	8.12
phosphodiesterase 4B, cAMP-specific	7.82
tumor necrosis factor, alpha-induced protein 2	5.77
phorbol-12-myristate-13-acetate-induced protein 1	5.29
Clk-associating RS-cyclophilin	5.17
T cell receptor alpha locus	4.93
jagged 1 (Alagille syndrome)	4.42
B-cell translocation gene 1, anti-proliferative	4.17
Ric (Drosophila)-like, expressed in many tissues	4.15
thioredoxin reductase 1	3.9
regulator of G-protein signalling 16	3.88
Pirin	3.78
KIAA0410 gene product	3

Genes listed were expressed over three fold by treatment with IPA for 24 hours, identified by microarray analysis described in "materials and methods".

Figure Ⅳ-5. Effects of differentiation inducers on the expressions of S100 family members.

(A) Expressions of S100P mRNA were examined by RT-PCR analysis. HL-60 cells were untreated or treated with 0.1mM IPA, 400 nM ATRA, or 24 nM VD_3 for 4 hours. The levels of GAPDH expression are shown to demonstrate that equal amounts of RNA were used for RT-PCR. (B) Comparison of mRNA expression among S100 family by RT-PCR analysis. HL-60 cells were untreated or treated with 0.1mM IPA, 400 nM ATRA, or 24 nM VD_3 for 1 day. Graph depicts relative levels of mRNA after normalizing to GAPDH mRNA levels.

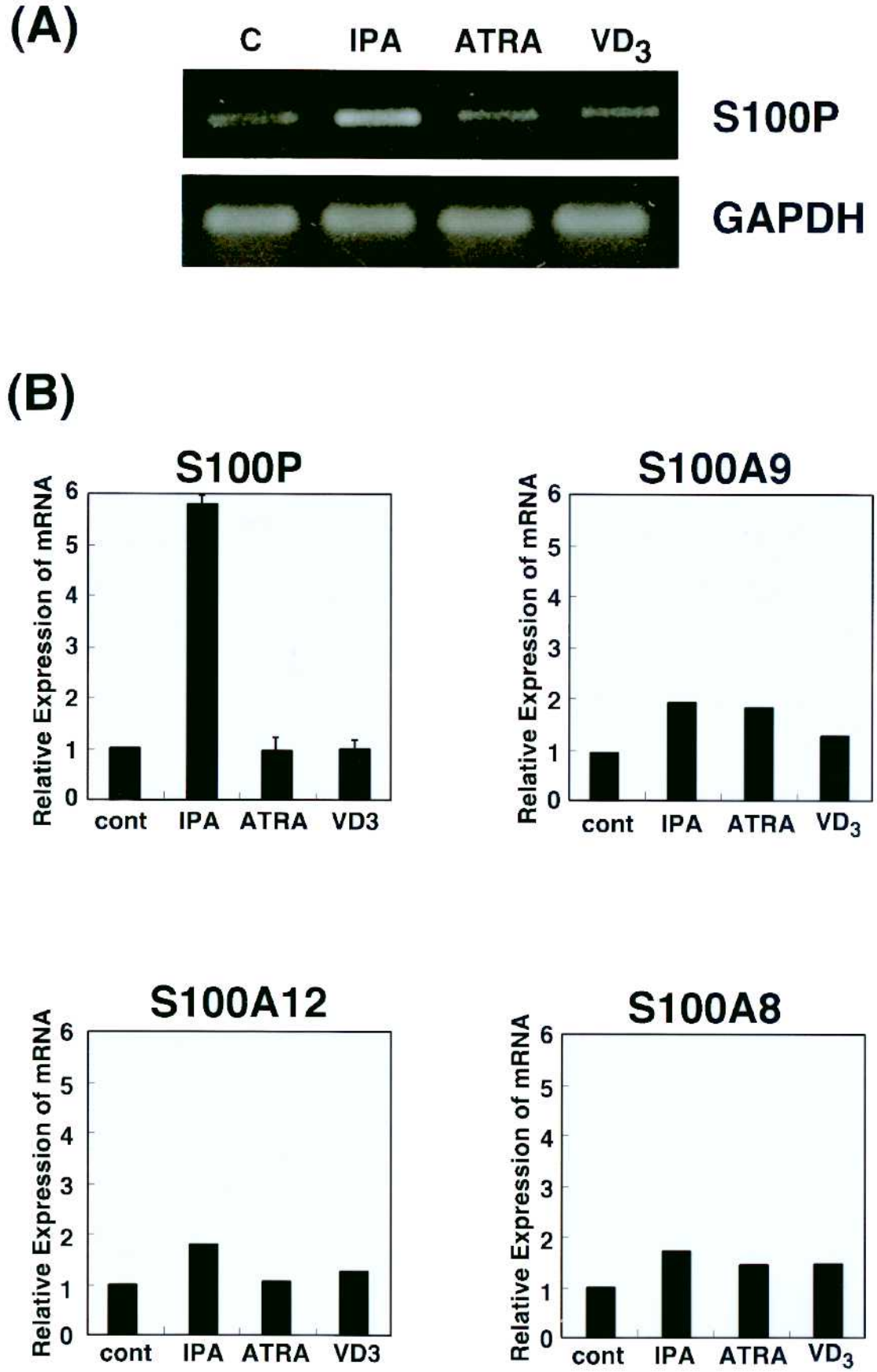
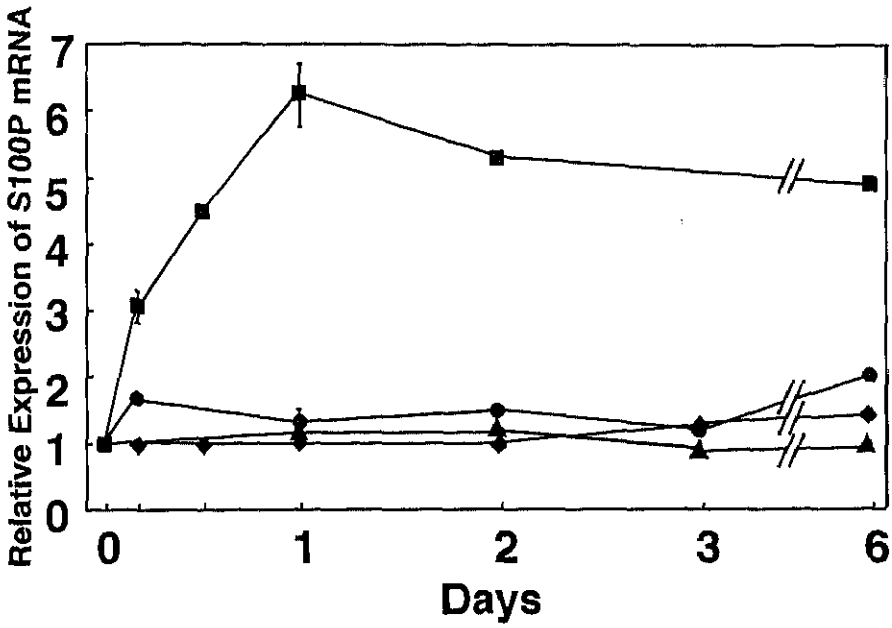

(A)
C
IPA
ATRA
VD3
S100P
GAPDH
(B)
S100P
S100A9
S100A12
S100A8
Relative Expression of mRNA
0
1
2
3
4
5
6
cont
IPA
ATRA
VD3

Figure Ⅳ-6. Effects of differentiation inducers on the expression of S100P mRNA in HL-60 cells.

(A) HL-60 cells were cultured without (◆) or with 0.15mM IPA (■), 400 nM ATRA (●) and 24 nM VD_3 (▲) for various days. Expression levels of S100P mRNA were determined by RT-PCR analysis. Graph depicts relative levels of S100P mRNA after normalizing to GAPDH mRNA levels.

(B) Non-association of S100P mRNA expression and growth inhibition. HL-60 cells were cultured with various concentrations of drugs and differentiation inducers; IPA(●), MJ(■), cotylenin A(◆), ATRA(▲), VD3(◎),
dimethyl sulfoxide (◇), actinomycin D (□), hydroxy urea (○) and aphidicolin (△) for 1 day. Graph depicts relative levels of S100P mRNA after normalizing to GAPDH mRNA levels.

(A)


Relative Expression of S100P mRNA
0
1
2
3
4
5
6
7
0
1
2
3
6
Days

(B)

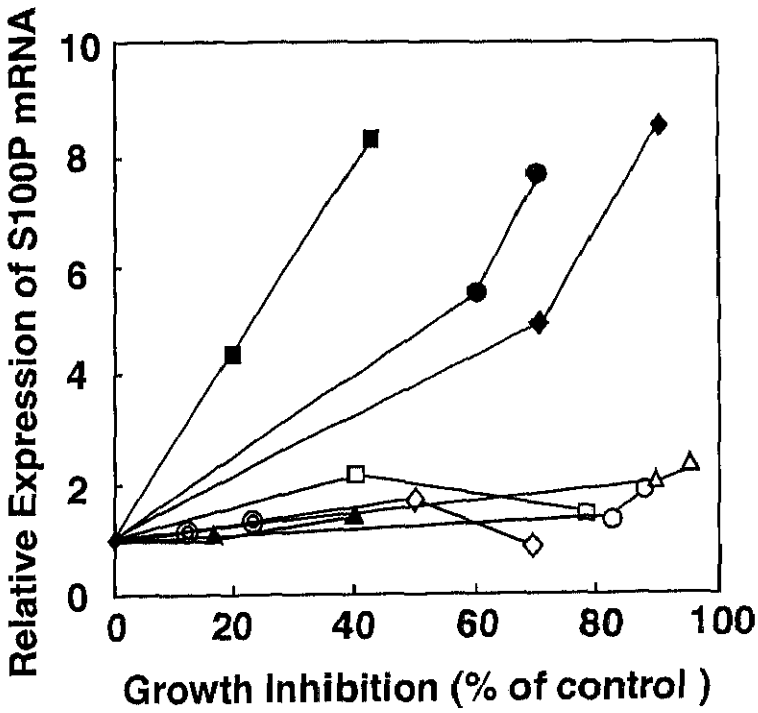
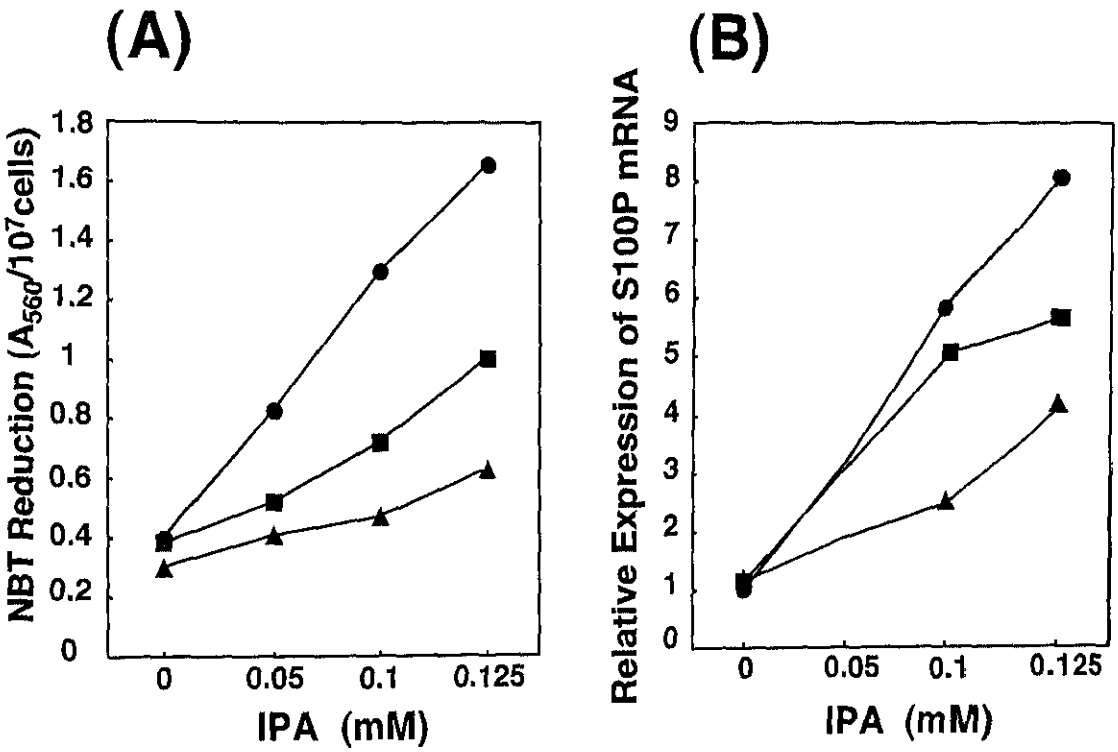

Relative Expression of S100P mRNA
0
2
4
6
8
10
0
20
40
60
80
100
Growth Inhibition (% of control)

Figure Ⅳ-7. Association of differentiation and expression of S100P mRNA induced by IPA in HL-60 cells.

Cells were cultured with various concentrations of IPA in the absence (●), or presence of $1.3x10^{-7}$M (■) and $2.6 x10^{-7}$M (▲) PD98059for 4 days. (A) Effect of PD98059 on the NBT reduction induced by IPA. (B) Effect of PD98059 on the IPA-induced expression of S100P mRNA in HL-60 cells. Total RNA was isolated form aliquot of cells used in (A), and expression levels were determined by RT-PCR analysis. Graph depicts relative levels of S100P mRNA after normalizing to GAPDH mRNA levels.


(A)
NBT Reduction (A_{560}/10^7cells)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.125
IPA (mM)
(B)
Relative Expression of S100P mRNA
9
8
7
6
5
4
3
2
1
0
0
0.05
0.1
0.125
IPA (mM)