Part Ⅱ Effects of cytokinin ribosides on the differentiation and apoptosis of HL-60 cells

Effect of cytokinin ribosides on the growth and differentiation of HL-60 cells

As described in Part Ⅰ, cytokinins such as kinetin, IPA, and BA induce to undergo granulocytic differentiation of human myeloid leukemia cells. Their riboside derivatives such as kinetin riboside, isopentenyladenosine (IPAR), benzyladenosine (BAR) are also known as cytokinins in many plants (Fig. Ⅱ-1), indicating that cytokinin ribosides have the almost same biological effects in plants (73).

Therefore, in this part, I examined the abilities of cytokinin ribosides to inhibit the growth and induce the differentiation of HL-60 cells. The cells were cultured with various concentrations of the analogs for 5 days, and the IC_{50} concentrations of the analogs were calculated . The riboside derivatives such as kinetin riboside, IPAR and BAR were more potent than the respective riboside-lacking compounds (Table. Ⅱ).

Next, I examined the abilities of these analogs to induce differentiation of HL-60 cells. Although the growth-inhibitory activities of cytokinin ribosides were much greater than those of their cytokinins, the differentiation-inducing effects of cytokinin ribosides were much weaker (Fig. Ⅱ-2). These results indicate that cytokinin ribosides are highly effective in inhibiting cell growth, whereas cytokinins are effective at inducing NBT reduction of HL-60 cells.

To investigate the differential effects of cytokinins and cytokinin ribosides on the growth and differentiation in HL-60 cells, I examined the morphologic changes of cells treated with IPA and IPAR (Fig. Ⅱ-3). HL-60 cells that had been treated with IPAR for 24 hours showed some characteristics of apoptosis (chromatin condensation, nuclear fragmentation and the formation of apoptotic bodies) (Fig. Ⅱ-3B). On the other hand, IPA induced the morphologic differentiation of HL-60 cells into granulocytes without apparent apoptosis after 6 days (Fig. Ⅱ-3C).

Induction of functional markers of apoptosis

Upon induction of apoptosis, cells express functional apoptosis markers, (Ⅰ) annexin V-FITC binding (Ⅱ) DNA ladder formation (Ⅲ) activation of caspase-3 (Ⅳ) intracellular ATP content (Ⅴ) disruption of the mitochondrial transmembrane potential (Ⅵ) generation of ROS. Then, I confirmed that cytokinin ribosides induce to undergo apoptosis in HL-60 cells by measurement of these functional characteristics specific for apoptosis.

(Ⅰ) Detect of annexin V binding

Some of the earliest apoptotic changes occur at the cell surface. One of the better understood cell surface modification is exposure of phosphatidylserine (PS). PS is confined to the inner layer of the lipid bilayer. However, after induction of apoptosis, under the influence of translocases, PS is flipped from the inner to the outer bilayer rendering the molecule available for detection. Annexin V is a blood clotting factor that exhibits a high specificity for PS binding. The coupling of annexin V to fluorescein

generates a direct, rapid, and simple method for the detection of apoptosis on unfixed cells (95).

Untreated cells showed no annexin V staining, whereas the cells progressively became annexin V-positive when treated with IPAR (Fig. Ⅱ-4A) or kinetin riboside (data not shown). On the other hand, IPA did not affect the annexin V binding even in a high concentration.

(Ⅱ) Detection of DNA fragmentation

DNA fragmentation occurs as one of the final stages of cell death and has long been considered a hallmark of apoptosis and one of the defining biochemical events of the pathway (96, 108).

Significant DNA fragmentation due to inter-nucleosomal cleavage occurred less than 12 hours after exposure to IPAR in a dose dependent manner. However, DNA fragmentation was not observed in HL-60 cells that were treated with a high concentration of IPA (Fig. Ⅱ-4B).

(Ⅲ) Activation of Caspase3

An apoptotic signal requires activation of caspases, members of a family of cystein proteases that are evolutionarily conserved determinants of cell death (14, 32 and 35).

IPAR or kinetin riboside increaesd caspase3 activity in a dose dependent manner, but IPA or kinetin did not (Fig. Ⅱ-5A). This activation was observed within 6 hours and persisted for at least 12 hours in a concentration-dependent manner (Fig. Ⅱ-5B).

(Ⅳ) Changes in the intracellular ATP content by IPAR

To examine the possibility that the actions of cytokinins and/or cytokinin ribosides may be involved in ATP metabolism, I examined the intracellular ATP content of HL-60 cells treated with IPA and IPAR (Fig. Ⅱ-6). Intracellular ATP was maintained in cells treated with IPA, whereas the ATP content dramatically decreased within 2 hours in cells treated with IPAR (Fig. Ⅱ-6A), and this decrease was concentration-dependent (Fig. Ⅱ-6B). Similar results were obtained when the cells were treated with kinetin or kinetin riboside (data not shown). These results suggest that the apoptosis-inducing effect of cytokinin ribosides is closely related to ATP depletion.

(Ⅴ) Mitochondrial transmembrane potential

The energy generated during cellular respiration accumulates in the mitochondrial transmembrane space as an electron gradient called the mitochondrial transmembrane potential. This electrochemical gradient is often disturbed during apoptosis, and mitochondrial dysfunction has been proposed to represent a "point of no return" during cell death (66, 87 and 126).

Figure. Ⅱ-7A shows that cytokinin riboside-induced changes may be closely related to disruption of the mitochondrial membrane potential. IPAR increased the proportion of the cells with depolarized mitochondrial transmembrane potential in a dose-dependent manner (Fig. Ⅱ-7B). On the other hand, neither kinetin nor IPA essentially affected the membrane potential (Fig. Ⅱ-7A).

(Ⅴ) Generation of ROS

Disruption of the mitochondrial transmembrane potential is usually associated with increased mitochondrial ROS production and mitochondrial membrane damage (57). To confirm the accumulation of ROS in IPAR-treated cells, I measured the cellular ROS content by flow cytometry analysis using hydroethidine, a compound specifically converted by ROS to highly fluorescent ethidium. Fig. Ⅱ-8A shows that treatment of HL-60 cells with IPAR caused a significant increase in ROS, whereas treatment with IPA did not (Fig. Ⅱ-8B). Similar results were obtained from kinetin and kinetin riboside. Taken together, IPAR or kinetin riboside-induced apoptosis was confirmed by several apoptotic markers.

Kinetin and IPA significantly induced NBT reduction in other human myeloid leukemia cells, such as ML-1, NB4 and U937 cells, and kinetin riboside and IPAR effectively induced apoptosis of these cells (data not shown). These results show that the effects of cytokinins and cytokinin ribosides are distinguishable from their ability to induce differentiation or apoptosis in human myeloid leukemia cells.

Induction of differentiation of HL-60 cells by cytokinin ribosides along with antioxidants and caspase inhibitors.

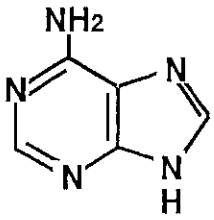
Cytokinins and cytokinin ribosides are similarly effective as plant redifferentiation-inducing hormones in most plant cells (75). Therefore, it is possible that cytokinin ribosides also induce the differentiation of human myeloid leukemia cells when apoptosis is prevented. Therefore, the antioxidant N-acetyl cysteine (NAC) or the O_2^- scavenger ambroxol was treated to prevent apoptosis induced by IPAR.

When HL-60 cells were incubated with IPAR in the presence of the antioxidant, NAC, morphological changes to apoptosis was prevented and morphological differentiation into granulocytes was observed in a dose dependent manner (Fig. Ⅱ-9A).

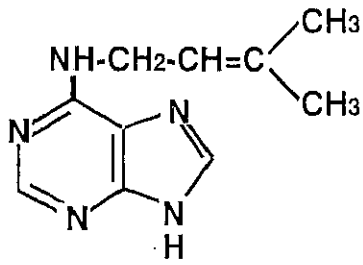
When HL-60 cells were incubated with IPAR in the presence of the O_2^- scavenger ambroxol, IPAR-induced apoptosis was significantly reduced and NBT reduction was greatly enhanced (Fig. Ⅱ- 9B). Morphological changes, CD11b expression and lysozyme induction n addition to NBT reduction was also induced by IPAR in the presence of ambroxol. On the other hand, neither ambroxol nor N-acetyl cysteine significantly affected IPA-induced NBT reduction (data not shown).

Furthermore, the differentiation-inducing effect of IPAR was also supported by experiments using caspase inhibitors. When HL-60 cells were incubated with kinetin riboside or IPAR in the presence of inhibitors of caspase-3, apoptosis was significantly reduced and expression of CD11b was induced (Fig. Ⅱ- 9C).

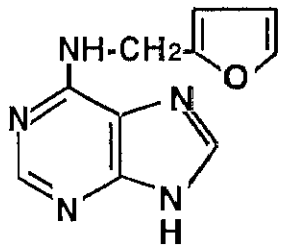
Figure Ⅱ-1. Structure of cytokinins and cytokinin ribosides



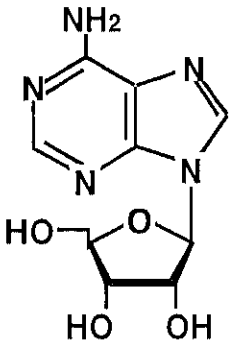
Adenine



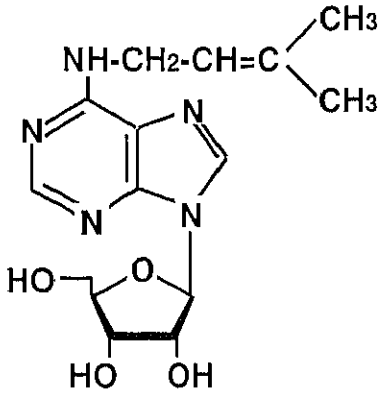
Isopentenyladenine



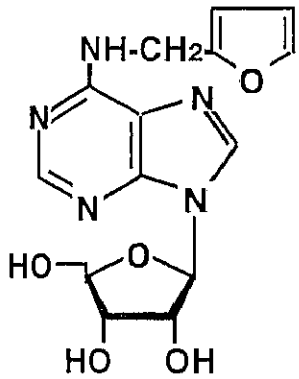
Kinetin



Adenosine



Isopentenyladenosine



Kinetin riboside

Table Ⅱ.

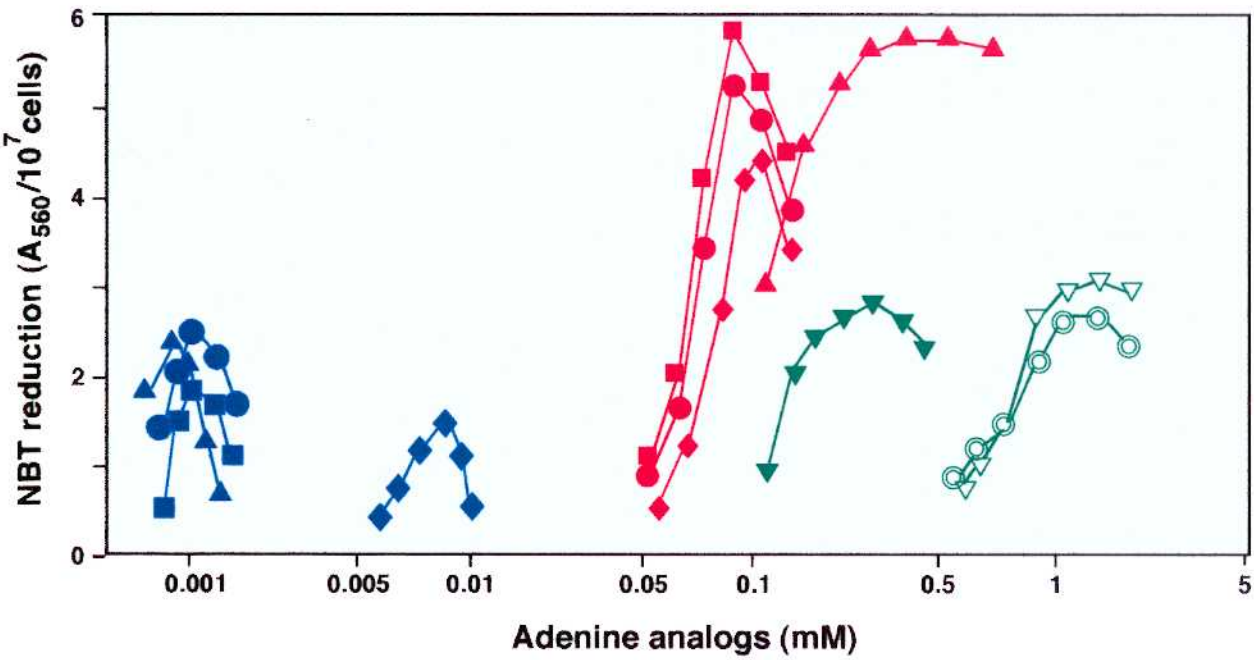
Effects of cytokinins and cytokinin ribosides on growth of HL-60 cells.

	Growth inhibition (IC50, mM)	
Analog	**-**	**+ribose**
Isopentenyladenine	47.6	0.972
Kinetin	48.8	0.981
2,6-diaminopurine	61.3	6.23
6-benzyladenine	67.6	0.706

Cells were cultured with various concentrations of the analogs for 5 days. Means of three separate experiments. IC_{50}, concentration of compound required for 50% inhibition of cell growth.

Figure Ⅱ-2. Effects of cytokinins and cytokinin nucleosides on induction of NBT reduction in HL-60 cells.

Cells were incubated with various concentrations of the compounds for 5 days. Values are means of four separate experiments.



■, isopentenyladenosine
●, kinetin riboside
▲, 6-benzyladenosine
◆, 2,6-diaminopurine deoxyriboside

■, isopentenyladenine
●, kinetin
▲, 6-benzyladenine
◆, 2,6-diaminopurine

▼, adenine
▽, adenosine
◎, deoxyadenosine

Figure Ⅱ-3. Morphologic changes in HL-60 cells by IPA and IPAR. Cells were cultured with (B) or without (A) 5 μM IPAR for 1 day. (C) Cells treated with 0.1 mM IPA for 6 days.

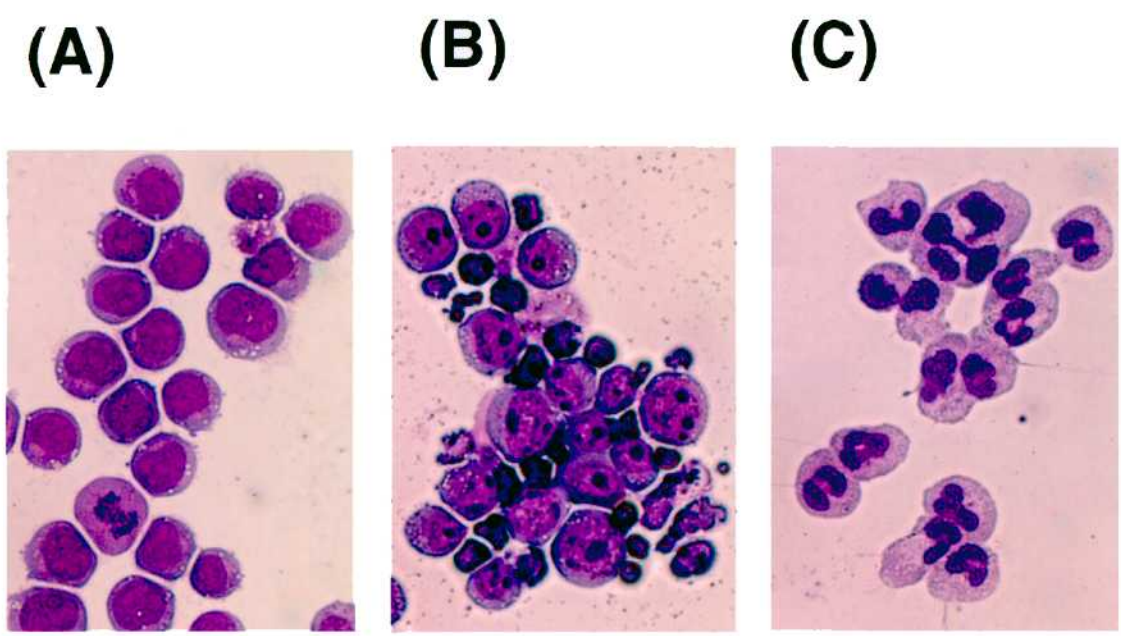
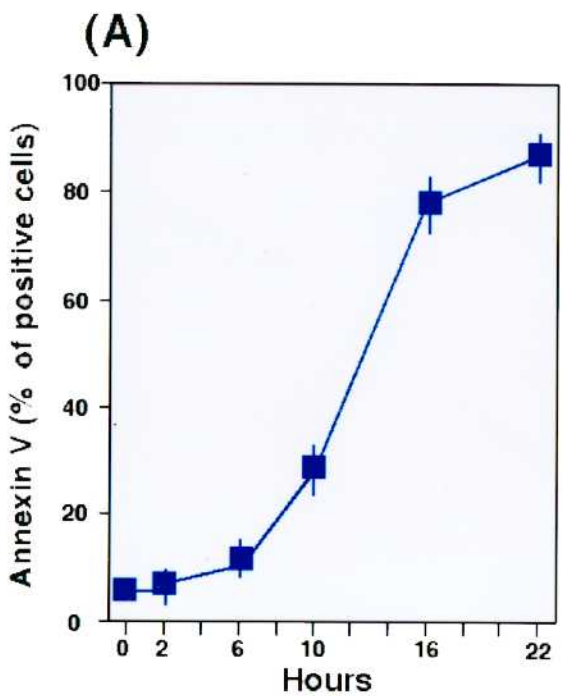

(A)
(B)
(C)

Figure Ⅱ-4. Induction of annexin V binding (A) and DNA fragmentation (B) by IPAR.

(A) HL-60 cells were cultured with 2.5 μM IPAR for various times. (B) HL-60 cells were cultured with various concentrations of IPAR (left) or IPA (middle) for 12 h or 2.5 μM IPAR for indicated times (right).


(A)
Annexin V (% of positive cells)
100
80
60
40
20
0
0 2 6 10 16 22
Hours

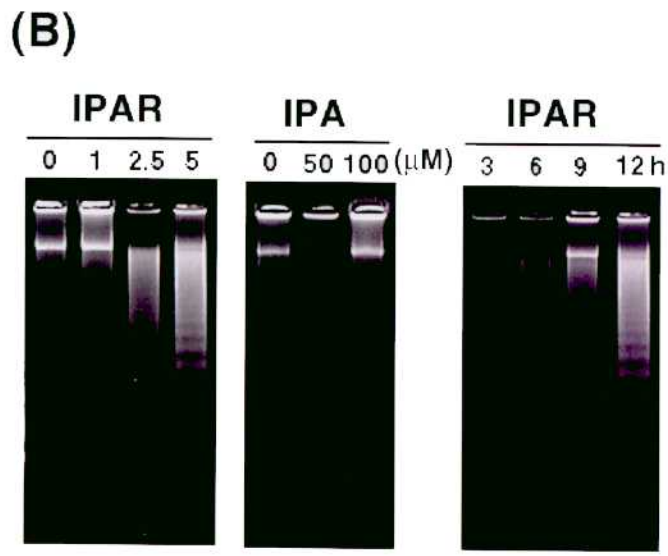

(B)
IPAR
0 1 2.5 5
IPA
0 50 100 (μM)
IPAR
3 6 9 12 h

Figure Ⅱ-5. Induction of caspase-3 activity by cytokinin ribosides. (A) HL-60 cells were treated with various concentrations of IPA (■), kinetin (●), IPAR (◆), or kinetin riboside (▲) for 12 hours. (B) Time-course of caspase-3 activation by IPAR. Cells were cultured with 0 (●), 2 (■), or 4 (▲)μM IPAR. Means ± SD of three separate experiments.

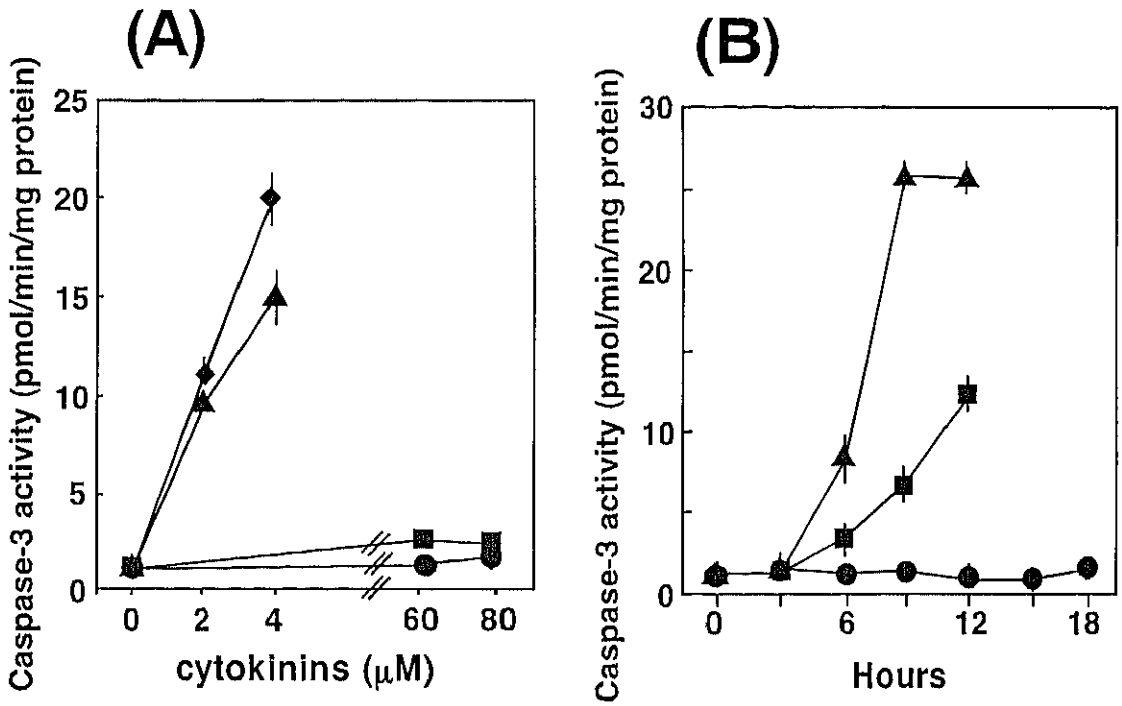

(A)
Caspase-3 activity (pmol/min/mg protein)
25
20
15
10
5
0
0
2
4
60
80
cytokinins (μM)
(B)
Caspase-3 activity (pmol/min/mg protein)
30
20
10
0
0
6
12
18
Hours

Figure Ⅱ-6. Effects of IPA and IPAR on intracellular ATP levels in HL-60 cells.

(A) Cells were treated with 0.1 mM IPA (◆) or 2μM IPAR(●), and ATP contents were measured at the indicated times. (B) Cells were treated with various concentrations of IPA(◆) or IPAR(●) for 6 hours. Each point represents the ration of ATP in treated cells to that in untreated cells at the time indicated. ATP content in untreated HL-60 cells is 1.78 nmoles/10^6cells.

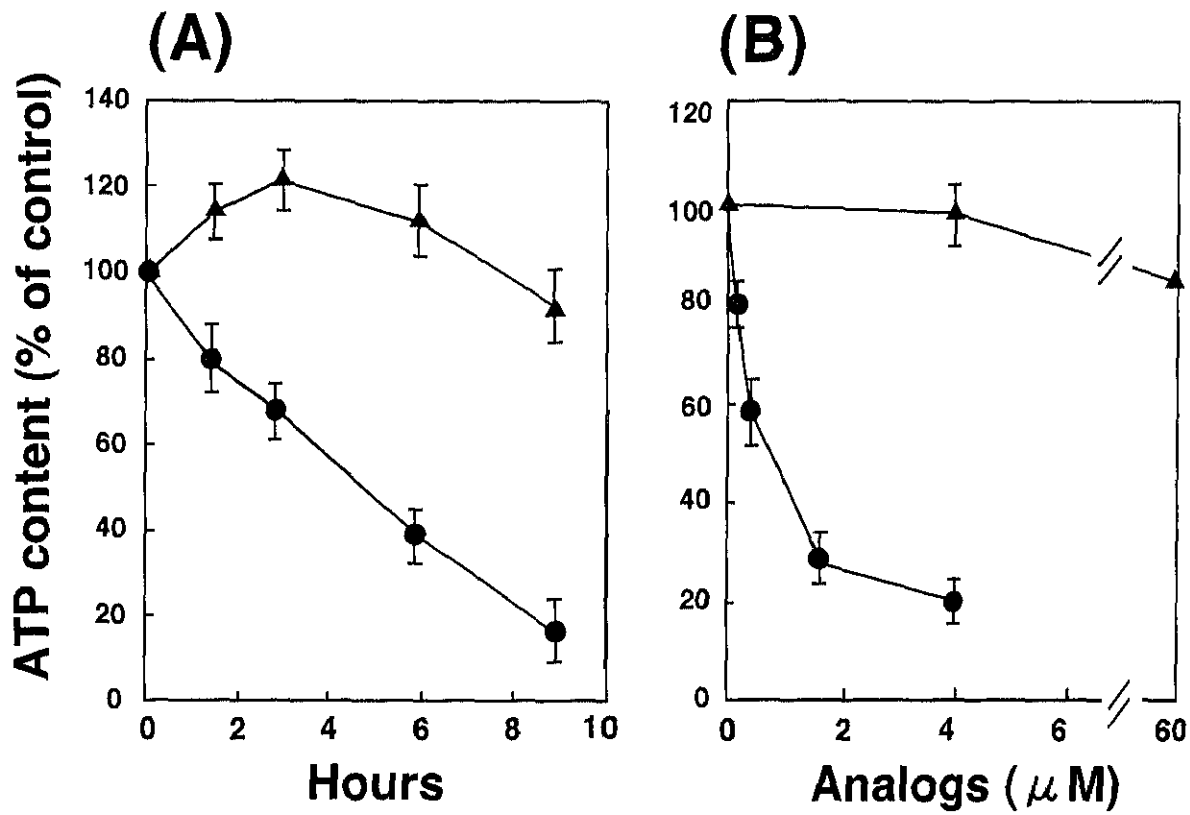

(A)
(B)
ATP content (% of control)
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Hours
120
100
80
60
40
20
0
0
2
4
6
60
Analogs (μM)

Figure Ⅱ-7. Effects of IPA and IPAR on mitochondrial membrane potential in HL-60 cells.

(A) Cells were treated with various concentrations of IPAR (■), IPA (▲), or kinetin (●) for 9 hours. (B) Cells were cultured with (■) or without (●) 4 mM IPAR for various times. Percentages of cells with reduced membrane potential. Means±SD of three separate experiments.

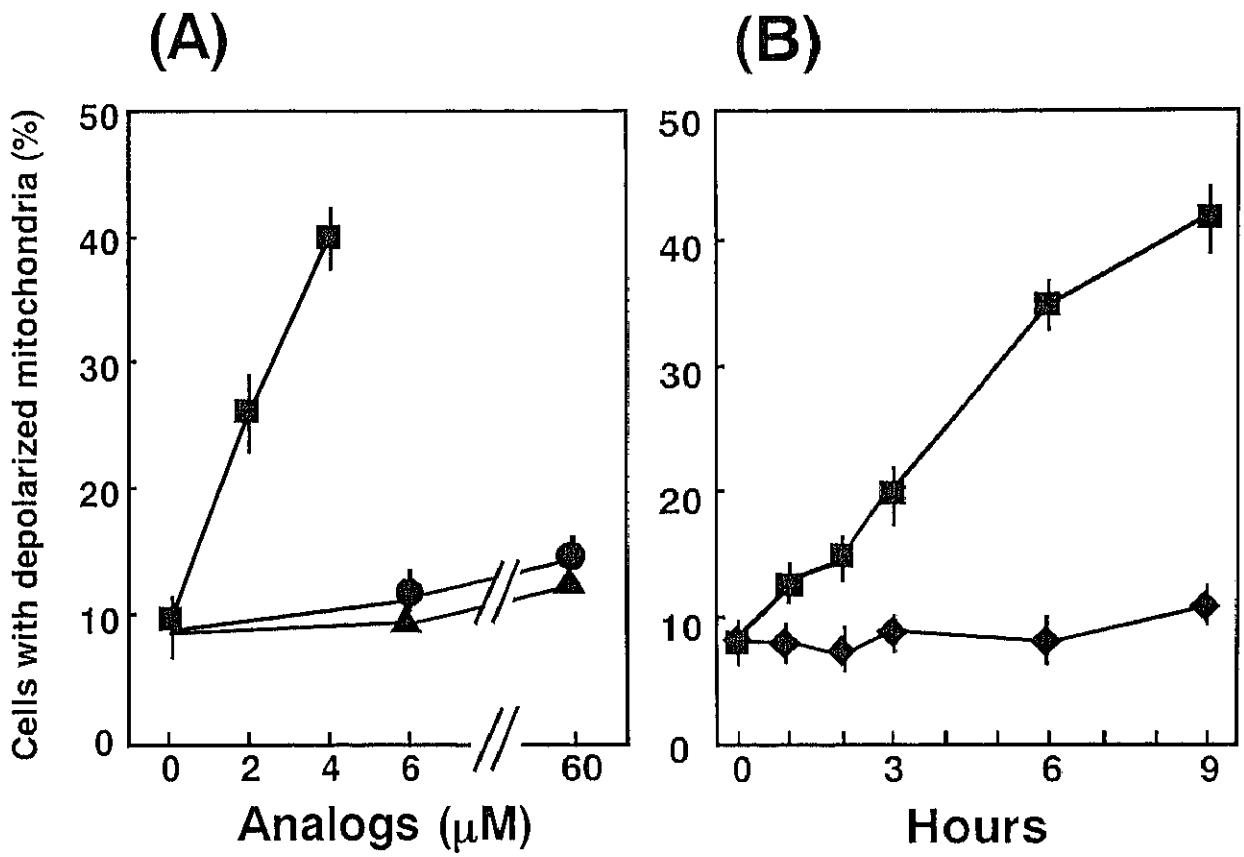
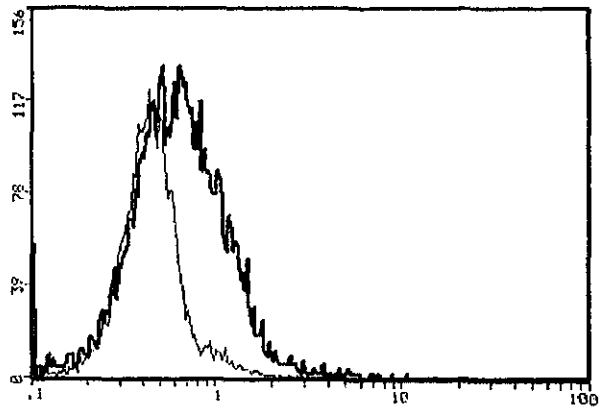

(A)
(B)
Cells with depolarized mitochondria (%)
50
40
30
20
10
0
0
2
4
6
60
Analogs (μM)
0
3
6
9
Hours

Figure Ⅱ-8. Effects of IPA or IPAR on ROS accumulation in HL-60 cells.

ROS accumulation in HL-60 cells treated with 5 μM IPAR (A) or 60 μM IPA for 10 hours (B) were measured by flow cytometry using the red laser channel. In each panels, dotted line represents untreated cells.

(A)



(B)

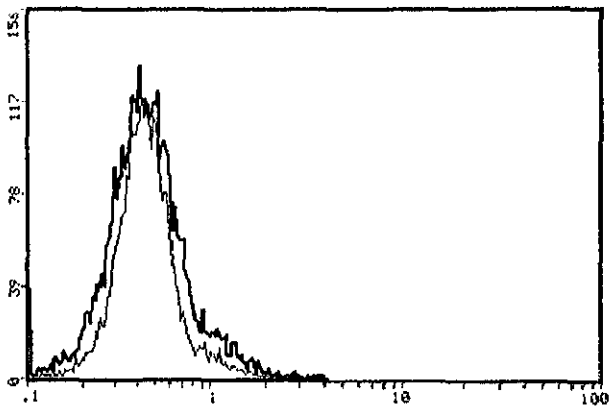
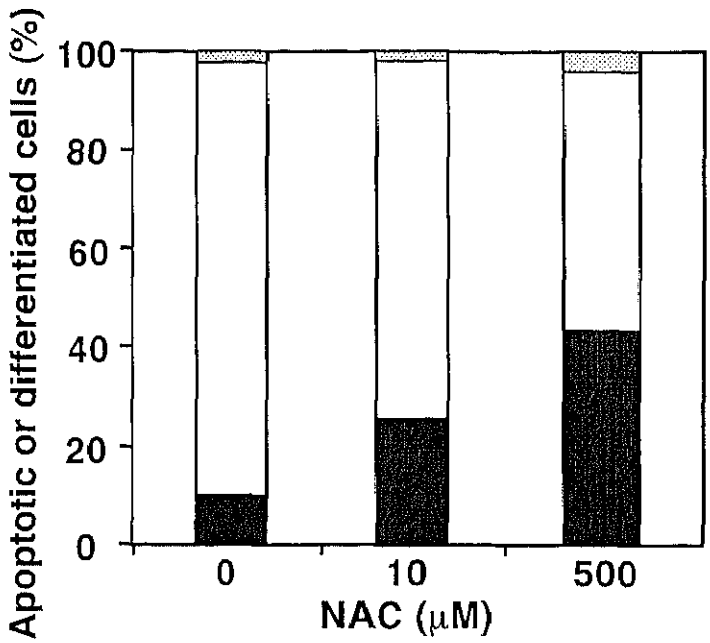
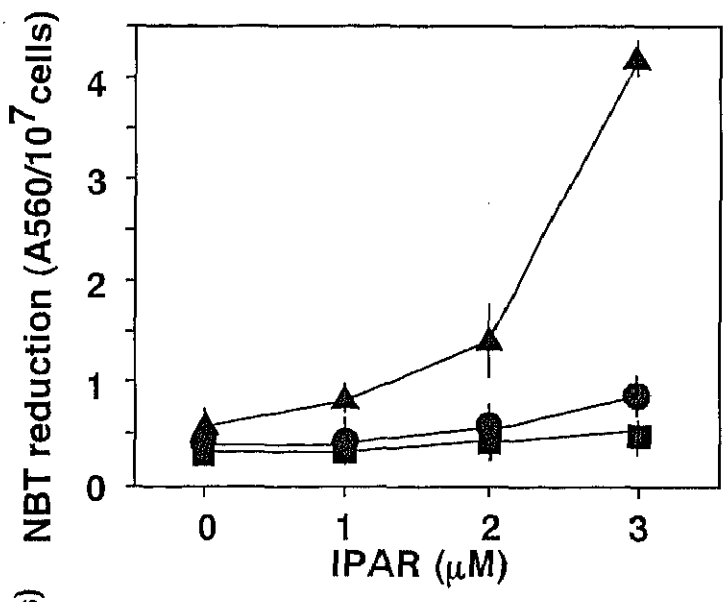


Figure Ⅱ-9. Differentiation of HL-60 cells treated with IPAR in the presence of O_2^- scavenger, antioxidant or caspase inhibitor.
(A) Effect of N-acetyl cysteine (NAC) on morphologic changes in IPAR-treated cells. Cells were treated with 5 μM IPAR in the presence or absence of various concentrations of NAC for 4 days. (□), apoptotic cells, (■), differentiated cells. (B) Effect of ambroxol on NBT reduction of IPAR-treated cells. Cells were cultured with various concentrations of IPAR in the presence of 0 (■), 10 (●), or 100 (▲) μM ambroxol for 4 days. (C) Effect of caspase inhibitor on the expression of CD11b of HL-60 cells treated with IPAR. Cells were cultured with IPAR in the presence of 0 (■), 1.5 (●), or 4.5 (▲) μM caspase inhibitor (FK-011) for 2 days. Means ± SD of three separate experiments.

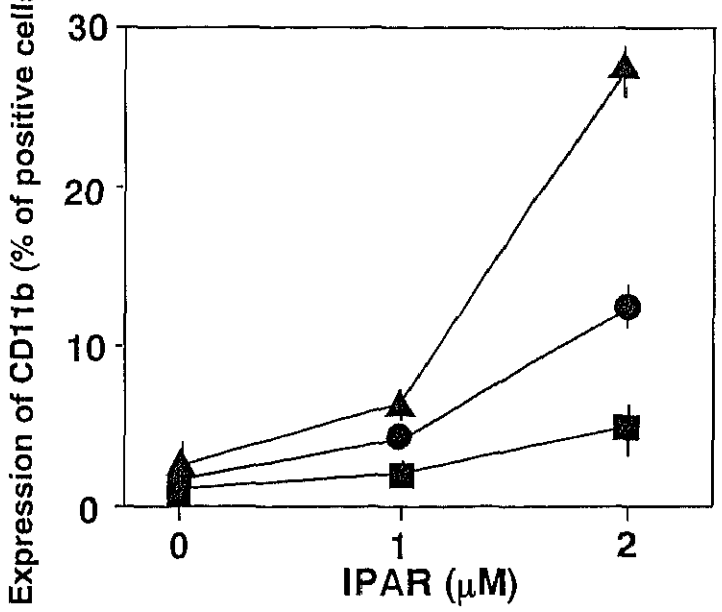
(A)


Apoptotic or differentiated cells (%)
100
80
60
40
20
0
0
10
500
NAC (μM)

(B)


NBT reduction (A560/10^7cells)
4
3
2
1
0
0
1
2
3
IPAR (μM)

(C)


Expression of CD11b (% of positive cells)
30
20
10
0
0
1
2
IPAR (μM)