

## **Part III Metabolism of cytokinins in human leukemia cells**

### **Identification of cytokinin metabolites in acid-soluble, RNA and DNA fractions**

Metabolism of cytokinins in HL-60 cells is described in this part. Since a radioactively labeled IPA is not commercially available, I examined intracellular uptake and metabolism of radioactively labeled BA ( $[^{14}\text{C}]\text{-BA}$ ), another potent cytokinin.

Kinetics of incorporation of  $[^{14}\text{C}]\text{-BA}$  into HL-60 cells was first examined. The cells which were treated with  $[^{14}\text{C}]\text{-BA}$  were fractionated into the acid-soluble fraction, RNA or DNA/protein fraction, and radioactivity was determined by liquid scintillation counting.

Most of the radioactivity incorporated was detected in acid-soluble fraction (Fig. III-1). Then, I analyzed the metabolites in acid-soluble fractions.

### **BA-nucleotides as main metabolites**

Adenine is metabolized into adenosine, then adenosine-5'-monophosphate (AMP), adenosine-5'-diphosphate (ADP) and ATP. Cytokinins, the adenine analogs might be metabolized in a similar manner. The acid-soluble extracts of cells incubated with  $[^{14}\text{C}]\text{-BA}$  for various time were subjected to thin layer chromatography to separate BA, BAR and BA-nucleotides. Radioactivity was determined by autoradiography, and the results demonstrated that most of the incorporated  $[^{14}\text{C}]\text{-BA}$  was rapidly converted to nucleotides (Fig. III-2).

## **Effect of adenosine (Ado) or an inhibitor of Ado kinase on the growth and differentiation of HL-60 cells in the presence of cytokinins**

As cytokinins are adenine analogs, I examined the combined effect of cytokinins and other adenine analogs. Although a low concentration of cytokinin or Ado alone was hardly effective at inhibiting the growth and inducing the differentiation of HL-60 cells, the combined treatment of cytokinin and Ado significantly induced the growth inhibition and differentiation (Fig. III-3A and B). Cytokinin-induced NBT reduction was not essentially affected by 2'-deoxyadenosine (dAdo), adenine, or other adenine analogs, suggesting that the enhancing effect of Ado is rather specific. Similar results were obtained when other human myelomonocytic leukemia cell lines such as NB-4, HT-93, U937, THP-1, and KOCL48 cells were cultured with cytokinins and/or Ado.

Next, the effect of 5'-amino-dAdo, an inhibitor of Ado kinase (16), on the growth and differentiation of HL-60 cells treated with IPA was examined. This inhibitor reduced the growth inhibition and differentiation induced by IPA (Fig. III-3C and D). These results indicate that the differentiation-inducing effects of cytokinins are closely related to Ado metabolism that requires phosphorylation by Ado kinase or its related enzymes.

## **Association of formation of BA-nucleotides with induction of differentiation**

Fig. III-3 shows that the action of cytokinins in HL-60 cells may be at least partly associated with Ado metabolism. To determine an association

between metabolisms of cytokinins and the cytokinin-induced differentiation, the conversion of labeled BA into nucleotides was investigated in the presence of Ado or 5'-amino-dAdo, the differentiation-enhancing or -inhibitory agent, respectively. Acid-soluble extracts of the HL-60 cells incubated with [<sup>14</sup>C]-BA in the presence or absence of Ado were subjected to thin layer chromatography. The conversion of [<sup>14</sup>C] - BA into nucleotides was significantly increased by Ado (Fig. III-4A). On the other hand, 5'-amino-dAdo decreased the conversion of [<sup>14</sup>C] - BA into nucleotides (Fig. III-4B). The conversion of [<sup>14</sup>C] - BA into BA or BAR were scarcely affected by Ado and 5'-amino-dAdo. These results are consistent with the effects of Ado or 5'-amino-dAdo on differentiation (Fig. III -3) and suggest that incorporation of cytokinin into nucleotides is an important step in the cytokinin-induced differentiation.

### **Incorporation of BA into DNA/RNA is not associated with the differentiation**

A possibility that cytokinins may function after incorporation into DNA or RNA has been reported in plants (51, 78 and 112). Therefore, I examined the association between the biological activities of cytokinin and its incorporation into DNA/RNA in the leukemia cells. HL-60 cells were treated with [<sup>14</sup>C] - BA for 12 hours, and RNA and DNA were isolated using Isogen according to the manufacturer's instructions. The labeled RNA was electrophoresed on agarose gel and transferred to a nitrocellulose membrane. The radioactivity was uniformly distributed in all the RNA fractions (Fig. III-5A left and 5B left), suggesting that the radioactive nucleotides were non-selectively incorporated into all of the RNA species. Although Ado was

treated to enhance the differentiation, the distribution of incorporated labeled RNA was not affected (Fig. III-5A right and 5B right).

Furthermore, it was determined whether [ $^{14}\text{C}$ ] - BA is incorporated into nucleic acid as free BA or adenine, which is deficient in benzyl group. The radioactive nucleotides in DNA isolated from HL-60 cells incubated with [ $^{14}\text{C}$ ]-BA for 24 hours was treated with nuclease and phosphatase. Subsequently, they were subjected to thin layer chromatography to separate adenine/Ado or BA/BAR. The most of the radioactive nucleotides in RNA and DNA were converted to adenine nucleotides, not BA nucleotides (Fig. III-5C). Similar results were obtained from the radioactive nucleotides in RNA. These results indicate that modification of RNA and DNA by BA does not occur during the cytokinin-induced differentiation of HL-60 cells. Similar results were obtained when the cells were incubated for 3 or 12 hours (data not shown), suggesting that the elimination of a benzyl group from radioactive nucleotides occurs before their incorporation into RNA/DNA. On the other hand, most of the radioactive nucleotides in acid-soluble fractions were BA-nucleotides (Fig. III-5D) and the amounts were closely correlated to induction of the differentiation. These results suggest that formation of BA-nucleotides is an important process for the differentiation.

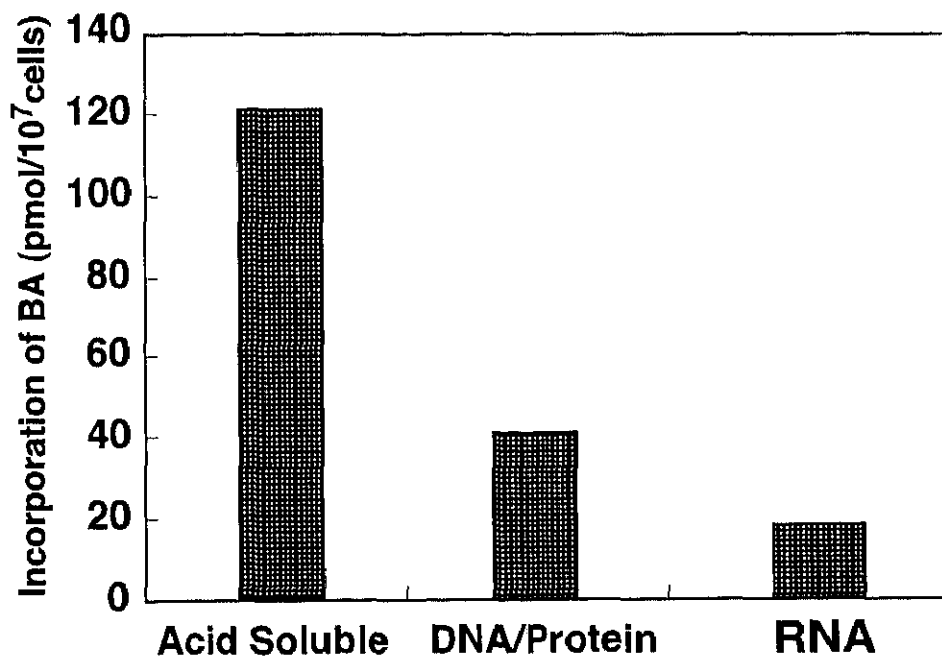
### **Separation of BA-nucleotides by column chromatography**

Cytokinin was converted to cytokinin nucleotides quickly after the intracellular uptake into HL-60 cells. There are three states for the nucleotides such as mono-, di-, or triphosphate, depending on the number of phosphate groups. In order to examine at which states of nucleotides were mainly formed, HL-60 cells were extracted at 24 hours after treatment with

[<sup>14</sup>C]-BA and separated by Dowex-1 column. The BAR-monophosphate was predominant in HL-60 cells (Fig. III-6).

**Figure III -1.** Incorporation of BA into the acid-soluble fraction, RNA and DNA/protein of HL-60 cells.

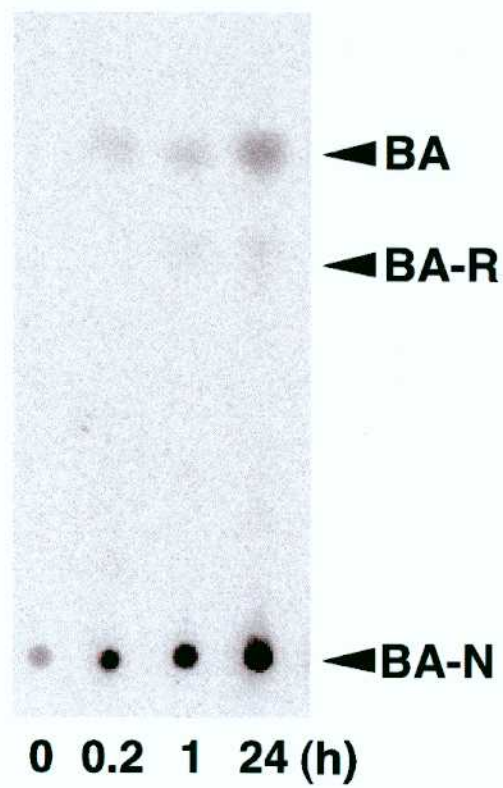
HL-60 cells were treated with 37kBq of [<sup>14</sup>C]-BA for 3 hours. Acid-soluble fraction, RNA and DNA/protein were extracted by the methods described in materials and methods. Each aliquot were then taken for the assay of radioactivity. Radioactivity was determined by liquid scintillation counting. Results are given as the mean of three separate experiments.



**Figure III -2.** Intracellular uptake of BA and synthesis of its nucleotides in HL-60 cells.

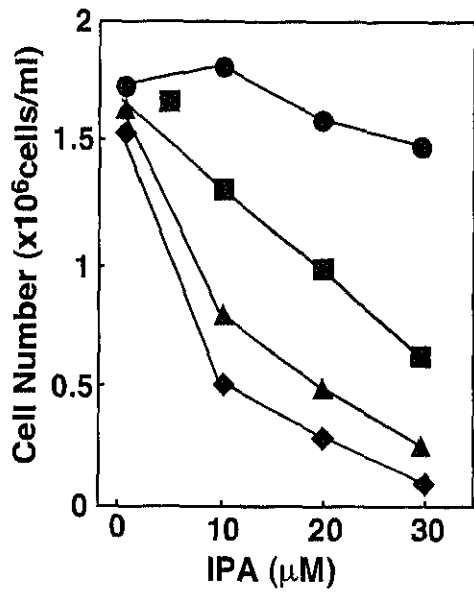
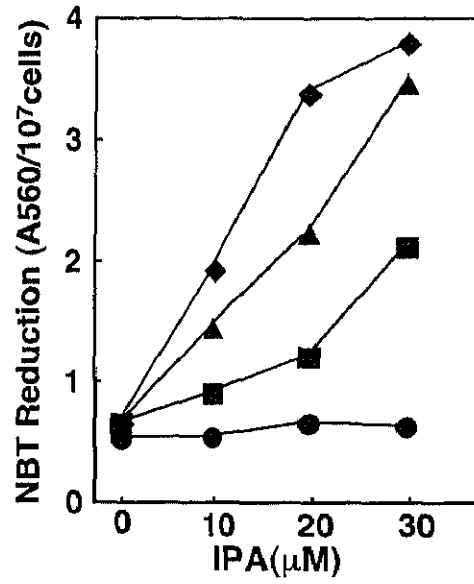
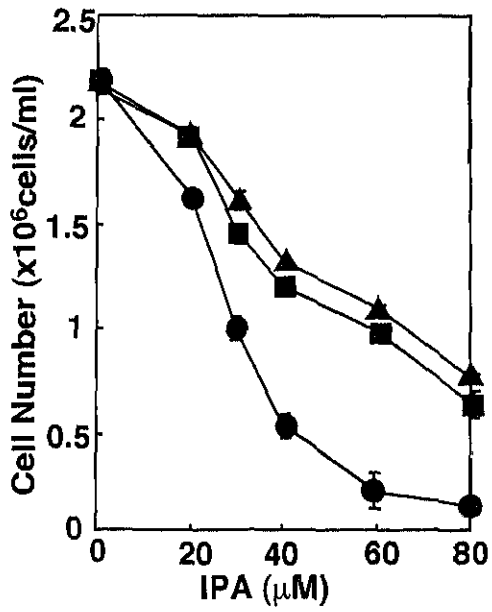
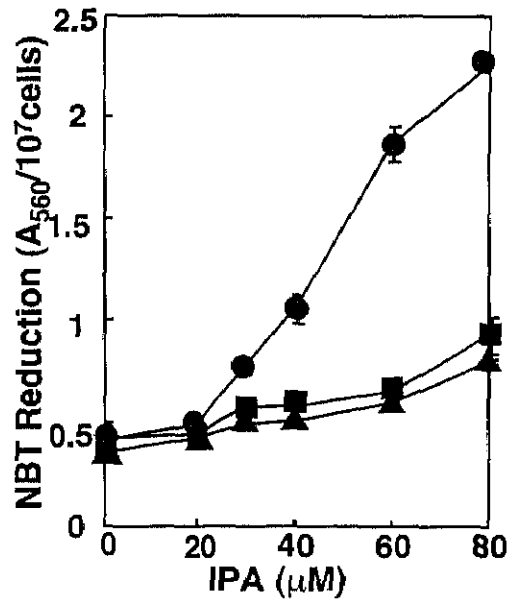
Cells were incubated with 37 kBq of [<sup>14</sup>C]-BA for various durations. After incubation, cells were washed twice with cold PBS and extracted with tetrahydrofuran. Nucleotides were separated from nucleobases and nucleosides by thin layer chromatography (n-butanol/water, 86:14, v/v). The zones corresponding to authentic compounds were evaluated by autoradiography. BA-N, benzyladenine nucleotides.





**Figure III-3.** Effects of Ado (A, B) and 5'-amino-dAdo (C, D) on growth inhibition and differentiation in IPA-treated HL-60 cells.

(A,B) Cells were cultured with various concentrations of IPA in the presence of 0 (●), 10(■), 20 (▲), or 30 (◆)  $\mu\text{M}$  Ado. Cell number (A) and NBT reduction (B) were examined after 5 days culture. (C,D) Cells were cultured with various concentrations of IPA in the presence of 0 (●), 1 (■), or 2 (▲)  $\mu\text{M}$  5'-amino-dAdo. Cell number (C) and NBT reduction (D) were examined after 5 days culture. Results are given as the mean  $\pm$  SD of three separate experiments.

**(A)****(B)****(C)****(D)**

**Figure III-4.** Effect of Ado and 5'-amino-dAdo on the synthesis of BA nucleotides.

Cells were incubated with 37 kBq of [<sup>14</sup>C]-BA in the presence or absence of 0.1 mM Ado for 3 hours (A) or 1 μM 5'-amino-dAdo for 1 day (B).

Similar results were obtained three times and typical results are shown.

BA-N, benzyladenine nucleotides.

**(A)**



**(B)**



**Figure III-5.** Incorporation of [ $^{14}\text{C}$ ]-BA into RNA and DNA.

(A, B) Electrophoretic pattern of RNA from HL-60 cells treated with 37 kBq of [ $^{14}\text{C}$ ]-BA for 12 hours in the presence (+) or absence (-) of 0.1 mM Ado. Staining with ethidium bromide (A) and autoradiography (B). (C, D) Digestion of labeled DNA or acid-soluble fractions. Labeled DNA (C) or labeled acid-soluble fractions (D) were treated with (+) or without (-) nuclease and phosphatase. An aliquot was applied to a chromatographic sheet, which was developed in a solvent system of chloroform/methanol/water (80/20/1).

**(A)**



**(B)**



Ado - +

- +

**(C)**

- +



**(D)**

- +



nuclease &  
phosphatase

BA-R  
BA

Ado  
Adenine

BA-N  
Adenine-N

**Figure III -6.** Chromatographic separation of BA nucleotides.

The labeling interval was 24 hours. The total tetrahydrofuran-extracted materials were put on a Dowex-1 column. The following eluents were used in succession; (1) water, (2) 0.1 M formic acid, (3) 1 M formic acid, (4) 4 M formic acid, (5) 4 M formic acid-0.2 M ammonium formate, (6) 4 M formic acid-0.4 M ammonium formate, (7) 4 M formic acid-0.8 M ammonium formate, and (8) 88% formic acid. Arrows indicate when the eluents were changed. Arrowheads indicate when the reference materials were eluted.



