The Influence of the Protein Kinase A System in Differentiation of HL-60-Eo Cells to Eosinophils Induced by Histamine

Kenichi Shimada, Tomoyuki Abe, Mitsunobu Mio and Chiaki Kamei*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan

Received June 27, 2001 Accepted September 7, 2001

ABSTRACT—The influence of the protein kinase A (A kinase) system in differentiation of HL-60-Eo cells to eosinophils induced by histamine was studied. Although 8-Cl-cAMP caused inhibitions of proliferation and [3H]thymidine uptake of HL-60-Eo cells similarly to histamine, no significant eosinophilic differentiation was observed. Histamine as well as 8-Cl-cAMP caused elevation of A kinase activity. However, KT-5720, an inhibitor of A kinase, had no effect on histamine-induced eosinophil differentiation. RI/G61 antisense oligodeoxynucleotide caused significant inhibition of HL-60-Eo cell growth, but RII/G62 antisense oligodeoxynucleotide had no effect. On the other hand, neither of the antisense oligodeoxynucleotides showed potentiating effects on growth inhibition induced by histamine. In addition, RI/G61 and RII/G62 antisense oligodeoxynucleotides caused neither differentiation to eosinophils itself nor potentiation of histamine-induced differentiation. From these findings, it was concluded that A kinase is not correlated directly with differentiation of HL-60-Eo cells to eosinophils.

Keywords: Histamine, 8-Cl-cAMP, Eosinophil differentiation, HL-60-Eo, KT-5720

A subclone of HL-60 cells (HL-60-Eo cells), which preferentially differentiate to eosinophils under histamine stimulation, was obtained by long-term culture under slightly alkaline conditions (pH 7.6) (1, 2). During the course of our investigations of the reason for the differentiation of HL-60-Eo cells to eosinophils, we found that the H2-agonist imipramide, dimaprit and 4-methylhistamine also increased the number of differentiated cells, although the H1-agonist 2-methylhistamine had no effect. The rank order of potency was histamine > imipramide > dimaprit > 4-methylhistamine (3). The rank order of potency on the differentiation of HL-60-Eo cells to eosinophils was somewhat different from those observed with pharmacological effects in other tissues. For instance, imipramide is 16.8-fold more potent than histamine in rat induction of gastric juice secretion (4). In addition, imipramide is 48.1- and 9.3-fold more potent than histamine on the guinea pig atrium and the rat uterus, respectively. On the other hand, H2 receptors are closely linked to the activation of adenylate cyclase, and their stimulation results in elevation of intracellular cAMP levels. Therefore, the present study was performed to clarify the role of the protein kinase A (A kinase) system in differentiation of HL-60-Eo cells to eosinophils induced by histamine.

MATERIALS AND METHODS

Cells

HL-60 cells (Japanese Cancer Research Resources Bank, Tokyo) were cultured under alkaline (pH 7.6) condition for 2 months. Thereafter, the cells were cultured in soft agar until the colonies became visible (HL-60-Eo cells). HL-60-Eo cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. In the differentiation studies, the cells were suspended in serum-free RPMI-1640 medium containing 500 μM insulin, 500 μM transferrin and 6 μM selenious acid, and they were cultured in a CO2 incubator at 37°C for 6 days (2).

Determination of cell proliferation

HL-60-Eo cells (1 × 105 cells/ml) were cultured in each well of 24-well plastic plates in the presence of test compounds at 37°C in a CO2 incubator for 6 days. Thereafter, 0.3% trypan blue was added to each well. Viable cells, visualized by the exclusion of trypan blue, were counted using a hemocytometer.

[3H]Thymidine uptake

Aliquots of 100 μl of HL-60-Eo cells (1 × 105 cells/ml)
in the medium used for the differentiation test were cultured in each well of 96-well plastic plates in the presence of test compounds at 37°C in a CO2 incubator for 6 days. Thereafter, 11 μl of [3H]thymidine (10 μCi/ml) was added to each well and incubation was continued for 4 h. After stopping the reaction by cooling, the mixture was filtrated through a Whatman GF/C filter, and then the filter was washed 3 times each with ethanol and water. The radioactivity remaining on the filter was measured using a liquid scintillation counter (LSC-1000; Aloka, Tokyo).

**Determination of cell differentiation**

Morphological changes in the cells were determined microscopically after May-Grünwald-Giemsa staining. Classification of cells that showed eosinophil specific granule on smears was carried out according to the criteria reported by Tasaka et al. (2).

**Determination of A kinase activity**

HL-60-Eo cells were cultured in the presence of histamine or 8-Cl-cAMP dissolved in RPMI-1640 medium for a variety of incubation periods and the cells were disrupted by sonication in disruption medium (1 mM theophylline, 0.2 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 20 mM 2-mercaptoethanol, 1% bovine serum albumin, 20 mM Tris-HCl, pH 7.5). After centrifugation at 20,000 × g for 1 h at 4°C, 80-μl aliquots of the supernatant were added to mixtures containing 120 μl of assay medium (400 μg/ml histone type II, 16.7 mM MgCl2, 100 μM ATP, 20 mM Tris-HCl, pH 7.5) and 20 μl of [γ-32P]ATP solution (2 μCi/20 ml, 500 μM), and the resultant mixtures were incubated for 10 min at 37°C in the presence or absence of 10 μM of cAMP. The reaction was terminated by addition of 20 μl of ice-cold 20% TCA and the mixtures were filtrated through a Whatman 3 MM filter. The radioactivity of phosphorylated histone remaining on the filter was determined using a liquid scintillation counter (LSC-1000).

**Introduction of antisense oligodeoxynucleotide**

A cell suspension (5 × 105 cells/ml) for cell differentiation was cultured in each well of 24-well plastic plates, and RIIα antisense oligodeoxynucleotide, 5'-GGC-GGT-ACT-GCC-AGA-CTC-CAT-3' (5), and RIIβ antisense oligodeoxynucleotide, 5'-CGC-CGG-GAT-CTC-GAT-GCT-CAT-3' (6), were added and incubated for 2 days at 37°C in a CO2 incubator. Thereafter, 5 μM histamine and 225 nM antisense oligodeoxynucleotide were added to the cell suspensions (1 × 105 cells/ml). Two days after commencement of differentiation, antisense oligodeoxynucleotide was also added at a concentration of 225 nM and incubation was continued under the above conditions.

**Chemicals**

The compounds used were obtained from the following sources indicated in parentheses: RPMI-1640 medium (Nissui, Tokyo); fetal bovine serum, insulin, transferrin, selenious acid, theophylline, phenylmethanesulfonyl fluoride, KT-5720 ((R,R,9S,11S)-(−)-9-hydroxy-9-n-hexyloxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b, 11a-triazadibenzo[a,g]cyloocta[c,d,e]trinden-1-one), 8-Cl-cAMP (Sigma Chemical Co., St. Louis, MO, USA); [3H]thymidine (Du Pont, Wilmington, DE, USA); [γ-32P]ATP (Amersham, Little Chalfont, UK); RIIα antisense oligodeoxynucleotide, RIIβ antisense oligodeoxynucleotide (Takara, Hyogo); and histamine dihydrochloride (Wako, Osaka). Other chemicals used were of reagent grade and were purchased from commercial sources.

**Statistical analysis**

Data are expressed as means ± S.E.M. One-way analysis of variance with Dunnett’s test was carried out to determine the statistical significance of differences. Probability values of less than 0.05 were considered significant.

**RESULTS**

**Effects of histamine and 8-Cl-cAMP on eosinophilic proliferation**

8-Cl-cAMP at concentrations of 0.1 and 1 μM showed no significant effect on proliferation of HL-60-Eo. However, at a concentration of 5 μM it caused a significant decrease in the number of cells. Histamine at concentrations of 1 and 5 μM showed a significant decrease in the number of cells. The potency of 8-Cl-cAMP in decreasing the cell number was almost the same as that of histamine (Fig. 1A).

**Effects of histamine and 8-Cl-cAMP on [3H]thymidine uptake**

8-Cl-cAMP at concentrations of 0.1 and 1 μM showed no significant effect, but at 5 μM, it significantly inhibited [3H]thymidine uptake. Histamine at concentrations of 1 and 5 μM caused significant inhibition of [3H]thymidine uptake (Fig. 1B).

**Effects of histamine and 8-Cl-cAMP on eosinophilic differentiation**

8-Cl-cAMP caused no increase in the number of differentiated cells even at a concentration of 5 μM. On the other hand, histamine at concentrations of 1 and 5 μM significantly increased the number of differentiated cells (Table 1).

**Effects of histamine and 8-Cl-cAMP on A kinase activity**

A kinase activity increased immediately after addition of histamine (5 μM) and lasted for 30 min, and the activity...
was restored to basal level after 2 h of histamine stimulation. Significant differences were observed after 15 and 30 min of stimulation with histamine. 8-Cl-cAMP (5 µM) also caused an increase in A kinase activity. A kinase activity was increased immediately after addition of 8-Cl-cAMP, and this elevated activity lasted for 3 h (Fig. 2).

Effects of KT-5720 on histamine-induced eosinophilic differentiation
To identify the role of A kinase in histamine-induced eosinophil differentiation, the effects of KT-5720 were studied. KT-5720 showed no effect on histamine-induced eosinophilic differentiation even at a concentration of 5 µM (Fig. 3).

Effects of A kinase regulatory subunit antisense oligodeoxynucleotides on cell growth and cell differentiation
RIα antisense oligodeoxynucleotide significantly inhibited growth of HL-60-Eo cells, while RIIα antisense oligodeoxynucleotide had no significant effect. On the other hand, neither RIα nor RIIβ antisense oligodeoxynucleotide influenced the inhibition of proliferation induced by histamine (Fig. 4). In the differentiation study, neither antisense oligodeoxynucleotide showed any effect on differentiation of eosinophils. In addition, RIα and RIIβ antisense oligodeoxynucleotides showed no potentiating effect on differentiation to eosinophils induced by histamine (Table 2).

DISCUSSION
In the present study, 8-Cl-cAMP was shown to cause a significant decrease in the cell number and inhibition of

**Table 1. Effects of histamine and 8-Cl-cAMP on eosinophilic differentiation of HL-60-Eo cells**

| Drugs          | Differential count (%) |
|----------------|------------------------|
| Control        | 9.7 ± 1.7              |
| Histamine (1 µM)| 34.7 ± 1.3**          |
| Histamine (5 µM)| 61.5 ± 3.9**          |
| 8-Cl-cAMP (5 µM)| 14.0 ± 1.0            |

Each value represents the mean ± S.E.M. of 5 separate experiments. **: Significantly different from the control (P<0.01).
[3H]thymidine uptake in HL-60-Eo cells. The effect of 8-
Cl-cAMP (5 μM) was almost the same as that of histamine
stimulation. Yokozaki et al. (7) reported that 8-Cl-cAMP
causen growth inhibition of HL-60 cells. They found that
IC50 of 8-Cl-cAMP on growth inhibition of HL-60 cells
was 0.4 μM. On the other hand, in the present study the
IC50 of 8-Cl-cAMP on cell growth inhibition in HL-60-Eo
cells was about 5 μM. The difference between Yokozaki
et al.’s finding and our present study may be attributable
to the difference of the cell number and proliferation ability
of cells used in the experiment.

On the other hand, histamine at concentrations of 1 and
5 μM caused significant differentiation of HL-60-Eo cells
to eosinophils. We also found that the H2-agonists impro-
midine and 4-methylhistamine also induced differentiation
of HL-60-Eo cells to eosinophils (3). Nonaka et al. (8)
reported that histamine induced differentiation of HL-60
cells to neutrophils under acidic conditions, and this differ-
entiation was inhibited by H2-antagonists such as cimeti-
dine, ranitidine and famotidine. These findings suggested
that differentiation of HL-60-Eo cells or HL-60 cells to
eosinophils or neutrophils induced by histamine is mediat-
ed by H2 receptors via the A kinase system. However, as
shown in the present study, 8-Cl-cAMP did not induce
differentiation of HL-60-Eo cells to eosinophils. Therefore,
we investigated the role of A kinase in differentiation
of HL-60-Eo cells to eosinophils in detail. Both histamine
and 8-Cl-cAMP caused increases in A kinase activity in
HL-60-Eo cells. In addition, we also found previously that
histamine caused increases in cAMP levels in HL-60-Eo
cells (9). These two alternations induced by histamine
seemed to be almost parallel, but as described in the text,
KT-5720, an inhibitor of A kinase, caused no observable
effect on histamine-induced eosinophil differentiation.
Therefore, A kinase may or may not be responsible for
differentiation of HL-60-Eo cells to eosinophils. Seifert
et al. (10) and Mitsuhashi et al. (11) reported that histamine
increases cytosolic Ca2+ in HL-60 cells. Therefore, it seems
likely that Ca may be responsible for differentiation of HL-
60-Eo cells. On the other hand, there are two types of
A kinase, type I and type II, in mammalian cells (12, 13). Rohlff et al. (14) reported that in HL-60 cells, a kinase type I comprised more than 90% and a kinase type II less than 10% of the total A kinase activity. These isomers of A kinase are distinguished by their different R subunits, RI and RII, that interact with the identical C subunit (14). Therefore, we examined the effects of A kinase regulatory subunit antisense oligodeoxynucleotides on cell growth and cell differentiation. RI<sub>α</sub> antisense oligodeoxynucleotide, which showed a slight potentiation of A kinase activity (data not shown), caused inhibition of cell growth in HL-60-Eo cells (15), but RII<sub>β</sub> antisense oligodeoxynucleotide had no effect on cell growth. In addition, neither RI<sub>α</sub> nor RII<sub>β</sub> antisense oligodeoxynucleotides showed any potentiating effect on inhibition of proliferation induced by histamine. RI<sub>α</sub> and RII<sub>β</sub> antisense oligodeoxynucleotides showed neither differentiation of eosinophils nor potentiation of inhibition of proliferation induced by histamine. RI<sub>α</sub> and RII<sub>β</sub> antisense oligodeoxynucleotides showed neither differentiation of eosinophils nor potentiation of histamine-induced differentiation. Tortora et al. (15) reported that RI<sub>α</sub> antisense oligodeoxynucleotide decreased proliferation of HL-60 cells. These findings are essentially the same as those of the present study using HL-60-Eo cells. From these results, it seems likely that proliferation of HL-60 cells and HL-60-Eo cells are closely related to the expression of A kinase type I.

Tortora et al. (15) also found that differentiation of HL-60 cells to monocytes was also induced by RI<sub>α</sub> antisense oligodeoxynucleotide. However, in the present study, RI<sub>α</sub> antisense oligodeoxynucleotide did not induce differentiation of HL-60-Eo cells to eosinophils. In addition, RI<sub>α</sub> antisense oligodeoxynucleotide showed no potentiating effect on histamine-induced differentiation. These findings suggested that there are some differences in the mechanisms of differentiation of HL-60 cells to neutrophils and HL-60-Eo cells to eosinophils subsequent to the A kinase system.

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