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HISTAMINE-INDUCED DIFFERENTIATION OF HL-60 CELLS

THE ROLE OF cAMP AND PROTEIN KINASE A

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Abstract—When HL-60 cells were stimulated with histamine, a significant differentiation of the cells toward neutrophils was elicited. Histamine increased phagocytic activity, but it reduced myeloperoxidase activity of HL-60 cells. Histamine-induced differentiation in HL-60 cells was inhibited not only by H_2 antagonists, such as cimetidine, ranitidine and famotidine, but also by an inhibitor of protein kinase A (A kinase), KT-5720. Histamine increased the cAMP level and A kinase activity in HL-60 cells; both increases preceded the cell differentiation. Histamine also enhanced phosphorylation of a 160 kD protein in HL-60 cells, while H_2 antagonists and KT-5720 inhibited this phosphorylation. The results of the present study indicate that an activation of A kinase via H_2 receptor stimulation may cause the phosphorylation of a 160 kD protein and that this phosphorylation is probably involved in the process leading to differentiation of HL-60 cells.

Granulocytopenia has been reported as one of the adverse effects of H_2 antagonists in clinical use [1, 2]. Since it has been indicated that stimulation of an H_2 receptor induces a differentiation of bone marrow cells to neutrophils, it is supposed that the granulocytopenia caused by H_2 antagonists may be exerted via an H_2 receptor blockade of bone marrow cells [3, 4]. Furthermore, it has been shown that in the differentiation of murine neutrophil progenitors the site of action of histamine is distributed among various differentiation stages ranging from colony-forming units in culture to metamyelocytes [4, 5]. However, in the bone marrow, neutrophil progenitors are diversely differentiated over a wide range of stages, making it difficult to determine the stage at which histamine acts to differentiate the immature cells and to elucidate the mechanism of this action. To eliminate these problems, it seemed reasonable to use the cells remaining in the same differentiation stages. In the present study, HL-60 cells, a cell line of human promeylocytic leukemia, were employed to analyse the mechanism of action by which histamine induced the differentiation to neutrophils.

MATERIALS AND METHODS

Cell culture. HL-60 cells, transferred from the Japanese Cancer Research Resources Bank, were suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS†), 100 U/mL of penicillin and 100 μg/mL of streptomycin and cultivated at 37° in a CO_2 incubator with 5% CO_2 in humidified air. In the protein phosphorylation experiments, the cells were cultured in phosphorus-free RPMI-1640 medium and the pH of the medium was adjusted with 10 mM HEPES to 7.4.

Determination of cell differentiation. HL-60 cells were incubated with test compounds dissolved in RPMI-1640 medium containing 10% FCS for 6 days and the morphological changes that occurred during the incubation period were determined microscopically using specimens stained with May–Grünward–Giemsa solution. Classification of immatured cells on smear was carried out according to the criteria established by Bessis [6].

Phagocytic activity, which increases in parallel with cell differentiation, was also determined. After incubation of HL-60 cells with test compounds for various periods of time, 200 μL of HL-60 cell suspension were poured into a mixture containing 200 μL of yeast suspended solution (2.5×10^8 yeast cells/mL) and 100 μL of fresh rat serum and incubated for 30 min at 37°. After centrifugation at 2000 rpm for 20 min at 4°, cells engulfing the yeast were counted.

Measurement of myeloperoxidase activity. Myeloperoxidase activity was measured according to the method described by Romeo *et al.* [7] with slight modifications. HL-60 cells were incubated with various concentrations of histamine for 6 days at 37° in a 5% CO_2 incubator. The cells (1×10^5 cells) were disrupted in 1 mL of lysis buffer [0.1% Triton X-100, 100 mM phosphate buffer, 0.1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride, pH 7.0] and then 2 mL of a reaction mixture consisting of 13 mM guaiacol, 0.02% acetyltrimethylammonium bromide, 100 mM phosphate buffer (pH 7.0) and 3.75 mM 3-amino-1*H*-1,2,4-triazole were added. The reaction was initiated by adding 50 μL of 20 mM H_2O_2. Peroxidase activity was expressed as moles of tetraguaiacol formed per minute per 10^6 cells.

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† Abbreviations: FCS, fetal calf serum; TCA, trichloroacetic acid; A kinase, protein kinase A.

Table 1. Effect of histamine on the differentiation of HL60 cells during 6 days of culture

Days of culture	Concentration of histamine (μM) 0	0.1	1	10
0	7.7 ± 1.3			
1	3.3 ± 0.3	0.3 ± 4.7	8.3 ± 0.7	12.0 ± 1.5
2	5.7 ± 1.5	6.0 ± 0.1	7.7 ± 0.9	8.0 ± 3.0
3	7.0 ± 0.9	4.7 ± 0.9	9.0 ± 0.1	14.0 ± 1.2*
4	15.3 ± 4.6	20.7 ± 2.2	23.7 ± 1.2*	24.3 ± 1.2*
5	19.0 ± 3.6	21.3 ± 2.2	32.0 ± 1.2*	28.0 ± 0.1*
6	18.0 ± 4.4	23.0 ± 1.5	30.3 ± 2.7*	41.7 ± 2.0†

Each value is the % of differentiated cells, mean ± SEM (N = 4).
* and † indicate statistical significance in comparison with non-stimulated group on each day at $P < 0.05$ and $P < 0.01$, respectively.

Since the activity of eosinophil peroxidase decreases in the presence of 3-amino-1*H*-1,2,4-triazole [7], the remaining enzyme activity corresponds to that of myeloperoxidase.

[^{3}H]*Thymidine uptake.* A suspension of 200 μL of HL-60 cells (1×10^5 cells/mL) in RPMI-1640 medium containing 10% FCS was cultured in each well of a 96-well plastic plate in the presence of test compounds at 37° in a CO_2 incubator for various incubation periods. Thereafter, 10 μL of [^{3}H]methyl thymidine (10 μCi/mL) were added to each well and incubated for 24 hr. After the addition of 20 μL of non-labeled thymidine (50 mM), the reaction mixture was filtrated through a Whatman GF/C filter and the filter was washed twice with phosphate-buffered saline (137 mM NaCl, 20 mM sodium phosphate buffer; pH 7.4). The radioactivity remaining on the filter was measured by means of a liquid scintillation counter (Aloka, LSC-1000).

Measurement of intracellular cAMP content. HL-60 cells (10^6 cells/500 μL) were incubated with test compounds at 37° in a CO_2 incubator for various periods of time and the cells were disrupted by adding 500 μL of 5% trichloroacetic acid (TCA). After centrifugation, the supernatant was extracted with water-saturated ether to remove TCA and the specimen was evaporated completely *in vacuo*. The cAMP content of each sample was determined using a radioimmunoassay kit (Yamasa).

Determination of protein kinase A (A kinase) activity. HL-60 cells were cultivated in several concentrations of histamine dissolved in RPMI-1640 medium for a variety of incubation periods and the cells were disrupted by sonication in a 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 100,000 *g* for 1 hr at 4°, 80 μL of the supernatant were added to a mixture containing 100 μL of assay medium (400 μg/mL histone type II, 16.7 mM $MgCl_2$, Tris-HCl; pH 7.5) and 20 μL of [γ-^{32}P]ATP solution (2 μCi/20 μL, 500 μM), and the resultant mixture was incubated for 10 min at 37° in the presence or absence of 10 μM of cAMP. The reaction was terminated by addition of 200 μL of ice-cold 20% TCA and the mixture was filtrated through a Whatman C-81 phosphocellulose filter. The radioactivity of phosphorylated histone remaining on the filter was determined by means of a liquid scintillation counter (Aloka, LSC-1000).

Protein phosphorylation. HL-60 cells suspended in a phosphate-free RPMI-1640 medium were preincubated with 50 μCi/5 × 10^6 cells/mL of [γ-^{32}P]orthophosphoric acid at 37° for 15 min. The cells were washed twice and resuspended in the same medium. Thereafter, incubation was carried out in the presence or absence of test compounds for various periods of time. The reaction was terminated by addition of the same volume of SDS–PAGE sample buffer, after which cellular proteins were denaturated by heating at 80° for 5 min. SDS–PAGE (4–20% polyacrylamide) was carried out according to the method of Laemmli [8]. After electrophoresis,

Table 2. Effect of histamine on the myeroperoxidase activity in HL-60 cells after 6 days of culture

Concentration of histamine (μM)	Myeloperoxidase activity (×10^6 tetraguaiacol/min/10^6 cells)
0	16.90 ± 0.97
0.1	15.48 ± 1.17
1	11.40 ± 0.62*
10	10.55 ± 0.77*

The enzymatic activity was measured after 6 days of culture and represented as the formed amount of tetraguaiacol/min/10^6 cells.
Each value represents the mean ± SEM (N = 4).
* Indicates statistical significance at $P < 0.01$.

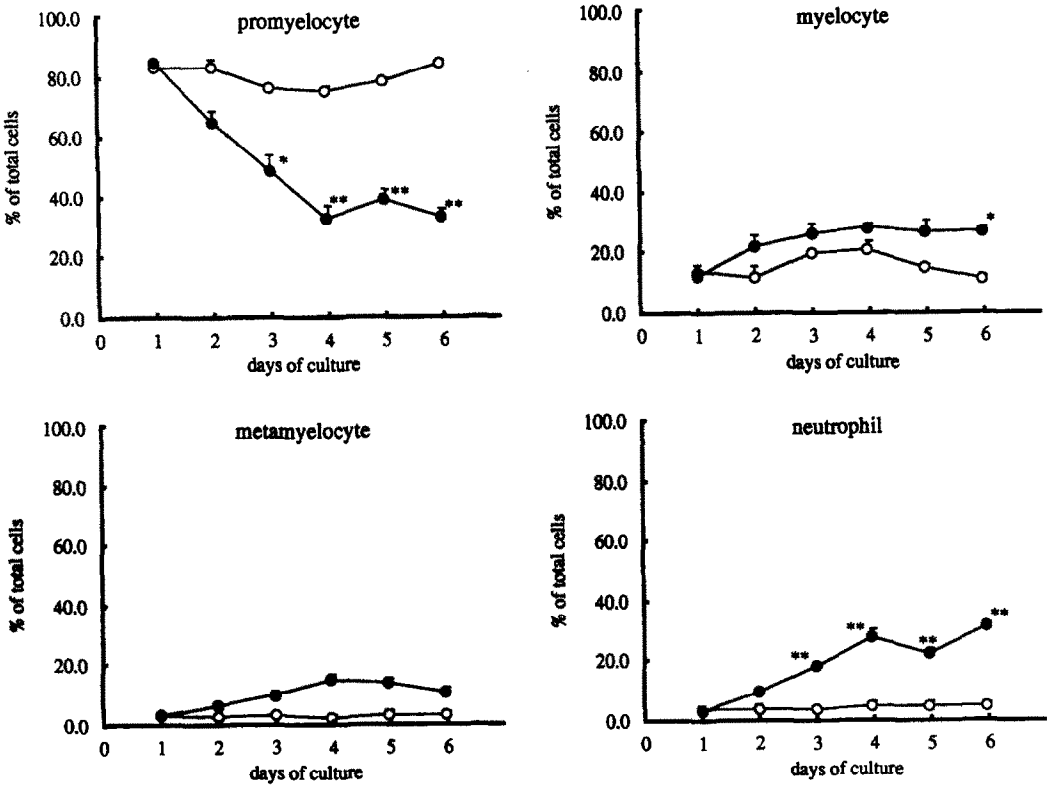

Fig. 1. Morphological changes in HL-60 cells treated with histamine. The cells were incubated with histamine for 6 days. Each point represents the mean ± SEM (N = 3). * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively. (○) Control, (●) histamine (1 μM).

the gel was stained with Coomassie brilliant blue R-250 and exposed to HyperFilm (Amersham) at −70° to obtain an autoradiogram.

Chemicals. The compounds used were as follows (sources are indicated in parentheses): RPMI-1640 medium (Nissui, Tokyo, Japan), phosphorus-free RPMI-1640 medium (Nissui), FCS (Hezelton, Lenaxa, KS, U.S.A.), penicillin (Toyo Jozo, Shizuoka, Japan), streptomycin (Meiji, Tokyo, Japan), [^{3}H]methyl thymidine (Amersham International, Amersham, U.K.), cAMP (Sigma Chemical Co., St Louis, MO, U.S.A.), cAMP radioimmunoassay kit (Yamasa, Tokyo, Japan), theophylline (Sigma), phenylmethanesulfonyl fluoride (Sigma) bovine serum albumin (Sigma), histone type II (Sigma), [γ-^{32}P]ATP (Amersham), [γ-^{32}P]orthophosphoric acid (Amersham), histamine (Wako Pure Chemicals, Osaka Japan), cimetidine (Smith Klein & French, Welwyn, U.K.), ranitidine (Glaxo, Greenford, U.K.), famotidine (Yamanouchi, Tokyo, Japan), pyrilamine (Rhône-Poulenc, Paris, Japan), KT-5720 (Kyowa, Tokyo, Japan). Other chemicals used were reagent grade and were purchased from commercial sources.

Statistical analysis. One way analysis of variance with Dunnett's test was carried out to determine the statistical significance.

RESULTS

Before the addition of histamine, 92.3 ± 3.8% (N = 4) of the total HL-60 cells were promyelocytic cells. When histamine was added to the incubation medium of HL-60 cells, a significant differentiation of the cells was elicited in a dose-dependent manner during 6 days of culture (Table 1). In this case, the number of differentiated cells included in the cells differentiated at various stages from myelocytes to neutrophils. The populational changes in HL-60 cells induced by 1 μM of histamine are indicated in Fig. 1. As the number of promyelocytes decreased, those of metamyelocytes and neutrophils increased, indicating that histamine induced the differentiation of HL-60 cells. The stimulative effect of histamine on the differentiation of HL-60 cells was remarkable after three days of culture. The myeloperoxidase activity of HL-60 cells was significantly reduced by histamine treatment in a dose-dependent manner (Table 2). Since myeloperoxidase activity decreased in accordance with cell differentiation from promyelocytes to neutrophils [9], this also may indicate that histamine effectively induced differentiation.

In order to analyse the mechanism of the intracellular signal transduction in HL-60 cells after histamine stimulation, changes in the cAMP content of the cells were measured. As indicated in Fig. 2, addition of histamine (1 μM) to the incubation medium immediately elicited an increase in the cAMP content of HL-60 cells, reaching a plateau 30 min after stimulation (Fig. 2). In addition, A kinase activity increased almost in parallel with the

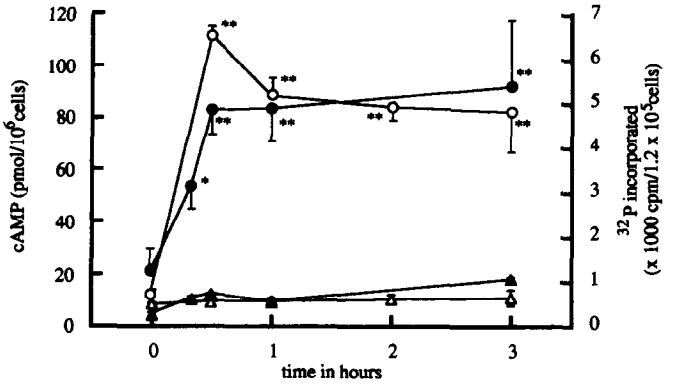

Fig. 2. Changes in intracellular cAMP levels and cAMP-dependent A kinase activity in HL-60 cells in association with histamine treatment. (○, △) cAMP content; the cells were incubated for 3 hr with or without 1 μM of histamine, respectively. (●, ▲) cAMP-dependent A kinase activity; the cells were incubated for 3 hr with or without 1 μM of histamine, respectively; after a disruption of the cells, the A kinase activity was determined in the presence of exogenous cAMP (10 μM). Each point represents the mean ± SEM (N = 3). * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.

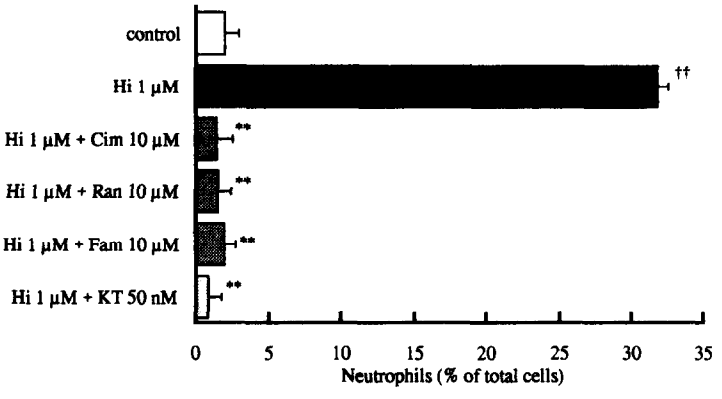

Fig. 3. Effect of H_2 antagonists and KT-5720 on the differentiation of HL-60 cells to neutrophils in the presence of histamine. The cells were cultured with 1 μM of histamine in the presence or absence of test compounds for 6 days. Each column represents the mean ± SEM (N = 3). †† Indicates significant difference from the control group at $P < 0.01$. ** Indicates significant difference from the histamine-treated group at $P < 0.01$. Hi, histamine; Cim, cimetidine; Ran, ranitidine; Fam, famotidine; KT, KT-5720.

increase in cAMP level; in both cases a rapid increase took place after the addition of histamine.

In the present experiment, KT-5720, an A kinase inhibitor [10], also effectively inhibited the differentiation of HL-60 cells at a concentration of 50 nM (Fig. 3). As shown in Fig. 4, yeast phagocytic activity, which is indicative of neutrophil differentiation, was significantly increased by histamine at 10^{-6} M. The histamine-induced increase in yeast phagocytic activity of HL-60 cells was remarkably inhibited by H_2 antagonists at equimolar concentrations to histamine, although pyrilamine, an H_1 antagonist, did not affect histamine-induced phagocytosis, even at a concentration of 10^{-5} M (Fig. 4).

When added to the incubation medium of HL-60 cells, KT-5720 alone slightly enhanced [^{3}H]thymidine uptake into HL-60 cells, as shown in Fig. 5. This may suggest that KT-5720 enhanced cellular proliferation by inhibiting a spontaneous differentiation of HL-60 cells. In connection with this, KT-5720 dose-dependently inhibited the A kinase activity of resting HL-60 cells as well as those stimulated by histamine (Fig. 6). This may suggest that the inhibitory effect of KT-5720 is actually exerted by an inhibition of A kinase activity.

Since it was assumed that the activation of A kinase may play some important role in the cell differentiation induced by histamine, histamine-induced protein phosphorylation was examined. As

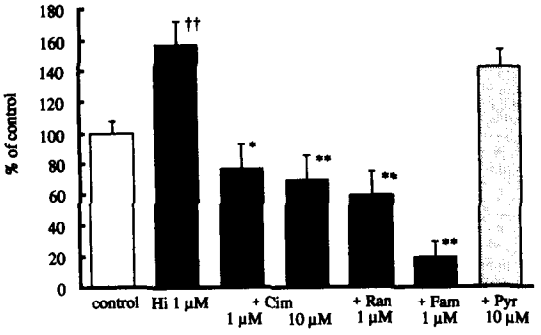

Fig. 4. Effects of histamine antagonists on the phagocytic activity of HL-60 cells engulfing the yeast provoked by histamine (4 days of culture). The cells were cultured with 1 μM of histamine in the presence or absence of test compounds. Each column represents the mean ± SEM (N = 3). †† Indicates significant difference from the control group at $P < 0.01$. * and ** indicate significant difference from the histamine-treated group at $P < 0.05$ and $P < 0.01$, respectively. Hi, histamine; Cim, cimetidine; Ran, ranitidine; Fam, famotidine; Pyr, pyrilamine.

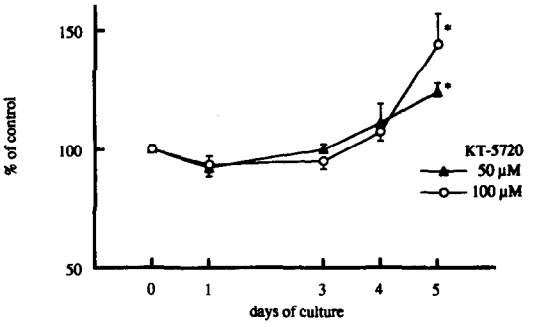

Fig. 5. Effect of KT-5720 on [^{3}H]thymidine incorporation into HL-60 cells. The cells were treated with KT-5720 and cultured for 5 days. Each point represents the mean ± SEM (N = 3). * indicates significant difference from the control at $P < 0.05$.

shown in Fig. 7, histamine at 1 μM strongly phosphorylated the 160 kD protein band. The 160 kD protein phosphorylation due to histamine was remarkably inhibited in the presence of either cimetidine or KT-5720; conversely, this may suggest that both H_2 receptor stimulation and A kinase activation are somehow crucial to the phosphorylation of the 160 kD protein.

DISCUSSION

Previous studies indicate that histamine induces a differentiation of neutrophil ancestor cells to mature neutrophils in association with its inhibitory influence on the proliferation of immature cells [4, 5]. It has also been reported that chronic injection of histamine, sustaining blood histamine levels slightly higher than the physiological blood concentration, induces a marked granulocytosis [3]. On the other hand, Gespach *et al.* [11] have shown that histamine causes an increase in intracellular cAMP in HL-60 cells via an H_2 receptor. In the present study, it became clear that histamine stimulates a differentiation of HL-60 cells to neutrophils and enhances phagocytic activity. Since promyelocytes do not exhibit phagocytic activity, this result may indicate that the number of phagocytic cells, such as myelocytes, metamyelocytes and neutrophils, increased in response to histamine. In connection with this, it has been reported that myeloperoxidase activity is present in primary granules of granulocytes and in myeloid precursors, most abundantly in promyerocytes [9]. The decrease in myeloperoxidase activity in response to histamine indicated that histamine provokes the differentiation of HL-60 cells to mature cells which exhibit less enzyme activity than promyelocytes [12].

The stimulatory effect of histamine on the differentiation of HL-60 cells was inhibited by H_2 antagonists but not by an H_1 antagonist. In many cells, it has been shown that histamine increases intracellular cAMP levels via H_2 receptors [13]. As indicated in the present study, rapid increases in both the cAMP content of and A kinase activity in HL-60 cells were elicited by histamine at concentrations which promote cell differentiation. Since the activity of A kinase is highly dependent upon the cAMP concentration, it was assumed that an early increase in cAMP may result in the subsequent activation of A kinase. In accordance with this, KT-5720, an A kinase inhibitor, was effective in inhibiting the differentiation of HL-60 cells elicited by histamine. It has been reported that the K_i value of KT-5720 on protein kinase A is 56 nM, while its K_i values on protein kinase C, protein kinase G and myosin light chain kinase are higher than 2000 nM [10]. Moreover, it has been shown that compounds which cause an increase in cAMP content, such as dibutyryl-cAMP, prostaglandin E_2 and theophylline, markedly promote the differentiation of neutrophil progenitors [14]; it was supposed that an increase in cAMP and A kinase activity due to histamine stimulation may be essential to trigger the differentiation of HL-60 cells. In addition, KT-5720 also enhanced a spontaneous proliferation of HL-60 cells, as shown in Fig. 5. Since HL-60 cells are spontaneously differentiated in the culture medium, it was assumed that when spontaneously activated A kinase was inhibited by KT-5720, a proliferation of cells was elicited while differentiation was inhibited.

In contrast, it has been reported that dimethyl sulfoxide and retinoic acid, which potently induce the differentiation of HL-60 cells to neutrophils, did not increase the cAMP content [15, 16]. However, it is reported also that dimethyl sulfoxide and retinoic acid may act directly on the microcircumstances of A kinase, which is located very near the site of action of cAMP, thus translocating the regulatory subunit of A kinase from cytosol to the membrane fraction, which would be a proper trigger for A kinase activation [14, 17]. However, in the case of histamine stimulation, an activation of A kinase is

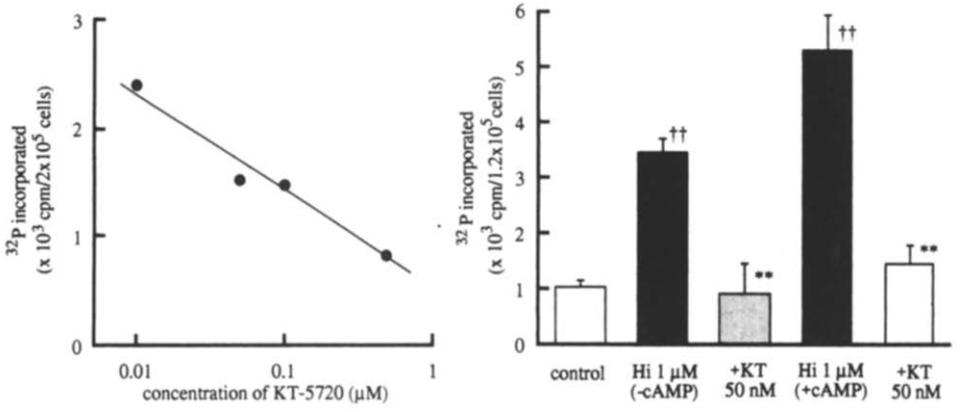

Fig. 6. Effect of KT-5720 on the cAMP-dependent A kinase activity in resting HL-60 cells (left) and histamine-treated HL-60 cells (right). The cells were incubated with KT-5720 both in the presence and absence of histamine (1 μM) for 3 hr. Thereafter, the A kinase activity was determined as indicated in the text. Each point or column represents the mean ± SEM (N = 3). †† Indicates significant difference from the control group at $P < 0.01$ and ** indicates significant difference from the histamine-treated group.

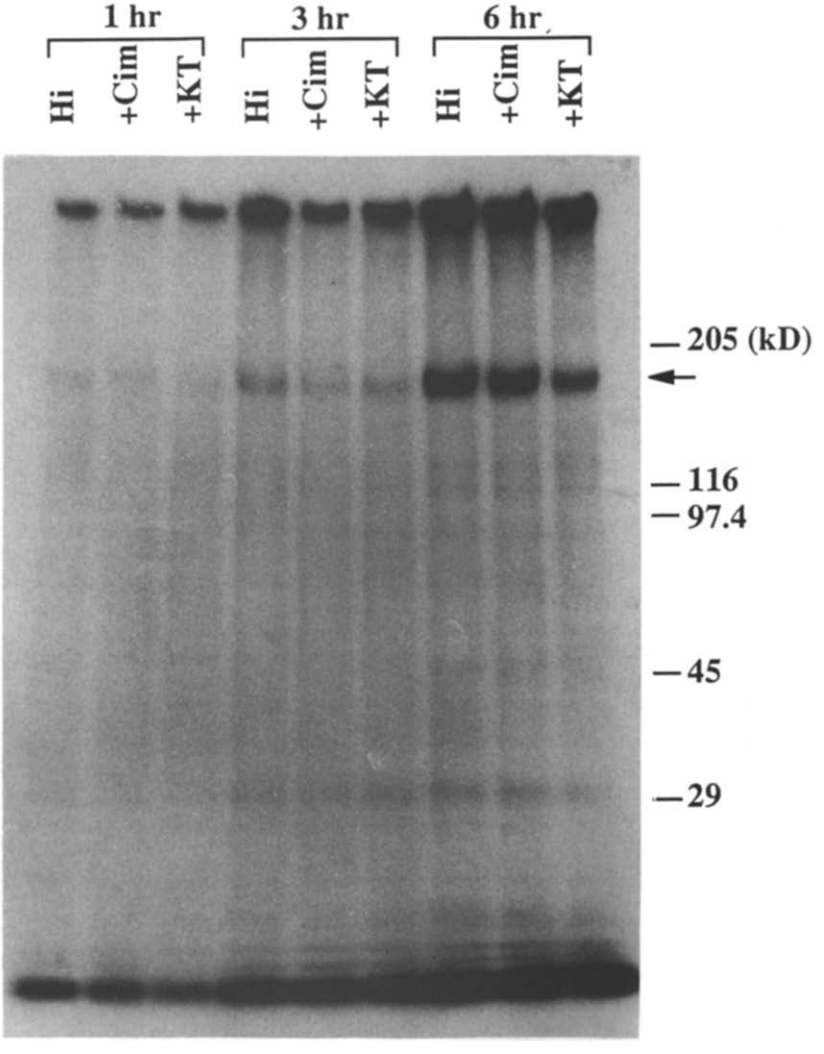

Fig. 7. Effects of cimetidine and KT-5720 on the protein phosphorylation in HL-60 cells elicited by histamine. The cells were stimulated with histamine (1 μM) in the presence or absence of test compounds for 6 hr. Cim, cimetidine (10 μM); KT, KT-5720 (50 nM). Arrow indicates 160 kD protein band.

intimately related to an increase in cAMP content and this may trigger the differentiation of neutrophil progenitors.

In the present study, the differentiation of HL-60 cells was observed after 3 days of culture with histamine. After the promyelocytes, myelocytes are capable of proliferation, though metamyelocytes and neutrophils are not. Therefore, in the early stages of histamine stimulation, the myelocyte population increased; this increase continued throughout 6 days of incubation as shown in Fig. 1.

The present examination of protein phosphorylation indicates that histamine induced the phosphorylation of a 160 kD protein in HL-60 cells, while phosphorylation was inhibited by H_2 antagonists and the A kinase inhibitor. This strongly suggests that phosphorylation of this protein is exerted by an activation of A kinase in association with H_2 receptor stimulation. It was assumed that this 160 kD protein may be related to the differentiation of HL-60 cells. The phosphorylation of the 160 kD protein spontaneously proceeded to a small degree in resting cells, suggesting that a spontaneous differentiation of HL-60 cells took place. On the other hand, it has been reported that phorbol esters, which are capable of eliciting the differentiation of HL-60 cells to macrophages and monocytes, induced the phosphorylation of a 64 kD and a 68 kD protein via activation of protein kinase C [18, 19]. Different phosphorylated proteins and protein kinases in HL-60 cells may lead the differentiation in different directions. Based on these findings, it was concluded that the 160 kD protein in HL-60 cells may play some critical role specifically in the differentiation toward neutrophils.

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