Effects of Dexamethasone and Aminophylline on Survival of Jurkat and HL-60 Cells

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The effects of dexamethasone and aminophylline on survival of Jurkat T-lymphocytic leukemia cells and HL-60 promyelocytic leukemia cells were investigated. Dexamethasone (10, 100 µM) and aminophylline (1, 100 µM) induced apoptosis in Jurkat and HL-60 cells in a concentration-dependent manner. Treatment with a combination of dexamethasone (10 nM) and aminophylline (1 µM) significantly increased the number of apoptotic HL-60 cells, but not that of Jurkat cells, compared with dexamethasone (10 nM) or aminophylline (1 µM) treatment alone. Dexamethasone and aminophylline also increased the number of phospho-histone H2B (Ser14)-positive Jurkat and HL-60 cells. Phospho-histone H2B (pH2B)-positive HL-60 cells were significantly increased by treatment with a combination of dexamethasone (10 nM) and aminophylline (1 µM), although no such effect was observed in Jurkat cells. On the other hand, simultaneous treatment with 10 nM dexamethasone and 1 µM aminophylline activated the 36-kDa MBP kinase, pro-apoptotic protein kinase in HL-60 cells. The activation of 36-kDa MBP kinase by dexamethasone and aminophylline was supported by studies showing an increase in the number of pH2B-positive and apoptotic Jurkat and HL-60 cells upon exposure to these drugs. Thus treatment with a combination of dexamethasone and aminophylline accelerates apoptosis of HL-60 cells via activation of 36-kDa MBP kinase and H2B phosphorylation.

Key words dexamethasone; apoptosis; phospho-histone H2B; HL-60; Jurkat

Glucocorticoids are currently the most potent anti-inflammatory drugs available and, whether administered systemically or by inhalation, are first-line drugs for the treatment of many respiratory diseases such as asthma. Theophylline is also known to have an anti-inflammatory action that may account for its clinical effectiveness in the reduction of inflammatory cells in the airway, in addition to its bronchodilative effects. There is increasing evidence that not only glucocorticoid, but also theophylline, attenuates eosinophil accumulation in the airways of asthmatic patients.1−3

Glucocorticoids and theophylline shorten the half-life of some inflammatory cells such as eosinophils. The survival of these cells depends on the presence of cytokines such as interleukin 5 and GM-CSF. Glucocorticoids and theophylline block the synthesis of these cytokines and so trigger apoptosis, but the molecular mechanism of this effect has yet to be elucidated.4−6

Several studies have shown that Mst1 (mammalian ste20-like kinase 1), a group II germinal center kinase, generates a catalytically active 36-kDa fragment during apoptosis.7−9 The 36-kDa Mst1 has been reported to catalyse the phosphorylation of histone H2B at Ser14, which is associated with chromosome condensation during apoptosis.10,11 The promotion of apoptosis of eosinophils and CD4+ T cells, but not neutrophils, is reportedly associated with the activation of 36-kDa Mst1.12,13

In the present study, we investigated the effect of dexamethasone and aminophylline (a theophylline dimer) on survival of T-lymphocytic leukemia Jurkat and promyelocytic leukemia HL-60 cells.

MATERIALS AND METHODS

Cell Culture Jurkat (clone E6-1, ATCC, VA, U.S.A.) and HL-60 (clone 15, ATCC, VA, U.S.A.) cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 2 mM l-glutamine in a 5% CO2 incubator at 37°C. The medium was refreshed every 2 or 3 d. HL-60 (clone 15) cell line is known to differentiate eosinophils, neutrophils and monocytes.

Determinatation of Apoptosis Apoptotic cells were quantified using Annexin V flow cytometry as described by Verves et al.14 The Annexin V assay exploits the fact that an early event during apoptosis of many cells is a loss of membrane lipid asymmetry, resulting in exposure of phosphatidylserine in the outer leaftlet of the plasma membrane. Briefly, cells were incubated with FITC-conjugated Annexin V and counterstained with propidium iodide (PI) in order to allow exclusion of necrotic cells. The cells were subsequently analyzed using a flow cytometer (Beckman Coulter Japan, Tokyo, Japan).

Detection of Phospho-Histone H2B (Ser14)-Positive Cells Cells grown on chamber slides (Asahi Technoglass) were fixed in 4% paraformaldehyde and then washed in PBS. Following permeabilization with 0.2% Triton X-100, cells were blocked in 2% goat serum PBS (blocking solution), then incubated with the H2B S14 phospho antibodies in block solution at 37°C. The cells were then washed three times with blocking solution and incubated for 1 h in goat anti-rabbit rhodamine (TRITC)-conjugated secondary antibody. After further washes, coverslips were applied with mounting medium. Phospho-H2B (Ser14) positive cells were determined under fluorescence microscopy.

Determination of Protein Kinase Activity by ‘in-gel’ Renaturation Kinase Assay After incubation with the indicated agents, 5×105 cells were lysed for 20 min on ice in 200 µl of immunoprecipitation buffer (10 mM Tris–HCl pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 2 mM sodium orthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotonin, 10 µg/ml pepstatin A, 1.25 mM NaN3 and 1 mM sodium pyrophosphate). Cell debris was removed by centrifugation at 12,000 × g for 15 min.
16000×g for 10 min at 4°C prior to addition of Laemmli sample buffer. The cell extract was used for MBP 'in-gel' renaturation kinase assay. The samples were separated on 10% SDS polyacrylamide gels containing 0.2 mg/ml MBP. After electrophoresis, SDS was removed by several washes with 20% isopropanol alcohol in 50 mM Tris–HCl, pH 8.0, followed by washing with 50 mM Tris–HCl, pH 8.0, and 5 mM 2-mercaptoethanol. Proteins were denatured by incubation with 6 M guanidine-HCl, 5 mM 2-mercaptoethanol, and 50 mM Tris–HCl, pH 8.0, and subsequently renatured by several washes over 12 to 18 h with 5 mM 2-mercaptoethanol, 0.04% Tween 40, and Tris–HCl, pH 8.0. The gels were then re-equilibrated by washing with 40 mM HEPES, pH 8.0, 10 mM MgCl₂, and 2 mM dithiothreitol. For the kinase reaction the gels were incubated for 3 h with 40 mM HEPES, pH 8.0, 10 mM MgCl₂, 55 μM ATP, 0.1 μM PKA inhibitor, and 925 kBq [³²P]ATP. The reaction was stopped and excess [³²P]ATP removed by washing in 5% trichloroacetic acid and 1% sodium pyrophosphate over 12 to 18 h. The gels were then dried, and radioactivity was detected by autoradiography.

**Chemicals** The following drugs and analytical reagents were used: FITC-conjugated Annexin V (Becton Dickinson Biosciences Japan, Tokyo, Japan), anti-human monoclonal antibodies against Fas (clone CH-11; Medical and Biological Laboratories, Nagoya, Japan), anti-phospho-histone H2B [Ser14] (Upstate, Lake Placid, NY, U.S.A.), goat F(ab')₂ fragment anti-rabbit IgG (H+L)-TRITC (Beckman Coulter Japan, Tokyo, Japan), γ[³²P]adenosine triphosphate ([γ⁳²P]ATP; >185 TBq/mmol; Perkin Elmer Life Sciences Japan, Tokyo, Japan), protein molecular weight marker (Amersham Biosciences Japan, Tokyo, Japan), Z-Asp-CH₂-DCB (carboxybenzoxyl-L-aspart-1-yl-[(2,6-dichlorobenzo)loxy]-methane; Peptide Institute, Osaka, Japan), dexamethasone, aminophylline, myelin basic protein, propidium iodide, fetal bovine serum (FBS), RPMI 1640 medium, aprotinin, pepstatin A, leupeptin, and all other chemicals (Sigma, St. Louis, MO, U.S.A.).

**Statistical Analysis** The data are expressed as means±S.E.M. and differences between them were analyzed either by Student's t test or ANOVA followed by the Newman–Keuls multiple comparison test, as appropriate, performed using the PRISM 4 software program (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical significance was defined as p<0.05.

**RESULTS**

Dexamethasone (10 nm and 1 μM, 24 h) and aminophylline (1 and 100 μM, 24 h) promoted apoptosis of Jurkat and HL-60 cells in a concentration-dependent manner. Apoptotic HL-60 cells were significantly increased by combination treatment with dexamethasone (10 nm) and aminophylline (1 μM) for 24 h in comparison with dexamethasone (10 nm) or aminophylline (1 μM) alone, but no such effect was observed in Jurkat cells. The numbers of apoptotic Jurkat and HL-60 cells were also significantly increased by anti-Fas antibody (Fig. 2). The apoptosis induced by anti-Fas antibody, dexamethasone, aminophylline, and combination treatment with dexamethasone and aminophylline, was significantly reduced by the caspase inhibitor Z-Asp-CH₂-DCB (100 μM) in both Jurkat and HL-60 cells. Typical Annexin V staining profiles were shown in Fig. 1.

To determine further apoptotic signals, we observed the phosphorylation of histone H2B using immunofluorescence. Typical fluorescence photomicrographs of pH2B-positive Jurkat and HL-60 cells were shown in Fig. 3. Figure 4A shows that pH2B-positive Jurkat cells (7.8±1.4%, n=5) were increased by treatment with 1 μM dexamethasone (28.5±7.7%, n=4), 100 μM aminophylline (38.3±9.5%, n=4), 100 ng/ml anti-Fas (65.5±7.8%, n=4) and combination treatment with 10 nm dexamethasone and 1 μM aminophylline (30.6±9.1%, n=4) for 24 h. On the other hand, pH2B-positive HL-60 cells (8.0±1.0%, n=5) were also increased by treatment with 1 μM dexamethasone (36.3±6.4%, n=4), 100 μM aminophylline (45.1±4.0%, n=4), 500 ng/ml anti-Fas (63.4±6.9%, n=4), and combination treatment with 10 nm dexamethasone and 1 μM aminophylline (54.9±7.3%, n=4) for 24 h (Fig. 4B). These results appear to reflect the effects of these drugs on apoptosis.

Phosphorylation of histone H2B is catalyzed by the 36-kDa MBP kinase, Mst1. We determined whether 36-kDa MBP kinase was activated by dexamethasone and aminophylline using the MBP 'in-gel' renaturation kinase assay. In both Jurkat and HL-60 cell lysates, we detected a 63-kDa MBP kinase, but not the 36-kDa molecule. The 36-kDa MBP kinase appeared after treatment with anti-Fas antibody for 4 h in both Jurkat and HL-60 cells. The activity was obtained up to 24 h (data not shown). The weak 36-kDa MBP kinase signals were detected by treatment with dexamethasone (1 μM), aminophylline (100 μM) and a combination of dexamethasone (10 nm) and aminophylline (1 μM) for 4 h, but were not enhanced by simultaneous treatment with anti-Fas antibody (100 ng/ml) and these drugs in Jurkat cells (Fig. 5A). In contrast, although the kinase signals were not detected after treatment with dexamethasone (1 μM) for 4 h, the signals were detected after treatment with aminophylline (100 μM) or a combination of dexamethasone (10 nm) and aminophylline (1 μM) for 4 h in HL-60 cells. Simultaneous treatment with anti-Fas antibody (500 ng/ml) and these drugs enhanced the 36-kDa MBP kinase signals in comparison with antibody treatment alone (Fig. 5B).

**DISCUSSION**

In the present study we investigated the effects of the anti-inflammatory drugs dexamethasone and aminophylline on survival of T-lymphocytic leukemia Jurkat and promyelocytic leukemia HL-60 cells. We demonstrated for the first time that a combination of dexamethasone (10 nm) and aminophylline (1 μM) at relatively low concentration accelerates the activation of 36-kDa MBP kinase, H2B phosphorylation and apoptosis in HL-60 cells, but not in Jurkat cells.

High concentrations of dexamethasone and aminophylline promoted apoptosis in both Jurkat and HL-60 cells, supporting the findings of previous studies. Although the mechanisms of these effects are still unclear, the authors speculate that they are correlated with protein kinase A activation or Bcl-2 attenuation. Anti-Fas antibody is widely known to act as an apoptosis inducer in various cell types. In HL-60 cells, the sensitivity of apoptosis to the antibody was relatively weak compared with that in Jurkat cells. O’Gorman et al. have reported that anti-Fas antibody-induced
Fig. 1. Typical Annexin V Staining Profiles in Jurkat (a—g) and HL-60 (h—n) Cells.
a and h, control; b, treated with 100 ng/ml anti-Fas antibodies; i, treated with 500 ng/ml anti-Fas antibodies; c and j, treated with 10 nM dexamethasone; d and k, treated with 1 mM dexamethasone; e and l, treated with 1 mM aminophylline; f and m, treated with 100 μM aminophylline; g and n, combination of 10 nM dexamethasone and 1 mM aminophylline.

Fig. 2. Apoptosis of Jurkat (A) and HL-60 (B) Cells is Mediated by Anti-Fas Antibody (100 or 500 ng/ml), Dexamethasone (Dex; 10, 1000 nM), Aminophylline (Am; 1, 100 μM) and Combination Treatment with Dex (10 nM) and Am (1 μM)

Cells were pretreated for 24 h with the indicated drugs and then analysed using flow cytometry. The data are presented as the percentage of apoptosis (mean±S.E.M. of 5—7 independent determinations). Closed column, treated with 100 μM Z-Asp-CH2-DCB.
* Significant difference from control value at \(p<0.05\).
† Significant difference between each other at \(p<0.05\).
* Significant difference from the value without Z-Asp-CH2-DCB at \(p<0.05\).

Fig. 3. Typical Fluorescence Photomicrographs of Phospho-H2B at Ser 14-Positive Jurkat (a—e) and HL-60 (f—j) Cells

a and f, control; b, treated with 100 ng/ml anti-Fas antibodies; g, treated with 500 ng/ml anti-Fas antibodies; c and h, treated with 1 μM dexamethasone; d and i, treated with 100 μM aminophylline; e and j, treated with combination of 10 nM dexamethasone and 1 μM aminophylline.
apoptosis was protected by phosphatidylinositol 3-kinase (PI3K) in HL-60 cells.

We also obtained evidence that apoptosis induced by combination treatment with dexamethasone and aminophylline in HL-60 cells involves phosphorylation of histone H2B. Chromatin condensation, as well as phosphatidylserine translocation, is an important process during apoptosis. Anti-Fas antibody, dexamethasone, aminophylline, and a combination of dexamethasone and aminophylline, enhanced the phosphorylation of histone H2B in HL-60 cells. In addition, anti-Fas antibody and aminophylline also enhanced the phosphorylation of histone H2B in Jurkat cells. These results confirmed the abilities of these drugs to induce apoptosis.

Studies using HL-60 cells have shown that induction of apoptosis using a range of anticancer drugs is associated with caspase-mediated cleavage and release of the catalytic domain of Mst1, which can be detected as a 36-kDa activity by 'in-gel' renaturation kinase assay employing MBP as a substrate. Furthermore, 36-kDa Mst1 is activated during apoptosis by anti-Fas antibody in human eosinophils and CD4+ T-lymphocytes, but not in human neutrophils. In the present study, activation of 36-kDa MBP kinase by anti-Fas antibody, dexamethasone and aminophylline also paralleled our observation of histone H2B phosphorylation. Cheung et al. demonstrated that catalytic 36-kDa Mst1 phosphorylated histone H2B in HL-60 cells. Therefore, we speculate that 36-kDa MBP kinase might be a Mst1-like kinase, though further experiments will be required to confirm this. A small amount of 36-kDa MBP kinase activity was detected after treatment with dexamethasone for 4 h in Jurkat cell lysate but not in HL-60 cell lysate, though the reason for this is unclear.

Asthma is characterized by persistent inflammation in the large and small airways, and by CD4+ T-lymphocytes, eosinophils and macrophages in subepithelial airway walls. Reduced levels of apoptosis have been observed in eosinophils and macrophages in the airway walls of patients with asthma. Dexamethasone and theophylline are known to accelerate apoptosis of eosinophils and lymphocytes. Furthermore, several studies have reported additive effects of low-dose theophylline (same concentration as that used in the present study) with moderate-to-high-dose inhaled corticosteroid therapy, in terms of peak flow and FEV1. Taken together, the effects of these drugs might be correlated with their abilities to induce apoptosis. Moreover, HL-60 cells, especially clone 15, are able to differentiate to eosinophils. Thus, those clinical evidences supported our present observations in Jurkat and HL-60 cells.

It remains to be shown mechanisms of the HL-60 selective synergistic activity of dexamethasone and aminophylline. As described above, we thought that the combination treatment of dexamethasone and aminophylline possibly inhibited the protective pathways to apoptosis such as PI3K.

In conclusion, HL-60 but not Jurkat cells show a significant increase in the number of apoptotic cells after combination treatment with dexamethasone (10 nM) and aminophylline (1 μM), in comparison with dexamethasone or aminophylline alone. The apoptosis is mediated through cleavage of a caspase-dependent 63-kDa MBP kinase, which might yield a catalytic 36-kDa Mst1-like kinase. These ef-
fects of dexamethasone and aminophylline seem to contribute to their antitumor or anti-inflammatory activities.

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