Glucocorticosteroids Induce DNA Fragmentation in Human Lymphoid Leukemia Cells

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The present study was undertaken to investigate the potential role of glucocorticoid-induced DNA damage in the lysis of human lymphoid leukemia cells by glucocorticoids. Lymphoblasts were isolated from patients with acute lymphoblastic leukemia (ALL) or chronic myelogenous leukemia (CML) in blast crisis and cultured in vitro with or without dexamethasone. DNA was then purified from the cells and analyzed by agarose gel electrophoresis. Only high molecular weight (mol wt) DNA was present in cells cultured without dexamethasone, but a ladder of DNA fragments ranging in size from 180 to 200 bp was present in cells cultured with dexamethasone. The DNA fragments were multiples of 180 to 200 bp, suggesting an internucleosomal site of DNA cleavage. The same pattern of DNA fragmentation was detected in normal thymocytes isolated from adrenalectomized rats following in vivo treatment with dexamethasone and in S49 mouse thymoma cells after in vitro incubation with dexamethasone. Dexamethasone-induced DNA fragmentation preceded overt loss of viability in glucocorticoid-sensitive cells but was not detected in two variants of the S49 cell line that are glucocorticoid resistant owing to glucocorticoid receptor defects. The results suggest that glucocorticoids kill human lymphoblastic leukemia cells and both normal and malignant murine thymocytes by a common mechanism that involves glucocorticoid induction of an endonucleolytic activity with cleavage of genomic DNA.

MATERIALS AND METHODS

Reagents. Ultrapure phenol was from Bethesda Research Laboratories, Gaithersburg, MD. High-pressure liquid chromatography (HPLC) grade chloroform was from Fisher, Orangeburg, NY. Agarose was from International Biotechnologies, New Haven, CN. Proteinase K, RNase (type I-A-S), ethidium bromide, dexamethasone, and other chemicals were from Sigma, St Louis.

Cell lines. Methods of cell culture and the properties of the cell lines have been described previously. The S49 cell line was established from a lymphoma induced in a BALB/c/St mouse and retains the properties of thymocytes, including H-2, Thy-1.2, and thymus leukemia antigens. Two variants of the S49 cell line, S49.22r and S49.143r, were the gift of Dr Keith Yamamoto, University of California, San Francisco. These variants are resistant to glucocorticoid-induced lysis because of defective glucocorticoid receptor function. Receptors in S49.22r cells (nt variant) do not translate to the nucleus and do not bind to DNA. Receptors in S49.143r cells (nt variant) are reduced in size and bind to DNA with an abnormally increased affinity. In most experiments, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glucose, penicillin, and streptomycin. Cells were maintained at 37°C in a 5% CO2/95% air atmosphere and were used for experiments during the exponential phase of growth.

Rat thymocytes. Male Sprague-Dawley rats, eight to ten weeks of age, were adrenalectomized and then three days later were injected intraperitoneally (IP) with either vehicle or 5 mg/kg dexamethasone. Six hours later, the animals were killed and thymus glands were removed and rinsed with ice-cold phosphate-buffered saline, pH 7.8 (PBS; 140 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L Na2HPO4, 15 mmol/L KH2PO4). The procedure for isolating...
ing thymocytes was modified after the method of Compton and Cidlowski.\(^9\) Thymus glands were minced and then passed through a fine wire mesh into 30 mL ice-cold PBS. Thymocytes were separated from RBCs and other contaminating cellular elements by Ficoll-Hypaque gradient centrifugation as previously described.\(^2\) The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

**Human leukemia cells.** Lymphoblasts were isolated from the peripheral blood or bone marrow of four children with leukemia. Patient 1 had Philadelphia chromosome-positive CML in lymphoid blast crisis. Approximately 80% of the cells in peripheral blood were lymphoblasts by morphology and were positive for terminal deoxynucleotidyl transferase (TdT). Patients 2 through 4 had untreated, newly diagnosed ALL, confirmed by morphology, histochemical staining, and serologic markers. Patient 1 reentered chronic phase and patients 2 through 4 entered complete remission after treatment with combination chemotherapy regimens including prednisone. The use of human subjects in this study was approved by the Institutional Review Board for Human Investigation of University Hospitals of Cleveland.

Cells were isolated from heparinized peripheral blood or bone marrow by Ficoll-Hypaque density-gradient centrifugation as previously described.\(^3\) DNA was purified from cells either immediately or after short-term in vitro culture in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L glutamine, penicillin, and streptomycin.

**Cell viability.** Cells were counted with a hemocytometer, and viability was determined by the ability of cells to exclude trypan blue dye.

**DNA purification and analysis.** DNA was isolated from 50 to 90 million cells by a modification of the method of Compton and Cidlowski.\(^9\) Cells were washed twice with ice-cold PBS and pelleted by centrifugation at 150 g for ten minutes at 4\(^\circ\)C. Cell pellets were resuspended in 1.0 to 1.5 mL buffer containing 0.2 mol/L Tris, pH 8.0/0.1 mol/L EDTA, and then an equal volume of buffer containing 0.2 mol/L Tris pH 8.0/0.1 mol/L EDTA-2% (wt/vol) sodium dodecyl sulfate (SDS) was added. Proteinase K was added to achieve a final concentration of 50 \(\mu\)g/mL, and then the cell lysate was incubated for 6 to 12 hours in a water bath at 37\(^\circ\)C. The cell lysate was extracted once with phenol, three to four times with phenol/chloroform, and finally with chloroform.

The aqueous phase was dialyzed at 4\(^\circ\)C against three 4-L exchanges of buffer containing 50 mmol/L Tris, pH 8.0/10 mmol/L EDTA 10 mmol/L NaCl, and then incubated with 100 \(\mu\)g/mL RNase for two hours at 37\(^\circ\)C. The cell lysate was extracted once with phenol, three to four times with phenol/chloroform until the interface was clear, and finally with chloroform.

The aqueous phase was dialyzed at 4\(^\circ\)C against three 4-L exchanges of buffer containing 10 mmol/L Tris, pH 8.0/10 mmol/L EDTA 10 mmol/L NaCl, and then incubated with 100 \(\mu\)g/mL RNase for two hours at 37\(^\circ\)C. DNA was extracted once with phenol, twice with phenol/chloroform, and finally with chloroform, and then was dialyzed at 4\(^\circ\)C against two 4-L exchanges of buffer containing 10 mmol/L Tris, pH 8.0/1 mmol/L EDTA. The concentration of DNA was estimated from the OD\(_{260}\) and 4 to 10 \(\mu\)g DNA was subjected to electrophoresis on 1.8% agarose gels at 25 V. An HaeIII digest of \(\phi X174\) (Bethesda Research Laboratories) was applied to each gel to provide mol-wt standards of 1,353, 1,078, 872, 603, 310, 271 to 281, 234, 194, 118, and 72 base pairs (bp). After completion of electrophoresis, DNA in the gel was stained by soaking the gel for 15 minutes in 90 mmol Tris, pH 8.25/90 mmol/L boric acid/2.5 mmol/L disodium EDTA (TBE buffer) containing 1 \(\mu\)g/mL ethidium bromide. The gel was then destained by soaking in TBE buffer for 30 minutes.

**RESULTS**

To determine the effect of glucocorticoids on the integrity of DNA in normal rat thymocytes, thymus glands were removed from adrenalectomized rats that had been injected six hours previously with either vehicle or 5 mg/kg dexamethasone, and DNA purified from the thymocytes was analyzed by agarose gel electrophoresis (Fig 1). Only high-mol-wt DNA was detected in thymocytes from animals treated with vehicle, but DNA from animals treated with dexamethasone was extensively fragmented, with fragments as small as 180 to 200 bp detected, consistent with earlier observations by other investigators.\(^4\)\(^-\)\(^9\) Dexamethasone induced a ladder of DNA fragments in which the individual fragments appeared to be integer multiples of 180 to 200 bp, consistent with an internucleosomal site of DNA cleavage. Dexamethasone-induced DNA fragmentation preceded cell death, since there was no difference between vehicle-treated and dexamethasone-treated rats in terms of weights of thymus glands or number of thymocytes recovered. Moreover, the proportion of nonviable thymocytes, defined by the uptake of trypan blue dye, was not significantly different in vehicle-treated and dexamethasone-treated animals (12% and 10%, respectively) six hours after injection of dexamethasone, although the proportion of nonviable cells was markedly different (10% and 75%, respectively) 24 hours after injection of dexamethasone.

Figure 1 also shows that dexamethasone induces the same pattern of DNA fragmentation in S49 mouse thymoma cells. In this experiment, wild-type S49 cells were incubated in vitro with 0.1 \(\mu\)mol/L dexamethasone for 24 hours before DNA purification and analysis. At this time, the proportion of nonviable cells, defined by uptake of trypan blue dye, was 2% for vehicle-treated cells and 9% for dexamethasone-treated cells. The reduction in cell viability observed for cells cultured for 24 hours with dexamethasone is consistent with the sensitivity of wild-type S49 cells to glucocorticoid-induced cell lysis.\(^5\)\(^-\)\(^9\) After incubation with dexamethasone...
for 48 hours, the number of thymocytes in culture was reduced two- to threefold as compared with control cultures and 45% to 50% of cells were nonviable, as defined by the uptake of trypan blue dye.

Figure 2 shows that dexamethasone induces DNA fragmentation in glucocorticoid-sensitive wild-type S49 cells but not in two glucocorticoid-resistant S49 variants (nt- and nt') that have defective glucocorticoid receptor function (a more detailed description of these cell lines is given in the Materials and Methods section). This result indicates that normal glucocorticoid-induced receptor function is a requirement for glucocorticoid-induced DNA fragmentation, consistent with knowledge that glucocorticoid-induced lymphocytolysis is mediated through the glucocorticoid receptor. DNA fragmentation in wild-type S49 cells was detected as early as six hours after addition of dexamethasone to cultures and did not appear to increase further by 12 hours. This is consistent with the time course of glucocorticoid-induced DNA fragmentation in murine thymocytes reported previously by other investigators. The proportion of nonviable cells, defined by uptake of trypan blue dye, in vehicle-treated and dexamethasone-treated cells was 3% and 4%, respectively, at six hours and 7% and 15%, respectively, at 12 hours. Therefore, glucocorticoid-induced DNA fragmentation precedes overt cell death.

To determine if glucocorticoids induce DNA fragmentation in human lymphoblastic leukemia cells and if the pattern of DNA fragmentation is the same as that observed in murine thymocytes, we determined the effect of dexamethasone on the integrity of DNA in lymphoblasts isolated from one patient with CML in lymphoid blast crisis (patient 1) and from three patients with ALL (patients 2 through 4). The results of studies using cells from patient 1 are shown in Fig. 3. Patient 1 had a large number of lymphoblasts in the peripheral blood and we were therefore able to analyze DNA after both in vivo and in vitro exposure to glucocorticoids. DNA fragmentation was not detected either before or 24 hours following initiation of in vivo treatment with 60 mg/m² oral prednisone and 1.5 mg/m² vincristine. However, DNA fragmentation was detected after in vitro incubation for five hours with 0.01 and 0.1 µmol/L dexamethasone. There was no increase in the uptake of trypan blue dye by cells at this time, indicating that DNA fragmentation preceded overt cell death. DNA fragmentation did not appear to increase after more prolonged exposure to dexamethasone (11 and 21 hours), although cell viability was reduced (not shown). The ladder of DNA fragments induced by dexamethasone was the same as that observed in murine thymocytes and is consistent with an internucleosomal site of DNA cleavage.

Lymphoblasts from patients 2 through 4 were also incubated in vitro with 0.1 µmol/L dexamethasone prior to DNA purification and analysis by agarose gel electrophoresis (Fig 4). These patients did not have large numbers of blasts in the peripheral blood, and repeat bone marrow aspirations could not be performed to assess the in vivo effect of glucocorticoid therapy. Dexamethasone induced the same pattern of DNA fragmentation in lymphoblasts from patient 2 as in lymphoblasts from patient 1. Again, dexamethasone-induced DNA fragmentation preceded overt cell death. Lymphoblasts from patients 3 and 4 were incubated with 0.1 µmol/L dexamethasone for 14 and 17 hours, respectively, without evidence of DNA fragmentation. DNA isolated from these samples was concentrated by ethanol precipitation so that 26 µg could be

Fig 2. Effect of dexamethasone on the integrity of DNA in glucocorticoid-sensitive and glucocorticoid-resistant mouse thymoma cells. Wild-type (wt) glucocorticoid-sensitive S49.1 cells and two glucocorticoid-resistant variants, S49.22r (nt-) and S49.143r (nt'), were cultured for six or 12 hours with either vehicle (-) or 0.1 µmol/L dexamethasone (+) prior to DNA purification. Shown is ethidium bromide-stained DNA after agarose gel electrophoresis; 4 µg DNA was applied to each lane of the gel. Mol-wt standards (S) range in size from 1,353 to 72 bp.

Fig 3. Effect of dexamethasone on the integrity of DNA in human lymphoblastic leukemia cells. Left panel: Lymphoblasts were isolated from the peripheral blood of patient 1 either before (lane 1) or 24 hours after initiation of treatment with prednisone and vincristine (lane 2). DNA was immediately purified from the cells and analyzed by agarose gel electrophoresis. Right panel: Lymphoblasts were isolated from the peripheral blood of patient 1 before treatment and incubated in vitro for five hours with vehicle (lane 3), 0.01 µmol/L dexamethasone (lane 4), or 0.1 µmol/L dexamethasone (lane 5). DNA was then purified and analyzed by agarose gel electrophoresis. Shown are the ethidium bromide-stained gels. Mol-wt standards (S) range in size from 1,353 to 72 bp.
viability was induced by dexamethasone. Cells were incubated without dexamethasone (not shown). DNA fragmentation was not detected in any of the samples when bromide-stained gels. Mol-wt standards range in size from 1,353 to 72 bp. Lane a, Patient 2; lane b, patient 3; lane c, patient 4.

DNA fragmentation was not detected in lymphoblasts isolated from one patient following in vivo treatment with prednisone and vincristine; DNA fragments were detected in cells from the same patient, however, after in vitro exposure to dexamethasone. There are several possible explanations for this result. First, lymphoblasts affected by glucocorticoids may be cleared rapidly from the circulation. Second, the concentration of glucocorticoid achieved in vivo may not have been comparable to that achieved in vitro. Third, the difference between in vivo and in vitro responses may reflect the greater potency of dexamethasone as compared with prednisone. Fourth, lymphoblasts may differ in terms of their sensitivity to different types of glucocorticoid compounds, as suggested by previous studies using murine thymocytes.

The findings of the present study must be interpreted in light of what is already known about the general mechanism of steroid hormone action. Most, if not all, effects of glucocorticoids on cells are mediated through the glucocorticoid receptor and involve regulation of gene transcription (reviewed in ref 27). The glucocorticoid receptor is a regulatory protein that binds to certain sequences in the genome and thereby regulates the transcription of specific genes. Based on this general model of glucocorticoid action, the mechanism by which glucocorticoids induce lymphocytolysis can be separated into two sequential steps. The first step involves interaction of the glucocorticoid with its receptor; the second step involves regulation of gene transcription by the glucocorticoid receptor complex.

With regard to the first step, considerable evidence shows that glucocorticoid-induced lysis of both murine thymocytes and human ALL cells is mediated through the glucocorticoid receptor and that quantitative or qualitative receptor defects give rise to glucocorticoid resistance. The results of the present study show that glucocorticoids do not induce DNA fragmentation in murine thymocytes that have defective glucocorticoid receptor function, indicating that glucocorticoid-induced DNA fragmentation is mediated through the glucocorticoid receptor.

With regard to the second step, genetic complementation experiments using glucocorticoid-sensitive and glucocorticoid-resistant mouse thymoma cells indicate that glucocorticoid-induced gene product(s) mediate glucocorticoid-induced cytolysis. The concept that glucocorticoid-induced lymphocytolysis involves regulation of gene transcription is supported by two other types of evidence. First, glucocorticoid-induced nuclear disruption and DNA fragmentation in murine thymocytes is blocked by inhibitors of macromolecular synthesis. Second, glucocorticoid-resistant thymoma cells can be converted to a glucocorticoid-sensitive phenotype by DNA demethylation. Based on these observations, Gasson and Bourgeois proposed the existence of a glucocorticoid-regulated "lysis gene." Evidence that DNA fragmentation is a central event in glucocorticoid-induced lymphocytolysis suggests that the lysis gene product may be either an endonuclease or a factor that activates an existing endonuclease. In support of this model, two groups of investigators recently detected glucocorticoid-induced endonuclease activities in murine thymocytes.

Based on the findings of the present study, we propose that activation of an endonucleolytic activity thatcleaves...
GLUCOCORTICOID-INDUCED DNA FRAGMENTATION

Genomic DNA may be an initiating event in the lysis of ALL cells by glucocorticoids. Further experimentation will be required to characterize the endonuclease responsible for glucocorticoid-induced DNA fragmentation in ALL cells. Ultimately, assays for glucocorticoid-induced endonucleolytic activity may prove a useful means of monitoring glucocorticoid therapy and predicting the responsiveness of individual patients to glucocorticoids.

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