Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 30-37

MOLECULAR CHANGES ASSOCIATED WITH INDUCTION OF CELL DEATH IN A HUMAN T-CELL LEUKAEMIA LINE: PUTATIVE NUCLEASES IDENTIFIED AS HISTONES

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Received May 28, 1989

Following treatment of the human T-cell leukaemia line, CEM-C7, with the glucocorticoid, dexamethasone, a rapid decrease in viability occurred after 40 h which coincided with fragmentation of DNA in these cells. A similar pattern of DNA fragmentation was observed when these cells were γ-irradiated or treated with cycloheximide. Distinct morphological changes occurred after treatment, indicating a form of cell death, regulated from within, termed apoptosis. A set of nuclear proteins ranging in size from 10-18 kDa appeared by 40 h following treatment with dexamethasone. Treatment of cells with γ-irradiation or cycloheximide also produced the same protein pattern. This set of proteins, and a doublet approximately 55 kDa in size, had apparent nuclease activity which was not observed in untreated cells. However, protein microsequencing of these bands in the 10-18 kDa region revealed that they were histone proteins. These results cast doubt on a recent report which provided evidence that these proteins were induced nucleases.

The death of thymocytes after treatment with glucocorticoids is associated with distinct morphological and molecular changes termed apoptosis or programmed cell death (1). During this process there is a rapid reduction in cell volume accompanied by loss of microvilli, blebbing of the cell surface, and condensation of the nuclear chromatin (2). Cells may remain intact or fragment into membrane bound apoptotic bodies which are either phagocytosed or go onto secondary necrosis. The morphologic changes in the nucleus are associated with the breakdown of chromatin into fragments that increase in size by approximately 200 bp, corresponding in size to the internucleosomal distance (3,4). Evidence suggests that this fragmentation is the result of activation of a Ca^{2+}/Mg^{2+} dependent endonuclease that degrades the exposed linker region between the nucleosomal cores (5). This process of regulated cell death (apoptosis) is dependent on active protein synthesis (2). Indeed recent studies have shown induction of nuclear proteins possessing nuclease activity in rat thymocytes treated with glucocorticoids (6). DNA fragmentation and

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apoptosis are not restricted to thymocytes treated with glucocorticoids but have been observed when lymphocytes are exposed to low doses of γ-irradiation (7), after removal of interleukin 2 from dependent T-cell lines (8), in target cells following attack by cytotoxic T-cells (9), following treatment of tumours with certain chemotherapeutic agents (10) and after isolation of human leukaemic cells from the blood (11,12).

This study was designed to define events at the morphological and molecular level in glucocorticoid-treated, human leukaemic cells using as a model system the glucocorticoid-sensitive human T-cell leukaemia line, CEM- C7.

MATERIALS AND METHODS

Cells and cell culture

CEM-C7 cells were a generous gift from Dr G. Melnykovych at the Veteran's Administration Center, Kansas City. Cells were grown as suspension cultures in RPMI 1640 supplemented with 10% foetal calf serum (Flow Laboratories) in an atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were maintained at concentrations ranging from 10^5 to 2 x 10^6 cells/ml. Cell numbers and viability were determined on a hemocytometer using 0.4% trypan blue.

In all experiments, cells were resuspended in tissue culture medium at a concentration of 5 x 10^5 cells/ml. The glucocorticoid, dexamethasone, was used at a final concentration of 10^{-6} M, and cycloheximide (Sigma) at a concentration of 50 μg/ml. γ-irradiation was delivered from a ^{60}Co source (Gamma Cell, Atomic Energy Commission of Canada) at a dose rate of 16 Gy/min.

DNA and Protein Extraction

DNA was extracted according to established methods (13) - approximately 10 μg/lane was electrophoresed in 1.4% agarose gels at 15 mA for 15 h.

Protein was extracted essentially as described by Compton & Cidlowski (6). All steps were performed at 4°C. Nuclei were isolated, using hypotonic shock, from approximately 2 x 10^8 cells by incubation in 1.5 mM $MgCl_2$ for 30 min. They were then pelleted at 200g for 10 min, washed in 1.5 mM $MgCl_2$ and repelleted. Protein was extracted from chromatin by addition of 2 ml of 0.6 M NaCl, 1 mM EDTA, 0.02% NaN_3, 0.5 μg/ml pepstatin A, 0.5 μg/ml aprotinin and 0.5 mM N-ethylmalemide and mixed for 1 h. The extract was centrifuged at 180,000g for 1 h and the supernatant dialyzed overnight against 20 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 0.02% NaN_3, 0.5 μg/ml aprotinin, 0.5 mM N-ethylmalemide and 11 μg/ml phenylmethylsulfonyl fluoride. The precipitate that formed inside the dialysis tubing was pelleted at 12,000g for 5 sec and resuspended in 40 mM Tris HCl (pH 7.4), 1% SDS and 50% glycerol. Histone proteins were extracted from untreated CEM-C7 cells for comparison with protein patterns appearing after dexamethasone treatment. Nuclei were prepared as described above, extracted with buffer containing 0.6M NaCl, and the resulting nuclear pellet further extracted with 20mM H_2SO_4. This was followed by centrifugation at 180,000g for 1h, the supernatant recovered and histones precipitated by the addition of 6 volumes of acetone. Histones were finally dissolved in resuspension buffer as described above.

Polyacrylamide gel electrophoresis

Ten micrograms of protein were mixed with loading buffer, boiled for 5 min and electrophoresed in a Bio-Rad Protean™ II Slab Cell in a 13% SDS-polyacrylamide gel (SDS-PAGE) using standard procedures (14). Gels were stained with Coomassie Blue and molecular weights determined using low molecular weight markers (Bio- Rad). Activity gels, for the detection of nucleases (15) were prepared as described for SDS-PAGE except that 20 μg/ml of

salmon sperm DNA was added to the gel solution before pouring. After electrophoresis, SDS was removed from the gel by washing overnight in 40 mM Tris HCl, (pH 7.4) and 2 mM $MgCl_2$ with several changes. Activity was detected by staining the gel with ethidium bromide and visualized using a UV transilluminator. This type of gel assay will also detect proteins that bind DNA.

Microsequencing of immobilized proteins

Nuclear extracts from dexamethasone treated cells were prepared as described above and samples were separated by SDS-PAGE according to Moos *et al.* (16). Microsequencing was performed using the Edman degradation method in an Applied Biosystems 470A Gas Phase Sequencer.

RESULTS

Chromatin cleavage

The human leukaemic cell line CEM-C7 was treated with dexamethasone in order to determine the time course of events leading to cell death. Exposure to 10^{-6} M dexamethasone had no appreciable effect on cell viability up to 30 h but by 40 h a rapid drop in viability was evident (Fig. 1). As expected, after exposure to radiation a marked effect on cell viability was observed with increasing dose, and also with cycloheximide in the presence or absence of dexamethasone. In order to check for DNA fragmentation, cells were harvested at 0, 24, 36, 42 and 48 h after treatment and DNA isolated and analyzed by agarose gel electrophoresis (Fig. 2). DNA fragmentation was not evident until 40 h and increased up to 48 h (lane 5). The fragments observed increased in size by approximately 200 bp. This pattern was also seen when cells were treated with 20 Gy of γ-irradiation or 50 μg/ml cycloheximide (Fig. 2, lanes 6, 7). The appearance of fragmentation following treatment with dexamethasone

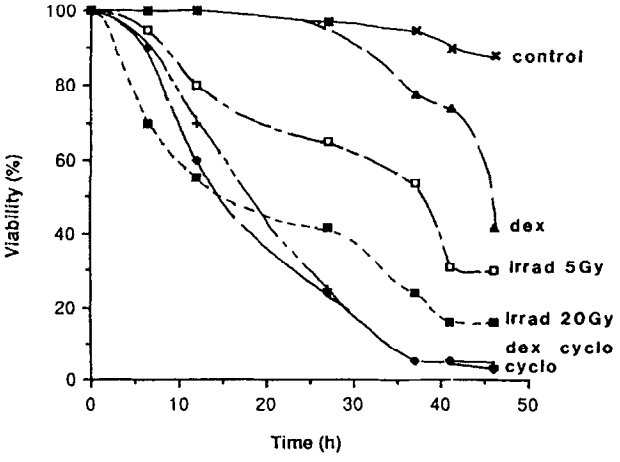

Figure 1. Time course of CEM-C7 viability following various treatments to induce apoptosis and DNA fragmentation. Cells were incubated in medium alone (control, ✕), with 10^{-6} M dexamethasone (dex, ▲), 10^{-6} M dexamethasone and 50 μg/ml cycloheximide (dex+cyclo, ✚), 50 μg/ml cycloheximide (cyclo, ◆), treated with γ-irradiation (irrad. 5 Gy, □; 20 Gy, ■).

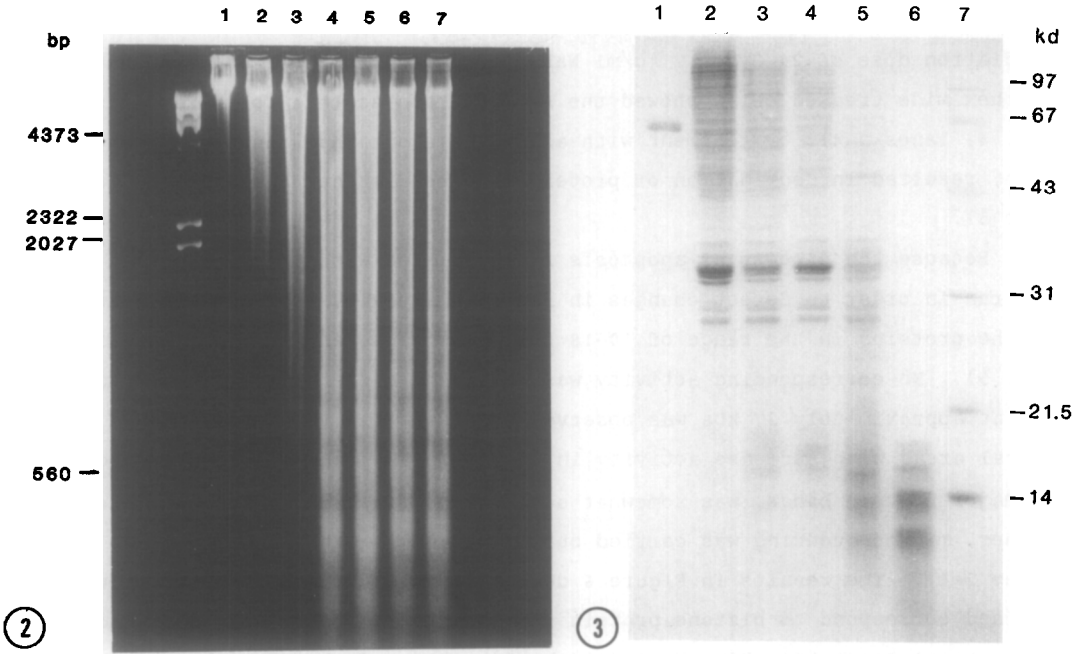

Figure 2. Time course of DNA fragmentation in CEM-C7 cells following addition of dexamethasone. DNA was extracted at the times indicated and electrophoresed in 1.4% agarose gels. Individual fragments are indicated by the horizontal bars. Lane 1, 0 h; lane 2, 24 h; lane 3, 36 h; lane 4, 40 h; lane 5, 48 h incubation with dexamethasone; lane 6, 48 h following 20 Gy γ-irradiation; lane 7, 48 h following addition of 50 μg/ml cycloheximide.

Figure 3. Time course of appearance of nuclear proteins in CEM-C7 cells following addition of 10^{-6} M dexamethasone. Protein was extracted at the times indicated, electrophoresed in 13% SDS-polyacrylamide gels and stained with Coomassie Blue. Lane 1, control DNase; lane 2, untreated cells; lane 3, 24 h; lane 4, 36 h; lane 5, 42 h; lane 6, 48 h incubation with dexamethasone; lane 7, molecular weight markers.

corresponded to the rapid drop in cell viability (Fig. 1). Examination of these cells by electron microscopy showed apoptosis as the dominant form of cell death (results not shown).

Protein changes during apoptosis

It has been shown previously that apoptosis is dependent on protein synthesis (2). Cells were therefore examined for induction of new proteins or increases in the levels of those already present following treatment with dexamethasone. Nuclear protein was extracted and run on SDS-PAGE. The only proteins that appeared to be induced were a group of 6 with a molecular weight range of 10-18 kDa (Fig. 3). Some of the protein bands were faintly visible in untreated cells and most of the bands could be seen even at 36 h but by 40 h a marked increase occurred which continued up to 48 h. At the same time as these proteins increased other proteins appeared to be lost, and by 48 h only the low molecular weight proteins were visible with Coomassie Blue staining. Protein

was also extracted from cells treated with; 50 μg/ml cycloheximide, a γ-irradiation dose of 20 Gy or 2 μg/ml NaN_3. Protein from γ-irradiated and cycloheximide treated cells showed the same pattern as that for dexamethasone (Fig. 4, lanes 2-4). Treatment with azide, an agent that causes necrotic cell death, resulted in degradation of protein with no distinct bands being visible (lane 5).

Because the process of apoptosis is associated with DNA degradation, gels were run in order to detect changes in nuclease activity in these extracts. All the proteins in the range of 10-18 kDa showed apparent nuclease activity (Fig. 5). No corresponding activity was observed in the control sample and a band at approximately 37 kDa was observed for DNase 1. The appearance of several areas with nuclease activity in the 10-18 kDa range, corresponding to the major protein bands, was somewhat surprising. In order to investigate this further, microsequencing was carried out on 3 bands of the 6 appearing in Fig 4 (lanes 2-4). The results in Figure 6 demonstrate that the N-terminus sequences obtained correspond to histone protein sequences. The uppermost band (Fig 4) is identical to histone H3, the middle band to H2B and the lower band is H2B

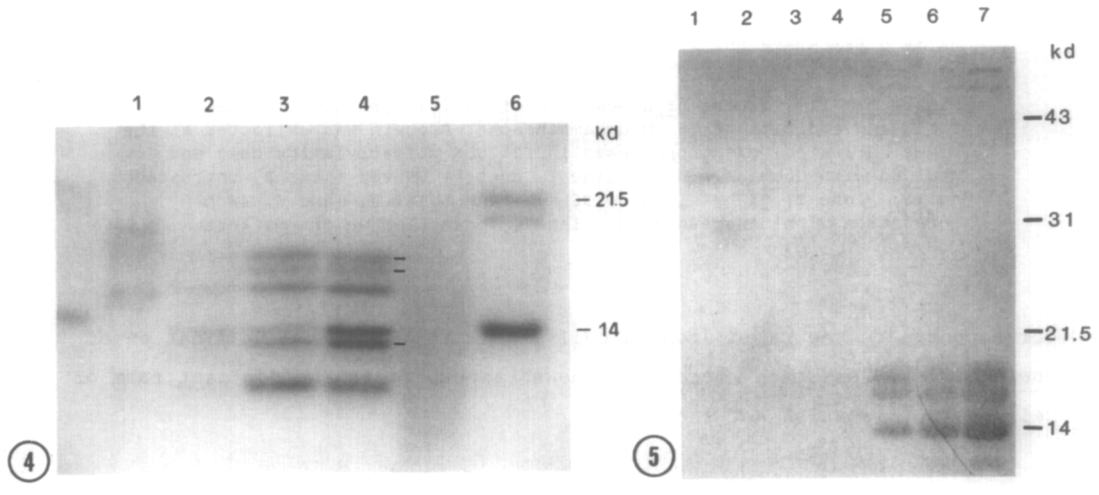

Figure 4. Nuclear proteins extracted from CEM-C7 cells following various treatments to induce apoptosis. Protein was extracted 48 h following the treatments indicated, electrophoresed in 13% SDS-polyacrylamide gels and stained with Coomassie Blue. Lane 1, untreated cells; lane 2, 10^{-6} M dexamethasone; lane 3, 50 μg/ml cycloheximide; lane 4, 20 Gy γ-irradiation; lane 5, 2 μg/ml NaN_3; lane 6, molecular weight markers.

Figure 5. Appearance of nuclease (DNA-binding protein) in CEM-C7 cells following addition of 10^{-6} M dexamethasone. Protein was extracted at the times indicated and electrophoresed in 13% SDS-polyacrylamide gels containing 20 μg/ml salmon sperm DNA. Following removal of SDS and incubation for 24h in the presence of Ca^{2+}, gels were stained with ethidium bromide and visualized using ultraviolet light to identify areas of activity. Lane 1, control DNase; lane 2, untreated cells; lane 3, 24 h; lane 4, 36 h; lane 5, 40 h; lane 6, 44 h; lane 7, 48 h incubation with dexamethasone.

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Sequence 1    ?  -Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser --
Histone H3    Ala-Arg-Thr-Lys-Gln-Thr-Ala-Arg-Lys-Ser --
              1   2   3   4   5   6   7   8   9   10

Sequence 2    ?  -Glu-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys --
Histone H2B   Pro-Glu-Pro-Ala-Lys-Ser-Ala-Pro-Ala-Pro-Lys-Lys --
              1   2   3   4   5   6   7   8   9   10  11  12

Sequence 3    Ala-Val-Thr-Lys-Ala-Gln-Lys-Lys --
Histone H2B   Ala-Val-Thr-Lys-Ala-Gln-Lys-Lys --
              17  18  19  20  21  22  23  24
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Figure 6. Comparison of sequence obtained from protein bands to known histone sequence. Proteins sequenced are indicated by horizontal bars on Figure 4 and are presented here in order from top to bottom (1-3). Residues are numbered from the N-terminus.

with 16 amino acids cleaved from the N-terminus. Comparison of the set of low molecular weight proteins with histones purified from untreated CEM-C7 cells showed that the patterns were very similar (Fig 7A). In addition it is clear from the results in Figure 7B that both the histone preparation (lane 4) and the extracts from dexamethasone treated cells (lane 3) have bands in the same positions on the activity gel. These bands were observed in the absence of added Ca^{2+}, immediately after removal of SDS. These results demonstrate that DNA-protein binding is being detected and not nuclease activity in the 10-18 kDa region. Furthermore when dexamethasone treated extracts were fractionated on sephadex G50 two protein peaks were obtained, one, where the histones were eluted (Kav 0.7) contained no nuclease activity while fractions from the other, at the void volume, did contain activity (results not shown). The latter

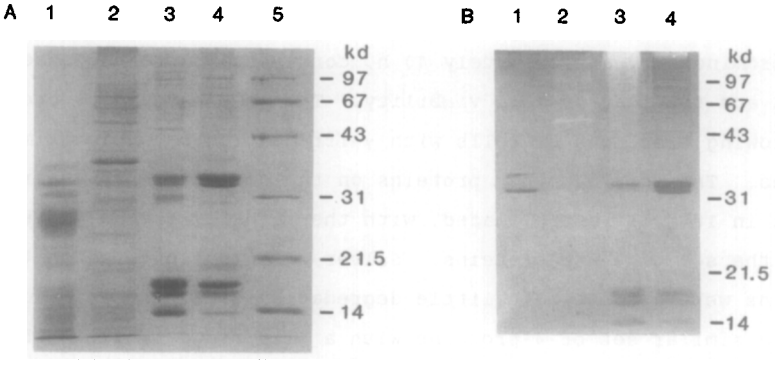

Figure 7. Comparison of nuclear proteins, extracted from CEM-C7 cells following addition of 10^{-6} M dexamethasone, with histone proteins. Nuclear protein was extracted 48h following treatment and along with histone protein electrophoresed in 13% SDS-polyacrylamide gels and stained with (A) Coomassie Blue or (B) ethidium bromide, for detection of nuclease activity or DNA-protein binding. Lane 1, control DNase; lane 2, untreated cells; lane 3, 48h incubation with dexamethasone; lane 4, histone protein. DNA-protein binding was determined immediately after removal of SDS without added Ca^{2+}.

activity could correspond to a doublet observed at 55kDa (Fig 5) which only appeared following incubation of the gel in the presence of Ca^{2+}.

DISCUSSION

Cell death is an important component in the normal functioning of the immune system (4). Two distinct forms of cell death are known, the first termed necrosis, results from physical or chemical injury while the other, apoptosis, results from programming from within the cell to self-destruct in response to external influences. In this study the human T-cell leukaemia line, CEM- C7, following treatment with dexamethasone showed morphological changes characteristic of apoptosis as the mode of cell death.

Another characteristic of apoptosis is degradation of chromatin into fragments of DNA, increasing in size by multiples of approximately 200 bp, corresponding in size to the internucleosomal distance (3). DNA fragmentation was observed in CEM-C7 cells and occurred at approximately 40 h after addition of dexamethasone and increased in extent up to 48 h. The onset of fragmentation at 40 h corresponded to a rapid decrease in viability beginning at this time. In this study, irradiation of CEM-C7 cells also led to DNA fragmentation. The viability of these cells decreased much earlier than was observed following treatment with dexamethasone. After 40 h, there was no further decrease in viability indicating that only the radioresistant cells remained.

Since glucocorticoids act by induction or inhibition of specific gene transcription (17) and since apoptosis requires active protein synthesis, nuclear proteins were analyzed for the presence of induced proteins. Analysis by SDS-PAGE demonstrated that a set of 6 proteins, with a size range of 10-18 kDa, became prominent by approximately 40 h, corresponding to the onset of DNA fragmentation and the loss of cell viability. The same pattern of proteins was observed following treatment of cells with γ-irradiation or cycloheximide using rat thymocytes. The loss of other proteins on the gel is due to their low concentration in 10μg of sample loaded, with the majority of the protein in the sample being the set of 6 new proteins. Silver staining revealed that the normal proteins were present with little degredation. Compton and Cidlowski (6) observed a similar set of 4 proteins with a size range of 12-19 kDa as well as a 30-32 kDa protein doublet, obvious by 5-6 h after treatment with dexamethasone. They provided evidence, based on DNase activity gels, that these proteins expressed prominent DNase activity. It seems likely from our sequence data and size comparison that these bands correspond to the core histones (12-19 kDa group) and histone H1 (30-32 kDa doublet). The higher molecular weight of H1 is due to anomalous migration. The DNase activity is

not real but rather due to DNA-protein binding which causes ethidium bromide to be excluded from DNA in these positions giving rise to a dark band against a fluorescent background which could be misinterpreted as nuclease activity. Gruol *et al.*(18) have recently reported that histones could score positively in this gel system by virtue of their capacity to exclude the ethidium stain.

While the lower molecular weight (histone) bands appeared immediately on the DNA activity gels, a 55 kDa doublet was only evident after 24hr incubation in the presence of Ca^{2+}. It is possible that the doublet represents genuine nuclease activity or DNA binding proteins that slowly renature during incubation. Such nucleases could play a role in the DNA fragmentation associated with apoptosis in CEM-C7 cells.

The apparent induction of nucleases reported by Compton and Cidlowski (6) and the set observed in this study appear to be due to extraction of histone proteins from nuclei at considerably lower salt concentrations than normal. In addition, one of these histones was specifically cleaved at a Lys-Ala bond, 16 residues from the N-terminus. Such changes to the histones may play an important role in the process of DNA fragmentation in apoptosis.

Acknowledgments: We acknowledge the assistance of Mr.B.Harmon for electron microscopy. This work was supported in part by funds from the National Health and Medical Research Council, the Queensland Cancer Fund and the University of Queensland Cancer Research Fund.

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