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Hoechst 33342 Induces Apoptosis and Alters Tata Box Binding Protein/DNA Complexes in Nuclei from BC3H-1 Myocytes

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Hoechst 33342 and Hoechst 33258 bind to adenine-thymine rich regions of the minor groove of DNA. Hoechst 33342, but not Hoechst 33258, induces BC3H-1 myocyte cell death and DNA fragmentation into an internucleosomal pattern characteristic of apoptosis. Hoechst 33342 has been shown to inhibit endogenous nuclear topoisomerase I activity. Another enzymatic activity utilizing the minor groove of DNA, the initiation of RNA polymerase II activity by formation of a TATA box binding protein/TATA box promoter complex, is shown to be altered using a gel mobility shift assay. A [^{32}P]-labeled 24-oligonucleotide containing a TATA box element formed one molecular weight complex in control and Hoechst 33258 treated cells. The presence of Hoechst 33342 (26.7 μM) decreased the amount of the control complex and increased the presence of lower molecular weight species suggesting degradation of nuclear TBP and/or release of other transcription factors from the complex creating a smaller sized molecular complex which retains TATA box binding capacity. These results suggest that the pathway utilized to induce apoptosis in BC3H-1 myocytes may also involve the alteration of normal TBP/DNA complex formation and reduction in the initiation of new transcription.

Cell death occurs by two processes, necrosis or apoptosis, whose cytological and biochemical characteristics are distinct. Apoptosis or programmed cell death is a complex process which involves a variety of signal transduction pathways leading to the degradation of cellular protein and DNA (1, 2). Although it appears that the downstream biochemical events in apoptosis are the same, such as phosphatidylserine translocation, DNA fragmentation, and activation of caspases, different apoptotic agents activate unique signal transduction pathways within the cell (1, 3). Determination of primary cellular sites of action for an inducer of apoptosis is required to determine the specific apoptotic intracellular mechanism(s) utilized to cause cell death.

Bisbenzimides (Hoechst 33342 or Hoechst 33258) are cell-permeable, adenine-thymine binding fluorescent dyes, which are used to stain DNA for evaluating the cell cycle, apoptosis, and quantifying viable cells by flow cytometry (4, 5). Recently, Hoechst 33342 but not Hoechst 33258 has been reported to induce apoptosis in BC3H-1 myocytes and hepatoma cells in a dose-dependent and time-dependent manner (6, 7). Hoechst 33342 initiates apoptosis in BC3H-1 myocytes by a pathway which is independent of the inhibition of previously initiated RNA synthesis (actinomycin-D) or protein synthesis (cycloheximide), and p53 expression (8). However, Hoechst 33342-induced apoptosis is associated with mitochondrial dysfunction and inhibition of endogenous nuclear topoisomerase I activity (7,8).

The single strand breaks created in DNA by topoisomerase I are initiated after the enzyme binds to the minor groove of DNA (9), the site of Hoechst 33342 binding (10). This study demonstrates that Hoechst 33342, but not Hoechst 33258, may inhibit another process which occurs at the minor groove; i.e., the initiation of new transcription by RNA polymerase II. Gel mobility shift analyses of nuclear extracts of BC3H-1 myocytes treated with Hoechst 33342 and a [^{32}P]-labeled 24 base pair oligonucleotide containing the TATA box promoter region demonstrates decreased formation of TATA box binding protein (TBP)/24-oligonucleotide complex and an increase of a smaller molecular weight TBP/24-oligonucleotide complex. Since the formation of the TBP/DNA complex is the initial step required for RNA polymerase II activation, the inhibition of both topoisomerase I and new transcription may play an important role in Hoechst 33342-induced apoptosis in BC3H-1 myocytes.

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MATERIALS AND METHODS

Cell culture and treatment. The murine muscle cell line (BC3H-1) was grown in Dulbecco's Modified Eagles medium (DMEM) (Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum (FBS) (Biocell Laboratories, Inc., Rancho Dominguez, CA) and incubated at 37°C in a humidified atmosphere of 5% CO_2 as previously described (11). For experiments, cells were plated at a density of 1×10^4 cells/mL and were cultured for 2 days with 80 to 90% confluence prior to treatment with Hoechst 33342 or Hoechst 33258 (Sigma, St Louis, MO). Hoechst 33342 or Hoechst 33258 were dissolved in distilled water at 25 mg/mL and added to DMEM with 2% FBS at a final concentration of 26.7 μM for 3, 6, and 12 hours.

Determination of cell viability. Cell viability was determined by trypan blue dye exclusion (12). The number of trypan-blue-negative cells was considered the number of viable cells. The effect of Hoechst 33342 and Hoechst 33258 was determined in triplicate. Statistical significance was determined by analysis of variance.

Analysis of DNA fragmentation. After incubation with Hoechst 33342 or Hoechst 33258 described above, BC3H-1 myocytes were washed twice with phosphate buffered saline (PBS) and precipitated by centrifugation. Lysis buffer (0.2 M Tris/HCl, pH 8.0; 0.1 M sodium ethylenediaminetetraacetic acid [EDTA]; 1% sodium dodecyl sulfate; and 100 mg/L proteinase K) was added, and the cells were incubated for 4 hours at 55°C. The lysates were extracted twice with an equal volume of phenol and once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Deoxyribonucleic acid (DNA) was precipitated with 0.05 volume of 5 M NaCl and 2.5 volumes of absolute ethanol and sedimented at 10,000 rpm for 10 minutes. The DNA pellet was dried and dissolved in Tris/EDTA buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA) containing 20 mg/L ribonuclease A and incubated for 1 hour at 37°C. The DNA was finally extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). DNA samples were analyzed by electrophoresis on 1.5% agarose gels, and the results were visualized by staining with ethidium bromide (6).

Preparation of nuclear extracts. BC3H-1 myocyte nuclear extracts used for gel mobility shift assay were prepared by a modification of the procedure previously described (13). Treated and untreated BC3H-1 myocytes were washed twice in ice-cold PBS. The cells were then resuspended in buffer A (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.05% Nondet P-40, 1% aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), and homogenized briefly on ice. After incubation on ice for 15 minutes, the homogenate was centrifuged at 14,000 rpm in a microcentrifuge at 4 °C for 10 minutes. The pellet was washed twice in buffer A and resuspended in buffer B (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 6.25% glycerol) containing protease inhibitors. After incubation on ice for 40 minutes, the nuclear suspension was centrifuged at 14,000 rpm in a microcentrifuge at 4 °C for 15 minutes. The protein concentrations of collected supernatant were determined by Bicichoninic Acid Protein Assay reagent (Pierce, Rockford, IL).

Gel (electrophoretic) mobility shift assay. Double stranded 5′-GAAGGGGGGCTATAAAAGGGGGTG-3′ oligonucleotide, positioned at −18 to −41 of the adenovirus major late promoter containing a TATA element (9), was synthesized by Bio-Synthesis Inc (Lewisville, TX). The oligonucleotide probe was end-labeled with [γ-^{32}P]-ATP (NEN Life Science Products Inc., Boston, MA) by using T4 polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany) and purified with ProbeQuant G-50 Micro Columns (Pharmacia Biotech, Piscataway, NJ). The binding reaction of TATA box binding protein to the 24-oligonucleotide took place in 20 mM Tris/HCl, pH 7.5, 10 mM sodium acetate, 0.5 mM EDTA, 5% glycerol including 5 μg total protein of the nuclear extract, and 1×10^4 cpm of end-labeled the [^{32}P]-labeled 24-oligonucleotide probe. The incubation was carried out for 1 hour at room temperature. DNA-protein complexes were separated by electrophoresis on 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer (Amresco, Solon, Ohio) and visualized by autoradiography (14).

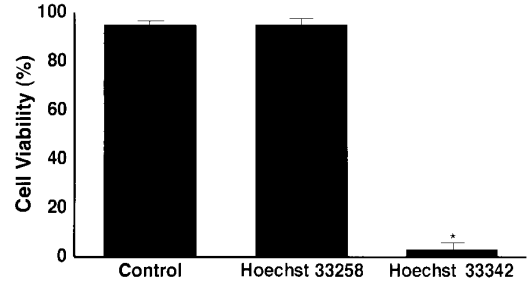

FIG. 1. Effect of Hoechst 33342 and Hoechst 33258 on cell viability of BC3H-1 myocytes. BC3H-1 myocytes initially cultured in DMEM/10% FBS were incubated with 26.7 μM Hoechst 33342 or Hoechst 33258 in DMEM/2% FBS for 12 hours. Each bar indicates mean + standard deviation from three separate experiments. *, $P<0.0001$.

RESULTS

Effect of Hoechst 33342 and Hoechst 33258 on BC3H-1 Cell Viability

Cell viability was measured by trypan blue exclusion after BC3H-1 myocytes were incubated with 26.7 μM of Hoechst 33342 or Hoechst 33258 for 12 hours. Hoechst 33342 significantly induced BC3H-1 myocyte death at 26.7 uM after 12 hour treatment when compared to the control cells (Figure 1). However, Hoechst 33258 failed to induce BC3H-1 myocyte death (Figure 1).

Analysis of DNA Fragmentation

Since an internucleosomal DNA fragmentation pattern is a biochemical hallmark of apoptosis (15), genomic DNA extracted from BC3H-1 myocytes treated with 26.7 μM of Hoechst 33342 or Hoechst 33258 for 3 or 6 hours was analyzed by electrophoresis in 1.5% agarose gel in order to confirm if Hoechst 33342 or Hoechst 33258 induces cell death via apoptosis. No DNA fragmentation ladder was observed when cells were treated with 26.7 μM Hoechst 33258 for 3 or 6 hours. Distinctive DNA fragmentation ladders were observed when cells were treated with 26.7 μM Hoechst 33342 for 3 or 6 hours (Figure 2). These results demonstrate that Hoechst 33342 induces cell death via apoptosis, while Hoechst 33258 fails to degrade DNA.

Effect of Hoechst 33342 and Hoechst 33258 on Formation of TBP/DNA Complexes

Gel mobility shift assays are a facile way to determine the specifity of protein binding to DNA, and to provide a means of quantitating DNA/protein complex formation. In the present study, the gel mobility shift assay was used to determine if Hoechst 33342 or

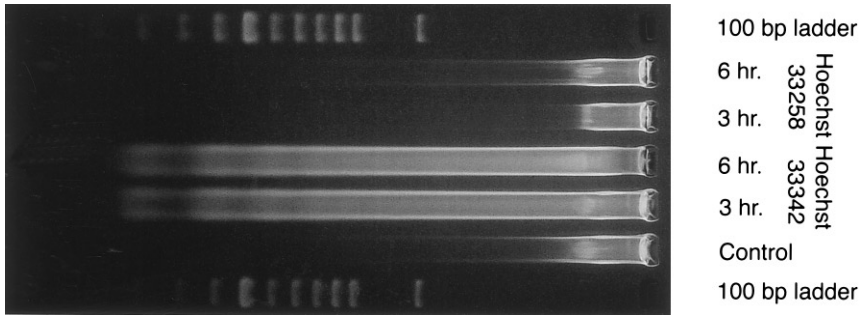

FIG. 2. Agarose gel electrophoresis of DNA from BC3H-1 myocytes incubated in DMEM/2% FBS treated with 26.7 μM Hoechst 33342 or Hoechst 33258 for 3 or 6 hours. Genomic DNA from the cells was extracted and electrophoresed.

Hoechst 33258 alter the binding of TBP and/or subsequent transcription factors (TFIID, TFIIB, TFIIE, etc.) to a TATA element contained within a [^{32}P]-labeled 24 base pair oligonucleotide. Our results demonstrate that Hoechst 33342 significantly attenuates formation of normal TBP/24-oligonucleotide complex (Band I in Figure 3) and increased that of smaller molecular weight TBP/24-oligonucleotide complex (Band II in Figure 3) after 3 hour treatment of Hoechst 33342 when compared to the control group. There are no significant changes in the TBP/24-oligonucleotide complex (Band I) in Hoechst 33258-treated group (Figure 3).

DISCUSSION

In the present study, our results demonstrate that Hoechst 33342 induces cell death via the apoptotic pathway associated with the typical cleavage of genomic DNA into internucleosomal-sized fragments. In contrast, the structurally related Hoechst 33258 has no effect on BC3H-1 myocyte viability or DNA fragmentation.

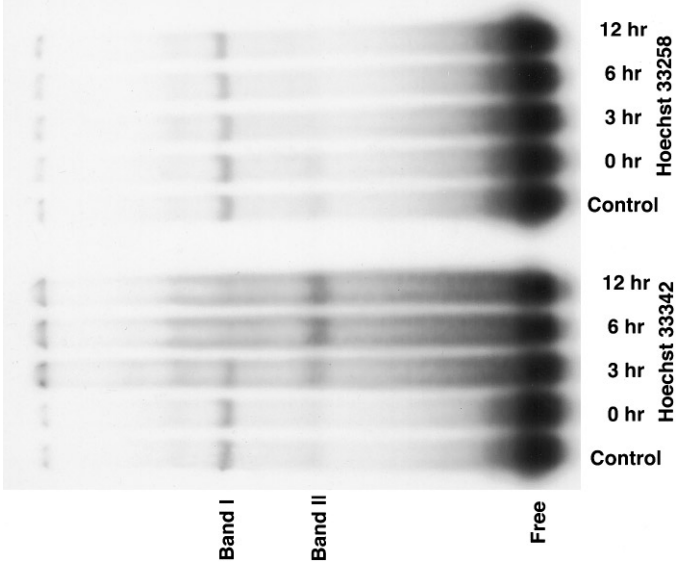

FIG. 3. Effect of Hoechst 33342 or Hoechst 33258 on formation of TBP/[^{32}P]-labeled 24-oligonucleotide complex. The nuclear extracts were prepared from untreated and Hoechst 33342-treated or Hoechst 33258-treated BC3H-1 myocytes. Nuclear extracts (5 μg total protein) were incubated with [^{32}P]-labeled double-stranded oligonucleotide to determine the formation of TBP/DNA complexes. The reaction mixtures were then analyzed by electrophoresis in 4% polyacrylamide gel.

Both dyes bind in the minor groove of DNA covering at least 6 base pairs and a minimum of 4 AT sequences are required for tight drug-DNA interaction (10). Several enzymatic activities are initiated in the minor goove of DNA including DNA polymerase, RNA polymerase II, and topoisomerase I and II. Both Hoechst 33342 and Hoechst 33258 inhibit topoisomerase I *in vitro* (16,17) and Hoechst 33342 inhibits the enzyme *in vivo* in nuclear extracts from drug-treated BC3H-1 (8) and hepatoma cells (7). TBP is a general transcription factor required for the initiation of transcription in the minor groove of DNA by RNA polymerase II. Following the binding of TBP to its TATA box promoter region, additional transcription factors (TFIID, TFIIB, TFIIE and others) will be added to the complex before new RNA synthesis begins. Since Hoechst 33258 is an effective inhibitor of the formation of the TBP/DNA complex *in vitro* (9), we investigated the effect of Hoechst 33342 and Hoechst 33258 on the ability of endogenous TBP and transcription factors from drug-treated or control nuclei to bind to a [^{32}P]-labeled 24-oligonucleotide containing a TATA promoter element by gel mobility shift assay.

As demonstrated in our results, Hoechst 33342 significantly decreased formation of normal size TBP/24-oligonucleotide complex and increased that of a smaller molecular weight TBP/24-oligonucleotide complex (Band II, Figure 3), while no changes in the TBP/24-oligonucleotide complex were observed in Hoechst 33258-treated group. The appearance of the smaller molecular weight TBP/24-oligonucleotide complex may be attributed to protease (caspase) degradation of TBP secondary to protease activation or protease inhibitor inactivation producing a smaller molecular weight TBP which retained its TATA box binding capacity. Also, transcription factor(s) included in band I may not bind to the proteolytically cleaved TBP. Both of these mechanisms would lead to a lower molecular

weight protein component in the TBP/24-oligonucleotide complex. The nature of this hypothetical protease remains unknown, although inhibitors of serine and cysteine proteases have failed to prevent Hoechst 33342-induced apoptosis in BC3H-1 myocytes (8). This reduced sized TBP/DNA complex *in vivo* may reduce new transcription but would not interfere with transcription initiated prior to the addition of Hoechst 33342 and the RNA synthesis inhibitor, actinomycin D (8).

Differences in the specific changes in cellular metabolism induced by Hoechst 33342 or presence of Hoechst 33342 but not in the presence of Hoechst 33258 (18); Hoechst 33342 decreases, while Hoechst 33258 increases, the rate of channel-mediated Ca^{2+} efflux from junctional sarcoplasmic reticulum vesicles (19). However, the only structural difference between the two dyes is that Hoechst 33258 has a hydroxy group where Hoechst 33342 has an ethoxy group. This structural difference confers on Hoechst 33342 greater lipophilic properties and greater cell membrane permeability (20). Based on the data presented here and previous observations (16-20), we propose a model to illustrate the process of Hoechst 33342-induced apoptotic cell death in BC3H-1 myocytes. Hoechst 33342 readily enters BC3H-1 myocytes and forms a stable Hoechst 33342/DNA complex at AT-rich regions in the minor groove. The binding of TBP to TATA elements is blocked, which results in accumulation of TBP in nuclei. Failure of RNA transcription triggers activation of endogenous proteases and nucleases that digest proteins (TBP) and genomic DNA leading to apoptosis. Since Hoechst 33258 is less cell membrane permeable compared to Hoechst 33342, the intracellular concentration of Hoechst 33258 fails to achieve the amount required to trigger apoptosis. This mechanism may be enhanced by Hoechst 33342's ability to inhibit endogenous topoisomerase I activity and disrupt mitochondrial function (7,8). Therefore, the pathway employed by Hoechst 33342 to induce apoptosis may involve a variety of intracellular metabolic perturbations.

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