Apoptosis Induced by Anthracycline Antibiotics in P388 Parent and Multidrug-resistant Cells

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ABSTRACT

The effect of the topoisomerase II inhibitor doxorubicin and its non-cross-resistant analogue annamycin on DNA degradation and programmed cell death was examined in murine leukemia P388 cells. P388 parental cells exposed to various concentrations of doxorubicin and annamycin for 24 h showed a reduction in cell volume and condensation of nuclear structures. Similar changes were observed in P388/Dox cells exposed to 10 μM doxorubicin or annamycin for 24 h but not in cells exposed to 10 μM doxorubicin. Time course studies demonstrated that DNA fragmentation was detected 12 h after incubation with 1 μM doxorubicin or annamycin, while loss of membrane integrity appeared at 24 h, thus indicating that DNA degradation was a preceding event. DNA fragmentation caused by doxorubicin and annamycin was inhibited by the RNA synthesis inhibitor actinomycin D, the protein synthesis inhibitor cycloheximide, and the endonuclease inhibitor auranofin but not by cycloheximide and aurintricarboxylic acid, thus suggesting that the apoptotic process caused by these drugs requires gene expression, synthesis of new proteins, and activation of endogenous nucleases. In contrast, DNA cleavage was not affected by incubating cells with 1 μM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, thus indicating that intracellular calcium depletion does not affect anthracycline-induced apoptosis. The results obtained demonstrate that the cell killing effect of anthracyclines is mediated, at least in part, by the induction of apoptosis.

INTRODUCTION

Doxorubicin is one of the most widely used anticancer agents because of its broad spectrum of antitumor activity, including solid tumors and hematological malignancies (1–3). During the last few years, a variety of doxorubicin analogues have been synthesized in an attempt to overcome the cumulative dose-limiting cardiotoxicity and resistance that certain tumors develop with repeated treatment (4, 5). We have studied a series of doxorubicin analogues that have a high affinity for cell membranes and selected annamycin (Fig. 1) for further studies based on its greater in vivo antitumor activity and lack of cross-resistance with doxorubicin in a variety of resistant cell lines (6). Previous studies on the mechanism of action of both drugs have demonstrated that annamycin-induced cytotoxicity is related, at least in part, to topoisomerase II-associated DNA lesions (7).

Apoptosis plays an important role in embryonic development, metamorphosis, hormone-dependent atrophy, and tumor growth, as a physiological event regulating the cell number or eliminating damaged cells (8–11). Cells undergoing apoptosis in normal and neoplastic tissues display the following morphological and biochemical features: reduced cell volume, condensed chromatin in the nucleus, formation of internucleosomal DNA fragmentation, and finally, loss of membrane integrity as well as generation of apoptotic bodies containing intact organelles and plasma membrane (12, 13). The molecular mechanism of apoptosis remains unknown; it has been proposed to be linked to the activation of specific genes and the synthesis of new proteins (14–17). Recent data have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents such as cisplatin (18, 19), cytarabine (20), the topoisomerase I inhibitor camptothecin (18), and topoisomerase II inhibitors such as amsacrine, etoposide, and teniposide (21–23).

We report here the cytotoxic effect of the topoisomerase II inhibitors doxorubicin and annamycin on P388-sensitive and -resistant cell lines and provide evidence that these drugs induce multinucleosomal DNA fragmentation and may exert their cytotoxicity, at least in part, by triggering programmed cell death. In addition, we also present the effects of RNA and protein synthesis and endonuclease inhibitors, as well as the effect of depletion of intracellular calcium, on anthracycline-induced DNA fragmentation and apoptosis.

MATERIALS AND METHODS

Chemicals. Doxorubicin was purchased from Ben Venue Laboratories (Bedford, OH), and a stock solution (2 μM) was prepared in normal saline. Annamycin was synthesized as previously described (24). Actinomycin D, cycloheximide, ATA, 12-O-tetradecanoylphorbol-13-acetate, and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 and trypan blue were purchased from Gibco/BRL (Gaithersburg, MD). DNA molecular marker and RNase were obtained from Boehringer Mannheim (Indianapolis, IN). [3H]Thymidine (2.11 GBq/nmol) was obtained from Amersham (Arlington Heights, IL). All other chemicals were purchased from Fisher (Pittsburgh, PA).

Cell Culture and Drug Treatment. P388-sensitive and multidrug-resistant cells (P388/Dox) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 units penicillin, and 100 μg streptomycin at 37°C in 5% CO2/95% air. Exponentially growing cells (0.5 × 10⁶ cells/ml) were exposed to various concentrations of doxorubicin and annamycin for different periods of time. After treatment, cells were harvested by centrifugation at 800 × g for 5 min and washed twice with cold Ca²⁺-, Mg²⁺-free PBS.

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2 To whom requests for reprints should be addressed, at Department of Head, Neck, and Thoracic Medical Oncology, Box 80, 1515 Holcombe Boulevard, Houston, TX 77030.

3 The abbreviations used are: annamycin, 2′,3′-OH-3′-epi-4-demethoxydoxorubicin; ATA, auranofin; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PBS, phosphate-buffered saline.

Doxorubicin (DOX)  Annamycin (AN)

Fig. 1. Chemical structures of doxorubicin and annamycin.
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Fig. 2. Concentration response of doxorubicin (DOX) and annamycin (AN) on the viability of P388 parental and multidrug-resistant cells (P388/Dox). Cell cultures were exposed to 0.1–10 μM doxorubicin or annamycin for 24 h. Viable cells were counted by the trypan blue exclusion method with the use of a hemocytometer, and the cell survival was expressed as the percentage of viable cells relative to control. Points, mean of three independent experiments; bars, SD.

Cell Viability and Morphological Assessment. Viable cells were counted in a hemocytometer by 0.2% trypan blue dye exclusion. The survival fraction in treated cultures was expressed as percentage of control, in which an equal volume of saline was added. P388 and P388/Dox cells were exposed to 1–10 μM doxorubicin and annamycin for 24 h. Drugs were removed and cells were washed twice with Ca²⁺, Mg²⁺-free PBS and resuspended in PBS. Aliquots of cell suspensions were taken and fixed onto microscope slides by cyto spin (Shandon, UK), stained with Wright-Giemsa dye, and examined under a light microscope.

Assessment of DNA Cleavage. DNA cleavage in control and treated P388 and P388/Dox cells was quantitatively determined by the method described by Kolber et al. (25) with slight modifications. In brief, exponentially growing cells (1 × 10⁶ cells/mL) were prelabeled with 1 μCi [³²P]thymidine for 24 h, washed three times with cold Ca²⁺, Mg²⁺-free PBS, and chased for 2 h with fresh medium containing 1 mM cold thymidine supplemented with 10% fetal calf serum. Labeled cells were exposed to various concentrations of doxorubicin and annamycin for different periods of time. Cells were harvested by centrifugation at 800 × g for 5 min and washed three times with PBS. Cell pellets were suspended in 0.5 ml hypotonic lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.2% Triton X-100. After incubating at room temperature for 30 min, samples were centrifuged at 12,000 × g for 30 min. Pellets were washed once with 0.5 ml lysis buffer. The radioactivity in supernatant (detergent-soluble low-molecular-weight DNA) and in pellet (intact chromatin DNA) was determined by liquid scintillation counter. The percentage of DNA fragmentation was calculated with the following formula:

\[
\% \text{ DNA fragmentation} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant} + \text{cpm in pellet}} \times 100
\]

Gel Electrophoresis of Fragmented DNA. To assess the pattern of DNA cleavage caused by doxorubicin and annamycin, agarose gel electrophoresis was performed as described by Cohen and Duke (15). In brief, control and drug-treated cells (2 × 10⁶ cells) were lysed with 0.5 ml lysis buffer containing 0.2% Triton X-100 at room temperature for 30 min. The supernatant fractions were collected by centrifugation at 12,000 × g for 30 min. The DNA in these fractions was precipitated overnight with 100 μl 5 M sodium chloride and 0.5 ml 2-isopropanol at -20°C. The DNA was dissolved in 20 μl of 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA buffer. Ten units RNase were added to the samples before incubating at 60°C for 60 min. After the addition of an equal volume of loading buffer, the samples were subjected to electrophoresis on an

Fig. 3. Light microscopic examination of sensitive P388 and resistant P388/Dox cells exposed to doxorubicin and annamycin. Exponentially growing P388 cells were treated with 1 μM doxorubicin or annamycin, and P388/Dox cells were treated with 10 μM drugs. Following a 24-h incubation period, cells were fixed onto microscope slides with cyto spin, stained with Wright-Giemsa dye, and observed under a light microscope (× 400). A, untreated P388 cells; B, P388 cells treated with 1 μM doxorubicin; C, P388 cells treated with 1 μM annamycin; D, untreated P388/Dox cells; E, P388/Dox cells treated with 10 μM doxorubicin; F, P388/Dox cells treated with 10 μM annamycin.
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Fig. 4. Effect of doxorubicin (DOX) and annamycin (AN) treatment on DNA cleavage in P388 (A) and P388/Dox (B) cells. P388 and P388/Dox cells were prelabeled with [14C]thymidine for 24 h and chased in fresh medium containing 1 μM nonradioactive thymidine for 3 h. The labeled cells were treated with various concentrations of drugs for 24 h and then lysed with lysis buffer containing 0.2% Triton X-100. The detergent-soluble and intact chromatin DNA were separated, and DNA fragmentation was determined as described in “Materials and Methods.” Points, means of four independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, difference from controls. Agarose gel analysis of drug-induced internucleosomal DNA cleavage in P388 (C) and P388/Dox (D) cells. Cells were incubated in the presence of 0.1–10 μM doxorubicin or annamycin for 24 h. The detergent-soluble cleaved DNA in supernatant was isolated and subjected to electrophoresis on a 2% agarose gel as described in “Materials and Methods.”

Statistical Analysis. Results were analyzed by a standard paired Student’s t test. The significance is a calculated P value of less than 0.05.

RESULTS

Cytotoxicity. Survival of murine leukemia P388 and P388/Dox cells following 24-h continuous exposure to 0.1–10 μM doxorubicin and annamycin was determined by counting the number of viable cells with trypan blue dye exclusion (Fig. 2). In sensitive P388 cells, 1 and 10 μM doxorubicin decreased survival to 28% and 15%, respectively, compared with controls, and 1 or 10 μM annamycin decreased survival to 30% and 18%, respectively, compared with controls. Under the same experimental conditions, the survival of P388/Dox cells was not decreased by either concentration of doxorubicin but was reduced to 55% and 30% of control value when exposed to 1 and 10 μM annamycin, respectively. These results are consistent with our earlier report, in which annamycin showed a lack of cross-resistance with doxorubicin (6, 7).

Morphological Changes. Compared with untreated cells (Fig. 3A), P388 parental cells treated with 1 μM doxorubicin or annamycin for 24 h showed a reduction in cell volume, an increased cytoplasmic:nuclear ratio, condensation of nuclear chromatin, and nuclear fragmentation in some cells (Fig. 3, B and C). Minor morphological alterations were observed in P388/Dox cells exposed for 24 h to 10 μM doxorubicin as compared with control cells (Fig. 3, D and E), while P388/Dox cells exposed to 10 μM annamycin showed the morphological changes described above, which were consistent with apoptosis (Fig. 3F).

Assessment of DNA Cleavage. To quantitatively determine DNA degradation, we used P388 and P388/Dox cells that were prelabeled with [14C]thymidine and treated with various concentrations of doxorubicin or annamycin. DNA cleavage in P388 cells was highly dependent on drug concentration (Fig. 4A). At 0.1 μM, the extent of DNA degradation was a little higher than that of untreated cells; at 1 μM, the extents of DNA cleavage caused by doxorubicin and annamycin were 37% and 30% (P < 0.01), respectively. However, at higher concentrations, the extent of DNA cleavage was only 18% in cells treated with 10 μM annamycin and lower than the control value in cells treated with 10 μM doxorubicin. Analysis by agarose gel electrophoresis fur-

2% agarose gel in 100 mM Tris-HCl (pH 8.0), 100 mM boric acid, and 1.5 mM EDTA buffer at 50 V for 3 h. The agarose gel was stained with ethidium bromide, and the resulting DNA fragmentation pattern was visualized by UV illumination.

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Fig. 5. Time course of DNA fragmentation following treatment with doxorubicin (DOX) or annamycin (AN) in P388 parental and resistant cells. A, P388 cells incubated in the absence of or in the presence of 1 μM doxorubicin or annamycin for the indicated time. B, P388/Dox cells treated with 10 μM doxorubicin or annamycin for different periods of time. After incubation for the indicated time period, aliquots of cells were taken and DNA fragmentation was determined as described in “Materials and Methods.” Points, mean of four independent experiments; bars, SD. C and D, the agarose gel analysis of drug-induced DNA cleavage in P388 and P388/Dox cells at different time points. DNA isolation and gel analysis were performed as described in “Materials and Methods.”

Fig. 5 shows the time course of DNA cleavage in [14C]thymidine-labeled P388 and P388/Dox cells. In sensitive cells, a considerable amount of DNA fragmentation was detected after 6 h of continuous incubation with 1 μM doxorubicin or annamycin, and the extent of DNA fragmentation was directly related to the incubation time. The amount of spontaneous DNA degradation in untreated cells was very low compared with treated cells (Fig. 5A). In P388/Dox cells, the DNA cleavage curves in controls and cells treated with 10 μM doxorubicin had a similar pattern indicating no time dependence. In contrast, the DNA fragmentation in P388/Dox cells treated with 10 μM annamycin was markedly related to the incubation time (Fig. 5B). By agarose gel analysis, the intensity of the DNA fragments caused by doxorubicin and annamycin in P388 and P388/Dox cells at different time points basically confirmed the results of extent of DNA cleavage in radiolabeled cells (Fig. 5, C and D).

**Determination of Membrane Integrity.** Since some reports indicate that DNA fragmentation occurs before the loss of membrane integrity in apoptosis (11-13), we also determined the loss of membrane integrity by trypan blue exclusion in untreated cells and cells treated with 1 μM drugs at different time points. No loss of membrane integrity was observed by trypan blue exclusion 12 h after incubation with anthracyclines (Fig. 6), while about 15% DNA fragmentation could be detected at that time (Fig. 5). Thus, DNA cleavage in doxorubicin- and annamycin-treated cells precedes the loss of membrane integrity by at least 12 h.

**Effect of Ca2+ Depletion on DNA Cleavage.** Some investigators have demonstrated that DNA fragmentation in apoptosis is dependent upon endonucleases activated by Ca2+ and Mg2+ and that deprivation of intracellular calcium can prevent DNA breakdown and the apop-
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A. P388 Sensitive Cells

B. P388 Resistant Cells

Table 1 Effect of EGTA and 12-0-tetradecanoylphorbol-13-acetate on doxorubicin- and annamycin-induced DNA fragmentation in P388 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX or AN only</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>DOX</td>
<td>40.1 ± 7.5</td>
</tr>
<tr>
<td>AN</td>
<td>26.0 ± 6.0</td>
</tr>
<tr>
<td>EGTA (1 mM)</td>
<td></td>
</tr>
<tr>
<td>EGTA only</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>DOX + EGTA</td>
<td>39.0 ± 2.0</td>
</tr>
<tr>
<td>AN + EGTA</td>
<td>26.0 ± 9.0</td>
</tr>
<tr>
<td>TPA (0.1 µM)</td>
<td></td>
</tr>
<tr>
<td>TPA only</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>DOX + TPA</td>
<td>38.0 ± 4.0</td>
</tr>
<tr>
<td>AN + TPA</td>
<td>30.0 ± 3.0</td>
</tr>
</tbody>
</table>

All cultures incubated for 24 h. All concentrations of doxorubicin and annamycin were 1 µM.

All data represent the mean ± SD of three independent experiments.

DOX, doxorubicin; AN, annamycin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

not affected by intracellular calcium depletion. By agarose gel analysis, deprivation of intracellular calcium did not affect anthracycline-induced DNA fragmentation (Fig. 7). These findings are consistent with reports by other investigators (29). Thus, topoisomerase II inhibitor-induced DNA cleavage in apoptosis may not be mediated by the regulation of intracellular calcium.

Tomei et al. (30) showed that 12-O-tetradecanoylphorbol-13-acetate, a protein kinase C activator, can prevent apoptosis in some experimental models, suggesting that apoptosis may be regulated by the signal transduction pathway. We attempted to determine whether 12-O-tetradecanoylphorbol-13-acetate could protect against anthracycline-induced DNA fragmentation and found that DNA fragmentation was not inhibited by this agent (Table 1). Thus, doxorubicin- and annamycin-induced DNA fragmentation and apoptosis may not be regulated by this signal transduction pathway.

Effect of Inhibition of Protein and RNA Synthesis on DNA Cleavage. Because apoptosis is an active process, it requires the synthesis of new RNA and proteins. Some studies show that inhibition of protein synthesis with cycloheximide and inhibition of RNA synthesis with actinomycin D can prevent cells from undergoing apoptosis (14, 15, 17, 28). We determined whether these inhibitors could prevent doxorubicin- and annamycin-induced DNA fragmentation in P388 cells. Table 2 shows that pretreatment with 2 µg/ml actinomycin D for 2 h partially protected against DNA breakdown as compared with cells treated only with doxorubicin or annamycin, although actinomycin D by itself also induced some DNA fragmentation. As shown in Table 2, pretreatment with 50 µg/ml cycloheximide for 2 h did not prevent doxorubicin- and annamycin-induced DNA cleavage. However, cells continuously incubated for 24 h with cycloheximide and doxorubicin or annamycin showed a marked decrease in drug-induced DNA cleavage. Incubation with cycloheximide alone resulted in 4.2% DNA degradation. Agarose gel electrophoresis analysis con-

Fig. 6. Time course of loss of membrane integrity in P388 (A) and P388/Dox (B) cells that were untreated (CON) or treated with doxorubicin (DOX) or annamycin (AN). Following incubation, aliquots of cells were taken, and the cell membrane integrity was determined by trypan blue dye exclusion as described in "Materials and Methods." Points, mean of three independent experiments with triplicate samples; bars, SD.

Fig. 7. Effects of depletion of intracellular calcium on doxorubicin (DOX)- and annamycin (AN)-induced DNA cleavage in P388 cells. P388 cells were incubated in the presence of 1 µM doxorubicin or annamycin either in combination with or without 1 mM EGTA for 24 h. The isolation of cleaved detergent-soluble DNA and agarose gel analysis were performed as described in "Materials and Methods."
Table 2 Effect of actinomycin D and cycloheximide on doxorubicin- and annamycin-induced DNA fragmentation in P388 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fragmentation (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX or AN only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated (24 h)</td>
<td>2.4 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>DOX*</td>
<td>32.6 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>AN*</td>
<td>24.0 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>Act D (pretreatment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (Act D only 2 h)</td>
<td>10.0 ± 2.8*</td>
<td>71</td>
</tr>
<tr>
<td>2-h Act D followed by DOX*</td>
<td>9.4 ± 2.6*</td>
<td>71</td>
</tr>
<tr>
<td>2-h Act D followed by AN*</td>
<td>14.4 ± 6.0*</td>
<td>40</td>
</tr>
<tr>
<td>CHX (pretreatment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (CHX only 2 h)</td>
<td>2.2 ± 0.5</td>
<td>19</td>
</tr>
<tr>
<td>2-h CHX followed by DOX*</td>
<td>26.0 ± 7.4</td>
<td>19</td>
</tr>
<tr>
<td>2-h CHX followed by AN*</td>
<td>22.0 ± 5.8</td>
<td>8</td>
</tr>
<tr>
<td>CHX + anthracycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (CHX only 24 h)</td>
<td>4.2 ± 0.1</td>
<td>87</td>
</tr>
<tr>
<td>DOX + CHX*</td>
<td>4.3 ± 0.6*</td>
<td>87</td>
</tr>
<tr>
<td>AN + CHX*</td>
<td>4.0 ± 0.6*</td>
<td>83</td>
</tr>
</tbody>
</table>

* Data represent the mean ± S.D. of three independent experiments.
* DOX, doxorubicin; AN, annamycin; Act D, actinomycin D; CHX, cycloheximide.
* All DOX and AN treatments were for 24 h using 1 μM concentration.
* P < 0.01, t test of difference between drug only and drug plus Act D or CHX.
* P < 0.05.
* Cells treated with 1 μM anthracycline plus 50 μg/ml CHX and incubated for 24 h.

confirmed the protective effect of actinomycin D and cycloheximide on doxorubicin- and annamycin-induced DNA fragmentation in P388 cells (Fig. 8).

Effect of the Endonuclease Inhibitor ATA on DNA Cleavage. When P388 cells were incubated with 100 μM ATA alone or in combination with 1 μM doxorubicin or annamycin for 24 h, the extent of drug-induced DNA cleavage was almost completely abolished, as demonstrated by the determination of cleaved radiolabeled DNA or the visualization of DNA fragments in ethidium bromide-stained agarose gel (Table 3; Fig. 9).

We also attempted to determine whether inhibition of DNA cleavage by ATA and cycloheximide could prevent or delay doxorubicin- and annamycin-induced programmed cell death in P388 cells. As shown in Fig. 10, cell survival at 12–24 h incubation with 1 μM doxorubicin and annamycin in the presence of 100 μM ATA was about 80% and 60%, respectively, while the survival of cells treated with doxorubicin and annamycin without ATA was 47% and 30%, respectively. However, after a 48–72 h incubation, no significant differences in cell survival between doxorubicin- and annamycin-treated cells with or without ATA were observed.

In the case of P388 cells incubated for 72 h in the presence of 50 μg/ml cycloheximide, the number of viable cells remained unchanged, thus suggesting that cycloheximide has a cytostatic effect, blocks cell proliferation, and does not induce cell death. Interestingly, cycloheximide showed a markedly protective effect against doxorubicin- and annamycin-induced cytotoxicity, as compared with cells treated only with doxorubicin and annamycin (Fig. 11). These results are in agreement with previous reports by others (31, 32).

DISCUSSION

Apoptosis is one of the two major modes of death in mammalian cells. However, the intracellular effector mechanisms that regulate apoptosis are largely unknown. Cells dying from apoptosis display DNA fragmentation at internucleosomal sites followed by altered nuclear morphology and finally loss of membrane integrity. Although all apoptotic cells exhibit these common features, it is not well established whether all apoptotic-related cell deaths occur by a common mechanism (8, 9, 11, 33). In view of increasing evidence that a variety of chemotherapeutic agents, including some topoisomerase II inhibitors, can trigger programmed cell death (22, 23, 33), we decided to investigate whether anthracycline compounds can induce DNA fragmentation and apoptosis.

In the present work, we attempted to determine whether doxorubicin and annamycin could induce internucleosomal DNA fragmentation and trigger the process of programmed cell death. The results presented herein using two DNA fragmentation assay techniques show that doxorubicin and annamycin, at the appropriate concentration (1 μM), pronouncedly induce DNA degradation in P388 cells, whereas at higher concentrations (10 μM), they display an increased cytotoxicity, suggesting that other mechanisms may be involved at higher drug concentrations and that endonucleases or other factors essential to apoptosis are activated only at lower concentrations.

Fig. 8. Left, fragmented DNA extracted from P388 cells pretreated with 2 μg/ml actinomycin D (Act. D) for 2 h and then exposed to 1 μM doxorubicin (DOX) or annamycin (AN) for 24 h resolved on a 2% agarose gel. Right, analysis of fragmented DNA from P388 cells pretreated with 50 μg/ml cycloheximide (CHX) for 2 h and then treated with 1 μM doxorubicin or annamycin for 24 h (a) or from P388 cells treated for 24 h with cycloheximide combined with either doxorubicin or annamycin (b).
Table 3  Effect of endonuclease inhibitor ATA on doxorubicin- and annamycin-induced DNA fragmentation in P388 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fragmentation (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX or AN only</td>
<td>2.0 ± 0.6</td>
<td>-</td>
</tr>
<tr>
<td>DOX</td>
<td>35.3 ± 3.8</td>
<td>-</td>
</tr>
<tr>
<td>AN</td>
<td>21.0 ± 4.2</td>
<td>-</td>
</tr>
<tr>
<td>ATA + anthracycline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATA only (100 µM)</td>
<td>1.6 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>DOX + ATA</td>
<td>2.5 ± 0.5*</td>
<td>92</td>
</tr>
<tr>
<td>AN + ATA</td>
<td>9.0 ± 1.8*</td>
<td>38</td>
</tr>
</tbody>
</table>

* All cells were incubated for 24 h. Anthracycline concentrations were 1 µM in all experiments.

** Data represent the mean ± SD of three independent experiments.

* DOX, doxorubicin; AN, annamycin.

* P < 0.01, t test of difference between drug alone and plus 100 µM ATA.

* P < 0.05.

Because apoptosis is an active process requiring the expression of specific gene(s) and the synthesis of new proteins, we studied the effect of several RNA and protein synthesis inhibitors on doxorubicin- and annamycin-induced DNA fragmentation. We found that P388 cells pretreated with 5 µg/ml actinomycin D for 2 h, washed with PBS, and reincubated in the presence of doxorubicin and annamycin for 24 h showed a reduction in drug-induced DNA fragmentation from 24% to 9.4% (P < 0.01) for doxorubicin and 32% to 14% (P < 0.05) for annamycin. Furthermore, we also compared the DNA fragmentation induced by doxorubicin and annamycin in P388 cells with that in P388/Dox cells and found that even at higher concentrations doxorubicin was unable to induce DNA degradation in resistant cells, while annamycin caused DNA breakdown and altered cell morphology in both cell lines. We previously determined the formation of drug-topoisomerase II-cleavable complexes by a sodium dodecyl sulfate-potassium chloride precipitation method and found that doxorubicin was able to induce such complexes only in sensitive cells, while annamycin was effective in both cell lines. These findings suggest that DNA fragmentation and induction of apoptosis in cells exposed to anthracyclines may be mediated through interaction with DNA and topoisomerase II, formation of the covalent complex, and alteration of the conformation of supercoiled DNA in chromatin, thus allowing the endogenous nuclease(s) and/or inducers to more easily access the linker regions in the nucleosome and initiate DNA cleavage and the process of apoptosis.

Fig. 9. Agarose gel analysis of the inhibitory effect of ATA on doxorubicin (DOX-) and annamycin (AN)-induced DNA fragmentation in P388 cells. P388 cells were incubated in the absence or presence of 100 µM ATA with 1 µM doxorubicin or annamycin for 24 h. The detergent-soluble fragmented DNA was prepared, subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination, as described in "Materials and Methods."

Fig. 10. Growth kinetics of P388 cells exposed to 1 µM doxorubicin (DOX-) or annamycin (AN) in combination with or without 100 µM ATA. Exponentially growing P388 cells were treated with anthracyclines alone or in combination with 100 µM ATA, and at the indicated times viable cell counts were determined by trypan blue exclusion in a hemocytometer. Points, means of three separated experiments with duplicate samples; bars, SD. CON, control.

Fig. 11. Growth curves of doxorubicin (DOX-) and annamycin (AN)-treated P388 cells in the absence or presence of cycloheximide (CHX). P388 cells were exposed to 1 µM doxorubicin or annamycin with or without 50 µg/ml cycloheximide. After incubation, the viable cells were determined as described above. Points, means of three independent experiments with duplicate samples; bars, SD.

ananny, thus suggesting that the inhibition of RNA synthesis can partially block anthracycline-induced DNA cleavage. These results are consistent with other reports (13, 15, 27, 28). When P388 cells were pretreated with 50 μg/ml cycloheximide for 2 h, DNA cleavage was not spontaneously induced or prevented in cells subsequently treated with doxorubicin or annamycin. However, DNA cleavage was markedly suppressed when cells were exposed to doxorubicin or annamycin for 24 h in the presence of 50 μg/ml cycloheximide, although this agent induced a small amount of DNA fragmentation by itself (Table 2). Moreover, 50 μg/ml cycloheximide had a cytostatic effect and provided full protection from anthracycline-induced cell death (Fig. 11). By examination under light microscopy, the presence of cycloheximide with or without doxorubicin or annamycin for 72 h resulted in a reduction in cell volume with full preservation of membrane integrity (data not shown). There are several possible explanations for the prevention by cycloheximide of drug-induced DNA degradation and apoptosis. One possibility is the inhibition of some critical proteins that may act as cofactors in triggering apoptosis. A second possibility is inhibition of the synthesis of topoisomerase II or reduction of enzyme activity (31). Finally, a third possibility is blockade at the G0 or G1 phase, thus resulting in loss of susceptibility of the cells to undergo apoptosis at the S and S/G2 phase (32, 34, 35).

The endonuclease inhibitor ATA markedly prevented anthracycline-induced DNA fragmentation and partially reduced the drug-induced cytotoxic effect (Table 3; Figs. 9 and 10). These results further confirm that DNA cleavage and cell death following treatment with doxorubicin and annamycin are mediated, at least in part, by activation of endonucleases.

Cohen and Duke reported that increasing levels of Ca2+ in immature thymocytes could activate DNA cleavage and that depletion of intracellular Ca2+ inhibited the apoptotic process (15, 25-27). In this work, we demonstrated that depletion of intracellular Ca2+ by the addition of 1 mm EGTA to the culture medium did not affect doxorubicin- and annamycin-induced DNA fragmentation in P388 cells. These results are in agreement with a report by Bertrand et al. (29), who showed that depletion of Ca2+ can protect against the cytotoxic effect of etoposide but does not inhibit drug-induced DNA fragmentation.

In summary, our results show that the anthracyclines doxorubicin and annamycin at the appropriate concentrations cleave the chromatin DNA at linker regions of nucleosomes and trigger apoptosis. Apoptosis induced by topoisomerase II inhibitors like anthracyclines is associated with formation of cleavable complexes with nuclear topoisomerase and requires the synthesis of new proteins and RNA as well as the activation of endonucleases, while it is independent of intracellular Ca2+.

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